Regulation of T cell differentiation by the TGF-β inhibitor Smad7 and non-Smad pathways

Dissertation

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My family

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1. Introduction

1.1 The immune system - an overview

The main role of the immune system is to protect the host from infection. Host defense against infection requires dramatically different responses, depending on the character of the pathogen and on the tissue under attack (Chaplin 2010). The presence of infection is detected by components of the innate immune system, among others neutrophils, eosinophils, natural killer (NK) cells and macrophages. This detection system during the innate immune response uses a series of pattern recognition receptors. Key components of such receptors are the "toll-like receptors" (TLRs), which recognize a panel of microbial molecules (Gewirtz 2003).The response of the innate immune system is unspecific but relatively fast and often the infection is cured. If not, the second line of defence is provided by the adaptive immune system which consists of T and B lymphocytes which are very specific. Innate immunity to microbes stimulates adaptive immune responses and can influence the nature of the adaptive responses to make them optimally effective against different types of microbes. Immune cells of the adaptive immune system build a memory effect after encountering an antigen for the first time and if these cells are confronting the same antigen again, the immune response is faster and more specific. T cells play a central role in orchestrating the immune response. Further, they are instrumental in eliminating intracellular pathogens (viruses, some bacteria) through the generation of cytotoxic T cells. B cells defend against extracellular pathogens by producing antibodies. Immune cells of the adaptive immune system constitute the foundation of the defense network to protect the host. Any perturbation in this network severely endangers the efficiency of the immune system to protect the host (Gorska and Alam 2003).

The immune system is normally focused on responding to foreign materials and has an inbuilt tendency to avoid attacking self-tissues. But when this process goes wrong, the immune system can attack self-tissues resulting in autoimmune disease. The perplexing issue of what allows the immune system to attack self-tissues is a continuing focus of research. In patients with an autoimmune disorder, the immune system cannot recognize the difference between healthy body tissue and antigens, which can result in an immune response that destroys normal body tissues. What

causes the immune system to no longer distinguish the difference between healthy body tissues and antigens is unknown. One theory is that some microorganisms (Lee et al. *2011* and Berer *et al.* 2011) or drugs may trigger some of these changes, especially in people who have genes that make them more likely to get autoimmune disorders. Multiple sclerosis (MS), which is an autoimmune disease of the central nervous system, is associated with activated microglia (Jack *et al.* 2005) and infiltrating $CD8^+$ T cells found in lesions (Babbe *et al.* 2000). The experimental autoimmune encephalomyelitis (EAE), which is the animal model for MS is also associated with infiltrated macrophages and auto-reactive T cells (Gold and Lassmann 2006, Lassmann 1983). These auto-reactive T cells are mainly interferon γ (IFN-γ) and interleukin 17 (IL-17) producing T cells which can cause inflammation in the target organ (Goverman 2009). Mast cells and B cells also play a role in autoimmunity (Benoist and Mathis 2002, Lassmann and van Horssen 2011). Immune cells are also involved in allergic diseases. Allergy involves an exaggerated response of the immune system. When the immune system of a healthy person responds to normally harmless substances in the environment allergic reactions take place. T helper cells 1 (Th1), Th2, Th17 and regulatory T cells (Tregs) are known to be involved in allergic reactions (Schmidt-Weber 2008). Natural killer cells and mast cells also play an important role in allergic inflammation (Erten *et al.* 2008, Taube and Stassen 2008). Moreover, the effector Th2 and Th17 cells promote experimental airway asthma whereas Tregs play regulatory function (Finotto 2008).

1.1.1 Generation and maturation of T cells

T cell progenitors arise from hematopoietic stem cells in the bone marrow and migrate to the thymus. Their development requires signals from nonhematopoietic stromal cells including various types of thymic epithelial cells and mesenchymal fibroblasts. These cells reside in distinct anatomic locations in the thymus, and movement of precursor cells between these microenvironments is critical for the perception of differentiation signals (Anderson and Jenkinson 2001). Differentiation is characterized by the temporally coordinated expression of cell surface proteins on the thymocyte, including CD4, CD8, CD44, and CD25. Upon entry into the thymus, precursors lack expression of T cell receptor (TCR), CD4 and CD8 and are called double negative (DN) thymocytes. In general, thymocyte maturation can be divided

into three broad categories based on co-receptor surface expression: 1. an early double negative (DN) $CD4^-CD8^-$ stage, 2. a predominant double positive (DP) $CD4^+CD8^+$ stage, and 3. mature $CD4^+$ or $CD8^+$ single positive (SP) cell stage (von Boehmer and Fehling 1997). Lind *et al.* in 2001, showed that progenitor cells enter the thymus at the cortico-medullary junction (CMJ) and move through the cortex as the cells progress through DN1 ($CD44^+/CD25^-$) and DN2 ($CD44^+/CD25^-$), arriving at the sub capsular zone as they enter DN3 ($CD44^-/CD25^+$) (MacDonald *et al.* 2001) and finally DN4 ($CD44^-/CD25^-$) (Godfrey *et al.* 1993). Immature DN thymocytes upregulate the co-receptors following TCRβ locus re-arrangement and preTCR or β-selection. TCRα chain rearrangement is initiated and TCRαβ heterodimers are expressed on the cell surface at the DP stage. At this point, these thymocytes become eligible for both positive and negative selection. T cells that express MHC class II restricted receptors are positively selected to the CD4 lineage, while T cells expressing class I–restricted TCRs are generally selected to the CD8 lineage (von Boehmer and Fehling 1997). The intrathymic developmental process is determined by positive- and negative-selection events shaping the pre-immune T cell repertoire. Positive selection promotes survival of thymocyte clones expressing TCRs binding to self-antigens presented on the MHC, whereas negative selection removes those with no or high-affinity binding to self-peptide MHC, because the latter could potentially cause autoimmune diseases (Starr *et al.* 2003). The selection takes place mainly in the medulla where T cells encounter with strongly activating self-ligands on haematopoietic and dendritic cells (Germain 1994, Biddison *et al.* 1982). Finally, properly selected mature T cells leave the thymus for peripheral lymphoid organs such as lymph nodes and spleen through the blood stream and rarely come back to their organ of origin (Sprent and Surh 2009). While recirculating between blood and peripheral tissue mature T cells are encountered to specific antigen and are induced to proliferate and differentiate into effector T cell subsets.

1.1.2 T cell subsets and their role in adaptive immune system

$CD4^+$ T cells play important roles in the cellular arm of the adaptive immune system. They participate in autoimmunity, asthma, and allergic responses as well as in tumor immunity. In addition, $CD4^+$ T cells promote humoral immunity by helping B cells in making antibody and, maintain $CD8^+$ T cells response as well as regulate

macrophage function. Depending on the particular cytokine milieu, naive $CD4^+$ T cells can differentiate upon T cell receptor (TCR) activation into one of several lineages of T helper cells, including Th1, Th2, Th17, and induced Tregs (iTregs) (Zhu *et al*. 2010a) (Figure 1).

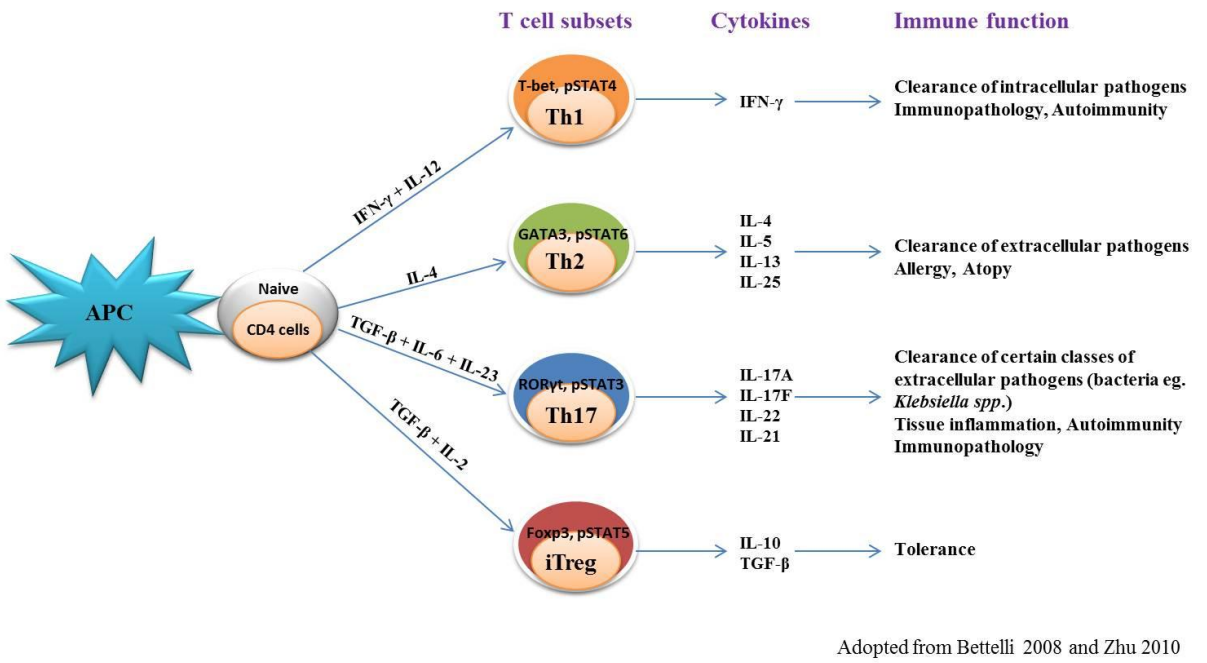


Figure 1: Overview of T cell subset differentiation, cytokine production and effector functions during adaptive immunity and pathologic conditions. Upon TCR activation driven by antigen presenting cells (APC), naïve $CD4^+$ T cells differentiate to distinct T cell subsets in the presence of corresponding cytokines and activation of transcriptional regulators and STAT proteins. T cell subsets perform their immune functions with their respective cytokines they produce.

Until few years ago, $CD4^+$ T cells were considered to be subdivided into two independent subsets, Th1 and Th2 (Mossmann and Coffman 1989). They could be distinguished mainly by the cytokines produced, but also through the expression of different patterns of cell surface molecules. Th1 cells produce large quantities of interferon (IFN)-γ, interleukin (IL)-2 and TNF and are predominantly involved in the clearance of intracellular pathogens through the activation of macrophages and induction of immunoglobulin class switching to complement-fixing antibodies. Th1 cells are also involved in cell-mediated and delayed-type hypersensitivity responses. The signature cytokines of Th2 cells are IL-4, IL-5, IL-9, IL-10, IL-13 and IL-25 and participate in the elimination of extracellular pathogens and parasites through the induction of immunoglobulin class switching to IgG1 and IgE (Mossmann *et al*. 1989, Fort *et al.* 2001). Atopic disorders are associated with elevated levels of allergen-

specific IgE in the serum. Recent reports indicate that enhanced allergen-specific IgE production in atopic disease results from selective activation of allergen specific Th2 cells producing IL-4 but little or no IFN-γ (Kapsenberg *et al.* 2002).

The differentiation of Th1 cells is initiated by activation of T cells in the presence of IFN-γ which is secreted by already differentiated Th1 cells, NK and NKT cells and IL-12, which is mainly produced by monocytes and dendritic cells, and results in the activation of STAT-4 and STAT1 and the Th1 specific transcription factor T-bet (Dardalhon *et al.* 2008a). T-bet is a member of the T-box family of transcription factors and is considered to be the master regulator of Th1 differentiation. Subsequently, T-bet induces the production of IFN-γ and the activation of the transcription factor H2.0-like homeobox (Hlx) and Runx3 (Wilson *et al.* 2009). TCR-signalling represses the up-regulation of the IL12Rβ2 subunit in an NFAT-dependent manner (Afkarian *et al.* 2002). The termination of TCR-signalling finally allows the up-regulation of IL12Rβ2. As a consequence, STAT4 activation through IL-12 signalling together with T-bet, Hlx and Runx3 activate the *ifnγ* locus and thereby positively enhances STAT1 signalling (Schulz *et al.* 2009). The ability of IFN-γ to stimulate T-bet expression and the ability of T-bet to enhance IFN-γ transcription sets up a positive feedback loop which drives differentiation of T cells towards the Th1 phenotype. The stability of the phenotype is further enhanced by the cooperation of Runx3 with T-bet in silencing of the *il4* gene in Th1 cells by binding to the *il4* silencer and by binding to the *ifnγ* promoter to further promote IFN-γ production (Djuretic *et al.* 2007, Naoe *et al.* 2007).

The differentiation of Th2 cells is induced by IL-4 provided by mast cells, basophils, NKT cells, eosinophils or previously differentiated Th2 cells and regulated by the Th2 specific transcription factor GATA-3 and activation of STAT-6 (Dardalhon *et al.* 2008b). Gata3 is a transcription factor that acts as a master regulator of Th2 differentiation, enhancing expression of IL-4, IL-5 and IL-13, which are located in the same genetic locus. Gata3 induces the transcription of the long form of viral musculoaponeurotic fibrosarcoma oncogene homolog (c-MAF), which additionally helps to activate *il4* transcription (Kurata *et al.* 1999, Ouyang *et al.* 2000). This activation results in a strong autocrine feedback loop that activates *il4, il5* and *il13*. Furthermore, IL-4 appears to repress IL-12 signalling through inhibition of IL12Rβ2

expression, thus antagonizing Th1 differentiation and stabilizing the Th2 phenotype (Szabo *et al.* 1997).

Th1 cells were also described to be the pathogenic subset in experimental autoimmune encephalomyelitis (EAE), an autoimmune disease of the central nervous system (CNS) (Sospedra and Martin 2005) and in diabetes of NOD mice (Nicholson *et al.* 2006). Th1 specific cytokines such as IFN-γ are present in CNS inflammatory lesions at the peak of EAE but decrease during remission. T helper cells invading the CNS at the peak of EAE express IFN-γ (Ben-Nun *et al.* 1981; Pettinelli *et al.* 1981, Renno *et al.* 1995). However, IFN-γ KO mice develops EAE (Ferber *et al.* 1996), which indicates that IFN-γ has a paradoxical effect on EAE. One reason might be the failure of encephalitogenic $CD4^+$ T cells to convert into $CD4^+CD25^+Foxp3^+$ Tregs in IFN-γ KO mice during EAE (Wang *et al.* 2006b). In the same study it was shown that reduced suppression activity of Tregs was also detected in IFN-γ KO mice during EAE.

IL-12, a key cytokine in the development of Th1 cells, is a heterodimeric cytokine composed of two subunits, p35 and p40. The of the role of Th1 cells in autoimmunity was challenged when it was shown that IL-12p35-/- mice were susceptible to EAE or collagen induced arthritis whereas IL-12p40-/- mice were resistant to the development of EAE (Gran *et al.* 2002, Becher *et al.* 2002, Cua *et al.* 2003, Murphy *et al.* 2003). It was shown later that the p40 subunit was shared with another cytokine, IL-23, which consists of p40 plus a unique subunit p19 (Oppman *et al.* 2000). Interestingly, loss of IL-12p40 or IL-23p19 results in complete resistance to EAE suggesting that it is IL-23 and not IL-12 that is necessary for the induction of EAE and potentially other autoimmune diseases (Cua *et al.* 2003). Later it was found that IL-23 could drive the expansion of an IL-17-producing T cell population subsequently termed Th17 cells, which could induce more severe EAE upon adoptive transfer than IL-12-driven Th1 cells (Langrish *et al.* 2005). Furthermore, when IL-23 is not available to maintain and expand a population of already primed Th17 cells, EAE is markedly attenuated (Cua *et al.* 2003, Veldhoen *et al.* 2006a).

Subsequently, interleukin 17 (IL-17) producing Th17 cells were classified as an additional effector $CD4^+$ T cell subset on the basis of their independence of the transcription factors GATA-3 and T-bet that, together with the marker cytokines IFN-γ and IL-4, define Th1 and Th2 cells, respectively (Harrington *et al.* 2005, Park *et al.*

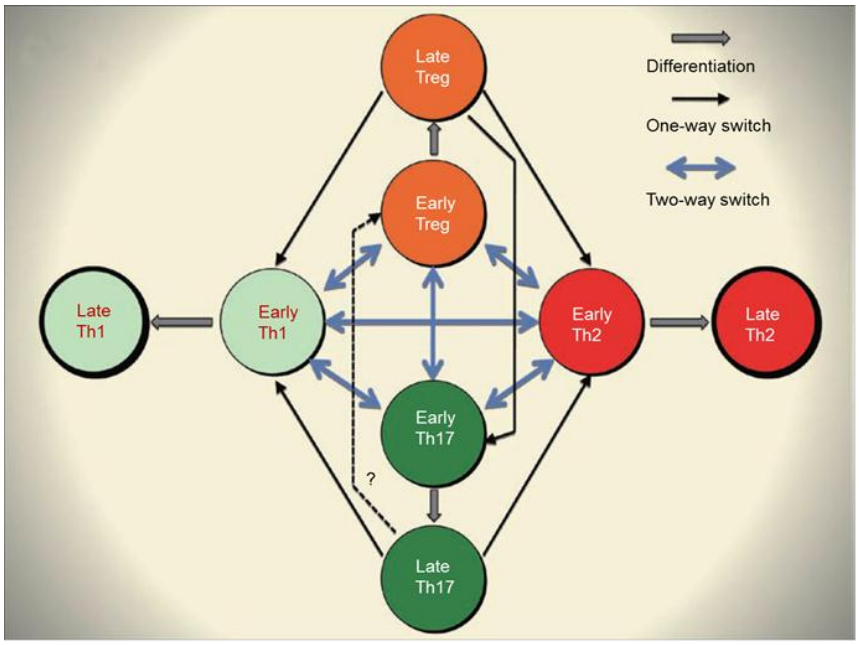
2005). Since IL-23 receptor is not expressed on naive T cells, IL-23 cannot act on naive T cells to induce their differentiation into Th17 cells. Indeed, three independent studies have demonstrated that the combination of IL-6, which is a proinflammatory cytokine together with TGF-β induces the differentiation of Th17 cells from murine naive T cells both in vitro and in vivo (Bettelli *et al.* 2006; Mangan *et al.* 2006, Veldhoen *et al*. 2006a). Contradictory data are reported regarding the necessity of TGF-β for human Th17 differentiation (Anne *et al.* 2008, Das *et al.* 2009). The differentiation is induced by transcription factors mainly ROR-γt, STAT3, IRF-4 (Korn *et al.* 2009) and many more. Th17 cells are characterized by the secretion of the cytokines IL-22, IL-21, IL-17A and IL-17F (Littman and Rudensky 2010).

Regulatory T cells are, a subset of $CD4^+$ T cells which express the IL-2 receptor α-chain (CD25) (Sakaguchi *et al.* 1995). Tregs also express increased levels of CD5 and cytotoxic T-lymphocyte antigen 4 (CTLA4) in resemblance of activated T cells (Takahashi *et al.* 2000, Sakaguchi *et al.* 1985). Being consistent with these findings, more research was conducted to find the genetic mechanisms underlying differentiation and function of Treg cells. These studies were facilitated by the discovery of the X chromosome encoded transcription factor Foxp3 and its loss of-function mutations in humans leading to a severe multi-organ autoimmune and inflammatory disorder immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) and similarly devastating widespread lesions in the scurfy mouse mutant strain (Brunkow *et al.* 2001, Bennett *et al.* 2001, Wildin *et al.* 2001, Chatila *et al.* 2000). Upon activation, naïve T cells are transformed to Tregs by TGF-β induced Foxp3 transcription (Chen *et al.* 2003, Fu *et al.* 2004). These Tregs control homeostasis of peripheral $CD4^+$ T cells (Almeida *et al.* 2002, Banz *et al.* 2003).

1.1.2.1 Trans-differentiation of T cell subsets

Even if different $CD4^+$ T cell subsets are classified based on the different effector cytokines they produce, they can also secret some common cytokines e.g. IL-2, IL-9 and IL-10. In addition, the pattern of cytokine secretion may switch from one lineage towards another under certain circumstances, suggesting that T helper cells are plastic. Deleting Gata3 from Th2 cells allows the production of IFN-γ (Zhu *et al.* 2004). After 2-3 rounds of stimulation, Th2 cells fail to produce INF-γ (Zhu *et al*.

2010b). The plasticity of Th1/Th2 cells seems to depend on their differentiation state (Murphy *et al.* 1996). Therefore, although fully differentiated Th1 cells cannot turn on IL-4 expression, Th1 cells primed *in vitro* for one round are able to produce IL-4 when they are switched to Th2 culture conditions (Zhu *et al*. 2004). When TGFβ is given to Th2 cells, IL-4 production is suppressed, while IL-9 is induced (Veldhoen *et al*. 2008, Dardalhon *et al*. 2008b). On the other hand, reduction of FoxP3 in Tregs renders them able to gain a Th2 phenotype (Wan *et al.* 2007). Gfi1 deletion from Th2 cells results in active epigenetic modifications at Th17- and iTreg-related gene loci, including Rorc, Il23r, and CD103 (Zhu *et al.* 2009). Tregs cultured under Th1 conditions gain the capacity to produce IFN-γ (Wei *et al.* 2009). Tregs can also be converted to pathogenic memory T cells in vivo if the Foxp3 expression on Tregs is not stable (Zhou *et al.* 2009). Tregs can be self-induced to become IL-17-producing cells in the absence of TGFβ but with the help of IL-6 signalling (Xu et al. 2007). The presence of IL-17-producing $Foxp3^+$ cells, both in mice and humans is also reported (Lochner *et al.* 2008, Voo *et al.* 2009). Transferring Tregs into a lymphopenic host also results in downregulation of Foxp3 which results in IL-17 and IFN-γ producing effector cells (Komatsu *et al*. 2009). Soon it has become clear that Th17 cells have considerable plasticity and readily acquire the ability to produce IFN-γ in addition to IL-17 production or completely shut off IL-17 production in vitro (Annunziato *et al.* 2007, Bending *et al.* 2009, Lee *et al.* 2009, Martin-Orozco *et al.* 2009, Shi *et al.* 2008). Upon adoptive transfer of *in vitro* differentiated encephalitogenic Th17 cells from IL-17F-reporter-positive mice either to RAG-deficient (T and B lymphocyte deficient mice) or to wild type mice it was shown that these Th17 cells partially loose IL-17 expression and adopt a Th1 phenotype (Kurschus *et al.* 2010). However, none of the aforementioned cytokines produced by Th17 cells have been found to be mandatory for the development of EAE (Kreymborg *et al.* 2007, Hofstetter *et al.* 2005, Haak *et al.* 2009, McGeachy *et al*. 2007, Coquet *et al.* 2008, Sonderegger *et al.* 2008a). Recently it has been shown that the main factor of encephalitogenictiy of Th17 cells might be GM-CSF, a cytokine produced by Th17 cells and driven by ROR-γt (Sonderegger *et al*. 2008b, El-Behi *et al.* 2011, Codarri *et al*. 2011). A summary of the plasticity of T helper cells is shown in Figure 2.



Adopted from Zhu et al. 2010

Figure 2: Plasticity of T helper cell subsets. At the early stages, Th1 and Th2 can be transdifferentiated to other lineages, whereas the majority of Th17 and Treg cells remain plastic throughout their differentiation process. Transdifferentiation depends on the differentiation state, cell types and the cytokine milieu.

1.2 The Transforming growth factor-β (TGF-β)

In 1975, Holley discovered that the interplay between several polypeptide hormones and hormone-like growth factors which are present in tissue fluids largely controls the growth of normal cells. Using normal rat kidney fibroblasts, it was demonstrated that the growth factor cocktail actually consisted of two distinct polypeptide growth factors, coined transforming growth factor (TGF)- α and -β (Roberts *et al*. 1981, Anzano *et al.* 1983). TGF-α displayed mitogenic activity though, it later became clear that TGF-β served as a potent growth inhibitor in most other cell types (Roberts and Sporn 1990). TGF-β serves as the prototype for the large and still growing TGF-β superfamily, consisting of more than 30 members which include bone morphogenetic proteins (BMPs), activins, inhibins, anti-mullerian hormone (AMH) and growth and differentiation factors (GDFs) (Massague 1990).

Three homologous TGF-β isoforms are presents in mammals, TGF-β1, TGF-β2, and TGF-β3, encoded by different genes (Govinden and Bhoola 2003). Besides forming homodimers, it has been reported that heterodimers can also form between TGF-β1 and TGF-β2, and between TGF-β2 and TGF-β3 (Cheifetz *et al.* 1987, Ogawa *et al.*

1992). The three TGF-β isoforms affect TGF-β signalling in a rather similar and redundant way *in vitro,* but display different *in vivo* expression patterns and functions (Roberts and Sporn 1992). TGF-β1 is the predominant isoform in the immune system (Li *et al.* 2006a).

1.2.1 Control of TGF-β production and activation

TGF-β is synthesized as a precursor protein, which is inactive and cannot bind to TGF-β receptors until being activated. After released from cells, the precursor protein associates with latency-associated protein (LAP) and form a small inactive complex. In the extracellular matrix, this complex is bound by latent TGF-β-binding protein (LTBP), a component of the extracellular matrix that is necessary for the secretion and storage of TGF-β (Li and Flavell 2008). The latent TGF-β can be activated either by enzymatic proteolysis, executed by plasmin, integrin, or thrombin, or through a conformational change (Nunes *et al.* 1997, ten Dijke *et al.* 2007).

1.2.2 TGF-β super family signalling

Transmembrane proteins are receptors for polypeptide growth factors and are able to transduce the extracellular information across the plasma membrane into an intracellular signal. The TGF-β and related factors signal through a group of trans-membrane protein serine/threonine kinases known as the TGF-β receptor family (Figure 3). The signal initiated by the TGF-β superfamily ligands is transduced by type I and type II serine/threonine kinase receptors into the intracellular space. Type I and type II receptors exist as homodimers at the cell surface in the absence of ligands, yet have an inherent heteromeric affinity for each other (Greenwald *et al.* 2004). Most ligands bind with high affinity to the type II or type I receptor, while others bind efficiently only to heteromeric receptor combinations.

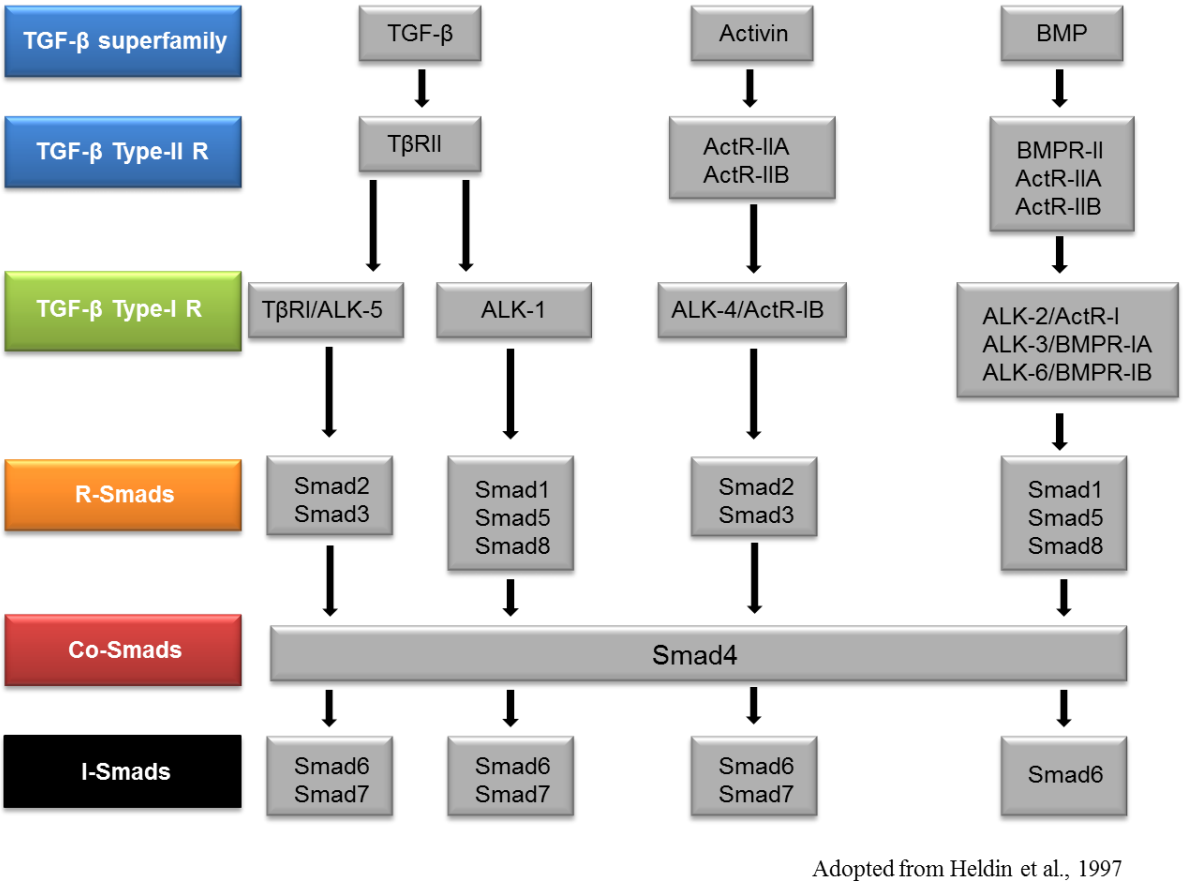


Figure 3: TGF-β superfamily members and their signalling molecules.

Interestingly, by using single-molecule microscopy to visualize TGF-βRII labelled with green fluorescent protein, it has been demonstrated that TGF-βRII is actually present on the cell surface as a monomer in the absence of TGF-β; TβRI also exists as a monomer on the cell surface in its non-active form, and TGFβ binding to TβRII results in the dimerization of both receptors (Zhang *et al.* 2009, 2010; Huang *et al.* 2011). Furthermore, TGFβ treatment causes a substantial increase in the number of dimeric receptors on the cell surface in a form of TβRII: TβRI heterodimers instead of TβRII2:TβRI2 heterotetramers (Huang *et al.* 2011).

The TGF-β superfamily ligands bind type II receptor, forming a heterodimeric complex which can recruit and activate the type I receptor by phosphorylating serine and threonine residues located primarily in the GS domain (Souchelnytskyi *et al.* 1996, Wrana *et al.* 1994). TGF-*β*1, TGF-*β*3 and activins bind efficiently to their respective type II receptors, T*β*RII and ActRII/ActRIIB, without the need for a type I

receptor though, the ligand contacts both receptor ectodomains to stabilize the type II-type I receptor complex (Boesen *et al.* 2002, Greenwald *et al.* 2004, Hart *et al.* 2002). TGF-β2 (Rodriguez *et al.* 1995), as well as BMP2 and BMP7, have affinity for both type I and II receptors, and associate with the receptor complex through co-operative binding (Massague 1998). The activated receptor can recruit downstream signalling molecules, known as Smad proteins.

1.2.2.1 The Smad family of signal transducers

Smad proteins, the only substrates for type I receptor kinases known to have a signalling function, were first identified as the products of the *Drosophila Mad* and *C. elegans Sma* genes, which lie downstream of the BMP-analogous ligand-receptor systems in these organisms (Patterson and Padgett 2000, Whitman 1998). Even earlier, in a genetic screen looking for enhancers of a weak decapentaplegic (dpp) maternal phenotype in Drosophila, a new gene mad (mothers against dpp) was isolated (Raftery *et al.* 1995, Sekelsky *et al.* 1995). This was followed by the discovery of three Mad14 homologues: sma-2, sma-3 and sma-4 in *C. elegans* (Savage *et al.* 1996). Mutations of these *sma* genes resembled the small body sized phenotype observed in the Daf4 mutants (type II serine/threonine receptor) of *C. elegans*. The vertebrate homologues of the *mad*-and *sma*-genes are called Smad proteins. Smad proteins are ubiquitously expressed throughout development and in all adult tissues (Flanders *et al.* 2001, Luukko *et al.* 2001). The proteins derived from these genes can be divided into three different subclasses**,** i) receptor activated Smad proteins (R-Smad proteins), ii) common mediator Smad proteins (Co-Smad proteins), and iii) inhibitory Smad proteins (I-Smad proteins) depending on their diverse roles in signalling (Figure 3). Smad proteins have two conserved domains, the N-terminal Mad homology 1 (MH1) and the C-terminal Mad homology 2 (MH2) domain**.** The MH1 domain is highly conserved among R-Smad proteins and Co-Smad proteins (Heldin *et al.* 1997). The R-Smad proteins, Smad2 and Smad3 mediate signals from TGF-β and activin ligands through the TβR-I/Alk-5 and ActR-IB receptors, respectively (Eppert *et al.* 1996, Macias-Silva *et al.* 1996, Zhang *et al*. 1996). BMP signalling is mediated through R-Smad proteins 1, 5 and 8 which become phosphorylated and activated by the ActR-I, BMPR-IA or BMPR-IB receptors (Thomsen 1996) (Figure 3). The determinant of specificity between the R-Smads and

their interaction with either TGF-β/activin or BMP receptors is the L3 loop region within the MH2 domain (Lo *et al.* 1998). However, some reports suggest that Smad1, 5, and 8 might be promiscuous towards the TGF-β receptors as well (Lux *et al.* 1999, Macias-Silva *et al.* 1998, Oh *et al.* 2000). The Co-Smad, Smad4 protein (also known as DPC4, deleted in pancreatic carcinomas), appears to play a critical role in both BMP- and TGF-β/activin-mediated pathways.

The affinity of Smad4 for R-Smad proteins can be increased through phosphorylation of the C-terminal part of R-Smad proteins (Souchelnytskyi *et al.* 1997). Moreover, Smad4 has a unique Smad activation domain (SAD) in the linker region, which governs transcriptional activation via the co-activator p300 (de Caestecker *et al.* 2000).

1.2.2.2 The canonical Smad signalling pathway

In the ligand induced complex, the type II receptor phosphorylates the GS domain and this activates the type I receptor, which catalyses R-SMAD phosphorylation. Phosphorylation decreases the affinity of R-Smad proteins for SARA (SMAD anchor for receptor activation) and increases their affinity for co-Smad proteins. The resulting R-Smad and co-Smad heteromeric complex is translocated to the nucleus where it regulates transcription of target genes (Lo *et al.* 1999) (Figure 4). Phosphorylation of the C-terminal serine residues in R-Smad proteins by type I receptor kinases is a crucial step in TGF-β family signalling (Abdollah *et al.* 1997, Macías-Silva *et al.* 1996, Souchelnytskyi *et al.* 1997). The two most C-terminal serine residues become phosphorylated and, together with a third, non-phosphorylated serine residue, form an evolutionarily conserved SSXS motif in all R-Smad proteins (Abdollah *et al.* 1997, Souchelnytskyi *et al*. 1997)**.** Substrate specificity is determined by the L45 loop in the type I receptors and by the L3 loop in the R-Smad MH2 domain. Thus, TGF-β and activin receptors phosphorylate Smad2 and Smad3, and BMP receptors phosphorylate Smad1, Smad5 and Smad8 (Chen *et al.* 1998). Smad3 and Smad4 have been shown to interact directly with specific DNA sequences via their MH1 domain (Dennler *et al.* 1998, Vindevoghel *et al.* 1998, Yingling *et al.* 1997). In order to fully activate transcription of the target promoters, the Smad protein complexes must recruit additional factors, like the transcription factor components AP-1 (Liberati *et al.* 1999), DNA-binding adaptors like FAST-1 (Chen *et al.* 1996), or co-activators

such as CBP/p300 (Feng *et al*. 1998, Janknecht *et al*. 1998, Nishihara *et al.* 1998, Shen *et al.* 1998, Topper *et al.* 1998). R-Smad proteins that move into the nucleus may return to the cytoplasm, but their ubiquitination - and proteasome-dependent degradation in the nucleus provides a way to terminate TGF-β responses (Lo *et al.* 1999).

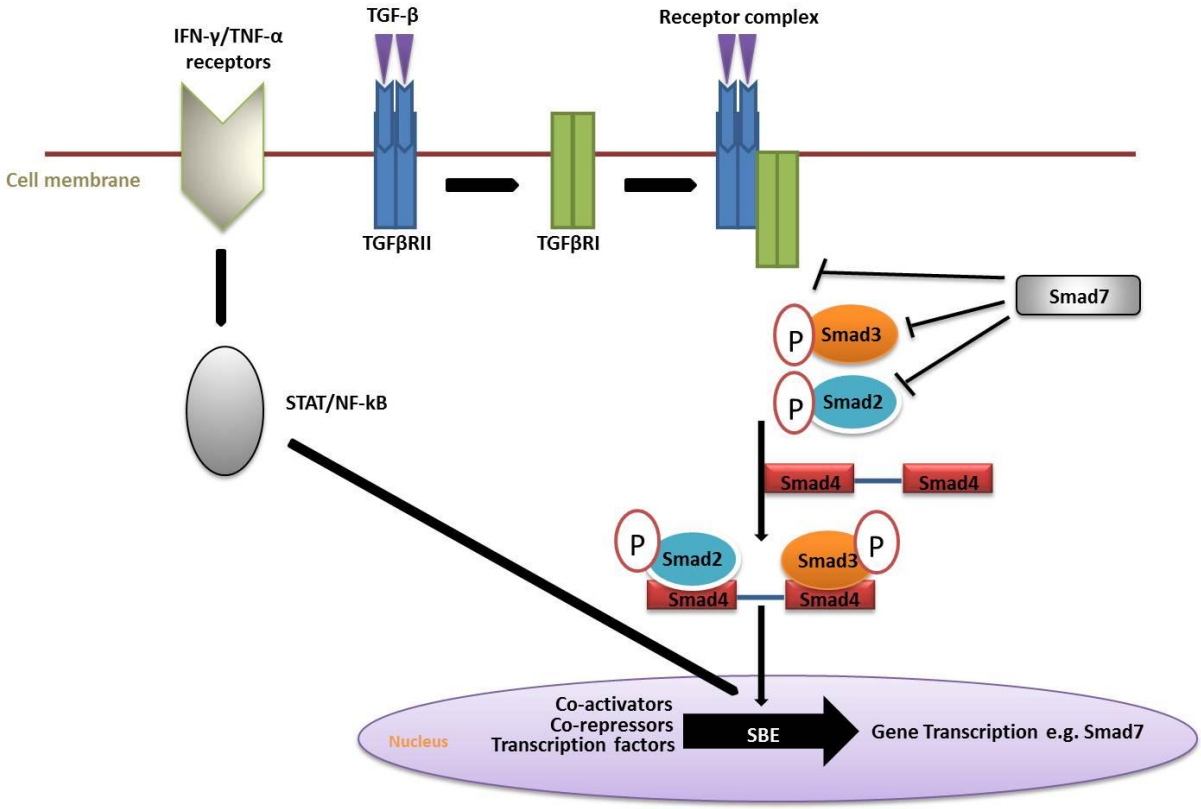


Figure 4: The TGF-β induced Smad signalling pathway and its inhibitor Smad7. Upon receptor ligand activation the TGF-β-receptor complex phosphorylates Smad2 and Smad3 which in turn form a heterodimeric complex with Smad4. This complex then translocates to nucleus to bind Smad binding element (SBE) and transcribes the target genes e.g. Smad7. Subsequently, in a negative feedback loop, Smad7 egresses from the nucleus to the cytoplasm and blocks the phosphorylation of Smad2 and Smad3. In addition, Smad7 breaks down TGF-βRI by lysozomal and proteosomal pathway. Smad7 can also be upregulated by IFN-γ and TNF-α through STAT and NF-kB pathway activation, respectively.

To date, two Inhibitory Smad (I-Smads) proteins have been identified in mammals, Smad6 and Smad7 (Imamura *et al.* 1997, Nakao *et al.* 1997, Topper *et al.* 1997). In contrast to R-Smad expression, the expression of the inhibitory Smad6 or Smad7 is highly regulated by extracellular signals. Although differentially controlled during development, R-Smad proteins and Smad4 are expressed in most, if not all, cell types (Massague 2000, Itoh *et al*. 2000, Moustakas 2001). Inhibitory Smad proteins have been characterised as inhibitors of TGF-β/activin and BMP signalling and have

been proposed to function in negative feed-back loops, since the expression of Smad6 and Smad7 is induced by TGF-β/activin and BMPmembers (Christian and Nakayama 1999). Accordingly, the downregulation of Smad6 and Smad7 expression during adipocyte differentiation may result from concomitant loss of autocrine TGF-β and BMP signalling (Choy *et al.* 2000).

Activation of the epidermal growth factor (EGF) receptor and possibly other tyrosine kinase receptors, interferon-γ signalling through STAT (signal transducer and activator of transcription) proteins, and activation of NF-kB by tumour-necrosis factor-α, also induce Smad7 expression, leading to inhibition of TGF-β signalling (Massague 2000, Itoh *et al.* 2000, Moustakas 2001).

It has been shown that I-Smad proteins can interact stably with the type I receptor and block further activation of R-Smad proteins (Imamura *et al.* 1997, Nakao *et al.* 1997, Souchelnytskyi *et al.* 1998). For Smad6, an additional mechanism has been suggested, where Smad6 competes with Smad4 for binding to Smad1, thereby preventing the formation of a functional heteromeric Smad1/Smad4 complex (Hata *et al.* 1998). Smad7 is considered as a general inhibitor of TGF-β superfamily-induced responses; whereas Smad6 is thought to preferentially block BMP mediated signalling (Itoh *et al.* 1998), although this is controversial (Imamura *et al.* 1997).

In addition, Smad7 antagonizes TGF-β signalling through other mechanisms. BAMBI (BMP and activin membrane-bound inhibitor) along with activated TβRI and Smad7 forms a ternary complex and synergizes with Smad7 to antagonize TGF-β signalling by interfering with the recruitment of R-Smad proteins (Yan *et al.* 2009). Smad7 also functions as an adaptor protein to induce the degradation of TβRI. Once TGF-β signalling is activated, Smad7 associates with Smurf1/2 in the nucleus, together they translocate into the cytoplasm, and Smad7 then binds to activated TβRI (Kavsak *et al.* 2000, Ebisawa *et al.* 2001, Suzuki *et al.* 2002). E2-conjugating enzyme UbcH7 (ubiquitin-conjugating enzyme 7), HSP90 (heat-shock protein 90) and two further HECT-type E3 ubiquitin ligases, NEDD4–2 and WWP1/Tiul1 may also enhance the Smad7/Smurf2-mediated ubiquitination and degradation of TβRI (Ogunjimi *et al.* 2005, Wrighton *et al.* 2008, Seo *et al.* 2004, Komuro *et al.* 2004, Kuratomi *et al.* 2005). In addition, Smad7 can engage the phosphatase GADD34 (growth-arrest and

DNA-damage-inducible protein 34) and PP1c (protein phosphatase 1c) to control the TβRI receptor activity (Shi *et al*. 2004). Besides, there are many other proteins which interact with Smad7 and regulate TβRI activity and/or stability, including STRAP (serine/threonine kinase receptor-associated protein), SIK (salt-inducible kinase), AIP4 (atrophin 1-interacting protein 4), YAP65 (Yes-associated protein 65), Cas-L (Crk-associated substrate lymphocyte type) and Hic5 (H2O2-inducible clone 5) (Datta *et al.* 2000, Kowanetz *et al.* 2008, Lallemand *et al.* 2005, Ferrigno *et al*. 2002, Inammoto *et al.* 2007, Wang *et al.* 2008). In some cell lines, including Hep3B, HeLa, Mv1Lu (mink lung epithelial cells), and human normal lung epithelial HPL-1 cells, Smad7 also antagonizes TGF-β signalling in the nucleus by interfering with the functional R-Smad proteins/Smad4–DNA complex formation on target gene promoters (Zhang *et al.* 2007). Smad7 may also affect the TGF-β target gene transcription by regulating the epigenetic status of chromatin since Smad7 is found to be able to associate with the histone deacetylases HDAC1 and SIRT1 and the acetyltransferase p300 (Gronroos *et al.* 2002, Simonsson *et al.* 2005, Kume *et al.* 2007). Arkadia promotes the degradation of Smad7 where Axin may act as an adaptor between Arkadia and Smad7, thus Arkadia enhances TGF-β signalling (Koinuma *et al.* 2003, Liu *et al.* 2006). Finally, Jab1/CSN5 regulates the stability of Smad7 and thereby controls TGF-β signalling (Kim *et al.* 2004a).

1.2.2.3 The alternative non-Smad signalling pathways

As of now, it has been well understood that the TGF-β pathway performs through its receptors kinases and intracellular Smad signalling (=canonical signalling pathway), though recent studies have shown that non-Smad pathways are also involved in signalling downstream of the TGF-β receptors (=non-canonical signalling pathway). Indeed, non-Smad signalling proteins which take part in the TGF-β signalling cascade were identified prior to the discovery of the Smad proteins (Yue and Mulder 2000).

There are three general mechanisms by which non-Smad signalling pathways are involved in physiological responses to TGF-β: (i) non-Smad signalling pathways directly modify (e.g. phosphorylate) the Smad proteins and thus modulate the activity of the central effectors; (ii) Smad proteins directly interact and modulate the activity of other signalling proteins (e.g. kinases), thus transmitting signals to other pathways;

and (iii) the TGF-β receptors directly interact with or phosphorylate non-Smad proteins, thus initiating parallel signalling that cooperates with the Smad pathway in eliciting physiological responses (Moustakas and Heldin 2005).

Non-canonical signalling cascades activated by TGF-β include the TGF-β-activated kinase-1 (Ono *et al.* 2003), Ras (Yue and Mulder 2001), various Rho proteins (Edlund *et al.* 2002, Mucsi *et al.* 1996), c-Jun NH2-terminal kinase (Engel *et al.* 1999 and Hocevar *et al.* 1999), extracellular signal-regulated kinase (Munshi *et al.* 2004), p38 (Kim *et al.* 2004b), and phosphatidylinositol 3-kinase (PI3K) (Chen *et al.* 1998, Horwitz *et al.* 2004, Bakin *et al.* 2000, Kim *et al.* 2004c) (Figure 5).

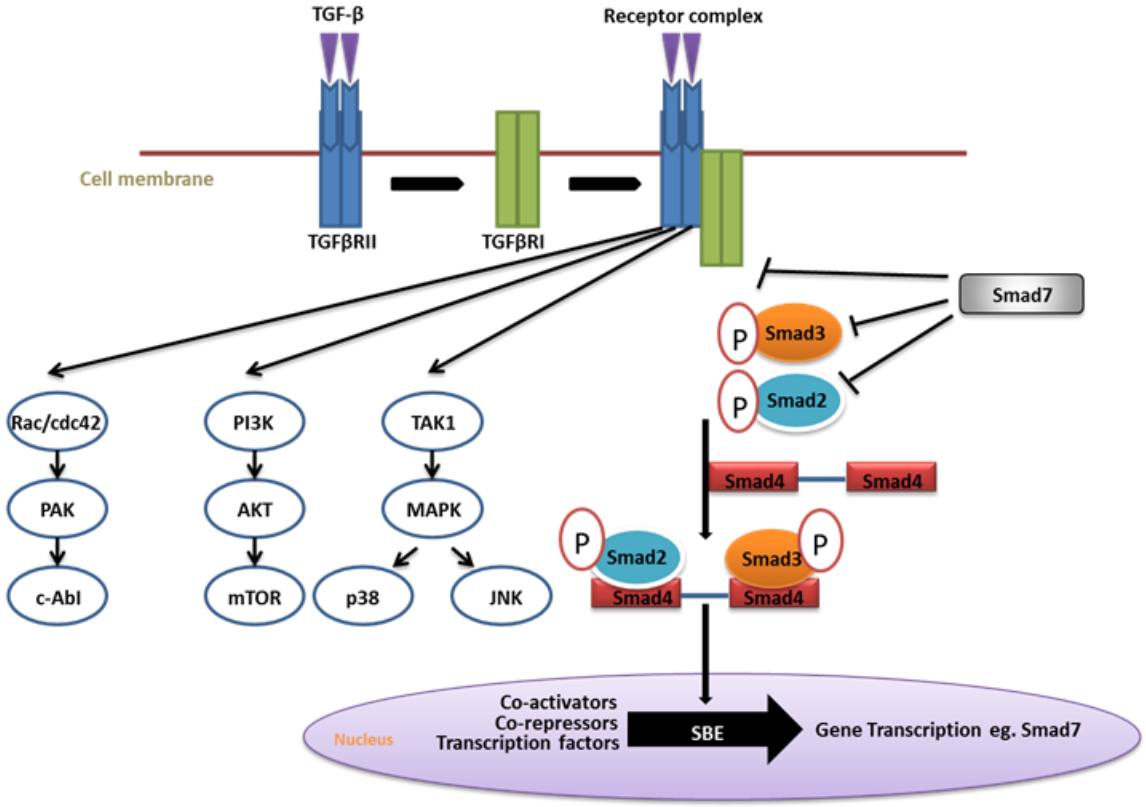


Figure 5: TGF-β signalling through Smad-independent pathways. Apart from the canonical TGF-β-Smad pathway, TGF-β can also activate other pathways like Rac/cdc42, PI3K/AKT/mTOR, and TAK1/MAPK etc. to accomplish its cellular functions. Well defined pathways are shown only.

Activation with slow kinetics in some cases may result from Smad-dependent transcription responses, but the rapid activation (5–15 min) in other cases suggests independence from transcription (Massague 2000). Studies using Smad4-deficient cells, or dominant-negative Smad proteins, support the possibility of MAPK pathway activation that is independent from Smad proteins (Engel *et al.* 1999). In addition,

mutated TGF-β type I receptors, defective in Smad activation, activate p38 MAPK signaling in response to TGF-β (Yu *et al.* 2002). The mechanisms of ERK, JNK or p38 MAPK activation by TGF-β and its biological consequences are poorly characterized. Rapid activation of Ras by TGF-β in epithelial cells may implicate Ras in TGF-β-induced ERK-MAPK signaling (Yue *et al.* 2000). The rapid guanosine 5'-triphosphate loading of Ras in response to TGF-β in epithelial cells may cause recruitment of Raf, a MAP kinase kinase kinase (MAP3K), to the plasma membrane and lead to activation of ERK through MEK1. Subsequently, rapid activation of ERK by TGF-β was observed in epithelial cells (Hartsough *et al.* 1995), breast cancer cells (Frey *et al.* 1997), and fibroblasts (Mucsi *et al*. 1996). The canonical TGFβ-Smad signalling pathway leading to growth inhibition is inhibited by the ERK-MAPKs, as they both negatively affect the activities of the transcription factors that cooperate with the Smad proteins (Feng and Derynck 2005, Lo *et al.* 2001) and the nuclear translocation of the Smad proteins, by phosphorylation of their linker regions (Guo and Wang 2009, Kretzschmar *et al.* 1997). The tyrosine kinase activity of TβRII suggests that the receptor by itself could promote non-Smad signalling pathways. Moreover, the expression levels and ratio of the TβRII/TβRI heterooligomers might be important for the determination of the downstream specificity of the activated TβRII/TβRI complex (Huang *et al.* 2011; Zhang *et al.* 2009, 2010). TGFβ selectively activates ERK1/2 in certain cell types and provides direct evidence for TβRI-independent TβRII signalling to a R-Smad-independent pathway. In dermal cells, high TβRII expression levels selectively activate Erk1/2 but not in epidermal cells since the expression levels of TβRII are 7- to 18-folds higher in dermal cells than in epidermal cells. Upregulation of TβRII expression in epidermal cells to a similar level as that in dermal cells switches TGFβ-induced ERK1/2 inhibition to ERK1/2 activation. Knockdown of TβRI/Alk5 does not block activation of ERK1/2, in dermal cells. Higher expression of TβR1 in epidermal cells shows no change in ERK activation (Bandyopadhyay *et al*. 2011). In addition, ERK substrates, such as AP-1 family members, can interact and function in conjunction with Smad proteins to regulate gene expression (Davies *et al.* 2005, Zhang *et al.* 1998, Hall *et al.* 2003). Probably the best-characterized non-Smad pathways are the JNK and p38 MAPK signaling cascades. TGFβ-induced activation of the p38 and JNK MAPK pathways has been implicated in the regulation of apoptosis, cell migration, and the Epithelial-

mesenchymal transition (Adhikari *et al.* 2007, Heldin *et al.* 2009, Sorrentino *et al*. 2008, Yamashita *et al.* 2008). Like ERK, JNK and p38 are activated by TGF-β through the MAP kinase kinases (MKKs), specifically MKK4 and MKK3/6, respectively (Weston *et al.* 2007, Frey *et al*. 1997, Engel *et al.* 1999, Hocevar *et al.* 1999, Hanafusa *et al.* 1999, Sano *et al.* 1999, Bhowmick *et al.* 2001, Yu *et al.* 2002). Experiments with a dominant-negative form of Smad3 or using Smad3- or Smad4-deficient cells show that Smads are dispensable for the TGF-β-induced activation of JNK (Engel *et al.* 1999, Hocevar *et al.* 1999), suggesting that the MAPK pathway is activated by TGF-β independently of Smad proteins. A direct demonstration of Smad independence was based on the utilization of a mutant TβRI receptor with an altered L45 loop (A nine amino acid sequence between kinase subdomains IV and V in type I receptors), which renders the receptor defective in Smad binding and activation, but allows an intact kinase activity. This mutant type I receptor is still capable of mediating TGF-β-induced activation of JNK and p38 MAPK (Yu *et al.* 2002, Itoh *et al.* 2003). TGF-β-induced TGF-β-activated kinase1 (TAK1) activation has recently been shown to cause activation of the NFκB pathway in osteoclasts, leading to the transcription of pro-survival genes (Gingery *et al*. 2008). TAK1 is a critical activator upstream of p38 in osteoblasts (Greenblatt *et al*. 2010). TAK1 regulates cell survival, differentiation, and inflammatory responses through its activity on p38, JNK and components of the NFκB pathway, thus regulating a number of specific transcription factors (Adhikari *et al.* 2007, Rincon and Davis 2009, Wagner and Nebreda 2009). TAK1 is shown to be absolutely required for TGF-β-induced JNK and NFκB activation by using TAK1-deficient mouse embryonic fibroblasts (Shim *et al.* 2005). Smad7 also facilitates TGF-β-induced activation of the p38 and JNK MAPK pathways by inhibiting the TGF-β-Smad signalling cascade (Kamiya *et al.* 2010, Yan and Chen 2011).

TGF-β can also activate phosphatidylinositol-3-kinase (PI3K), as indicated by phosphorylation of its effector AKT (Bakin *et al.* 2000, Vinals *et al.* 2001). This mechanism appears to be independent of Smad2/3 activation (Wilkes *et al.* 2005). Both TβRII and TβRI appear to be required for the activation of the PI3K pathway, and TβRI has also been found to associate with the p85 subunit of PI3K (Yi *et al.* 2005). In addition, TGF-β may also induce activation of PI3K indirectly through TGF-β-induced TGF-α expression and subsequent activation of EGF receptor signalling

(Vinals and Pouyssegur 2001). On the other hand, TGF-β is shown to down-regulate PI3K/AKT signalling activity through Smad-dependent expression of the lipid phosphatase src homology 2-containing inositol phosphatase (SHIP) in haematopoietic cells (Valderrama *et al.* 2002). The recent identification of TGFβ-induced regulation of the mammalian target of rapamycin (mTOR) pathway suggests that TGFβ utilizes this pathway to regulate cell survival, metabolism, migration, and invasion (Lamouille and Derynck 2007, 2011). The Rho-like GTPases, including RhoA, Rac and Cdc42, play important roles in rapid regulation of the cytoskeleton in cells, cell motility, and gene expression through a variety of effectors (Jaffe and Hall 2005). TGF-β rapidly activates RhoA-dependent signalling pathways to induce stress fiber formation and mesenchymal characteristics in epithelial cells and primary keratinocytes (Bhowmick *et al.* 2001, Edlund *et al.* 2002). The rapid activation of RhoA induced by TGF-β is likely to be independent of Smad2 and/or Smad3, as suggested by the rapid onset and the inability of a dominant-negative Smad3 mutant to block RhoA activities in epithelial cells (Bhowmick *et al.* 2001). Besides RhoA, TGF-β can also induce activation of the Cdc42 GTPase. Activation of Cdc42 by TGF-β appears to be independent of Smad proteins, because blocking either Smad2 or Smad3 phosphorylation, or both simultaneously, does not affect activation of the p21-activated kinase (PAK) 2, which acts downstream of Cdc42 (Wilkes *et al.* 2003). Smad7 appears to be required for TGF-β-mediated Cdc42 activation (Edlund *et al.* 2004), but whether Smad7 works here as tight junction accessory protein is not known.

1.3 Regulation of T cell mediated immunity by TGF-β

Mice deficient in TGF-β1 develop a multiorgan autoimmune inflammatory disease and die a few weeks after birth (Shull *et al.* 1992, Kulkarni *et al.* 1993). Various transgenic mice whose T cells are unstable to respond specifically to TGF-β1 have also been shown to develop autoimmunity, indicating that TGF-β1 signalling is essential for T cell homeostasis (Gorelik *et al.* 2000a, Marie *et al*. 2006, Li *et al.* 2006b). The pathology in Tgfbr2-conditional-knockout mice was accompanied by the early activation and expansion of $CD4^+$ and $CD8^+$ T cells. (Li *et al.* 2006b, Marie *et al.* 2006). The pleiotropic cytokine TGF-β plays a critical role in thymic T cell development, in peripheral T cell homeostasis, tolerance to self-antigens and T cell

differentiation during cell mediated immune regulation (Li and Flavell 2008). TGF-β controls inflammatory responses through the regulation of chemotaxis, activation, and survival of lymphocytes, natural killer cells, dendritic cells, macrophages, mast cells, and granulocytes (Li *et al.* 2006a). In addition, it also exerts immunosuppressive functions (Becker *et al.* 2006). TGF-β affects T cell proliferation, and survival (Gorelik *et al.* 2002a, Cerwenka *et al.* 1999). The effect of TGF-β on T cells is context dependent since many regulatory signals, including co-stimulatory molecules and inflammatory cytokines, influence TGF-β regulation on T cell function (Li *et al*. 2006a).

1.3.1 TGF-β signalling during T cell proliferation and T cell survival

TGF-β inhibits T cell proliferation *in vitro* (Kehrl *et al.* 1986), by inhibiting the expression of IL-2 through the suppression of IL-2 transcription (Brabletz *et al.* 1993). In contrast, TGF-β co-stimulated splenic T cells proliferate in the presence of immobilized anti-CD3 antibody. This bi-functional capability of TGF-β on T cell growth regulation is largely controlled via IL-2 and IL-4 independent pathways (Lee and Rich 1991). TGF-β inhibits TCR-stimulated proliferation of naive T cells. However, in the presence of CD28, TGF-β inhibits T cell apoptosis and promotes T cell expansion (Sung *et al.* 2003, Gunnlaugsdottir *et al.* 2005). The antiapoptotic effect of TGF-β is associated with reduced c-myc expression that results in reduced levels of FasL (Genestier *et al.* 1999). IFN-γ participates in T cell Activation Induced Cell Death by inducing caspase 8 expression (Refaeli *et al.* 2002) and TGF-β downregulates IFN-γ expression in response to TCR stimulation which results in reduced apoptosis (Bommireddy *et al.* 2003).

1.3.2 TGF-β and T helper cell differentiation

Upon activation, naive $CD4^+$ T cells differentiate under polarizing conditions into various helper T cells subsets (Murphy and Reiner 2002). TGF-β inhibits Th1 and Th2 differentiation in vitro. In particular, TGF-β inhibits Th2 development via inhibition of IL-4 and/or GATA-3 expression (Gorelik *et al.* 2000b, Heath *et al.* 2000). The effects of TGF-β on Th1 development are less clear. Some reports have described TGF-β inhibits Th1 development by suppressing T-bet expression (Gorelik *et al.*

2002b), others have described that TGF-β enhances Th1 differentiation (Lingnau *et al.* 1998, Smeltz *et al*. 2005).

A combination of TGF-β and IL-6 results in upregulation of expression of the transcription factor ROR-γt as well as Th17 differentiation (Ivanov *et al.* 2006). A recent study indicates that Th17 cells also produce TGF-β *in vivo* and that this TGF-β plays a role to maintain Th17 cells in an autocrine manner (Gutcher *et al.* 2011). TGF-β also plays a role in the generation and expansion of Tregs (Horwitz *et al.* 2003). There are mainly two types of Tregs, e.g. natural Tregs and inducible Tregs. The differentiation of naturally occurring $CD4^+Foxp3^+$ Treg (nTreg) cells in the thymus is regulated by TCR affinity. Experiments with TCR transgenic mouse models reveal that engagement of agonist self-peptides induces not only T cell negative selection but also nTreg cell differentiation (Apostolou *et al.* 2002, Jordan *et al.* 2001, Kawahata *et al.* 2002, Walker *et al.* 2003). Mechanism by which TGF-β controls nTreg cell differentiation and homeostasis remain poorly understood. Studies with mice with T cell-specific deletion of the TGF-β type II receptor (TGFβRII) gene show that TGF-β signalling in T cells is dispensable for the development of nTreg cells in 12- to 16-day-old mice (Li *et al*. 2006b, Marie *et al.* 2006). However, another study reveals an earlier requirement for TGF-β signalling in nTreg cell development. Conditional deletion of the TGF-β type I receptor (Tgfbr1) gene in T cells blocks thymic nTreg cell differentiation in 3- to 5-day-old mice but triggers nTreg cell expansion in mice older than 1 week (Liu *et al*. 2008). It is postulated that TGF-β signalling is required for the induction of Foxp3 gene expression and nTreg cell lineage commitment in neonatal mice similar to iTreg cells (Liu *et al*. 2008). A recent report shows that TGF-β signalling is not necessary for nTreg cell lineage commitment; rather TGF-β promotes nTreg cell survival by antagonizing T cell negative selection (Quyang *et al.* 2010).

Some of the early evidence of peripheral conversion of naïve conventional $CD4^+$ T cells into $Foxp3^+$ T cells originated from adoptive transfer experiments in which polyclonal $CD4^+CD25^+$ naïve T cells were injected into lymphopenic mice or mice containing a monoclonal T cell repertoire devoid of nTreg cells (Curotto de Lafaille *et al.* 2004, Furtado *et al.* 2002). TGF-β converts naïve $CD4^+CD25^-$ precursors to T regulatory cell by inducing Foxp3 transcription (Chen *et al.* 2003, Fu *et al.* 2004). The

mechanism by which TGF-β induces transcription of Foxp3 involves cooperation of the transcription factors STAT3 and NFAT at a Foxp3 gene enhancer element (Fantini *et al*. 2004, Josefowicz and Rudensky 2009).

Numerous *in vitro* studies of both human and mouse $CD4^+$ T cell differentiation suggest the existence of two additional types of $CD4^+$ T cell that have suppressive properties, these cells are known as Tr1 and Th3 cell, which produce IL-10 and TGF-β1, respectively (Thomas *et al*. 2005, Roncarolo *et al*. 2006). It has been reported that TGF-β1 facilitates the differentiation of both Tr1 and Th3 cells (Levings *et al.* 2002, Weiner 2001). A summary of the effects of TGF-β in T helper cell differentiation and homeostasis is shown in Figure 6.

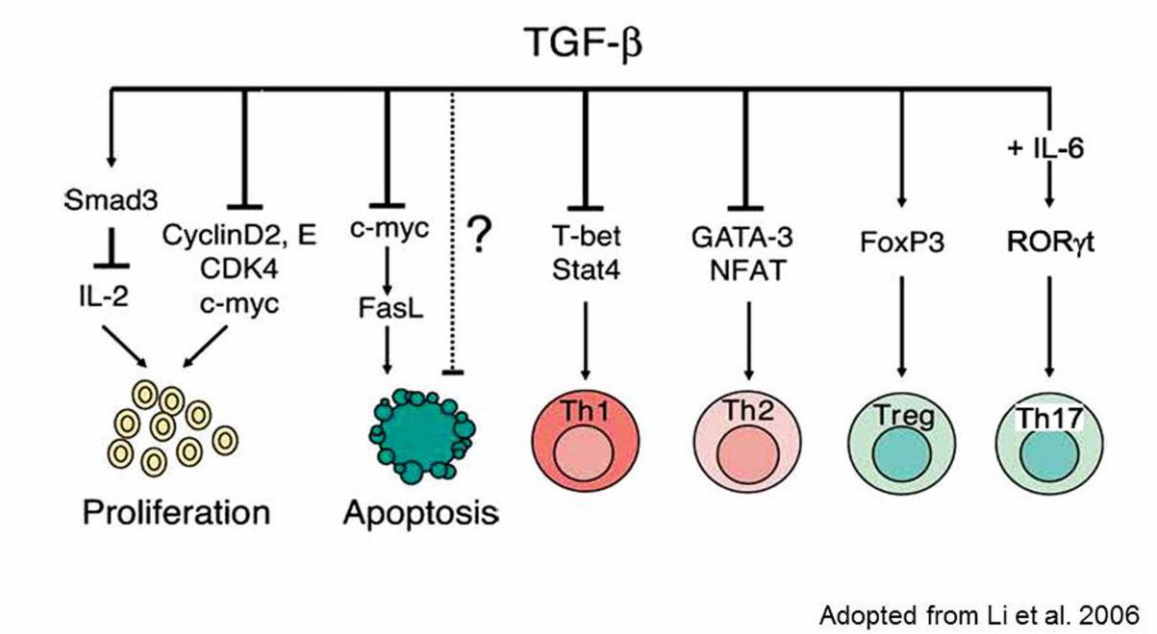


Figure 6: Effects of TGF-β on T cell differentiation and homeostasis. TGF-β inhibits T cell proliferation by blocking IL-2 production via Smad3 and blocking the function of Cyclin E, and c-myc. TGF-β blocks activation induced cell death by inhibiting c-myc induced FasL expression and through other mechanisms yet to be found. TGF-β inhibits Th1 and Th2 differentiation by blocking T-bet/STAT4 and GATA3/NFAT transcription. TGF-β induces Treg and, together with IL-6, Th17 differentiation by enhancing Foxp3 and ROR-γt transcription respectively.

1.3.2.1 Smad dependent and independent regulation of T helper cell differentiation by TGF-β

As mentioned earlier, TGF-β signals through both Smad dependent and independent pathways. Accumulating reports indicate that TGF-β is required to orchestrate T cell immunity, but whether various TGF-β–mediated effects on T cells are equally dependent on Smad signalling is not well understood. Evidences reveal that Smad3 is essential for the suppressive effect of TGF-β on IL-2 production and T cell proliferation (McKarns *et al.* 2004). Smad3 is also required for the suppressive effects of TGF-β on Th2 type cytokine productions and Th2 type disease in the skin (Anthoni *et al*. 2008). Smad2-KO mice are embryonic-lethal (Nomura *et al.* 1998), and Smad3-KO mice exhibit inflammatory diseases (Yang *et al.* 1999). T-cell-specific Smad2 conditional KO mice show unexpected overlapping functions of Smad2 and Smad3 in TGF-β-induced Foxp3 induction as well as Treg suppression (Takimoto *et al.* 2010). Smad2/Smad3-double KO mice, but not single KO mice, develop fatal inflammatory diseases, with higher IFN-γ production and reduced Foxp3 expression in $CD4^+$ T cells in the periphery (Takimoto *et al.* 2010). TGF-β mediated induction of Foxp3, as well as suppression of IFN-γ and IL-2 is partially impaired in Smad2- and Smad3-deficient T cells, and is completely eliminated in Smad2/3-double KO T cells (Takimoto *et al.* 2010). By using T cell specific Smad2 and Smad3 conditional knockout mice, recent studies reveal that neither Smad2 nor Smad3 alone are sufficient for the differentiation of Th17 cells or Th17 cell mediated EAE (Lu *et al.* 2010). p38 (Lu *et al.* 2010, Noubade *et al.* 2011) and ERK MAPK pathways which are independent of TGF-β-Smad cascade, positively regulate Th17 differentiation as well as modulate EAE and iTreg differentiation, respectively (Lu *et al.* 2010). It was also reported that ERK signalling negatively regulates Th17 development (Tan and Lam 2010).

1.4 Role of TGF-β and Smad7 in experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is a T cell-mediated demyelinating disease of the CNS that is frequently used as a model for the human disease multiple sclerosis (Sospedra and Martin 2005). EAE can be induced in susceptible mice by adoptive transfer of myelin-reactive $CD4^+$ T cells or by

immunization with myelin antigens. The course of EAE can be subdivided into an initiation stage involving activation and expansion of myelin-specific T cells in the periphery, which then cross the blood brain barrier (BBB), an effector stage involving re-activation of myelin-specific T cells in the CNS, resulting in cytokine-induced chemokine expression in the CNS-resident cells and a stage of remission and repair in which the immune response is down-regulated (McFarland and Martin 2007, Steinman 2001). Early studies show that administration of exogenous TGF-β1 to mice or treatment of myelin basic protein (MBP)-specific T cells with TGF-β is able to prevent or inhibit EAE (Johns *et al.* 1999, Racke *et al.* 1999, Kuruvilla *et al*. 1991). Increased expression of TGF-β1 mRNA or protein is associated with remission of the disease (Racke *et al.* 1992, Issazadeh *et al.* 1995). Administration of neutralizing antibody to TGF-β also enhances the clinical severity of the disease (Johns *et al.* 1993). TGF-β1 functions as an effector cytokine of Th3 cells which secrete high amounts of TGF-β1 and protect mice from EAE. Anti-TGF-β1 antibody treatment abrogates the protection (Chen *et al.* 1994). In T cells, TGF-β1 induces both Foxp3 and ROR-γt in the presence of IL-2 and IL-6, respectively. IL-6, as a pro-inflammatory cytokine inhibits Foxp3 expression, whereas $Foxp3^+$ Treg cells are increased in IL-6 knockout mice, which are resistant to EAE induction (Tang *et al.* 2004, Bettelli *et al*. 2006, Korn *et al.* 2008), indicating that TGF-β1 decreases the severity of the disease by upregulating Foxp3 expression in the absence of IL-6. Deletion of the *Tgfb1* gene from activated T cells and Treg cells, but not Treg cells alone, abrogates Th17 cell differentiation which results in almost complete protection from EAE. In this model, it was shown that Th17 cells also produce TGF-β *in vivo* and that this TGF-β plays a role to maintain Th17 cells in an autocrine manner which helps to maintain the disease activity (Gutcher *et al.* 2011). Studies with CD4dnTGFbRII showed that TGFβ is required for Th17 differentiation and EAE induction (Veldhoen *et al.* 2006b). TGF-β production by CNS resident glial cells induces TGF-β signalling in neurons and in inflammatory T cells which results in earlier onset of EAE (Luo *et al.* 2007). Although it has been shown that TGF-β has paradoxical effects in EAE, the overall effect of TGF-β seems to be immunosuppression. As the cytokine TGF-β directly regulates the differentiation, maintenance and the function of effector T and regulatory T cells and also their cytokine production, a context-dependent regulation of TGF-β signalling is indispensable to control EAE. Smad7 can be a suitable

candidate since Smad7 negatively regulates TGF-β signalling in a negative feedback loop. Smad7 expression is upregulated in the spinal cord of SJL/J mice and DA rats with EAE and systemic administration of Smad7-antisense oligonucleotides results in significantly milder disease course in these animals (Kleiter *et al.* 2007). Thus, the regulation of Smad7 expression is instrumental to study the role of TGF-β regulated T helper cell differentiation and their function in the context of EAE. To this end, mice with a T cell specific Smad7 deletion (CD4Cre-Smad7$^{fl/fl}$) were made, which showed a significantly reduced EAE disease onset as compared to control mice (Figure 7A). In the presence of TGF-β, activated T cells from CD4Cre-Smad7$^{fl/fl}$ mice showed enhanced TGF-β signalling in T cells with an upregulation of Smad2 phosphorylation (Figure 7B).

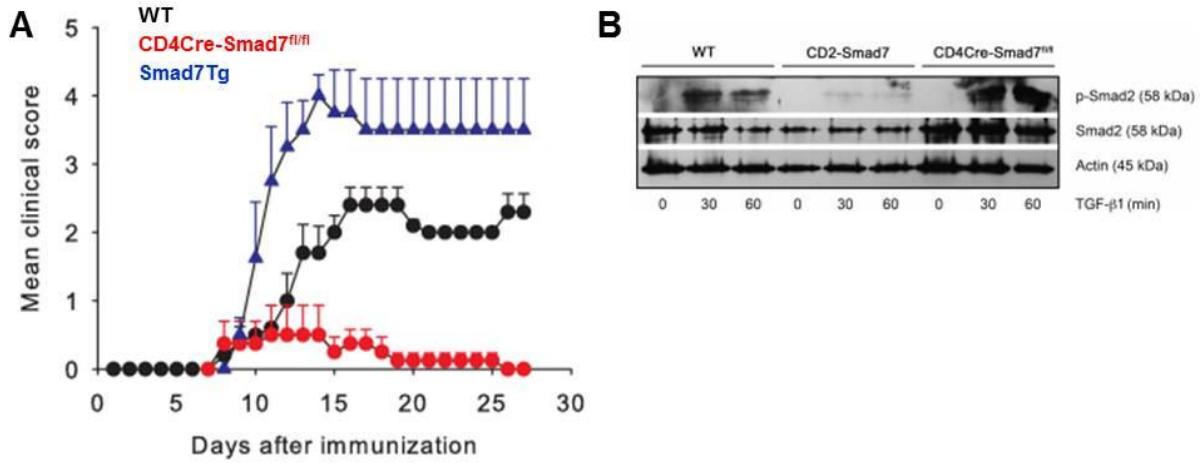


Figure 7: Reduced EAE disease course in CD4Cre-Smad7$^{fl/fl}$ mice and enhanced TGF-β signalling in CD4Cre-Smad7$^{fl/fl}$ T cells**.** (A) Clinical scores of MOG ($_{33–55}$)-induced EAE (n=5) of WT, CD4Cre-Smad7$^{fl/fl}$ and Smad7Tg mice. The difference in mean clinical scores for the CD4Cre-Smad7$^{fl/fl}$ and the control groups were statistically significant from Day 14 to 27 (P<0.05, ANOVA). Results are presented as mean values ±SEM and are representative of two different experiments. (B) MACS sorted naïve CD4^{+} T cells from WT, CD4CreSmad7$^{fl/fl}$ and Smad7Tg mice were stimulated with anti-CD3 and anti-CD28 in the presence of TGF-β1 (2ng/ml) for the indicated time points. Protein samples were prepared from stimulated cells and phosphorylation of Smad2 was checked at different time points by immunoblotting (Kleiter *et al.* 2010).

2. Objectives

Mice with a T cell-specific Smad7 deletion show a significantly reduced phenotype of experimental autoimmune encephalomyelitis and increased TGF-β signalling in Smad7-deficient T cells. Having these results as a basement, the aim of this thesis was to study TGF-β-induced Smad-dependent and independent signalling pathways in T cell differentiation. First, the immune phenotype of mice with a T cell-specific deletion of Smad7 should be studied. Second, it should be elucidated whether an altered expression of Smad7 determines T helper cell differentiation and function, in particular the suppressive capacity of regulatory T cells. Third, the role of TGF-β-induced Smad-dependent and Smad-independent intracellular signalling pathways in Th17 differentiation should be characterized, to finally identify and confirm pathways that are involved in controlling T helper cell differentiation and their effector functions.

3. Materials and methods

3.1 Materials

Consumables

Product	Supplier
Amersham Hyperfilm ™ ECL	GE Healthcare, München
BD Plastikpak™, syringe (1ml)	Becton Dickinson, Heidelberg
BD Microlance ™ 27G	Becton Dickinson, Heidelberg
BD Microlance ™ 20G	Becton Dickinson, Heidelberg
BD Falcon™ Cell Strainer, 40 µm	Becton Dickinson, Heidelberg
BD Falcon™ Cell Strainer, 70 µm	Becton Dickinson, Heidelberg
Blotting paper	A.Hartenstein, Würzburg
Combitips	Eppendorf, Hamburg
Disposable gloves	Hartman, Heidenheim
MACS® Cell Separation Columns, MS	Miltenyi Biotec, Bergisch Gladbach
MACS® Cell Separation Columns, LS	Miltenyi Biotec, Bergisch Gladbach
MACS® Cell Separation Columns, LD	Miltenyi Biotec, Bergisch Gladbach
Nitrocellulose Membrane	Whatman, Dassel
Petridishes	Becton Dickinson, Heidelberg
Pipette tips	Gilson, Middleton, USA
	Sarstedt, Nürmbrecht
Pipette Tipps with filters	Biozym, Hessisch Oldendorf
Polystyrene tubes 15 ml	Sarstedt, Nürmbrecht
Polystyrene tubes 50 ml	Sarstedt, Nürmbrecht
RT-PCR-Tubes: Optical Cap + Tube	Stratagene, Agilent Technologies,
	Oberhaching
Test plate (12-well/24-well/96-well)	Omnilab, Schubert & Weiß, München
	TPP, Schweiz
Vials	Eppendorf, Hamburg
	Falcon BD, Heidelberg
	Gibco BRL, Karlsruhe
	Sarstedt, Nürmbrecht

Table 1: Basic consumables

Reagents and chemicals

Product	Supplier
Agarose, electrophoresis grade	AppliChem, Darmstadt
Albumin bovine (BSA) Fraction V	Biomol, Hamburg
Ammoniumpersulfat (APS)	Sigma, Taufkirchen
Ammoniumchlorid (NH_4CL) 0,15M	Sigma, Taufkirchen
Bromophenol-blue	Merck, Darmstadt
Collagenase	Sigma, Taufkirchen
Dimethyl Sulfoxide (DMSO)	Sigma, Taufkirchen
DNase	Worthington Biochemicals, England
Dulbecco`s PBS	PAA, Pasching, Österreich
Ethylenediaminetetraacetic acid (EDTA)	Sigma, Taufkirchen
Ethanol	J.T.Backer, Deventer, Niederlande
Ethidium bromide	Sigma, Taufkirchen
Fetal Calf Serum (FCS)	Biochrom, Berlin
Ficoll 400	Amersham Pharmacia, Freiburg
Glutamin 200 mM	PAN Biotech GmbH, Aidenbach
Glycerin	Merck, Darmstadt
Glycine	Merck, Darmstadt
Hank's Buffered Salt Solution (HBSS)	Gibco, Invitrogen, Karlsruhe
Heparin-Natrium (5000 IE/ml)	Ratiopharm, Ulm
(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 1M	Gibco Invitrogen, Karlsruhe
Isopropanol	AppliChem, Darmstadt
Ketanest (Ketamin) 100 mg/ml	Bela-Pharm GmbH&Co KG, Vechta
LymphoPrep	Axis-Shield PoCAS, Oslo, Norwegen
Mannide monooleate	Sigma, Taufkirchen
Methanol	Merck, Darmstadt
MOG $_{35-55}$ peptide	Pepceuticals, Nottingham, England
Mycobacteria tuberculosis H37RA	Difco, BD Bioscience, Heidelberg
Non-Essential Amino Acid (NEAA)	PAN Biotech GmbH, Aidenbach
Nonidet P-40	Sigma, Taufkirchen
Penicillin/Streptomycin 100 µg/ml	PAN Biotech GmbH, Aidenbach

Pertussis Toxin	Calbiochem, San Diego, USA
Phosphotase-Inhibitor, PhosSTOP	Roche, Mannheim
Phenylmethylsulfonyl fluoride (PMSF)	Sigma, Taufkirchen
Ponceau S solution	Sigma, Taufkirchen
Potassiumbicarbonate ($KHCO_3$) 10mM	Merck, Darmstadt
Precision Plus Protein Dual Color Standards	Biorad, München
Protease-Inhibitor, Complete Mini	Roche, Mannheim
Proteinase K	Roche, Switzerland
Restore Western Blot Stripping Buffer	Thermo Scientific, Karlsruhe
Rompun (Xylazine) 20 mg/ml	Serumwerk, Bernburg
Roswell Park Memorial Institute medium (RPMI)1640	Gibco Invitrogen, Karlsruhe
Sodium-Deoxycholate	Sigma, Taufkirchen
Sodium-Pyruvat 100mM	Gibco Invitrogen, Karlsruhe
Sodiumchlorid (NaCl)	VWR, Darmstadt
Sodium Dodecyl Sulfate (SDS)	Merck, Darmstadt
Tetramethylethylenediamine (TEMED) Plus One	GE Healthcare, München
Tris-Base	Sigma, Taufkirchen
Trypanblue	Sigma, Taufkirchen
Trypsin	Sigma, Taufkirchen
Polysorbate 20 (or TWEEN 20)	Sigma, Taufkirchen

Table 2: Reagents and chemicals for cell culture and molecular biology

Pharmacological inhibitors

Inhibitor	**Pathway or molecule blocked**	**Supplier**
LY 294002	AKT, PI3K	Calbiochem, Merck, Darmstadt
LY 294002	AKT, PI3K	JS Research Chemicals Trading Wedel
PD98059	ERK, MAPK	Calbiochem, Merck, Darmstadt
Rapamycin	mTOR	Calbiochem, Merck, Darmstadt
Rapamycin	mTOR	JS Research Chemicals Trading

		Wedel
SP600125	JNK	Calbiochem, Merck, Darmstadt
SB202190	p38	Sigma, Taufkirchen
U0126	MEK1 & MEK2	Calbiochem, Merck, Darmstadt

Table 3: Pharmacological inhibitors for cell culture

Kits and PCR master mix

Product	**Supplier**
Bio-Rad Protein Assay Kit	Bio-Rad laboratories GmbH, Munich
Brilliant® II SYBR® Green QPCR Master Mix	Stratagene, Agilent Technologies, Oberhaching
Brilliant® QPCR Master Mix	Stratagene, Agilent Technologies, Oberhaching
CD4 (L3T4) Micro Bead kit, mouse	Miltenyi Biotec, Bergisch Gladbach
$CD4^+CD62L^+$ T Cell Isolation kit II, mouse	Miltenyi Biotec, Bergisch Gladbach
CD25 Micro Bead kit, mouse	Miltenyi Biotec, Bergisch Gladbach
CD90.2 T cell depletion kit, mouse	Miltenyi Biotec, Bergisch Gladbach
Immobilon Western, Chemoluminescent HRP Substrate	Millipore, Schwalbach
QuantiTect® Reverse Transcription Kit	Qiagen, Hilden
Rneasy® Micro Kit	Qiagen, Hilden
TGF-beta signalling phospho-specific antibody microarray	Fullmoon Biosystem, BioCat GmbH, Heidelberg
Wizard SV Genomic DNA purification system	Promega, Mannheim
X 5 Green Go Taq kit	Promega, Mannheim

Table 4: Kits for cell culture and molecular biology

Cytokines and antibodies

Antibody or Cytokine	**Clone**	**Concentration used**	**Supplier**
Anti-mouse CD3	145-2C11	1µg/ml	BD Bioscience, Heidelberg
Anti-mouse CD28	37.51	1ng/ml	BD Bioscience, Heidelberg
Anti-mouse IL-4	11B11	10µg/ml	BD Bioscience, Heidelberg
Anti-mouse IFN-γ	XMG1.2	10µg/ml	BD Bioscience, Heidelberg
r-mIL-12		20ng/ml	R&D Systems, Wiesbaden

r-mIL-6		20ng/ml	R&D Systems, Wiesbaden
r-mIL-2		10ng/ml	R&D Systems, Wiesbaden
rh-TGF-β		0, 0.4, 2ng/ml	R&D Systems, Wiesbaden

Table 5: Cytokines and antibodies for cell culture

RT-PCR-primer (Murine) :

Primer	Sequence	Supplier
FoxP3	QT00138369	Qiagen, Hilden
IFNγ	QT01038821	Qiagen, Hilden
IL17a	QT001103278	Qiagen, Hilden
RORyt f	GGTGATAACCCCGTAGTGGA	Invitrogen, Karlsruhe
RORyt r	TCAGTCATGAGAACACAAATTGAA	Invitrogen, Karlsruhe
Serpine1 (PAI-1)	QT00154756	Qiagen, Hilden
Smad7	QT00124607	Qiagen, Hilden
T-bet	Mn00450960_m1	Applied Biosystems, Darmstadt
18s rRNA f	CGGCTACCACATCCAAGGAA	Invitrogen, Karlsruhe
18s rRNA r	GCTGGAATTACCGCGGCT	Invitrogen, Karlsruhe

Table 6: Primers for gene expression analysis

Sequence from 3` to 5`, f = forwards, r = reverse, QT = QuantiTect Primer Assay

Western Blot-antibodies

Product	Supplier
Actin	Sigma, Taufkirchen
AKT	Assay Biotech, Acris Antibodies GmbH, Herford
p-AKT (Tyr474)	Assay Biotech Acris Antibodies GmbH, Herford
Goat-anti- mouse IgG HRP	Chemicon International, Millipore, Schwalbach
m-TOR	Cell Signalling, New England Biolabs GmbH, Frankfurt am Main
p-mTOR (Thr2446)	Millipore, Schwalback
PI3K	Cell Signalling, New England Biolabs GmbH, Frankfurt am Main
p-PI3K p85 (Tyr458)/p55	Cell Signalling, New England Biolabs GmbH, Frankfurt am Main
Smad2	Cell Signalling, Beverly, USA
p-Smad2 (Ser465/467)	Cell Signalling, Beverly, USA

Table 7: Antibodies for Immunoblotting

Fluorescence conjugated-antibodies

Specificity	Clone	Supplier
CD4	GK.1.5/4	BD Pharmingen, Heidelberg
CD8	53-6.7	BD Pharmingen, Heidelberg
CD25 (IL2Ra)	7D4	BD Pharmingen, Heidelberg
CD62L (L-Selectin)	MEL-14	BD Pharmingen, Heidelberg
CD69	H1.2F3	BD Pharmingen, Heidelberg
CD11c	HL3	BD Pharmingen, Heidelberg
CD19	ID3	BD Pharmingen, Heidelberg
CD44	KM114	BD Pharmingen, Heidelberg
CD90.2 (Thy 1.2)	CFO-1	BD Pharmingen, Heidelberg
FoxP3	FJK16s	eBioscience, Frankfurt
IFN-γ	XMG1.2	BD Pharmingen, Heidelberg
IL-17A	TC11-18H10	eBioscience, Frankfurt

Table 8: Antibodies for Flow cytometric analysis

Enzyme-linked immunosorbent assay kits

Product	Supplier
IFN-γ	Biozol, Eching
IL-2	BD Bioscience, Heidelberg
IL-4	BD Bioscience, Heidelberg
IL-10	BD Bioscience, Heidelberg
IL-12	BD Bioscience, Heidelberg
IL-17	Biozol, Eching
TGF-β	R&D Systems, Wiesbaden

Table 9: ELISA kits for measuring cytokine from supernatant

Technical equipment

Blotting chamber	Biometria, Göttingen
Centrifuge 5417 R	Eppendorf, Hamburg
Emax Precision Microplate Reader	Molecular Devices, Union City, USA
Incubator HERA Cell	Heraeus Instruments GmbH,

Megafuge 1.0 R	Heraeus Instruments GmbH, Hanu
Mighty Small, multiple gel caster	Hoefer, Biostep, Jahnsdorf
Neubauer counting chamber	VWR, Darmstadt
Olympus CK 30 (Mikroscop)	Olympus, Hamburg
Perfect Spin 24 (Zentrifuge)	PeqLab, Erlangen
Photometer Gene Quant II	Amersham/Pharmacia Biotech, Freiburg
Pipet boy	Integra Biosciences, Fernwald
Power Supply - EPS 601	Amersham/Pharmacia Biotech, Freiburg
SE 260 Gelelektrophoresekammer	Hoefer, Biostep, Jahnsdorf
Thermocycler Mastercycler gradient	Eppendorf, Hamburg
Thermomixer comfort	Eppendorf, Hamburg
Vortex-Genie 2	Scientific Industries, New York, USA

Table 10: Basic laboratory equipments

Computer software

Adobe Photoshop C2 version 9.0	Adobe Systems GmbH, München
Flow Jo FACS analysis software	Tree Start, Inc. Ashland, OR, USA
GraphPad Prism 5	GraphPad Software Inc., USA
Microsoft Office	Microsoft Corporation
Mx3005P QPCR System	Stratagene, Agilent Technologies, Oberhaching

Table 11: Software for data analysis

Medium and buffer

T cell medium:	
RPMI 1640	450 ml
1M HEPES	7.6 ml
100 mM Na-Pyruvate	5 ml
NEAA (100x)	5 ml
200 mM Glutamin	5 ml
100 µg/ml Penicillin/Streptomycin	5 ml
FCS (10%)	50 ml
2-Mercaptoethanol (14.3 mol/l)	1.7 ml

MACSbuffer:	
Dulbecco`s PBS	500 ml
BSA (5mg/ml)	2.5 g
0.5 M EDTA	2 ml
ACK lysis buffer:	**Total volume 1 L**
0.15 M Amoniumchlorid (NH_4CL)	8,.9 g
10 mM Potassiumbicarbonate ($KHCO_3$)	1 g
0.1 mM EDTA	37. mg
Distilled Water	800 ml
Hydrochloric acid (HCL)	pH was adjusted to 7.2-7.4
Wash buffer T-TBS:	**Total volume 1 L**
Tris-Base	6.05 g
Sodiumchloride (NaCl)	8.76 g
Distilled Water	800 ml
Hydrochloric acid (HCL)	pH was adjusted to 7.5
TWEEN 20 (final conc 0.1%)	1 ml
Radioimmunoprecipitation assay (RIPA) buffer:	**Total volume 100 ml**
10% Nonidet P-40	10 ml
10% Na-Deoxycholate	2.5 ml
100 mM EDTA	1 ml
Tris-Base in dest. H_20 (pH=7.4)	790 mg
Sodiumchloride (NaCl) in dH_20 (pH=7.4)	900 mg
Distilled Water	Up to 100 ml
Protease-Inhibitor, Complete Mini	1 Tablet per 10 ml
Phosphotase-Inhibitor, PhosSTOP	1 Tablet per 10 ml
300 mM PMSF (just before use)	33.3 µl per 10 ml

Protein loading-buffer:	10 ml
0.625 M Tris/HCL pH 6.8	2 ml
Sodiumdodecylsulfate (SDS)	0.2 g
85% Glycerin	5 ml
2-Mercaptoethanol (14.3 mol/l)	0.5 ml
Bromophenol-Blue	0.001 g
Distilled Water	2.4 ml
Western blot running buffer:	**Total volume 1 L**
Tris-Base	30.2 g
Glycine	144 g
Distilled Water	800 ml
Hydrochloric acid (HCL)	pH was adjusted to 8,8
10% Sodiumdodecylsulfate (SDS)	100 ml
Western blot transfer buffer:	**Total volume 1 L**
Tris-Base	3.03 g
Glycine	11.26 g
100% Methanol	100 ml
Distilled Water	800 ml
Hydrochloric acid (HCL)	pH was adjusted to 8.3
Western blot stacking gel buffer:	**Total volume 1 L**
Tris-Base	61 g
Distilled Water	800 ml
Hydrochloric acid (HCL)	pH was adjusted to 6.8
Western blot separating gel buffer:	
Tris-Base	182 g
dest. Wasser	800 ml
Hydrochloric acid (HCL)	pH was adjusted to 8.8

PBS/EDTA solution:	
0.5 M EDTA pH=7.4	1 ml
PBS	500 ml
PBS/Heparin solution:	
Heparin 5000 IE/ml	500 µl
PBS	500 ml
FACS buffer:	
Dulbecco`s PBS	500 ml
BSA (10mg/ml)	5 g
Sodium azide	2.5g

Table 12: Medium and buffers for cell culture and molecular biology

3.2. Methods in cell biology

3.2.1 Animals

CD4Cre-Smad7$^{fl/fl}$ (Kleiter *et al.* 2010) and CD2-Smad7 (Dominitzki *et al.* 2007) mice, both with C57BL/6 genetic background and control wild type C57BL/6 mice (Charles River) were used for experiments. The mouse strain TGF-βRII (Chytil *et al.* 2002) was crossed to CD4Cre (Lee *et al.* 2001). Unless indicated, mice were around 6-8 weeks old and of both sexes. All experiments were conducted in compliance with the guidelines of the Central Animal Testing Facilities (CTL) of the University of Regensburg. Ethical approval for the in vivo animal experiments was obtained from the Regierung der Oberpfalz (AZ 54-2531.1-27/05).

3.2.2 Preparation of single cell suspensions

The animals were sacrificed with CO_2 and single cell suspension from lymphoid organs, spleen, additionally thymus when indicated, were prepared by homogenizing the organs mechanically and passing suspensions through a 70 and a 40 µM mesh (Falcon strainer). Primary cell suspensions were washed with PBS twice. Contaminant erythrocytes in the suspension were lysed by incubation with 1x ACK lysis buffer for 2 min at room temperature. Immediately, PBS with 10% FCS 20 ml was added to the suspension in order to stop the lysis reaction. Next, cells were spun down for 5 minutes at 550 g at 4^0C. This step was repeated once again. Cells were then resuspended in PBS once and counted after staining with Trypan blue dye which allowed for exclusions of dead cells. Cell suspensions were either kept on ice in PBS or processed according to the further steps. Preparation of the cell suspension and all subsequent cell culture work was conducted in a laminar flow cabinet. Sterile working tools were used.

3.2.3 Cell sorting and analysis

3.2.3.1 Magnetic cell sorting

Specific cell populations were sorted for negative and positive fractions according to the cell population of interest by magnetic cell sorting (MACS technique; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturers protocol. These include the incubation of cell suspensions with microbeads which are antibodies conjugated with magnetic particles of a size of 50nm. During incubation, these antibodies bind to surface molecules of the cells. Cells are then allowed to

pass through a column which is positioned in a strong magnetic field. Negative populations are collected from the flow through and for positive fractions; the column is transferred to a new collection tube and filled with usually 5 ml MACS buffer. The liquid in the column is then flushed out firmly by a plunger. For instance, if CD4 microbeads are used, negative population will contain other lymphocytes than $CD4^+$ T cells and positive population will contain only $CD4^+$ T cells.

Briefly, for example, in the case of naïve $CD4^+CD62L^+$ T cells, a total lymphocyte cell suspension was incubated with 100µl $CD4^+$ T cell Biotin-Antibody Cocktail II and 400µl MACS buffer per 10^8 cells for 10 minutes at 4-8^{0}C in 50 ml falcon tube. Next, 300 µl MACS buffer and 200 µl Anti-Biotin microbeads were added per 10^8 cells, mixed well and incubated for 15 minutes at 4-8^{0}C. More MACS buffer were added to the falcon tube to make the total volume 40 ml. Cells were then washed by centrifuging at 550 g for 5 minutes at 4^{0}C. Cells were resuspended in 500 µl MACS buffer per 10^8 cells. Next, cells went through magnetic separation and the unlabelled pre-enriched $CD4^+$ T cell fraction was collected. This fraction was incubated with 200µl CD62L microbeads and 800 µl MACS buffer per 10^8 cells for 15 minutes at 4-8^{0}C. Cells were washed with additional MACS buffer in a volume of 40 ml by centrifuging at 550 g for 5 minutes at 4^{0}C. Cells were resuspended in 500 µl MACS buffer per 10^8 cells. Naïve $CD4^+CD62L^+$ T cells were obtained by magnetic separation. Purity of the selected population was always checked by flow cytometry (FACS Calibur). From one mouse around 5 x 10^6 naïve T cells were obtained which were 97% pure (for one representative figure, see Appendix: S1).

3.2.3.2 FACS cell sorting

Naïve T cells $CD4^+CD62L^+CD25^-$ were sorted by FACS Aria as well. Cells were first MACS purified to enrich $CD4^+$ T cells and subsequently surface stained for CD4, CD62L and CD25 with fluorochrome conjugated antibodies. The cells were then sorted using FACSAria to get $CD4^+CD25^-CD62L^+$ naïve cells unless stated otherwise.

3.2.4 Flow cytometry

Flow cytometry is a method based on laser detection of single cell suspensions in a flow sheath. Surface or intracellular markers of a particular cell type are labelled with antibodies which are artificially conjugated with flurochromoes. In principle, when the cells are analysed by flow cytometry, the cells expressing the marker for which the antibody is specific will manifest fluorescence. Cells lacking the marker will not manifest fluorescence. Cells stained with fluorochrome conjugated antibodies are excited by a laser beam emit the light in a different wavelength, which is detected by photomultipliers since Flow cytometers use the principle of hydrodynamic focusing for presenting cells to a laser. By changing the excitation light and using more than one fluorochrome, it is possible to analyse several parameters of the sample at any one time. This forms the basis of multicolour fluorescence studies.

3.2.4.1 Surface marker staining

After making the cell suspension from organs or tissues (see **3.2.2**), cells were counted and 0.1-1x10^6 cells were used for staining. First, cells were washed with PBS by spinning down with 600 g at 4°C for 5 min. Cells were resuspended in 200µl dPBS and 2% FCS buffer (FACS buffer) and centrifuged with 600 g at 4°C for 5 min. Cells were resuspended in 50µl FACS buffer containing the fluorochrome conjugated antibodies and incubated for 15 minutes in the dark at 4°C. Cells were then washed once with FACS buffer at 600 g at 4^0C for 5 minutes. After discarding the supernatant, the pellet was resuspended in 200µl FACS buffer for flow cytometric analysis by FACS Calibur (BD Bioscience). Data were analysed by Flow Jo software.

3.2.4.2 Intracellular and intranuclear marker staining

Intracellular and nuclear staining was performed to check the transcription factor expression and cytokine production of different T cell subsets after 5 days. For intracellular FACS analysis of IL-17, IL-4 and IFNγ producing T cells, cells were stimulated for 4h with PMA (50 ng/ml), Ionomycin (500 ng/ml) and 1µg/ml Golgi stop (all from BD Bioscience) in T cell medium at 37°C, 5%CO2. Intracellular staining was performed by using BD Bioscience staining kit for intracellular staining of IFNγ and IL17 (BD Cytofix/CytopermTMplus Fixation/Permeabilization kit with BD golgiPlugTM; BD Bioscience, Germany) according to the manufacturers' protocols. Briefly, for cytokine staining, after harvesting, cells were washed once with FACS buffer at 600 g

at 4^0C for 5 minutes. Fc receptors were blocked by anti-FcR-antibody in FACS buffer for 10min. Next, cells were stained for surface markers with FACS buffer for 15 minutes. Cells were then washed once with FACS buffer at 600 g at 4^0C for 5 minutes. Cells were resuspended with 100µl Cytofix/Cytoperm buffer and incubated for 30min at 4°C in the dark in order to fix surface markers. The reaction was stopped by 1x Perm/Wash buffer followed by two washing steps with Perm/Wash buffer in order to make pores through the cell wall so that intracellular fluorochrome conjugated antibodies can bind to the intracellular molecules. Subsequently, cells were incubated with the fluorochrome conjugated antibodies (e.g. IFNγ, IL17) diluted with Perm/Wash buffer for 20min at 4°C in the dark. Cells were washed twice in the Perm/wash buffer at 600 g at 4^0C for 5 minutes. Afterwards, cells were resuspended in 200µl FACS buffer for FACS analysis. For the transcription factor FoxP3 staining, FoxP3 staining kit from eBioscience was used. Cells were directly (no PMA, Ionomycin, BrefeldinA stimulation) stained for surface markers in FACS buffer for 15 minutes. Cells were then washed once with FACS buffer at 600 g at 4^0C for 5 minutes and incubated for 2h in Fix/Perm buffer (prepared according to the protocol provided in the kit) at 4°C in the dark. Cells were washed with 1x permeabilization buffer at 600 g at 4^0C for 5 minutes. Subsequently, cells were incubated with the fluorochrome conjugated antibodies (PE anti-FoxP3) diluted with permeabilization buffer for 20min at 4°C in the dark. Afterwards, cells were washed twice with Permeabilization buffer at 600 g at 4^0C for 5 minutes and resuspend in 200µl FACS buffer for flow cytometric analysis by FACS Calibur and Data were analysed by Flow Jo software.

3.2.5 Carboxyfluorescein succinimidyl ester staining

In order to check the proliferation of T cells, carboxyfluorescein succinimidyl ester (CFSE) staining was performed. CFSE is partitioned equally among daughter cells with each division. Since CFSE has bright fluorescence, it is possible to check cell proliferation in each division by FACS analysis. $10x10^6$ cells were washed with ice cold PBS (no FCS) in 15ml tube and resuspended in 500µl of PBS and 500µl of CFSE (2µM) in order to set the final concentration of 1µM CFSE in the total volume and incubated at dark at room temperature for 3 minutes. Immediately, 10 ml of 10% FCS was added to the suspension to stop the reaction and centrifuged at 550 g, 4^0C for 5 minutes. This washing step was repeated once again. Cells were then

resuspended in 10 ml T cell medium and centrifuged at 550, 4^0C for 5 minutes and resuspended again in the T cell medium and used for further experiment.

3.2.6 T helper cell subset differentiation *in vitro*

3.2.6.1 Th1 cell differentiation

Naïve $CD4^+CD25^-CD62L^+$ T cells were sorted either by FACS Aria or magnetic separation. $0.3x10^6$ cells in 200µl/well were stimulated in T cell medium supplemented with anti-CD3 (1µg/ml), anti-CD28 (10ng/ml), anti-IL4 (10µg/ml), rm-IL-12 (20ng/ml) in round bottom 96-well plates for 5 days at 37° and 5% CO_2. After 5 days of incubation, supernatants were analysed for IFN-γ production by ELISA. In addition, IFN-γ production was also checked by flow cytometry.

3.2.6.2 Th2 cell differentiation

Naïve $CD4^+CD25^-CD62L^+$ T cells were sorted either by FACS Aria or magnetic separation. $0.3x10^6$ cells in 200µl/well were stimulated in T cell medium supplemented with anti-CD3 (1µg/ml), anti-CD28 (10ng/ml), anti-IFN-γ (10µg/ml), rm-IL-4 (10ng/ml) in round bottom 96-well plates for 5 days at 37° and 5% CO_2. After 5 days of incubation, supernatants were analysed for IL-4 production by ELISA. In addition, IL-4 production was also checked by flow cytometry.

3.2.6.3 Th17 cell differentiation

Naïve $CD4^+CD25^-CD62L^+$ T cells were sorted either by FACS Aria or magnetic separation. $0.3x10^6$ cells in 200µl/well were stimulated in T cell medium supplemented with anti-CD3 (1µg/ml), anti-CD28 (10ng/ml), rh-TGFβ1 (2ng/ml), rm-IL-6 (20ng/ml), anti-INFγ (10µg/ml) in round bottom 96-well plates for 5 days at 37° and 5% CO_2. After 5 days of incubation, supernatants were analysed for IL-17 production by ELISA. In addition, IL-17 production was also checked by flow cytometry.

3.2.6.4 Treg cell differentiation

Naïve $CD4^+CD25^-CD62L^+$ T cells were sorted either by FACS Aria or magnetic separation. $0.3x10^6$ cells in 200µl/well were stimulated in T cell medium supplemented with anti-CD3 (1µg/ml), anti-CD28 (10ng/ml), rmIL-2 (10ng/ml) and rh-TGF-β (2ng/ml) in round bottom 96-well plates for 5 days at 37° and 5% CO_2. After 5

days of incubation, cells were harvested and surface stained for CD4, CD25 and for intranuclear FoxP3 to confirm Treg differentiation by flow cytometry.

3.2.7 Mixed lymphocyte reaction

In order to elucidate whether T cell specific Smad7 deletion affects the suppressive function of Tregs and the capability for T cells to proliferate, co-culture experiments between Tregs and T responder cells were performed.

3.2.7.1 Cell suspension

Spleens and LNs were collected from 8-12 weeks old male and female mice. Cell suspension was made as described in section (3.2.2).

3.2.7.2 Cell sorting procedure

The following sub types from mouse T cells were sorted by MACS beads and FACS Aria:

Mice	**Sub type mouse T cells**	**Function**
WT	$CD4^+CD25^-$ $CD4^+CD25^+$ $CD4^-CD25^-CD90.2^-$	Responder T cells Tregs APC
Smad7KO	$CD4^+CD25^-$ $CD4^+CD25^+$	Responder T cells Tregs
Smad7Tg	$CD4^+CD25^-$ $CD4^+CD25^+$	Responder T cells Tregs

Cell suspensions from all these three strains were prepared in a concentration of $10x10^6$ cells/100µl and washed once with FACS buffer and incubated with following antibodies:

Antibody	Stock conc.	Amount used per 100µl	Incubation time on ice
Fc-receptor blocker	0.5mg/ml	1µl	5 minutes before addition of anti-CD25 PE
anti-CD25 PE	0.2mg/ml	0.5µl	20 minutes at dark

Afterwards, cells were washed once with FACS buffer and incubated with 10µl anti-PE beads per $10x10^6$ cells for 20 minutes on ice and then washed once with MACS buffer and resuspended in 500 µl MACS buffer per 10^8 cells. Cells were passed through magnetic bead separation and both $CD25^+$ (from 3 different mice) and $CD25^-$ T cells were collected.

$CD25^+$ T cells from all mice were washed once with FACS buffer and incubated with 1µl anti-CD4-APC (stock conc. 0.2mg/ml) per $10x10^6$ cells/100µl and 0.5µl anti-CD25-PE per $10x10^6$ cells/100µl for 20 minutes on ice at dark. Cells were washed and resuspended in FACS buffer (<$20x10^6$ cells/300µl FACS buffer and >$20x10^6$ cells/500µl FACS buffer) for FACS Aria sorting. **$CD4^+CD25^+$** Tregs were sorted by FACS Aria. Purity was over 98% (for one representative figure, see Appendix: S2).

$CD25^-$ T cells were incubated with 10µl anti-CD4 (L3T4) micobeads per $10x10^6$ cells/100µl in MACS buffer for 20 minutes on ice. Cells were then passed through magnetic separation. Both $CD4^+CD25^-$ (responder T cells) and $CD4^-CD25^-$ fractions were collected.

$CD4^+CD25^-$ responder T cells were then washed once with FACS buffer and incubated with 1µl anti-CD4-APC per $10x10^6$cells/100µl and 0.5µl anti-CD25-PE per $10x10^6$ cells/100µl for 20 minutes in dark and resuspended in FACS buffer and then went through FACS Aria sorting in order to get pure $CD4^+CD25^-$ responder T cells.

$CD4^-CD25^-$ cell fractions were incubated with 10µl anti-CD90.2 (a T lymphocyte marker) per $10x10^6$ cells/90µl for 20 minutes on ice and then washed once with MACS buffer and resuspended in 500 µl MACS buffer per 10^8 cells. Cells were passed through magnetic separation with LD columns and only the negative fraction (flow through) was collected which were regarded as $CD4^-CD25^-CD90.2^-$ and used as APC (antigen presenting cells). 10,000 cells of this APC were incubated with FACS antibodies against CD4, CD25 and CD90.2 for 20 minutes at dark on ice and

purity was checked by FACS Calibur. Usually, >95% purity was obtained.

$CD4^-CD25^-CD90.2^-$ APC were irradiated at 3000 rad for 316 seconds. This cell fraction was then used as pure APC for the co-culture. APCs were irradiated so that these cells would not proliferate in the culture but would activate T responder cells.

3.2.7.3 Co-culture procedure

Each condition of co-cultures were made in a total volume of 200 µl in round bottom 96 well plates and incubated for 3 days at 37° and 5% CO_2.

Conditions for Co-culture

Treg (Regulatory/suppressor T cells)……50,000 cells/50µl

Tresp (Responder T cells)……...50,000 cells/50µl

APC (Antigen presenting cells)……..100,000 cells/50µl

Anti-CD3 (final conc. is 0.5µg/ml) added in…..50µl T cell medium

3.3. Methods in molecular biology

3.3.1 Genomic DNA extraction

The wizard SV Genomic DNA purification system from Promega was used for DNA extraction. Mice tail tips (0.5 cm length) were cut off and digested in 1.5 ml microcentrifuge tube with 275 µl of Digestion Solution Master Mix (Nuclei Lysis Solution 200 µl, 0.5M EDTA of pH 8.0 50 µl, Proteinase K of 20mg/ml, 20 µl, and RNase A solution of 4mg/ml, 5 µl) to each tube. Sample tubes were incubated overnight for 18 hours in a 55^0C heat block. Wizard SV lysis buffer 250 µl was added to each tube and mixed well by vortexing. Samples were transferred to Wizard SV minicolumn assembly and spun down at 13,000 g for 3 minutes. Liquid in the collection tube was discarded. 650 µl of Wizard SV wash solution (with 95% ethanol added) was added to each assembly and spun down at 13,000 x g for 1 minute. Liquid in the collection tube were discarded. This washing step was repeated 4 times. Liquid in the collection tube was discarded and the complete assembly was spun down without adding anything for 2 minutes at 13,000 g to dry the binding matrix. The Wizard SV minicolumn was transferred to a new 1.5 ml microcentrifuge tube (collection tube) and supplemented with 250 µl of room temperature nuclease-free water and incubated for 2 minutes at room temperature and spun down at 13,000 g for 1 minute. Liquid containing purified DNA remained in the collection tube. An additional 250 µl of room temperature nuclease-free water was added to the minicolumns and incubated for 2 minutes at room temperature and spun down at 13,000 g for 2 minutes. Purified DNA was collected from the collection tube and stored at -20 to -80^0C unless it was used immediately.

3.3.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a common detection method of creating copies of specific fragments of DNA. PCR rapidly amplifies a single DNA molecule into many millions of molecules. The method is based on binding of appropriate oligonucleotide primers (18-75 nucleotides) to DNA fragments. Binding of primers to DNA templates is catalysed by the heat resistant taq polymerase (X 5 green Go Taq kit, Promega, Mannheim) which synthesizes the DNA starting from the 3’ end of the primer. In this thesis, PCR was performed to screen experimental mice for presence of targeted alleles or transgenes.

3.3.2.1 Typing of CD4Cre-Smad7$^{fl/fl}$, Smad7 Tg and TGFβRIIf$^{fl/fl}$ mice

Materials:

Materials	Amount in µl
X 5 Green Go Taq reaction buffer	6
dNTPs	0.3
Primer	1
Taq Polymerase	0.3
Molecular biology H_20	21.4
Genomic DNA	1

Table 13: List of reagents for PCR.

Total volume used for PCR analysis was 30 µl containing 10pmol of each primer. PCR protocols were followed as shown in (Table 14).

A) Smad7flox

	Temp.	Time	Cycle number
Initial denaturation	95.0	3´	
Denaturation	95.0	45´´	35
Annealing	61.8	30´´	
Elongation	72.0	45´´	
final elongation	72.0	5´	

Primer:
Smad7fl sen 5´-GTCAGGTTGGATCACCATGCC-3´
Smad7fl ase 5´-GACTGCCTGGAGAAGTGTGTC-3´

Fragment size:

WT	413 bp
HET (Smad7fl/+)	413, 568 bp
KO (Smad7fl/fl)	**568 bp**

B) CD4Cre

	Temp.	Time	Cycle number
Initial denaturation	95.0	3´	
Denaturation	95.0	45´´	35
Annealing	56.65	30´´	
Elongation	72.0	45´´	
final elongation	72.0	5´	

Primer:
CD4Cre sen 5´-CCCAACCAACAAGAGCTC-3´
CD4Cre ase 5´-CCCAGAAATGCCAGATTACG-3´

Fragment size:

WT	300 bp
CD4Cre	**600 bp**

C) Smad7Tg-strain (CD2-Smad7)

	Temp.	Time	Cycle number
Initial denaturation	95.0	1´	
Denaturation	95.0	1´	35
Annealing	55.0	1´	
Elongation	72.0	1´	
Final elongation	72.0	7´	

Primer sequence: **Fragment size:**

CD2: 5´-CCCAGCTTTCCCTGAAAGTG-3´ WT no band
Smad7: 5´-CCGTAAGATTCACAGCAACAC-3´TG 650-700bp

D) TGFβRII-strain (TGFβRII$^{fl/fl}$)

	Temp.	Time	Cycle number
Initial denaturation	94.0	4´	
Denaturation	94.0	45´´	35
Annealing	68.0	45´´	
Elongation	72.0	1´	
final elongation	72.0	8´	

Primer:

mEIIf: 5' –GCA GGC ATC AGG ACC TCA GTT TGA TCC-3'
mSAr: 5' –AGA GTG AAG CCG TGG TAG GTG AGC TTG-3'

Fragment size:

WT	556 bp
HET (TGFbRIIfl/+)	556, 711 bp
KO (TGFbRIIfl/fl)	**711 bp**

Table 14: Conditions for genotyping.

3.3.2.2 Agarose gel electrophoresis

Agarose gel electrophoresis is the easiest and commonest way of separating and analysing DNA. Here, the purpose of the gel is to quantify the size of a particular band. In principle, negatively charged DNA migrates in an electric field towards the anode, resulting in DNA to be separated in a size dependent manner. The DNA is visualised in the gel by addition of ethidium bromide. This binds strongly to DNA by intercalating between the bases and absorbs invisible UV light and transmits the

energy as visible orange light. The size of DNA fragments was measured by loading a size marker 3 µl (DNA molecular weight marker VIII, Roche Diagnostics; GeneRulerTM 1Kb plus DNA ladder, Fermentas; 1Kb DNA ladder, Invitrogen) to the 2% Agarose gel along with the DNA samples. After agarose gel electrophoresis, the following principle was used to screen experimental mice (Table 15).

Strain name	**Band size**	**Band size**
CD4Cre-Smad7$^{fl/fl}$	CD4Cre 600 bp	Smad7 flox 568 bp
CD4Cre-Smad7$^{fl/+}$	CD4Cre 600 bp	Smad7 flox 413 and 568 bp
Smad7Tg	CD2 no band	Smad7 650-700 bp

Table 15: Corresponding band size of WT, CD4Cre-Smad7$^{fl/fl}$ and Smad7Tg mice

3.3.3 Gene expression analysis

3.3.3.1 Preparation of ribonucleic acid

In order to quantify the target gene expression, as a first step, ribonucleic acid (RNA) was extracted from the experimental samples. RNAse free equipments and reagents were used since most ribonucleases are very stable and can contaminate the gene expression. Samples and reagents were always kept in ice. To ensure a good quality, full-length RNA was used as starting material.

3.3.3.2 Ribonucleic acid isolation

To isolate RNA from purified cells, the RNeasy® Micro Kits from Qiagen was used. RW1 and RPE buffer were put on ice prior to use. After stimulation, cells were harvested and washed twice with ice cold PBS at 14000 g, 4^0C for 15 mintues in 1.5 ml Eppendorf tube and resuspended in 350µl RLT buffer supplemented with 1% 2-mercaptoethanol and mixed well until a viscous solution was formed. 350 µl of 70% cold ethanol was added to each sample and mixed well so that no solid particle was viewed. This solution was then transferred to RNeasy MinElute spin column placed in a 2 ml collection tube (supplied) and spun down at 8,000 g for 30 seconds. Flow through was discarded. RW1 buffer 350 µl was added and spun down at 8,000 g for 30 seconds. Flow through was discarded. DNase1 10 µl and RDD buffer 70 µl were mixed per sample and pipetted directly on the membrane of the column of each sample tube and incubated at room temperature for 15 minutes. In this step, all the genomic DNA were destroyed. Subsequently, the columns were washed twice with RW1 buffer 350 µl by spinning down at 8,000 g for 30 seconds. Flow through was

discarded. The coloumns were then resuspended in RPE washing buffer 500 µl and spun down at 8,000 g for 30 seconds and flow through was discarded. Columns were resuspended in 80% ethanol 500 µl and spun down at 8,000 g for 2 minutes and the flow through was discarded. The columns in new 2 ml collection tubes (lid was open) were spun down without adding anything in order to dry the columns at 14,000g for 5 minutes. The flow-through and collection tubes were discarded. The columns where RNA lies were then placed to new 1.5 ml collection tubes. RNase free water 14 µl were added directly on the membrane in the column and incubated for 1 minute on ice to elute RNA. The colums were then spun down at 14,000 g for 1 minute. RNA solution was accumulated in the 1.5 ml collection tubes. In order to check the purity, RNA was mixed well with 10 mM Tris-C1 buffer at a ratio of 1:30 and RNA-concentration was measured by UV-photometer. The purity was measured by calculating the ratio of absorbance at 260 nm for RNA and absorbance at 280 nm for proteins. A ratio between 1.8 to 2.2 of RNA was considered to be useful. RNA was stored at -20 to -80^{0}C unless it was used immediately.

3.3.3.3 Complementary deoxyribonucleic acid synthesis

The cDNA synthesis was performed using the QuantiTect® Reverse Transcription Kit from Qiagen. Up to 1 µg of RNA was mixed with 2 µl of genomic DNA wipeout buffer and filled with a variable amount of RNase-free water to set the volume 14 µl and then incubated for 3 min at 42° C placed in a thermal cycler. After that, reverse transcription master mix 6 µl was added to the RNA solution and incubated for 15 minutes at 42^{0}C.

The master mix contained the following elements:

1 µl Quantiscript Reverse Transcriptase
4 µl Quantiscript RT buffer
1 µl RT Primer Mix
For the cDNA synthesis the following temperature profile was used for the thermo cycler:
42°C 15 min.
95°C 3 min.
4°C 5 min.
The cDNA were then stored at -20^{0}C unless it was used immediately.

3.3.3.4 Quantitative Real Time PCR

Real-time quantitative PCR (qPCR) allows the sensitive, specific and reproducible quantification of nucleic acids. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). Different samples can be compared if the Ct values of specific probes are normalized to Ct values of a standard “housekeeping gene”. The relative mRNA amounts were determined by normalization to the 18S mRNA content. The relative expression of transcripts was calculated using the ΔΔCT method (Livak and Schmittgen 2001).

SYBR green system

SYBR Green is a dye that intercalates with double-stranded DNA. This intercalation causes the SYBR Green to emit light. The qPCR machine detects the fluorescence and software calculates Ct values from the intensity of the fluorescence. The following protocol and temperature condition was used.

10 min.	95°C	1 cycle
30 sec.	95°C	35-45 cycles
1 min.	55°C	
1 min.	72°C	
1 min.	95°C	1 cycles
30 sec.	variabel	
30 sec.	95°C	

Annealing temperatures for qPCR

QuantiTect Primer Assay (Qiagen)	55°C
Primer from Applied Biosystems	55°C
RORc	62°C
RORyt	68°C
18s-RNA	67°C

3.3.4 Western blot analysis

Western blotting is an analytical method to characterize the molecular weight and amount of a specific protein from a complex mixture (e.g. crude cell extract) of proteins. This method horizontally separates proteins by gel electrophoresis based on their size and subsequently vertically transfers these proteins onto a nitrocellulose or PVDF membrane. The nitrocellulose is then soaked in blocking buffer (3-5% skimmed milk solution or BSA 3-5%) to "block" the nonspecific binding of proteins. Afterwards, the nitrocellulose is incubated with the specific antibody to detect the protein of interest. The nitrocellulose is then incubated with a second antibody, which is specific for the first antibody. The second antibody will typically have a covalently attached enzyme provided with a chromogenic substrate such as horseradish peroxidase, HRP. The chemoluminescence is then detected at the end with a photo film.

3.3.4.1 Protein extraction

Proteins were extracted from differentiated T helper cells. Extraction of protein was performed on ice. All buffers and solutions were kept cold at 4^0C to prevent degradation of proteins. Cells were harvested and washed twice with ice cold PBS at 14,000 g for 10 minutes at 4^0C. Supernatants were discarded. Subsequently, the cell pellet (typically 2-5 million cells) was resuspended in 100-200 µl RIPA extraction buffer. Pipetting up and down was done to mix the cell pellet and buffer uniformly. Afterwards, a 1 ml syringe with a 20 Gauge needle was used to mix the solution well until a clear viscous and homogenous solution was viewed. Then the solution was incubated for half an hour on ice and vortexed at 5 minutes intervals, followed by spinning down at 14,000 g for 15 minutes at 4^0C. Supernatants (protein lie here) were collected to new tubes and stored at -20^0C unless used immediately.

3.3.4.2 Measurement of protein concentration

In order to measure the protein concentration a Bradford protein assay kit (Bio-Rad laboratories GmbH, Munich) was used. 5µl of protein and BSA standards (from 0 to 2 mg/ml) were pipetted in a 96 well plate. 25 µl of the mixture of substance S (1:50) and substance A were added. Finally, 200 µl substance B was added to the wells. The plate was incubated for 15 minutes at room temperature and readings were obtained by measuring at 650 nm by Emax Precision Microplate Reader. Protein

concentration of samples was calculated by the BSA-protein standard curve using Microsoft Excel.

3.3.4.3 SDS-polyacrylamide-gel

Polyacrylamide gel electrophoresis (PAGE), a polymer of acrylamide monomer, is a method used to separate proteins according to their size. Gels were made in a “Multiple Gel Caster” by Hoefer. First the separation gel and then the stacking gel, in which a plastic comb was placed, were made. Samples of proteins were administered in the pockets of the stacking gel. Different proteins migrated differently due to differences in their secondary, tertiary or quaternary structure. SDS, an anionic detergent, was used in SDS-PAGE to reduce proteins to their primary (linearized) structure and label them with uniform negative charges which mean proteins with the negative charges moved towards the positive pole when placed in an electric field. Depending on which proteins to be detected, separation gels of different acrylamide concentration were used. The table shows the recipes for the different gels.

Separation gel

Protein size in kDa	> 100	~ 80	40-70	30-40	20-30	< 20
% Acrylamide	7,5	10	12,5	15	17	20
Total volume in ml	10	10	10	10	10	10
Water in ml	4,7	4	3,1	2,3	1,5	0,675
10% SDS in ml	0,1	0,1	0,1	0,1	0,1	0,1
buffer in ml	2,5	2,5	2,5	2,5	2,5	2,5
30% Acrylamid in ml	2,5	3,3	4,2	5	5,8	6,65
100% TEMED in µl	7,5	7,5	7,5	7,5	7,5	7,5
10% APS in µl	75	75	75	75	75	75

Table 16: Contents and amounts of reagents for separation gels

Stacking gel (5% Acrylamid)

Total volume: 3 ml	1xGel	2xGel	4xGel
Water in µl	1830	3660	7320
Buffer in µl	750	1500	3000
30% Acrylamid in µl	390	780	1560
10% SDS in µl	30	60	120
10% APS in µl	15	30	60
100% TEMED	5	10	20

Table 17: Contents and amounts of reagents for stacking gels

3.3.4.4 Gel electrophoresis

For gel electrophoresis, 10-20 µg proteins were used per lane. First the protein solution was mixed with loading buffer (1:5) and incubated at 95^0C for 10 minutes in a heating chamber. In this step, proteins were denaturized meaning they lost most of their secondary and tertiary structure which increases binding of antibodies to the protein. After denaturation, proteins were applied to the SDS-PAGE gel. In the first gel pocket the protein standard (whose size was known) Dual Colour Standard from Bio-Rad was pipetted. The negatively charged proteins migrated from top to bottom through the polyacrylamide gel and separated according to their molecular weight. The gel was run for about 1 h at 150 V and 45 mA.

3.3.4.5 Blotting

The separated proteins were transferred from the gel to nitrocellulose membranes. First two blotting papers, the membrane, the gel containing protein and then two blotting papers were soaked with transfer buffer and placed one after the other in a semidry Blotter from Biometra whose bottom layer was connected to the anode. Then the blotting chamber was covered which was connected to the cathode. The chamber was run for 50-60 minutes at 110V and 400mA. After the blotting procedure, the membrane was exposed to Ponceau S solution to check the protein bands as well as whether the transfer process was carried out well. The membrane was washed several times with T-TBS.

3.3.4.6 Blocking

The membrane was then placed in a plastic box for one hour with 3-5% milk powder or 3-5% BSA in TBS-T on a shaker (40-60 rpm) at room temperature. This step was done to prevent unspecific binding of the antibody.

3.3.4.7 Protein labeling and detection

After blocking, the membrane was incubated with the first antibody (diluted with 0.3-5% milk powder or 0.3-5% BSA in TBS-T) which was specific to the protein of interest for 15 hours at 4^0C on a shaker (40-60 rpm). Then the membrane was washed three times with T-TBS, each time 5 minutes on the shaker (40-60 rpm) to get rid of the unbound antibody. Subsequently, the secondary antibody (diluted with 0.3-5% milk powder or 0.3-5% BSA in TBS-T) conjugated with HRP was added and incubated for one hour on a shaker (40-60 rpm) at room temperature. Then the membrane was washed three times with T-TBS, each time 5 minutes on the shaker (40-60 rpm) to get rid of the unbound antibody from the membrance. The membrane then was incubated with a chemiluminescence solution (Amersham hyperfilm TM ECL) according to the manufacturers' protocol for 1 minutes on a shaker (40-60 rpm). Then protein bands were detected by The ImageQuant LAS 4000 (GE Health Care, München) or a film.

3.3.4.8 Membrane stripping

To be able to use the same membrane for detection of other proteins, the membrane was incubated with 7 ml stripping buffer (Thermo Scientific) on a shaker on a water bath at 30^0C for 5-7 minutes. Then the membrane was washed three times with T-TBS, each time for 5 minutes on the shaker (40-60 rpm). ECL solution was added to the membrane and checked by ImageQuant to ensure that membranes were washed well. Then the membrane was blocked and incubated with antibodies as described above.

3.3.5 Enzyme-linked immunosorbent assay

In order to check secretion of cytokines in supernatants of lmyphocyte cultures, the ELISA technique was performed. Flat bottom 96 well plates were coated with unlabeled capture antibodies (primary antibody of interest, see table 9) diluted with coating buffer at 1:200 ratio over night at 4^0C. Wells were washed 4 times. Plates

were incubated for 1 hour at room temperature with assay diluent 200µl per well to block non-specific binding sites. Wells were washed 4 times. Samples were diluted (1:10 or 1:100) with assay diluent and plated as 100µl per well. Plates were sealed and incubated at room temperature for 2 hours or overnight at 4^0C. Wells were washed 4 times. Plates were incubated with detection antibody diluted with assay diluent at 1:200 ratios for 1 hour at room temperature. Wells were washed 4 times. Diluted enzyme conjugate streptavidin-HRP (optimal concentration was pre-determined) with assay diluent was added to the plate at 100µl/well. Plates were sealed and incubated for 30 minutes at room temperature. Wells were washed 5 times this time. 100µl of TMB solution (substrate) were plated per well and incubated for 20-30 minutes for color development. To stop the color reaction, 50µl stop solution 2N H2SO4 was added to each well. After 15 minutes, optical density for each well was measured with a microplate reader at 450nm.

3.3.6 Protein array for detection of protein phosphorylation

Protein arrays are rapidly becoming established as a powerful means to identify protein–protein interactions, to check protein phosphorylation, to identify the substrates of proteins kinases, to identify transcription factor protein-activation, or to identify the targets of biologically active small molecules. To locate reactive proteins on a proteome chip, small molecule probes are labelled with either fluorescent, affinity, photochemical, or radioisotope tags. Fluorescent labels are generally preferred, as they are safe and effective and are compatible with readily available microarray laser scanners.

In this thesis, protein microarrays were used to characterize the phosphorylation status of proteins obtained from polarized T cells. Using the TGF-β signalling phospho-specific antibody microarray, (Fullmoon Biosystems, Sunnyvale, USA), a total of 44 proteins can be detected both in phosphorylated and non-phosphorylated form, 3 proteins can be detected only in phosphorylated form and 18 proteins can be detected only in the non-phosphorylated form by Streptavidin- Cy3 (Appendix IV: Table 22 and 23). All proteins have 6 replicates. The process of protein detection is based on the principle of a sandwich ELISA method.

3.3.6.1 Protein Extraction from Th17 differentiated T cells

Naive $CD4^+CD62L^+$ T cells were sorted by magnetic separation as described in **3.2.3** from female Smad7Tg mice with C57BL/6 genetic background and from female WT-C57BL/6 mice. Subsequently, naïve T cells were polarized to Th0 and Th17 phenotypes. After 48 hours, cells were harvested and washed three times with ice cold PBS in 1.5 ml eppendorf tubes at 1,000 g for 10 minutes at 4^0C. Supernatants were collected to measure the IL-17 production to check whether Th17 differentiation worked. IL-17 production was also checked by flow cytometry. Protein extraction buffer (RIPA buffer contained 1 tablet of each phospho and protease inhibitor per 10 ml) was added to the tubes at a concentration of 200 µl/$5x10^6$ cells. Pipetting up and down was done to mix the cell pellet and solution uniformly. Subsequently, a 1 ml syringe with a 20 Gauge needle was also used to mix the solution well until a clear viscous and homogenous solution was viewed. Then the solution was incubated for half an hour on ice and vortexed at 5 minutes intervals, followed by spinning down at 14000 rpm for 15 minutes at 4^0C. Supernatant (proteins lie here) were collected both for array and western blot analysis. Protein concentration was measured by Bradford assay as described in **3.3.4.2.**

3.3.6.2 Reagent preparation

The following REAGENTS (from Fullmoon Biosystems, USA) were warmed prior to use

Blocking Reagent Coupling Reagent Wash buffer	25-30^0 C in a water bath.
Biotin Detection Buffer DMF Dry Milk Labelling Buffer Stop Reagent	Room temperature

Table 18: Reagents for phospho protein array

The washing solution was diluted 10 times with ddH_20 and mixed well. The blocking solution was made by adding 1.8g of Dry Milk to 60 ml ddH_20; the coupling solution was made by adding 0.36g of Dry Milk to 12 ml ddH_20.

3.3.6.3 Protein labelling

The Biotin reagent was centrifuged briefly before use. 100 µl of DMF (*N, N-Dimethylformamide*) was added to 1 mg of Biotin reagent to make a concentration of 10 µg/ul. Aliquots of the protein samples were adjusted to 10-25 µl containing 40-100 µg of proteins by vacuum speed. In all experiments, concentration of the proteins was always within the range of 2-10 µg/µl. 25-40 µl Labelling Buffer were added to the protein sample to bring the volume to 50 µl. Subsequently, 1.5 µl of the Biotin/DMF solution was added to the protein samples. Protein samples were then mixed and incubated at room temperature for 2 hours and vortexed at 15 minutes intervals. Reaction was stopped by adding 25 µl of stop reagent and incubated for 30 minutes at room temperature with mixing or shaking.

3.3.6.4 Blocking of array slides

Antibody microarrays were warmed to room temperature for 30 to 45 minutes before usage. Slides were placed in 100x15 mm petri dishes and 30 ml of Blocking Solution was added. Each slide was submerged in one petri dish in the Blocking Solution having the barcode label of the slides faced up. Slides were incubated on an orbital shaker rotating at 55 rpm for 30 to 45 minutes at room temperature. Slides were then washed extensively with Milli-Q grade water by placing one slide into one 50 ml conical Falcon tube filled with 45 ml water and vigorous shaking with hands for 10 seconds. Tubes were filled with fresh water after pouring off the previous water and washed again. Washing was repeated 5 to 10 times to ensure that any blocking solution residues were removed from the slide surface. Excessive water on the slide surface was removed by shaking off and by tipping the edge of the slide on a paper towel. The next step was started immediately so that slides did not dry out completely.

3.3.6.5 Coupling

6 ml of Coupling Solution and the biotin labelled proteins (40-100 µg) were placed into a 15 ml tube per protein samples and vortexed briefly to mix. Each slide was placed in an individual well of the Coupling Chamber, with the arrays facing up. 6 ml of protein coupling Solution were slowly poured over the slide and mixed to ensure that the slides were completely submerged. The Coupling Chamber was covered and incubated on an orbital shaker rotating at 35 rpm for 1-2 hours at room temperature.

After incubation, slides were transferred to each 100x15 mm Petri dish containing 30 ml of 1x Wash Solution and incubated on an orbital shaker rotating at 55 rpm for 10 minutes at room temperature. The washing solution was discarded. This step was repeated twice. Slides were then washed extensively with Milli-Q grade water by placing one slide into one 50 ml conical Falcon tube filled with 45 ml of water by inverting the tube up and down or shaking by hand for 10 seconds. Tubes were filled with fresh water after pouring off the previous water and washed again. Washing was repeated 5 to 10 times. Excessive water on the slide surface was removed by shaking off and by tipping the edge of the slide on a paper towel. The next step was started immediately so that slides did not dry out completely.

3.3.6.6 Detection

Cy3-Streptavidin (0.5 mg/ml) 60 µl was added to 60 ml of Detection Buffer. 30 ml of the Cy3-Streptavidin solution was poured into a 100x15 mm Petri dish per array slide. Then each array slide was submerged in the Cy3-Streptavidin solution and incubated on an orbital shaker rotating at 55 rpm for 30 to 45 minutes at room temperature in the dark or covered with aluminium foil. After incubation, slides were transferred to each 100x15 mm Petri dish containing 30 ml of 1x Wash Solution and incubated on an orbital shaker rotating at 55 rpm for 10 minutes at room temperature. The washing solution was discarded. This step was repeated twice. Slides were then washed extensively with Milli-Q grade water by placing one slide into one 50 ml conical Falcon tube filled with 45 ml of water by shaking with hand for 10 seconds. Tubes were filled with fresh water after pouring off the previous water and washed again. Washing steps were repeated 5 to 10 times. Slides were kept in 50 ml tube, put in the eppendorf centrifuge 5810R and spun down at 450 g for 1 minute at room temperature to dry out the slides to make them ready for scanning.
Slides were scanned with Axon GenePix 4400A microarray scanner (Department of Pathology, University of Regensburg). Each slide was analysed by the software GenePix Pro 7126 at 760nm wavelength and raw data were produced by the software in gpr files according to the GAL file (provided by Fullmoon Biosystems). The following raw data were provided for each spot of the array (= specific proteins and positive and negative controls):

1. Median intensity of fluorescence
2. Mean intensity of fluorescence
3. Median intensity of background of the slide
4. Mean intensity of background of slide
5. Median intensity of fluorescence subtracted from median intensity of background of slide
6. Mean intensity of fluorescence subtracted from mean intensity background of slide
7. Furthermore, the whole image was provided in jpeg form.

3.3.6.7 Analysis of raw data

Processing of samples

For analysis of protein phosphorylation, the median fluorescence intensity of array spots was used. First, the median background signal was subtracted from the median fluorescence intensity for each spot. Any negative values with a background signal higher than the foreground signal were set to zero. Next, potential outliers from the 6 replicate samples that were statistically inconsistent with the other replicates (e. g., due to an artefact) were identified using Z score with α=0.05. The Z score was calculated as the difference between the potential outlier value y_i and the mean of the replicates y_{mean} divided by the standard deviation SD.

$$Z = \frac{(y_{mean} - y_i)}{SD}$$

Measurements with Z= >1.96 or <-1.96 were excluded from further analysis.

Finally, the adjusted mean of 6 replicates were computed after the outliers were removed.

Normalization of arrays

To be able to compare the median fluorescence signals of different arrays (e.g. comparison between an experimental stimulation on one array to the control stimulation on a second array), normalization was done. The total median intensity of all positive values (= values with median fluorescence intensity higher than the background signal) from individual arrays were calculated. The signal intensities of

phospho and non phospho proteins were normalized to the respective total median intensity of each array:

1. Non-P $Th0_{norm}$ = non-phosphorylated proteins Th0 / total median intensity of all proteins on Th0 array
2. P $Th0_{norm}$ = phosphorylated proteins Th0 / total median intensity of all proteins on Th0 array
3. Non-P $Th17_{norm}$ = non-phosphorylated proteins Th17 / total median intensity of all proteins on Th17 array
4. P $Th17_{norm}$ = phosphorylated proteins Th17 / total median intensity of all proteins on Th17 array

P and Non-P stands for phosphorylated and non-phosphorylated protein respectively.

Calculation of protein ratios to detect protein expression and phosphorylation status:

The following ratios were calculated to measure the degree of protein expression and protein phosphorylation in control and experimental samples:

A. Non-phosphorylated protein ratio (Th17/Th0) = (Non-P $Th17_{norm}$ x 100) / Non-P $Th0_{norm}$

B. Phosphorylated protein ratio (Th17/Th0) = (P $Th17_{norm}$ x 100) / P $Th0_{norm}$

C. Signal ratio of phosphorylated protein to non-phosphorylated protein (Th0)= P $Th0_{norm}$ / Non-P $Th0_{norm}$

D. Signal ratio of phosphorylated protein to non-phosphorylated protein (Th17)= P $Th17_{norm}$ / Non-P $Th17_{norm}$

E. Signal ratio of phosphorylated protein to non-phosphorylated protein (Th17 / Th0) = D (Signal ratio of phosphorylated protein to non-phosphorylated protein Th17) / C (Signal ratio of phosphorylated protein to non-phosphorylated protein Th0)

$$phosphorylation\text{-}ratio = \frac{phospho_{experiment}}{unphospho_{experiment}} \Big/ \frac{phospho_{control}}{unphospho_{control}}$$

Ratios >150% were defined as an increase of protein concentration (equation A, B) or phosphorylation (equation C, D), ratios of <50% as decrease of protein concentration or dephosphorylation, respectively. Ratio between 50% and 150% were defined as no change. Calculation E finally was not used because of the high

variance of the phosphorylation ratio of proteins between different experiments detected.

After calculation of the protein ratios A-D, 10 categories were made to describe the expression and phosphorylation status of proteins during Th17 differentiation. They were as follows:

1	Both Non-P and P proteins absent
2	Non-P>P proteins, Th0=Th17
3	Non-P>P proteins, only in Th17 or Th17>Th0
4	Non-P>P proteins, only in Th0 or Th0>Th17
5	Non-P=P proteins, Th0= Th17
6	Non-P= P proteins, only in Th17 or Th17>Th0
7	Non-P= P proteins, only in Th0 or Th0>Th17
8	P> Non-P proteins, Th0= Th17
9	P> Non-P proteins, only in Th17 or Th17>Th0
10	P> Non-P proteins, only in Th0 or Th0>Th17

Table 19. Categories to describe the expression and phosphorylation status of proteins during Th0 and Th17 differentiation. P stands for phosphorylation.

3.4 Statistical calculations

To test for statistical significance of differences between two subpopulations, Student's *t*-test was performed. One way ANOVA was performed by the Tukey's multiple comparisons test to identify differences between individual subpopulations. A value of $P<0.05$ was considered statistically significant. All statistical tests were performed either with Microsoft excel or SigmaStat 3.0 software (SPSS Inc.) or GraphPad Prism 5.

4. Results

4.1 Characterization of mice with a T cell specific deletion of Smad7

TGF-β has an essential role in T cell homeostasis (Li *et al*. 2006b). To elucidate the effects of a deletion of the TGF-β signalling inhibitor Smad7 in T cells on the development, maturation and differentiation of T cells, we carried out flow cytometric analyses from naïve CD4Cre-Smad7$^{fl/fl}$ (Kleiter *et al.* 2010) and control mice.

4.1.1 Thymic T cell development

TGF-β produced by thymic epithelial cells limits the development progression of CD4$^-$ CD8$^-$ double negative thymocytes to CD4$^+$CD8$^+$ double positive thymocytes (Suda and Zlontnik 1992, Plum 1995). We hypothesized that enhanced TGF-β signalling in T cells with a Smad7 deletion might alter T cell development. To this point, frequencies of CD4$^+$ and CD8$^+$ T cells were checked. No difference was found regarding the distribution of double positive (CD4$^+$CD8$^+$) and single positive CD4$^+$ T cells, but the maturation of CD8$^+$ thymocytes was around two fold increased in CD4Cre-Smad7$^{fl/fl}$ mice as compared to control mice (Figure 8).

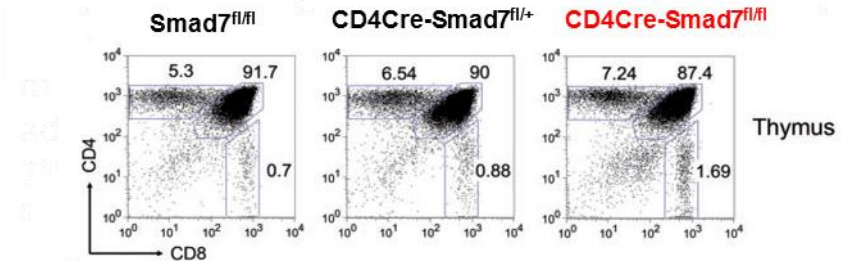


Figure 8: Thymic T cell development in mice with a T cell-specific Smad7 deletion. *Ex vivo* thymocytes were collected from naïve Smad7$^{fl/fl}$, CD4Cre-Smad7$^{fl/+}$ and CD4Cre-Smad7$^{fl/fl}$ mice and were surface stained to check the frequency of CD4$^+$ and CD8$^+$ T cells present in the thymus. The figure shows cell populations as CD4$^+$ vs CD8$^+$ T cells gated on live thymocytes. The different T cell populations are characterized by CD4$^-$CD8$^-$ for double negative, CD4$^+$CD8$^+$ for double positive, CD4$^+$CD8$^-$ for single positive CD4 T cells and CD4$^-$CD8$^+$ for single positive CD8 T cells. The percentage of gated cells is indicated. Three female mice of 6-8 weeks age were used per group. The figure is representative of 3 independent experiments with similar results.

4.1.2 Distribution of T cell receptor positive CD4$^+$ and CD8$^+$ T cells in the thymus

Stimulation of the T cell receptor (TCR) is required during T cell development in particular when cells are differentiating to be single positive cells such as CD4$^+$ or

CD8$^+$ T cells. To investigate whether Smad7 has a role on altered TCR expression, *ex vivo* cells from mice were surface stained with TCR-β, CD4 and CD8. There was no difference found in the expression of TCR-β on CD4$^+$ and CD8$^+$ cells between CD4Cre-Smad7$^{fl/fl}$ mice and controls (Figure 9). Summarized results of 3 independent experiments are shown in Table 20.

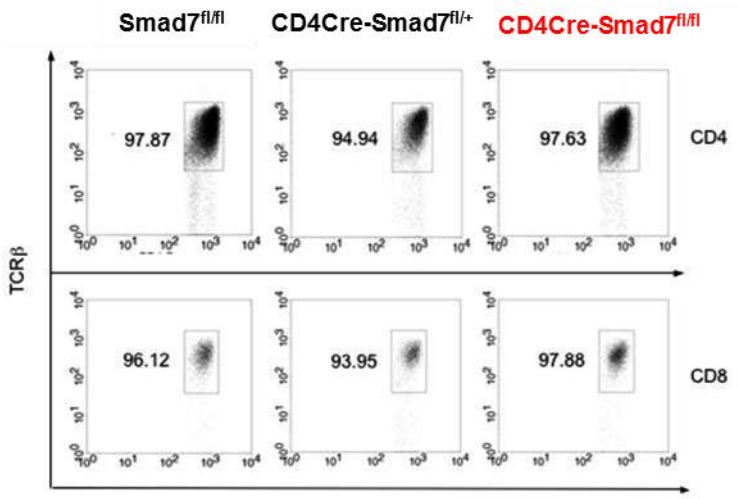


Figure 9: TCRβ expression of thymic CD4$^+$ and CD8$^+$ T cells in mice with a T cell-specific Smad7 deletion. *Ex vivo* thymocytes were collected from naïve Smad7$^{fl/fl}$, CD4Cre-Smad7$^{fl/+}$ and CD4Cre-Smad7$^{fl/fl}$ mice and were surface stained for TCRβ, CD4 and CD8. Cells were gated on live CD4$^+$ and CD8$^+$ thymocytes. This figure shows CD4$^+$ vs TCRβ$^+$ T cells and CD8$^+$ vs. TCRβ$^+$ T cells. The percentage of gated cells is indicated. Three female mice of 6-8 weeks age were used per group. The figure is representative of 3 independent experiments with similar results.

4.1.3 Development of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells

Both naturally occurring regulatory T cells (nTreg), which are derived from the thymus (Sakaguchi *et al.* 2008) and inducible regulatory T cells (iTregs) from the periphery (Baecher-Allan *et al*. 2004) are defined by the surface markers CD4$^+$, CD25$^+$ and the intranuclear marker Foxp3$^+$. TGF-β converts CD4$^+$CD25$^-$ precursors to Treg cells by inducing Foxp3 transcription (Chen *et al.* 2003, Fu *et al.* 2004). So, it was worth to check whether Smad7 has a role in inducing Treg cells, since Smad7 is an inhibitor of TGFβ signalling. Therefore, *ex vivo* thymocytes and splenocytes from indicated mice were stained for the markers CD4 and CD25 and for the FoxP3. Deletion of Smad7 did not change the amount of Tregs both in the thymus and the spleen as compared to controls (Figure 10). Summarized results of 3 independent experiments are shown in Table 20.

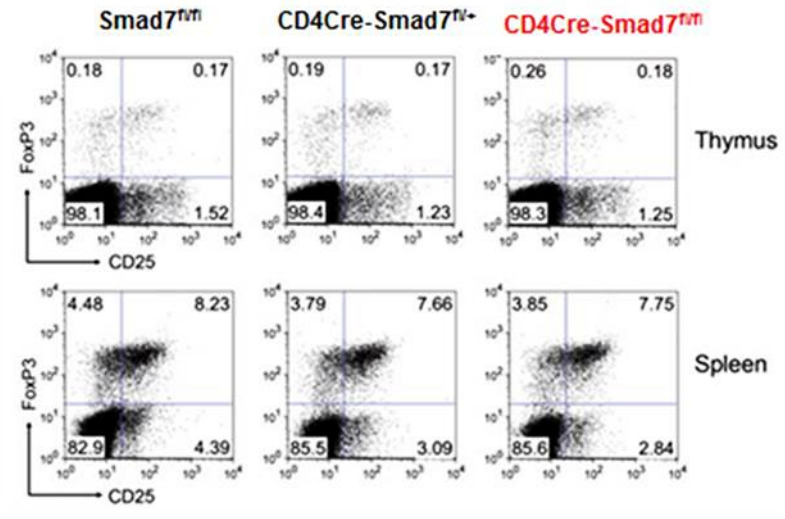


Figure 10: Frequencies of Foxp3$^+$ regulatory T cells in the thymus and spleen of mice with a T cell-specific Smad7 deletion. *Ex vivo* thymocytes and splenocytes were collected from naïve Smad7$^{fl/fl}$, CD4Cre-Smad7$^{fl/+}$ and CD4Cre-Smad7$^{fl/fl}$ mice and were stained for surface markers for CD4, CD25 and for the intranuclear marker FoxP3. Cells were gated on lymphocytes and CD4$^+$ T cells. The percentage of gated cells is indicated. Three female mice of 6-8 weeks age were used per group. The figure is representative of 3 independent experiments with similar results.

4.1.4 Distribution of T cells and B cells in the spleen

We also checked whether Smad7 influences the distribution of CD4$^+$ and CD8$^+$ T cells in the periphery. No difference was found in the distribution of CD4$^+$ and CD8$^+$ cells in the periphery between naïve CD4Cre-Smad7$^{fl/fl}$ mice and controls (Figure 11). Summarized results of 3 independent experiments are shown in Table 20.

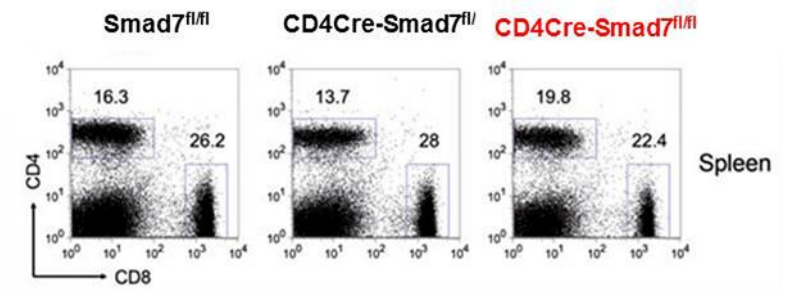


Figure 11: Frequencies of CD4$^+$ and CD8$^+$ T cells in the periphery of mice with a T cell-specific Smad7 deletion. *Ex vivo* splenocytes were collected from naïve Smad7$^{fl/fl}$, CD4Cre-Smad7$^{fl/+}$ and CD4Cre-Smad7$^{fl/fl}$ mice and were surface stained for CD4 and CD8. Cells were gated on live splenocytes CD4$^+$ vs CD8$^+$. The percentage of gated cells is indicated. Three female mice of 6-8 weeks age were used per group. The figure is representative of 3 independent experiments with similar results.

Distribution of T and B cell in the periphery was also checked. *Ex vivo* splenocytes from CD4Cre-Smad7$^{fl/fl}$ and control mice were stained with the surface markers CD19 (B cell marker) and CD90.2 (T cell marker). The deficiency of Smad7 did not

influence the frequencies T and B cells or the T to B cells ratio in the spleen (Figure 12).

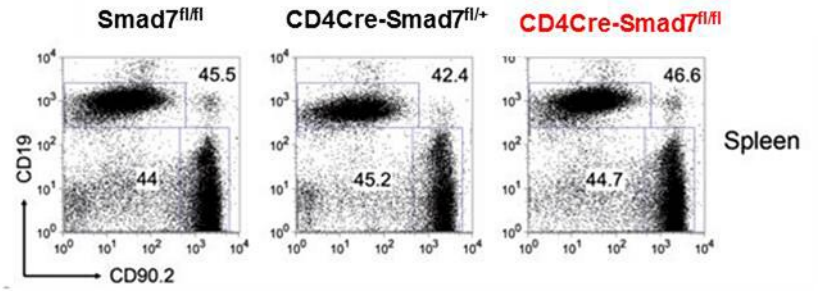


Figure 12: Frequencies of T and B cells in the spleen of mice with a T cell-specific Smad7 deletion. *Ex vivo* splenocytes of naïve Smad7$^{fl/fl}$, CD4Cre-Smad7$^{fl/+}$ and CD4Cre-Smad7$^{fl/fl}$ mice were stained for the surface markers B220 and CD90.2. Gate was set on live lymphocytes. The percentage of gated cells is indicated. Three female mice of 6-8 weeks age were used per group. The figure is representative of 3 independent experiments with similar results.

4.1.5 Activation status of T cells in the periphery

TGFβ regulates proliferation and the activation status of T lymphocytes (Rubtsov and Rudensky 2007). To check whether deletion of Smad7 in T cells influences the activation status, *ex vivo* cells from lymph nodes were stained with the surface markers CD69, CD4 and CD8. The molecule CD69 is not expressed on the surface of peripheral resting T cells, but is found on activated T cells (Testi, D'Ambrosio *et al.* 1994). Frequencies of activated CD4$^+$ and CD8$^+$ T cells were significantly reduced in the periphery of CD4Cre-Smad7$^{fl/fl}$ mice as compared to controls (Figure 13). Summarized results of 3 independent experiments are shown in Table 20.

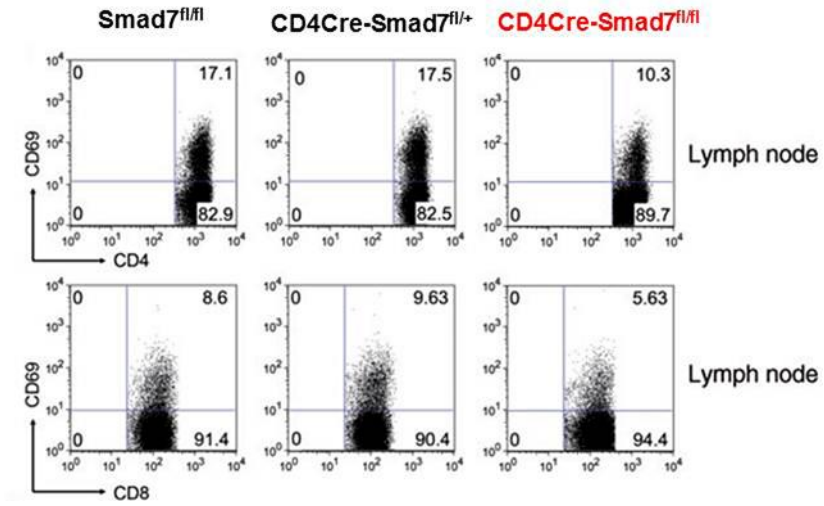


Figure 13: Frequencies of activated T cells in the periphery of mice with a T cell-specific Smad7 deletion. Cells derived from lymph nodes of naïve mice with the genotype Smad7$^{fl/fl}$, CD4Cre-Smad7$^{fl/+}$ and CD4Cre-Smad7$^{fl/fl}$ were stained with the surface markers CD69, CD4 and CD8. Live cells were gated on CD4 or CD8 single positive cells. This figure shows CD69 vs. CD4 or CD8 positive cells. The percentage of gated cells is indicated in the figure. Activated T cells are positive for CD69. Three female mice of 6-8 weeks age were used per group. The figure is representative of 3 independent experiments with similar results.

4.1.6 Distribution of naïve and memory T cells

Upon activation, naïve T cells (CD4$^+$CD44^{low}CD62L$^+$) become memory T cells (CD4$^+$CD44$^+$CD62L^{low}). In order to check whether altered Smad7 expression affects the frequencies of naïve and memory T cells, lymphocytes from lymph nodes were stained with the markers CD62L and CD44. The frequency of naïve T cells and memory T cells was unaltered in CD4Cre-Smad7$^{fl/fl}$ mice as compared to controls (Figure 14).

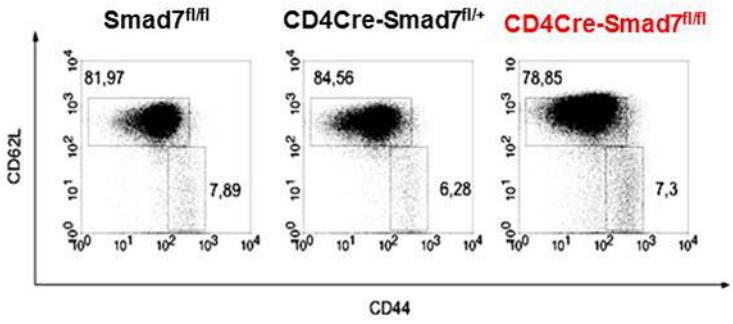


Figure 14: Frequencies of naïve and memory T cells in the periphery of mice with a T cell-specific Smad7 deletion. *Ex vivo* cells from lymph nodes of naïve Smad7$^{fl/fl}$, CD4Cre-Smad7$^{fl/+}$ and CD4Cre-Smad7$^{fl/fl}$ mice, were stained with the surface markers CD4, CD44 and CD62L. The gate was set on live CD4$^+$ T cells. The percentage of gated cells is indicated. Three female mice of 6-8 weeks age were used per group. The figure is representative of 3 independent experiments with similar results.

Lymphocyte population frequencies.

Genotype	SP CD4$^+$	SP CD8$^+$	DP CD4$^+$CD8$^+$	CD4$^+$TCR$^+$	CD8$^+$TCR$^+$	CD4$^+$ CD8$^+$TCR$^+$	CD4$^+$FoxP3$^+$	CD4$^+$CD69$^+$	CD8$^+$CD69$^+$
Smad7$^{fl/fl}$	7.21 ± 0.57	1.27 ± 0.11	89.41 ± 0.80	98.19 ± 0.27	94.23 ± 2.22	3.11 ± 0.49	0.48 ± 0.18	11.67 ± 1.87	8.05 ± 1.81
CD4Cre-Smad7$^{fl/+}$	6.00 ± 1.05	1.13 ± 0.28	90.58 ± 1.44	95.48 ± 2.05	92.88 ± 2.97	3.35 ± 0.45	0.35 ± 0.00	10.57 ± 1.50	7.10 ± 1.31
CD4Cre-Smad7$^{fl/fl}$	6.35 ± 0.78	1.51 ± 0.27	89.43 ± 1.33	97.35 ± 1.15	93.80 ± 4.27	3.43 ± 0.66	0.47 ± 0.19	11.41 ± 3.33*	7.90 ± 2.59*

Table 20: Lymphocyte population frequencies. Thymocyte populations from 8-wk-old CD4Cre-Smad7$^{fl/fl}$ and control mice (n=4-7) were measured by flow cytometry for the indicated surface markers adn are shown % of total thymocytes. Results are presentated as mean ± SD. * *P* <0.05 (ANOVA):

4.2 The role of T cell Smad7 in regulatory T cell function

4.2.1 Does the expression level of Smad7 influence the suppressive capacity of regulatory T cells?

It is well established that TGF-β is an immunosuppressive cytokine involved in the regulatory properties of Tregs (Nakamura *et al.* 2004, Maloy *et al.* 2003). By using transgenic CD2-ΔkTβRII mice it was shown that TGF-beta signalling is required for the in vivo expansion and for the maintenance of the immunosuppressive capacity of regulatory $CD4^+CD25^+$ T cells (Huber *et al*. 2004). Another study showed that TGF-β signalling is essential for maintenance of the suppressive function of $CD4^+CD25^+$ Tregs *in vitro and in vivo*, (Su *et al*. 2008). However, it was also reported that in TGF-$β1^{-/-}$ mice, $CD4^+CD25^+$ Tregs were able to develop up to an age of 2 weeks and that autocrine TGF-β1 production was not essential for these cells to exhibit suppressive activity *in vivo* (Mamura *et al.* 2004). Furthermore, because of the rapid onset of inflammation in TGF-$β1^{-/-}$ mice (Shull *et al.* 1992), it is difficult to study Treg cell function over time in TGF-β1-/- mice. T cells from CD4Cre-$Smad7^{fl/fl}$ mice produce more TGF-β upon TCR stimulation (Kleiter *et al*. 2010) which could increase the suppressive function of Tregs. So, comparing the function of Tregs from WT mice with Tregs from CD4Cre-$Smad7^{fl/fl}$ and Smad7Tg mice should be helpful to characterize the role of TGF-β signalling in the suppressive capacity of Tregs.

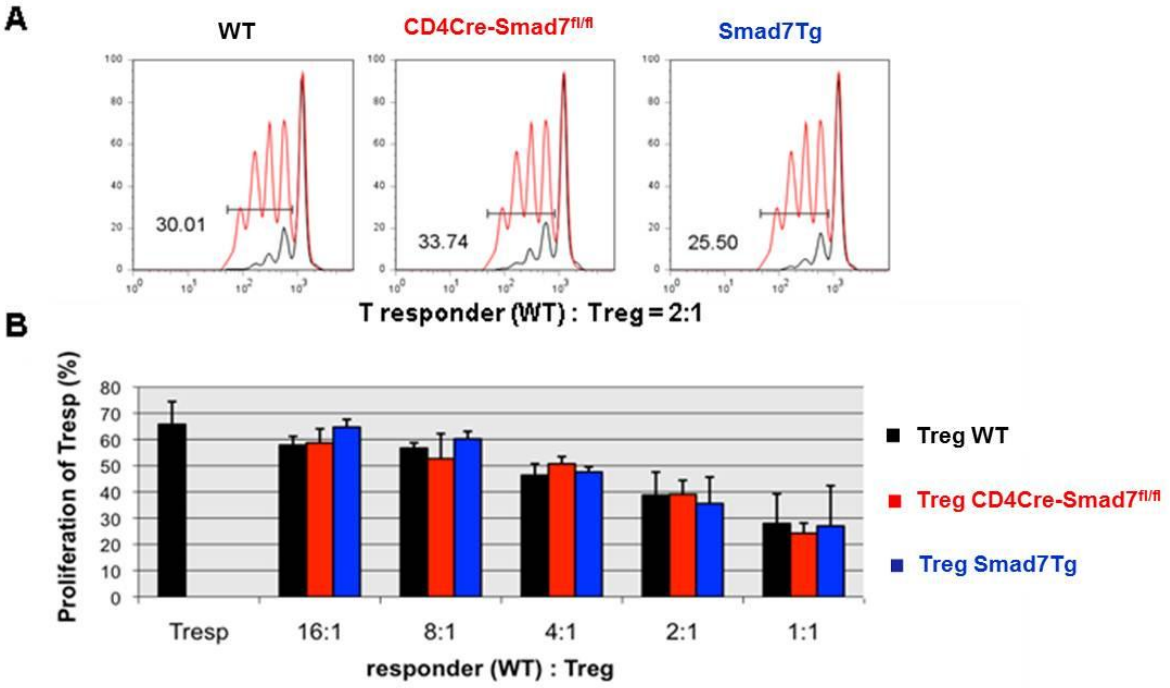


Figure 15: Altered Smad7 expression does not influence the functional capacity of regulatory T cells. T responder cells ($CD4^+CD25^-$) from WT and Treg cells ($CD4^+CD25^+$) from WT, CD4Cre-$Smad7^{fl/fl}$ and Smad7Tg mice were sorted as described in methods and were co-cultured at different ratios as indicated for 3 days, stimulated with antigen presenting cells and anti-CD3 (0.5µg/ml). T responder cells were labelled with CFSE before putting them in the culture. On day 4^{th}, cells were harvested, stained with the surface markers CD4 and CD25 and propidium iodide (PI) which is a marker to distinguish dead cells was added to each samples few minutes before the samples were measured by flow cytometry. Cells were gated on $CD4^+$ which was gated on live (PI negative) cells. $CD25^+$ (Treg marker) was used to track down the frequency of Tregs present in each condition (not shown). In the co-culture experiment, number of T responder cells was constant. Tregs were diluted to the indicated ratio. (A) The histogram shows one representative figure of CFSE staining of T responder cells of all co-culture experiments. Black and red lines show the proliferation of T responder cells after 24 hours and on day 3, respectively. (B) A summary of three experiments is shown. For each experiment, 6 female mice of 6-8 weeks age were used from WT to obtain Tregs and responder T cells and 4 female mice of 6-8 weeks age were used per other two genotypes to obtain Tregs. Values represent the mean ± SEM of 3 experiments.

An allogeneic co-culture was made to check whether an altered expression level of Smad7 influences the suppressive function of Tregs on WT responder T cell proliferation. Without Tregs about 70% activated T responder cells were proliferating after 3 days. With increasing number of Tregs the proliferation of T responder cells was downregulated, meaning Tregs were suppressing responder T cell proliferation in general. Like WT Tregs, Tregs with a Smad7 deletion (CD4Cre-$Smad7^{fl/fl}$) or overexpression (Smad7Tg) had the same suppressive capacity (Figure 15B). Furthermore, there was no difference in TGF-β production in the supernatants of mixed lymphocyte reactions with Tregs from the three different genotypes and WT

responder T cells (data not shown). This experiment showed that Smad7 does not influence the capacity of Tregs to suppress T cell proliferation.

4.2.2 Does the expression level of Smad7 determine the proliferation of responder T cells in mixed lymphocyte reactions?

Enhanced Smad7 protein expression was found to be critical in EAE in SJL/J and B6 mice and in effector T cells upon antigen stimulation (Kleiter *et al.* 2007). Systemic treatment of mice with Smad7-antisense oligonucleotides downregulates Smad7 mRNA expression levels in *ex-vivo*-treated primary mouse lymph node cells (LNC) and causes a reduction of antigen specific proliferation and encephalitogenicity of lymph node cells. It was also reported that responder T cells from Smad7Tg mice show a higher proliferation as compared to WT responder T cells in response to Treg suppression in vitro (Fantini *et al.* 2009). Moreover, T cells not able to respond to TGF-β1 due to an overexpression of the dominant negative form of the TGF-β receptor II (dnTGF-βRII) are resistant to Treg-mediated suppression (Fahlen *et al.* 2005). Thus, we wanted to check whether altered expression of the TGF-β inhibitor Smad7 influences the proliferation of responder T cells in mixed lymphocyte reactions with Tregs. An allogeneic co-culture with T responder cells from WT, CD4Cre-Smad$^{fl/fl}$, or Smad7Tg mice and Tregs from WT mice was made. T responder cells from Smad7Tg mice seemed to proliferate more, but the difference was not statistically significant (Figure 16A). Interestingly, Smad7Tg responder T cells already proliferated without the addition of Tregs to a high extent than control T responder cells, arguing that a general hyper-proliferation, rather than a specific inhibition of Treg suppression took place. It is also worth to notice that Smad7 deficient T responder cells did not show a higher suppression than WT T responder cells, which suggests that either TGF-β is not the main suppressive effector mechanism of Tregs or if TGF-β is needed for the effector mechanism of Tregs, it does not signal through the Smad signalling cascade.

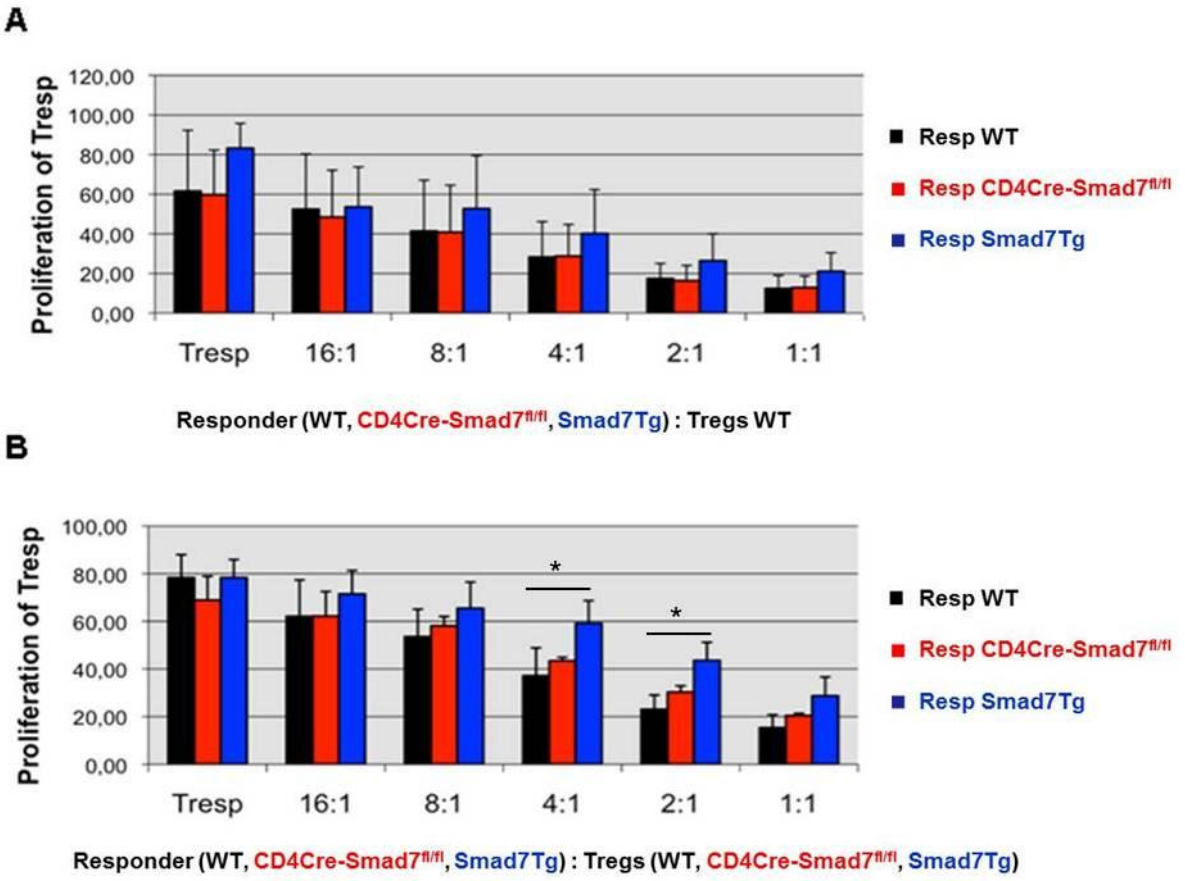


Figure 16: Expression of Smad7 controls the proliferation of responder T cells. T responder cells ($CD4^+CD25^-$) and Tregs cells ($CD4^+CD25^+$) sorted as described in methods were co-cultured at different ratios as indicated for 3 days, stimulated with antigen presenting cells and anti-CD3 (0.5µg/ml). T responder cells were labelled with CFSE before putting them in the culture. On day 4, cells were harvested, stained with the surface markers CD4 and CD25 and PI was added to each sample few minutes before the samples were measured by flow cytometry. Cells were gated on $CD4^+$ which was gated on live (PI negative) cells. $CD25^+$ (Tregs marker) was used to track down the frequency of Tregs present in each condition (not shown). Number of T responder cells was constant. Tregs were diluted to the indicated ratio. (A) A summary of three independent experiments was shown. Co-cultures were made with T responders from WT, CD4Cre-$Smad^{fl/fl}$ and Smad7Tg mice and Tregs from WT mice. (B) A summary of three independent experiments was shown. Co-cultures were made with T responders and Tregs both from WT, CD4Cre-$Smad^{fl/fl}$ and Smad7Tg mice. 8 female mice of 6-8 weeks age were used from WT and 6 female mice of 6-8 weeks age were used per other two genotypes. Values represent the mean ± SEM of 3 experiments. * P <0.05 (ANOVA).

Furthermore, co-cultures from responder and suppressor cells of one genotype were made, meaning WT T responder cells and Tregs; CD4Cre-$Smad^{fl/fl}$ T responder cells and Tregs; Smad7Tg T responder cells and Tregs. T responder cells from Smad7Tg mice showed a trend of higher proliferation compared to WT and CD4Cre-$Smad^{fl/fl}$ T responder cells but the difference between Smad7Tg and WT at 4:1 and 2:1 condition is statistically significant (Figure 16B). These results indicate that expression of Smad7 makes T responder cells resistant to the suppressive effect of

Tregs. Noticeably, Smad7 deficient T responder cells did not show a higher suppression than WT T responder cells which could mean either TGF-β is not required for Tregs suppressive function or TGF-β signals through other pathways than Smad signalling cascade.

4.3 In vitro T helper cell differentiation in T cells with altered Smad7 expression

4.3.1 Th1 differentiation.

As mentioned earlier, TGF-β suppresses Th1 differentiation by downregulating the expression of T-bet which is the transcription factor responsible for the induction of Th1 differentiation (Gorelik *et al*. 2002b). In order to determine whether Smad7, the negative regulator of TGF-β signalling, plays a role in Th1 differentiation, naïve $CD4^+CD62L^+$ T cells from WT, CD4Cre-$Smad7^{fl/fl}$ and Smad7Tg mice, were sorted and differentiated under Th1 polarizing conditions for 5 days with increasing concentrations of TGF-β. In cultures of WT T cells, IFN-γ production indicating Th1 differentiation was gradually reduced with increasing concentrations of TGF-β. Expression of IFN-γ and T-bet on the mRNA level was also reduced in CD4Cre-$Smad7^{fl/fl}$ and Smad7Tg Th1 cultures with addition of TGF-β (2ng/ml). Without addition of TGF-β, lowest IFN-γ production was detected in CD4Cre-$Smad7^{fl/fl}$ and highest in Smad7Tg Th1 cultures as compared to WT controls. There are two possibilities to explain this phenomenon found in CD4Cre-$Smad7^{fl/fl}$ T cell culture.

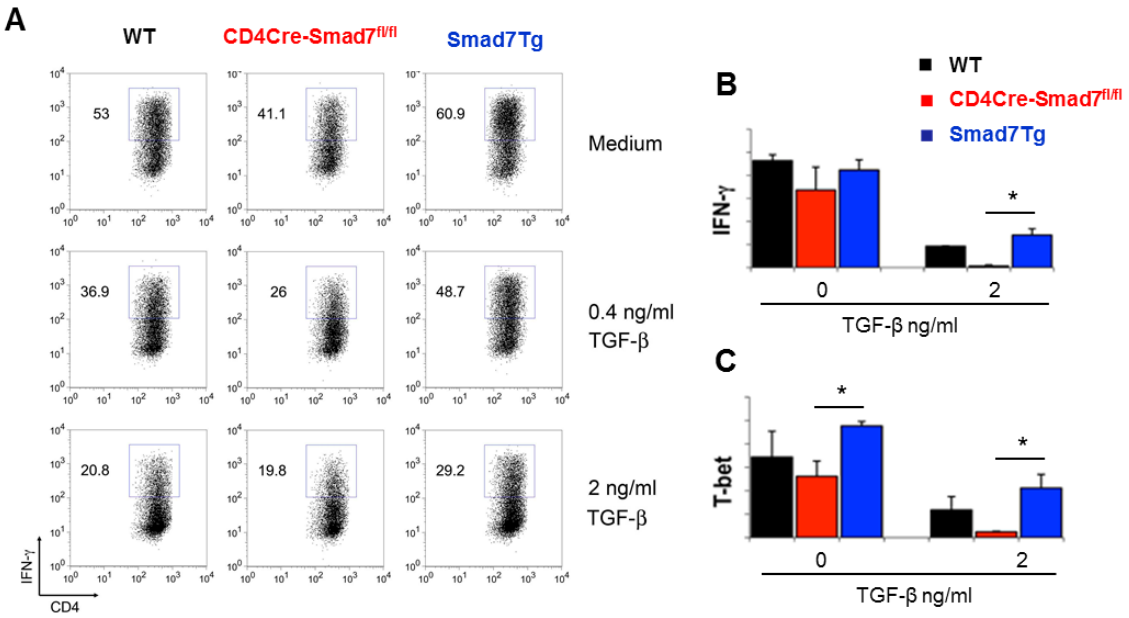


Figure 17: Decreased Th1 differentiation in CD4Cre-Smad7$^{fl/fl}$ T cell cultures. Naïve T cells from WT, CD4Cre-Smad7$^{fl/fl}$ and Smad7Tg mice were cultured under Th1 differentiating conditions using a-CD3, a-CD28, rIL-12, a-IL-4 with increasing concentrations of TGF-β for 5 days. Cells were restimulated with PMA, ionomycin and golgi stop for 4 hours. (A) The figure is representative of 3 independent experiments with similar result. After restimulation, cells were harvested, surface stained with the CD4 marker followed by intracellular staining for IFN-γ and analysed by flow cytometry. IFN-γ is the signature cytokine for Th1. Cells were gated on lymphocytes. The percentage of gated cells is indicated. (B and C) After 5 days of differentiation with and without TGF-β (2ng/ml) treatment, cells were harvested and RNA was isolated from these cells. Real Time PCR (RT-PCR) was performed to check the mRNA expression of IFN-γ and the transcription factor T-bet which induces Th1 differentiation. In RT-PCR, expression is presented relative to the 18S-RNA content and normalized on the unstimulated control. Three female mice of 6-8 weeks age were used per group. Values represent the mean ± SEM of 3 experiments. * *P* <0.05 (ANOVA).

Firstly, T cell medium containing TGF-β or TGF-β in the supernatant produced by T cells themselves could have downregulated IFN-γ production. Secondly, Smad7 could have an intrinsic direct role in promoting Th1 differentiation as highest IFN-γ production was detected in Smad7Tg T cell cultures. Lowest and highest expression of IFN-γ and T-bet was also detected on the mRNA level in the CD4Cre-Smad7$^{fl/fl}$ and Smad7Tg Th1 cultures respectively. Interestingly, Th1 cultures from all genotypes equally responded to TGF-β found in both flow cytometric analysis (Figure 17A) and real time PCR (Figure 17B and C) even if canonical TGF-β-Smad signalling cascade was blocked in Smad7Tg T cells which indicates TGF-β can also suppress Th1 differentiation through TGF-β-Smad independent pathways.

To investigate whether the increased or decreased susceptibility to TGF-β determined Th1 differentiation in Smad7-altered T cells or whether Smad7 had an

intrinsic property to regulate Th1 differentiation further experiment were done. Naïve $CD4^+CD62L^+$ T cells from wild type, CD4Cre-$Smad7^{fl/fl}$ and Smad7Tg mice, were sorted and differentiated under Th1 polarizing conditions for 5 days in serum free medium where TGF-β was absent, with or without a neutralizing anti-TGF-β antibody (5 µg/ml). IgG antibody was used as control. After addition of Th1-polarizing cytokines a higher frequency of activated $INF\gamma^+CD25^+$ T cells was found in cultures from Smad7Tg mice as compared to CD4Cre-$Smad7^{fl/fl}$ and WT control. After addition of a neutralizing TGF-β antibody, IFN-γ production was almost 2 fold increased in WT and CD4Cre-$Smad7^{fl/fl}$ T cells but remained unchanged in Smad7Tg T cells (Figure 18).

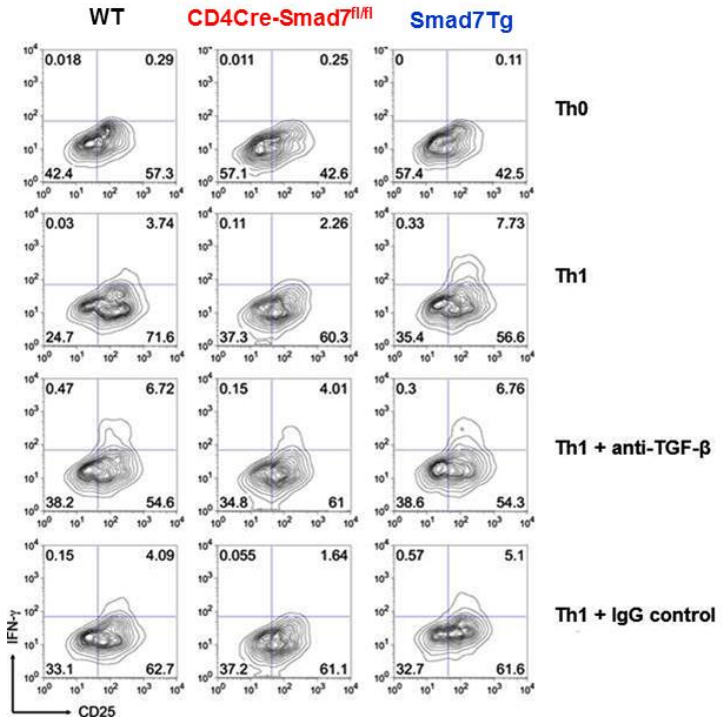


Figure 18: The expression level of Smad7 determines Th1 differentiation. Naïve T cells from WT, CD4Cre-$Smad7^{fl/fl}$ and Smad7Tg mice were cultured under Th1 differentiating conditions using a-CD3, a-CD28, rIL-12, a-IL-4 with or without anti-TGF-β (5µg/ml) for 5 days. Cells were then restimulated with PMA, ionomycin and golgi stop for 4 hours. Afterwards, cell were harvested, surface stained with CD4 and CD25 marker and finally intracellular staining for IFN-γ was performed and analysed by flow cytometry. Cells were gated on lymphocytes and $CD4^+$ T cells. 2 female mice of 6-8 weeks old per group were used. Data are representative of 2 independent experiments.

These results implicate that in addition to regulating the susceptibility towards TGF-β, Smad7 might have an intrinsic role in regulating Th1 differentiation, unrelated to the canonical TGF-β-Smad signalling pathway.

4.3.2 Th2 differentiation.

TGF-β suppresses Th2 differentiation by downregulating the expression of GATA-3 which is the transcription factor responsible for the induction of Th2 differentiation (Li *et al.* 2006a). In order to determine whether altered Smad7 expression plays a role in Th2 differentiation by regulating TGF-β signalling, naïve $CD4^{+}CD62L^{+}$ T cells from wild type, CD4Cre-$Smad7^{fl/fl}$ and Smad7Tg mice, were sorted and differentiated under Th2 polarizing conditions for 5 days with increasing concentrations of TGF-β. In WT T cell cultures, IL-4 production indicating Th2 differentiation was as expected gradually reduced with increasing concentrations of TGF-β (Figure 19). Without addition of TGF-β, the lowest IL-4 production was detected in CD4Cre-$Smad7^{fl/fl}$ and the highest in Smad7Tg T cell cultures as compared to WT controls. Two explanations are possible for this above phenomenon found in CD4Cre-$Smad7^{fl/fl}$ T cell cultures. First, TGF-β present in the T cell medium or produced by T cells which might downregulate IL-4 production. Alternatively, Smad7 might have an intrinsic direct role in promoting Th2 differentiation as highest IL-4 production was detected in Smad7Tg T cell cultures. Interestingly, like Th1 differentiated T cells, Th2 polarized T cells from all genotypes equally responded to TGF-β even when the canonical TGF-β-Smad signalling cascade was blocked in Smad7Tg T cells. This indicated that TGF-β can also suppress Th2 differentiation through TGF-β-Smad independent pathways.

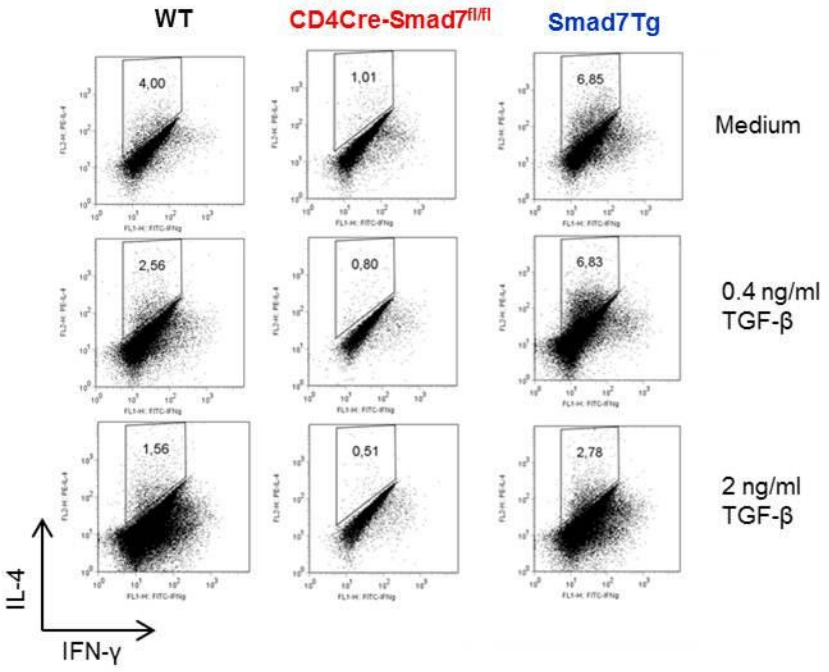


Figure 19: Decreased Th2 differentiation in CD4Cre-Smad7$^{fl/fl}$ T cell cultures. Naïve T cells from WT, CD4CreSmad7$^{fl/fl}$ and Smad7Tg mice were cultured under Th2 differentiating conditions using a-CD3, a-CD28, rIL-4, a-IFN-γ with increasing concentrations of TGF-β for 5 days. Cells were then restimulated with PMA, ionomycin and golgi stop for 4 hours. Afterwards, cells were harvested, surface stained with the CD4 marker and finally intracellular staining for IFN-γ and IL-4 was performed and cells analysed by flow cytometry. IL-4 is the signature cytokine for Th2. Cells were first gated on lymphocytes and then on $CD4^+$ T cells. The percentage of gated cells is indicated. 2 female mice of 6-8 weeks age were used per group. Data are representative of 2 independent experiments.

4.3.3 Th17 differentiation.

TGF-β along with IL-6 induces Th17 differentiation (Ivanov *et al.* 2006). Enhanced or reduced intracellular TGF-β-Smad signalling in T cells with Smad7 deletion or overexpression, respectively, might play a role in Th17 differentiation. To investigate this, naïve $CD4^+CD62L^+$ T cells from wild type, CD4Cre-Smad7$^{fl/fl}$ and Smad7Tg mice, were sorted and differentiated under Th17 polarizing conditions for 5 days with increasing concentrations of TGF-β.

T cells were polarized to a Th17 phenotype by adding TGF-β in all 3 genotypes, WT, CD4Cre-Smad7$^{fl/fl}$ and Smad7Tg (Figure 20A). Increased Th17 differentiation was found in CD4Cre-Smad7$^{fl/fl}$ T cell cultures and decreased Th17 differentiation in Smad7Tg T cell cultures as compared to WT cultures. Regarding mRNA expression, IL-17A and ROR-γt was also upregulated in CD4Cre-Smad7$^{fl/fl}$ T cell cultures (Figure 20B and C). Interestingly, Th17 differentiation was detected in Smad7Tg T cell cultures even though the TGF-β-Smad signalling cascade was blocked, which suggests that Th17 differentiation in Smad7Tg T cell culture was induced by TGFβ but not exclusively propagated by the canonical TGF-β-Smad signalling pathway.

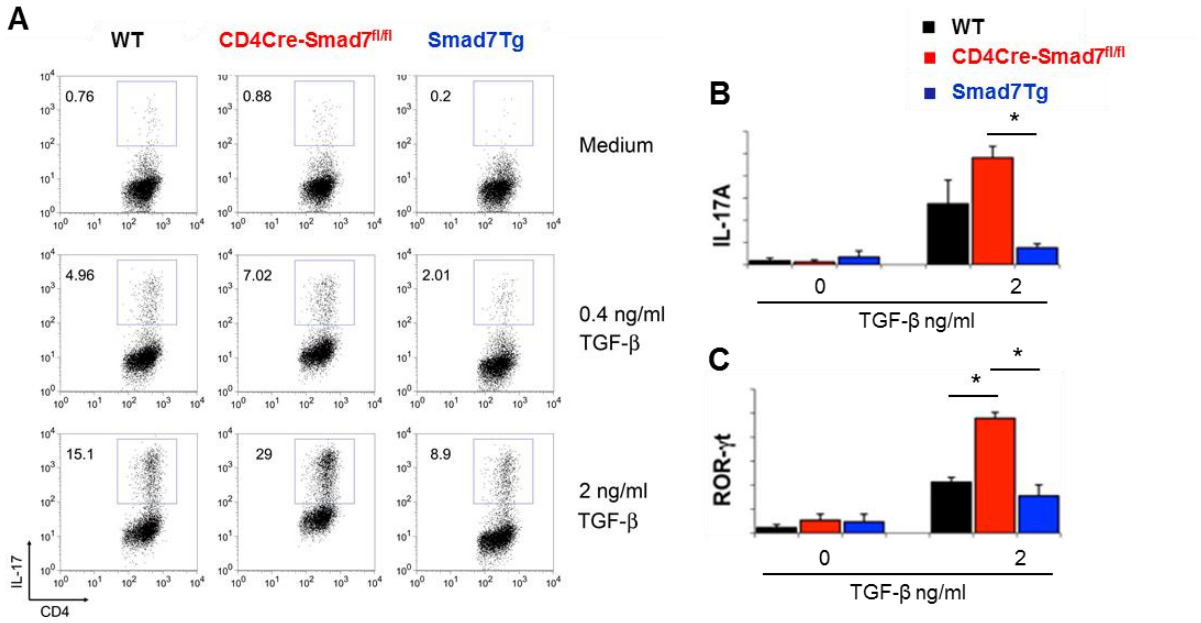


Figure 20: Increased Th17 differentiation in CD4Cre-Smad7$^{fl/fl}$ T cell cultures. Naïve T cells from WT, CD4Cre-Smad7$^{fl/fl}$ and Smad7Tg mice were cultured under Th17 differentiating conditions using a-CD3, a-CD28, rIL-6, a-IL-4, a-IFN-γ with increasing concentrations of TGF-β for 5 days. Cells were restimulated with PMA, ionomycin and golgi stop for 4 hours. (A) The figure is the representative of 3 independent experiments with similar results. Afterwards, cells were harvested, surface stained with the CD4 marker and finally intracellularly stained for IL-17 and analysed by flow cytometry. IL-17 is the signature cytokine for Th17. Cells were gated on lymphocytes. The percentage of gated cells is indicated. (B and C) After 5 days of differentiation with and without TGF-β (2ng/ml) treatment, cells were harvested, RNA was isolated from these cells. Real Time PCR (RT-PCR) was performed to check the mRNA expression of IL-17A and the transcription factor ROR-γt which induces Th17 differentiation. In RT-PCR, expression is presented relative to the 18S-RNA content and normalized on the unstimulated control. 3 female mice of 6-8 weeks age were used per group. Values represent the mean ± SEM of 3 experiments. * *P* <0.05 (ANOVA).

4.3.4 Treg differentiation.

TGF-β positively regulates Treg differentiation (Horwitz *et al.* 2003) and genetic deletion of Smad2 and Smad3 strongly reduces Foxp3 expression and Treg differentiation (Takimoto *et al.* 2010 and Lu *et al.* 2010). Altered TGF-β signalling in T cells with Smad7 deletion or overexpression should have an impact on the signal transduction processes leading to Treg differentiation. Naïve CD4^{+}CD62L^{+} T cells from wild type, CD4Cre-Smad7$^{fl/fl}$ and Smad7Tg mice, were sorted and differentiated under Treg polarizing conditions for 5 days with indicated increasing concentrations of TGF-β.

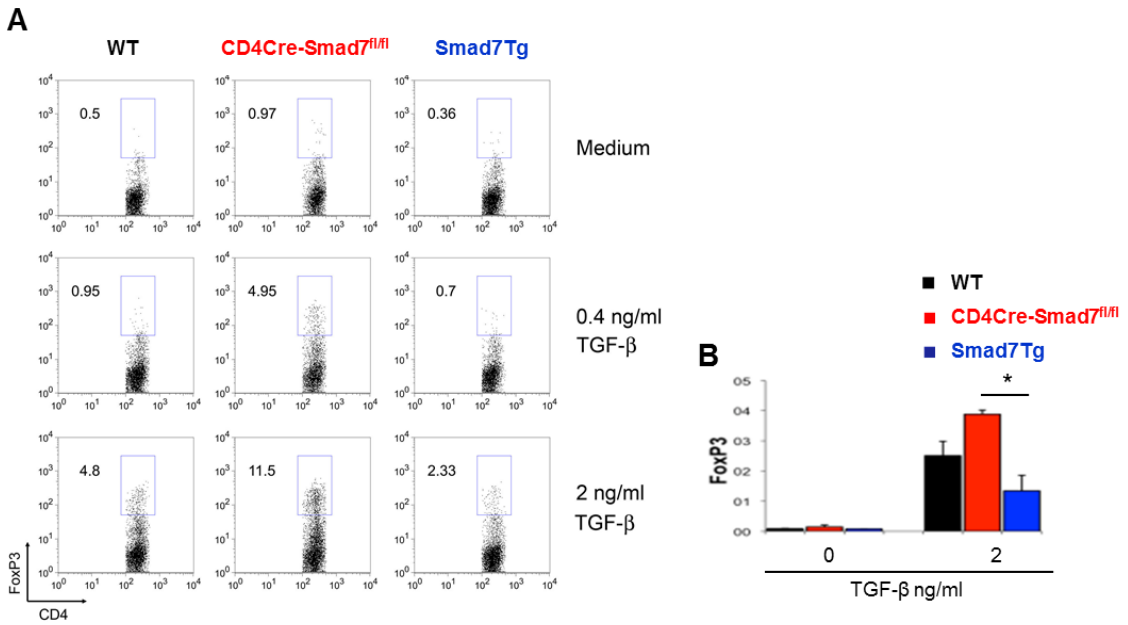


Figure 21: Increased Treg differentiation in CD4Cre-Smad7$^{fl/fl}$ T cell cultures. Naïve T cells from WT, CD4Cre-Smad7$^{fl/fl}$ and Smad7Tg mice were sorted and cultured under Tregs differentiating conditions using a-CD3, a-CD28 with increasing concentrations of TGF-β for 5 days. (A) The figure is representative of 3 independent experiments with similar results. Cells were harvested; surface stained with the CD4 marker and finally intranuclearly stained for Foxp3. Foxp3 is the signature molecule and also transcription factor for Tregs. Cells were gated on lymphocytes. The percentage of gated cells is indicated as analysed by flow cytometry. (B) After 5 days of differentiation with and without TGF-β (2ng/ml) treatment, cells were harvested, RNA was isolated from these cells. Real Time PCR (RT-PCR) was performed to check the mRNA expression of the transcription factor Foxp3 which induces Treg differentiation. In RT-PCR, expression is presented relative to the 18S-RNA content and normalized on the unstimulated control. 3 female mice of 6-8 weeks age were used per group. Values represent the mean ± SEM of 3 experiments. * *P* <0.05 (ANOVA).

Increased Treg differentiation was found with an increased concentration of TGF-β in T cell cultures of all genotypes (Figure 21A). Treg differentiation was upregulated in CD4Cre-Smad7$^{fl/fl}$ T cells which was also confirmed by an upregulation of Foxp3-mRNA expression (Figure 21B). Apart from the canonical signalling pathway TGF-β might induce Treg differentiation through Smad independent pathways since Treg differentiation was detected in Smad7Tg T cell cultures, even if with low abundance.

4.3.5 Smad7 expression in T cells during Th1 and Th17 differentiation.

Considering the results of the previous experiments, it was clear that the expression level Smad7 plays role in T helper cell differentiation. So, it was worth to check whether Smad7 expression was upregulated during Th1 and downregulated during Th17 differentiation, respectively. To this point, naïve T cells were sorted from WT mice and polarized under Th1 and Th17 differentiation for the indicated time and Smad7 expression was checked at the mRNA level by RT-PCR to see the time kinetic during Th1 and Th17 differentiation.

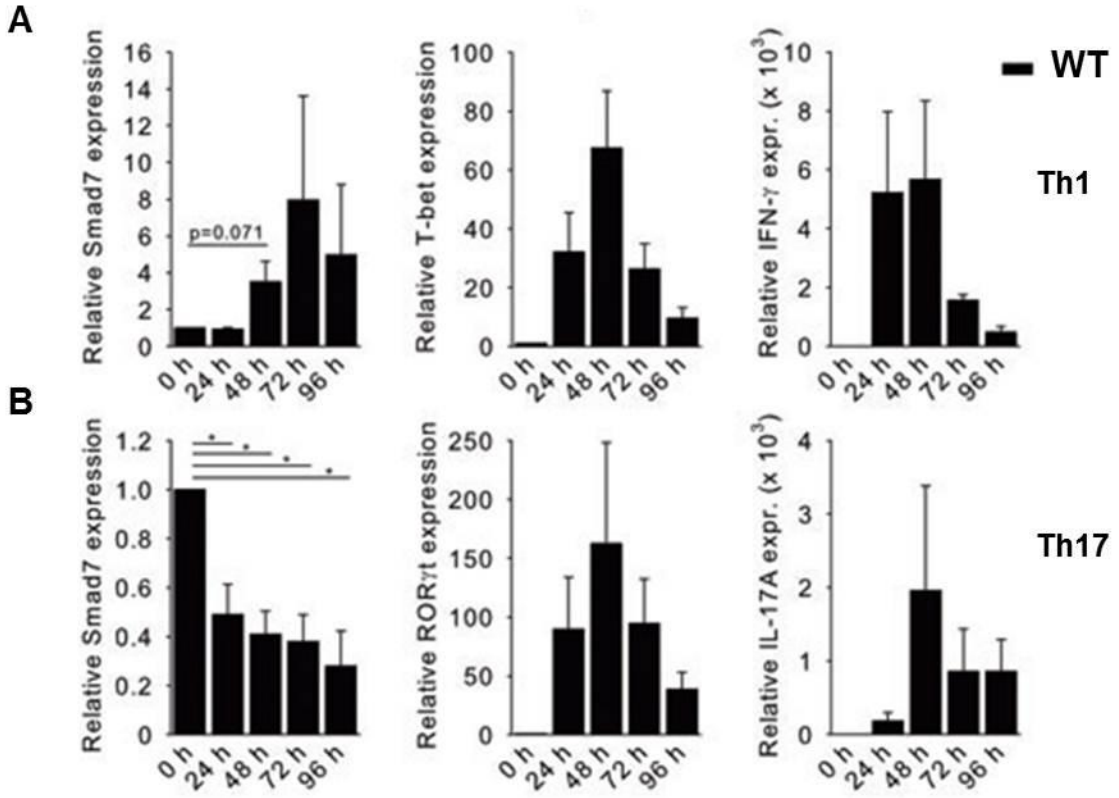


Figure 22: Time kinetic of Smad7 expression during Th1 and Th17 differentiation. Naïve T cells from WT mice were cultured under Th1 and Th17 differentiating conditions. Cells were harvested at the indicated time points. RNA was isolated and RT-PCR was performed to check Smad7 expression during Th1 and Th17 differentiation. (A) The expression of Th1 associated transcription factor and genes was checked. (B) Smad7 expression was checked during Th17 differentiation. The expression of Th17 associated transcription factors and genes were checked. In RT-PCR, expression is presented relative to the 18S-RNA content and normalized on the unstimulated control (=1.0 arbitrary units). 3 female mice of 6-8 weeks old were used. Data are representative of 3 independent experiments. Values represent the mean ± SEM of 3 experiments. **P* <0.05 (ANOVA).

During Th1 differentiation, the expression of the transcription factor T-bet and the cytokine IFN-γ was highest after 48 hours and Smad7 expression also increased after 48-96 hours (Figure 22). This result shows that upregulation of Smad7 occurs

after priming of Th1 differentiation and probably helps to maintain the Th1 phenotype. During Th17 differentiation, the expression of the transcription factor ROR-γt and the cytokine IL-17A was also highest after 48 hours but Smad7 expression was downregulated very early which suggests that TGF-β signal might be important to induce Th17-specific genes like ROR-γt and IL-17.

4.4 Investigation of Smad-independent signalling pathways during Th17 differentiation.

4.4.1 TGF-β signals are not exclusively transmitted by Smad proteins during Th17 differentiation.

Upon binding to TGF-βRI and TGF-βRII, TGF-β signalling results in the activation of downstream signal cascade by either Smad-dependent or Smad-independent pathways (Li *et al.* 2006a). The canonical Smad-dependent pathway involves phosphorylation of Smad2 and Smad3, which translocate into the nucleus in a complex with Smad4 and transcribe or suppress target genes, for example Smad7. In this study, Th17 differentiation was also detected in Smad7Tg T cell cultures (Figure 20), despite the complete blockade of TGF-β-Smad signalling as shown by absent Smad2 phosphorylation (Kleiter *et al.* in 2010). Hence, Th17 cells might also be differentiated through non-Smad signalling pathways downstream of TGF-βR signalling. In order to confirm this hypothesis, Th17 differentiation was performed in T cells either devoid of the TGF-βRII, treated with an ALK-5 inhibitor (SB-431542) or with overexpression of Smad7. SB-431542 blocks Smad2 and Smad3 phosphorylation by inhibiting the TGF-βRI kinase phosphorylation (Inman *et al.* 2002).

In this experiment, Th0 served as a control to show that without TGF-β and IL-6, naïve T cells do not differentiate into Th17 cells. DMSO, the solvent of SB-431542 was added to the control Th17 cultures to be able to specifically analyse the effect of the inhibitor during Th17 differentiation. After inhibiting TGF-βRI kinase activity by the ALK-5 inhibitor, Th17 differentiation strongly decreased (Figure 23A and B). Smad7Tg and CD4Cre-TGFβRII $^{fl/fl}$ T cells showed 6.23% and 0.00% Th17 differentiation respectively. This figure implies that in order for naïve T cells to differentiate into Th17 cells, Smad2 and Smad3 phosphorylation was essential but not exclusively mandatory. This means without Smad2 and Smad3 phosphorylation

naïve T cells can also differentiate into Th17 cells but to a smaller extent. Importantly, in both cases (with and without Smad2 and Smad3 phosphorylation) binding of TGF-β to the TGF-βRII was indispensable for Th17 differentiation as CD4Cre-TGFβRII $^{fl/fl}$ T cells did not differentiate into Th17 at all. The relative mRNA expression of the transcription factor ROR-γt was also reduced in WT T cells treated with the ALK-5 inhibitor and Smad7Tg T cells and absent in CD4Cre-TGFβRII $^{fl/fl}$ T cells, conforming the results of IL-17 expression shown by flow cytometry (Figure 23C). In summary, this experiment shows that naïve T cells need TGF-β to differentiate into the Th17 subset and that Smad-independent signalling pathways are involved in this process. In order to find molecules or pathways that are involved in this TGF-β-Smad independent pathway during Th17 differentiation, TGF-β signalling phosphorylated protein antibody array was performed.

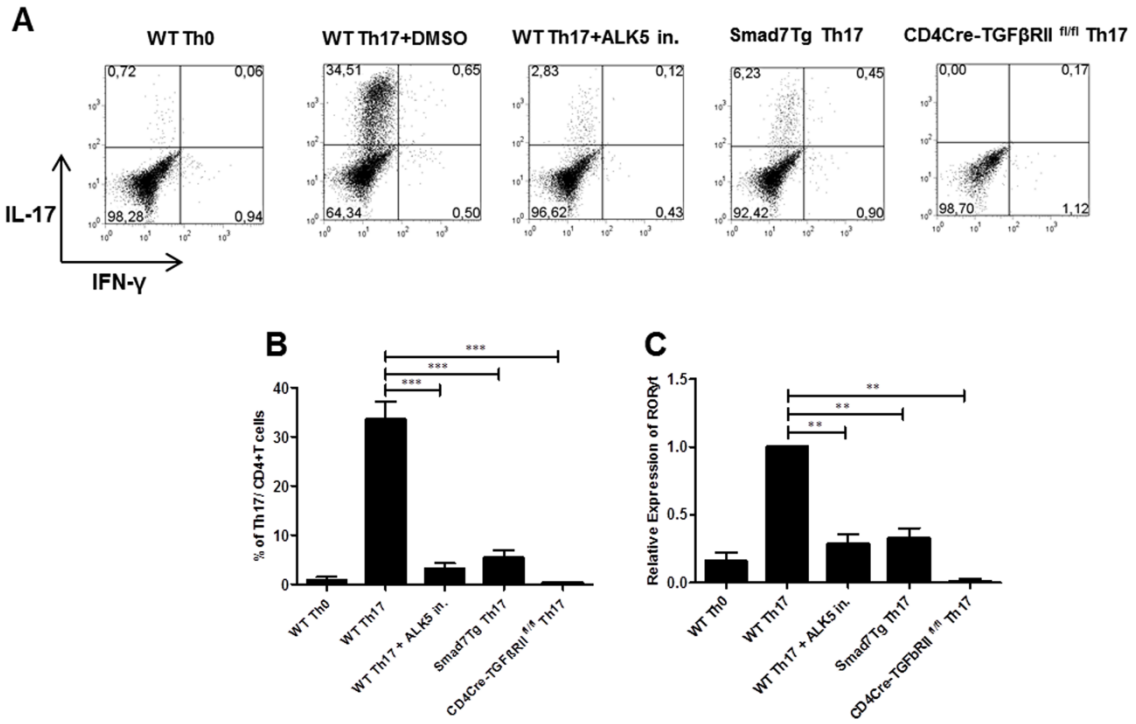


Figure 23: TGF-β signals through non-Smad pathways during Th17 differentiation. Naïve T cells from WT, Smad7Tg and CD4Cre-TGFβRII $^{fl/fl}$ mice were stimulated under Th17 differentiating conditions using a-CD3, a-CD28, rIL-6, a-IL-4, a-IFN-γ and TGF-β (2ng/ml) for 5 days. In case of ALK-5 inhibitor treatment, cells were pre-incubated with the 1 µM SB-431542 for half an hour on ice before putting the cells into the culture. T cells used for control Th17 differentiation were also pre-incubated with equivalent amount of DMSO as the amount was used for SB-431542 as solvent. (A) One representative figure of 3 independent experiments with similar results. After 5 days of differentiation, cells were restimulated with PMA, ionomycin and golgi stop for 4 hours. Cells were harvested, surface stained with the CD4 marker, intracellularly stained for IL-17 and INF-γ and analysed by flow cytometry. IL-17 and IFN-γ are the signature cytokine for Th17 and Th1, respectively. Cells were gated

on lymphocytes and $CD4^+$ T cells. The percentage of gated cells is indicated. (B) Summary of flow cytometric analysis of 3 independent experiments of figure A. (C) After 5 days of differentiation, cells were harvested, RNA was isolated and Real Time PCR (RT-PCR) was performed to check the mRNA expression of the transcription factor ROR-γt which induces Th17 differentiation. In RT-PCR, expression is presented relative to the 18S-RNA content and normalized on the unstimulated control (=1.0 arbitrary units). Three female mice of 6-8 weeks age were used per group. Values represent the mean ± SEM of 3 experiments. ***, *P value* <0.001. **, *P* value <0.01 (Student's *t* test).

4.4.2 Phosphorylation of signalling proteins downstream of the TGF-β receptor during Th17 differentiation.

Our previous results showed that TGF-β positively regulates Th17 differentiation, mainly through intracellular Smad proteins, but also to a smaller extent through Smad-independent signalling. Although several alternative pathways downstream of TGF-β receptor, e.g. MAPK signalling, PI3K and AKT signalling and so on are well characterized (Yoshimura *et al.* 2010), the Smad-independent pathways involved in Th17 differentiation remain elusive. To this point, the phosphorylation status of signalling proteins downstream of TGF-β receptors during Th17 differentiation was analysed and identified pathways were verified by western blotting and functional assays. A diagram shows briefly the whole procedure (Figure 24, see also methods).

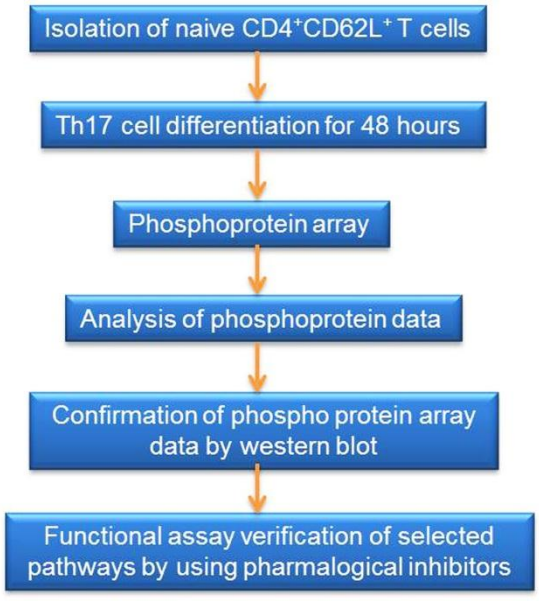


Figure 24: A schematic diagram of the experimental procedures used to identify non-Smad signalling pathways during Th17 differentiation.

4.4.2.1 Time kinetic of Th17 differentiation

First, a time kinetic of ROR-γt expression was done, to identify the best time point for the analysis of signal transduction during Th17 differentiation. After 48h, ROR-γt expression was highest and dropped thereafter (Figure 25A), whereas a terminal differentiation, shown by IL-17 production, peaks at around 120h (Lazarevic *et al.* 2011). Therefore, we chose 48h for further analysis, since T cells are still in the process of Th17 differentiation, driven by ROR-γt, but not terminally differentiated yet. Before performing each array, IL-17 production by Th17 cells was checked after 48 hours of differentiation by flow cytometry (Summary Figure 25B; for a representative FACS plot, see Appendix: S3).

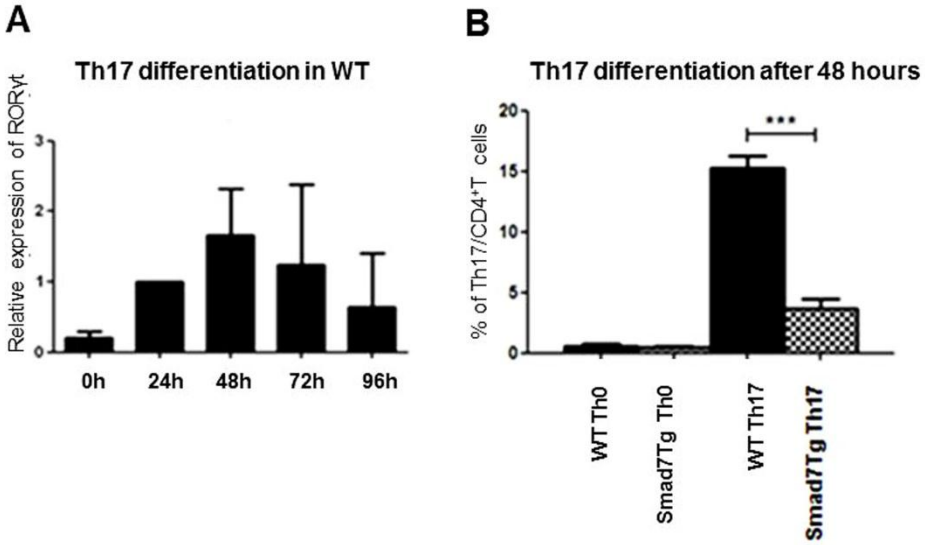


Figure 25: Time kinetic of Th17 differentiation used for phospho protein arrays. Naïve T cell from WT and Smad7Tg mice were stimulated under Th17 polarizing conditions using a-CD3, a-CD28, rIL-6, a-IL-4, a-IFN-γ and TGF-β (2ng/ml) for up to 4 days. (A) A summary of 3 independent experiments. Cells were harvested at different time points and RT-PCR was performed to check ROR-γt expression. Expression is presented relative to the 18S-RNA content and normalized to the 24h stimulation (=1.0 arbitrary units). (B) A summary of 4 WT and 3 Smad7Tg Th17 differentiation experiments was shown. Cells were harvested after 48 hours of differentiation and IL-17 production was checked by performing intracellular FACS staining. Two female mice of 6-8 weeks age were used for each experiment (A). 3 female mice of 6-8 weeks age were used per group for each array. Values represent the mean ± SEM of all experiments. ***, *P value* <0.001 (Student's *t* test).

4.4.2.2 Protein arrays for the detection of the phosphorylation status during Th17 differentiation

To characterize the phosphorylation status of proteins during Th0 and Th17 differentiation from both WT and Smad7Tg T cells, the TGF-β signalling phospho-specific antibody array from Fullmoon Biosystem was used. Using this phospho protein antibody array, a total of 44 proteins can be detected both in phosphorylated and non-phosphorylated form, 3 proteins can be detected only in phosphorylated form and 18 proteins can be detected only in non-phosphorylated form (see Appendix: S4). In this thesis, Full Moon Biosystems' antibody array kit was used since it provides the major reagents required to perform protein extraction, labeling, conjugation and detection. This array allowed simultaneous detection of plenty of proteins on a single slide without performing numerous immunoprecipitation and/or Western blot analyses, which was cost effective, less time consuming and it also reduced the number of variables that could affect our results. All arrays are printed on standard size microscope slides. Each slide consists of an array of well-characterized antibodies with six replicates and multiple positive and negative controls to maximize data reliability. Each set of antibody arrays contains two slides (two identical arrays) - one slide is for the control sample and the other is for experimental sample. The antibodies are covalently immobilized on high quality glass surface coated with 3-D polymer materials to ensure high binding efficiency and specificity. GAL files are also provided by the company to extract data. For each array experiment, two slides (one for Th0 and the other one for Th17 differentiation) per genotype were used. A total of 5 independent array experiments were done. In the first experiment, Th17 was compared to Th0 differentiation in WT T cells. In the next 4 experiments, Th17 was compared to Th0 differentiation in both WT and Smad7Tg T cells. One set of arrays for WT and Smad7Tg T cells, had to be excluded from analysis for technical reasons (streak artefact; background signal too high). One example of protein expression is shown, where the protein expression of p38 (phospho-Thr180) is upregulated during Th17 differentiation in both WT and Smad7Tg T cells (Figure 26).

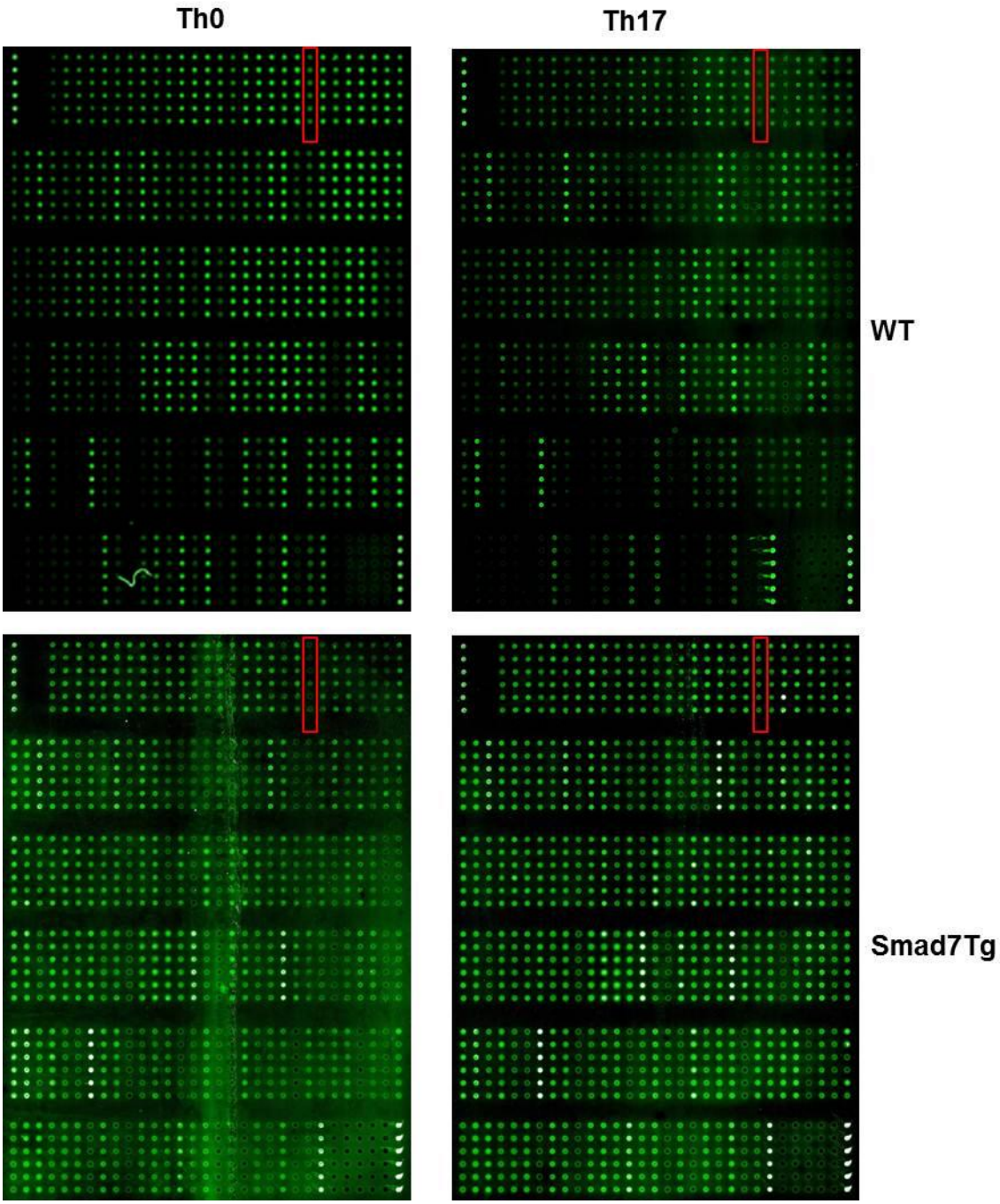


Figure 26: Comparison of protein expression between WT and Smad7Tg T cells during Th0 and Th17 differentiation. TGF-β signalling phospho-specific antibody microarray. Protein array was performed after 48 hours of Th17 differentiation with both WT and Smad7Tg T cells. Six replicates of each antibody including positive and negative controls are included. Spots in bright white color are the positive controls and black spots are negative controls. Higher intense green light indicates higher expression of proteins. Less intense green light depicts decreased protein expression. The red marking indicates the protein expression of p38 (phospho-Thr180). Figure represents one complete array.

In both Th0 and Th17 condition, we saw a high variance in the background median intensities and in median intensities of phosphorylated and non-phosphorylated proteins when compared to their replicate experiments on corresponding arrays (see Appendix: S5). Normalization of total signalling intensities between individual arrays allowed us to get rid of this problem. Sometimes, both phosphorylated and non-phosphorylated forms of proteins were not detected in Th0 and Th17 conditions. Other proteins were phosphorylated in Th0 but not in Th17 or in both condition or in Th17 but not in Th0. In order to be able to compare the phosphorylation status of specific proteins during Th17 differentiation between independent experiments, ten different categories describing protein expression and phosphorylation were made (Table 19, see methods). Briefly, proteins were first grouped according to their phosphorylation status and subsequently according to their expression during Th17 differentiation. Based on category 9, which is defined as an increased phosphorylation and expression during Th17 differentiation, Th17-specific proteins were detected which showed increased phosphorylation in at least two experiments in WT T cells and Smad7Tg T cells, or both listed (Table 21).

Increase of phosphorylation during Th17 differentiation:

Protein phosphorylated Th17 vs Th0	**WT**	**Smad7Tg**
AKT1 (Phospho-Ser246)	Yes	No
AKT1 (Phospho-Tyr474)	Yes	Yes
AKT2 (Phospho-Ser474)	Yes	No
ERK1-p44/42 MAP Kinase (Phospho-Tyr204)	Yes	No
mTOR (Phospho-Thr2446)	Yes	Yes
mTOR (Phospho-Ser2448)	Yes	No
Myc (Phospho-Ser62)	Yes	No
P38 MAPK (Phospho-Thr180)	Yes	Yes
PAK1 (Phospho-Thr212)	Yes	No
PAK1/2/3 (Phospho-Thr423/402/421)	Yes	No
PAK2 (Phospho-Ser192)	Yes	No
PAK3 (Phospho-Ser154)	Yes	No
PKC alpha (Phospho-Tyr657)	Yes	No
PKC theta (Phospho-Ser676)	Yes	No

PKC beta/PKCB (Phospho-Ser661)	Yes	No
PKC delta (Phospho-Thr505)	Yes	Yes
PKC zeta (Phospho-Thr560)	Yes	No
Rac1/cdc42 (Phospho-Ser71)	Yes	No
Ras-GRF1 (Phospho-Ser916)	Yes	No
SAPK/JNK (Phospho-Tyr185)	Yes	No
Smad2 (Phospho-Ser250)	Yes	No
Smad2 (Phospho-Ser467)	Yes	No
Smad3 (Phospho-Ser213)	Yes	No

Table 21: Proteins with increased phosphorylation during Th17 differentiation as compared to the control Th0 condition.

In the 4 different experiments using WT T cells, 26 proteins, 26 proteins, 25 proteins and 9 proteins were more phosphorylated during Th17 than during Th0 differentiation in array1, array2, array3 and array 4, respectively. Using Smad7Tg T cells, 2 proteins, 2 proteins, and 23 proteins were found to be more phosphorylated during Th17 differentiation as compared to the control Th0 condition in array2, array3 and array4, respectively. The numbers of proteins based on the other categories are summarized in Figure 27.

WT

Array 1

Statistics	Category	Protein #
	1	13
	2	4
Th17	3	7
Th0	4	0
	5	18
Th17	6	16
Th0	7	0
	8	20
Th17	9	26
Th0	10	0

Array 2

Statistics	Category	Protein #
	1	11
	2	7
Th17	3	18
Th0	4	0
	5	6
Th17	6	33
Th0	7	0
	8	2
Th17	9	26
Th0	10	1

Array 3

Statistics	Category	Protein #
	1	31
	2	11
Th17	3	9
Th0	4	0
	5	11
Th17	6	12
Th0	7	1
	8	4
Th17	9	25
Th0	10	0

Array 4

Statistics	Category	Protein #
	1	0
	2	12
Th17	3	0
Th0	4	2
	5	35
Th17	6	4
Th0	7	12
	8	26
Th17	9	9
Th0	10	4

Smad7Tg

Array 2

Statistics	Category	Protein #
	1	1
	2	6
Th17	3	0
Th0	4	0
	5	50
Th17	6	19
Th0	7	0
	8	18
Th17	9	2
Th0	10	8

Array 3

Statistics	Category	Protein #
	1	28
	2	11
Th17	3	2
Th0	4	15
	5	7
Th17	6	2
Th0	7	7
	8	4
Th17	9	2
Th0	10	26

Array 4

Statistics	Category	Protein #
	1	6
	2	12
Th17	3	0
Th0	4	2
	5	20
Th17	6	18
Th0	7	8
	8	14
Th17	9	23
Th0	10	1

Figure 27: An overview of the categorized results of the phospho protein arrays. In each array, 104 proteins were categorized based on their phosphorylation status and expression during Th17 differentiation according to the Table 19. The total number of proteins falling into each category is shown.

Most proteins from both WT and Smad7Tg T cells which were identified to have an elevation of phosphorylation during Th17 differentiation belonged to specific signalling pathways, in particular AKT1, ERK1, mTOR, p38 MAPK, PAK, PKC and the Smad family (Table 21). Using WT T cells, during Th17 differentiation as compared to Th0 condition, AKT1 (Phospho-Ser246), AKT1 (Phospho-Tyr474), AKT2 (Phospho-Ser474) were found to be more phosphorylated in 3 arrays, ERK1-p44/42 MAP Kinase (Phospho-Tyr204), mTOR (Phospho-Thr2446), mTOR (Phospho-Ser2448), Myc (Phospho-Ser62) in 2 arrays, p38 MAPK (Phospho-Thr180) in 3 arrays, PAK1 (Phospho-Thr212), PAK1/2/3 (Phospho-Thr423/402/421), PAK2 (Phospho-Ser192) in 2 arrays, PAK3 (Phospho-Ser154) in 3 arrays, PKC alpha (Phospho-Tyr657), PKC theta (Phospho-Ser676), PKC beta/PKCB (Phospho-Ser661), PKC delta (Phospho-Thr505), PKC zeta (Phospho-Thr560) in 2 arrays, Rac1/cdc42 (Phospho-Ser71) in 3 arrays, Ras-GRF1 (Phospho-Ser916) in 2 arrays, SAPK/JNK (Phospho-Tyr185) in 3 arrays, Smad2 (Phospho-Ser250) and Smad2 (Phospho-Ser467) were found to be phosphorylated in different single experiment. Smad3 (Phospho-Ser213) was detected with increased phosphorylation in 2 arrays (Figure 28).

Using Smad7Tg T cell, AKT (Phospho-Ser473), AKT (Phospho-Thr308), AKT (Phospho-Tyr326), AKT2 (Phospho-Ser474), ERK3 (Phospho-Ser189), LIMK1 (Phospho-Thr508), MKK3/MAP2K3 (Phospho-Thr222), mTOR (Phospho-Ser2481), mTOR (Phospho-Ser2448), Myc (Phospho-Ser62), p38 MAPK (Phospho-Thr180), p38 MAPK (Phospho-Tyr322), PAK1/2/3 (Phospho-Ser141), PKC delta (Phospho-Thr505), PKC theta (Phospho-Ser676), PKC zeta (Phospho-Thr410), PKC zeta (Phospho-Thr560), SAPK/JNK (Phospho-Thr183), SAPK/JNK (Phospho-Tyr185), Smad1 (Phospho-Ser187) , Smad1 (Phospho-Ser465), Smad2 (Phospho-Ser250), Smad2 (Phospho-Ser467), Smad2/3 (Phospho-Thr8) were found to be phosphorylated during Th17 differentiation as compared to Th0 condition (Figure 28).


WT
Smad7Tg
Array 1
Array 2
Array 3
Array 4
Array 2
Array 3
Array 4
Abl1 (Phospho-Thr 754/735)
Abl1 (Phospho-Tyr204)
Abl1 (Phospho-Tyr412)
AKT (Phospho-Ser473)
AKT (Phospho-Thr308)
AKT (Phospho-Tyr326)
AKT1 (Phospho-Ser124)
AKT1 (Phospho-Ser246)
AKT1 (Phospho-Thr450)
AKT1 (Phospho-Thr72)
AKT1 (Phospho-Tyr474)
AKT2 (Phospho-Ser474)
c-Abl (Phospho-Tyr245)
c-Abl (Phospho-Tyr412)
Cofilin (Phospho-Ser3)
ERK1-p44/42 MAP Kinase (Phospho-Thr202)
ERK1-p44/42 MAP Kinase (Phospho-Tyr204)
ERK3 (Phospho-Ser189)
ERK8 (Phospho-Thr175/Tyr177)
Gab2 (Phospho-Tyr643)
JNK1/2/3 (Phospho-Thr183/Tyr185)
LIMK1 (Phospho-Thr508)
MKK3 (Phospho-Ser189)
MKK3/MAP2K3 (Phospho-Thr222)
MKK6 (Phospho-Ser207)
mTOR (Phospho-Ser2448)
mTOR (Phospho-Ser2481)
mTOR (Phospho-Thr2446)
Myc (Phospho-Ser373)
Myc (Phospho-Ser62)
Myc (Phospho-Thr358)
Myc (Phospho-Thr58)
p38 MAPK (Phospho-Thr180)
p38 MAPK (Phospho-Tyr182)
p38 MAPK (Phospho-Tyr322)
PAK1 (Phospho-Ser204)
PAK1 (Phospho-Thr212)
PAK1/2 (Phospho-Ser199)
PAK1/2/3 (Phospho-Ser141)
PAK1/2/3 (Phospho-Thr423/402/421)
PAK2 (Phospho-Ser192)
PAK2 (Phospho-Ser20)
PAK3 (Phospho-Ser154)
PI3-kinase p85-alpha (Phospho-Tyr607)
PI3-kinase p85-subunit alpha/gamma (Phospho-Tyr467/199)
PKC alpha (Phospho-Tyr657)
PKC alpha/beta II (Phospho-Thr638)
PKC beta/PKCB (Phospho-Ser661)
PKC delta (Phospho-Ser645)
PKC delta (Phospho-Thr505)
PKC epsilon (Phospho-Ser729)
PKC theta (Phospho-Ser676)
PKC theta (Phospho-Thr538)
PKC zeta (Phospho-Thr410)
PKC zeta (Phospho-Thr560)
PP2A-a (Phospho-Tyr307)
Rac1/cdc42 (Phospho-Ser71)
Ras-GRF1 (Phospho-Ser916)
Rho/Rac guanine nucleotide exchange factor 2 (Phospho-Ser885)
S6 Ribosomal Protein (Phospho-Ser235)
SAPK/JNK (Phospho-Thr183)
SAPK/JNK (Phospho-Tyr185)
SEK1/MKK4 (Phospho-Ser80)
SEK1/MKK4 (Phospho-Thr261)
Shc (Phospho-Tyr349)
Shc (Phospho-Tyr427)
Smad1 (Phospho-Ser187)
Smad1 (Phospho-Ser465)
Smad2 (Phospho-Ser250)
Smad2 (Phospho-Ser467)
Smad2 (Phospho-Thr220)
Smad2/3 (Phospho-Thr8)
Smad3 (Phospho-Ser204)
Smad3 (Phospho-Ser208)
Smad3 (Phospho-Ser213)
Smad3 (Phospho-Ser425)
Smad3 (Phospho-Thr179)
SP1 (Phospho-Thr739)
TAK1 (Phospho-Thr184)
1
2
3
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6
7
8
9
10

Figure 28: Heat map showing the phosphorylation status of different proteins during Th17 differentiation from both WT and Smad7Tg T cells. TGF-β signalling specific phospho protein antibody arrays (from Fullmoon Biosystems') were used to identify proteins which are phosphorylated during Th17 differentiation. To this point, proteins were extracted from Th0 and Th17 differentiated cells after 48 hours. Proteins were categorized into 10 groups according to their expression and phosphorylation status (P and Non-P stands for phosphorylated and non-phosphorylated proteins, respectively): 1. Both Non-P and P proteins absent; 2. Non-P>P proteins, Th0=Th17; 3. Non-P>P proteins, only in Th17 or Th17>Th0; 4. Non-P>P proteins, only in Th0 or Th0>Th17; 5. Non-P=P proteins, Th0= Th17; 6. Non-P= P proteins, only in Th17 or Th17>Th0; 7. Non-P= P proteins, only in Th0 or Th0>Th17; 8. P> Non-P proteins, Th0= Th17; 9. P> Non-P proteins, only in Th17 or Th17>Th0; 10. P> Non-P proteins, only in Th0 or Th0>Th17.

Our main goal was to find proteins which signal through TGF-β-Smad independent pathways during Th17 differentiation. Hence, we searched for proteins which were phosphorylated both in WT and Smad7Tg T cells during Th17 differentiation. There were 4 proteins which were found to be more phosphorylated both in WT and Smad7Tg T cells as compared to Th0 condition. They were AKT1 (Phospho-Tyr474), mTOR (Phospho-Thr2446), p38 MAPK (Phospho-Thr180) and PKC delta (Phospho-Thr505) (Figure 28). Since T cell specific Smad7 expression did not affect the phosphorylation status of these proteins, we concluded that these proteins play a role during Th17 differentiation via TGF-β-Smad independent pathways. AKT and mTOR belong to PI3K/AKT/mTOR pathway (Yap *et al.* 2008), p38 belongs to MAPK pathways (Yu *et al*. 2002). PKC-delta is known to be an effector of the PI3K pathway and p38 MAPK pathway (Deb *et al.* 2003, Uddin *et al.* 2002). Array data indicates that PI3K/AKT/mTOR and p38 MAPK, which are non-Smad pathways, were activated during Th17 differentiation and proteins belong to these pathways were phosphorylated on specific sites.

4.4.2.3 Confirmation of array results by immunoblotting

It was found by the antibody protein array that AKT1 (Phospho-Tyr474) and mTOR (Phospho-Thr2446) are phosphorylated during Th17 differentiation. These proteins belong to one single pathway, the PI3K/AKT/mTOR pathway. So, it was worth to confirm these results by immunoblotting. Phosphorylation of PI3K was also checked by western blot since PI3K is the upstream kinase of AKT and mTOR (Weichhart and Säemann 2008).

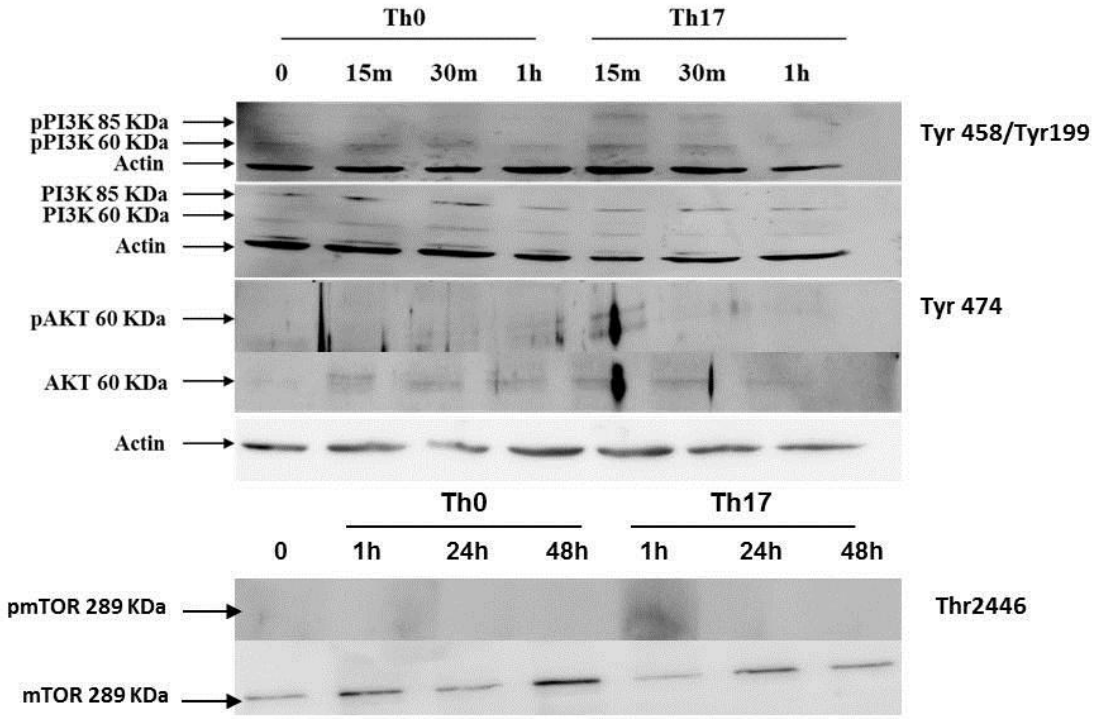

Figure 29: PI3K/AKT/mTOR kinase phosphorylation during Th17 differentiation. Sorted naïve T cells from WT mice were stimulated under Th17 polarizing condition using a-CD3, a-CD28, rIL-6, a-IL-4, a-IFN-γ and TGF-β (2ng/ml). Differentiation was stopped by harvesting cells at different time points as indicated and proteins were extracted for immunoblotting. Actin was used as control. Data are the representative of two independent experiments.

PI3K was phosphorylated on Tyr 458/Tyr199 at 15 and 30 minutes after induction of Th17 differentiation, AKT was phosphorylated on Tyr474 at 15 minutes after induction of Th17 differentiation and mTOR was phosphorylated on Thr2446 1h after induction of Th17 differentiation (Figure 29).

4.4.2.4 Functional role of the PI3K/AKT/mTOR pathway during Th17 differentiation.

Additionally to the Smad pathway which is critical for many aspects of TGF-β signalling, Smad-independent responses have also been documented (Engel *et al*. 1999, Hocevar *et al.* 1999, Derynck and Zhang 2003). A previous study has shown that AKT and mTOR regulate TGF-β dependent Smad3 activation in NRP-152 cells (Song *et al.* 2006). However, the exact mechanisms how Smad-independent signalling pathways integrate with each other and / or Smad signalling remain unknown.

As stated before, using antibody arrays, AKT and mTOR proteins were found to be more phosphorylated during Th17 than during the control Th0 condition. To examine whether the entire PI3K/AKT/mTOR pathway plays a functional role in Th17 differentiation, pharmacological inhibitors which block specific kinases were used.

After sorting, naïve T cells were pre-incubated with different concentrations of rapamycin which inhibits the kinase activity of mTOR (Kim *et al.* 2002) and LY294002 which blocks the kinase activity of PI3K and AKT (Vlahos *et al.* 1994) for an hour on ice prior to adding them in the culture to inhibit the PI3K/AKT/mTOR pathway. Treatment with 100nM of rapamycin showed no difference in the frequency of Th17 differentiation as compared to control Th17+DMSO. However, IL-17 production started decreasing from 48.37% to 21.48% with 1μM rapamycin treatment. With 10 and 20μM of rapamycin treatment, cells did not produce IL-17 at all (Figure 30A), which clearly indicates mTOR kinase signalling is required to induce Th17 differentiation and the effect of rapamycin in Th17 differentiation is dose dependent. LY294002 was found to be more sensitive in Th17 differentiation. It could block the IL-17 production from 48.37% to 13.48% with 100nM concentration. Treatment with 1μM of LY294002 blocked IL-17 production completely (Figure 30A). The effect of LY294002 in Th17 differentiation was also found to be dose dependent. IL-17 production in the supernatant was also reduced with the same frequency by both inhibitors as it was detected by flow cytometry (Figure 30C). Summaries of the results of three independent experiments analysed by flow cytometry and ELISA are also shown, respectively (Figure 30B and C). These results suggest that pharmacological inhibition of PI3K/AKT/mTOR pathway reduces Th17 differentiation in vitro.

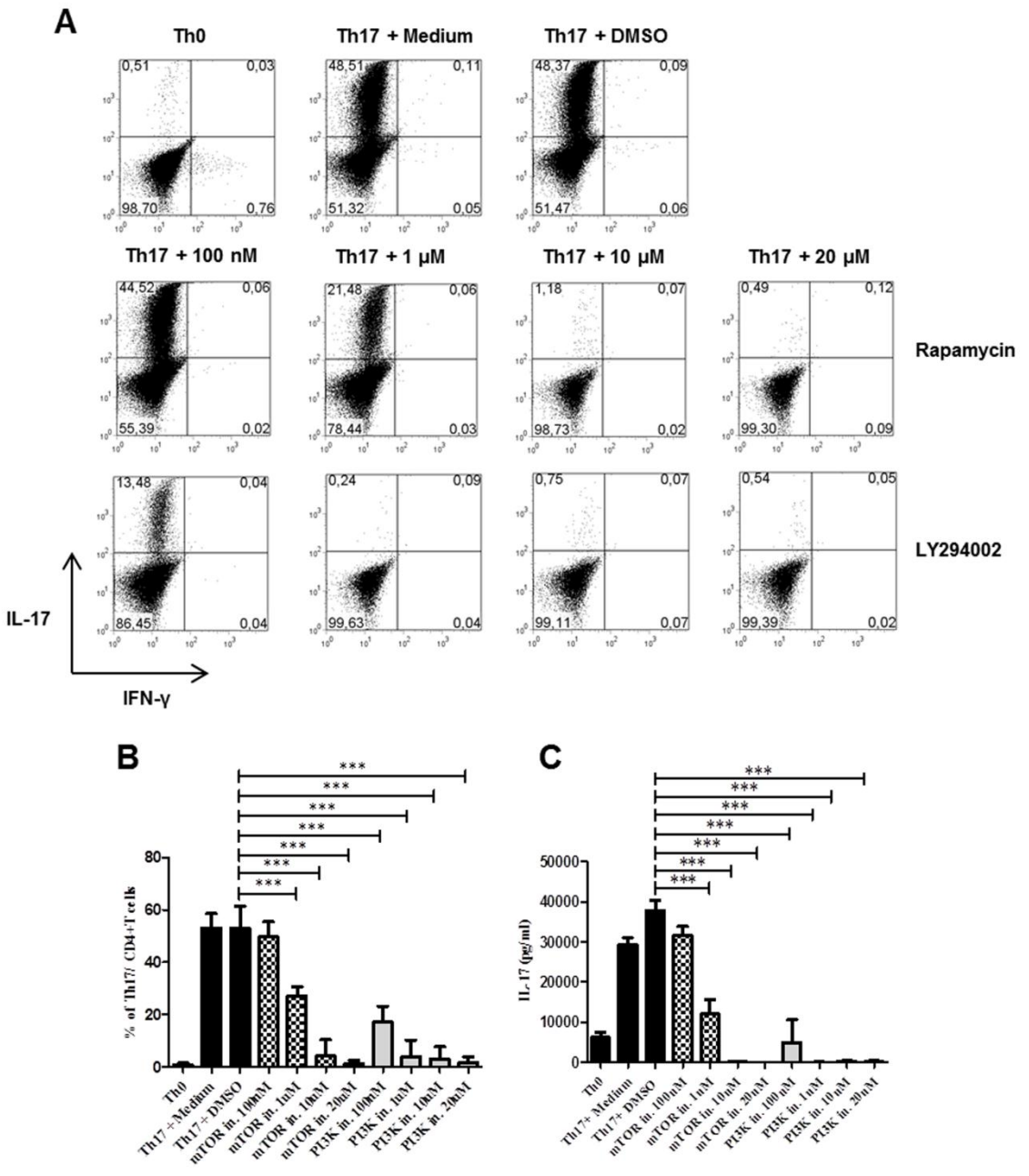

Figure 30: The non-Smad pathway PI3K/AKT/mTOR is involved in Th17 differentiation. Naïve T cells from WT mice were stimulated under Th17 differentiating conditions using a-CD3, a-CD28, rIL-6, a-IL-4, a-IFN-γ and TGF-β (2ng/ml) for 5 days. Naïve T cells were pre-incubated with Rapamycin (mTOR inhibitor) and LY294002 (PI3K inhibitor) with the indicated concentrations for one hour on ice before they were added in the culture. (A & B) After 5 days of differentiation, cells were harvested, supernatants were collected and cells were restimulated with PMA, ionomycin and golgi stop for 4 hours. Afterwards, cells were harvested, surface stained with CD4 and finally intracellularly stained for IL-17 and INF-γ. Cells were analysed by flow cytometry. IL-17 and IFN-γ are the signature cytokine for Th17 and Th1 respectively. Cells were first gated on lymphocytes and then $CD4^+$ T cells. The percentage of gated cells is indicated. (C) IL-17 production by Th17 cells was measured by ELISA. Three female mice of 6-8 weeks age were used. (A) Data are representative of 3 independent experiments. (B and C) Summaries of 3 independent experiments are shown. Values represent the mean ± SEM of 3 experiments. ***, *P* value <0.001 (ANOVA).

We also checked whether the functional role of PI3K/AKT/mTOR pathway during Th17 differentiation is independent of Smad signalling. In this experiment, treatment with 1μM rapamycin, IL-17 production was reduced from 24.82% to 11.95% which showed the reduction is 50% (Figure 31A). 50% reduction of IL-17 was also found in WT Th17 cells with 1μM rapamycin treatment in the previous experiment. With LY294002 treatment, IL-17 production was also reduced from 24.82% to 9.80% which indicated the reduction is more than 50% (Figure 31A). Same result was also found in the WT Th17 differentiation with 100nM LY294002 in the previous experiment. So, the presence of Smad7 on T cells did not affect the inhibitory effect of rapamycin and LY294002 during Th17 differentiation. IL-17 production in the supernatant was also reduced with the treatment of these inhibitors with the same frequency as it was found in the FACS analysis (Figure 31C). Summary of the FACS and ELISA figures of three independent experiments along with control WT Th17 differentiation is also shown, respectively (Figure 31B and C). These results imply that PI3K/AKT/mTOR pathway functions during Th17 differentiation independently of Smad signalling cascade. However, in the previous figure with the WT Th17 differentiation, it was found that 10μM of either inhibitor blocked IL-17 production completely. But with Smad7Tg cultures, T cells produced IL-17 even after treating with 10μM of both inhibitors which means along with PI3K/AKT/mTOR pathway, other non-Smad pathways are also functionally involved in Th17 differentiation.

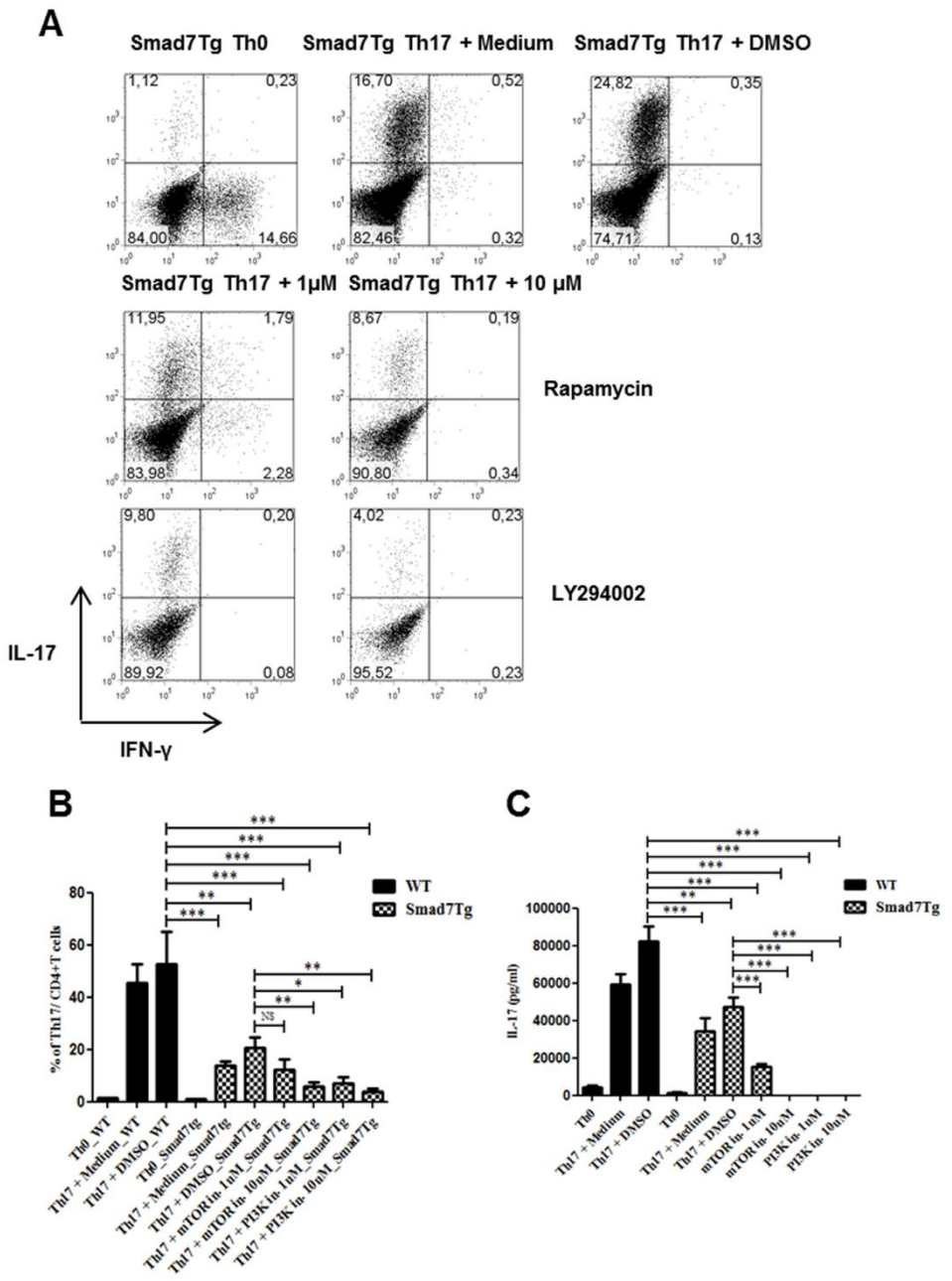

Figure 31: The PI3K/AKT/mTOR pathway regulates Th17 differentiation independently of TGF-β-Smad signalling. Naïve T cells from WT and Smad7Tg mice were stimulated under Th17 differentiating conditions using a-CD3, a-CD28, rIL-6, a-IL-4, a-IFN-γ and TGF-β (2ng/ml) for 5 days. Naïve T cells from Smad7Tg mice were pre-incubated with mTOR and PI3K inhibitor with indicated concentrations for one hour on ice before they were added in the culture. (A & B) After 5 days of differentiation, cells were harvested, supernatants were collected and cells were restimulated with PMA, ionomycin and golgi stop for 4 hours. Cells were then harvested, surface stained with CD4 and finally intracellularly stained for IL-17 and INF-γ and cells analysed by flow cytometry. IL-17 and IFN-γ are the signature cytokine for Th17 and Th1 respectively. Cells were gated on lymphocytes and $CD4^+$ T cells. The percentage of gated cells is indicated. (C) IL-17 production in the supernatant by Th17 cells measured by ELISA. Three female mice of 6-8 weeks age were used. (A) Data are representative of 3 independent experiments. (B and C) Summaries of 3 experiments are shown. Values represent the mean ± SEM of 3 experiments. ***, P value <0.001, ** *P* value<0.01, **P* value<0.05 (ANOVA).

4.4.2.5 Functional role of the MAPK pathways during Th17 differentiation.

Since the AKT/mTOR inhibitors rapamycin and LY294002 at 10 µM concentrations could not block IL-17 production completely, as shown in Figure 31A, additional pathways were examined. As it was found in the array that p38 (Thr180) was phosphorylated during Th17 differentiation, the MAPK signalling pathway was blocked to check its function in Th17 differentiation. Hence, naïve T cells from WT mice were stimulated under Th17 differentiating condition for 5 days with or without p38 inhibitor (SB202190) and JNK inhibitor (SP00125). Respective naïve T cells were pre-incubated with p38 and JNK inhibitor with indicated concentrations.

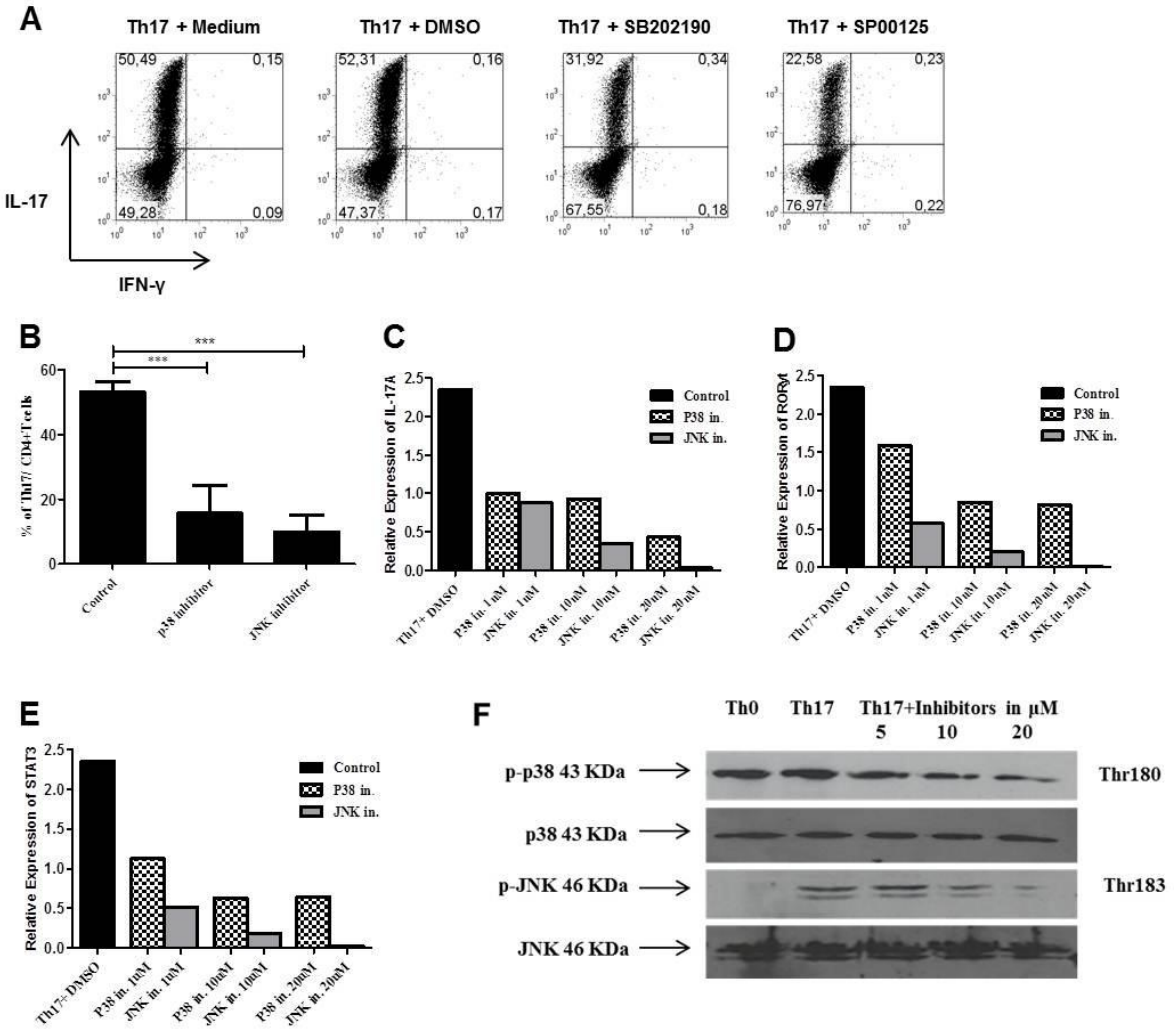

Figure 32: The MAPK pathways positively regulate Th17 differentiation. Naïve T cells from WT mice were stimulated under Th17 differentiating conditions using a-CD3, a-CD28, rIL-6, a-IL-4, a-IFN-γ and TGF-β (2ng/ml) for 5 days. Some naïve T cells were pre-incubated with p38 (SB202190) 20μM and JNK (SP00125) 10μM inhibitor for one hour on ice before they were added in the culture. (A & B) After 5 days of differentiation, cells were harvested and restimulated with PMA, ionomycin and golgi stop for 4 hours. Afterwards, cells were harvested, surface stained with CD4 and finally intracellularly stained for IL-17 and INF-γ and analysed by flow cytometry. IL-17 and IFN-γ are the signature cytokine for Th17 and Th1 respectively. Cells were gated on lymphocytes and $CD4^+$. The percentage of gated cells is indicated. (C, D & E) Relative mRNA expression of Th17 associated genes IL-17, ROR-γt and STAT3. Expression is presented relative to18S-RNA and normalized on the unstimulated control (=1.0 arbitrary units). (F) Phosphorylation status of p38 and JNK in Th17 differentiation condition using a-CD3, a-CD28, rIL-6, a-IL-4, a-IFN-γ at 15 minutes. Three female mice of 6-8 weeks age were used. (A) Data are representative of 3 independent experiments (A). (B) Summary of three independent experiments of (A). Values represent the mean ± SEM of 3 experiments. ***, *P* value <0.001 (Student's *t* test).

Blockade of the p38 and JNK kinases (another member of MAPK pathway) resulted in the reduction of IL-17 production from 52.31% to 31.92% and to 22.58%, respectively. Summary of the FACS analysis of three independent experiments is shown (Figure 32A&B). Expression of the Th17 associated genes IL-17, ROR-γt and STAT3 were also downregulated accordingly after p38 and JNK inhibitor treatment to the same frequency as IL17 production was reduced (Figure 32C, D, and E). In order to check the phosphorylation status of p38 and JNK during Th17 differentiation, naïve T cells were stimulated under Th17 differentiation condition for 15 minutes and immunoblotting was done. Subtle upregulation in phosphorylation of p38 on Thr180 and clear upregulation in phosphorylation of JNK on Thr183 was detected in Th17 differentiation as compared to control Th0. Phosphorylation of p38 and JNK was reduced when cells were treated with both p38 (20μM) and JNK (10μM) inhibitors during Th17 differentiation (Figure 32F). Taken together, inhibition of p38 as well as of the MAPK JNK resulted in reduced Th17 differentiation, probably through TGF-β-Smad independent pathways.

5 Discussion

The immune system is subject to homeostatic regulation, which ensures that the total number of lymphocytes in the periphery is kept more or less at a constant level. Among the large numbers of T cells in the periphery, T cells exhibit structural diversity, i.e., the expression of a diverse repertoire of T cell receptors (TCRs), and functional diversity, i.e., the presence of T cells at naïve, effector, and memory developmental stages. TGF-β plays a critical role in thymic T cell development, in peripheral T cell homeostasis and T cell differentiation (Li and Flavell 2008).

5.1 T cell development and Treg function in mice with a T cell specific deletion of Smad7

In order to investigate whether increased TGF-β signalling affects these events, we first checked the thymic T cell development in CD4Cre-Smad7$^{fl/fl}$ mice. The frequency of double positive CD4$^+$ and CD8$^+$ T cells was unchanged but single positive CD8$^+$ T cells were 2 fold increased in CD4Cre-Smad7$^{fl/fl}$ mice compared to control littermates. This is in line with the finding that TGF-β signalling positively regulates CD8$^+$ thymocyte development (Li *et al.* 2006a). However, mice with a T cell specific deletion of Smad2 and Smad3 and, genomic Smad3 knockout show normal T cell development both in thymus and spleen (Takimoto *et al.* 2010, Wang *et al*. 2006a). In the periphery, the ratio of CD4$^+$ and CD8$^+$ T cells was unchanged in CD4Cre-Smad7$^{fl/fl}$ mice compared to controls. TGFβ inhibits the proliferation of B lymphocyte precursors and induces apoptosis in immature and mature B lymphocytes of different stages (Lomo *et al.* 1995). In CD4Cre-Smad7$^{fl/fl}$ mice, the development of B cells was unchanged compared to controls which resemble the finding that mice lacking the TGFβ-receptor show no alteration in B cell development (Cazac and Roes 2000).

The concept of T cell-mediated neuroinflammation implicates the invasion of the CNS by autoreactive T cells and a loss of suppressive capability of Tregs both in the periphery and the CNS. TGF-β plays a central role in Treg development and expansion (Horwitz *et al.* 2003) and Tregs control the homeostasis of peripheral CD4$^+$ T cells (Almeida *et al.* 2002, Banz *et al*. 2003). TGF-β is not essential for thymic nTreg generation (Quyang *et al*. 2010). In line with these results, flow cytometric analysis showed that there was no difference in thymic nTregs

development in CD4Cre-Smad7$^{fl/fl}$ mice compared to control littermates. On the other hand, TGF-β plays a positive role in the induction of iTregs (Chen *et al.* 2003, Fu *et al.* 2004) and generation of iTregs takes place in the spleen. In contrast, analysis showed no difference in iTregs generation in the periphery of CD4Cre-Smad7$^{fl/fl}$ mice and control littermates. Surprisingly, inhibition of TGFβ signalling in T cells by a genetic deletion of Smad2 results in increased natural and induced Treg populations (Takimoto *et al.* 2010). Increased induced Tregs numbers were also found in Smad3 knockout mice (Wang *et al.* 2006a). These contradictory findings implicate that tight regulation of TGFβ and TGF-β-Smad signalling is important for the generation of thymic and induced Tregs and that non-Smad pathways might also be involved in the development of these T cell subsets.

One recent study showed that myelin specific Tregs in CNS fail to prevent EAE although these Tregs could suppress MOG-specific encephalitogenic T cells *in vitro.* The authors concluded that controlling tissue inflammation is required for Tregs to suppress inflammation (Korn *et al.* 2007a). We checked whether altered Smad7 expression affects the efficiency of Tregs to suppress the proliferation of responder T cells or the proliferation capacity of responder T cells in response to Tregs suppression. By doing co-culture experiments between suppressor Tregs and responder T cells, we showed that a Smad7 deletion or overexpression in T cells did not change the suppression capability of Tregs. This finding was unexpected because Smad7 deficient T cells were shown to produce more TGF-β (Kleiter *et al.* 2010) and an increase of TGF-β mediated Foxp3 positive cells is responsible for attenuated EAE in IL-6 KO mice (Tang *et al.* 2004, Bettelli *et al.* 2006, Korn *et al*. 2007a). We also checked the effect of Smad7 on proliferation of responder T cells with the same co-culture system. We found that responder T cells from Smad7Tg mice were less prone to suppression by WT Tregs or Tregs with an increased Smad7 expression than responder T cells from WT and CD4Cre-Smad7$^{fl/fl}$ mice. This finding is in line with recent reports that responder T cells from Smad7Tg mice are less responsive to Treg suppression (Fantini *et al.* 2009). Moreover, T cells with overexpression of the dominant negative form of the TGF-β receptor II (dnTGF-βRII) are resistant to Treg-mediated suppression (Fahlen *et al.* 2005).

Naïve T cells become activated in the periphery before they migrate to the CNS and TGF-β regulates the status of activated T cells (Rubtsov and Rudensky 2007). Ex vivo analysis showed that in CD4Cre-Smad7$^{fl/fl}$ mice with increased TGF-β signalling in T cells, both CD4^{+} and CD8^{+} peripheral T cells were less activated than control littermates. This result is also supported by the finding that inhibition of TGF-β signalling in TGF-βRII deficient T cells leads to an increased activation of CD4^{+} and CD8^{+} T cells (Marie *et al*. 2006). Blockade of TGF-β signalling in CD4dnTGF-βRII mice also results in more activated CD4^{+} and CD8^{+} T cells (Gorelik and Flavell 2000a). Massive activation and expansion of T cells is also observed in TGF-β1-deficient mice (Shull *et al.* 1992). T cells undergoing an activation process exhibit a change in expression of the surface markers CD62L and CD44 (Shimizu *et al.* 1989). Naïve T cells are CD62L^{hi}CD44^{low} whereas activated memory T lymphocytes are CD62L^{neg}CD44^{hi}. There was no difference found in naïve CD62L^{hi}CD44^{low} T cells as well as in activated memory CD62L^{neg}CD44^{hi} T cells in CD4Cre-Smad7$^{fl/fl}$ mice in comparison with control littermates. But in contrast, reduced naïve CD62L^{hi}CD44^{low} CD8^{+} T cells were found in CD4dnTGF-βRII mice (Gorelik and Flavell 2000a) and very low number of naïve T cells were observed in T cell specific Smad2 and Smad3 double knockout mice (Takimoto *et al.* 2010). Smad7Tg mice were not included as control since these mice were not available at this time in our lab.

5.2 Effect of Smad7 on *in vitro* T helper cell differentiation

Many previous studies indicate that TGF-β is essential to orchestrate T cell immunity, but it is not clear whether various TGF-β–mediated effects on T cells are equally dependent on Smad signalling. TGF-β suppresses Th1 differentiation (Li *et al.* 2006a). Studies with T cells from CD4dnTGF-βRII mice in which TGF-β signalling is blocked only on T cells, show that the canonical TGF-β-Smad signalling is essential for TGF-β induced suppression of Th1 differentiation. Inhibition of TGF-β-Smad signalling blocks T-bet expression, the master transcription factor for Th1 differentiation, but leaves the expression of the IL-12 receptor β2 chain unaffected (Gorelik *et al.* 2002b). TGF-β-receptor II (TGF-βRII) deficient CD4^{+} T cells exhibit an enhanced Th1 differentiation program (Marie *et al.* 2006). Experiments with Smad2 and Smad3 deficient T cells show that TGF-β–mediated suppression of Th1 cell differentiation is dependent on TGF-β-Smad signalling (Takimoto *et al*. 2010). We

showed that a T cell specific Smad7 deletion affects T helper cell differentiation *in vitro.* CD4Cre-Smad7$^{fl/fl}$ T cells showed a decreased frequency of IFN-γ producing Th1 cells. Decreased expression of IFN-γ was associated with a decreased T-bet expression. On the other hand, Th1 responses were elevated in Smad7Tg T cells. In time kinetic experiments, we found that Smad7 expression was correlated to T-bet during Th1 but not to ROR-γt expression during Th17 differentiation. So, Smad7 might have an intrinsic influence on Th1 differentiation. It is known that Th1 cells, but not Th2 and Th17 cells overexpress Smad7 (Veldhoen *et al.* 2006a). Aggravated Th1 response in CNS mononuclear cells was detected in the CNS from Smad7Tg EAE mice (Kleiter *et al*. 2010). In Th1 differentiation, it was found that difference in INF-γ production in WT, CD4Cre-Smad7$^{fl/fl}$ and Smad7Tg T cells was not so pronounced which left a question whether other pathways than TGF-β-Smad are involved in the suppression of Th1 differentiation.

Decreased frequency of IL-4 producing Th2 cells was detected in CD4Cre-Smad7$^{fl/fl}$ T cells. This supports the finding that TGF-β signalling downregulates Th2 differentiation (Gorelik and Flavell 2002, Heath *et al.* 2000). Recently it was also shown that the TGF-β induced adaptor protein Ndfip1 suppresses IL-4 producing Th2 mediated inflammation (Beal *et al.* 2011)

TGF-β positively regulates Th17 differentiation (Ivanov *et al*. 2006) and these Th17 cells may also play a role in the induction of EAE (Siffrin *et al*. 2010, El-Behi *et al.* 2011). TGF-β signalling through Smad2 and Smad3 is required for effective Th17 cell development (Takimoto *et al.* 2010) since neither Smad2 nor Smad3 alone are sufficient for Th17 differentiation (Lu *et al.* 2010). Ex vivo analysis of CD4dnTGF-βRII and CD4Cre-TGF-βR1$^{fl/fl}$ mice reveals a reduced proportion of IL-17 producing T cells in the lamina propria but there are no differences detected in the absolute number of Th17 cells in these two mice compared to wild type controls (Goreschi *et al*. 2010). By modulating TGF-β signalling through altered Smad7 expression in T cells, we showed that enhanced TGF-β signalling results in an increased proportion of Th17 cells and decreased TGF-β signalling in Smad7Tg T cells in a reduction of Th17 cells as compared to wild type controls. Time kinetic experiments showed that the level of Smad7 expression was rapidly decreased after induction of Th17

differentiation *in vitro.* So, Smad7 is reciprocally involved in Th1 and Th17 differentiation as showed by *in vitro* experiments.

TGF-β plays a positive role in the generation and expansion of Tregs (Horwitz *et al.* 2003). So, we examined Tregs differentiation *in vitro* and found more $CD4^{+}Foxp3^{+}$ T cells in CD4Cre-$Smad7^{fl/fl}$ T cells as compared to WT T cells. In Smad7Tg T cells, the lowest frequency of Treg differentiation was detected, suggesting that TGF-β signalling is crucial for the formation of induced Tregs. This result is supported by the recent finding where it was shown that Smad7 expression is downregulated in Treg development (Fantini *et al.* 2004). A possible mechanism is that trans-signalling of the proinflammatory cytokine IL-6 induces Smad7 expression in naïve T cells from Smad7Tg mice (Dominitzki *et al.* 2007). There are also reports which show that a blockade of Smad7 converts autoreactive T cells to cells with a regulatory function (Nakao *et al.* 2000, Monteleone *et al.* 2001). Tregs which mediate type V collagen-induced tolerance to lung allografts do not express Smad7 (Mizobuchi *et al.* 2003). Reports suggest that Smad3 but not Smad2, is critical for the induction of Foxp3 (Tone *et al.* 2008). More recent findings indicate that induction of $Foxp3^{+}$ Treg by TGF-β is partially impaired in Smad2 and Smad3 deficient T cells, and is completely abolished in Smad2/Smad3-double KO T cells (Lu *et al.* 2010, Takimoto *et al.* 2010). In our experiment we showed that TGF-β signalling contributed to Treg differentiation. However, a small proportion of Tregs was also observed in Smad7Tg T cells, which gives rise to the possible involvement of other pathways apart from TGF-β-Smad signalling. Studies with pharmacological inhibitors of ERK and JNK as well as studies with ERK1 and JNK2 KO T cells show that ERK and JNK pathways positively regulate Treg differentiation (Lu *et al.* 2010).

5.3 Are TGF-β and Smad proteins required for Th17 helper cells differentiation?

TGF-β along with IL-6 induces the differentiation of Th17 cells from murine naive T cells both in vitro and in vivo (Bettelli *et al*. 2006, Mangan *et al.* 2006, Veldhoen *et al.* 2006a). Absence of Th17 differentiation in CD4dnTGF-βRII T cells indicates the necessity of TGF-β signalling in Th17 development (Veldhoen *et al*. 2006b). Some reports also show that Smad2 is crucial for Th17 differentiation (Malhotra *et al.* 2010, Martinez *et al.* 2010). Others have demonstrated that Smad3 deficiency leads to

enhanced Th17 cell development in vitro and in vivo (Martinez *et al.* 2009). In addition, using mice deficient in individual Smad proteins, it was shown that neither Smad2 nor Smad3 nor Smad4 are required for Th17 differentiation (Yang *et al*. 2008a, Jana *et al.* 2009, Martized *et al*. 2009, Lu *et al*. 2010). Moreover, recently it was shown that Th17 cells can be differentiated in the complete absence of TGF-β signalling (Ghoreschi *et al*. 2010). Regarding human Th17 cells, there are reports indicating that TGF-β is not required for human naïve T cells to differentiate into Th17 cells (Chen *et al*. 2007, Acosta-Rodriguez *et al.* 2007). Experiments with Stat6$^{-/-}$T-bet$^{-/-}$ T cells revealed that simultaneous inhibition of Th1 and Th2 differentiation is necessary for Th17 differentiation where TGF-β did not show any effect on Th17 development (Das *et al.* 2009). Naïve T cells from Stat6$^{-/-}$T-bet$^{-/-}$/dnTGF-βRII mice differentiate into Th17 cells in the presence of TGF-β but treatment with anti-TGF-β neutralizing antibody has no effect on ROR-γt expression and the differentiation of IL-17 producing Th17 cells, hence IL-6 is considered to be the main differentiating factor (Das *et al.* 2009). In contrast, experiments with a TGF-βR1 inhibitor (ALK-5) and T cell specific Smad2 and Smad3 double knockout mice showed that TGF-β-Smad signalling is important for Th17 differentiation but not for ROR-γt expression (Lu *et al*. 2010, Takimoto *et al.* 2010). Taken together, it is clear that TGF-β-Smad signalling is an important regulator for Th17 differentiation, but ROR-γt expression seems to be independent of Smad signalling. This indicates that other Smad-independent pathways might be involved in Th17 differentiation. We found that T cells deficient in the TGF-βRII did not differentiate into the Th17 phenotype at all, indicating that TGF-β/TGF-βR signalling is important for Th17 differentiation which is in line with the previous findings. It was also shown that TGF-β signalling through TGFβRI is required for generation of both Th17 and iTreg cells (Yang *et al.* 2008b). We found a low proportion of IL-17 producing Th17 cells when TGFβRI and the Smad2/3 signalling cascade were blocked. We also detected reduced ROR-γt expression by Th17 cells. Thus, in our case, expression of ROR-γt was not independent of the Smad signalling pathway, which is in contrast to previous findings.

Taken together, we showed that TGF-β/TGF-βR signalling is crucial for naïve T cells to obtain a Th17 phenotype, mainly through Smad signalling, but also to a lesser degree through TGF-β induced non-Smad pathways.

Experiments with Smad2 and Smad3 double deficient T cells elucidated that Eomesodermin, a transcription factor, downregulates Th17 differentiation (Ichiyama et al. 2011). Expression of eomesodermin was suppressed by the TGF-β induced Smad-independent JNK pathway (Ichiyama *et al.* 2011). We performed phospho protein antibody array with WT and Smad7Tg Th17 cells in order to find TGF-β induced, non-Smad signalling pathways which are involved in Th17 differentiation. Protein phosphorylation was measured by a series of calculations both in WT and Smad7Tg Th17 polarized T cells.

Four proteins, AKT1 (Phospho-Tyr474), mTOR (Phospho-Thr2446), p38 MAPK (Phospho-Thr180) and PKC delta (Phospho-Thr505) were found to be phosphorylated both in WT and Smad7Tg proteins during Th17 differentiation, which means these four proteins were activated by TGF-β through non-Smad signalling pathways. Using immunoblotting, the phosphorylation status of AKT1 (Phospho-Tyr474), mTOR (Phospho-Thr2446), p38 MAPK (Phospho-Thr180) proteins was detected quite early during the Th17 differentiation process. Phosphorylation of PKC delta (Phospho-Thr505) and of the other indicated proteins at later time points, e.g. 48 hours, could not be included in this thesis. However, further experiments examining later time points should be done in future.

5.4 The PI3K/AKT/mTOR pathway and Th17 differentiation

PI3K as well as mTOR pathways are two key cellular signalling pathways that affect broad aspects of cellular functions, including metabolism, growth and survival. (Deane *et al.* 2004, Wullschleger *et al.* 2006). Although initially viewed as two separate pathways, it has been shown that PI3K and mTOR signalling are connected via the serine/threonine kinase AKT (Sekulic *et al*. 2000). AKT, is one of the most important survival kinases involved in regulating a similarly wide array of cellular processes as PI3K and mTOR, including metabolism, growth, proliferation and apoptosis (Brazil *et al*. 2004). The entire PI3K/AKT/mTOR pathway has been long known to be important in regulating adaptive immune cell activation. For example, different PI3K heterodimers, but also mTOR, critically control cell survival, proliferation, B- and T-cell receptor (BCR and TCR, respectively) signalling and chemotaxis in B and T lymphocytes (Koyasu *et al.* 2003, Okkenhaug *et al.* 2003).

Mice lacking PI3K signals have defective thymocyte survival (Sasaki *et al.* 2000, Webb *et al.* 2005, Swat *et al.* 2006), while constitutive activation of AKT increases thymocyte survival (Jones *et al.* 2000). PI3K/mTOR regulates T cell proliferation by controlling autocrine IL-2 production by T cells (Colombetti *et al.* 2006). Activated $CD4^+$ T cells can become immune effectors that drive immune responses (Ansel *et al.* 2006) or differentiate into regulatory T cells, which dampen immune responses (Sakaguchi *et al.* 2008) and PI3K/AKT/mTOR signaling controls these events (Sauer *et al.* 2008, Haxhinasto *et al.* 2008). T lymphocyte trafficking is also regulated by PI3K/AKT and the nutrient-sensing mTOR pathway (Sinclair *et al.* 2008, Waugh *et al.* 2009). The adipocyte-derived proinflammatory hormone leptin increases the activity of the AKT/mTOR pathway which is required for MOG-specific autoreactive $CD4^+$ T cells to induce EAE (Galgani *et al.* 2010). It has also been reported that PI3K/AKT/mTOR kinase signalling blocks regulatory T cell differentiation by inhibiting Foxp3 expression (Merkenschlager and Von Boehmer 2010). However, when AKT signals through protein kinase B, it induces the function of natural Tregs and regulatory T cells formation (Pierau *et al.* 2009).

PI3Ks are divided into four classes (IA, IB, II, and III) based on their subunit composition (Cantrell *et al.* 2001). Class IA and IB PI3Ks are the best understood in the immune system and are the subject of this discussion (Fruman *et al.* 2007). Class IA PI3Ks are heterodimers consisting of a regulatory adaptor subunit (p85α, p55α, p50α, p85β, or p55γ) and a catalytic subunit (p110α, p110β, or p110δ) (Fruman *et al.* 2007). Class IB PI3Ks differ in their subunit composition, as they are heterodimers of the catalytic subunit p110γ paired with a regulatory subunit p101 or p84 (Andrews *et al.* 2007). There are three isoforms of AKT, AKT1, AKT2, AKT3, the isoforms 1 and 2 have the highest expression in thymocytes (Juntilla *et al*. 2007). mTOR signalling acts through two sub complexes: TORC1 (TOR Complex 1) and TORC2 (Guertin *et al*. 2006). TORC1 is rapamycin sensitive and contains Rheb (a small GTPase), the regulatory-associated protein of mTOR (raptor), G protein b-subunit like protein (GbL), and the proline-rich Protein Kinase B (PKB)/AKT substrate 40 kDa (PRAS40). TORC1 activation results in the phosphorylation and activation of, among other targets, the ribosomal S6 kinase (S6K1), and is thought to be associated with ribosome biogenesis, autophagy, and protein translation (Sabatini 2006). TORC2

contains, in addition to mTOR and GbL, the rapamycin-insensitive companion of mTOR (rictor) and mammalian stress-activated protein kinase interacting protein-1 (mSin1) (Guertin *et al.* 2006). Although it was initially thought that rapamycin exclusively inhibited mTORC1 activation, it has become clear that it can also inhibit mTORC2 (Zeng *et al.* 2007).

In the phospho protein antibody arrays, two members of the PI3K/AKT/mTOR pathway were found to be phosphorylated during Th17 differentiation which are AKT1 (Phospho-Tyr474) and mTOR (Phospho-Thr2446). We showed that inhibition of both mTOR and PI3K by rapamycin and LY294002, respectively, resulted in reduced Th17 differentiation. Of note, there is no selective AKT inhibitor available. LY294002 has the specificity for both PI3K and AKT according to the manufacturer. The effect of blocking was found to be dose dependent. It was already reported that rapamycin blocks Th17 differentiation but promotes Treg differentiation (Kopf *et al.* 2007), which might be due to rapamycin mediated IL-6 signal transduction inhibition (Kahan *et al.* 1991). Two recent studies examining a T cell specific mTOR deletion, particularly mTORC1, showed impaired Th1 and Th17 differentiation but induced Treg differentiation (Delgoffe *et al.* 2009, 2011). Until now the specific sites of mTOR phosphorylation remained unknown. In our study, we showed that mTOR was phosphorylated at Thr2446 through TGF-β-Smad independent signalling and positively regulated Th17 differentiation. As we found that mTOR can also be phosphorylated at other sites, it may very well be that additional phosphorylation sites of mTOR, e.g. Ser2448 and Ser2481, may contribute to the regulation of T helper cell differentiation. Constitutive AKT activity blocks Foxp3 induction (Haxhinasto *et al.* 2008). mTORC2 activates AKT by phosphorylating it on Ser473, and loss or inhibition of mTORC2 inactivates AKT and consequently promotes Foxp3 induction (Delgoffe *et al.* 2009). A recent study utilizing Rictor knockout T cells demonstrated that the differentiation of Th1 and Th2 is highly dependent on TORC2 signalling, leading to activation of AKT and PKC-θ respectively, whereas the development of Th17 cells seems to be unimpaired by the lack of TORC2 activity (Lee *et al.* 2010). We detected AKT1 to be phosphorylated on Tyr474 in both WT and Smad7Tg Th17 polarized cells, which indicates that AKT1p474 is involved in non-Smad signalling during Th17 differentiation.

5.5 The MAPK pathways and Th17 differentiation

Besides the canonical TGF-β-Smad pathway, TGF-β can also activate Smad-independent pathways, such as MAPKs, in T cells (Zhang 2009b). MAPK pathways are involved in T helper cell differentiation. Naïve $CD4^+$ T cells stimulated with TGF-β significantly increase phosphorylation of ERK and JNK but not p38 (Lu *et al.* 2010). Experiments with pharmacological inhibitors of JNK or ERK as well as ERK1 and JNK2 KO mice show attenuated Foxp3 expression in TGF-β primed $CD4^+$ T cells, whereas inhibition of p38 does not have any effect on Foxp3 expression (Lu *et al.* 2010). On the contrary, another study showed that TGF-β activates the p38 MAPK in naïve T cells and plays a role in the conversion of naïve T cells to iTregs (Huber *et al.* 2008). The JNK pathway is also involved in Th17 differentiation (Lu *et al.* 2010). TGF-β induces the JNK pathway, which in turn suppresses the expression of eomesodermin, a transcription factor negatively regulating Th17 differentiation (Ichiyama *et al.* 2011). Recent studies with pharmacological inhibition showed that p38 positively regulates Th17 differentiation (Lu *et al*. 2010, Li *et al.* 2010, Gulen *et al.* 2010, Commodaro *et al.* 2010, Noubade *et al*. 2011). The MAPK p38 can be phosphorylated at various residues and the specific site of phosphorylation during Th17 differentiation was unclear. Performing phospho protein assay, we showed that the p38 MAPK pathway was phosphorylated on Thr180 both in WT and Smad7Tg Th17 differentiation. So, during Th17 differentiation, p38 was phosphorylated only on Thr180 via non-TGF-β-Smad signalling pathway. We also showed site specific JNK phosphorylation during Th17 differentiation. In addition, doing functional assay, we found that pharmacological inhibition of JNK and p38 MAPK pathways resulted in a decreased proportion of IL-17 producing Th17 cells. In the protein array, we also found that protein kinase C delta (PKCδ) was phosphorylated on Thr505 both during WT and Smad7Tg Th17 differentiation. PKCδ is the first PKC isotype of the novel subfamily nPKC to be identified, which is expressed ubiquitously among cells and tissues. Both binding of diacylglycerol/phorbol ester and tyrosine phosphorylation is known to regulate enzymatic activation of PKCδ (Kikkawa *et al.* 2002). Evidences indicate that PKCδ plays a critical negative role in cellular functions, e.g. by inhibiting proliferation plus promoting cell death (Gschwendt 1999). For example, PKCδ plays a role in TCR-induced negative regulation of IL-2 cytokine production and T cell proliferation (Gruber *et al.* 2005). It has been already reported that PKCδ-deficient

mice have an increased susceptibility to autoimmune disease. Spleen and lymph nodes of PKCδ-deficient mice are enlarged and show a marked increase in the production of IgG antibodies that are specific for nuclear antigens (Mecklenbrauker *et al.* 2002, Miyamoto *et al.* 2002). PKCδ is activated during engagement of the Type I IFN receptor and associates with Stat1, which appears to be critical for phosphorylation of Stat1 on serine 727. Being activated, PKCδ induces activation of the p38 MAP kinase (Uddin *et al.* 2002). The precise role of PKCδ activated MAPK pathway in T helper cell differentiation remains unknown.

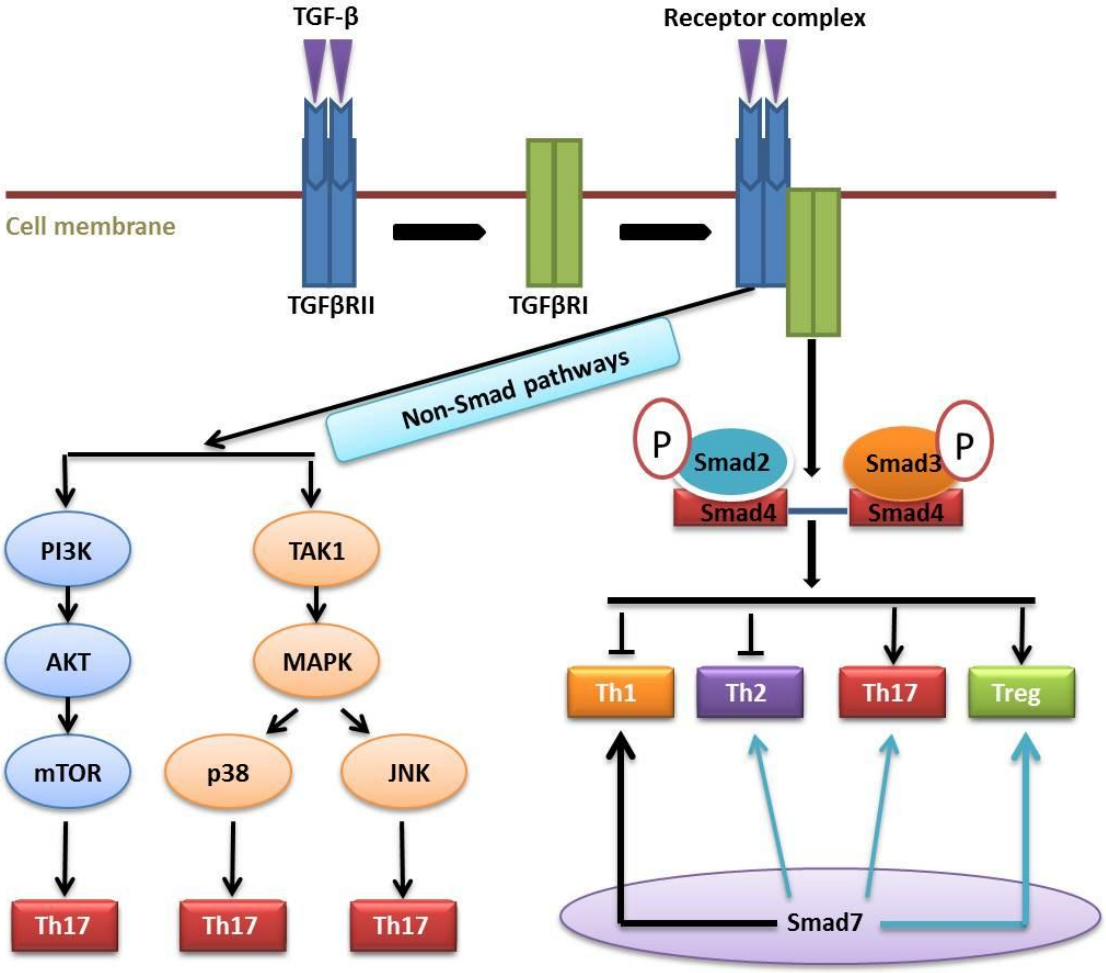

Figure 33: Schematic model of TGF-β-induced Smad and non-Smad signalling pathways in T helper cell differentiation. TGF-β downstream signalling through Smad proteins inhibits Th1, Th2 and induces Th17 and Tregs differentiation. Additionally, TGF-β-induced non-Smad signalling through PI3K/AKT/mTOR and p38, JNK MAPK pathways positively regulates Th17 differentiation. Smad7 has an intrinsic role in upregulating Th1 differentiation. It remains unknown if there is a direct role of Smad7 on Th1, Th17 and Treg development.

In summary, Smad7 regulates T helper cell differentiation by modulating canonical TGF-β signalling and by its own direct intrinsic effects. The results of this thesis divulge the potential involvement of non-Smad signalling pathways in T helper cell differentiation. Further investigation of these pathways, particularly during experimental autoimmune diseases, might be worth to find diagnostic measures and therapeutic agents for the treatment of T helper cell induced diseases.

6. Summary

The cytokine transforming growth factor (TGF-β) has a pivotal role in T cell differentiation. Regulation of intracellular signalling pathways during T cell differentiation has been given a great attention recently. Under certain conditions TGF-β inhibits T helper (Th1) and Th2 differentiation or induces regulatory T cell and Th17 development, mainly through the Smad-dependent signalling pathway. In addition to that, growing evidence implicates the involvement of non-Smad signalling pathways in T cell differentiation. Smad7 is the intracellular inhibitor of the canonical TGF-β signalling cascade, but its role in T cell differentiation was unknown.

In order to investigate the role of Smad7 in T cell differentiation, we used mice with a T cell specific Smad7 deletion, which allowed us to study the function of Smad7 exclusively in T cells. These Smad7 conditional knockout mice show normal immune homeostasis but less activated T cells in the periphery as compared to control wild type littermates. Smad7 deletion in T cells results in decreased Th1, Th2 and increased Th17 and regulatory T cell differentiation. Smad7 shows no effect on the suppressive capacity of regulatory T cells but exerts effects on the proliferation of responder T cells when challenged with regulatory T cell suppression. Furthermore, Smad7 plays a direct role in priming Th1 differentiation which is associated with T-bet transcription. Upon blocking the canonical TGF-β-Smad signalling pathway, naive T cells differentiate into Th17 cells to a smaller extent. Using a TGF-β downstream signalling phospho-protein antibody array, we show that PI3K/AKT/mTOR and p38 MAPK signalling pathways, which are independent of the canonical TGF-β-Smad cascade, are activated at specific phosphorylation sites during Th17 differentiation. Inhibition of these pathways leads to decreased IL-17 production by Th17 cells.

In essence, Smad7 is a major regulator of T cell differentiation. Besides the canonical signalling cascade of TGF-β, naïve T cells use non-Smad pathways to differentiate into the Th17 subset. Signalling through PI3K/AKT/mTOR and p38 MAPK pathways plays a crucial role in this event. This study provides insight into molecular mechanisms of T helper cell differentiation and thus helps to elucidate the pathophysiology of autoimmune diseases like multiple sclerosis which could eventually lead to development of new diagnostic or therapeutic procedures.

7. References

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8. General list of Abbreviations

ActR	activin receptor
APC	antigen presenting cell
ALK	activin receptor-like kinase
AMH	anti-Müllerian hormone
BBB	blood brain barrier
BMP	bone morphogenetic protein
bp	base pair
^{0}C	temperature in degrees Celsius
CD	cluster differentiation
cDNA	complementary DNA
CIA	collagen induced arthritis
c-MAF	musculoaponeurotic fibrosarcoma oncogene homolog
Cre	site-specific recombinase
DMSO	Dimethyl sulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleotide-triphosphate
EAE	Experimental autoimmune encephalomyelitis
EDTA	ethylene-diaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorting
FasL	Fas ligand
FITC	fluorescein-isothiocyanate
Foxp3	forkhead box P3
g	gram & gravitational force
GFP	green fluorescent protein

GM-CSF	granulocyte-macrophage colony-stimulating factor
h	hour & human
HEPES	N-2—hydroxyethylpiperazine-N’-2-ethanesulfonic acid
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iTreg	inducible regulatory T cells
JNK	jun N-terminal kinase
kb	kilobase pair
KDa	kilodalton
L	liter
LAP	latency-associated protein
Loxp	recognition sequence for Cre
LTBP	latent TGF-β-binding protein
M	molar
MACS	magnetic cell sorting
MHC	major histocompatibility complex
MH1/2	Mad homology1/2
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
min	minute
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NaCI	sodium chloride
NaOH	sodium hydroxide
OD	optical density

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrine
PI3K	The phosphatidylinositol-3 kinase
PMA	phorbol 12-myristate 13-acetate
qPCR	quantitative reverse transcriptase real time PCR
r	recombinant
RNA	ribonucleic acid
ROR	retinoid-related orphan receptor
RT	room temperature
Runx3	Runt-related transcription factor 3
s	second
SAD	Smad activation domain
SARA	Smad anchor for receptor activation
SBE	Smad binding element
SDS	sodium dodecyl sulfate
SMURF	Smad ubiquitylation regulatory factor
Smad7	Smad7 family member 7
STAT	signal transducer of activated cells
TAK1	TGF-β-activated kinase1
TCR	T cell receptor
TGF-β	Transforming growth factor β1
Th	T helper
TLR	toll like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell

TβR	TGF-β receptor
v/v	volume per volume
w/v	weight per volume
WT	wild type

9. List of tables

10. List of figures

11. Appendix

S1: Purity of naïve T cells ($CD4^+CD62L^+$) - 97.35% pure

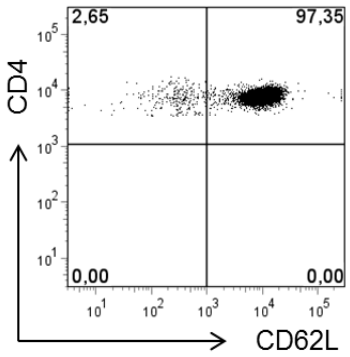

Figure 34: Purity of naïve T cells. Single cell suspensions were made from spleen and LNs from WT, CD4Cre-Smad7$^{fl/fl}$ and Smad7Tg mice. From the cell suspension, naïve T cells were sorted either by magnetic beads or FACS sorting. This is one representative figure (WT naïve T cells) of all naïve $CD4^+CD62L^+$ T cells sorted by magnetic beads. Cells were gated on lymphocytes. The percentage of gated cells is indicated.

S2: Purity of Tregs ($CD4^+CD25^+$) - 98.86% pure

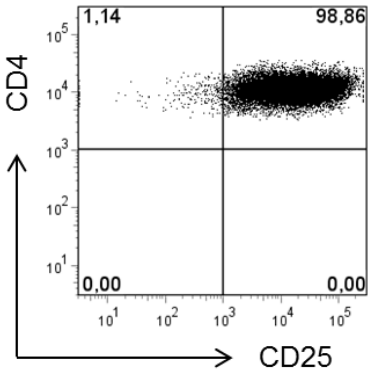

Figure 35: Purity of Treg cells. Single cell suspensions were made from spleen and LNs from WT, CD4Cre-Smad7$^{fl/fl}$ and Smad7Tg mice. From the cell suspension, Treg cells were sorted either by magnetic beads or FACS sorting. This is one representative figure (WT Treg cells) of all Treg cells sorted by magnetic beads. Cells were gated on lymphocytes. The percentage of gated cells is indicated.

S3. Th17 differentiation in vitro from WT and Smad7Tg mice after 48 hours

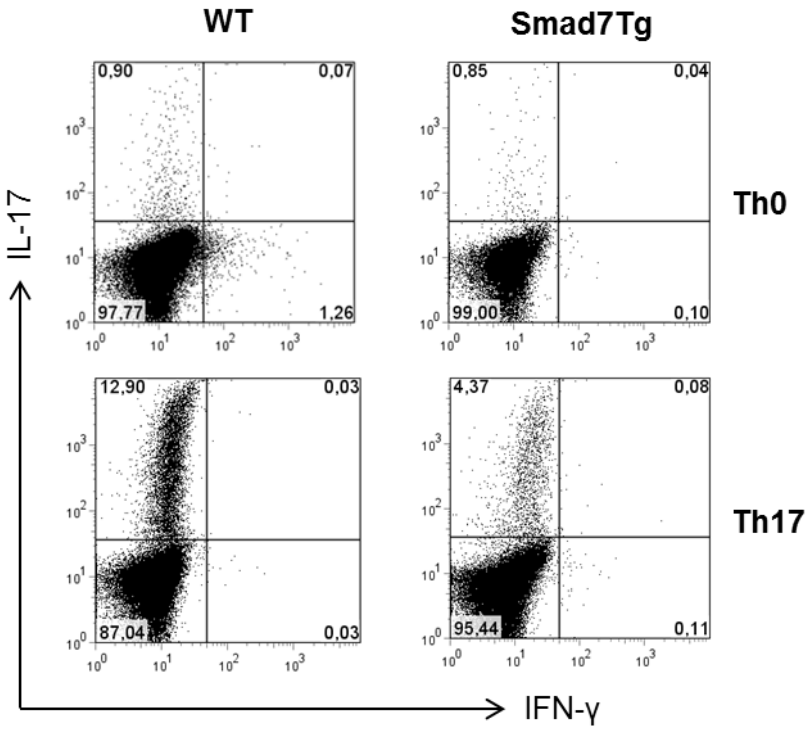

Figure 36: Th17 differentiation from WT and Smad7Tg T cells after 48 hours. Naïve T cells were sorted from spleen and LNs from WT and Smad7Tg mice and cultured for 48 hours in Th17 differentiation condition using a-CD3, a-CD28, rIL-6, a-IL-4, a-IFN-γ. This is one representative figure of all Th17 differentiations done for the phospho protein arrays. Cells were gated on lymphocytes and $CD4^+$ T cells. The percentage of gated cells is indicated.

S4. List of proteins detected in phosphorylated and non-phosphorylated form on the TGF-β phospho antibody microarray kit from Fullmoon Bioscience.

A) Proteins in both phosphorylated and non-phosphorylated form

Number	Protein	Phosphorylation site	Antibody used to detect non-phosphorylated form
1	Abl1	Thr754/735, Tyr204, Tyr412	Ab-754/735, Ab-Tyr204,
2	AKT	Ser473, Thr308, Tyr326	Ab-473, Ab-Thr308, Ab-Tyr326
3	AKT1	Ser124, Ser246, Thr450 Thr72, Tyr474	Ab-124, Ab-246, Ab-450 Ab-72, Ab-474
4	AKT2	Ser474	Ab-474
5	c-Abl	Tyr245, Tyr412	Ab-412
6	cofilin	Ser3	Ab-3
7	ERK1-p44/42 MAP Kinase	Thr202, Tyr204	Ab-202, Ab-204
8	ERK3	Ser189	Ab-189
9	ERK8	Thr175/Tyr177	
10	Gab2	Tyr643	Ab-623
11	JNK1/2/3	Thr183/Tyr185	Ab-183/185
12	LIMK1	Thr508	Ab-508
13	MAP3K1/MEKK1	Thr1381	
14	MKK3	Ser189	Ab-189
15	MKK3/MAP2K3	Thr222	Ab-222
16	MKK6	Ser207	Ab-207
17	mTOR	Ser2448, Ser2481,	Ab-2448, Ab-2481,

		Thr2446	Ab-2446
18	Myc	Ser373, Ser62, Thr358, Thr58	Ab-373, Ab-62, Ab-358, Ab-58,
19	p38 MAPK	Thr180, Tyr182, Tyr322	Ab-180, Ab-182, Ab-322
20	PAK1	Ser204, Thr212	Ab-204, Ab-212
21	PAK1/2	Ser199	Ab-199
22	PAK1/2/3	Ser141, Thr423/402/421	Ab-141, Ab-423/402/421
23	PAK2	Ser192, Ser20	Ab-192, Ab-197,
24	PAK3	Ser154	Ab-154
25	PI3-kinasep85-alpha	Tyr607	
26	PI3-kinase p85-subunit alpha/gamma	Tyr467/199	Ab-467/199
27	PKC alpha	Tyr657	Ab-657
28	PKC alpha/beta II	Thr638	Ab-638
29	PKC beta/PKCB	Ser661	Ab-661
30	PKC delta	Ser645, Thr505	Ab-645, Ab-505
31	PKC epsilon	Ser729,	Ab-729
32	PKC theta	Ser676, Thr538	Ab-676, Ab-538
33	PKC zeta	Thr410, Thr560	Ab-410, Ab-560
34	PP2A-a	Tyr307	Ab-307
35	Rac1/cdc42	Ser71	Ab-71
36	Ras-GRF1	Ser916	Ab-916
37	Rho/Rac guanine nucleotide	Ser885	Ab-885

	exchange factor 2		
38	S6 Ribosomal Protein	Ser235	Ab-235
39	SAPK/JNK	Thr183, Tyr185	Ab-183, Ab-185
40	SEK1/MKK4	Ser80, Thr261	Ab-80, Ab-261
41	Shc	Tyr349, Tyr427	Ab-349, Ab-427
42	Smad1	Ser187, Ser465	Ab-187, Ab-465
43	Smad2	Ser250, Ser467, Thr220	Ab-250, Ab-467, Ab-220 Ab-255, ab-245,
44	Smad2/3	Thr8	Ab-8
45	Smad3	Ser204, Ser208, Ser213, Ser425, Thr179	Ab-204, Ab-213, Ab-425, Ab-179
46	SP1	Thr739	Ab-739
47	TAK1	Thr184	Ab-184

Table 22: List of proteins which can be detected in phosphorylated and non-phosphorylated forms

B) Protein only in non-phosphorylated form

Number	Unphosphorylated form
1	Beta actin
2	CBP (Inter)
3	ERK1/2 (N-term)
4	GAPDH
5	JNKK (MKK4) (Inter)
6	p300/CBP (C-term)
7	RAS(p21 H and K) (Inter)

8	RhoA (Ab-188)
9	S6K (Inter)
10	S6K-alpha 6 (Inter)
11	Smad1/5/9 (Inter)
12	Smad4 (Inter)
13	TGF alpha (inter)
14	TGF beta1 (inter)
15	TGF beta2 (inter)
16	TGF beta3 (inter)
17	TGFBR1 (Ab-165)
18	TGFBR2 (Ab-250)

Table 23: List of proteins which can be detected in non-phosphorylated form

S5. Array raw data

Raw data of all arrays are presented in tables. Equations in the columns 11, 12,13, 14 and 15 (with letters A, B, C, D and E) correspond to the equations shown in the methods sections (page 61), by which protein expression and phosphorylation status were calculated. Categories in the interpretation (column 16) were made based on columns 11-14. Column 15 was not used because this calculation resulted in high variance in protein phosphorylation between experiments.

Array1 WT (1.1)

Non Phospho Protein	Phospho Protein	Non Phospho Protein Median Intensity		Phospho Protein Median Intensity		Non Phospho Protein Normalized Data		Phospho Protein Normalized Data		Non Phospho Protein Ratio Th17/Th0 (A)	Phospho Protein Ratio Th17/Th0 (B)	Signal Ratio P-Protein to Non-P-Protein (C for Th0) (D for Th17)		P- to Non-P Ratio Th17/Th0 (E)	Interpretation
		Th0	Th17	Th0	Th17	Th0	Th17	Th0	Th17	>150, <50	>150, <50	Th0	Th17	>150, <50	Category 1-10
Abl1 (Ab-754/735)	Abl1 (Phospho-Thr754/735)	5187	12823	0	1115	1,52	1,66	0,00	0,14	109					
Abl1 (Ab-204)	Abl1 (Phospho-Tyr204)	11821	25261	6247	12450	3,46	3,27	1,83	1,61	95	88	0,53	0,49	93	**2**
Abl1 (Ab-204)	Abl1 (Phospho-Tyr412)			7119	12303			2,08	1,59		76	0,60	0,49	81	**5**
AKT (Ab-473)	AKT (Phospho-Ser473)	0	4396	4297	11976	0,00	0,57	1,26	1,55		123		2,72		**8**
AKT (Ab-308)	AKT (Phospho-Thr308)	0	2905	3470	11023	0,00	0,38	1,01	1,43		140		3,79		**8**
AKT (Ab-326)	AKT (Phospho-Tyr326)	0	1023	4327	10820	0,00	0,13	1,27	1,40		111		10,58		**8**
AKT1 (Ab-124)	AKT1 (Phospho-Ser124)	4474	8265	4974	9391	1,31	1,07	1,45	1,21	82	84	1,11	1,14	102	**5**
AKT1 (Ab-246)	AKT1 (Phospho-Ser246)	0	6750	3411	10983	0,00	0,87	1,00	1,42		142		1,63		**8**
AKT1 (Ab-450)	AKT1 (Phospho-Thr450)	3428	10351	1901	10023	1,00	1,34	0,56	1,30	134	233	0,55	0,97	175	**6**
AKT1 (Ab-72)	AKT1 (Phospho-Thr72)	5437	8237	0	4163	1,59	1,07	0,00	0,54	67		0,00	0,51		**3**
AKT1 (Ab-474)	AKT1 (Phospho-Tyr474)	0	1494	1727	7050	0,00	0,19	0,51	0,91		181		4,72		**9**
AKT2 (Ab-474)	AKT2 (Phospho-Ser474)	0	799	0	6251	0,00	0,10	0,00	0,81				7,82		**9**
c-Abl (Ab-412)	c-Abl (Phospho-Tyr245)			2366	9518			0,69	1,23		178	0,59	0,69	117	**6**
c-Abl (Ab-412)	c-Abl (Phospho-Tyr412)	4028	13892	0	7035	1,18	1,80	0,00	0,91	153		0,00	0,51		**3**
cofilin (Ab-3)	cofilin (Phospho-Ser3)	231	7757	0	4964	0,07	1,00	0,00	0,64	1485		0,00	0,64		**6**
ERK1-p44/42 MAP Kinase (Ab-202)	ERK1-p44/42 MAP K(P-Thr202)	0	762	0	1361	0,00	0,10	0,00	0,18				1,79		**9**
ERK1-p44/42 MAP Kinase (Ab-204)	ERK1-p44/42 MAP K(P-Tyr204)	0	5349	4226	14427	0,00	0,69	1,24	1,87		151		2,70		**9**
ERK3 (Ab-189)	ERK3 (Phospho-Ser189)	0	0	5051	11762	0,00	0,00	1,48	1,52		103				**8**
ERK3 (Ab-189)	ERK8 (Phospho-Thr175/Tyr177)			3218	8168			0,94	1,06		112				**5**
Gab2 (Ab-623)	Gab2 (Phospho-Tyr643)	0	0	6172	16485	0,00	0,00	1,80	2,13		118				**8**
JNK1/2/3 (Ab-183/185)	JNK1/2/3 (P-Thr183/Tyr185)	1753	8083	0	3865	0,51	1,05	0,00	0,50	204		0,00	0,48		**3**
LIMK1 (Ab-508)	LIMK1 (Phospho-Thr508)	0	4323	4415	12391	0,00	0,56	1,29	1,60		124		2,87		**8**
MKK3 (Ab-189)	MKK3 (Phospho-Ser189)	14	7751	3846	10011	0,00	1,00	1,12	1,29	24487	115	274,71	1,29	0	**5**
MKK3/MAP2K3 (Ab-222)	MKK3/MAP2K3 (P-Thr222)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
MKK6 (Ab-207)	MKK6 (Phospho-Ser207)	0	3609	5300	10851	0,00	0,47	1,55	1,40		91		3,01		**8**
mTOR (Ab-2448)	mTOR (Phospho-Ser2448)	0	749	126	8960	0,00	0,10	0,04	1,16		3145		11,96		**9**
mTOR (Ab-2481)	mTOR (Phospho-Ser2481)	0	581	2217	10641	0,00	0,08	0,65	1,38		212		18,31		**9**
mTOR (Ab-2446)	mTOR (Phospho-Thr2446)	0	0	1604	8906	0,00	0,00	0,47	1,15		246				**9**
Myc (Ab-373)	Myc (Phospho-Ser373)	0	6955	1580	10299	0,00	0,90	0,46	1,33		288		1,48		**9**
Myc (Ab-62)	Myc (Phospho-Ser62)	0	1576	4037	11074	0,00	0,20	1,18	1,43		121		7,03		**8**
Myc (Ab-358)	Myc (Phospho-Thr358)	3799	10126	1385	10315	1,11	1,31	0,41	1,33	118	329	0,36	1,02	279	**5**
Myc (Ab-58)	Myc (Phospho-Thr58)	65	9252	0	2693	0,02	1,20	0,00	0,35	6295		0,00	0,29		**3**
p38 MAPK (Ab-180)	p38 MAPK (Phospho-Thr180)	0	0	0	2239	0,00	0,00	0,00	0,29						**9**
p38 MAPK (Ab-182)	p38 MAPK (Phospho-Tyr182)	9370	21770	1769	7927	2,74	2,82	0,52	1,03	103	198	0,19	0,36	193	**3**
p38 MAPK (Ab-322)	p38 MAPK (Phospho-Tyr322)	0	3843	4978	10877	0,00	0,50	1,46	1,41		97		2,83		**8**
PAK1 (Ab-204)	PAK1 (Phospho-Ser204)	2	1556	0	4535	0,00	0,20	0,00	0,59	34410		0,00	2,91		**9**
PAK1 (Ab-212)	PAK1 (Phospho-Thr212)	0	2625	1769	7735	0,00	0,34	0,52	1,00		193		2,95		**9**
PAK1/2 (Ab-199)	PAK1/2 (Phospho-Ser199)	0	5970	1469	8109	0,00	0,77	0,43	1,05		244		1,36		**9**
PAK1/2/3 (Ab-141)	PAK1/2/3 (Phospho-Ser141)	0	2228	4048	10474	0,00	0,29	1,18	1,35		114		4,70		**8**
PAK1/2/3 (Ab-423/402/421)	PAK1/2/3 (P-Thr423/402/421)	0	0	2597	8895	0,00	0,00	0,76	1,15		151				**9**
PAK2 (Ab-192)	PAK2 (Phospho-Ser192)	929	2104	2499	9275	0,27	0,27	0,73	1,20	100	164	2,69	4,41	164	**9**
PAK2 (Ab-197)	PAK2 (Phospho-Ser20)	0	1370	0	938	0,00	0,18	0,00	0,12				0,68		**6**
PAK3 (Ab-154)	PAK3 (Phospho-Ser154)	0	1154	3022	11413	0,00	0,15	0,88	1,48		167		9,89		**9**
PI3-kinase p85-subunit alpha/gamma (A	PI3-kinase p85-alpha (P-Tyr607)			4450	9611			1,30	1,24		96	0,83	1,13	136	**5**
PI3-kinase p85-subunit alpha/gamma (A	PI3-ki p85-sa/g(P-Tyr467/199)	5380	8538	3151	9263	1,57	1,10	0,92	1,20	70	130	0,59	1,08	185	**5**
PKC alpha (Ab-657)	PKC alpha (Phospho-Tyr657)	0	0	6727	13904	0,00	0,00	1,97	1,80		91				**8**
PKC alpha/beta II (Ab-638)	PKC alpha/beta II (P-Thr638)	0	0	3240	10504	0,00	0,00	0,95	1,36		143				**8**
PKC beta/PKCB (Ab-661)	PKC beta/PKCB (P-Ser661)	0	3	0	4723	0,00	0,00	0,00	0,61				1574,33		**9**
PKC delta (Ab-645)	PKC delta (Phospho-Ser645)	2603	9689	3002	6972	0,76	1,25	0,88	0,90	165	103	1,15	0,72	62	**5**

Array1 WT (1.2)

PKC delta (Ab-505)	PKC delta (Phospho-Thr505)	0	0	0	291	0,00	0,00	0,00	0,04						**9**
PKC epsilon (Ab-729)	PKC epsilon (Phospho-Ser729)	0	0	4493	12473	0,00	0,00	1,31	1,61		123				**8**
PKC theta (Ab-676)	PKC theta (Phospho-Ser676)	0	1132	4252	10669	0,00	0,15	1,24	1,38		111		9,42		**8**
PKC theta (Ab-538)	PKC theta (Phospho-Thr538)	13939	20629	5033	10797	4,08	2,67	1,47	1,40	65	95	0,36	0,52	145	**2**
PKC zeta (Ab-410)	PKC zeta (Phospho-Thr410)	774	6983	5198	12903	0,23	0,90	1,52	1,67	399	110	6,72	1,85	28	**8**
PKC zeta (Ab-560)	PKC zeta (Phospho-Thr560)	0	800	743	6977	0,00	0,10	0,22	0,90		415		8,72		**9**
PP2A-a (Ab-307)	PP2A-a (Phospho-Tyr307)	1875	10161	4774	7721	0,55	1,31	1,40	1,00	240	72	2,55	0,76	30	**5**
Rac1/cdc42 (Ab-71)	Rac1/cdc42 (Phospho-Ser71)	0	3222	3736	13049	0,00	0,42	1,09	1,69		154		4,05		**9**
Ras-GRF1 (Ab-916)	Ras-GRF1 (Phospho-Ser916)	0	896	1682	6923	0,00	0,12	0,49	0,90		182		7,73		**9**
Rho/Rac guanine nucleotide exchange	Rho/Rac g n efactor 2 (P-Ser885)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
S6 Ribosomal Protein (Ab-235)	S6 Ribosomal Protein (P-Ser235)	0	3745	0	6200	0,00	0,48	0,00	0,80				1,66		**9**
SAPK/JNK (Ab-183)	SAPK/JNK (Phospho-Thr183)	0	3586	745	7685	0,00	0,46	0,22	0,99		456		2,14		**9**
SAPK/JNK (Ab-185)	SAPK/JNK (Phospho-Tyr185)	0	2547	3103	8992	0,00	0,33	0,91	1,16		128		3,53		**9**
SEK1/MKK4 (Ab-80)	SEK1/MKK4 (Phospho-Ser80)	2395	3890	2119	10617	0,70	0,50	0,62	1,37	72	222	0,88	2,73	308	**9**
SEK1/MKK4 (Ab-261)	SEK1/MKK4 (Phospho-Thr261)	10084	25809	4613	15645	2,95	3,34	1,35	2,02	113	150	0,46	0,61	133	**2**
Shc (Ab-349)	Shc (Phospho-Tyr349)	4807	10691	5714	12921	1,41	1,38	1,67	1,67	98	100	1,19	1,21	102	**5**
Shc (Ab427)	Shc (Phospho-Tyr427)	6066	13643	6353	13136	1,77	1,76	1,86	1,70	99	91	1,05	0,96	92	**5**
Smad1 (Ab-187)	Smad1 (Phospho-Ser187)	0	0	5867	9937	0,00	0,00	1,72	1,29		75				**8**
Smad1 (Ab-465)	Smad1 (Phospho-Ser465)	1102	7727	4306	9621	0,32	1,00	1,26	1,24	310	99	3,91	1,25	32	**5**
Smad2 (Ab-250)	Smad2 (Phospho-Ser250)	0	0	0	2229	0,00	0,00	0,00	0,29						**9**
Smad2 (Ab-467)	Smad2 (Phospho-Ser467)	0	4165	3791	7728	0,00	0,54	1,11	1,00		90		1,86		**8**
Smad2 (Ab-220)	Smad2 (Phospho-Thr220)	3031	8755	2839	6934	0,89	1,13	0,83	0,90	128	108	0,94	0,79	85	**5**
Smad2/3 (Ab-8)	Smad2/3 (Phospho-Thr8)	0	1037	3588	8689	0,00	0,13	1,05	1,12		107		8,38		**8**
Smad3 (Ab-204)	Smad3 (Phospho-Ser204)	3390	9478	0	2213	0,99	1,23	0,00	0,29	124		0,00	0,23		**3**
Smad3 (Ab-204)	Smad3 (Phospho-Ser208)			0	2522			0,00	0,33			0,00	0,27		**6**
Smad3 (Ab-213)	Smad3 (Phospho-Ser213)	0	0	0	2809	0,00	0,00	0,00	0,36						**9**
Smad3 (Ab-425)	Smad3 (Phospho-Ser425)	1087	8629	360	8499	0,32	1,12	0,11	1,10	351	1044	0,33	0,98	297	**6**
Smad3 (Ab-179)	Smad3 (Phospho-Thr179)	0	3953	0	3957	0,00	0,51	0,00	0,51				1,00		**6**
SP1 (Ab-739)	SP1 (Phospho-Thr739)	0	3597	4652	14253	0,00	0,47	1,36	1,84		136		3,96		**8**
TAK1 (Ab-184)	TAK1 (Phospho-Thr184)	0	2065	0	0	0,00	0,27	0,00	0,00				0,00		**3**
Beta actin		11279	20439			3,30	2,64			80					**5**
CBP (Inter)		0	0			0,00	0,00								**1**
ERK1/2 (N-term)		2962	10713			0,87	1,39			160					**6**
GAPDH		0	0			0,00	0,00								**1**
JNKK (MKK4) (Inter)		0	0			0,00	0,00								**1**
	MAP3K1/MEKK1 (P-Thr1381)			0,00	3240,00			0,00	0,42						**6**
p300/CBP (C-term)		0	0			0,00	0,00								**1**
PAK4/PAK5/PAK6 (Ab-474)		2316	5426			0,68	0,70			104					**5**
RAS(p21 H and K) (Inter)		0	0			0,00	0,00								**1**
RASE (Inter)		3667	6369			1,07	0,82			77					**5**
RASF4 (Inter)		0	1674			0,00	0,22								**1**
RhoA (Ab-188)		2930	9067			0,86	1,17			137					**5**
S6K (Inter)		0	0			0,00	0,00								**1**
S6K-alpha 6 (Inter)		0	0			0,00	0,00								**1**
Smad1/5/9 (Inter)		0	1748			0,00	0,23								**6**
Smad2 (ab-245)		0	0			0,00	0,00								**1**
Smad2 (Ab-255)		359	5740			0,10	0,74			707					**6**
Smad4 (Inter)		0	0			0,00	0,00								**1**
TGF alpha (inter)		523	5007			0,15	0,65			423					**6**
TGF beta receptor II (inter)		289	3428			0,08	0,44			525					**6**
TGF beta1 (inter)		0	142			0,00	0,02								**6**
TGF beta2 (inter)		4126	8081			1,21	1,05			87					**5**
TGF beta3 (inter)		0	0			0,00	0,00								**1**
TGFBR1 (Ab-165)		0	5205			0,00	0,67								**6**
TGFBR2 (Ab-250)		118	6231			0,03	0,81			2335					**6**
negative marker		0	0			0,00	0,00								
positive marker		46019	48191			13,46	6,23			46					

Array2 WT (1.1)

Non Phospho Protein	Phospho Protein	Non Phospho Protein Median Intensity		Phospho Protein Median Intensity		Non Phospho Protein Normalized Data		Phospho Protein Normalized Data		Non Phospho Protein Ratio Th17/Th0 (A)	Phospho Protein Ratio Th17/Th0 (B)	Signal Ratio P-Protein to Non-P-Protein (C for Th0) (D for Th17)		P- to Non-P Ratio Th17/Th0 (E)	Interpretation
		Th0	Th17	Th0	Th17	Th0	Th17	Th0	Th17	>150, <50	>150, <50	Th0	Th17	>150, <50	Category 1-10
Abl1 (Ab-754/735)	Abl1 (Phospho-Thr754/735)	0	21853	0	13349	0,00	2,39	0,00	1,46				0,61		**6**
Abl1 (Ab-204)	Abl1 (Phospho-Tyr204)	4348	42522	0	9136	1,08	4,65	0,00	1,00	431		0,00	0,21		**3**
Abl1 (Ab-204)	Abl1 (Phospho-Tyr412)			664	7879			0,16	0,86		523	0,15	0,19	121	**6**
AKT (Ab-473)	AKT (Phospho-Ser473)	0	17450	0	1549	0,00	1,91	0,00	0,17				0,09		**3**
AKT (Ab-308)	AKT (Phospho-Thr308)	0	9910	0	11474	0,00	1,08	0,00	1,26				1,16		**6**
AKT (Ab-326)	AKT (Phospho-Tyr326)	2986	9962	0	1864	0,74	1,09	0,00	0,20	147		0,00	0,19		**2**
AKT1 (Ab-124)	AKT1 (Phospho-Ser124)	12745	24163	0	8897	3,16	2,64	0,00	0,97	84		0,00	0,37		**2**
AKT1 (Ab-246)	AKT1 (Phospho-Ser246)	0	4507	0	11269	0,00	0,49	0,00	1,23				2,50		**9**
AKT1 (Ab-450)	AKT1 (Phospho-Thr450)	0	13802	0	10555	0,00	1,51	0,00	1,15				0,76		**6**
AKT1 (Ab-72)	AKT1 (Phospho-Thr72)	14913	24355	0	12037	3,70	2,66	0,00	1,32	72		0,00	0,49		**2**
AKT1 (Ab-474)	AKT1 (Phospho-Tyr474)	0	374	0	8651	0,00	0,04	0,00	0,95				23,13		**9**
AKT2 (Ab-474)	AKT2 (Phospho-Ser474)	0	0	0	1612	0,00	0,00	0,00	0,18						**9**
c-Abl (Ab-412)	c-Abl (Phospho-Tyr245)			0	13001			0,00	1,42				0,68		**6**
c-Abl (Ab-412)	c-Abl (Phospho-Tyr412)	0	19137	0	15057	0,00	2,09	0,00	1,65				0,79		**6**
cofilin (Ab-3)	cofilin (Phospho-Ser3)	0	14595	0	4627	0,00	1,60	0,00	0,51				0,32		**3**
ERK1-p44/42 MAP Kinase (Ab-202)	ERK1-p44/42 MAP Kinase (Phosph	2120	7163	0	0	0,53	0,78	0,00	0,00	149		0,00	0,00		**2**
ERK1-p44/42 MAP Kinase (Ab-204)	ERK1-p44/42 MAP Kinase (Phosph	0	1535	0	10295	0,00	0,17	0,00	1,13				6,71		**9**
ERK3 (Ab-189)	ERK3 (Phospho-Ser189)	0	257	0	0	0,00	0,03	0,00	0,00				0,00		**1**
ERK3 (Ab-189)	ERK8 (Phospho-Thr175/Tyr177)			0	1019			0,00	0,11				3,96		**6**
Gab2 (Ab-623)	Gab2 (Phospho-Tyr643)	0	2982	0	43130	0,00	0,33	0,00	4,72				14,46		**9**
JNK1/2/3 (Ab-183/185)	JNK1/2/3 (Phospho-Thr183/Tyr18	0	729	0	15105	0,00	0,08	0,00	1,65				20,72		**9**
LIMK1 (Ab-508)	LIMK1 (Phospho-Thr508)	0	0	2287	14672	0,00	0,00	0,57	1,61		283				**9**
MKK3 (Ab-189)	MKK3 (Phospho-Ser189)	0	5709	0	7159	0,00	0,62	0,00	0,78				1,25		**6**
MKK3/MAP2K3 (Ab-222)	MKK3/MAP2K3 (Phospho-Thr222)	968	14701	0	0	0,24	1,61	0,00	0,00	669		0,00	0,00		**3**
MKK6 (Ab-207)	MKK6 (Phospho-Ser207)	0	6644	0	1534	0,00	0,73	0,00	0,17				0,23		**3**
mTOR (Ab-2448)	mTOR (Phospho-Ser2448)	0	1238	0	1467	0,00	0,14	0,00	0,16				1,18		**6**
mTOR (Ab-2481)	mTOR (Phospho-Ser2481)	2154	14792	10309	25640	0,53	1,62	2,56	2,81	303	110	4,79	1,73	36	**8**
mTOR (Ab-2446)	mTOR (Phospho-Thr2446)	0	0	0	18708	0,00	0,00	0,00	2,05						**9**
Myc (Ab-373)	Myc (Phospho-Ser373)	0	21054	0	13810	0,00	2,30	0,00	1,51				0,66		**6**
Myc (Ab-62)	Myc (Phospho-Ser62)	0	2192	0	3627	0,00	0,24	0,00	0,40				1,65		**9**
Myc (Ab-358)	Myc (Phospho-Thr358)	0	33684	0	11300	0,00	3,69	0,00	1,24				0,34		**3**
Myc (Ab-58)	Myc (Phospho-Thr58)	0	23100	0	1199	0,00	2,53	0,00	0,13				0,05		**3**
p38 MAPK (Ab-180)	p38 MAPK (Phospho-Thr180)	0	0	0	4280	0,00	0,00	0,00	0,47						**9**
p38 MAPK (Ab-182)	p38 MAPK (Phospho-Tyr182)	0	20647	0	5439	0,00	2,26	0,00	0,60				0,26		**3**
p38 MAPK (Ab-322)	p38 MAPK (Phospho-Tyr322)	1978	13759	0	0	0,49	1,51	0,00	0,00	306		0,00	0,00		**3**
PAK1 (Ab-204)	PAK1 (Phospho-Ser204)	10983	22065	0	25240	2,73	2,41	0,00	2,76	89		0,00	1,14		**2**
PAK1 (Ab-212)	PAK1 (Phospho-Thr212)	0	5449	0	10804	0,00	0,60	0,00	1,18				1,98		**9**
PAK1/2 (Ab-199)	PAK1/2 (Phospho-Ser199)	0	9168	6017	11133	0,00	1,00	1,49	1,22		82		1,21		**8**
PAK1/2/3 (Ab-141)	PAK1/2/3 (Phospho-Ser141)	0	6073	0	9140	0,00	0,66	0,00	1,00				1,51		**6**
PAK1/2/3 (Ab-423/402/421)	PAK1/2/3 (Phospho-Thr423/402/4	0	1015	0	13390	0,00	0,11	0,00	1,46				13,19		**9**
PAK2 (Ab-192)	PAK2 (Phospho-Ser192)	7132	21227	4337	30846	1,77	2,32	1,08	3,37	131	313	0,61	1,45	239	**9**
PAK2 (Ab-197)	PAK2 (Phospho-Ser20)	8351	14393	0	5976	2,07	1,57	0,00	0,65	76		0,00	0,42		**2**
PAK3 (Ab-154)	PAK3 (Phospho-Ser154)	10352	18188	1925	30598	2,57	1,99	0,48	3,35	77	700	0,19	1,68	905	**9**
PI3-kinase p85-subunit alpha/gamma (A	PI3-kinase p85-alpha (Phospho-Tyr607)			0	7194			0,00	0,79				2,19		**6**
PI3-kinase p85-subunit alpha/gamma (A	PI3-kinase p85-subunit alpha/gam	0	3289	0	9175	0,00	0,36	0,00	1,00				2,79		**9**
PKC alpha (Ab-657)	PKC alpha (Phospho-Tyr657)	0	0	1844	13675	0,00	0,00	0,46	1,50		327				**9**
PKC alpha/beta II (Ab-638)	PKC alpha/beta II (Phospho-Thr63	2023	6170	0	6411	0,50	0,68	0,00	0,70	134		0,00	1,04		**5**
PKC beta/PKCB (Ab-661)	PKC beta/PKCB (Phospho-Ser661)	8750	16402	0	25133	2,17	1,79	0,00	2,75	83		0,00	1,53		**9**
PKC delta (Ab-645)	PKC delta (Phospho-Ser645)	0	11408	13356	11782	0,00	1,25	3,32	1,29		39		1,03		**10**

Array2 WT (1.2)

PKC delta (Ab-505)	PKC delta (Phospho-Thr505)	0	2310	0	4202	0,00	0,25	0,00	0,46				1,82		**9**
PKC epsilon (Ab-729)	PKC epsilon (Phospho-Ser729)	0	6406	0	7911	0,00	0,70	0,00	0,87				1,23		**6**
PKC theta (Ab-676)	PKC theta (Phospho-Ser676)	0	0	0	3156	0,00	0,00	0,00	0,35						**9**
PKC theta (Ab-538)	PKC theta (Phospho-Thr538)	35918	41126	9171	14378	8,92	4,50	2,28	1,57	50	69	0,26	0,35	137	**5**
PKC zeta (Ab-410)	PKC zeta (Phospho-Thr410)	0	0	2090	8508	0,00	0,00	0,52	0,93		179				**9**
PKC zeta (Ab-560)	PKC zeta (Phospho-Thr560)	0	4203	0	0	0,00	0,46	0,00	0,00				0,00		**3**
PP2A-a (Ab-307)	PP2A-a (Phospho-Tyr307)	0	11629	0	11296	0,00	1,27	0,00	1,24				0,97		**6**
Rac1/cdc42 (Ab-71)	Rac1/cdc42 (Phospho-Ser71)	0	3184	0	5269	0,00	0,35	0,00	0,58				1,65		**9**
Ras-GRF1 (Ab-916)	Ras-GRF1 (Phospho-Ser916)	183	9594	883	9457	0,05	1,05	0,22	1,03	2310	472	4,83	0,99	20	**6**
Rho/Rac guanine nucleotide exchange f	Rho/Rac guanine nucleotide excha	0	1031	0	16953	0,00	0,11	0,00	1,85				16,44		**9**
S6 Ribosomal Protein (Ab-235)	S6 Ribosomal Protein (Phospho-Se	0	3369	0	2096	0,00	0,37	0,00	0,23				0,62		**6**
SAPK/JNK (Ab-183)	SAPK/JNK (Phospho-Thr183)	0	7228	0	8967	0,00	0,79	0,00	0,98				1,24		**6**
SAPK/JNK (Ab-185)	SAPK/JNK (Phospho-Tyr185)	0	0	0	3830	0,00	0,00	0,00	0,42						**9**
SEK1/MKK4 (Ab-80)	SEK1/MKK4 (Phospho-Ser80)	9308	17908	0	2238	2,31	1,96	0,00	0,24	85		0,00	0,12		**2**
SEK1/MKK4 (Ab-261)	SEK1/MKK4 (Phospho-Thr261)	297	35742	0	6230	0,07	3,91	0,00	0,68	5302		0,00	0,17		**3**
Shc (Ab-349)	Shc (Phospho-Tyr349)	0	8209	0	2699	0,00	0,90	0,00	0,30				0,33		**3**
Shc (Ab427)	Shc (Phospho-Tyr427)	1774	16194	0	1992	0,44	1,77	0,00	0,22	402		0,00	0,12		**3**
Smad1 (Ab-187)	Smad1 (Phospho-Ser187)	0	3109	0	382	0,00	0,34	0,00	0,04				0,12		**3**
Smad1 (Ab-465)	Smad1 (Phospho-Ser465)	0	8515	865	11737	0,00	0,93	0,21	1,28		598		1,38		**9**
Smad2 (Ab-250)	Smad2 (Phospho-Ser250)	0	0	0	666	0,00	0,00	0,00	0,07						**1**
Smad2 (Ab-467)	Smad2 (Phospho-Ser467)	0	10948	0	2010	0,00	1,20	0,00	0,22				0,18		**3**
Smad2 (Ab-220)	Smad2 (Phospho-Thr220)	0	19952	0	2492	0,00	2,18	0,00	0,27				0,12		**3**
Smad2/3 (Ab-8)	Smad2/3 (Phospho-Thr8)	0	7021	0	3756	0,00	0,77	0,00	0,41				0,53		**6**
Smad3 (Ab-204)	Smad3 (Phospho-Ser204)	0	6752	0	1625	0,00	0,74	0,00	0,18				0,24		**3**
Smad3 (Ab-204)	Smad3 (Phospho-Ser208)			0	3360			0,00	0,37				0,50		**6**
Smad3 (Ab-213)	Smad3 (Phospho-Ser213)	0	0	3258	21226	0,00	0,00	0,81	2,32		287				**9**
Smad3 (Ab-425)	Smad3 (Phospho-Ser425)	0	12351	0	10933	0,00	1,35	0,00	1,20				0,89		**6**
Smad3 (Ab-179)	Smad3 (Phospho-Thr179)	0	10213	0	2808	0,00	1,12	0,00	0,31				0,27		**3**
SP1 (Ab-739)	SP1 (Phospho-Thr739)	0	0	0	2571	0,00	0,00	0,00	0,28						**9**
TAK1 (Ab-184)	TAK1 (Phospho-Thr184)	0	7724	0	8091	0,00	0,85	0,00	0,89				1,05		**6**
Beta actin		22810	45217			5,66	4,95			87					**5**
CBP (Inter)		0	1772			0,00	0,19								**6**
ERK1/2 (N-term)		0	17246			0,00	1,89								**6**
GAPDH		0	0			0,00	0,00								**1**
JNKK (MKK4) (Inter)		0	0			0,00	0,00								**1**
	MAP3K1/MEKK1 (Phospho-Thr138	0	15784			0,00	1,73								**6**
p300/CBP (C-term)		0	0			0,00	0,00								**1**
PAK4/PAK5/PAK6 (Ab-474)		9591	18840			2,38	2,06			87					**5**
RAS(p21 H and K) (Inter)		0	0			0,00	0,00								**1**
RASE (Inter)		5280	16961			1,31	1,86			142					**5**
RASF4 (Inter)		0	0			0,00	0,00								**1**
RhoA (Ab-188)		0	11206			0,00	1,23								**6**
S6K (Inter)		0	0			0,00	0,00								**1**
S6K-alpha 6 (Inter)		0	0			0,00	0,00								**1**
Smad1/5/9 (Inter)		0	2217			0,00	0,24								**6**
Smad2 (ab-245)		0	0			0,00	0,00								**1**
Smad2 (Ab-255)		0	12057			0,00	1,32								**6**
Smad4 (Inter)		0	0			0,00	0,00								**1**
TGF alpha (inter)		1178	8917			0,29	0,98			334					**6**
TGF beta receptor II (inter)		3717	12831			0,92	1,40			152					**6**
TGF beta1 (inter)		0	7869			0,00	0,86								**6**
TGF beta2 (inter)		9988	27338			2,48	2,99			121					**5**
TGF beta3 (inter)		0	4562			0,00	0,50								**6**
TGFBR1 (Ab-165)		0	8315			0,00	0,91								**6**
TGFBR2 (Ab-250)		0	14684			0,00	1,61								**6**
negative marker		0	0			0,00	0,00								
positive marker		35376	39318			8,78	4,30			49					

Array3 WT (1.1)

Non Phospho Protein	Phospho Protein	Non Phospho Protein Median Intensity		Phospho Protein Median Intensity		Non Phospho Protein Normalized Data		Phospho Protein Normalized Data		Non Phospho Protein Ratio Th17/Th0 (A)	Phospho Protein Ratio Th17/Th0 (B)	Signal Ratio P-Protein to Non-P-Protein (C for Th0) (D for Th17)		P- to Non-P Ratio Th17/Th0 (E)	Interpretation
		Th0	Th17	Th0	Th17	Th0	Th17	Th0	Th17	>150, <50	>150, <50	Th0	Th17	>150, <50	Category 1-10
Abl1 (Ab-754/735)	Abl1 (Phospho-Thr754/735)	194	260	0	0	0,53	0,43	0,00	0,00	80,88		0,00	0,00		**2**
Abl1 (Ab-204)	Abl1 (Phospho-Tyr204)	9178	19234	0	646	25,18	31,84	0,00	1,07	126		0,00	0,03		**2**
Abl1 (Ab-204)	Abl1 (Phospho-Tyr412)			530	1063			1,45	1,76		121	0,06	0,06	96	**5**
AKT (Ab-473)	AKT (Phospho-Ser473)	0	94	159	413	0,00	0,16	0,44	0,68		157		4,39		**9**
AKT (Ab-308)	AKT (Phospho-Thr308)	164	616	161	536	0,45	1,02	0,44	0,89	227	201	0,98	0,87	89	**6**
AKT (Ab-326)	AKT (Phospho-Tyr326)	0	0	0	597	0,00	0,00	0,00	0,99						**9**
AKT1 (Ab-124)	AKT1 (Phospho-Ser124)	1160	1373	0	360	3,18	2,27	0,00	0,60	71		0,00	0,26		**2**
AKT1 (Ab-246)	AKT1 (Phospho-Ser246)	0	0	363	493	0,00	0,00	1,00	0,82		82				**8**
AKT1 (Ab-450)	AKT1 (Phospho-Thr450)	390	985	0	689	1,07	1,63	0,00	1,14	152		0,00	0,70		**6**
AKT1 (Ab-72)	AKT1 (Phospho-Thr72)	932	1504	0	0	2,56	2,49	0,00	0,00	97		0,00	0,00		**2**
AKT1 (Ab-474)	AKT1 (Phospho-Tyr474)	0	0	0	36	0,00	0,00	0,00	0,06						**9**
AKT2 (Ab-474)	AKT2 (Phospho-Ser474)	0	0	0	528	0,00	0,00	0,00	0,87						**9**
c-Abl (Ab-412)	c-Abl (Phospho-Tyr245)			0	239			0,00	0,40			0,00	0,12		**6**
c-Abl (Ab-412)	c-Abl (Phospho-Tyr412)	833	1953	0	187	2,29	3,23	0,00	0,31	141		0,00	0,10		**3**
cofilin (Ab-3)	cofilin (Phospho-Ser3)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
ERK1-p44/42 MAP Kinase (Ab-202)	ERK1-p44/42 MAP K(P-Thr202)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
ERK1-p44/42 MAP Kinase (Ab-204)	ERK1-p44/42 MAP K(P-Tyr204)	0	75	366	1093	0,00	0,12	1,00	1,81		180		14,57		**9**
ERK3 (Ab-189)	ERK3 (Phospho-Ser189)	0	0	0	899	0,00	0,00	0,00	1,49						**9**
ERK3 (Ab-189)	ERK8 (Phospho-Thr175/Tyr177)			112	1600			0,31	2,65		862				**6**
Gab2 (Ab-623)	Gab2 (Phospho-Tyr643)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
JNK1/2/3 (Ab-183/185)	JNK1/2/3 (P-Thr183/Tyr185)	0	756	0	0	0,00	1,25	0,00	0,00				0,00		**3**
LIMK1 (Ab-508)	LIMK1 (Phospho-Thr508)	0	0	565	1104	0,00	0,00	1,55	1,83		118				**8**
MKK3 (Ab-189)	MKK3 (Phospho-Ser189)	0	197	0	0	0,00	0,33	0,00	0,00				0,00		**3**
MKK3/MAP2K3 (Ab-222)	MKK3/MAP2K3 (P-Thr222)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
MKK6 (Ab-207)	MKK6 (Phospho-Ser207)	85	0	31	741	0,23	0,00	0,09	1,23	0	1443	0,36			**9**
mTOR (Ab-2448)	mTOR (Phospho-Ser2448)	0	0	0	345	0,00	0,00	0,00	0,57						**9**
mTOR (Ab-2481)	mTOR (Phospho-Ser2481)	0	0	544	1023	0,00	0,00	1,49	1,69		113				**8**
mTOR (Ab-2446)	mTOR (Phospho-Thr2446)	0	0	0	337	0,00	0,00	0,00	0,56						**9**
Myc (Ab-373)	Myc (Phospho-Ser373)	0	355	0	297	0,00	0,59	0,00	0,49				0,84		**6**
Myc (Ab-62)	Myc (Phospho-Ser62)	0	0	124	433	0,00	0,00	0,34	0,72		211				**9**
Myc (Ab-358)	Myc (Phospho-Thr358)	7455	10020	0	635	20,45	16,59	0,00	1,05	81		0,00	0,06		**2**
Myc (Ab-58)	Myc (Phospho-Thr58)	0	194	0	24	0,00	0,32	0,00	0,04				0,12		**3**
p38 MAPK (Ab-180)	p38 MAPK (Phospho-Thr180)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
p38 MAPK (Ab-182)	p38 MAPK (Phospho-Tyr182)	17172	20089	0	284	47,11	33,26	0,00	0,47	71		0,00	0,01		**2**
p38 MAPK (Ab-322)	p38 MAPK (Phospho-Tyr322)	0	404	0	746	0,00	0,67	0,00	1,24				1,85		**9**
PAK1 (Ab-204)	PAK1 (Phospho-Ser204)	0	284	193	406	0,00	0,47	0,53	0,67		127		1,43		**5**
PAK1 (Ab-212)	PAK1 (Phospho-Thr212)	0	1167	0	395	0,00	1,93	0,00	0,65				0,34		**3**
PAK1/2 (Ab-199)	PAK1/2 (Phospho-Ser199)	0	500	38	225	0,00	0,83	0,10	0,37		357		0,45		**3**
PAK1/2/3 (Ab-141)	PAK1/2/3 (Phospho-Ser141)	0	316	154	262	0,00	0,52	0,42	0,43		103		0,83		**5**
PAK1/2/3 (Ab-423/402/421)	PAK1/2/3 (P-Thr423/402/421)	740	1125	740	313	2,03	1,86	2,03	0,52	92	26	1,00	0,28	28	**7**
PAK2 (Ab-192)	PAK2 (Phospho-Ser192)	0	148	607	955	0,00	0,25	1,67	1,58		95		6,45		**8**
PAK2 (Ab-197)	PAK2 (Phospho-Ser20)	90	731	0	0	0,25	1,21	0,00	0,00	490		0,00	0,00		**3**
PAK3 (Ab-154)	PAK3 (Phospho-Ser154)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
PI3-kinase p85-subunit alpha/gamma (A	PI3-kinase p85-alpha (P-Tyr607)			302	600			0,83	0,99		120		1,32		**5**
PI3-kinase p85-subunit alpha/gamma (A	PI3-ki p85-s al/g (P-Tyr467/199)	0	454	7	189	0,00	0,75	0,02	0,31		1629		0,42		**3**
PKC alpha (Ab-657)	PKC alpha (Phospho-Tyr657)	0	0	198	986	0,00	0,00	0,54	1,63		301				**9**
PKC alpha/beta II (Ab-638)	PKC alpha/beta II (P-Thr638)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
PKC beta/PKCB (Ab-661)	PKC beta/PKCB (P-Ser661)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
PKC delta (Ab-645)	PKC delta (Phospho-Ser645)	0	141	0	121	0,00	0,23	0,00	0,20				0,86		**6**

Array3 WT (1.2)

PKC delta (Ab-505)	PKC delta (Phospho-Thr505)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
PKC epsilon (Ab-729)	PKC epsilon (Phospho-Ser729)	0	0	0	234	0,00	0,00	0,00	0,39						**9**
PKC theta (Ab-676)	PKC theta (Phospho-Ser676)	0	0	111	405	0,00	0,00	0,30	0,67		220				**9**
PKC theta (Ab-538)	PKC theta (Phospho-Thr538)	24412	31190	326	1014	66,97	51,64	0,89	1,68	77	188	0,01	0,03	243	**2**
PKC zeta (Ab-410)	PKC zeta (Phospho-Thr410)	0	530	249	528	0,00	0,88	0,68	0,87		128		1,00		**5**
PKC zeta (Ab-560)	PKC zeta (Phospho-Thr560)	0	0	0	206	0,00	0,00	0,00	0,34						**9**
PP2A-a (Ab-307)	PP2A-a (Phospho-Tyr307)	4698	6509	63	382	12,89	10,78	0,17	0,63	84	366	0,01	0,06	438	**2**
Rac1/cdc42 (Ab-71)	Rac1/cdc42 (Phospho-Ser71)	0	0	57	841	0,00	0,00	0,16	1,39		890				**9**
Ras-GRF1 (Ab-916)	Ras-GRF1 (Phospho-Ser916)	0	0	30	389	0,00	0,00	0,08	0,64		783				**9**
Rho/Rac guanine nucleotide exchange f	Rho/Rac gu nu ex f2 (P-Ser885)	0	0	0	364	0,00	0,00	0,00	0,60						**9**
S6 Ribosomal Protein (Ab-235)	S6 Ribosoma Protein (P-Ser235)	0	0	0	14	0,00	0,00	0,00	0,02						**1**
SAPK/JNK (Ab-183)	SAPK/JNK (Phospho-Thr183)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
SAPK/JNK (Ab-185)	SAPK/JNK (Phospho-Tyr185)	0	0	0	421	0,00	0,00	0,00	0,70						**9**
SEK1/MKK4 (Ab-80)	SEK1/MKK4 (Phospho-Ser80)	2456	5121	0	0	6,74	8,48	0,00	0,00	126		0,00	0,00		**2**
SEK1/MKK4 (Ab-261)	SEK1/MKK4 (Phospho-Thr261)	8631	8018	0	0	23,68	13,27	0,00	0,00	56		0,00	0,00		**2**
Shc (Ab-349)	Shc (Phospho-Tyr349)	2384	4180	0	1255	6,54	6,92	0,00	2,08	106		0,00	0,30		**2**
Shc (Ab-427)	Shc (Phospho-Tyr427)	1414	2072	287	1033	3,88	3,43	0,79	1,71	88	217	0,20	0,50	246	**6**
Smad1 (Ab-187)	Smad1 (Phospho-Ser187)	0	0	243	1345	0,00	0,00	0,67	2,23		334				**9**
Smad1 (Ab-465)	Smad1 (Phospho-Ser465)	0	690	56	1067	0,00	1,14	0,15	1,77		1150		1,55		**9**
Smad2 (Ab-250)	Smad2 (Phospho-Ser250)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
Smad2 (Ab-467)	Smad2 (Phospho-Ser467)	0	0	0	611	0,00	0,00	0,00	1,01						**9**
Smad2 (Ab-220)	Smad2 (Phospho-Thr220)	0	1170	0	897	0,00	1,94	0,00	1,49				0,77		**6**
Smad2/3 (Ab-8)	Smad2/3 (Phospho-Thr8)	0	273	406	1375	0,00	0,45	1,11	2,28		204		5,04		**9**
Smad3 (Ab-204)	Smad3 (Phospho-Ser204)	54	784	0	0	0,15	1,30	0,00	0,00	876		0,00	0,00		**3**
Smad3 (Ab-204)	Smad3 (Phospho-Ser208)			0	0			0,00	0,00			0,00	0,00		**1**
Smad3 (Ab-213)	Smad3 (Phospho-Ser213)	0	0	0	4	0,00	0,00	0,00	0,01						**1**
Smad3 (Ab-425)	Smad3 (Phospho-Ser425)	0	12	0	345	0,00	0,02	0,00	0,57				28,75		**9**
Smad3 (Ab-179)	Smad3 (Phospho-Thr179)	0	12	0	0	0,00	0,02	0,00	0,00				0,00		**1**
SP1 (Ab-739)	SP1 (Phospho-Thr739)	0	0	4	608	0,00	0,00	0,01	1,01		9173				**9**
TAK1 (Ab-184)	TAK1 (Phospho-Thr184)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
Beta actin		18667	27077			51,21	44,83			88					**5**
CBP (Inter)		0	0			0,00	0,00								**1**
ERK1/2 (N-term)		31	658			0,09	1,09			1281					**6**
GAPDH		0	0			0,00	0,00								**1**
JNKK (MKK4) (Inter)		0	0			0,00	0,00								**1**
	MAP3K1/MEKK1 (P-Thr1381)			0	87			0,00	0,14						**6**
p300/CBP (C-term)		0	0			0,00	0,00								**1**
PAK4/PAK5/PAK6 (Ab-474)		0	141			0,00	0,23								**6**
RAS(p21 H and K) (Inter)		0	0			0,00	0,00								**1**
RASE (Inter)		497	1040			1,36	1,72			126					**5**
RASF4 (Inter)		0	0			0,00	0,00								**1**
RhoA (Ab-188)		4763	5302			13,07	8,78			67					**5**
S6K (Inter)		0	0			0,00	0,00								**1**
S6K-alpha 6 (Inter)		0	0			0,00	0,00								**1**
Smad1/5/9 (Inter)		0	0			0,00	0,00								**1**
Smad2 (ab-245)		0	0			0,00	0,00								**1**
Smad2 (Ab-255)		3132	4414			8,59	7,31			85					**5**
Smad4 (Inter)		0	0			0,00	0,00								**1**
TGF alpha (inter)		849	1364			2,33	2,26			97					**5**
TGF beta receptor II (inter)		0	12			0,00	0,02								**1**
TGF beta1 (inter)		0	0			0,00	0,00								**1**
TGF beta2 (inter)		0	0			0,00	0,00								**1**
TGF beta3 (inter)		0	0			0,00	0,00								**1**
TGFBR1 (Ab-165)		0	638			0,00	1,06								**6**
TGFBR2 (Ab-250)		1027	2174			2,82	3,60			128					**5**
negative marker		0	0			0,00	0,00								**5**
positive marker		27447	32524			75,30	53,85			72					**5**

Array4 WT (1.1)

Non Phospho Protein	Phospho Protein	Non Phospho Protein Median Intensity		Phospho Protein Median Intensity		Non Phospho Protein Normalized Data		Phospho Protein Normalized Data		Non Phospho Protein Ratio Th17/Th0 (A)	Phospho Protein Ratio Th17/Th0 (B)	Signal Ratio P-Protein to Non-P-Protein (C for Th0) (D for Th17)		P- to Non-P Ratio Th17/Th0 (E)	Interpretation
		Th0	Th17	Th0	Th17	Th0	Th17	Th0	Th17	>150, <50	>150, <50	Th0	Th17	>150, <50	Category 1-10
Abl1 (Ab-754/735)	Abl1 (Phospho-Thr754/735)	10092	2967	1806	993	2,42	1,23	0,43	0,41	51	95	0,18	0,33	187	**2**
Abl1 (Ab-204)	Abl1 (Phospho-Tyr204)	25429	17061	7584	2611	6,10	7,06	1,82	1,08	116	59	0,30	0,15	51	**2**
Abl1 (Ab-204)	Abl1 (Phospho-Tyr412)			11060	4357			2,65	1,80		68	0,43	0,26	59	**5**
AKT (Ab-473)	AKT (Phospho-Ser473)	3092	1579	4550	2507	0,74	0,65	1,09	1,04	88	95	1,47	1,59	108	**8**
AKT (Ab-308)	AKT (Phospho-Thr308)	2645	1423	6499	3043	0,63	0,59	1,56	1,26	93	81	2,46	2,14	87	**8**
AKT (Ab-326)	AKT (Phospho-Tyr326)	1608	793	10138	5936	0,39	0,33	2,43	2,45	85	101	6,30	7,49	119	**8**
AKT1 (Ab-124)	AKT1 (Phospho-Ser124)	1940	1688	2556	1566	0,47	0,70	0,61	0,65	150	106	1,32	0,93	70	**5**
AKT1 (Ab-246)	AKT1 (Phospho-Ser246)	4612	1302	6175	2960	1,11	0,54	1,48	1,22	49	83	1,34	2,27	170	**9**
AKT1 (Ab-450)	AKT1 (Phospho-Thr450)	8633	4256	2668	1991	2,07	1,76	0,64	0,82	85	129	0,31	0,47	151	**2**
AKT1 (Ab-72)	AKT1 (Phospho-Thr72)	6220	6103	3321	1586	1,49	2,52	0,80	0,66	169	82	0,53	0,26	49	**2**
AKT1 (Ab-474)	AKT1 (Phospho-Tyr474)	2561	698	4486	2375	0,61	0,29	1,08	0,98	47	91	1,75	3,40	194	**9**
AKT2 (Ab-474)	AKT2 (Phospho-Ser474)	2946	1418	4114	2118	0,71	0,59	0,99	0,88	83	89	1,40	1,49	107	**5**
c-Abl (Ab-412)	c-Abl (Phospho-Tyr245)			6819	3655			1,64	1,51		92	0,67	0,71	106	**5**
c-Abl (Ab-412)	c-Abl (Phospho-Tyr412)	10234	5184	5529	2778	2,46	2,14	1,33	1,15	87	87	0,54	0,54	99	**2**
cofilin (Ab-3)	cofilin (Phospho-Ser3)	6045	3254	5692	2594	1,45	1,35	1,37	1,07	93	79	0,94	0,80	85	**5**
ERK1-p44/42 MAP Kinase (Ab-202)	ERK1-p44/42 MAPK(P-Thr202)	5824	2936	11680	6405	1,40	1,21	2,80	2,65	87	95	2,01	2,18	109	**8**
ERK1-p44/42 MAP Kinase (Ab-204)	ERK1-p44/42 MAPK(P-Tyr204)	2846	1115	9983	5282	0,68	0,46	2,40	2,18	68	91	3,51	4,74	135	**8**
ERK3 (Ab-189)	ERK3 (Phospho-Ser189)	425	97	425	5627	0,10	0,04	0,10	2,33	39	2282	1,00	58,01	5801	**9**
ERK3 (Ab-189)	ERK8 (Phospho-Thr175/Tyr177)			8051	3341			1,93	1,38		72	18,94	34,44	182	**5**
Gab2 (Ab-623)	Gab2 (Phospho-Tyr643)	485	11368	17057	11368	0,12	4,70	4,09	4,70	4040	115	35,17	1,00	3	**6**
JNK1/2/3 (Ab-183/185)	JNK1/2/3 (P-Thr183/Tyr185)	6702	2683	2453	1631	1,61	1,11	0,59	0,67	69	115	0,37	0,61	166	**2**
LIMK1 (Ab-508)	LIMK1 (Phospho-Thr508)	4155	2035	6739	3280	1,00	0,84	1,62	1,36	84	84	1,62	1,61	99	**8**
MKK3 (Ab-189)	MKK3 (Phospho-Ser189)	5402	2931	6323	3034	1,30	1,21	1,52	1,25	94	83	1,17	1,04	88	**5**
MKK3/MAP2K3 (Ab-222)	MKK3/MAP2K3 (P-Thr222)	340	177	879	214	0,08	0,07	0,21	0,09	90	42	2,59	1,21	47	**10**
MKK6 (Ab-207)	MKK6 (Phospho-Ser207)	4058	2207	10639	4486	0,97	0,91	2,55	1,86	94	73	2,62	2,03	78	**8**
mTOR (Ab-2448)	mTOR (Phospho-Ser2448)	2453	1132	6743	3012	0,59	0,47	1,62	1,25	80	77	2,75	2,66	97	**8**
mTOR (Ab-2481)	mTOR (Phospho-Ser2481)	658	409	7851	3438	0,16	0,17	1,88	1,42	107	75	11,93	8,41	70	**8**
mTOR (Ab-2446)	mTOR (Phospho-Thr2446)	66	0	4044	3322	0,02	0,00	0,97	1,37	0	142	61,27			**9**
Myc (Ab-373)	Myc (Phospho-Ser373)	4090	2418	5332	2418	0,98	1,00	1,28	1,00	102	78	1,30	1,00	77	**5**
Myc (Ab-62)	Myc (Phospho-Ser62)	3364	1455	9582	1455	0,81	0,60	2,30	0,60	75	26	2,85	1,00	35	**10**
Myc (Ab-358)	Myc (Phospho-Thr358)	7833	6274	5163	6274	1,88	2,59	1,24	2,59	138	209	0,66	1,00	152	**6**
Myc (Ab-58)	Myc (Phospho-Thr58)	4644	3336	2087	3336	1,11	1,38	0,50	1,38	124	276	0,45	1,00	223	**6**
p38 MAPK (Ab-180)	p38 MAPK (Phospho-Thr180)	1201	236	1201	1306	0,29	0,10	0,29	0,54	34	187	1,00	5,53	553	**9**
p38 MAPK (Ab-182)	p38 MAPK (Phospho-Tyr182)	21392	15178	21392	3394	5,13	6,28	5,13	1,40	122	27	1,00	0,22	22	**4**
p38 MAPK (Ab-322)	p38 MAPK (Phospho-Tyr322)	2296	1228	2296	4536	0,55	0,51	0,55	1,88	92	341	1,00	3,69	369	**9**
PAK1 (Ab-204)	PAK1 (Phospho-Ser204)	1070	527	6125	3770	0,26	0,22	1,47	1,56	85	106	5,72	7,15	125	**8**
PAK1 (Ab-212)	PAK1 (Phospho-Thr212)	4729	1839	3421	2390	1,13	0,76	0,82	0,99	67	120	0,72	1,30	180	**5**
PAK1/2 (Ab-199)	PAK1/2 (Phospho-Ser199)	6172	2071	2340	1602	1,48	0,86	0,56	0,66	58	118	0,38	0,77	204	**5**
PAK1/2/3 (Ab-141)	PAK1/2/3 (Phospho-Ser141)	3576	1029	5335	2949	0,86	0,43	1,28	1,22	50	95	1,49	2,87	192	**9**
PAK1/2/3 (Ab-423/402/421)	PAK1/2/3 (P-Thr423/402/421)	431	0	4329	2630	0,10	0,00	1,04	1,09	0	105	10,04			**8**
PAK2 (Ab-192)	PAK2 (Phospho-Ser192)	1102	551	8197	4997	0,26	0,23	1,97	2,07	86	105	7,44	9,07	122	**8**
PAK2 (Ab-197)	PAK2 (Phospho-Ser20)	888	442	2420	670	0,21	0,18	0,58	0,28	86	48	2,73	1,52	56	**8**
PAK3 (Ab-154)	PAK3 (Phospho-Ser154)	1373	548	9583	5920	0,33	0,23	2,30	2,45	69	106	6,98	10,80	155	**9**
PI3-kinase p85-subunit alpha/gamma (A	PI3-kinase p85-alpha (P-Tyr607)			3046	2053			0,73	0,85		116	0,38	0,90	237	**5**
PI3-kinase p85-subunit alpha/gamma (A	PI3-kp85-suba/g (P-Tyr467/199)	8012	2278	3472	2278	1,92	0,94	0,83	0,94	49	113	0,43	1,00	231	**5**
PKC alpha (Ab-657)	PKC alpha (Phospho-Tyr657)	113	84	11663	6984	0,03	0,03	2,80	2,89	128	103	103,21	83,14	81	**8**
PKC alpha/beta II (Ab-638)	PKC alpha/beta II (P-Thr638)	465	239	7874	3989	0,11	0,10	1,89	1,65	89	87	16,93	16,69	99	**8**
PKC beta/PKCB (Ab-661)	PKC beta/PKCB (P-Ser661)	984	373	5816	2823	0,24	0,15	1,40	1,17	65	84	5,91	7,57	128	**8**
PKC delta (Ab-645)	PKC delta (Phospho-Ser645)	10109	4961	5785	1755	2,43	2,05	1,39	0,73	85	52	0,57	0,35	62	**4**

Array4 WT (1.2)

PKC delta (Ab-505)	PKC delta (Phospho-Thr505)	497	303	1984	494	0,12	0,13	0,48	0,20	105	43	3,99	1,63	41	**10**
PKC epsilon (Ab-729)	PKC epsilon (Phospho-Ser729)	893	383	11048	5866	0,21	0,16	2,65	2,43	74	92	12,37	15,32	124	**8**
PKC theta (Ab-676)	PKC theta (Phospho-Ser676)	3083	977	11266	4527	0,74	0,40	2,70	1,87	55	69	3,65	4,63	127	**8**
PKC theta (Ab-538)	PKC theta (Phospho-Thr538)	9614	8915	9597	5616	2,31	3,69	2,30	2,32	160	101	1,00	0,63	63	**5**
PKC zeta (Ab-410)	PKC zeta (Phospho-Thr410)	7268	2950	8462	3018	1,74	1,22	2,03	1,25	70	61	1,16	1,02	88	**5**
PKC zeta (Ab-560)	PKC zeta (Phospho-Thr560)	1660	671	6842	2763	0,40	0,28	1,64	1,14	70	70	4,12	4,12	100	**8**
PP2A-a (Ab-307)	PP2A-a (Phospho-Tyr307)	11355	5213	5143	2848	2,72	2,16	1,23	1,18	79	95	0,45	0,55	121	**2**
Rac1/cdc42 (Ab-71)	Rac1/cdc42 (Phospho-Ser71)	3606	1922	8463	3990	0,87	0,79	2,03	1,65	92	81	2,35	2,08	88	**8**
Ras-GRF1 (Ab-916)	Ras-GRF1 (Phospho-Ser916)	2166	1329	2645	947	0,52	0,55	0,63	0,39	106	62	1,22	0,71	58	**5**
Rho/Rac guanine nucleotide exchange f	Rho/Rac gnex factor2 (P-Ser885)	1292	0	2880	1478	0,31	0,00	0,69	0,61	0	88	2,23			**8**
S6 Ribosomal Protein (Ab-235)	S6 Ribosomal Protein (P-Ser235)	3500	2173	5071	2057	0,84	0,90	1,22	0,85	107	70	1,45	0,95	65	**5**
SAPK/JNK (Ab-183)	SAPK/JNK (Phospho-Thr183)	5381	3330	7421	3330	1,29	1,38	1,78	1,38	107	77	1,38	1,00	73	**5**
SAPK/JNK (Ab-185)	SAPK/JNK (Phospho-Tyr185)	3858	4958	9869	4958	0,93	2,05	2,37	2,05	222	87	2,56	1,00	39	**5**
SEK1/MKK4 (Ab-80)	SEK1/MKK4 (Phospho-Ser80)	4038	2826	9781	5178	0,97	1,17	2,35	2,14	121	91	2,42	1,83	76	**8**
SEK1/MKK4 (Ab-261)	SEK1/MKK4 (Phospho-Thr261)	24267	17726	8836	4601	5,82	7,33	2,12	1,90	126	90	0,36	0,26	71	**2**
Shc (Ab-349)	Shc (Phospho-Tyr349)	11974	6362	9513	3543	2,87	2,63	2,28	1,47	92	64	0,79	0,56	70	**5**
Shc (Ab427)	Shc (Phospho-Tyr427)	11435	7027	7485	2543	2,74	2,91	1,80	1,05	106	59	0,65	0,36	55	**2**
Smad1 (Ab-187)	Smad1 (Phospho-Ser187)	682	130	11486	5365	0,16	0,05	2,76	2,22	33	81	16,84	41,27	245	**9**
Smad1 (Ab-465)	Smad1 (Phospho-Ser465)	7183	4015	10365	3787	1,72	1,66	2,49	1,57	96	63	1,44	0,94	65	**5**
Smad2 (Ab-250)	Smad2 (Phospho-Ser250)	1168	351	3411	863	0,28	0,15	0,82	0,36	52	44	2,92	2,46	84	**10**
Smad2 (Ab-467)	Smad2 (Phospho-Ser467)	5315	1981	8313	3143	1,28	0,82	1,99	1,30	64	65	1,56	1,59	101	**8**
Smad2 (Ab-220)	Smad2 (Phospho-Thr220)	6883	2825	8082	2590	1,65	1,17	1,94	1,07	71	55	1,17	0,92	78	**5**
Smad2/3 (Ab-8)	Smad2/3 (Phospho-Thr8)	1556	722	9524	4107	0,37	0,30	2,29	1,70	80	74	6,12	5,69	93	**8**
Smad3 (Ab-204)	Smad3 (Phospho-Ser204)	8225	3323	2489	630	1,97	1,37	0,60	0,26	70	44	0,30	0,19	63	**2**
Smad3 (Ab-204)	Smad3 (Phospho-Ser208)			2655	797			0,64	0,33		52	0,32	0,24	74	**7**
Smad3 (Ab-213)	Smad3 (Phospho-Ser213)	0	0	1344	819	0,00	0,00	0,32	0,34		105				**8**
Smad3 (Ab-425)	Smad3 (Phospho-Ser425)	8437	3748	3337	2180	2,02	1,55	0,80	0,90	77	113	0,40	0,58	147	**2**
Smad3 (Ab-179)	Smad3 (Phospho-Thr179)	3763	1472	5311	1581	0,90	0,61	1,27	0,65	67	51	1,41	1,07	76	**5**
SP1 (Ab-739)	SP1 (Phospho-Thr739)	3452	1568	11045	5604	0,83	0,65	2,65	2,32	78	87	3,20	3,57	112	**8**
TAK1 (Ab-184)	TAK1 (Phospho-Thr184)	3315	939	604	226	0,80	0,39	0,14	0,09	49	64	0,18	0,24	132	**2**
Beta actin		3568	40273			0,86	16,66			1946					**6**
CBP (Inter)		1996	357			0,48	0,15			31					**7**
ERK1/2 (N-term)		1300	511			0,31	0,21			68					**5**
GAPDH		100	0			0,02	0,00			0					**7**
JNKK (MKK4) (Inter)		2046	494			0,49	0,20			42					**7**
	MAP3K1/MEKK1 (P-Thr1381)			3061	1615			0,73	0,67	110					**5**
p300/CBP (C-term)		124	68			0,03	0,03			95					**5**
PAK4/PAK5/PAK6 (Ab-474)		2185	1371			0,52	0,57			108					**5**
RAS(p21 H and K) (Inter)		2423	859			0,58	0,36			61					**5**
RASE (Inter)		9624	2771			2,31	1,15			50					**5**
RASF4 (Inter)		639	0			0,15	0,00			0					**7**
RhoA (Ab-188)		7993	5034			1,92	2,08			109					**5**
S6K (Inter)		129	0			0,03	0,00			0					**7**
S6K-alpha 6 (Inter)		996	161			0,24	0,07			28					**7**
Smad1/5/9 (Inter)		3699	978			0,89	0,40			46					**7**
Smad2 (ab-245)		321	0			0,08	0,00			0					**7**
Smad2 (Ab-255)		2997	1642			0,72	0,68			94					**5**
Smad4 (Inter)		119	0			0,03	0,00			0					**7**
TGF alpha (inter)		5395	2388			1,29	0,99			76					**5**
TGF beta receptor II (inter)		4168	1440			1,00	0,60			60					**5**
TGF beta1 (inter)		2013	556			0,48	0,23			48					**7**
TGF beta2 (inter)		7745	3566			1,86	1,47			79					**5**
TGF beta3 (inter)		1713	459			0,41	0,19			46					**7**
TGFBR1 (Ab-165)		5149	1594			1,24	0,66			53					**5**
TGFBR2 (Ab-250)		6412	2954			1,54	1,22			79					**5**
negative marker		0	0			0,00	0,00								
positive marker		49812	48945			11,95	20,24			169					

Array2 Smad7Tg (1.1)

Non Phospho Protein	Phospho Protein	Non Phospho Protein Median Intensity		Phospho Protein Median Intensity		Non Phospho Protein Normalized Data		Phospho Protein Normalized Data		Non Phospho Protein Ratio Th17/Th0 (A)	Phospho Protein Ratio Th17/Th0 (B)	Signal Ratio P-Protein to Non-P-Protein (C for Th0) (D for Th17)		P- to Non-P Ratio Th17/Th0 (E)	Interpretation
		Th0	Th17	Th0	Th17	Th0	Th17	Th0	Th17	>150, <50	>150, <50	Th0	Th17	>150, <50	Category 1-10
Abl1 (Ab-754/735)	Abl1 (Phospho-Thr754/735)	10775	24456	13174	20275	0,82	1,00	1,00	0,83	123	83	1,22	0,83	67,81	**5**
Abl1 (Ab-204)	Abl1 (Phospho-Tyr204)	36509	43697	9249	25553	2,77	1,79	0,70	1,05	65	150	0,25	0,58	231	**2**
Abl1 (Ab-204)	Abl1 (Phospho-Tyr412)			10058	28225			0,76	1,16		152	0,28	0,65	234	**6**
AKT (Ab-473)	AKT (Phospho-Ser473)	20451	26689	13744	19084	1,55	1,10	1,04	0,78	71	75	0,67	0,72	106	**5**
AKT (Ab-308)	AKT (Phospho-Thr308)	13759	20832	27311	25759	1,04	0,86	2,07	1,06	82	51	1,98	1,24	62	**10**
AKT (Ab-326)	AKT (Phospho-Tyr326)	12518	23456	11418	24901	0,95	0,96	0,87	1,02	101	118	0,91	1,06	116	**5**
AKT1 (Ab-124)	AKT1 (Phospho-Ser124)	34334	32771	22701	22961	2,60	1,35	1,72	0,94	52	55	0,66	0,70	106	**5**
AKT1 (Ab-246)	AKT1 (Phospho-Ser246)	4932	26681	17209	23152	0,37	1,10	1,31	0,95	293	73	3,49	0,87	25	**5**
AKT1 (Ab-450)	AKT1 (Phospho-Thr450)	13381	26932	29622	28440	1,01	1,11	2,25	1,17	109	52	2,21	1,06	48	**10**
AKT1 (Ab-72)	AKT1 (Phospho-Thr72)	36144	34401	10956	18280	2,74	1,41	0,83	0,75	52	90	0,30	0,53	175	**2**
AKT1 (Ab-474)	AKT1 (Phospho-Tyr474)	1793	17515	17740	22019	0,14	0,72	1,35	0,90	529	67	9,89	1,26	13	**8**
AKT2 (Ab-474)	AKT2 (Phospho-Ser474)	9190	16581	19728	24463	0,70	0,68	1,50	1,00	98	67	2,15	1,48	69	**8**
c-Abl (Ab-412)	c-Abl (Phospho-Tyr245)			17207	24854			1,31	1,02		78	0,63	0,71	112	**5**
c-Abl (Ab-412)	c-Abl (Phospho-Tyr412)	27187	34993	18954	27015	2,06	1,44	1,44	1,11	70	77	0,70	0,77	111	**5**
cofilin (Ab-3)	cofilin (Phospho-Ser3)	16788	28599	15427	24345	1,27	1,17	1,17	1,00	92	85	0,92	0,85	93	**5**
ERK1-p44/42 MAP Kinase (Ab-202)	ERK1-p44/42 MAP K(P-Thr202)	8743	21063	3837	14563	0,66	0,87	0,29	0,60	130	206	0,44	0,69	158	**6**
ERK1-p44/42 MAP Kinase (Ab-204)	ERK1-p44/42 MAP K(P-Tyr204)	10281	22495	19448	35092	0,78	0,92	1,48	1,44	118	98	1,89	1,56	82	**8**
ERK3 (Ab-189)	ERK3 (Phospho-Ser189)	2048	4303	9757	25147	0,16	0,18	0,74	1,03	114	140	4,76	5,84	123	**8**
ERK3 (Ab-189)	ERK8 (Phospho-Thr175/Tyr177)			7928	25432			0,60	1,04		174	3,87	5,91	153	**6**
Gab2 (Ab-623)	Gab2 (Phospho-Tyr643)	2576	6312	32078	43447	0,20	0,26	2,43	1,78	133	73	12,45	6,88	55	**8**
JNK1/2/3 (Ab-183/185)	JNK1/2/3 (P-Thr183/Tyr185)	6907	20377	17749	21893	0,52	0,84	1,35	0,90	160	67	2,57	1,07	42	**5**
LIMK1 (Ab-508)	LIMK1 (Phospho-Thr508)	15144	22170	20432	31953	1,15	0,91	1,55	1,31	79	85	1,35	1,44	107	**5**
MKK3 (Ab-189)	MKK3 (Phospho-Ser189)	19950	25442	15040	20235	1,51	1,05	1,14	0,83	69	73	0,75	0,80	105	**5**
MKK3/MAP2K3 (Ab-222)	MKK3/MAP2K3 (P-Thr222)	14186	8667	2676	8667	1,08	0,36	0,20	0,36	33	175	0,19	1,00	530	**5**
MKK6 (Ab-207)	MKK6 (Phospho-Ser207)	6618	16326	6679	22255	0,50	0,67	0,51	0,91	134	180	1,01	1,36	135	**5**
mTOR (Ab-2448)	mTOR (Phospho-Ser2448)	4988	12983	12021	24176	0,38	0,53	0,91	0,99	141	109	2,41	1,86	77	**8**
mTOR (Ab-2481)	mTOR (Phospho-Ser2481)	18419	19140	22620	32671	1,40	0,79	1,72	1,34	56	78	1,23	1,71	139	**6**
mTOR (Ab-2446)	mTOR (Phospho-Thr2446)	0	3820	25975	23433	0,00	0,16	1,97	0,96		49		6,13		**10**
Myc (Ab-373)	Myc (Phospho-Ser373)	25014	29296	22898	25520	1,90	1,20	1,74	1,05	63	60	0,92	0,87	95	**5**
Myc (Ab-62)	Myc (Phospho-Ser62)	2905	15887	6147	21487	0,22	0,65	0,47	0,88	296	189	2,12	1,35	64	**6**
Myc (Ab-358)	Myc (Phospho-Thr358)	29529	36529	23476	23833	2,24	1,50	1,78	0,98	67	55	0,80	0,65	82	**5**
Myc (Ab-58)	Myc (Phospho-Thr58)	24608	28733	18017	18845	1,87	1,18	1,37	0,77	63	57	0,73	0,66	90	**5**
p38 MAPK (Ab-180)	p38 MAPK (Phospho-Thr180)	0	3521	3995	19819	0,00	0,14	0,30	0,81		269		5,63		**9**
p38 MAPK (Ab-182)	p38 MAPK (Phospho-Tyr182)	28930	45627	8485	20198	2,19	1,87	0,64	0,83	85	129	0,29	0,44	151	**2**
p38 MAPK (Ab-322)	p38 MAPK (Phospho-Tyr322)	19118	23408	16059	26366	1,45	0,96	1,22	1,08	66	89	0,84	1,13	134	**5**
PAK1 (Ab-204)	PAK1 (Phospho-Ser204)	24223	27155	20381	28640	1,84	1,12	1,55	1,18	61	76	0,84	1,05	125	**5**
PAK1 (Ab-212)	PAK1 (Phospho-Thr212)	5615	24679	21069	23311	0,43	1,01	1,60	0,96	238	60	3,75	0,94	25	**5**
PAK1/2 (Ab-199)	PAK1/2 (Phospho-Ser199)	12176	28026	24910	24135	0,92	1,15	1,89	0,99	125	52	2,05	0,86	42	**10**
PAK1/2/3 (Ab-141)	PAK1/2/3 (Phospho-Ser141)	6392	22339	19881	24365	0,48	0,92	1,51	1,00	189	66	3,11	1,09	35	**8**
PAK1/2/3 (Ab-423/402/421)	PAK1/2/3 (P-Thr423/402/421)	456	1496	22272	26174	0,03	0,06	1,69	1,08	178	64	48,84	17,50	36	**8**
PAK2 (Ab-192)	PAK2 (Phospho-Ser192)	25443	29011	27155	31759	1,93	1,19	2,06	1,30	62	63	1,07	1,09	103	**5**
PAK2 (Ab-197)	PAK2 (Phospho-Ser20)	12187	18900	6934	17376	0,92	0,78	0,53	0,71	84	136	0,57	0,92	162	**5**
PAK3 (Ab-154)	PAK3 (Phospho-Ser154)	16252	20035	25688	29369	1,23	0,82	1,95	1,21	67	62	1,58	1,47	93	**8**
PI3-kinase p85-subunit alpha/gamma (A	PI3-kinase p85-alpha (P-Tyr607)			19337	22296			1,47	0,92		62	2,60	1,05	40	**5**
PI3-kinase p85-subunit alpha/gamma (A	PI3-k p85-sa/g (P-Tyr467/199)	7427	21260	19099	18841	0,56	0,87	1,45	0,77	155	53	2,57	0,89	34	**10**
PKC alpha (Ab-657)	PKC alpha (Phospho-Tyr657)	0	4900	19461	33436	0,00	0,20	1,48	1,37		93		6,82		**8**
PKC alpha/beta II (Ab-638)	PKC alpha/beta II (P-Thr638)	10857	13476	15311	26312	0,82	0,55	1,16	1,08	67	93	1,41	1,95	138	**8**
PKC beta/PKCB (Ab-661)	PKC beta/PKCB (P-Ser661)	15875	23969	17660	28993	1,20	0,98	1,34	1,19	82	89	1,11	1,21	109	**5**
PKC delta (Ab-645)	PKC delta (Phospho-Ser645)	15036	30807	9810	32974	1,14	1,27	0,74	1,35	111	182	0,65	1,07	164	**5**
PKC delta (Ab-505)	PKC delta (Phospho-Thr505)	7641	9337	3621	14141	0,58	0,38	0,27	0,58	66	211	0,47	1,51	320	**9**

Array2 Smad7Tg (1.2)

PKC epsilon (Ab-729)	PKC epsilon (Phospho-Ser729)	8339	15676	16185	26538	0,63	0,64	1,23	1,09	102	89	1,94	1,69	87	**8**
PKC theta (Ab-676)	PKC theta (Phospho-Ser676)	2755	17306	10902	28997	0,21	0,71	0,83	1,19	340	144	3,96	1,68	42	**8**
PKC theta (Ab-538)	PKC theta (Phospho-Thr538)	40717	45366	24900	33401	3,09	1,86	1,89	1,37	60	73	0,61	0,74	120	**2**
PKC zeta (Ab-410)	PKC zeta (Phospho-Thr410)	8280	25375	5746	27389	0,63	1,04	0,44	1,13	166	258	0,69	1,08	156	**6**
PKC zeta (Ab-560)	PKC zeta (Phospho-Thr560)	13749	20788	10123	22517	1,04	0,85	0,77	0,92	82	120	0,74	1,08	147	**5**
PP2A-a (Ab-307)	PP2A-a (Phospho-Tyr307)	9486	29409	17849	23784	0,72	1,21	1,35	0,98	168	72	1,88	0,81	43	**5**
Rac1/cdc42 (Ab-71)	Rac1/cdc42 (Phospho-Ser71)	9488	19979	10455	19018	0,72	0,82	0,79	0,78	114	99	1,10	0,95	86	**5**
Ras-GRF1 (Ab-916)	Ras-GRF1 (Phospho-Ser916)	11859	23706	17449	31597	0,90	0,97	1,32	1,30	108	98	1,47	1,33	91	**5**
Rho/Rac guanine nucleotide exchange f	Rho/Rac gnefactor 2 (P-Ser885)	2343	15061	22980	26416	0,18	0,62	1,74	1,09	348	62	9,81	1,75	18	**8**
S6 Ribosomal Protein (Ab-235)	S6 Ribosom Protein (P-Ser235)	7107	17400	8447	22924	0,54	0,71	0,64	0,94	133	147	1,19	1,32	111	**5**
SAPK/JNK (Ab-183)	SAPK/JNK (Phospho-Thr183)	8650	21277	8600	22620	0,66	0,87	0,65	0,93	133	142	0,99	1,06	107	**5**
SAPK/JNK (Ab-185)	SAPK/JNK (Phospho-Tyr185)	6202	20108	14455	28638	0,47	0,83	1,10	1,18	176	107	2,33	1,42	61	**10**
SEK1/MKK4 (Ab-80)	SEK1/MKK4 (Phospho-Ser80)	18564	29354	15387	20464	1,41	1,21	1,17	0,84	86	72	0,83	0,70	84	**5**
SEK1/MKK4 (Ab-261)	SEK1/MKK4 (Phospho-Thr261)	30836	36143	17248	24565	2,34	1,48	1,31	1,01	63	77	0,56	0,68	122	**5**
Shc (Ab-349)	Shc (Phospho-Tyr349)	14967	35705	6154	21727	1,14	1,47	0,47	0,89	129	191	0,41	0,61	148	**2**
Shc (Ab427)	Shc (Phospho-Tyr427)	18009	33925	12545	25988	1,37	1,39	0,95	1,07	102	112	0,70	0,77	110	**5**
Smad1 (Ab-187)	Smad1 (Phospho-Ser187)	5521	15578	12062	28338	0,42	0,64	0,91	1,16	153	127	2,18	1,82	83	**8**
Smad1 (Ab-465)	Smad1 (Phospho-Ser465)	15289	26413	13194	31307	1,16	1,08	1,00	1,29	94	128	0,86	1,19	137	**5**
Smad2 (Ab-250)	Smad2 (Phospho-Ser250)	2622	13640	6344	21901	0,20	0,56	0,48	0,90	282	187	2,42	1,61	66	**8**
Smad2 (Ab-467)	Smad2 (Phospho-Ser467)	9189	25345	5441	26847	0,70	1,04	0,41	1,10	149	267	0,59	1,06	179	**6**
Smad2 (Ab-220)	Smad2 (Phospho-Thr220)	19622	34095	11123	27718	1,49	1,40	0,84	1,14	94	135	0,57	0,81	143	**5**
Smad2/3 (Ab-8)	Smad2/3 (Phospho-Thr8)	7134	20580	13422	35137	0,54	0,85	1,02	1,44	156	142	1,88	1,71	91	**8**
Smad3 (Ab-204)	Smad3 (Phospho-Ser204)	10441	26066	335	19685	0,79	1,07	0,03	0,81	135	3182	0,03	0,76	2354	**6**
Smad3 (Ab-204)	Smad3 (Phospho-Ser208)			4026	19934			0,31	0,82		268	0,39	0,76	198	**6**
Smad3 (Ab-213)	Smad3 (Phospho-Ser213)	0	443	26829	28947	0,00	0,02	2,03	1,19		58		65,34		**10**
Smad3 (Ab-425)	Smad3 (Phospho-Ser425)	11665	27156	26623	27380	0,88	1,12	2,02	1,12	126	56	2,28	1,01	44	**10**
Smad3 (Ab-179)	Smad3 (Phospho-Thr179)	9336	25323	8288	28587	0,71	1,04	0,63	1,17	147	187	0,89	1,13	127	**6**
SP1 (Ab-739)	SP1 (Phospho-Thr739)	5775	15407	21963	32215	0,44	0,63	1,67	1,32	144	79	3,80	2,09	55	**8**
TAK1 (Ab-184)	TAK1 (Phospho-Thr184)	8506	26395	9534	12535	0,65	1,08	0,72	0,51	168	71	1,12	0,47	42	**2**
Beta actin		33884	43203			2,57	1,77			69					**5**
CBP (Inter)		3236	15879			0,25	0,65			266					**6**
ERK1/2 (N-term)		18876	31785			1,43	1,31			91					**5**
GAPDH		0	0			0,00	0,00								**1**
JNKK (MKK4) (Inter)		5151	19862			0,39	0,82			209					**6**
	MAP3K1/MEKK1 (P-Thr1381)			16230	24430			1,23	1,00	82					**5**
p300/CBP (C-term)		0	4399			0,00	0,18								**6**
PAK4/PAK5/PAK6 (Ab-474)		25394	24767			1,93	1,02			53					**5**
RAS(p21 H and K) (Inter)		0	7378			0,00	0,30								**6**
RASE (Inter)		22302	28664			1,69	1,18			70					**5**
RASF4 (Inter)		6117	14132			0,46	0,58			125					**5**
RhoA (Ab-188)		11014	31442			0,84	1,29			155					**5**
S6K (Inter)		0	1257			0,00	0,05								**6**
S6K-alpha 6 (Inter)		1527	11475			0,12	0,47			407					**6**
Smad1/5/9 (Inter)		8217	23093			0,62	0,95			152					**6**
Smad2 (ab-245)		0	7371			0,00	0,30								**6**
Smad2 (Ab-255)		13645	25733			1,03	1,06			102					**5**
Smad4 (Inter)		0	594			0,00	0,02								**6**
TGF alpha (inter)		10172	25231			0,77	1,04			134					**5**
TGF beta receptor II (inter)		14837	28706			1,13	1,18			105					**5**
TGF beta1 (inter)		12650	22933			0,96	0,94			98					**5**
TGF beta2 (inter)		24568	38427			1,86	1,58			85					**5**
TGF beta3 (inter)		9246	20560			0,70	0,84			120					**5**
TGFBR1 (Ab-165)		10573	28679			0,80	1,18			147					**5**
TGFBR2 (Ab-250)		12602	31206			0,96	1,28			134					**5**
negative marker		0	0			0,00	0,00								**5**
positive marker		36580	41601			2,77	1,71			62					**5**

Array3 Smad7Tg (1.1)

Non Phospho Protein	Phospho Protein	Non Phospho Protein Median Intensity		Phospho Protein Median Intensity		Non Phospho Protein Normalized Data		Phospho Protein Normalized Data		Non Phospho Protein Ratio Th17/Th0 (A)	Phospho Protein Ratio Th17/Th0 (B)	Signal Ratio P-Protein to Non-P-Protein (C for Th0) (D for Th17)		P- to Non-P Ratio Th17/Th0 (E)	Interpretation
		Th0	Th17	Th0	Th17	Th0	Th17	Th0	Th17	>150, <50	>150, <50	Th0	Th17	>150, <50	Category 1-10
Abl1 (Ab-754/735)	Abl1 (Phospho-Thr754/735)	271	0	0	0	0,78	0,00	0,00	0,00	0,00		0,00			**4**
Abl1 (Ab-204)	Abl1 (Phospho-Tyr204)	14142	15607	296	0	40,64	49,86	0,85	0,00	123	0	0,02	0,00	0	**2**
Abl1 (Ab-204)	Abl1 (Phospho-Tyr412)			482	296			1,39	0,95		68	0,03	0,02	56	**5**
AKT (Ab-473)	AKT (Phospho-Ser473)	0	0	211	0	0,00	0,00	0,61	0,00		0				**10**
AKT (Ab-308)	AKT (Phospho-Thr308)	288	648	104	0	0,83	2,07	0,30	0,00	250	0	0,36	0,00	0	**3**
AKT (Ab-326)	AKT (Phospho-Tyr326)	116	0	548	72	0,33	0,00	1,57	0,23	0	15	4,72			**10**
AKT1 (Ab-124)	AKT1 (Phospho-Ser124)	1066	1342	243	0	3,06	4,29	0,70	0,00	140	0	0,23	0,00	0	**2**
AKT1 (Ab-246)	AKT1 (Phospho-Ser246)	0	0	230	0	0,00	0,00	0,66	0,00		0				**10**
AKT1 (Ab-450)	AKT1 (Phospho-Thr450)	517	117	0	0	1,49	0,37	0,00	0,00	25		0,00	0,00		**4**
AKT1 (Ab-72)	AKT1 (Phospho-Thr72)	1658	1010	0	587	4,76	3,23	0,00	1,88	68		0,00	0,58		**6**
AKT1 (Ab-474)	AKT1 (Phospho-Tyr474)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
AKT2 (Ab-474)	AKT2 (Phospho-Ser474)	0	0	144	0	0,00	0,00	0,41	0,00		0				**10**
c-Abl (Ab-412)	c-Abl (Phospho-Tyr245)			477	0			1,37	0,00		0	0,95	0,00	0	**7**
c-Abl (Ab-412)	c-Abl (Phospho-Tyr412)	503	565	0	0	1,45	1,81	0,00	0,00	125		0,00	0,00		**2**
cofilin (Ab-3)	cofilin (Phospho-Ser3)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
ERK1-p44/42 MAP Kinase (Ab-202)	ERK1-p44/42 MAPK(P-Thr202)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
ERK1-p44/42 MAP Kinase (Ab-204)	ERK1-p44/42 MAPK(P-Tyr204)	0	0	849	335	0,00	0,00	2,44	1,07		44				**10**
ERK3 (Ab-189)	ERK3 (Phospho-Ser189)	0	0	599	0	0,00	0,00	1,72	0,00		0				**10**
ERK3 (Ab-189)	ERK8 (Phospho-Thr175/Tyr177)			410	20			1,18	0,06		5				**7**
Gab2 (Ab-623)	Gab2 (Phospho-Tyr643)	0	0	8905	8319	0,00	0,00	25,59	26,58		104				**8**
JNK1/2/3 (Ab-183/185)	JNK1/2/3 (P-Thr183/Tyr185)	170	27	0	0	0,49	0,09	0,00	0,00	18		0,00	0,00		**4**
LIMK1 (Ab-508)	LIMK1 (Phospho-Thr508)	0	0	488	0	0,00	0,00	1,40	0,00		0				**10**
MKK3 (Ab-189)	MKK3 (Phospho-Ser189)	0	0	334	0	0,00	0,00	0,96	0,00		0				**10**
MKK3/MAP2K3 (Ab-222)	MKK3/MAP2K3 (P-Thr222)	0	0	0	623	0,00	0,00	0,00	1,99						**9**
MKK6 (Ab-207)	MKK6 (Phospho-Ser207)	0	0	596	118	0,00	0,00	1,71	0,38		22				**10**
mTOR (Ab-2448)	mTOR (Phospho-Ser2448)	0	0	90	0	0,00	0,00	0,26	0,00		0				**10**
mTOR (Ab-2481)	mTOR (Phospho-Ser2481)	0	0	0	27	0,00	0,00	0,00	0,09						**9**
mTOR (Ab-2446)	mTOR (Phospho-Thr2446)	0	0	67	0	0,00	0,00	0,19	0,00		0				**10**
Myc (Ab-373)	Myc (Phospho-Ser373)	103	0	42	0	0,30	0,00	0,12	0,00	0	0	0,41			**4**
Myc (Ab-62)	Myc (Phospho-Ser62)	0	0	15	0	0,00	0,00	0,04	0,00		0				**10**
Myc (Ab-358)	Myc (Phospho-Thr358)	8803	10891	306	0	25,30	34,80	0,88	0,00	138	0	0,03	0,00	0	**2**
Myc (Ab-58)	Myc (Phospho-Thr58)	128	0	0	0	0,37	0,00	0,00	0,00	0		0,00			**4**
p38 MAPK (Ab-180)	p38 MAPK (Phospho-Thr180)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
p38 MAPK (Ab-182)	p38 MAPK (Phospho-Tyr182)	21439	20250	170	0	61,61	64,70	0,49	0,00	105	0	0,01	0,00	0	**2**
p38 MAPK (Ab-322)	p38 MAPK (Phospho-Tyr322)	32	0	351	65	0,09	0,00	1,01	0,21	0	21	10,97		0	**10**
PAK1 (Ab-204)	PAK1 (Phospho-Ser204)	68	0	154	84	0,20	0,00	0,44	0,27	0	61	2,26		0	**8**
PAK1 (Ab-212)	PAK1 (Phospho-Thr212)	550	35	52	0	1,58	0,11	0,15	0,00	7	0	0,09	0,00	0	**4**
PAK1/2 (Ab-199)	PAK1/2 (Phospho-Ser199)	534	4	790	0	1,53	0,01	2,27	0,00	1	0	1,48	0,00	0	**7**
PAK1/2/3 (Ab-141)	PAK1/2/3 (Phospho-Ser141)	220	0	37	0	0,63	0,00	0,11	0,00	0	0	0,17		0	**4**
PAK1/2/3 (Ab-423/402/421)	PAK1/2/3 (P-Thr423/402/421)	1146	1309	0	0	3,29	4,18	0,00	0,00	127		0,00	0,00		**2**
PAK2 (Ab-192)	PAK2 (Phospho-Ser192)	0	0	400	203	0,00	0,00	1,15	0,65		56				**8**
PAK2 (Ab-197)	PAK2 (Phospho-Ser20)	180	313	0	0	0,52	1,00	0,00	0,00	193		0,00	0,00		**3**
PAK3 (Ab-154)	PAK3 (Phospho-Ser154)	0	0	344	175	0,00	0,00	0,99	0,56		57				**8**
PI3-kinase p85-subunit alpha/gamma (A	PI3-kinase p85-alpha (P-Tyr607)			203	27			0,58	0,09		15	0,48		0	**7**
PI3-kinase p85-subunit alpha/gamma (A	PI3-k p85-sa/g(P-Tyr467/199)	426	0	42	0	1,22	0,00	0,12	0,00	0	0	0,10		0	**4**
PKC alpha (Ab-657)	PKC alpha (Phospho-Tyr657)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
PKC alpha/beta II (Ab-638)	PKC alpha/beta II (P-Thr638)	0	0	158	0	0,00	0,00	0,45	0,00		0				**10**
PKC beta/PKCB (Ab-661)	PKC beta/PKCB (P-Ser661)	0	0	170	0	0,00	0,00	0,49	0,00		0				**10**
PKC delta (Ab-645)	PKC delta (Phospho-Ser645)	360	318	0	0	1,03	1,02	0,00	0,00	98		0,00	0,00		**2**

Array3 Smad7Tg (1.2)

PKC delta (Ab-505)	PKC delta (Phospho-Thr505)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
PKC epsilon (Ab-729)	PKC epsilon (Phospho-Ser729)	0	0	208	0	0,00	0,00	0,60	0,00		0				**10**
PKC theta (Ab-676)	PKC theta (Phospho-Ser676)	0	0	288	0	0,00	0,00	0,83	0,00		0				**10**
PKC theta (Ab-538)	PKC theta (Phospho-Thr538)	34130	35735	449	112	98,07	114,17	1,29	0,36	116	28	0,01	0,00	24	**2**
PKC zeta (Ab-410)	PKC zeta (Phospho-Thr410)	336	0	590	0	0,97	0,00	1,70	0,00	0	0	1,76		0	**10**
PKC zeta (Ab-560)	PKC zeta (Phospho-Thr560)	0	0	270	0	0,00	0,00	0,78	0,00		0				**10**
PP2A-a (Ab-307)	PP2A-a (Phospho-Tyr307)	9736	7680	58	0	27,98	24,54	0,17	0,00	88	0	0,01	0,00	0	**2**
Rac1/cdc42 (Ab-71)	Rac1/cdc42 (Phospho-Ser71)	0	0	345	0	0,00	0,00	0,99	0,00		0				**10**
Ras-GRF1 (Ab-916)	Ras-GRF1 (Phospho-Ser916)	0	0	81	0	0,00	0,00	0,23	0,00		0				**10**
Rho/Rac guanine nucleotide exchange f	Rho/Rac gnefactor 2 (P-Ser885)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
S6 Ribosomal Protein (Ab-235)	S6 Ribosom Protein (P-Ser235)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
SAPK/JNK (Ab-183)	SAPK/JNK (Phospho-Thr183)	0	0	62	0	0,00	0,00	0,18	0,00		0				**10**
SAPK/JNK (Ab-185)	SAPK/JNK (Phospho-Tyr185)	0	0	344	0	0,00	0,00	0,99	0,00		0				**10**
SEK1/MKK4 (Ab-80)	SEK1/MKK4 (Phospho-Ser80)	2610	26	320	26	7,50	0,08	0,92	0,08	1	9	0,12	1,00	816	**4**
SEK1/MKK4 (Ab-261)	SEK1/MKK4 (Phospho-Thr261)	8236	52	433	52	23,67	0,17	1,24	0,17	1	13	0,05	1,00	1902	**4**
Shc (Ab-349)	Shc (Phospho-Tyr349)	3563	3155	389	0	10,24	10,08	1,12	0,00	98	0	0,11	0,00	0	**2**
Shc (Ab427)	Shc (Phospho-Tyr427)	1892	1379	277	0	5,44	4,41	0,80	0,00	81	0	0,15	0,00	0	**2**
Smad1 (Ab-187)	Smad1 (Phospho-Ser187)	0	0	563	211	0,00	0,00	1,62	0,67		42				**10**
Smad1 (Ab-465)	Smad1 (Phospho-Ser465)	634	50	170	101	1,82	0,16	0,49	0,32	9	66	0,27	2,02	753	**4**
Smad2 (Ab-250)	Smad2 (Phospho-Ser250)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
Smad2 (Ab-467)	Smad2 (Phospho-Ser467)	45	0	92	0	0,13	0,00	0,26	0,00	0	0	2,04		0	**10**
Smad2 (Ab-220)	Smad2 (Phospho-Thr220)	538	104	510	0	1,55	0,33	1,47	0,00	21	0	0,95	0,00	0	**7**
Smad2/3 (Ab-8)	Smad2/3 (Phospho-Thr8)	161	85	1230	556	0,46	0,27	3,53	1,78	59	50	7,64	6,54	86	**10**
Smad3 (Ab-204)	Smad3 (Phospho-Ser204)	362	0	0	0	1,04	0,00	0,00	0,00	0		0,00			**4**
Smad3 (Ab-204)	Smad3 (Phospho-Ser208)			0	0			0,00	0,00			0,00			**1**
Smad3 (Ab-213)	Smad3 (Phospho-Ser213)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
Smad3 (Ab-425)	Smad3 (Phospho-Ser425)	133	0	0	0	0,38	0,00	0,00	0,00	0		0,00			**4**
Smad3 (Ab-179)	Smad3 (Phospho-Thr179)	229	0	0	0	0,66	0,00	0,00	0,00	0		0,00			**4**
SP1 (Ab-739)	SP1 (Phospho-Thr739)	0	0	0	0	0,00	0,00	0,00	0,00						**1**
TAK1 (Ab-184)	TAK1 (Phospho-Thr184)	106	0	0	0	0,30	0,00	0,00	0,00	0		0,00			**4**
Beta actin		14099	19876			40,51	63,50			157					**6**
CBP (Inter)		0	0			0,00	0,00								**1**
ERK1/2 (N-term)		501	82			1,44	0,26			18					**7**
GAPDH		0	0			0,00	0,00								**1**
JNKK (MKK4) (Inter)		0	0			0,00	0,00								**1**
	MAP3K1/MEKK1 (Phospho-Thr1381)			0	0			0,00	0,00						**1**
p300/CBP (C-term)		0	0			0,00	0,00								**1**
PAK4/PAK5/PAK6 (Ab-474)		0	0			0,00	0,00								**1**
RAS(p21 H and K) (Inter)		0	0			0,00	0,00								**1**
RASE (Inter)		758	486			2,18	1,55			71					**5**
RASF4 (Inter)		0	0			0,00	0,00								**1**
RhoA (Ab-188)		5800	5779			16,67	18,46			111					**5**
S6K (Inter)		0	0			0,00	0,00								**1**
S6K-alpha 6 (Inter)		0	0			0,00	0,00								**1**
Smad1/5/9 (Inter)		0	0			0,00	0,00								**1**
Smad2 (ab-245)		0	0			0,00	0,00								**1**
Smad2 (Ab-255)		4009	4722			11,52	15,09			131					**5**
Smad4 (Inter)		0	0			0,00	0,00								**1**
TGF alpha (inter)		1970	1797			5,66	5,74			101					**5**
TGF beta receptor II (inter)		0	0			0,00	0,00								**1**
TGF beta1 (inter)		0	0			0,00	0,00								**1**
TGF beta2 (inter)		2889	3202			8,30	10,23			123					**5**
TGF beta3 (inter)		0	0			0,00	0,00								**1**
TGFBR1 (Ab-165)		403	0			1,16	0,00			0					**7**
TGFBR2 (Ab-250)		1613	1348			4,64	4,31			93					**5**
negative marker		0	0			0,00	0,00								**5**
positive marker		26485	26172			76,11	83,62			110					**5**

Array4 Smad7Tg (1.1)

Non Phospho Protein	Phospho Protein	Non Phospho Protein Median Intensity		Phospho Protein Median Intensity		Non Phospho Protein Normalized Data		Phospho Protein Normalized Data		Non Phospho Protein Ratio Th17/Th0 (A)	Phospho Protein Ratio Th17/Th0 (B)	Signal Ratio P-Protein to Non-P-Protein (C for Th0) (D for Th17)		P- to Non-P Ratio Th17/Th0 (E)	Interpretation
		Th0	Th17	Th0	Th17	Th0	Th17	Th0	Th17	>150, <50	>150, <50	Th0	Th17	>150, <50	Category 1-10
Abl1 (Ab-754/735)	Abl1 (Phospho-Thr754/735)	9611	4483	1737	564	3,18	2,23	0,57	0,28	70	49	0,18	0,13	69,61	**2**
Abl1 (Ab-204)	Abl1 (Phospho-Tyr204)	14314	9821	642	5001	4,74	4,89	0,21	2,49	103	1172	0,04	0,51	1135	**2**
Abl1 (Ab-204)	Abl1 (Phospho-Tyr412)			7176	5143			2,37	2,56		108	0,50	0,52	104	**5**
AKT (Ab-473)	AKT (Phospho-Ser473)	2043	1458	0	3065	0,68	0,73	0,00	1,53	107		0,00	2,10		**9**
AKT (Ab-308)	AKT (Phospho-Thr308)	834	1459	0	2916	0,28	0,73	0,00	1,45	263		0,00	2,00		**9**
AKT (Ab-326)	AKT (Phospho-Tyr326)	1642	857	1625	3552	0,54	0,43	0,54	1,77	79	329	0,99	4,14	419	**9**
AKT1 (Ab-124)	AKT1 (Phospho-Ser124)	4047	2563	3236	1559	1,34	1,28	1,07	0,78	95	73	0,80	0,61	76	**5**
AKT1 (Ab-246)	AKT1 (Phospho-Ser246)	4035	3040	3480	2912	1,34	1,51	1,15	1,45	113	126	0,86	0,96	111	**5**
AKT1 (Ab-450)	AKT1 (Phospho-Thr450)	4558	3138	3594	1904	1,51	1,56	1,19	0,95	104	80	0,79	0,61	77	**5**
AKT1 (Ab-72)	AKT1 (Phospho-Thr72)	8146	6142	1790	918	2,70	3,06	0,59	0,46	113	77	0,22	0,15	68	**2**
AKT1 (Ab-474)	AKT1 (Phospho-Tyr474)	3833	1130	2556	1574	1,27	0,56	0,85	0,78	44	93	0,67	1,39	209	**5**
AKT2 (Ab-474)	AKT2 (Phospho-Ser474)	0	774	0	1837	0,00	0,39	0,00	0,91				2,37		**9**
c-Abl (Ab-412)	c-Abl (Phospho-Tyr245)			4525	2202			1,50	1,10		73	0,85	0,61	72	**5**
c-Abl (Ab-412)	c-Abl (Phospho-Tyr412)	5304	3593	0	2085	1,76	1,79	0,00	1,04	102		0,00	0,58		**6**
cofilin (Ab-3)	cofilin (Phospho-Ser3)	4401	2270	0	1687	1,46	1,13	0,00	0,84	78		0,00	0,74		**6**
ERK1-p44/42 MAP Kinase (Ab-202)	ERK1-p44/42 MAPK (P-Thr202)	1205	600	0	737	0,40	0,30	0,00	0,37	75		0,00	1,23		**6**
ERK1-p44/42 MAP Kinase (Ab-204)	ERK1-p44/42 MAPK (P-Tyr204)	0	1812	10053	5972	0,00	0,90	3,33	2,97		89		3,30		**8**
ERK3 (Ab-189)	ERK3 (Phospho-Ser189)	0	0	270	3091	0,00	0,00	0,09	1,54		1723				**9**
ERK3 (Ab-189)	ERK8 (Phospho-Thr175/Tyr177)			25	2306			0,01	1,15		13882				**6**
Gab2 (Ab-623)	Gab2 (Phospho-Tyr643)	516	148	13525	9093	0,17	0,07	4,48	4,53	43	101	26,21	61,44	234	**8**
JNK1/2/3 (Ab-183/185)	JNK1/2/3 (P-Thr183/Tyr185)	4061	2002	2680	1070	1,34	1,00	0,89	0,53	74	60	0,66	0,53	81	**5**
LIMK1 (Ab-508)	LIMK1 (Phospho-Thr508)	1725	1318	0	3085	0,57	0,66	0,00	1,54	115		0,00	2,34		**9**
MKK3 (Ab-189)	MKK3 (Phospho-Ser189)	2974	1969	737	2781	0,98	0,98	0,24	1,38	100	568	0,25	1,41	570	**6**
MKK3/MAP2K3 (Ab-222)	MKK3/MAP2K3 (P-Thr222)	394	299	133	0	0,13	0,15	0,04	0,00	114	0	0,34	0,00	0	**2**
MKK6 (Ab-207)	MKK6 (Phospho-Ser207)	2193	1690	2512	3143	0,73	0,84	0,83	1,57	116	188	1,15	1,86	162	**9**
mTOR (Ab-2448)	mTOR (Phospho-Ser2448)	0	543	0	2686	0,00	0,27	0,00	1,34				4,95		**9**
mTOR (Ab-2481)	mTOR (Phospho-Ser2481)	1124	543	5020	2327	0,37	0,27	1,66	1,16	73	70	4,47	4,29	96	**8**
mTOR (Ab-2446)	mTOR (Phospho-Thr2446)	0	0	4835	2064	0,00	0,00	1,60	1,03		64				**8**
Myc (Ab-373)	Myc (Phospho-Ser373)	4689	2008	0	2261	1,55	1,00	0,00	1,13	64		0,00	1,13		**6**
Myc (Ab-62)	Myc (Phospho-Ser62)	0	1196	0	3569	0,00	0,60	0,00	1,78				2,98		**9**
Myc (Ab-358)	Myc (Phospho-Thr358)	10056	7125	0	2624	3,33	3,55	0,00	1,31	107		0,00	0,37		**2**
Myc (Ab-58)	Myc (Phospho-Thr58)	4639	2261	0	1444	1,54	1,13	0,00	0,72	73		0,00	0,64		**2**
p38 MAPK (Ab-180)	p38 MAPK (Phospho-Thr180)	0	0	0	1243	0,00	0,00	0,00	0,62						**9**
p38 MAPK (Ab-182)	p38 MAPK (Phospho-Tyr182)	19379	12735	0	2560	6,41	6,34	0,00	1,27	99		0,00	0,20		**2**
p38 MAPK (Ab-322)	p38 MAPK (Phospho-Tyr322)	2328	941	2850	2936	0,77	0,47	0,94	1,46	61	155	1,22	3,12	255	**9**
PAK1 (Ab-204)	PAK1 (Phospho-Ser204)	1387	578	4050	1947	0,46	0,29	1,34	0,97	63	72	2,92	3,37	115	**8**
PAK1 (Ab-212)	PAK1 (Phospho-Thr212)	3793	1515	2986	1484	1,26	0,75	0,99	0,74	60	75	0,79	0,98	124	**5**
PAK1/2 (Ab-199)	PAK1/2 (Phospho-Ser199)	4056	1814	2632	1175	1,34	0,90	0,87	0,59	67	67	0,65	0,65	100	**5**
PAK1/2/3 (Ab-141)	PAK1/2/3 (Phospho-Ser141)	2628	1241	2815	2866	0,87	0,62	0,93	1,43	71	153	1,07	2,31	216	**9**
PAK1/2/3 (Ab-423/402/421)	PAK1/2/3 (P-Thr423/402/421)	0	105	3442	2338	0,00	0,05	1,14	1,16		102		22,27		**8**
PAK2 (Ab-192)	PAK2 (Phospho-Ser192)	1175	649	4590	2711	0,39	0,32	1,52	1,35	83	89	3,91	4,18	107	**8**
PAK2 (Ab-197)	PAK2 (Phospho-Ser20)	2403	695	1011	409	0,80	0,35	0,33	0,20	44	61	0,42	0,59	140	**4**
PAK3 (Ab-154)	PAK3 (Phospho-Ser154)	1172	483	6015	3228	0,39	0,24	1,99	1,61	62	81	5,13	6,68	130	**8**
PI3-kinase p85-subunit alpha/gamma (A	PI3-kinase p85-alpha (P-Tyr607)			3049	1869			1,01	0,93		92	0,60	0,64	107	**5**
PI3-kinase p85-subunit alpha/gamma (A	PI3-k p85-sa/g(P-Tyr467/199)	5109	2930	5109	1741	1,69	1,46	1,69	0,87	86	51	1,00	0,59	59	**7**
PKC alpha (Ab-657)	PKC alpha (Phospho-Tyr657)	0	0	4308	3103	0,00	0,00	1,43	1,55		108				**8**
PKC alpha/beta II (Ab-638)	PKC alpha/beta II (P-Thr638)	0	0	4330	2437	0,00	0,00	1,43	1,21		85				**8**
PKC beta/PKCB (Ab-661)	PKC beta/PKCB (P-Ser661)	3157	279	3157	1271	1,04	0,14	1,04	0,63	13	61	1,00	4,56	456	**7**
PKC delta (Ab-645)	PKC delta (Phospho-Ser645)	0	3213	0	2074	0,00	1,60	0,00	1,03				0,65		**6**

Array4 Smad7Tg (1.2)

PKC delta (Ab-505)	PKC delta (Phospho-Thr505)	0	246	0	236	0,00	0,12	0,00	0,12				0,96		**6**
PKC epsilon (Ab-729)	PKC epsilon (Phospho-Ser729)	643	172	4536	3238	0,21	0,09	1,50	1,61	40	107	7,05	18,83	267	**8**
PKC theta (Ab-676)	PKC theta (Phospho-Ser676)	0	888	0	3574	0,00	0,44	0,00	1,78				4,02		**9**
PKC theta (Ab-538)	PKC theta (Phospho-Thr538)	34388	16615	3826	3266	11,38	8,27	1,27	1,63	73	128	0,11	0,20	177	**2**
PKC zeta (Ab-410)	PKC zeta (Phospho-Thr410)	0	2642	316	3969	0,00	1,32	0,10	1,98		1890		1,50		**9**
PKC zeta (Ab-560)	PKC zeta (Phospho-Thr560)	930	478	0	1714	0,31	0,24	0,00	0,85	77		0,00	3,59		**9**
PP2A-a (Ab-307)	PP2A-a (Phospho-Tyr307)	11993	5213	2684	2087	3,97	2,60	0,89	1,04	65	117	0,22	0,40	179	**2**
Rac1/cdc42 (Ab-71)	Rac1/cdc42 (Phospho-Ser71)	1481	1277	4289	3511	0,49	0,64	1,42	1,75	130	123	2,90	2,75	95	**8**
Ras-GRF1 (Ab-916)	Ras-GRF1 (Phospho-Ser916)	1182	567	3781	2030	0,39	0,28	1,25	1,01	72	81	3,20	3,58	112	**8**
Rho/Rac guanine nucleotide exchange f	Rho/Rac gnef2 (P-Ser885)	949	37	3491	1209	0,31	0,02	1,16	0,60	6	52	3,68	32,68	888	**10**
S6 Ribosomal Protein (Ab-235)	S6 Ribosom Protein (P-Ser235)	3022	1331	0	1724	1,00	0,66	0,00	0,86	66		0,00	1,30		**6**
SAPK/JNK (Ab-183)	SAPK/JNK (Phospho-Thr183)	0	1420	0	2363	0,00	0,71	0,00	1,18				1,66		**9**
SAPK/JNK (Ab-185)	SAPK/JNK (Phospho-Tyr185)	0	942	0	3628	0,00	0,47	0,00	1,81				3,85		**9**
SEK1/MKK4 (Ab-80)	SEK1/MKK4 (Phospho-Ser80)	7069	3808	2753	2487	2,34	1,90	0,91	1,24	81	136	0,39	0,65	168	**2**
SEK1/MKK4 (Ab-261)	SEK1/MKK4 (Phospho-Thr261)	21752	15345	2641	2689	7,20	7,64	0,87	1,34	106	153	0,12	0,18	144	**2**
Shc (Ab-349)	Shc (Phospho-Tyr349)	1397	5619	2005	4276	0,46	2,80	0,66	2,13	605	321	1,44	0,76	53	**6**
Shc (Ab427)	Shc (Phospho-Tyr427)	6864	3912	1111	5268	2,27	1,95	0,37	2,62	86	714	0,16	1,35	832	**6**
Smad1 (Ab-187)	Smad1 (Phospho-Ser187)	0	0	1154	2925	0,00	0,00	0,38	1,46		381				**9**
Smad1 (Ab-465)	Smad1 (Phospho-Ser465)	2803	1853	0	3928	0,93	0,92	0,00	1,96	99		0,00	2,12		**9**
Smad2 (Ab-250)	Smad2 (Phospho-Ser250)	1080	302	0	1001	0,36	0,15	0,00	0,50	42		0,00	3,31		**9**
Smad2 (Ab-467)	Smad2 (Phospho-Ser467)	2708	1504	0	3169	0,90	0,75	0,00	1,58	84		0,00	2,11		**9**
Smad2 (Ab-220)	Smad2 (Phospho-Thr220)	5070	2217	0	2244	1,68	1,10	0,00	1,12	66		0,00	1,01		**6**
Smad2/3 (Ab-8)	Smad2/3 (Phospho-Thr8)	2116	1055	0	4015	0,70	0,53	0,00	2,00	75		0,00	3,81		**9**
Smad3 (Ab-204)	Smad3 (Phospho-Ser204)	4401	2566	0	1109	1,46	1,28	0,00	0,55	88		0,00	0,43		**2**
Smad3 (Ab-204)	Smad3 (Phospho-Ser208)			0	1509			0,00	0,75			0,00	0,59		**6**
Smad3 (Ab-213)	Smad3 (Phospho-Ser213)	0	0	2162	854	0,00	0,00	0,72	0,43		59				**8**
Smad3 (Ab-425)	Smad3 (Phospho-Ser425)	4535	2158	0	2295	1,50	1,07	0,00	1,14	72		0,00	1,06		**6**
Smad3 (Ab-179)	Smad3 (Phospho-Thr179)	3536	1153	0	1437	1,17	0,57	0,00	0,72	49		0,00	1,25		**6**
SP1 (Ab-739)	SP1 (Phospho-Thr739)	2375	1239	0	3809	0,79	0,62	0,00	1,90	79		0,00	3,07		**9**
TAK1 (Ab-184)	TAK1 (Phospho-Thr184)	4274	1136	321	0	1,41	0,57	0,11	0,00	40	0	0,08	0,00	0	**4**
Beta actin		15462	10301			5,12	5,13			100					**5**
CBP (Inter)		882	256			0,29	0,13			44					**7**
ERK1/2 (N-term)		303	4266			0,10	2,12			2119					**6**
GAPDH		0	11			0,00	0,01								**6**
JNKK (MKK4) (Inter)		846	270			0,28	0,13			48					**7**
	MAP3K1/MEKK1 (P-Thr1381)			4967	934			1,64	0,47						**7**
p300/CBP (C-term)		0	0			0,00	0,00								**1**
PAK4/PAK5/PAK6 (Ab-474)		2620	1683			0,87	0,84			97					**5**
RAS(p21 H and K) (Inter)		0	0			0,00	0,00								**1**
RASE (Inter)		4150	2020			1,37	1,01			73					**5**
RASF4 (Inter)		0	648			0,00	0,32								**6**
RhoA (Ab-188)		11683	4972			3,87	2,48			64					**5**
S6K (Inter)		0	0			0,00	0,00								**1**
S6K-alpha 6 (Inter)		0	0			0,00	0,00								**1**
Smad1/5/9 (Inter)		1692	713			0,56	0,36			63					**5**
Smad2 (ab-245)		0	0			0,00	0,00								**1**
Smad2 (Ab-255)		7362	2359			2,44	1,17			48					**7**
Smad4 (Inter)		0	0			0,00	0,00								**1**
TGF alpha (inter)		7391	2924			2,45	1,46			60					**5**
TGF beta receptor II (inter)		2204	1092			0,73	0,54			75					**5**
TGF beta1 (inter)		1359	385			0,45	0,19			43					**7**
TGF beta2 (inter)		8583	5093			2,84	2,54			89					**5**
TGF beta3 (inter)		767	260			0,25	0,13			51					**5**
TGFBR1 (Ab-165)		5602	1735			1,85	0,86			47					**7**
TGFBR2 (Ab-250)		9060	3474			3,00	1,73			58					**5**
negative marker		0	0			0,00	0,00								**5**
positive marker		62229	60820			20,59	30,29			147					**5**

12. Curriculum Vitae

Personal data:

Name: Md. Maruf Hasan

Born: 22, October, 1980 in Rangpur, Bangladesh

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Education:

Degree	Name of the Institution	Passing year
Master's in Bio- and food technology	University of Lund, Lund, Sweden	2007
Bachelor of Science in Biotechnology and Genetic Engineering	Khulna University, Khulna, Bangladesh	2002
Higher Secondary Certificate	Cantonment Public School and College, Rangpur, Bangladesh	1997
Secondary School Certificate	Rangpur Zilla School, Rangpur, Bangladesh	1995

Summer project:

Role of CD1d-dependent NKT cells in CIA and AIA and the joint histopathology. Supervisor: Prof. Shohreh Issazadeh Navikas. Neuroinflammation Unit, Department of Experimental Medicine. University of Lund, Lund, Sweden. 2006.

Master's thesis:

Neurons kill Glioblastoma cells via PD-L1 dependent signalling pathway. Supervisor: Prof. Shohreh Issazadeh Navikas. Neuroinflammation Unit, Department of Experimental Medicine. University of Lund, Lund, Sweden. 2007.

PhD thesis:

Regulation of T cell differentiation by the TGF-β inhibitor Smad7 and non-Smad pathways. Supervisor: Prof. Dr. Ingo Kleiter. Neuroimmunology, Department of Neurology. University of Regensburg, Regensburg, Germany. 2008-2012.

Scientific presentations and talks:

Neurons kill Glioblastoma cells via PD-L1 dependent signalling pathway.
Oral presentation, University of Copenhagen, Aug. 2006. **Copenhagen, Denmark.**

The TGF-beta inhibitor Smad7 controls T cell differentiation and susceptibility to experimental autoimmune encephalomyelitis.
Poster presentation, Keystone conference, Feb. 2009. **Vancouver, Canada.**

Regulation of T cell differentiation by the intracellular TGF-β inhibitor Smad7.
Poster presentation, International Congress of Immunology, Sept. 2009, **Berlin, Germany.**

Publications:

Teige A, Bockermann R, **Hasan M,** Olofsson K E, Liu Y, Issazadeh-Navikas S. (2010). CD1d-dependent NKT cells play a protective role in acute and chronic arthritis models by ameliorating antigen-specific Th1 responses. *J Immunol.* 185(1): 345-56.

Kleiter I, Song J, Lukas D, **Hasan M**, Neumann B, Croxford AL, Pedré X, Hövelmeyer N, Yogev N, Mildner A, Prinz M, Wiese E, Reifenberg K, Bittner S, Wiendl H, Steinman L, Becker C, Bogdahn U, Neurath MF, Steinbrecher A, Waisman A. (2010). Smad7 in T cells drives T helper 1 responses in multiple sclerosis and experimental autoimmune encephalomyelitis. Brain. 133(Pt 4): 1067-81.

Liu Y, Carlsson R, Ambjorn M, Badn W, **Hasan M**, Siesjö P, Issazadeh-Navikas S. Neuronal-Mediated killing of GL261 is Interferon-β and PD-L1 Dependent.
(Manuscript submitted).

Hasan M, Neumann B, Waisman A, Bogdahn U, Kleiter I. Regulation of Th17 differentiation by TGF-β- induced non- Smad signalling pathways. ***(Manuscript in preparation).***

Miscellaneous:

- Merit scholarship for 1 semester. University of Khulna, Khulna, Bangladesh.
- Summer scholarship. University of Lund, Lund, Sweden. 2006.
- Master's thesis scholarship. Bibi & Nils Jensen's Foundation, Sweden, 2007.
- Research assistant, Neuroinflammation Unit, Department of Experimental Medicine. University of Lund, Lund, Sweden. July 2007-November 2007.
- Travel Grant, International Congress of Immunology, Berlin, Germany. 2009.
- Business Development Executive. 2004-2005.
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  Company name : Genetix biotech Asia Pvt. Ltd. New Delhi, India
  Job training : Techniques in molecular biology,
  Genetix biotech Asia Pvt. Ltd. New Delhi, India

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14. Declaration

I hereby declare that I have made this dissertation independently, without unauthorized aids, and tools used have been fully specified. Parts of the dissertation were published in BRAIN in 2010 (see publication list in my CV). The acquired figures from other sources have been indicated.

Regensburg, date Md. Maruf Hasan