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„Molecular analysis of the protein tyrosine kinase Tec in Th17 cells “

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1. Abstract

Members of the Tec kinase family play an important role during lymphocyte development and activation. Mutations in this kinase family are the molecular cause of immunodeficiencies in humans and mice. During the last years Th17 cells received a lot of attention since they are not only responsible for the clearance of extracellular bacteria and fungi, which involves tissue inflammation, but also have been identified as key players for the development of auto-immune diseases. Tec, the founding member of the Tec kinase family, was shown to regulate a memory Th17 subset *in vivo* and Th17 differentiation *in vitro* in a T cell-intrinsic way. It has not been identified how Tec regulates Th17 differentiation and function. Further, several isoforms of Tec have been described, however which of these isoforms are important for regulating Th17 cells is not known. Therefore, one aim of this master´s thesis was to determine the expression and expression kinetic of Tec during Th17 cell differentiation using qRTPCR as well as immunoblotting approaches. Moreover, by using retroviral-mediated gene transduction approaches, we investigated how the enforced expression of Tec regulates Th17 differentiation. With these experimental strategies, we showed that Tec is specifically induced in Th17 cells with its highest expression levels on day 3 after Th17 differentiation. In addition, the Tec isoform a was predominantly expressed compared to the other isoforms under Th17 conditions, indicating an isoform-specific regulation of Tec expression. Furthermore, we observed that Tec functions as a dampener of Th17 cell differentiation by acting downstream of IL-6 receptor signaling and by regulating STAT-3 phosphorylation. Together, our data suggest that Tec might be a promising therapeutic target to modulate Th17 differentiation processes *in vivo*.

2. Zusammenfassung

Mehrere Vertreter der Familie der Tec Kinasen spielen eine wichtige Rolle während der Entwicklung und Aktivierung von Lymphozyten. Vielen Immundefekten im Menschen und in der Maus liegen auf molekularer Ebene Mutationen in Kinasen der Tec Familie zu Grunde. Th17 Zellen entwickelten sich in den letzten Jahren zu einer viel untersuchten Population der $CD4^+$ T-Helferzellen. Sie sind mitverantwortlich für Entzündungsreaktionen, welche zur Bekämpfung von Infekten mit extrazellulären Bakterien und Pilzen notwendig sind, spielen aber auch in der Entwicklung von Autoimmunerkrankungen eine entscheidende Rolle. Die Familie der Tec Kinasen ist nach einem ihrer Vertreter, Tec, benannt. Dabei handelt es sich um eine Kinase, die T-Zell spezifisch *in vivo* eine Population an Th17 Gedächtniszellen reguliert und *in vitro* Th17 Differenzierung unterstützt. Die Mechanismen hinter diesen Regulierungen in Th17 Zellen durch die Tec Kinase sind allerdings noch nicht näher beschrieben. Außerdem wurde noch nicht geklärt, welche der verschiedenen Isoformen der Tec Kinase für die Regulierung von Th17 Zellen verantwortlich ist. Das Ziel meiner Masterarbeit war es, die Expression und die Kinetik der Tec Kinase in Th17 Zellen mit Hilfe von quantitativer Echt-Zeit PCR und Immunblots zu untersuchen. Weiters führte ich retrovirale Gentransduktionen durch um die erzwungene Expression von Tec in Th17 Zellen zu erforschen. Wir konnten nachweisen, dass die Isoform a die höchsten Expressionswerte in kultivierten Th17 Zellen aufweist und dass die Expression von Tec in Th17 Zellen drei Tage nach deren Kultivierung ein Maximum erreicht. Außerdem konnten wir zeigen, dass Tec durch die Regulierung der STAT-3 Phosphorylierung unterhalb des IL-6 Signalweges als negativer Regulator der Th17 Differenzierung agiert. Diese Eigenschaft der negativen Regulation von Th17 Zellen lässt auf eine mögliche therapeutische Anwendung von Tec Kinasen hoffen.

3. Introduction

3.1. Innate and adaptive immunity

Immunity can be defined as the ability of an organism to resist diseases caused by infectious agents. This resistance is mediated by the immune system, a highly complex system of interacting cells and tissues. Classically, it is divided into two closely interconnected branches, the innate and the adaptive immune system. The innate immune system provides a first line of defence against many microbial infections and is therefore essential for the control of pathogens by the recognition of their pathogen associated molecular patterns (PAMPs). However, some pathogens cannot be recognized or controlled by the innate immune system, which therefore needs help from the adaptive immune system. In contrast to the innate immune system, the adaptive one is more versatile by providing a huge diversity of antigen receptors, which allow the recognition of any type of pathogen. The adaptive immune system relies on lymphocytes. A unique genetic mechanism, based on somatic recombination and allelic exclusion, ensures not only that there is a broad diversity within the lymphocyte populations leading to the potential recognition of millions of foreign antigens, but also that each lymphocyte carries receptors of only one specificity. Lymphocytes can be divided into T- and B cells, and T cells are further subdivided into T helper cells (Th cells) and cytotoxic T cells, based on the expression of CD4 or CD8 coreceptor molecules on their surface. B cells are responsible for the production of antibodies, while major histocompatibility complex (MHC) class II-restricted $CD4^+$ T cells fulfil their coordinative function of the immune response by their interaction with other immune cells such as B-cells, macrophages or dendritic cells and through the production of specific types of cytokines. MHC class I-restricted $CD8^+$ T cells have the ability to kill infected or malignant cells, and are therefore called cytotoxic T cells.

3.2. T cell development

While the development of immunocompetent B-cells takes place within the bone marrow microenvironment[1], hematopoietic stem cells (HSC), destined to become T cells, need to migrate into the thymus and develop immune-competence there[2]. These T cell progenitor cells, once arrived in the thymus, go through multiple developmental stages, which can be subdivided based on the expression of the CD4 and CD8 coreceptor molecules, starting with the double-negative (DN) stage in which they neither express CD4 nor CD8 coreceptor molecules. DN thymocytes can be subdivided into four subsets, defined by their expression of CD44 and CD25. Rearrangement of the *Tcrb* locus at DN stage 3 results in the expression of a functional TCRβ chain, which together with pre-Tα forms the pre-TCR. The formation of the pre-TCR leads to the proliferation and the expansion of these cells. This ensures that only cells that have generated a functional pre-TCR proceed for further differentiation. Further the cells induce CD4 and CD8

coreceptor expression and hence progress to the double-positive (DP) stage. At the DP stage the recombination of the *Tcra* locus leads to the expression of the TCRα chain of the mature αβTCR. At this stage T cells undergo positive selection in the cortex: only T cells with an appropriate TCR affinity towards self-peptide/MHC survive. Cells expressing a TCR unable to bind self-peptide/MHC molecules do not receive a survival signal and undergo apoptosis, a process also called “death by neglect”. Developing single-positive (SP) thymocytes are checked for self-reactivity in a process called negative selection, which takes place in the medulla. This process, also known as central tolerance, leads to apoptotic deletion of autoreactive T cells. During the positive selection process, DP T cells undergo CD4/CD8 lineage choice and develop either into $CD4^+$ or $CD8^+$ SP T cells [3,4].

Fully mature $CD4^+$ SP or $CD8^+$ SP thymocytes leave the thymus and emigrate into the periphery to constitute the naïve peripheral T cell pool. While $CD8^+$ T cells fulfil their function as cytotoxic T cells upon activation, activated $CD4^+$ T cells differentiate into further subsets (Th1, Th2, Th17, Tfh, peripheral-induced regulatory T cells and potentially more), depending on the type of antigen-presenting cells and cytokine environment. To coordinate a proper immune response, these subsets have very distinct effector functions. The functional differences of the T helper (Th) subsets can be explained by their unique cytokine profiles.

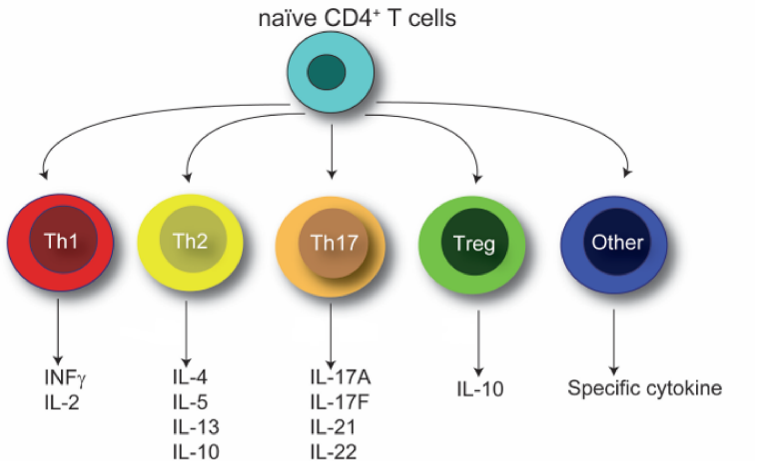

Figure 1 **Naïve $CD4^+$ T cells can differentiate in various subsets depending on the cytokine milieu.** The produced cytokines are listed below the corresponding subset. **(**Figure kindly provided by Nicole Boucheron)

Besides the two best described subsets of peripheral effector Th cells, Th1 and Th2[5], additional subsets of Th cells were characterized[6,7] (Figure 1). The main function of Th1 cells is the elimination of intracellular pathogens and the promotion of immunoglobulin-G (IG-G) production by B-cells. Th2 cells mediate the defence against helminthic infections by stimulating IG-E production and drive the activation of eosinophils[8]. Th17 cells are crucial in the protection against extracellular bacterial and fungal pathogens[6]. While most T cells express a CD3-associated αβTCR that

recognizes peptide/MHCII complexes presented by APCs, there are also subsets of unconventional T cells that differ in their TCR-specificity and TCR-repertoire diversity as well as in expression pattern of activation markers[9]. Belonging to the group of unconventional T cells are Natural killer T cells (NKT cells), which in general recognise glycolipids presented on the nonclassical MHC complex class CD1d molecule, and γδT cells, which recognise small phosphorylated molecules, alkyl amines, or lipids and have a limited TCR-repertoire diversity in many tissues[10]. Compared to conventional T cells, unconventional ones are only a minor population in the peripheral blood, but constitute a major population e.g. in the epithelium of the skin or the gastrointestinal tract[9].

3.3. Activation of $CD4^+$ T cells

Peripheral $CD4^+$ T cells require two signals presented by antigen presenting cells (APCs) to become fully activated. Signal 1, which is recognized by the TCR, is provided by a peptide-MHC class II complex. The second signal is provided by the co-stimulatory proteins B7-1 and B7-2 and recognized on T cells by CD28. After antigen recognition, TCR complexes cluster in the plasma membrane and together with CD28 and adhesion molecules form an immunological synapse. One of the first steps of T cell activation involves the phosphorylation of immunoreceptor tyrosine-based activation motives (ITAMs) by the co-receptor associated lymphocyte-specific protein tyrosine kinase (Lck), a protein tyrosine kinase (PTK) which belongs to the Src family[11]. Phosphorylation of ITAMs in the CD3 complex results in the recruitment of zeta-chain associated kinase 70 (ZAP-70)[12]. As a consequence, adaptor proteins such as linker for activated T cells (LAT) or SH2 domain-containing leukocyte protein of 76 kDa (Slp76), assemble into a large signaling complex that contains multiple proteins involved in the activation of the Ras-mitogen activated protein (MAP) kinase pathway, Ca^{2+} signaling and finally in the activation of transcription factors such as nuclear factor of activated T cells (NFAT-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) or activator protein 1 (AP-1). Once active, these transcription factors bind and activate the IL-2 promoter. One important protein recruited to LAT is Phospholipase Cγ1 (PLC γ1), which after activation via Itk or Tec (Figure 2) produces the second messenger diacylglycerol (DAG) and inositol trisphosphate (IP3) from phosphatidylinositol 4,5-bisphosphate (PIP2). IP3 leads to the release of Ca^{2+} from the endoplasmic reticulum (ER) into the cytoplasm[13] which triggers the opening of Ca^{2+} release activated Ca^{2+} (CRAC) channels at the cell membrane. Due to extracellular Ca^{2+} influx and thereby elevated intracellular Ca^{2+} concentration, Calcineurin is activated and dephosphorylates NFAT-1, allowing NFAT-1 migration to the nucleus[14]. DAG activates several proteins, including protein kinase C (PKC) which participates in the NF-κB pathway. Another important protein recruited to LAT is growth factor receptor bound protein 2 (Grb2) which binds the Ras-GDP/GTP exchange factor

SOS. Ras is the major determinant for MAP kinase signaling resulting in the activation of the transcription factor AP-1[15].

Taken together, NFAT-1, NF-κB and AP-1 are the main transcription factors active during T-cell activation, regulating many cellular processes like proliferation, differentiation and cytokine secretion.

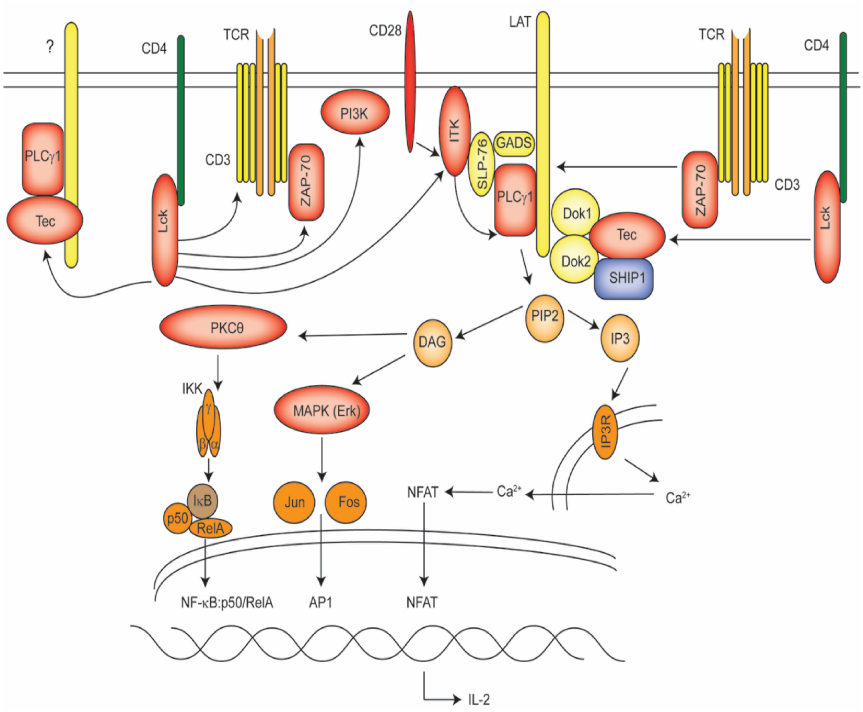

Figure 2 **Signaling pathways in an activated T cell.** Ligation of the TCR leads to a cascade of signaling events initiated by the activation of Lck, a Src-family kinase, which phosphorylates ITAMs on CD3, followed by the activation of ZAP-70 which phosphorylates the adaptors LAT and SLP-76. TCR signaling also activates PI3-kinase which catalyses the accumulation of PIP3. Binding of PIP3 via their PH domain mediates the recruitment to the plasma membrane of Tec and Itk. After their recruitment, Itk and Tec kinases get phosphorylated by Lck and phosphorylate in turn PLCγ1. While Itk associates with LAT and SLP-76, an association of Tec with these adaptor molecules could not be observed. Phosphorylation of PLCγ1 results in the cleavage of PIP2 into IP3 and DAG. While IP3 mediates an increase in intracellular calcium levels, important for the activation of NFAT, activation of DAG results in the activation of JUN amino-terminal kinase (JNK) and extracellular-signal-regulated kinase (Erk1/2), resulting in the upregulation of transcription factor AP-1. (Figure taken from Boucheron, 2018; 2: 5349-5357)

During the activation and differentiation of $CD4^+$ T cells cytokines, including interferons (IFNs) and interleukins (ILs) are key regulators. In concert with TCR and CD28 signaling, cytokine signaling is crucial for many T cell functions, in particular proliferation, subset differentiation, memory establishment and T cell recall. Cytokines signal through Janus kinase and signal transducer and activator of transcription (JAK/STAT) pathways. Cytokines are extracellular signaling polypeptides that can bind to specific transmembrane receptors. This receptor-ligand coupling results in the activation of the non-receptor protein tyrosine kinases JAK that is con-

stitutively associated with the cytoplasmic domain of the receptor. Besides their kinase domain, JAK molecules also contain a kinase like domain without catalytic activity which serves for their autoregulation[16]. Among several others the most important JAK phosphorylation targets are STAT proteins. STAT proteins are transcription factors (TF) which residue in the cytoplasm in an inactive form until phosphorylation of a single tyrosine, performed by JAK kinases, results in their dimerization via their scr-homology 2 (SH2) domain[17]. Activated STAT proteins migrate into the nucleus and direct transcription of target genes by binding of enhancer sequences[18].
In mammals several JAK kinases and STAT proteins have been described.

3.4. Differentiation and function of Th17 cells

Th17 cells are intensively studied, as they are involved in controlling extracellular bacteria and fungi infections via IL-17A cytokine production and the recruitment of neutrophils[19,20]. In a steady state, Th17 cells are mainly restrained to the intestine, where they have a crucial function in the protection against infection at the epithelial barrier. If dysregulated, Th17 cells are also linked with auto-immune responses[21]. IL-17, a pro-inflammatory cytokine, has been implicated in several auto-immune diseases such as rheumatoid arthritis[22] or psoriasis[23]. The ability of Th17 cells to trigger severe inflammation is what makes their tight regulation crucial.
In the presence of TGF-β and IL-6, TCR-stimulated naïve $CD4^+$ T cells differentiate into Th17 cells[24]. The TF Basic leucine zipper transcription factor ATF-like (BATF) and Interferon Regulatory Factor 4 (IRF4) are induced upon TCR and CD28 stimulation. BATF and IRF4 act as pioneer TF by mediating chromatin remodelling and nucleosome repositioning at Th17 specific genes[25], allowing binding of lineage specific TF like STAT-3. STAT-3 is activated by IL-6 signaling and Smad2 by TGF-β signaling. Smad2 cooperates with STAT-3 in the induction of Th17 cells by binding to open Th17-associated genes, leading to the expression of IL-21, IL-23, IL-1R1 and the Th17 master TF retinoic acid-related orphan receptor-γt (RORγt). TGF-β also induces the T regulatory (Treg) lineage-specific transcription factor Foxp3, IL-6 however orchestrates the down-regulation of Foxp3. In addition, beside IL-6 and TGF-β, other cytokines further support Th17 skewing and maturation. IL-23 is important for the expansion and maintenance of Th17 cells, but has also been associated with the induction of the transcription factor PR domain zinc finger protein 1 (prdm1) or Blimp1 and a subsequent programming of Th17 cells towards a pathogenic subset[26]. In addition, IL-1 was shown to play a critical role in the early differentiation of Th17 cells and conversion of $Foxp3^+$ Treg cells into Th17 cells[27]. Th17 cells are characterised by their production of IL-17A (also IL-17), IL-17F, IL-21, and IL-22. IL-17 directly interacts with NF-κB activator 1 (Act1) which results in the expression of several pro-inflammatory proteins such as CCL7 or CCL2[28].

3.5. Structure, activation and substrates of Tec family kinases

Tec kinase family (TFKs), a group of nonreceptor protein tyrosine kinases, are preferentially expressed in the hematopoietic system, but only three members of the family, Tec, Itk and Rlk, are expressed in T cells. N-terminally, Tec carries a pleckstrin homology (PH) domain followed by two proline-rich (PR) regions which together with the Btk homology (BH) domain form the so-called Tec homology (TH) domain (Figure 3). The TH domain is followed by the Src homology (SH) domains SH2 and SH3 and by a C-terminal kinase domain.

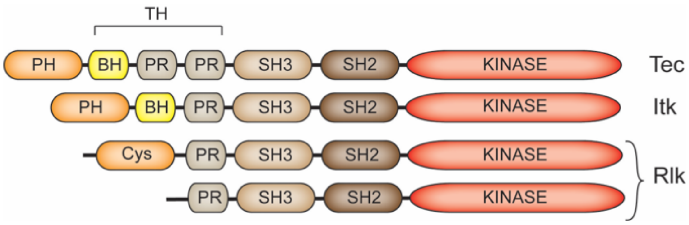

Figure 3 **Tec family kinase members Tec, Itk and Rlk are formed by modular segments.** Common to all three members are a PR domain, a SH3 domain, a SH2 domain and a kinase domain**.** (Figure taken from Boucheron et al., 2012; 31:133-154)

The TH domain is involved in the auto-regulation of Tec family kinases by intra- and intermolecular SH3/PR interactions. In Itk, these interactions are exclusively intramolecular, while in Tec they appear in an inter- and intramolecular fashion, due to the two PR regions. The PH domain is able to bind phospholipids but more importantly confers membrane localisation to Tec upon binding of PI3-kinase-generated phosphatidylinositol (3,4,5)-triphosphate (PIP3)[29] which acts as the first step of the full activation of Tec kinases in T cells. After TCR ligation and TFKs recruitment to the plasma membrane, Lck phosphorylates a critical tyrosine residue within the conserved activation loop in the kinase domain of TFKs. A further autophosphorylation of a tyrosine residue within the SH3 domain leads to full activation of TFKs. After their activation, both Itk and Tec are able to phosphorylate and thereby contribute to the activation of PLCγ1. While the activation via Itk requires the adaptor molecules SLP76 and LAT, PLCγ1 activation via Tec is independent of these adaptor molecules[30].

Tec is the founding member of the Tec kinase family. *Tec* is composed of 19 exons and maps to mouse chromosome 5[31] (Figure 4A). The most important splice variants are Tec splice variant 1, 2 and 3 (Figure 4B). Variant 1 represents the full length transcript, variant 2 carries a deletion before the ATG entry site and variant 3 carries a deletion in the SH3 domain.

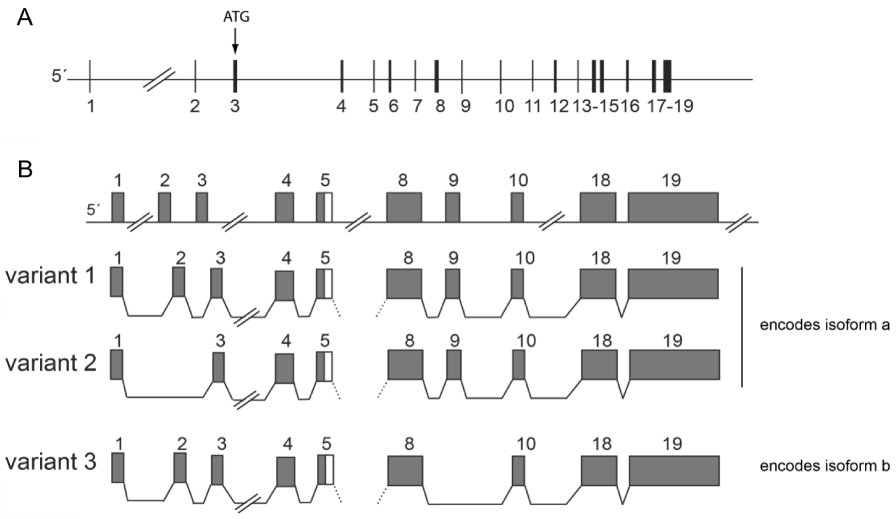

Figure 4 **The murine *Tec* gene is assigned to chromosome 5 and is composed of 19 exons.** (A) The ATG entry site is shown. (B) Presented are the most important splice variants described by the National Center for Biotechnology Information (NCBI). (Figure taken from Boucheron, 2018; 2: 5349-5357)

Two isoforms of Tec are predominantly expressed (Figure 5). Most studies were performed using Tec isoform a (Tec[a]) (encoded by splice variant 1 and 2) which is mostly expressed in the hematopoietic system. Tec isoform b (Tec[b]) (encoded by splice variant 3) was described as potentially more active, as it does not contain the inhibitory SH3 domain, and was shown to be expressed predominantly in liver.

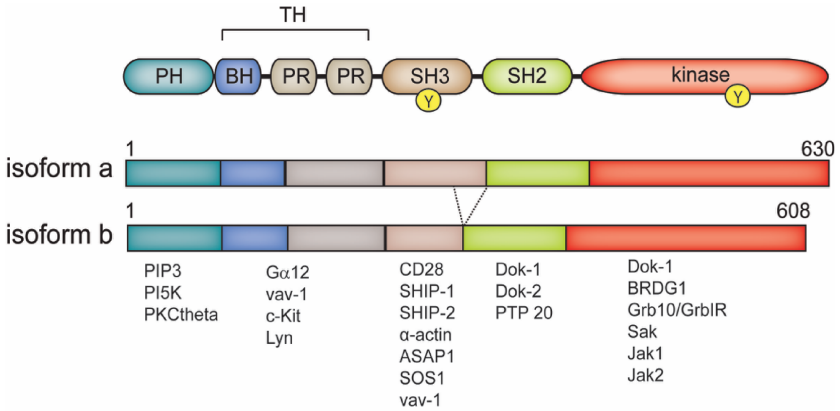

Figure 5 **Domain structure of Tec and knowing binding partners.** Shown are the two main isoforms of Tec with its domains: isoform a which has a length of 630 amino acids and is encoded by splice variant 1 or splice variant 2, and isoform b which has a 22 amino acid long deletion in the SH3 domain and is encoded by splice variant 3. The known binding partners for Tec are listed below the corresponding domain[68,69]. (Figure taken from Boucheron, 2018; 2: 5349-5357)

Compared to Itk, Tec has different interaction partners (Figure 5). In particular Tec was shown to interact with SHIP-1 and SHIP-2[32], and other known negative regulators like Dok proteins[33,34].

Additional interaction partners not assigned to a Tec domain were SOCS1 and SOCS3[35,36]. Other interaction partners of Tec are JAK proteins in cytokine signaling pathways[37,38].

3.6. Expression of Tec kinase and its role during T cell signaling

Initial studies, which were performed in T cell lines, suggested an important role for Tec during T cell activation. Studies performed in murine T cell hybridoma cell lines showed an interaction of Tec via its SH3 domain with CD28 (see Figure 5 for Tec interaction partners), followed by the activation of Tec in response to TCR and CD28 cross-linking, resulting in an enhanced transcription of both IL-2 and IL-4 promoters[39,40,41]. These studies implicated Tec in TCR/CD28-mediated pathways and in the activation of the *Il2* and *Il4* promoters. Another study showed reduced IL-2 production in murine splenocytes after downregulation of Tec expression by antisense approaches[42]. Moreover, overexpression of Tec, but not of Itk or Btk, in Jurkat T cells resulted in the activation of the NFAT pathway[43].

Despite the unique properties of Tec in T cell activation observed in T cell lines, Ca^{2+} flux and the overall tyrosine phosphorylation patterns were not altered in anti-CD3 activated *Tec*$^{-/-}$ murine primary $CD4^+$ T cells[44]. The normal activation pattern upon TCR stimulation in *Tec*$^{-/-}$ $CD4^+$ T cells can be explained in several ways. Compared to other members of the TFKs, Tec is expressed approximately 15-fold lower in naïve T cells and only upregulated upon T cell activation and in effector/memory T cells[44,43]. Tec expression was shown to be controlled by transcription factors Sp1 and PU-1. Another level of transcriptional control is provided by the NF-κB subunit p65/RelA, in a dose-dependent manner which might be responsible for the cell activation-state specific expression levels[45]. Another explanation for the lack of major immunological alterations in Tec-deficient mice might be that Itk or Rlk compensate for the absence of Tec in naïve T cells. The specific upregulation of Tec during T cell activation and in effector/memory T cell subsets indicates a role of Tec in the regulation of signaling pathways in effector T cells.

3.7. The role of Tec family kinases in T helper cells

Knock-out studies revealed a major role for Itk in T cell development, since *Itk*$^{-/-}$ mice display reduced numbers of peripheral T cells and a reduced $CD4^+$ to $CD8^+$ T cell ratio [46]. Moreover, the activation and proliferation of naïve $CD4^+$ T cells as well as their polarization into Th2 effector T cells is impaired in the absence of Itk[47,48]. Studies of *Itk*$^{-/-}$ *Rlk*$^{-/-}$ T cells indicate a partial compensation for the loss of Itk by Rlk in TCR-mediated signaling events[49] and a role of Rlk in Th1 polarization[50,51]. It was reported that Tec expression is up-regulated in effector T cells, in particular Th2 cells and therefore a specific role for Tec in Th2 cell differentiation and function was proposed[43]. However, in studies based on *Tec*$^{-/-}$ mice, *Tec*$^{-/-}$ $CD4^+$ T cells showed normal Th2 differentiation[44]. Other Tec kinases expressed in *Tec*$^{-/-}$ T cells might compensate for loss of Tec

during the activation of $CD4^+$ T cells and differentiation into Th2 cells. In the same study an increased $CD44^{hi}CD62L^-$ Th17 effector/memory subset was observed in *Tec*$^{-/-}$ mice as well as an enhanced IL-17A production in these cells upon activation with anti-CD3ε or phorbol-12-myristate-13-acetate (PMA) and ionomycin. An *in vivo* approach with *Streptococcus pneumonia* immunized and infected *Tec*$^{-/-}$ mice revealed elevated IL-17A expression levels in the lung which correlated with enhanced clearance of pneumococci and a reduction of lung inflammation in the absence of Tec[44]. Taken together Tec family kinases are important regulators of T cell development and function and also contribute to immune related diseases.

4. Aim of the Thesis

Tec was recently identified as a T cell-intrinsic dampener of Th17 differentiation. The aim of my master´s thesis was to investigate in more detail how Tec modulates Th17 differentiation and function.

Specific aim 1: Provide insight into the expression of Tec isoforms and the kinetic of Tec in Th17 cells

Several isoforms of Tec have been described, however which of these isoforms are important for regulating Th17 cells is not known. In this aim I wanted to study the expression of Tec isoforms in Th0 and Th17 cells with varying concentrations of the Th17-driving cytokines IL-6 and TGF-β. Furthermore, I wanted to determine the kinetic of Tec expression in Th17 cells by assessing Tec expression levels at different time points after activation.

Specific aim 2: Test the function of Tec isoform a (Tec^{a}) and Tec isoform a kinase dead (Tec^{a} KD) during Th17 differentiation

In this aim I wanted to specifically analyse the function of Tec^{a} and the role of the kinase domain in the function of Tec. I performed retroviral-mediated expression approaches of either Tec^{a} or Tec^{a} KD, expressing a non-functional kinase domain, in *Tec*$^{-/-}$ CD4^{+} T cells during their differentiation into Th17 cells.

5. Results

5.1. Tec dampens Th17 differentiation *in vivo* in a T cell-intrinsic way

To further test the role of Tec in Th17 cells, an *in vivo* transfer model with either wild-type (WT) or *Tec*$^{-/-}$ mice (OTII^{+} and CD45.2^{+}) donor mice and CD45.1^{+} recipient mice was employed. Donor mice were IL-17A fate mapping mice that have a Cre recombinase inserted into the *Il17a* locus. To monitor Cre activity, these mice where crossed with mice expressing enhanced yellow fluorescence protein (eYFP) from the *Rosa26* locus. This system allows the identification of cells that have or had switched on IL-17A expression[52]. After transfer of naïve OTII^{+} CD4^{+} T cells from WT or *Tec*$^{-/-}$ mice and immunization with Ovalbumine peptide in CFA (Figure 6A), flow cytometry analysis showed a significant increase in Th17 cells derived from *Tec*$^{-/-}$ donor mice (Figure 6B and 6C). These data, generated by my supervisor Dr. Nicole Boucheron, indicate a T cell-intrinsic function of Tec as a dampener of Th17 cells *in vivo*.

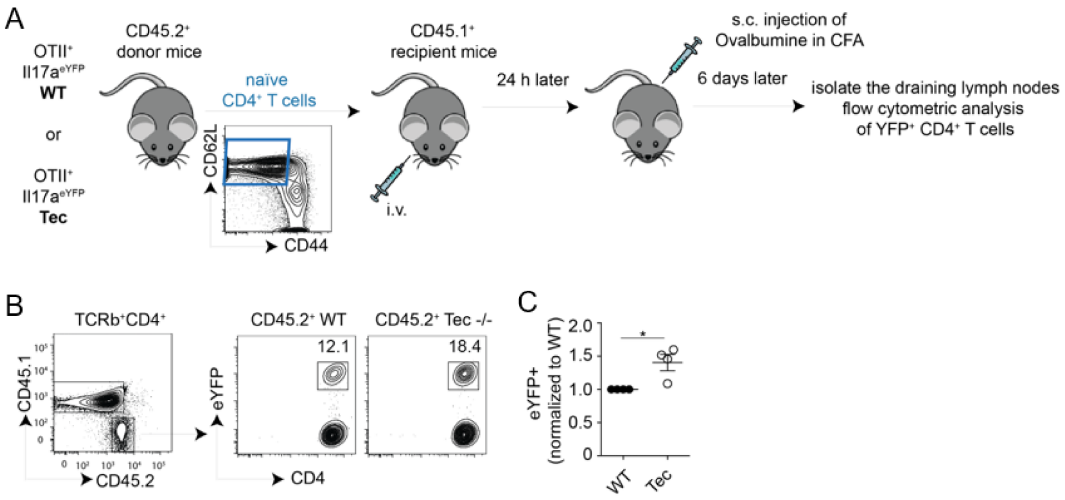

Figure 6 **The absence of Tec results in an increase of IL-17 producing cells.** (A) Schematic overview of the adaptive transfer model. Naïve CD4^{+} cells from either *Tec*$^{-/-}$ mice or WT mice were transferred by intravenous (i.v.) injection into recipient mice. 24h after injection mice received a subcutaneous injection of Ovalbumin (OVA) for OVA specific T cell activation of OT-II T cells. (B) Flow cytometric analysis of YFP^{+} CD4^{+} T cells isolated from draining lymph nodes. (C) Results from four independent experiments show a significant increase of eYFP^{+}, representing IL-17, in CD4^{+} cells from *Tec*$^{-/-}$ mice. (Figure kindly provided by Nicole Boucheron)

5.2. Enhanced IL-6 sensing in the absence of Tec

Next, to study the role of Tec during Th17 differentiation in more detail, the effect of IL-6 levels on abundance of IL-17 expressing cells and IL-17 expression in *Tec*$^{-/-}$ and WT CD4^{+} T cells was analysed in Th17 differentiation assays. The highest significant increase in IL-17 producing cells (Figure 7A and 7B), as well as in IL-17 secretion (Figure 7C) between *Tec*$^{-/-}$ and WT cells was achieved with IL-6 concentrations of 5 ng/ml and 10 ng/ml. At concentrations above 20 ng/ml of IL-6, *Tec*$^{-/-}$ and WT showed comparable levels of IL-17 producing cells. Since IL-6 activates

STAT-3[53], we determined STAT-3 phosphorylation levels. STAT-3 phosphorylation was increased in *Tec*$^{-/-}$ Th17 cells cultured for 2 days in presence of 5 ng/ml IL-6 compared to WT Th17 cells cultured under the same conditions (Figure 7F). We did not observe an increase of STAT-3 phosphorylation when cells were cultured with 20 ng/ml of IL-6.

Together, the data presented in 5.1 and 5.2 indicate that Tec is a negative regulator of Th17 cells and is acting downstream of the IL-6 receptor (IL-6R).

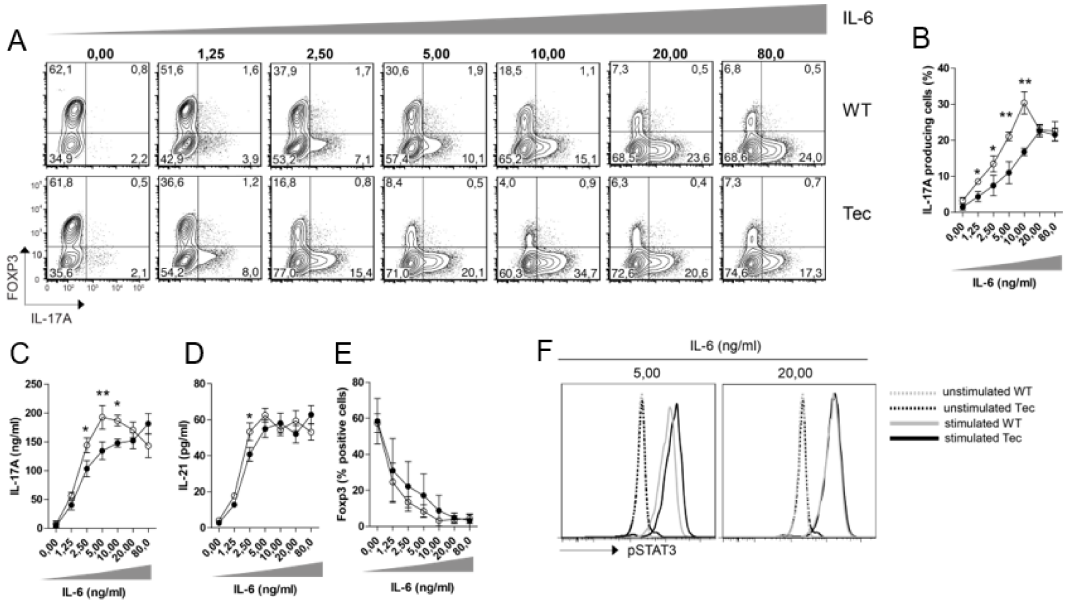

Figure 7 **Enhanced IL-6 sensing in the absence of Tec.** (A) Naïve CD4$^+$ T cells from WT and *Tec*$^{-/-}$ mice were cultured with 2 ng/ml TGFß1 and increasing amounts of IL-6 as indicated in the figure for three days followed by analysis of Foxp3 and IL-17A by flow cytometry. (B) Diagram represents the summary of five independent experiments as described in (A). (C) Secreted IL-17A was assessed by ELISA from cultures described in (A) and (B). (D) Secreted IL-21 was assessed by ELISA from cultures described in (A) and (B). (E) Diagram represents Foxp3 expression of five independent experiments as described in (A). (F) Naïve CD4$^+$ T cells from WT and *Tec*$^{-/-}$ mice were cultured with 2 ng/ml TGFß1 and either 5 or 20 ng/ml IL-6. STAT-3 phosphorylation was assessed by flow cytometry on day two of culture. (Figure kindly provided by Nicole Boucheron)

5.3. TGFβ1 and IL-6 induce the expression of Tec in CD4$^+$ T cells

In the next experiments, we wanted to determine the impact of TFGβ1 and IL-6 signaling on Tec expression. The western blot in figure 8 depicts the expression levels of Tec in murine CD4$^+$ T cells after their culture in the presence of various cytokines. IL-6 alone was not able to induce Tec expression. This is in contrast to TGFβ1, which induced low expression of Tec also in the absence of IL-6. When TGFβ1 as well as IL-6 were added to the culture, the expression of Tec was approximately three times higher than the levels of Tec expression induced by TGFβ1 alone. Adding IL-1 and IL-23 did not increase the expression levels of Tec. These data indicate that Tec is specifically induced under iTreg conditions (addition of TGFß1) and Th17 conditions (addition of IL-6 and TGFß1), with the highest level induced under Th17 conditions.

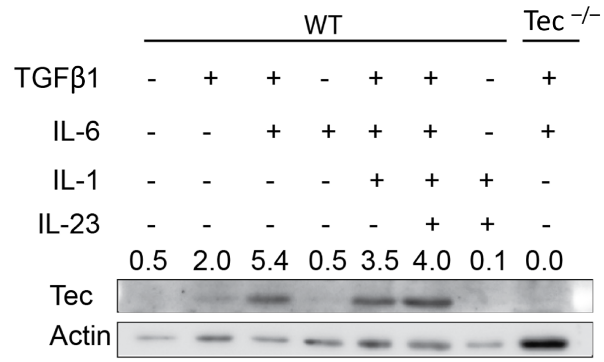

Figure 8 **TGFβ1 and IL-6 induce the expression of Tec in CD4⁺ T cells.** Murine T cells isolated from lymph nodes and spleens of WT or *Tec*$^{-/-}$ mice, were sorted into naïve CD4⁺ T cells and cultured with different cytokines. After 72 h of culture, the cells were lysed and blotted with anti-Tec antibody from Upstate or anti-Actin antibody respectively. Data are representative of two independent experiments.

Several Tec isoforms encoded by different splice variants of Tec have been described[54,31]. Next we analysed the expression levels of these individual variants of Tec under different TGFβ1 and IL-6 conditions. In absence of TGFβ1 and IL-6, or presence of only IL-6, none of the Tec variants were expressed (Figure 9). Addition of TGFβ1 alone induced expression of Tec variant 1 or 2 (indistinguishable by PCR) and variant 3. The expression of these variants was further enhanced under Th17 conditions (addition of IL-6 and TGFβ1). Variant 1 and variant 2, both encoding isoform a, displayed the highest expression level compared to variant 3, which encodes for isoform b, under Th17 conditions and therefore represents the predominantly expressed isoform of Tec.

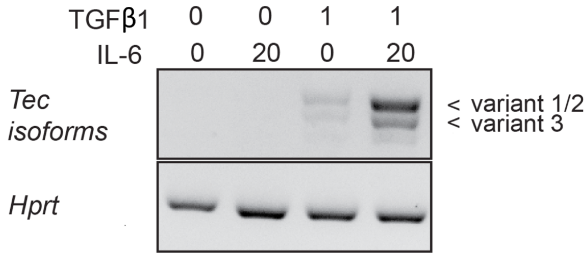

Figure 9 **Tec variant 1 and variant 2 are the dominant splice variants of Tec in Th17 cells.** Naïve CD4⁺ cells from WT mice were cultured under different TGFβ1 and IL-6 conditions for three days followed by RNA isolation and cDNA synthesis. *Tec* isoform PCR was performed and samples were analysed on a 2% agarose gel. *Hprt* was used as a house keeping gene. Data are representative of two independent experiments.

We next tested the concentrations of TGFβ1 and IL-6 that are necessary for a proper induction of Tec in T cells. Figure 10 shows that a concentration of 1 ng/ml of TGFβ1 was sufficient for a low induction of *Tec* mRNA expression. The addition of 20 ng/ml IL-6 was able to compensate for lower TGFβ1 concentrations (0.25 ng/ml), but did not induce Tec expression alone. A con-

centration of 1 ng/ml of TGFβ1 and 20 ng/ml of IL-6 showed the highest induction of Tec expression.

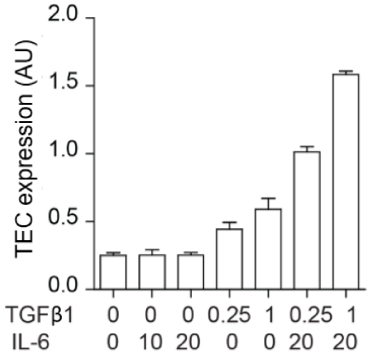

Figure 10 **A combination of TGFβ1 and IL-6 leads to a stronger induction of Tec compared to TGFβ1 alone.** Naïve CD4⁺ T cells were incubated with an increasing concentration of TGFβ1 and/or IL-6 and analysed by qRTPCR after three days of cultivation. Data are representative of two independent experiments.

We next investigated whether Tec protein expression was also induced by increasing TGFβ1 and constant IL-6 levels (Figure 11). The titration of TGFβ1 showed that a concentration of 0.1 ng/ml of TGFβ1 is already sufficient to induce the expression of Tec. Taken together these data indicate that Tec is induced by TGFβ1 in a concentration-dependent manner.

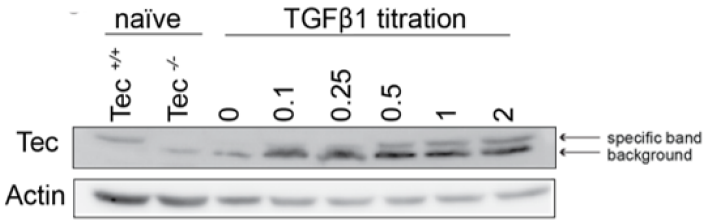

Figure 11 **Positive correlation between TGFβ1 concentration and the expression level of Tec.** Naïve CD4⁺ cells from either WT or *Tec*$^{-/-}$ mice were cultured with different concentrations of TGFβ1, harvested after 3 days and further analysed by western blot. Naïve cells were directly used for western blot and blotted with anti-Tec antibody (Upstate) or anti-Actin antibody as a loading control. The figure represents the results of two independent experiments.

5.4. Expression of Tec in Th17 cells starts after 24 h in culture and peaks after 72 h

Western blot and quantitative-Real-Time-PCR (qRTPCR) approaches both showed that the expression of Tec under Th17 conditions was already induced after 24 h of culture, increased after 48 h and peaked at 72 h (Figure 12). Results from qRTPCR showed that *Tec* mRNA levels after 72 h of culture under Th0 condition were not enhanced when compared to 5 h of culture under Th17 conditions, indicating that Tec was not induced under Th0 conditions within this time frame. To fulfil its function, Tec might need a specific signalling network. Candidates for interaction partners of Tec are SOCS proteins. In particular, SOCS-3 was shown to play a major role in downregulating Th17 responses[55]. With qRTPCR approaches we compared the expression kin-

etic of *Tec* and *Socs-3*. Expression levels of *Socs-3* were highest before activation, declined 5, 24 and 48 h after activation and increased again after 72 h of activation, showing a modulation of SOCS-3 expression during Th17 differentiation. Therefore, the kinetic of Tec and the kinetic of SOCS-3 show substantial differences which might argue against a possible interaction between them. However, further experiments are needed to determine Tec interaction partners.

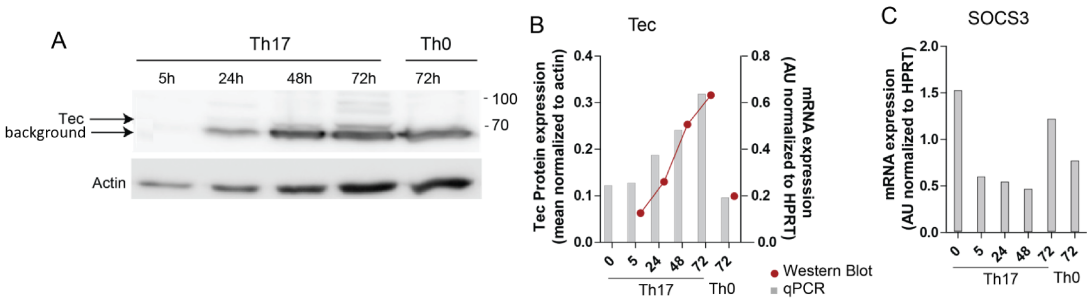

Figure 12 **Upregulation of Tec in Th17 cells starts after 24 h upon activation and peaks after 72 h.** The upregulation of Tec in Th17 cells was analysed on (A) protein level by western blot and on (B) mRNA level by qRTPCR. Naïve CD4$^+$ T cells were cultured under Th17 or Th0 conditions and harvested at defined time points as indicated. The arrow highlights the specific band and the star an unspecific band. Actin was used as a loading control (C). In parallel, expression of SOCS-3 in Th17 and Th0 cultures as described in (A) and (B) was assessed by qRTPCR. Data are representative of two independent experiments.

5.5. Retroviral-mediated expression of Tec

To analyse the individual isoforms of Tec and to obtain a better understanding of the kinase-specific function of Tec in Th17 cells, we expressed Tec^a or Tec^a KD in *Tec*$^{-/-}$ naïve CD4$^+$ T cells. Tec^a KD carries a point mutation at position 1379 (A to G), corresponding to amino acid 397 (Lys to Glu), in the region coding for the kinase domain and therefore expresses a non-functional kinase domain[39]. Figure 13 shows Tec expression in GFP$^+$ *Tec*$^{-/-}$ cells transduced with Tec^a respectively with Tec^a KD compared to no Tec expression in GFP$^-$ *Tec*$^{-/-}$ cells and to Tec expression in WT cells. The expression levels of Tec in GFP$^+$ *Tec*$^{-/-}$ cells and WT cells are comparable. In the control group transduction was performed with “empty” MSCV-EGFP virus. Neither GFP$^+$ nor GFP$^-$ cells from the control show Tec expression.

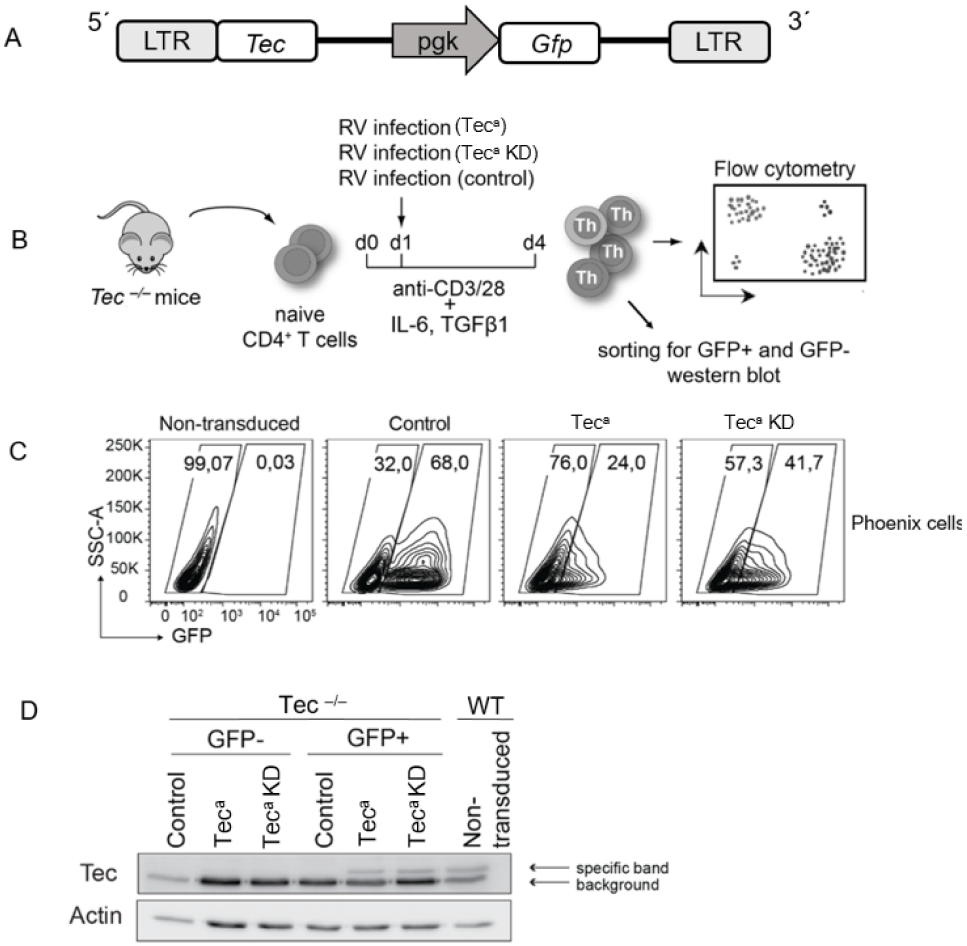

Figure 13 **Transient transfection of Phoenix cells and transduction of naïve CD4⁺ T cells with MSCV-EGFP.** (A) Schematic drawing of the retroviral construct. In the MSCV-EGFP vector, the LTR drives the expression of the *Tec* gene and the pgk promoter drives the *GFP* gene. The sequence encoding Tecᵃ represents splice variant 1 and was taken from NCBI (Gene ID:21682). (B) Experimental setup for the retroviral mediated expression of Tec. (C) The efficiency of the transient transfection of Phoenix cells was analysed by flow cytometry. *Tec*$^{-/-}$ naïve CD4⁺ T cells were transduced with Tecᵃ, Tecᵃ KD or an empty control retroviral vector, encoding a GFP expression cassette under pgk promoter, 24 h after culture under Th17 conditions. (D) The efficiency of the transduction was analysed after three days in culture by western blot with anti-Tec antibody (Upstate). Actin served as a loading control.

As shown in Figure 14 the reintroduction of Tecᵃ into *Tec*$^{-/-}$ T cells resulted in an increase of IL-17 production in the presence of low IL-6 levels (5 ng/ml). This increase was dependent on the kinase activity of Tec, as no difference in IL-17 production was observed with 5 ng/ml IL-6 with Tecᵃ KD. In the presence of higher IL-6 levels (from 10 ng/ml on) no significant difference in IL-17 production was observed.

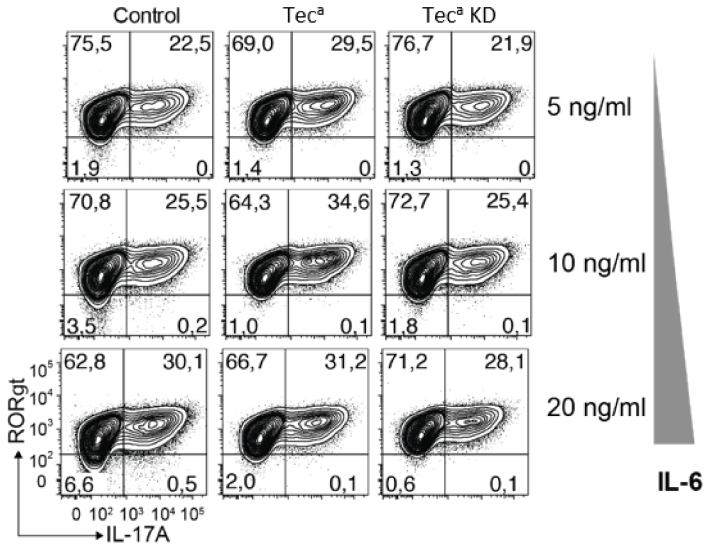

Figure 14 **Under low IL-6 concentration reintroduction of Tec^a in *Tec*$^{-/-}$ T cells results in an increase of IL-17 production.** *Tec*$^{-/-}$ naïve CD4+ T cells were transduced with Tec^a, Tec^a KD or control retroviral vector. After infection, cells were cultured for three days with 2 ng/ml TGFß1 and increasing concentrations of IL-6 ranging from to 20 ng/ml.

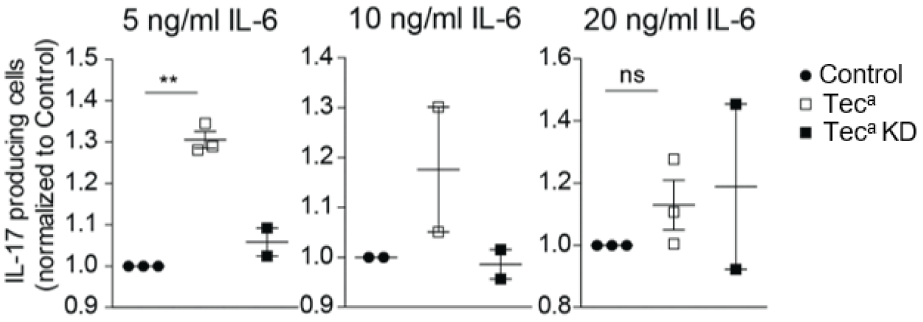

Figure 15 **Tec^a significantly increases the number of IL-17 producing cells under low IL-6 levels.** The data from three independent experiments was normalized to the control. Horizontal bars indicate mean values. An unpaired two-tailed Student's t-test was used for the statistical analysis. The P-values were defined as following: **, P < 0.01; n.s., not significant

Taken together the analysis of Tec$^{-/-}$ CD4$^+$ T cells, as shown in Figure 7, revealed Tec as a negative regulator of Th17 differentiation by dampening IL-6 signaling at low IL-6 levels. However, the retroviral-mediated expression studies indicate also a positive regulatory role for Tec in Th17 differentiation at low IL-6 levels. This suggests that the kinetic of Tec expression is crucial for the regulation of the IL-6 signaling pathway.

6. Discussion

Here we identified Tec, a member of the Tec kinase family, as a negative regulator of Th17 cells. Results from a study of my master´s thesis supervisor Dr. Nicole Boucheron revealed that Tec regulates a $CD44^{+}CD62L^{-}$ Th17 subset[44]. It was shown that the absence of Tec results in an increase of $CD44^{+}CD62L^{-}$ $CD4^{+}$ T cells that produce enhanced levels of IL-17, indicating a general bias toward a Th17 memory in *Tec*$^{-/-}$ mice[44]. In ongoing studies, by using adoptive transfer models, it was further revealed that the damping effect of Tec on Th17 differentiation *in vivo* is due to a T cell-intrinsic alteration (Figure 6). These results demonstrate an important function of Tec in the homeostasis of Th17 cells.

One question that arises from these studies is how Tec is regulating Th17 differentiation. The activation and inhibition of T cells is regulated by complex signalling pathways, which include the interaction of multiple signalling molecules. To provide better insight into the role of Tec in T cell receptor signalling of Th17 cells, we performed *in vitro* Th17 differentiation assays. In the first part of my thesis we analysed the role of Tec on IL-17A production in Th17 cells using increasing amounts of IL-6 and showed that the absence of Tec results in an increase of IL-17A under low IL-6 concentration. This suggests an enhanced IL-6 sensing of *Tec*$^{-/-}$ cells and indicates that Tec is acting downstream of the IL-6 receptor (IL-6R). When analysing STAT-3 phosphorylation of activated $CD4^{+}$ cells we observed an increase of STAT-3 phosphorylation in *Tec*$^{-/-}$ $CD4^{+}$ T cells cultured under Th17 conditions with low (5 ng/ml) IL-6 concentration. Phosphorylation of STAT-3 in *Tec*$^{-/-}$ $CD4^{+}$ T cells cultured under Th17 conditions with higher (20 ng/ml) IL-6 concentration showed no differences compared to WT. Our observation of Tec being a negative regulator of STAT-3 phosphorylation, an important transcription factor of Th17 cells, strengthens the hypothesis of Tec being a dampener of Th17 cells.

In the second part of my thesis, I investigated Tec expression levels under varying concentrations of the Th17-driving cytokines TGFβ1, IL-6, IL-1 and IL-23 and showed that Tec is specifically induced to high levels in Th17 cells. While IL-1 and IL-23 seem to have no impact on Tec expression, TGFβ1 and IL-6 were both required for maximal Tec expression. TGFβ1 was essential for Tec expression and induced Tec in a dose dependent way.

To provide insight into the kinetic of Tec expression in Th17 cells we measured the expression levels of Tec at different timepoints after activation. We showed that the expression of Tec is induced 24 hours after activation and peaks around 72 hours. The activation-induced and upregulated expression of Tec might be of high importance, especially as it was shown that the expression levels of Tec kinases are important in mediating their particular function[56].

While results from the *in vivo* transfer assay, the Th17 skewing *in vitro* assay and the STAT-3 phosphorylation assay suggested a negative regulatory role for Tec in Th17 cells, data from retroviral-mediated expression revealed also a positive regulatory role for Tec during Th17 differentiation. Tec gain-of-function studies in *Tec*$^{-/-}$ T cells cultured under Th17 conditions with Tec^{a} resulted in a significant increase of Th17 cells compared to the control group (complementation with “empty” MSCV-EGFP virus) and also compared to complementation with Tec^{a} kinase dead. These results indicate not only that the kinase domain is crucial for Tec to fulfil its function, but also that the function of Tec might be modulated either at the level of protein expression or at the level of its expression kinetic. The expression level of Tec^{a} was comparable to the expression level of Tec in WT cells upon Th17 differentiation, implying that the changed protein levels do not explain the discrepancy observed. Our data showed that under Th17 conditions Tec expression peaks after 72h, while during retroviral transduction it might be expressed much earlier. This indicates that the function of Tec might be modulated at the level of its expression kinetic. Many other factors are known to regulate Th17 cells. Tec might interact with other regulators, which are not yet upregulated or already downregulated in the setting of the retroviral-mediated expression assay. To fulfil its function, Tec might need a specific signaling network. Candidates for important interaction partner of Tec and regulator of Th17 cells are suppressors of cytokines signaling (SOCS). It is known that SOCS proteins can inhibit cytokine signaling in several ways. SOCS-1 and SOCS-3 are two members of the family which act by inhibiting JAK tyrosine kinase activity[57,58,53]. Recent studies showed that SOCS-3 binds the SH2 domain of the gp130-JAK1 complex which acts as a receptor complex for cytokines of the IL-6 family, and therefor blocks the IL-6 induced STAT-3 signal[59,60]. Several independent studies showed an association between SOCS proteins and Tec kinase either in human embryonic kidney cells (HEK) 293T cells for SOCS-1 and Tec or human and murine mesangial cells for SOCS-3 and Tec[35,36]. Studies on the upregulation of SOCS proteins in Th cells showed major differences between SOCS-1 and SOCS-3. While *Socs-1* mRNA levels are completely down in naïve Th cells and substantially increase 48h after stimulation, *Socs-3* mRNA levels are the highest in naïve Th cells and dramatically decline after stimulation[61]. We showed that the mRNA level of *Tec* gradually increased after T cell activation and finally peaked 72h after activation. Further, we demonstrated that the mRNA level of *Socs-3* was highest before activation, declined 5, 24 and 48 h after activation and increased again after 72 h of activation. Therefore, the kinetic of Tec rather resembles the kinetic of SOCS-1 than the kinetic of SOCS-3. SOCS-1 has been implicated in inhibiting IL-6 mediated STAT-3 activation[62, 63] and therefore also represents a possible interaction partner for Tec in the regulation of IL-6 sensing downstream of the IL-6R.

Src homology region 2 domain-containing tyrosine phosphatase-1 (SHP-1) might also be involved in the signaling network of Tec. SHP-1 was identified as a dampener of murine Th17 development[64]. IL-6 dose-response assays, comparable to Th17 skewing assays we performed with *Tec*$^{-/-}$cells, showed an increase in IL-17 in *Shp-1*$^{-/-}$ T cells cultured under Th17-inducing only under low IL-6 concentrations, ranging from 0.1 ng/ml to 0.5 ng/ml. Thus, like we observed for Tec, *Shp-1* deficient CD4^{+} T cells are hyper-responsive to low IL-6 levels. Besides IL-6, the cytokine IL-21 has also been identified to induce STAT-3 activation and thereby mediates Th17 cell differentiation along with TGFß1[65]. Th17 differentiation assays with *Shp-1*$^{-/-}$ T cells and WT T cells revealed that in contrast to the IL-6 response, *Shp-1*$^{-/-}$ cells continued to hyper-respond, also at higher IL-21 concentrations[64].

A study showed that in 293T cells and in Jurkat cell lines SH2-containing inositol phosphatase (SHIP-1) interacts preferentially with Tec, compared with other Tec family members[32]. SHIP-1 is also involved in the regulation of Th17 versus Treg differentiation[66]. This regulation was however not IL-6 dose-dependent. This implies that Tec and SHIP-1 might not act in the same signaling network downstream of the IL-6R. In line with different signaling pathways is the observation that Foxp3 positive cells were not increased in the absence of Tec under Th17 skewing conditions (Figure 7A).

In summary, our experiments demonstrate that Tec dampens Th17 differentiation in a T cell-intrinsic way and that Tec is specifically induced in iTreg and Th17 conditions, with highest expression in Th17 conditions. IL-6 dose-response assays revealed an enhanced IL-6 sensing in the absence of Tec. Among the key transcription factors that promote Th17 differentiation is STAT-3. Our results suggest Tec as a regulator of STAT-3 phosphorylation. Our data indicate a negative regulation and specific function of Tec in the differentiation of Th17 cells, downstream of the IL-6R. However, our data also indicate that a tight control of the kinetic of Tec expression is important, since enforced expression of Tec at early stages of T cell differentiation (as performed in our retroviral-mediated gene transduction experiments) might impair Th17 cell differentiation.

What is still unclear are the upstream signaling events that modulate the regulators of Th17 differentiation. We suggest a possible link of Tec to other regulators of Th17 cells, for example SOCS proteins or SHP-1. Future studies will aim on investigating how Tec is integrated into the signaling pathways regulating Th17 differentiation.

7. Material and Methods

7.1. Cell culture media

For Phoenix cells

Dulbecco´s Modified Eagle Medium (DMEM) (Sigma)
supplemented with:

10%	FBS (heat inactivated)
100 U/ml	Penicillin-Streptomycin (GE Healthcare)
1x	Glutamax (GIBCO)

For $CD4^+$ T cells

RPMI-1640 (Sigma)
supplemented with:

10%	FBS (heat inactivated)
100 U/ml	Penicillin-Streptomycin (GE Healthcare)
1x	Glutamax (GIBCO)
0.55 mM	β-mercapthoethanol (Sigma)

7.2. Cell lines

Phoenix cells

As a packaging cell line phoenix cells, originating from human embryonic kidney cells (HEK 293T cells) were used. These cells carry an expression plasmid with gag-pol and env, proteins essential for the assembly and production of retroviruses.

Phoenix cells stored at -80°C were thawed in a 37°C water bath, immediately mixed in 25 ml DMEM medium and centrifuged at 250 g for 4 min at 20°C. Cells were resuspended in DMEM media, transferred in surface treated 10 cm dishes (Therma Scientific™ Nunclon™ Delta surface) and cultured in 37°C incubator supplied with 5% CO_2. Depending on the confluency of the cells, they were split every three to four days. For splitting, the medium of the cultures was aspired and the cells were trypsinized by adding 1 ml 0.25% trypsin EDTA (GIBCO) per dish until the cells easily detached. Trypsinization was stopped by adding 9 ml of fresh DMEM to the suspension. To maintain the cell culture, cells were diluted 1:10 with DMEM.

Cell stocks were prepared by resuspending the cells in 50% FCS, 40% DMEM and 10% DMSO (ROTH) and subsequently transferring them into cryo-tubes (Cryoviral). Cells were frozen in isopropanol (Sigma) at -80°C and stored at -80°C.

7.3. Mice

Tec$^{-/-}$ mice were previously described and backcrossed to C57BL/6 for at least 10 generations.[67] The animals were bred and maintained in the animal facility of the Medical University of Vienna according to institutional guidelines. Cells were isolated from mice 6-12 weeks of age.

7.4. Purification of naïve CD4^{+} T cells

7.4.1. Preparation of a single cell suspension from spleen and lymph nodes

Mice were sacrificed by cervical dislocation and disinfected with 70% ethanol to minimize the risk of contamination. 6 well "Nuclon™ Delta Surface" plates (Thermo Scientific) with 70µm cell strainers were prefilled with 4 ml of cold PBS/2% FCS and place on ice. Spleens and lymph nodes were isolated, transferred into the cell strainers and mechanically homogenized using a 5 ml syringe plunger grinding against the strainer. The cell suspension was transferred into a 15 ml falcon tube. Cell strainers were rinsed with 5 ml of PBS/2% FCS to collect all remaining cells. The suspension was centrifuged at 4°C for 5 min at 320 g (standard setting). The resulting cell pellet was resuspended in 1 ml of 1x Pharm lyse buffer and incubated for 1 min at room temperature to deplete erythrocytes, followed by neutralization in PBS/2% FCS. Cells were pelleted at the standard settings and used further for magnetic-activated cells sorting.

7.4.2. Magnetic-activated cell sorting

Pooled cell suspension of lymph nodes and spleen were incubated with biotinylated anti-CD8α, anti-CD11b, anti-CD11c, anti-CD45R, anti-Ly-6G, anti-Ter-119, and anti-NK1.1 Abs in PBS/2% FBS for 15 min on ice. The antibody mix was prepared as shown in Table 1. The suspension was washed with 10 ml PBS/2% FCS, followed by centrifugation and decantation.

Biotin antibody	Target	Clone	Company	final conc/sample
Anti-mouse CD8a	Cytotoxic T cells	53-6.7	Biolegend	2 µg/ml
Anti-mouse CD 11b	Macrophages	M1/70	Biolegend	1.5 µg/ml
Anti-mouse CD 11c	Dendritic cells	N 418	Biolegend	1 µg/ml
Anti-mouse CD45R/B220	B cells	Ra 3-6B2	Biolegend	4 µg/ml
Anti-mouse Ly-6/Ly-6c (Gr1)	Granulocytes	RB6-8C5	Biolegend	2 µg/ml
Anti-mouse NK 1.1	Natural killer cells	PK 136	BD Pharmigen	1 µg/ml
Anti-mouse Ter 119/Erythroid cells	Erythrocytes	Ter 119	BD Pharmigen	1.5 µg/ml

Table 1 **Biotin antibodies for magnetic cell sorting**

Pelleted cells were incubated with 10 µl streptavidin beads (MagniSort SAV Negative Selection Beads, ebioscience)/mouse for 15 min on ice. After incubation, 3 ml of PBS/2% FCS were added and the cell suspension was transferred into a 5 ml polystyrene round bottom tube (Corn-

ing). The polystyrene tube was placed in the magnetic field of a magnetic manual cell separator for 2.5-3 min. The cell suspension, containing now mostly $CD4^+$ cells, was pipetted of into a 14 ml polystyrene round bottom tube (Corning), the bead pellet was resuspended in another 3 ml of PBS/2% FCS and put on the magnet again. The whole procedure was repeated twice. The cell suspension, collected in a 14 ml polystyrene tube was placed on the magnet for further 6 min, to get rid of all beads. The purity of the cells was assessed by flow cytometry and was routinely >96%.

7.4.3. Fluorescence activated cell sorting of naïve $CD4^+$ T cells

$CD4^+$ T cells were further sorted into $CD44^{low}CD62L^+$ (naïve) and $CD44^{high}CD62L^-$ populations on a FACSAria cytometer (BD Biosciences), and the purity of the populations was routinely >99% for naive cells and >97% for $CD44^{high}CD62L^-$ cells.

Antigen	Clone	Fluorochrom	Company	Concentration
B220 (CD45R)	RA36B2	FITC	eBioscience	0.5 mg/ml
CD4	RM4-5	PE-Cyanine 7	eBioscience	0.5 mg/ml
CD8a	53-6.7	APC	eBioscience	0.2 mg/ml
CD44	IM7	V500	BD Horizon	0.2 mg/ml
RORγt	Q31-378	PerCp-Cyanine 5	BD Pharmigen	0.2 mg/ml
IL 17	TC11-18H10.1	APC A	Biolegend	0.2 mg/ml

Table 2 **Surface antibodies for cell sorting**

7.5. Th17 cultures

Cells were cultured in 48 well "Nuclon™ Delta Surface" plates (Thermo Scientific) wells which were coated overnight on 4°C with anti-CD3ε (1 µg/ml, BD Pharmigen) plus anti-CD28 (3 µg/ml, BD Pharmigen) in PBS. Sorted naïve $CD4^+$ T cells were plated with a density of 0.25-0.3 × 10^6 cells/well and cultured in $CD4^+$ T cell medium supplemented with IL-6 (20 ng/ml) and TGFβ1 (2 ng/ml). Cells were cultured for three days in 37°C incubator supplied with 5% CO_2.

7.6. Complementation assay (Day 0 – Day 3)

7.6.1. Transient transfection

Day 0:

One day before the transfection, Phoenix cells were seeded at a density of 1.5 x 10^6 cells/plate in 10 cm cell culture dishes (Therma Scientific™ Nunclon™ Delta surface) and cultured in 37°C incubator supplied with 5% CO_2.

Day 1:

2x HEPES-buffered saline (Hebs) buffer:

10%	HEPES (Roth)
100 U/ml	NaCl
1.48 mM	Na_2HPO_4 (dibasic)
	dH_2O

The solution was adjusted to a pH of exactly 7.00 using NaOH and HCl, sterile filtered and stored at -20°C.

2 M $CaCl_2$ (Sigma)

Sterile filtered and stored at -20°C.

On the day of the transfection Phoenix cells should have reached a confluency of 50-60 %. For the transfection 2 ml of medium from each phoenix dish was collected and supplemented with 9 µl of 50 mM chloroquine (Sigma). For the transfection mix 1200 µl of dH_2O were pipetted into 5 ml round-bottom polystyrene tubes (Corning). To this 18 µg of either the control plasmid MSCV-EGFP or plasmids carrying Tec^a or Tec^a KD together with 7 µg of psi2 helper plasmid (a gift from Taniguchi lab, the University of Tokyo, Tokyo, Japan) was added. The mixture was supplemented with 170 µl of 2 M $CaCl_2$ and 1400 µl of Hebs buffer while bubbling with a Pasteur pipette. 6 min after adding Hebs buffer to the first tube, 2 ml of medium supplemented with chloroquine was added cautiously onto the phoenix dish. 12 min after the addition of Hebs buffer, the transfection mix was slowly dropped onto the phoenix cells, without falling drops or touching the plate. 5 min after the procedure the plates were checked for the formation of calcium precipitates. The cells were incubated for 7 h at 37°C in 5% CO_2 incubator, after which the medium was exchanged with 6 ml of fresh DMEM medium.

Day 2:

The next evening DMEM medium was exchanged with 6 ml RPMI $CD4^+$ T cell medium. The efficiency of the transfection was assessed by flow cytometry and was routinely >45%.

7.6.2. Naïve $CD4^+$ T cell isolation

Day 2:

Naïve $CD44^{low}CD62L^+$ $CD4^+$ T cells were isolated and Th17 cultures prepared as described in 7.4. respectively 7.5. The cells were activated with anti-CD3ε and anti-CD28 for three days with 2 ng/ml TGFß1 and with either 5 ng/ml, 10 ng/ml or 20 ng/ml IL-6. After 24 h the cells were retrovirally transduced with an empty control vector (MSCV-EGFP), Tec^a or Tec^a KD plasmids using spin infection.

7.6.3. Spin infection (retroviral transduction)

Day 3:

Viral supernatant of transfected phoenix cells was harvested with a syringe and filtered through 0.2 µm filter (Thermo Scientific) and supplemented with 5 ng/ml polybrene (Sigma). Supernatant from anti-CD3ε and anti-CD28 stimulated and Th17 polarised T cells was collected in falcons and kept on room temperature. 1 ml of virus infected supernatant was added to the T cells which then were centrifuged for 1 h 30 min at 600 g and 32°C with slow de- and acceleration rates (set to 2). After an incubation of 1 h at 37°C in 5% CO_2 incubator, the supernatant was removed from the T cells and the polarizing T cell medium containing cytokines was added. Cells were incubated at 37°C in 5% incubator for additional two days.

7.6.4. Harvesting and sorting of cells

Carine lysis buffer

20 mM	Tris - HCl (pH 8)
138 mM	NaCl
10 mM	EDTA
100 mM	NaF
1%	Nonident P-40 (Sigma)
10%	glycerol
2 mM	Na_3VO_4

Add phosphatase inhibitors right before usage at an 1:50 ratio.

After three days of incubation, T cells were harvested and either used for FACS analysis, real time PCR or for Western Blot. For the Western Blot they were sorted into GFP^+ and GFP^- population, pelleted and resuspended in 10 µ l of cold carine lysis buffer per 1 x 10^6 cells. Lysates were put on -80°C for at least one night, or stored until further usage.
For the real time PCR cells were pelleted and stored at -80°C.

7.6.5. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

10 % separating gel

2.5 ml	Tris (pH 8.8)
4 ml	dH_2O
3.3 ml	30% acrylamide mix
0.1 ml	10% SDS
0.1 ml	10% APS
0.004 ml	TEMED

5 % stacking gel

0.5 ml	1.5 M Tris (pH 6.8)
1.4 ml	dH_2O
0.33 ml	30% acrylamide mix
0.02 ml	10% SDS
0.02 ml	10% APS
0.002 ml	TEMED

Laemmli sample buffer (4 x BioRad)

277.8 mM	Tris-HCl (pH 6.8)
44.4%	glycerol
4.4%	LDS
0.02%	bromophenol blue

100 µl of 2-mercaptoethanol were added per 900 µl buffer.

1 x Running buffer (10 L)

10 g	SDS
33.3 g	Tris
144.2 g	glycine

Samples from the Complementation assay were centrifuged at 13.000rpm for 30 min. The supernatants were transferred to a fresh 1.5 ml Eppendorf tube and supplemented with 4 x Laemmli buffer (Bio-Rad). The samples were boiled at 95°C for 10 min and loaded on a gel (5% stacking, 10% separating) which was run in 1 x running buffer at 200 V until the marker (PageRuler prestained protein ladder, Thermo Scientific, 10-180 kDa) had separated nicely.

7.6.6. Western Blot

1 x Transfer buffer (10l)

37.88 g	Tris base
180.25g	glycine
	dH_2O

1 x Tris buffered saline (TBST)

55 mM	Tris HCl (pH 8)
100 mM	NaCl

0.1 % Tween 20

dH_2O

After separation, the proteins were electro-blotted on a PVDF membrane (BioRad) by wet transfer for 2 h on constant 200 mA in transfer buffer at 4°C. Subsequently the blot was blocked over night at 4°C, in TBST supplemented with 5% non-fat dried milk (Fixmilk instant). After blocking the membranes were washed in TBST for 30 min at room temperature and incubated in TBST supplemented with 5% non-fat dried milk and primary antibody over night at 4°C. After washing with TBST for 30 min at room temperature the membranes were incubated with 5% non-fat dried milk and secondary antibody for 2h at room temperature. After a final round of washing, the membranes were developed with Amersham ECL Plus Western Blotting Detection System (GE Healthcare) and imaged with Fujifilm LAS-4000.

Name	Target	Host	Company	Dilution
Anti-Tec	Amino acid residues 165-182 of mouse Tec	Rabbit polyclonal IgG	Upstate	1:1000
Anti-β actin	β actin	Mouse	Sigma	1:5000

Table 3 Primary antibodies for Western blotting

Name	Type	Company	Dilution
Peroxidase-conjugated Affinipure goat anti-mouse IgG	Polyclonal	Jackson ImmunoResearch	1:5000
Peroxidase-conjugated Affinipure goat anti-rabbit IgG	Polyclonal	Jackson ImmunoResearch	1:2000

Table 4 Secondary antibodies for Western blotting

7.6.7. Quantitative Real-Time PCR (qRTPCR)

7.6.7.1. RNA isolation and c-DNA synthesis

RNA was isolated using the RNeasy Mini kit from QIAGEN according to the manufacturer´s instructions. RNA was resuspended in a final volume of 60 µl of nuclease-free water. For an isopropanol precipitation the RNA samples were complemented with 1 x volume of RNA grade isopropanol (ROTH) and 1 µl of glycogen (Fermentas) and frozen over night at -80°C. The samples were centrifuged at 13.000 rpm for 30 min at 4°C and washed with 70% ethanol. After a centrifugation step of 10 min at 13.000 rpm at 4°C the supernatant was removed, the pellet air dried and dissolved in 8 µl of nuclease-free water.

For the DNA digestion the samples were incubated with 1 µl of 10 x reaction buffer with $MgCl_2$ (Fermentas) and 1 U of Dnase I, Rnase-free (1 U/µl, Thermo Scientific) at 37°C for 30 min. 1 µl of 50 mM EDTA (Thermo Scientific) were added for an incubation at 65°C for 10 min.
For the cDNA synthesis 1 µl of Primer (Oligo(dT)20 (Invitrogen) and 1 µl of dNTPs (10 mM each, Thermo Scientific) were added to each RNA sample and placed on 65°C for 10 min. To cool the samples down they were placed on ice for at least 1 min and mixed with 10 µl of synthesis mix.

10 µl synthesis mix:

2 µl	10 x reaction buffer (Invitrogen)
4 µl	25 mM $MgCl_2$ (25 mM, Invitrogen)
2 µl	0.1 M DTT (Invitrogen)
1 µl	RNaseOut (40 U/µl, Invitrogen)
1 µl	Superscript III RT (Invitrogen)

Synthesis program:

cDNA synthesis	50°C for 50 min
terminate reaction	65°C for 5 min
remove RNA	addition of 1 µl Rnase H (Invitrogen) 37°C for 20 min

cDNA was used for qRTPCR.

7.6.7.2. qRTPCR

For the qRTPCR cDNA samples were diluted 1:3.

Reaction	1x	
	3 µl	dH_2O
	0.5 µl	forward primer
	0.5 µl	reverse primer
	5 µl	iTaq Universal SYBR Green Supermix (BioRad)
	1 µl	cDNA

Primer	Sequence	Company
IL-17A fw	CTCGAGAAGGCCCTCAGACTA	Invitrogen
IL-17A rev	AGCTTTCCCTCCGCATTGACA	Invitrogen

Table 5 Primers for qRTPCR

7.6.8. Flow cytometry

Intracellular transcription factor and cytokine staining

Cultured cells were harvested, washed with PBS/2% FCS and resuspended in PBS/2% FCS containing Viability dye (1:1000 dilution). They were incubated on ice for 15 min and subsequently washed with 1 ml of PBS/2% FCS. The stainings were performed using the Fixation/Permeabilisation Solution Kit (BD Bioscience) according to the manufacturer's instructions. Cells were fixed with 100 µl of BD Cytofix while vortexing and incubated for 10 min on ice in the dark. For permeabilisation they were washed once with 1 ml of 1 x permeabilisation buffer and incubated with 50 µl 1 x permeabilization buffer for 10 min on ice in the dark. For intracellular transcription factor and cytokine staining 50 µl of permeabilisation buffer containing the appropriate antibodies were added and the cells incubated for 30 min on ice in the dark. Finally, the samples were washed once with 1 x permeabilisation buffer and once with PBS/2% FCS and measured on Fortessa (BD Bioscience).

Antigen	Clone	Fluorochrom	Company	Concentration
B220 (CD45R)	RA36B2	FITC	eBioscience	0.5 mg/ml
CD4	RM4-5	PE-Cyanine 7	eBioscience	0.5 mg/ml
CD8a	53-6.7	APC	eBioscience	0.2 mg/ml
CD44	IM7	V500	BD Horizon	0.2 mg/ml
RORyt	Q31-378	PerCp-Cyanine 5	BD Pharmigen	0.2 mg/ml
IL 17	TC11-18H10.1	APC A	Biolegend	0.2 mg/ml
CD25	PC 61	APC	BD Pharmigen	0.2 mg/ml
CD62 L	MEL14	APC Cy v780	Biolegend	0.2 mg/ml

Table 6 Surface and intracellular antibodies for flow cytometry

8. Bibliography

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9. List of Tables

10. Abbreviation

AP-1	activator protein 1
APS	ammonium persulfate
BATF	basic leucine zipper transcription factor ATF-like
$CaCl_2$	Calcium chloride
CRAC	Ca^{2+} release activated Ca^{2+}
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
eYFP	enhanced yellow fluorescent protein
GFP	green fluorescent protein
Grb2	growth factor receptor bound protein 2
HCl	hydrochloric acid
IL	interleukin
IL-6R	interleukin-6 receptor
IRF-4	interferon-regulatory factor 4
ITAMs	immunoreceptor tyrosine-based activation motives
i.v.	intravenous
LAT	linker for activated T cells
Lck	lymphocyte-specific protein tyrosine kinase
MHC	major histocompatibility complex
MSCV	plasmid murine stem cell virus system
NaCl	sodium chloride
NaOH	sodium hydroxide
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OVA	Ovalbumin
PAMPs	pathogen associated molecular patterns
PBS	phosphate buffered saline
PMA	*phorbol-12-myristate-13-acetate*
ROR-γt	RAR-related orphan receptor gamma
Slp76	SH2 domain-containing leukocyte protein of 76 kDa
SDS	sodium dodecyl sulfate
TCR	T cell receptor
Tec	tyrosine kinase expressed in hepatocellular carcinoma
TF	transcription factor

TFKs	Tec family kinases
TGF1ß	transforming growth factor 1ß
Tris	tris(hydroxymethyl)-aminomethan
Na_3VO_4	sodium vanadate
NaF	sodium fluoride
WT	wild-type
ZAP-70	zeta-chain associated kinase 70