STUDIES ON THE ROLE OF SOCS3 AND STAT3 IN DEVELOPMENT AND FUNCTION OF THE IMMUNE SYSTEM

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Studies on the role of SOCS3 and STAT3 in development and function of the immune system

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ABSTRACT

The immune system is composed of an innate and an adaptive response that protects us from the microbial attacks. The innate system provides a fast defense against infection. However, many microbes evade or overwhelm the innate host defenses and establish the infection in the host. In these cases, the innate immune system will be determinant in triggering the adaptive immune responses. The adaptive immunity consists of antibody-mediated immunity, and T cell-mediated immunity. T cells are the central object of study in this thesis.

The thymus is a specialized primary lymphoid organ for T cell development from hematopoietic stem cells. Histologically, thymus can be divided into two parts, the central medulla and the peripheral cortex. Cells in thymus consist of developing T cells termed thymocytes and stromal cells including cortical epithelial cells (cTEC), medullary epithelial cells (mTEC), as well as dendritic cells (DC). The crosstalk between thymocytes, TECs and DCs that occurs via cell-to-cell contact, cytokines and growth factors is critical for T cell development.

Several cytokines and growth factors activate the JAK/STATs signaling pathway after binding to their specific receptors. The activated STATs transcription factors will later translocate to the nucleus and initiate the transcription of a variety of genes. The “Suppressor of Cytokine Signaling” (SOCS) is a family of proteins including SOCS1-7 and CISH that hamper the activation of different STAT transcription factors. STAT3 has been shown to be critical for the differentiation of many immune cells including those of different T helper subsets. STAT3 is involved in both inflammatory and anti-inflammatory responses. SOCS3 is a negative regulator of STAT3 when activated by some (but not all) cytokines. The role of SOCS3 in T cell development in the thymus is unknown.

The purpose of this thesis is to study the role of SOCS3 in T cell development in thymus. The role of SOCS3, CISH and STAT3 in control of \textit{M.tuberculosis} infection was also addressed.

By using an inducible SOCS3 conditional knockout mouse model, we demonstrated that SOCS3 was required for T cell development. The thymus from mice with SOCS3 deletion was atrophied, with a 90% decrease of thymus cellularity, and a high frequency of apoptosis on double positive (DP) thymocytes and a reduced differentiation of DN thymocytes to single positive (SP) cells. Experiments with bone marrow radiation chimeras indicated that SOCS3 in thymic stromal cells was involved in the T cells development. In addition, hematoxylin and eosin (HE) and immunohistochemistry staining showed that the mice with SOCS3 deletion displayed a thymus with disorganized cortex and medulla. However, SOCS3 did not alter the number of TEC but was necessary for the TEC maturation. A transcriptome analysis of TECs indicated that genes involved in apoptosis and cell survival, negative selection and lympho-stromal interactions were regulated by SOCS3.
Tuberculosis (TB) caused by infection with *Mycobacterium tuberculosis* is one of the top 10 causes of death worldwide. Approximately 90–95% of infected individuals successfully contain *M. tuberculosis* but 5–10% infected individuals will develop active TB. The mechanisms behind reactivation are still not well understood. SOCS3 in myeloid cells was shown to be critical for the control of infection with *Mycobacterium tuberculosis*. Moreover, single nucleotide polymorphisms (SNPs) of cish gene were associated with an increased risk of TB.

Here we found mice deficient in STAT3 in myeloid cells had a decreased susceptibility to *M. tuberculosis*, with lower bacteria load in lungs and spleen, higher level of neutrophils infiltration and less area occupied by granuloma in lung. In STAT3 deficient mice, the levels of il-6, il-23, il-17 and il22 mRNA in lungs were increased during infection. STAT3 deficient bone marrow derived macrophages or dendritic cells expressed high levels of IL-6 and IL-23 at the protein and mRNA after *M. tuberculosis* infection. Furthermore, antigen-specific T cells released higher levels of IL-17 when co-cultured with *M. tuberculosis* infected APCs with STAT3 deficiency, indicating that STAT3 in APCs hampered Th17 differentiation and IL-17 secretion. The increased IL-17 levels resulted in an improved control of infection because neutralization of IL-17 receptor A in STAT3 deficient mice hampered bacterial control, as well as the neutrophil infiltration. Mice deficient in SOCS3 in myeloid cells or DCs were more susceptible to *M. tuberculosis* infection. The secretion of IL-17 by mycobacteria specific T cells was reduced when these cells were stimulated by mycobacteria-infected SOCS3 deficient APCs.

Additionally, we showed that the SOCS family member CISH was induced during *M. tuberculosis* infection. We found that cish<sup>−/−</sup> mice had higher bacteria load in spleens and lungs up to 2.5 weeks after infection but not later compared to controls. This was associated to a decreased expression of transcripts of *inos* and *tnf*, which mediate defense against *M. tuberculosis* early but not late after infection. In addition, rag1<sup>−/−</sup> mice adaptively transferred with cish<sup>−/−</sup> or control T cells displayed similar protection to infection with *M. tuberculosis*.

In conclusion, we demonstrated that SOCS3 expression in non-hematopoietic cells played a critical role for T cell development in the thymus and for maintenance of thymus architecture. On the other hand, STAT3 expression in myeloid cell hampers the control of *M. tuberculosis* infection by hampering Th17 stimulating cytokines secretion and the secretion of IL-17 by CD4+ T cells. In addition, we showed that CISH-mediated the control of *M. tuberculosis* in mice at an early stage of infection by the regulation of the innate immune responses.
LIST OF SCIENTIFIC PAPERS

I. **YU GAO**, Juan Basile, Akihiro Yoshimura, Berit Carow and Martin E. Rottenberg. SOCS3 expression in thymic epithelial cells is indispensable for T cell development. *Manuscript.*

II. **YU GAO**, Juan Basile, Caja Clashon, Dolores Gavier-Widen, Akihiro Yoshimura, Berit Carow and Martin Rottenberg. STAT3 expression by myeloid cells is detrimental for the T-cell-mediated control of infection with *Mycobacterium tuberculosis*. *Manuscript, Submitted.*


V. Vishnu Priya Bollampalli, Lívia Harumi Yamashiro, Xiaogang Feng, Damiën Bierschenk, **YU GAO**, Hans Blom, Birgitta Henriques-Normark, Susanne Nylén, Antonio Gigliotti Rothfuchs. BCG Skin Infection Triggers IL-1R-MyD88-Dependent Migration of EpCAMlow CD11bhigh Skin Dendritic cells to Draining Lymph Node During CD4+ T-Cell Priming. *Journal of PLOS Pathogens | DOI: 10.1371/journal.ppat.1005206*
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<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guérin</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow-derived dendritic cells</td>
</tr>
<tr>
<td>BMM</td>
<td>Bone marrow-derived macrophages</td>
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<tr>
<td>CLPs</td>
<td>Common lymphoid progenitors</td>
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<tr>
<td>CMJ</td>
<td>Cortico-medullary junction</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytidine-phosphatate-guanosin</td>
</tr>
<tr>
<td>cTECs</td>
<td>Cortical epithelial cells</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalitis</td>
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<tr>
<td>ETPs</td>
<td>Early T lineage progenitors</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
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<tr>
<td>KIR</td>
<td>Kinase inhibitory region</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LT</td>
<td>Lymphotoxin</td>
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<tr>
<td>M.tb</td>
<td>Mycobacterium tuberculosis</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MPPs</td>
<td>Multipotent progenitors</td>
</tr>
<tr>
<td>mTECs</td>
<td>Medullary epithelial cells</td>
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<tr>
<td>MyD88</td>
<td>Myeloid differentiation factor 88</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>nTreg</td>
<td>Natural Regulatory T cell</td>
</tr>
<tr>
<td>R</td>
<td>Receptor</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination activating gene</td>
</tr>
<tr>
<td>RNAK</td>
<td>Receptor activator of nuclear factor κ B</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signaling</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TECs</td>
<td>Thymic epithelial cells</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TM</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TSPs</td>
<td>Thymus-settling progenitors</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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1 INTRODUCTION

The immune system is a host defense system composed of a series of biological structures and processes within organism, which can detect many pathogens and harmful substances such as bacteria, virus, fungus and parasite and distinguish them from body's own healthy cells and tissues. The immune system is an interactive network consists with lymphoid tissues, immune cells, cytokines and humoral factors [1].

The immune system is classified into innate immune system and adaptive immune system according to the speed and specificity of the immune responses. The innate immune system including physical (skin, mucosa, epithelium), chemical (antimicrobial peptides, enzymes), and biological barriers, consists of a variety of immune elements such as inflammation, complement system mediated cascade events, neutrophils, monocytes, macrophages, dendritic cells, natural killer cells, γδ T cells, and different kinds of cytokines[2, 3].

Although the innate immune system can effectively prevent the penetration of pathogens into the body, some of them successfully evolved to escape the innate immune responses and thereby trigger the adaptive immune response. This response is composed of T lymphocytes- and B lymphocytes-mediated antigen-specific reactions. The hallmark of adaptive immunity is to provide a stronger immune response and immune memory so that each pathogen can be "remembered" by the immune system through specific epitopes. The specificity of adaptive immunity is the recognition of "non-self" antigens by antigen-specific receptors on T and B cells interacting with major histocompatibility complex (MHC) molecules via a process called antigen presentation[4, 5].

1.1 T cell development in thymus

T cells differentiate from hematopoietic stem cells localized in the bone marrow (BM). Unlike other hematologic cell lineages, T cell development occurs in the thymus, which provides an inductive environment for maturation of T cell progenitors and for commitment to different T cell lineages. The TCR rearrangement, and the positive/negative selections occur in the thymus [6, 7]. The earliest T cell progenitors,
the double negative (DN) cells lack expression of both CD4 and CD8 co-receptors. Depending on the differential expression of CD25, CD44 and CD117 receptors, the DN stage is further subdivided into four stages (DN1 through DN4)[8]. After the DN stage T cells will differentiate into DP (double positive cells) expressing both CD4 and CD8.

1.1.1 DN1 Cells

Thymus-settling progenitors (TSPs) comprise a heterogeneous mixture of multipotent progenitors (MPPs), common lymphoid progenitors (CLPs), CLP-like cells and T lineage-committed progenitors. The process of TSPs passing through postcapillary venules at the corticomedullary junction (CMJ) to enter the thymus is mediated by CCR7/CCR9 binding chemokines. The TSPs differentiate into early T lineage progenitors (ETPs) that migrate to the outer part of the cortex, before they progress through the different DN stages [9-12]. Differentiation to the DN1 stage (CD25−CD44high) occurs around the area of thymic entry. However, DN1 cells still keep the potential to differentiate to non-T lineages such as DCs, macrophages (MØ) and NK cells. Specific signaling pathways thus are required to restrict this potential and guide the T lineage commitment. Several studies have described that Notch signaling is required for the generation and differentiation of ETPs [13-17]. In addition, IL-7R and CD117 (the stem cell factor receptor) are crucial for the proliferative expansion of early pro-T cells and DN1 cells within the thymus [18, 19].

1.1.2 DN2 cells

DN2 thymocytes with CD25+ CD44+ CD117+ phenotype are developed from DN1 cells when they migrate from the CMJ toward the subcapsular area of the cortex, where they interact with cortical thymic epithelial cells (cTECs) and fibroblasts [20, 21]. The hallmarks for DN2 stage are the further restricted T-lineage commitment, and the initiation of gene rearrangement at the TCRγ, TCRδ, and TCRβ gene loci [22]. Based on the CD117 expression level, DN2 cells are divided into two subsets, DN2a (CD25+CD44+CD117int) and DN2b (CD25+CD44+CD117high) [23]. By using the eGFP/lck cre transgenic mouse model, Masuda et al. found that DN2a cells still maintain the potential to differentiate to DCs and NK cells, whereas DN2b subset can only develop to NK cells [24]. Later, a study by Li et al. demonstrated that the expression of zinc finger transcription factor Bcl11b could repress the NK cell differentiation potential on DN2b subset and determine T lineage commitment [25].
Similarly, according to the expression level of IL-7R, DN2 cells have been divided into two subpopulations, IL-7Rα\text{high} and IL-7Rα\text{low}, which are biased for the γδ and αβ T cell lineages, respectively [26]. However, several signaling pathways for T-lineage (γδ/αβ T cell) choice have been investigated, such as extracellular signal regulated kinase (ERK)- early growth response (Egr)- inhibitor of differentiation 3 (Id3) axis, with strong signal guiding the γδ and weak signal guiding the αβ T lineage commitment [27]. Not only T cell lineage commitment, but also the further development of thymocytes from early phases to up to the DN3 stage requires Notch signal. Notch signaling in all the early stages of T cell development damps other lineage potentials, such as B lymphocyte and myeloid cell differentiation[28]. IL-7/IL-7R signaling pathway is required for the survival of DN cells during early T-cell development[29]. IL-7 expressed by TECs binds to IL-7R to activate the Jak1 and JAK3/STAT5 signaling pathway, triggering the downstream gene Bcl-2 expression, which prolongs the survival of TCRβ+ cells (DN2 to DN3a). Moreover, the activation of transcription factor nuclear factor of activated T-cells (NFAT) c1 mediated by IL-7/JAK3/P-Tyr371 shown in DN2 and DN3 cells was reported to be the alternative way to trigger Bcl-2 anti-apoptosis signaling pathway[30].

1.1.3 DN3 cells

Within the subcapsular zone of cortex, DN2 cells differentiate into DN3 that shows CD25+ CD44low expression. The DN3 stage is marked by the rearrangement TCRβ, TCRδ and TCRγ [18]. According to the different events progression on this DN3 stage, DN3 cells are divided into two parts: DN3a and DN3b. DN3a cells are continually progressing on TCR rearrangement and completing the αβ and γδ T cells fate specification. Successful recombination of γδ TCR and αβ T cell fate specification will occur at DN3a stage[31]. On the other hand, an important checkpoint termed β-selection for αβ T cells development is ongoing during this phase. At this stage, DN3 cells that successfully complete the βTCR rearrangement and assemble with an invariant pre-Tα and a CD3 chain to generate the functional pre-TCR will be allowed to progress; otherwise they are eliminated by apoptosis[32, 33]. The assembly of the pre-TCR complex initiates the rapid cell proliferation, as well as the termination of CD25 expression and enter the DN3b stage [18]. A number of signaling pathways involved in DN3 stage have been investigated in many studies. In DN3a stage, IL-7R and PI3K/Akt axis have been demonstrated to be involved in cell survival and
metabolism [34, 35]. Furthermore, a study performed by Trampont et al. showed that CXCR4/CXCL12 could regulate the localization and promote the survival and proliferation of immature thymocytes, and they concluded that CXCR4 was a co-stimulator of the pre-TCR during β-selection [36].

### 1.1.4 DN4 and double positive (DP) cells

After the DN3 stage, thymocytes progress to the DN4 stage with the extinction of CD25 and CD44 expression. The DN4 cells migrate from subcapsular zone of cortex towards the medulla region. Following the pre-TCR formation and β-selection, DN4 cells initiate the expression of CD4 and CD8 and become DP cells. In this stage, thymocytes will re-express the RAG molecules to initiate the TCRα recombination [6, 19]. The immature DP thymocytes expressing T cell receptors that bind self-peptide–MHC complexes, which are presented by cTECs, will be positively selected. Depending on the MHC (I or II) that is presenting, they will commit to either CD4 or CD8 single positive (SP) cells. More than 90% of DP thymocytes die by neglect because of their useless TCRs [37].

### 1.1.5 CD4+ and CD8+ SP cells

SP cells migrate into the medulla region for the negative selection following the positive selection. During this selection, SP thymocytes carrying high-affinity TCRs that recognize tissue-restricted antigens (TRAs) presented by medullary epithelial cells (mTECs) and DCs would be eliminated. These cells otherwise will react with self antigens and generate autoimmune diseases[38]. The details of the positive and negative selection in the thymus are discussed below in the chapter of thymic epithelial cells.

Finally, the up-regulation of sphingosine-1-phosphate receptor 1 (S1P1) on SPs will allow them to egress from the thymus into the circulation [39], as shown in figure 1. The receptor acts as a chemotactic receptor following gradient concentrations of S1P between thymus and the blood[40].
1.1.6 Thymic epithelial cells

The interaction between developing αβ T cells and thymic antigen-presenting cells with self-peptide embedded in MHC molecules is a critical checkpoint for T cell development, during positive and negative selection. Positive selection, mediated by cTECs, provides survival signaling to DP thymocytes and completes the commitment from DP cells to either the CD4+ or the CD8+ T cell lineage. Instead, negative selection mediated by mTECs and DCs results in central tolerance by clonal deletion. It also mediates nTreg cell selection and differentiation [41]. As a consequence of the positive and negative selection, a functional and self-tolerant αβ T cell repertoire is built up. The cTECs and mTECs have been shown to be differentiated from common bipotent thymic epithelial cell progenitors (bTECp) [42-44]. The bTECp are characterized by the expression of EpCAM1, Mts20 and Mts24 in embryonal thymus.
A large number of molecules and transcription factors are required for TECs development and differentiation[47]. Jenkinson et al. showed that fibroblast growth factor (FGF) 7 and 10 expressed by thymus mesenchymal cells are necessary to support the proliferation of bTECp and lead to thymus growth [48]. The transcription factor forkhead box protein N1 (FOXM1) has been described as a critical regulator for TECs development in both the prenatal and the postnatal thymus [49]. FOXM1 regulates the activation of many genes [47]. By using chromatin immunoprecipitation sequencing (ChIP-seq) analysis, Žuklys and co-authors mapped FOXM1 target genes in postnatal TECs and identified a FOXM1 binding motif [50].

1.1.6.1 cTECs development and positive selection

The molecular mechanisms regulating cTECs differentiation are not completely understood. Surface molecules and transcription factors have been reported to identify the cTECs lineages. A murine study from Saba Shakib et al. showed that transcription factor FOXM1 initiated the development of cTECs from bTECp by up-regulating the expression of CD205 as well as the co-stimulatory molecule CD40. Levels of CD40, MHC II, CD205 and the delta like ligand 4 (DLL4), which binds to Notch1, could be used to identify different stages of cTECs development[51-53]. Functional cTECs will present self-antigens to immature DP cells to mediate the positive selection. Antigen processing in cTECs has been discussed in many studies. For MHC class I antigen presentation, two murine studies performed by Murata et al. and Florea et al. showed that cTECs express a chymotrypsin-like activity proteasome subunit termed β5t, which is a key factor required for the generation of the MHC class I peptide repertoire involved in positive T cell selection [54, 55]. In addition, Nitta et al. found that the generation of CD8+ T cells in the absence of β5t is reduced in spleens and lymph nodes obviously, with defective allogeneic and antiviral responses [56].

With regards to the MHC II antigen presentation, it seems to be mostly mediated by an unconventional pathway called macroautophagy, a process in which the cytoplasmic molecules are phagocytized into a double-membrane-limited organelle. The autophagosome fuses with endosomal and lysosomal vesicles resulting in degradation of the engulfed cytoplasmic proteins [57]. Interestingly, Nedjic et al. found that both cTECs and mTECs exhibited constitutive macroautophagy mediating the MHC II-restricted antigen processing and presentation for both positive and negative
Two proteases, cathepsin L1 and thymus-specific serine protease (TSSP) expressed by cTECs, have been shown to be involved in MHC II antigen processing. By using OT-II mice, a transgenic mouse model expressing MHC II-restricted TCR, Gommeaux et al. found that the CD4 T cells positive selection was clearly impaired in the absence of TSSP, whereas CD8 T cells selection was not affected in OT-I mouse model[59, 60].

1.1.6.2 mTECs development, negative selection and nTreg differentiation

Recently positive selected CD4 or CD8 SP thymocytes have to move to the medulla, where they interact with mTECs, which express a wide spectrum of self-peptides derived from TRAs. The mTECs present self antigens to SP thymocytes and signal those CD4 or CD8 SP cells with high-affinity αβTCR binding to self-peptide-MHC complex to die via apoptosis and thereby delete from the T cell repertoire and create the central tolerance, which is termed negative selection[61]. Most of the TRAs expression is regulated by AIRE, a critical transcriptional regulator in terminal differentiated mTECs. AIRE dependent or independent TRAs expression is highly based on epigenetic mechanisms (histone modification and CpG methylation), which is well reviewed by Abramson[62].

The factors and molecular mechanisms involved in mTECs development have been extensively studied. Thymocyte-TEC interactions by RANK/RANKL, CD40/CD40L, lymphotoxin (LT) α/β resulting NFκB activation are critical for mTECs differentiation[63]. RANK and CD40 are critical for the AIRE+ and UEA+ mTECs differentiation [64]. A study by Akiyama et al. showed that the number of UEA+ and Aire+ mTECs were severely reduced in RANK deficient embryonic thymus. They could also see that RANK and CD40 were involved in the development and maintenance of mTECs in the postnatal thymus [65, 66].

The role of the LT signaling pathway in mTECs development has been investigated in many studies by using LT receptor gene knockout mouse model. A study from Chin and colleagues claimed that LT signaling pathway is necessary for the expression of AIRE and its downstream target genes. They found that mice deficient in LTα or LTβR expressed lower level of AIRE and of several self-antigens in mTECs and consequently resulted in an impaired central tolerance[67]. Furthermore, by using the LTβ or LTβR deficient mouse model, Boehm et al. reported an impaired thymocytes-mTECs
crosstalk in the absence of LTβ/LTβR/NFκB-inducing kinase (NIK) axis leading to a reduced number of mTECs and a disorganized medulla area, accompanied with the signs of autoimmunity[68]. In addition, LTβ/LTβR signaling is necessary for the expression of chemokines CCL21 and CCL19, which mediate CCR7+ SP cells migration toward the medulla for negative selection [69].

Unlike antigen presentation by cTECs, TRAs expressed by mTECs can be presented to T cells either by mTECs themselves or by neighboring DCs. During MHC I antigen processing, peptides are generated by two proteasome subunits β5 and β5i expressed in mTECs[37]. For MHC II-restricted self-antigens processing, similar to cTECs, mTECs generate self-antigens via macroautophagy and present them to CD4+ T cells [58]. In addition, antigen transfer between mTECs and DCs represents an additional pathway for negative selection. Unlike the cortex, the thymic medulla is packed with bone marrow (BM)–derived APCs[70]. Taniguchi et al. found that mice with MHC II deficient bone marrow derived cells displayed a higher frequency of auto-reactive CD4+ T cells [71].

1.1.6.3 nTregs and mTECs

Lei and co-authors showed that mTECs could express the chemokine XCL1 to recruit XCR1 expressing CD11c+ thymic DCs. By using the xcl1 deficient mice, they found that CD11c+ thymic DCs recruited by AIRE+ mTECs played a minor role in negative selection but were required for nTreg differentiation, which is a critical subpopulation preventing autoimmune diseases [72]. nTregs are generated at the same time as conventional αβ T cells during the negative selection. Unlike conventional CD4+ T cells, nTreg cells have a TCR repertoire that is primarily auto-reactive [73]. CD25-Foxp3+ CD4 SP cells or CD25+Foxp3- CD4 SP cells have been described as the precursors of nTregs. Additionally, IL-2 and IL-15 are necessary for the nTregs development in the thymus[74, 75].

A study by Coquet and co-authors found that mTECs and CD8α+ conventional DCs in the thymic medulla performed CD27–CD70 co-stimulation to rescue developing Treg cells from apoptosis, subsequently inducing Foxp3 expression by TCR and CD28 signals[76]. In addition, Mahmud et al. reported that UEA+ mTECs with constitutive expression of GITRL, OX40L and TNF could promote the differentiation of Tregs from
Tregs progenitors that express tumor-necrosis factor (TNF) receptor superfamily (TNFRSF) including GITR, OX40 and TNFR2[77].

1.2 Basics of STAT and SOCS

Cytokines are a category of small proteins or peptides that are critical for cell signaling. Cytokines are mostly water-soluble proteins or glycoproteins with small molecular weight around 5 to 30 kDa[78].

According to the structural homology, cytokines can be divided into four types: A family of cell molecules with four α-helical bundles. The family is divided into three subfamilies including Interleukin-2 (IL-2) subfamily, Interferon subfamily and IL-10 subfamily; IL-1 cell molecular family mainly including IL-1 and IL-18; IL-17 cell molecular family and chemokines[79, 80]. Based on clinical and experimental functions, some of the cytokines are divided into: Type I helper T cell (Th1) cytokines, such as IFN-γ and TNF-α[81]; Type II helper T cell (Th2) cytokines such as IL-4, IL-13, IL-10, and transforming growth factor beta (TGF-β) [82]. These two types of cytokines tend to inhibit each other.

Many different kinds of cell types, including immune cells like B lymphocytes, T lymphocytes, macrophages, dendritic cells, neutrophils, natural killer cells and mast cells, endothelial cells, epithelial cells and fibroblasts could produce cytokines. Cytokines therefore are involved in immune responses against inflammation, infectious and auto-immune diseases, as well as embryonic development[83].

Depending on their two features including the widespread distribution of cellular sources and redundancy, cytokines can be differed from another critical cell signaling molecules, hormones. Hormones circulate in less variable concentration and tend to be released by specific kinds of cells[84].

The mode of cytokine action can be summarized as follows: autocrine, which means that cytokines act on the cells that release it; paracrine, which means that cytokines act on the cells around them and endocrine, which means cytokines spread to distant areas through circulation to influence different tissues[85].

To initiate the biological functions, cytokines bind to specific receptors on the cell surface that mediate their signal transduction. Janus kinases–signal transducers and
activators of transcription (JAK–STAT) signaling pathway is the widely used a large number of cytokines as well as growth factors and hormones.

1.2.1 STATs

The JAK-STAT signaling pathway is involved in signal transduction of many cytokines, growth factors, and hormones during many cellular processes [86]. The family of STAT proteins with seven components comprises a unique class of transcription factors with a length between 750 and 850 amino acids that are characterized by the presence of four functional parts as follows (Figure 2): 1, the Src homology 2 (SH2) domain, which specifically recognizes the phosphorylated state of tyrosine residues at the cytokine receptors; 2, a C-terminal transactivation site, which includes tyrosine phosphorylation sites; 3, the amino acids between residues 400 and 500 of STAT proteins that constitute the DNA-binding site; 4, a coiled structure that presents a predominantly hydrophilic surface area for interaction with other proteins [87].

![Figure 2: Structure and function domains for STATs](image)

**Figure 2:** Structure and function domains for STATs
Different cytokines that mediate their signaling via JAK/STATs signaling pathway are shown in **Table 1**. Interestingly, the use of the same type of STAT may render different biological consequences.

<table>
<thead>
<tr>
<th>STATs</th>
<th>Activated by</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAT1</td>
<td>IFN-α, β, γ</td>
<td>[88, 89]</td>
</tr>
<tr>
<td>STAT2</td>
<td>IFN-α, β</td>
<td>[90, 91]</td>
</tr>
<tr>
<td>STAT3</td>
<td>IL-6 family (IL-6, IL-11, IL-31, LIF, CNTF, CLC/CLF, NP, CT1, OSM), G-CSF, IL-10 family</td>
<td>[92, 93]</td>
</tr>
<tr>
<td>STAT4</td>
<td>IL-12, 23</td>
<td>[92, 94]</td>
</tr>
<tr>
<td>STAT5A</td>
<td>IL-2 family (IL-2, IL-4, IL-7, IL-9 and IL-15)</td>
<td>[95]</td>
</tr>
<tr>
<td>STAT5B</td>
<td>IL-2 family (IL-2, IL-4, IL-7, IL-9 and IL-15)</td>
<td>[95]</td>
</tr>
<tr>
<td>STAT6</td>
<td>IL-4 and IL-13</td>
<td>[96]</td>
</tr>
</tbody>
</table>

**Table 1**: Factors for STATs activation

The JAK-STAT system consists of three main components: **1.** the cytokine receptor, **2.** JAK kinases TYK2, JAK1, JAK2, JAK3 and **3.** the STAT proteins [97]. Cytokines binding to their respective receptors induce a receptor aggregation. JAKs constitutively bind specific chains of these receptors and the aggregation results in JAK juxtaposition and their activation via cross-phosphorylation [98]. JAKs also phosphorylate the STAT proteins. STATs bind to the tyrosine-phosphorylated cytokine receptors through their SH2 domains and become phosphorylated on their C-terminal tyrosine residues (p-Tyr or p-Ser) by the JAKs. Then phosphorylated STATs will dissociate from the receptor and mediate their dimerization. Different STATs form hetero- or homodimers. STAT dimers enter the nucleus, where they bind to specific DNA sequences in the promoter region of their target genes, and thus regulate their transcription [86]. The excessive stimulation mediated by cytokine-induced JAK/STATs signaling has been linked to several immune diseases [99]. For example, multiple sclerosis is due to the dysregulation of STAT1, STAT3 and STAT4 activation, which mediates the focal inflammatory infiltration into the central nervous system, demyelinating lesions, and pro-inflammatory cytokines production that activate immune cells and damage neurons and oligodendrocytes [100]. Thus, a tight control is
necessary to avoid the detrimental consequences of a pathogenic over stimulation. It has become clear that phosphotyrosine phosphatases (PTPs), protein inhibitors of activated STAT (PIAS), and suppressor of cytokine signaling (SOCS) proteins are the three classic inhibitory molecular families that contribute to the negative regulation of JAK/STAT-mediated cytokine signaling [101].

1.2.2 SOCS

SOCS proteins are major regulators of JAK-STAT signaling but they control only some of the cytokines that activate JAK/STAT. SOCS proteins have been shown to widely regulate over 30 cytokines, including Leukemia Inhibitory Factor (LIF), Granulocyte Colony Stimulating Factor (G-CSF), Interferon-γ (IFN-γ), Interleukin-6 (IL-6) and IL-10 [102]. The SOCS protein family consists of eight proteins, including SOCS1-7 and CIS [103]. All SOCS bind to different cytokine receptors. All eight proteins share a similar architecture that includes two functional domains: 1, a central SH2 domain, which binds to defined tyrosine-phosphorylated substrates on the cytokine receptor; and 2, a SOCS-box domain at their C-terminus, which binds elongins B and C and cullin 5, thereby leading to the ubiquitination and degradation of its bound receptor [104-106], as shown in Figure 3. In addition, the two most well known members of the SOCS family, SOCS1 and SOCS3, act through an additional mechanism: they use a short motif, named the kinase-inhibitory region (KIR) absent in the other members, to inhibit signaling by directly inhibiting the catalytic activity of JAKs [107, 108]. The suppressor of cytokine signaling-3 (SOCS3) protein is well known as a feedback inhibitor of the JAK/STAT3 pathway through binding to the shared interleukin-6 (IL-6) receptor subunit gp130 inhibiting STAT3 phosphorylation [109].
Figure 3: Names and structures of SOCS family

1.3 STAT3 and SOCS3

STAT3 is a critical transcription factor that transmits signals to the nucleus after the activation by cytokine, growth factors and other stimuli [110]. STAT3 consists of six components: a C-terminal domain for transactivation, an SH2 domain for dimerization, a coiled-coil domain for protein-protein interactions, a linker region, a DNA-binding domain, and N-terminal domain (NTD)[111]. Based on the components, the signaling pathways for STAT3 activation have been investigated in many studies. STAT3 could be activated by tyrosine phosphorylation (P-Tyr705) via Janus kinases (JAKs), which leads to the P-STAT3 dimer formation through reciprocal phosphotyrosine-SH2 domain interactions. The dimer will translocate to nucleus and bind to specific DNA elements, consequently inducing gene expression. Meanwhile, serine phosphorylation (P-Ser727) at a site within the transactivation domain is another way for STAT3 activation, which is mediated by several serine kinases such as ERK1, ERK2, p38 and JNK[112, 113]. A plenty of studies have reported that STAT3 within different tissues and cell types is involved in a broad variety of biological events, including acute-phase response induced by IL-6 in the liver, embryogenesis, cell growth and differentiation, pro-inflammatory and anti-inflammatory responses,
hematopoiesis, and tumor survival[110]. Excessive JAKs-STATs activation could mediate a detrimental effect on the organism, so that tight regulation by inhibitory molecules is necessary for the balance. JAKs-STATs pathways are controlled through several different mechanisms, including phosphotyrosine phosphatases (PTPs), protein inhibitor of activated STAT (PIAS) and SOCS proteins[114].

One function of SOCS3 is to inhibit signaling by IL-6 cytokine family by preventing JAK2-mediated activation of STAT3, which is involved in many immune diseases and cancer [115]. Structure-function studies have revealed the mechanism by which SOCS3 inhibits IL-6/JAK2/STAT3 signaling. Nadia et al. showed the crystal structure of a ternary complex between mouse SOCS3, JAK2 and a fragment of the IL-6 receptor β-chain demonstrating that SOCS3 can bind to JAK2 and the receptor at the same time with two opposing regions (SH2 domain and kinase-inhibitory region). SOCS3 inhibits the catalytic activity of JAK2 in two ways: 1. phosphotyrosine-binding groove on the SOCS3 SH2 domain occupies the receptor, then JAK2 can only bind in a phospho-independent manner to a non-canonical surface; and 2. KIR of SOCS3 occludes the substrate-binding groove on JAK2 and thereby blocks substrate association [116, 117], as shown in Figure 4.

Figure 4: SOCS3 binding sites on Jak and receptor

1.3.1 STAT3 and SOCS3 in myeloid cells

APCs have been viewed as a critical cell population in immune system, because these cells recognize and capture antigens, release a variety of cytokines, migrate to the
lymphoid organs and present to T cells in a way that triggers the immune response. In myeloid cells, such as macrophages and dendritic cells, STAT3 activation triggered by IL-6 and IL-10, can induce the pro-inflammatory and anti-inflammatory responses, respectively. The role of STAT3 in infection and immune diseases has been investigated by using stat3 gene conditional knockdown mouse model.

Inflammatory bowel disease (IBD) is characterized by a chronic inflammation of the gastrointestinal tract and is subdivided into two major phenotypes: Crohn’s disease (CD) and ulcerative colitis (UC). IBD is defined as the class of autoimmune diseases, in which the immune system attacks elements of the digestive system [118]. Intestinal macrophages and DCs form a unique group of tissue immune cells that are ideally situated at the interface of the host and the enteric luminal environment to appropriately respond to microbes and other potential stimuli [119]. Mice with a conditional knockdown for STAT3 in myeloid cells (Stat3\textsuperscript{lox/lox} LysMcre) displayed enhanced Th1 responses and developed chronic enterocolitis reminiscent of human IBD, with increased levels of inflammatory cytokines (TNF-α, IL-6, IL-12 and IFN-γ) from macrophages and dendritic cells. In vitro experiment showed that the suppressive effects of IL-10 on inflammatory cytokine production were completely abolished in bone marrow derived macrophages or peritoneal macrophages from Stat3 mutant mice [120, 121]. However, development of colitis as well as STAT3 activation was significantly reduced in IL-6–deficient mice treated with dextran sulfate sodium (DSS), suggesting that STAT3 plays an important role in the perpetuation of colitis[122].

Both IL-6 and IL-10 mediate pro-inflammatory or anti-inflammatory responses of myeloid cells respectively by signaling via the JAK2/STAT3 pathway [123, 124]. It has been shown that the modulation by SOCS3 of the IL-6 but not the IL-10 signaling pathway (SOCS3 cannot bind to the IL-10 receptor) is responsible for the contrasting immune responses although both cytokines utilize the same pathway [125]. Accordingly, IL-6 responses have been shown to resemble IL-10 responses in absence of SOCS3 [126]. SOCS3 is strongly induced by LPS among other innate receptor ligands, IL-6 and IL-10 in macrophages [127-129]. Mice that are deficient for SOCS3 in their myeloid cells (LysMCre-Socs3\textsuperscript{-/-} mice) showed higher plasma levels of pro-inflammatory M1 cytokines such as IL-1β, TNF-α and IL-6 that correlated with the prolonged STAT3 activation during a LPS-mediated sepsis model [130]. However,
mice with a conditional knockdown for SOCS3 or a mutation of the SOCS3 binding site in macrophages are resistant to endotoxemia due to a reduced production of inflammatory cytokines, which indicates that SOCS3 specifically blocks IL-6-induced anti-inflammatory responses [126]. In addition, Carow et al. reported that SOCS3 expression in *Mycobacterium tuberculosis*-infected macrophages and dendritic cells (DCs) prevented the IL-6/STAT3-mediated inhibition of TNF and IL-12 secretion and contributed to a timely antigen-specific T cell response *in vivo*, which is critical for tuberculosis control [131]. SOCS3 has been linked to inflammation and autoimmune diseases, such as rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis (MS) [132]. Experimental allergic encephalomyelitis (EAE) is an animal model of brain inflammation of human nervous system demyelinating diseases, including MS, which is an inflammatory demyelinating disease of the central nervous system (CNS). It is mostly used with rodents and is widely studied as an animal model of the human CNS demyelinating diseases[27]. By using a myelin oligodendrocyte glycoprotein-induced mouse model of MS, experimental autoimmune encephalomyelitis (EAE), Qin et al. demonstrated that mice with a SOCS3 deletion in macrophages exhibited enhanced STAT3 signaling, which led to the classical M1 macrophage polarization and provided the microenvironment to up-regulate Th1 and Th17 cell differentiation and induced neuronal death[133]. The severity of EAE can be decreased by intravenous injection of M2 macrophages or SOCS3-overexpressing DCs that induce a Th2 cell polarization suppressing Th1 and Th17 cell differentiation[133]. In the contrary, mice transferred with SOCS3-deficient DCs developed a less severe EAE, suggesting that SOCS3-deficient DCs are immunosuppressive or less able to stimulate autoimmune responses in vivo [134].

1.3.2 STAT3 and SOCS3 in T cells

STAT proteins and SOCS family members have been described as the necessary regulators for T helper cell differentiation[135], as shown in **Figure 5**.
For example, STAT3 activation in response to IL-6 in combination with transforming growth factor-β (TGF-β) and IL-23 leads to up-regulated expression of orphan nuclear receptors RORγt, which is the signature transcription factor for Th17 cells[136]. The hyper IgE syndrome (HIES) is a primary immune deficiency characterized by elevated serum IgE, rash and recurrent staphylococcal infections of the skin and lung. Autosomal dominant HIES results from STAT3 hypomorphic mutations and has a variety of connective tissue and skeletal abnormalities[137]. In some cases, hematopoietic cell transplantation can potentially reverse the severity of HIES [138]. Scott et al. constructed a mouse expressing a STAT3 mutations observed in HIES. In this model, bone marrow transplantation partly reduced the heightened inflammatory responses with high levels of IL-6, TNF-α, IL-12 and IFN-γ due to a non-efficient STAT3/IL-10 anti-inflammatory pathway[139].

At the same time, STAT3 has a critical pro-inflammatory role, since it is required for Th17 differentiation as well as IL-17 and IL-22 secretion, which ensures the antimicrobial peptide regenerating islet-derived protein 3 γ (Red3 γ) production and would control the recurrent *Citrobacter rodentium* infections seen in HIES [139].

**Figure 5:** STATs and SOCS proteins in T helper cell differentiation
Specifically, Th17 cells secrete different cytokines (IL-17A, IL-17F, IL-21, and IL-22) and their differentiation requires the activation of several transcription factors including STAT3 and RAR-related orphan receptor-γt (RORγt). The former is induced by IL-6, IL-23 and the latter by transforming growth factor-β (TGF-β). In HIES Th17 are thereby deficient and susceptible to infections which can be also observed upon mutations of IL-17 or rorgT in HIES[140].

Follicular T helper (Tfh) cells are a subset of CD4 T cells required for the T-dependent germinal center (GC) formation, which leads to the production of antigen-specific memory B and plasma cells [141]. IL-6, IL-21 and IL-27 signal via STAT3 and are required for the Tfh cell differentiation and GC formation during viral infections [142-145]. Human mutations in STAT3 as found in patients with HIES led to an impaired Tfh cell differentiation that could contribute to their impaired control of a variety of infections, including reactivation of latent viruses [145-147]. McIlwain et al. showed that STAT3 is involved in the maintenance of generation of T-follicular helper (Tfh) cells, germinal center reactions, and specific anti-virus IgG-secretion during LCMV infection [148].

Intestinal inflammation caused by pathogenic bacteria is a frequent and potentially life-threatening disease [149]. A meta-analysis of Crohn’s disease and ulcerative colitis genome-wide association scans have connected the transcription factor STAT3, which is widely expressed by different tissues and cell types, to intestinal pathology [150]. Backert et al. found that STAT3 in T cells mediated a protective role on intestinal epithelial cells (IECs) during infectious colitis. STAT3 promotes the Th17, Th22 cell differentiation and IL-17, IL-22 secretion, which stimulates IECs and promotes protection from enteropathogenic bacteria through maintenance of high induction antimicrobial peptides [151].

The role of STATs and SOCS proteins in T helper cell differentiation has been reviewed previously [135]. SOCS3 has been shown to negatively regulate IL-23-mediated STAT3 phosphorylation and Th17 polarization [152]. Recently, Th17 cells have been identified as one of the major pathogenic T cell subpopulations underlying the development of several autoimmune diseases [153]. STAT3 binds to both the IL-17A and IL-17F promoter [154]. By using overexpression models, SOCS3 has been implicated as a regulator of Th1/2 polarization via its ability to inhibit IL-12-induced
STAT4 activation [155, 156]. However, Chen et al. found that a deficiency for SOCS3 had little effect on Th1/2 polarization. Instead, SOCS3 negatively regulated Th17 cells differentiation through the down-regulation of IL-23-induced STAT3 activation[154].

Loss of SOCS3 in T cells during atherosclerosis increased both IL-17 and IL-10 production, and M2 macrophage polarization, and led to an unexpected IL-17-dependent reduction in lesion development and vascular inflammation. This identified a novel SOCS3-controlled IL-17 regulatory pathway in atherosclerosis and may have important implications for the understanding of the increased susceptibility to vascular inflammation in patients with dominant-negative STAT3 mutations and defective Th17 cell differentiation [157]. In contrast, in a model of experimental autoimmune uveitis (EAU), double-producing IL-17/IFN-γ CD4 T cells were markedly reduced in mice deficient for SOCS3 in T cells. This suggests that SOCS3 promotes the expansion of the IL-17/IFN-γ T cell subset associated with development of severe uveitis. Thus, SOCS3 is a potential therapeutic target in uveitis and other autoimmune diseases [158].

1.3.3 STAT3/SOCS3 and tuberculosis

IL-17 has been shown to be involved in the control of tuberculosis [159]. The frequency of Th17 cells in blood and/or pleural effusion samples of patients with active TB has been demonstrated to be lower than that in healthy donors or in individuals with latent TB infection [160]. IL-17 was involved in early Th1 cell recruitment, neutrophil infiltration and granuloma formation in tuberculosis patients [161-163]. SOCS3 is thought to inhibit STAT3-induced Th17 differentiation. Interestingly, SOCS3 expression in CD4 T cells can promote human CD161high Th17 cell polarization and IL-17 secretion during M. tuberculosis infection, which can be reversed by IL-7 treatment due to the down-regulation of SOCS3 expression [164]. On the other hand, in tuberculosis patients, PD-1 and STAT3 phosphorylation but not SOCS3 in CD4 T cells control the IL-23R expression and IL-17 secretion. PD-1 up-regulation induced by M. tuberculosis can dampen STAT3 phosphorylation in T cells, thereby inhibiting IL-23R expression and Th17 cell differentiation [165].
1.3.4 STAT3 in thymic epithelial cells

T cell development in thymus depends on the crosstalk between thymocytes and stromal cells including epithelial cells, macrophages and dendritic cells [166]. Thymic epithelial cells (TECs) have been demonstrated to be organized in a three dimensional architecture, the cortex and the medulla, where T cell precursors mature and differentiate[167].

Cortical epithelial cells (cTEC) regulate the early and intermediate stages for T cell development, including commitment of progenitors to the T cell lineage, the proliferation and the positive selection of developing thymocytes, selecting only those that recognize peptides on own MHC [21]. Medullary epithelial cells (mTEC) present tissue-restricted self-antigens (TRA) to delete the self-reactive T cells and preventing autoimmunity, which is called negative selection [37, 168]. Previous studies have demonstrated that the developing thymocytes and TECs interact in different manners. Thymocytes can express many surface or secreted molecules (CD40L, RANKL, LTα and LTβ), which critically control the growth and maturation of TECs[65, 66, 169-171].

Keratin 5 is expressed in mTECs. Using K5-Cre mice to delete Stat3, Satoh et al. generated the mouse model with Foxn1-Cre-driven inactivation of the Stat3 locus in TEC and showed that the thymus from juvenile and adult mice showed a normal size with a reduced medulla but a normal cortex[172]. More notably, Lomada et al. provided a transgenic mouse model with constitutively active Stat3 transgene (K5.Stat3C) highly expressed in the thymic medulla, which resulted in a selectively expansion of MHCIIloCD80lo immature mTECs. In summary, the phenotypes from previous studies generated by enforcing or preventing Stat3-mediated signaling in TECs reveal that Stat3 activation may play a key role in TECs maintenance and T cell development in thymus, but still requiring more precise studies [173].

1.4 CISH

Cytokine-inducible SRC homology 2 (SH2) domain protein (CISH) was the first member of the SOCS family to be characterized and has been shown to regulate the responses of immune cells to different cytokines such as IL-2, IL-3, IL-4, IL-15, GM-CSF and EPO[174-177]. Compared with SOCS1 and SOCS3, CISH does not have a kinase-inhibitory region; thereby it cannot inhibit the tyrosine-kinase activity of JAK proteins
directly. Instead, CISH binds to phosphorylated cytokine receptors and induces the ubiquitin degradation of the receptor. It has been shown that CISH can bind to the phosphorylated second tyrosine residue (Tyr401) of the EPO receptor, which is one of the two major STAT5 activation sites. More notably, Cis is an ubiquitinated protein, which can induce ubiquitin proteasome-dependent degradation of the activated EPO receptors [108].

CISH expression in CD4+ T cells has also been shown to be a negative modulator of STAT5 activation induced by IL-2[175, 178]. Further CISH is induced in T cells by IL-4 and inhibits the phosphorylation of STAT3, STAT5 and STAT6. Cish-deficient T cells showed enhanced differentiation into the TH2 and TH9 subsets of helper T cells[179] and ageing CISH−/− mice showed allergic pulmonary inflammation.

Recently, variant alleles of multiple CISH polymorphisms were found to be associated with susceptibility of each infectious disease. Associations of these CISH polymorphisms with susceptibility to infection with Hepatitis B have also been reported[180]. Clinic case control study performed by Khor et al. identified a panel of five CISH SNPs (at positions −639, −292, −163, +1320, and +3415) associated with increased susceptibility to bacteremia, tuberculosis, and malaria in Gambia, Hong Kong, Kenya, Malawi, and Vietnam[181]. Another two studies performed by Ji et al. and Zhao et al. showed that two of the five genetic variants (positions at -292 and +1320) in CISH gene appeared to increase susceptibility to TB in Chinese Han population[182, 183]. More notably, SNP-292 has been correlated to susceptibility in Chinese children[184]. The fact that CISH polymorphisms are associated with susceptibility to different infectious diseases suggests that CIS has a critical role in immune responses against various pathogens.
1.5 Mycobacterium tuberculosis

1.5.1 Epidemiology

*Mycobacterium tuberculosis* is a pathogenic bacterial species of the family Mycobacteriaceae and the causative agent of Tuberculosis (TB), which has existed for several millennia and still states a major global health problem nowadays[185]. About one-third of the world’s population has latent TB, in other words they have been infected by *M. tuberculosis* but have not developed disease and cannot transmit the infection. Latent TN asymptomatic individuals have a 10% lifetime risk of developing TB[186]. However, individuals with a compromised immune system, such as people living with HIV, malnutrition or diabetes, or smokers, have a much higher risk of developing active TB [187]. HIV and TB form a lethal combination. Report from WHO for TB case in 2015 showed that there were 10.4 million new (incidence) active TB cases worldwide, of which 1.8 million died from the disease, including 0.4 million among people with HIV[188].

1.5.2 Treatment

A combined and lengthy antibiotic treatment (4 months Isoniazid plus rifampicin plus pyrazinamide plus ethambutol followed by 2 months Isoniazid plus rifampicin) is mandatory in cases of active TB. Since the anti-TB medicines have been used for many years, *M. tuberculosis* strains that are resistant to one or more of the drugs have developed. Multidrug-resistant *M. tuberculosis* (MDR-TB) does not respond to both isoniazid and rifampicin, the two most powerful, first-line anti-TB drugs[189]. In some cases, more severe drug resistance can develop. Extensively drug-resistant TB (XDR-TB) is a more serious form of MDR-TB caused by bacteria that do not respond to the most effective second-line anti-TB drugs (fluoroquinolones (FQs) and second-line injectable drugs including amikacin, kanamycin, and capreomycin), often leaving patients without further treatment options[190].
1.5.3 Vaccine

The only existing vaccine to prevent tuberculosis and other mycobacterial infections is Bacille Calmette-Guérin (BCG), a live strain of mycobacterium bovis developed by Calmette and Guerin. Although BCG effectively protects against disseminated tuberculosis in young children, it only shows variable protection against pulmonary tuberculosis probably due to the presence of environmental mycobacterium, differences in the strains of BCG used, presence of helminths, UV light or the host genetics [191]. BCG does not prevent the infection with *M. tuberculosis* but depending on the population study will reduce the development of active TB. Therefore, there is an urgent need for new, safe and effective vaccines that prevent against all forms of TB.

1.5.4 Microbiology

First discovered in 1882 by Robert Koch, *Mycobacterium tuberculosis* is a non-motile rod-shaped bacterium. The rods are 2-4 micrometers in length and 0.2-0.5 um in width. It has a tough cell wall that prevents passage of nutrients into and excretion from the cell, therefore giving it the characteristic of slow growth rate, with doubling times on the order of 18 to 24 hours[192]. This is extremely slow compared to other bacteria, which can divide in a matter of minutes. *M. tuberculosis* requires oxygen to grow, does not produce spores, and is non-motile. The bacillus can withstand weak disinfectants and can survive in a dry state for weeks. Man is the only reservoir for *M. tuberculosis*. The cell wall structure of *M. tuberculosis* is a major determinant of virulence for the bacterium and probably account for resistance to desiccation. It contains peptidoglycan and lipids, which consist of three major components including mycolic acids, cord factor, and wax-D[193]. *M. tuberculosis* cannot be classified as either gram-positive or gram-negative because it is not stained by Gram and lacks LPS. Due to the unique cell wall structure, *M. tuberculosis* is identified by using one of the acid-fast stain methods, which is called Ziehl-Neelsen stain[194]. More recently, other species of non-tuberculous *mycobacteria* (NTM) causing clinical disease have been classified. The most common organism associated with pulmonary disease is the *Mycobacterium avium* complex (MAC), a slow growing NTM that consists of many subspecies including *silvaticum, avium, paratuberculosis,* and *hominissuis*. *M.kasassii*, another slow growing species, is the second most common cause of pulmonary. *M.*
abscessus, a rapidly growing NTM, is the third most common cause of lung disease. Thus, it is important to determine the pathogenic significance of the NTMs by studying the patient's clinical presentation[195].

1.5.5 Transmission

*M. tuberculosis* is carried in small airborne droplets, called droplet nuclei. Infectious droplet nuclei are generated when persons, who have pulmonary or laryngeal TB disease, cough, sneeze, talk or sing. Depending on the environment, these particles can remain suspended in the air for minutes to hours after expectoration[196]. Each one of these droplets may transmit the disease, since the infectious dose of tuberculosis is very small. *M. tuberculosis* is only transmitted through the air, not by surface contact, which means the infection cannot be acquired by shaking hands, making contact with toilet seats, sharing food or drink, sharing toothbrushes, or kissing. *M. tuberculosis* transmission occurs when a person inhales droplet nuclei containing bacteria. These droplet nuclei pass through the upper respiratory tract, and bronchi to reach the alveoli of the lungs[197].

1.5.6 Experimental models

*Mycobacterium tuberculosis* lacks natural hosts except humans, so surrogate models, which can mimic the development of the disease, are necessary to study the pathophysiology or design tools for treatment or prevention of the disease. There is a wide range of animal models of tuberculosis including mice, guinea pigs, rabbits, zebrafishes, minipigs and monkeys[198].

Mice are widely used as the animal model for tuberculosis due to many advantages including low cost, easy handling and gene modification, more availability for immunological tools and reagents[199]. Resistant strain C57BL/6 infected with *M. tuberculosis* via aerosol route can develop a steady state of infection and maintain for more than one year with stable bacteria load in lungs due to cell-mediated immune responses, which provides a perfect model to study the role of immune cells against infection through many well-established methods including gene manipulation and immunological reagent treatment[200]. However, the two most common used inbred laboratory strains (C57BL/6 and BALB/c) with *M. tuberculosis* infection mouse cannot develop well-structured granulomas and caseous necrosis and characteristic lesions.
in human pulmonary tuberculosis [201]. Models including C3HeB/FeJ and CBA/J mice, which can form granulomas with caseating necrosis, have been developed [202, 203].

Unlike murine infection, guinea pigs challenged with low dose *M. tuberculosis* by aerosol routine can develop the histopathology of granulomatous lesions, which is similar to that seen in human tuberculosis including the formation of well-structured granulomas, pulmonary lesions, dissemination and caseation necrosis[200]. With the advantage mentioned above, guinea pigs have been widely used as an animal model to characterize the morphologic and clinical features in human tuberculosis and anti-tuberculosis drug development. Additionally, the guinea pig model of tuberculosis is used extensively in the investigation of novel vaccines, since Mycobacterium bovis BCG vaccination dramatically prolongs survival after low-dose aerosol infection with virulent *M. tuberculosis* [204].

Zebrafish embryos infected with Mycobacterium marinum, an aquatic, close genetic relative of *M. tuberculosis*, has been described in many publications as an animal for tuberculosis due to natural advantages including the optical transparency, abundant genetic tools and in vivo imaging of the progression of infection[205]. Importantly, zebrafish embryo lacking the functional adaptive immune system in the first weeks of life can provide a unique tool for visualizing the dynamics of primary granuloma formation and dissemination to generate new secondary granulomas mediated by the innate immune responses in real time[206].

### 1.5.7 Innate immune responses against *M. tuberculosis*

*M. tuberculosis* is inhaled and traverse through the trachea, bronchus, bronchioles and eventually to the alveoli in the lung. Many cell types localized in the respiratory tract including airway epithelial cells (AECs), alveolar macrophages (AMs), dendritic cells (DCs), and mucus-secreting goblet cells get in contact with the bacteria. These cells might also constitute a first line defense against infection[197, 207]. But this has not been definitively established yet.

#### 1.5.7.1 Alveolar epithelial cells

AECs in primary, secondary and tertiary bronchi provide a barrier that prevents harmful invasion through physical and immunological ways. Surfactant proteins (A, D)
secreted by alveolar type II cells exhibit the capacity to bind to *M. tuberculosis* and regulate the interaction between *M. tuberculosis* and alveolar macrophages. The respiratory epithelium has been shown to predominately express many pattern recognition receptors (PRRs) such as Toll like receptors (TLR1-9), Dectin-1, C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain-containing protein 2 (NOD2), dendritic cell (DC)-specific intercellular adhesion molecule-3-grabbing non-integrin and mannose receptor which can recognize different kinds of cell components (lipoproteins, LPS, flagellin, ssRNA, CpG DNA, Alpha-glucan) presented by *M.tuberculosis* mediate the host-pathogen interaction through pathogen-associated molecular patterns (PAMPs) and trigger appropriate signaling pathways and subsequently release of cytokines, chemokines and antimicrobial peptides. AECs have been shown to play a role as antigen presenting cells (DCs and macrophages) by presenting *M.tuberculosis* antigen to major histocompatibility complex (MHC)-related protein 1-restricted mucosal associated invariant T (MAIT) cells and trigger them to produce IFN-γ, TNF-α and grazyme, which might be involved in *M.tuberculosis* clearance[208].

1.5.7.2 Alveolar macrophages

Alveolar macrophages (AMs) are one of the first cell types that encounter *M.tuberculosis* and play a critical role for first line defense against infection. AMs account for more than 90% of the cells collected from bronchoalveolar lavage and maintain the alveolar microenvironment by removing debris, dead cells, and microbes[209]. AMs also express a large number of PRRs such as the MR, TLRs, scavenger receptor (SR), complement receptors and Fcγ receptors, which mediate bacteria recognition and up-take, as well as cell activation[210, 211]. Unlike other macrophages populations, AMs have a high expression of nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ), which mediates the anti-inflammatory immune responses by secreting PGE2 and TGF-β and IL-10 [212]. Meanwhile, two surfactant-associated collectins, surfactant protein A (SP-A) and surfactant protein D (SP-D), have been shown to be involved in *M. tuberculosis*-AMs interaction, including the bacteria phagocytosis and the production of nitric oxide (NO), reactive oxygen species (ROS) and reactive nitrogen species (RNS) in response to stimuli [213].
Phagocytosis of *M. tuberculosis* by macrophages initiates a cascade of pro-inflammatory events involving the production of cytokines which stimulate the activation of phagocyte anti-microbial activities (such as IL-1, IL-6, IL-10, IL-12, TNF, IL-23) and chemokines (such as CCL-2, 3, 4, 5, CXCL8, CXCL10, CXCL13), that recruit blood polymorphonuclear and mononuclear leukocytes to the site of infection [212, 214].

The death of macrophages, including necrosis and apoptosis, has been widely investigated in the pathogenesis of TB. Within *M. tuberculosis*-infected macrophages, several pro-apoptotic genes, including Fas, Bim and caspase-3, -5, -7 and -8, have been shown to be up-regulated and trigger the macrophage apoptosis resulting in an ordered degradation of cellular contents and the formation of apoptotic vesicles, thereby preventing the dissemination of mycobacteria [215-217]. However, in order to avoid apoptosis, which hampers dissemination, several *M. tuberculosis* proteins such as SecA2 and NuoG have been shown to suppress macrophage apoptosis. Instead of apoptosis, some virulent *M. tuberculosis* strains can trigger mitochondrial inner membrane disruption and hampering the lysosomal and Golgi-mediated plasma membrane repair, thereby inducing necrosis, which may be an important factor in granuloma formation, inflammatory tissue damage and ultimately in the dissemination of the bacteria within different organs and in bacterial transmission[207, 218].

Alveolar and recruited inflammatory macrophages express various PRRs including Toll-Like Receptor (TLR)-2, TLR-4, TLR-8 and TLR-9; C-type Lectin Receptors (CLRs), such as dectin-1, mannose receptor and Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Non-Integrin (DC-SIGN); NLRs; and Stimulator of Interferon Genes (STING). In addition, the complement C3 receptors (CR1, CR3 and CR4) expressed on macrophages surface, mediate the *M. tuberculosis* phagocytosis through the interaction with cell components such as surface polysaccharides, lower order phosphatidylinositol and mannoses (PIMs) and glycopeptidolipids [219, 220]. Meanwhile, Fc receptors and scavenger receptors are also involved in mycobacterial recognition and endocytosis[221].
1.5.7.3 Neutrophils

Neutrophils are short-lived effector cells within the innate immune responses and the most abundant leukocytes in the blood[222]. Following infection, neutrophils are the first phagocytes to arrive from the circulation and contribute to clearance of pathogens [223]. Abadie et al. have shown that neutrophils infiltrate to the site of dermal BCG infection within 4 hours of inoculation in mice[224]. A large number of chemo-attractant cytokines or chemokines (including IL-8, G-CSF, GM-CSF, MIP-2, LL-37, LTB4, and IL-17[225]) have been suggested to participate in neutrophils recruitment.

Neutrophils express TLRs, G-protein-coupled receptors (GPCR), nucleotide-binding oligomerization domain-like receptors, C-type lectins, Fcγ and complement receptors, and thereby recognize and mount responses against pathogens [226, 227].

The killing of bacteria phagocytized in neutrophils is mediated through several mechanisms, including degranulation, the generation of reactive oxygen intermediates (ROI), and the formation of neutrophil extracellular traps (NETs), as well as many bactericidal peptides and proteins such as cathepsin G, elastase, defensins (e.g., human neutrophil proteins 1–3, HNP-1–3), cathelicidin LL-37, lactoferrin, and lysozyme[228, 229].

Results from studies on the neutrophil capacity to kill M. tuberculosis, in vitro and in vivo, are conflicting. In a study, Ramos and co-authors have shown that neutrophils infected with M. tuberculosis in vitro form NETs to trap mycobacteria but are unable to kill them[230]. The role of defensins in M. tuberculosis control has been investigated in several experimental studies. Martineau et al. showed that Black African participants (with high susceptibility to TB) had lower counts of neutrophils, as well as lower concentrations of circulating HNP1–3 and lipocalin 2 anti-microbial peptides than south Asian and white participants[231]. Cytokines and chemokines produced by neutrophils could mediate interactions with other immune cell types including macrophages, monocytes, DCs and T cells to control M. tuberculosis infection[224, 232-234]. Whereas early after infection neutrophils play a protective role and contribute to early priming of T cells in the draining lymph node, neutrophils accumulation late during infection has been shown to mediate susceptibility to M. tuberculosis (9-13).
1.5.7.4 Dendritic cells

DCs are crucial cell types viewed as the bridge between innate and adaptive immune responses. Monocyte-derived human DCs express several receptors including mannose receptors, CD11b, CD11c, and DC-SIGN, all of which have the capacity to recognize \textit{M. tuberculosis} molecules[235]. A study by Tailleux \textit{et al.} has shown that DC-SIGN expressed on human lung DCs is the major receptor mediating the \textit{M. tuberculosis} harboring and antigen presentation via recognition of mycobacteria-specific lipoglycan lipoarabinomannan (LAM) (ManLAM) and CCL19/CCl21 mediated migration, respectively[236]. Using a mouse model of TB, Wolf and co-authors have shown that myeloid DCs are a major cell population infected with \textit{M. tuberculosis} in the lungs and lymph nodes. Bacteria-infected DCs in the lung are transported to the draining lymph node by using a CCL19/21-dependent mechanism[237]. However, Wolf \textit{et al.} showed that delayed bacterial transported by DCs from lungs to the mediastinal lymph nodes resulted in delayed T cell activation[238].

1.5.7.5 Natural killer cells

Natural killer cells or NK cells are a type of cytotoxic lymphocyte critical to the innate immune system. The natural cytotoxicity receptor (NCR) NKp44 expressed on NK cells can directly bind to mycolic acids, a critical component of \textit{M. tuberculosis} cell wall[239]. In human TB, several studies have been shown to investigate the function of NK cells in TB control. Zhang \textit{et al.} showed that \textit{M.tuberculosis} activated NK cells can improve \(\gamma\delta\) T cell proliferation both via CD54-mediated cell-cell contact through the formation of immune synapse and pro-inflammatory cytokines including TNF-\(\alpha\), GM-CSF, and IL-12[240]. In addition, another study from Vankayalapati \textit{et al.} demonstrated that activated human NK cells release IFN-\(\gamma\), which triggers the secretion of IL-15 and IL-18 by \textit{M. tuberculosis} infected monocytes, thereby maintaining the frequency of \textit{M. tuberculosis}-responsive CD8+IFN-\(\gamma\)+T cells[241]. Meanwhile, they also showed that human NK cells could lyse \textit{M. tuberculosis}-infected monocytes and macrophages depending on the NKp46 expression[242]. However, another murine study by Junqueira \textit{et al.} showed that mice with NK cell depletion using NK 1.1 antibody displayed a comparable bacteria load in lungs after \textit{M.tuberculosis} infection[243].
1.5.8 Adaptive immune responses against *M. tuberculosis*

The adaptive immune responses are critical for the control of *M. tuberculosis*-infection in humans. T cells are of special importance for *M. tuberculosis* control. The mechanisms involved in the initiation of CD4+ T cell response to *M. tuberculosis* infection have been discussed in many studies using the murine infection model.

After aerosol infection, DCs in the lung will phagocytose *M. tuberculosis* and migrate to the lung-draining lymph node (also termed mediastinal lymph node, MLN), where T-cell priming can occur. A study by Khader *et al.* has demonstrated that mice lacking IL-12p40 fail to generate activated CD4+ T cells after *M. tuberculosis* infection, which is associated with the absence of expression of the chemokine receptor, CCR7 on DCs [244]. This is surprising since IL-12/ IL-23 (of which IL-12p40 is part of) are known to participate in the differentiation of Th1 or Th17 rather than in the migration of these cells.

Later, using the GFP-expressing *M. tuberculosis*, Wolf *et al.* showed that more than 65% of the infected cells in the MLN were CD11c<sup>high</sup>CD11b<sup>high</sup> myeloid DCs, which resemble the predominant infected cell population in the lungs. Surprisingly, Wolf and co-authors also showed that *M. tuberculosis*-infected DCs in MLN showed impaired activation of naive *M. tuberculosis*-specific CD4+ T cells. This was partially due to low expression levels of MHC class II[237]. On the other hand, Srivastava *et al.* showed that infected migratory DCs could release soluble and unprocessed *M. tuberculosis* antigens, which could be taken up and presented by uninfected resident lymph node DCs[245].

1.5.8.1 IFN-γ

It is well established that IFN-γ and TNF, expressed by Th1 CD4+ T cells, activate effector functions in macrophages that control intracellular *M. tuberculosis*. Mice with defect on CD4 T cells, IL-12, IFN-γ, or T-bet are highly susceptible to TB. People with inborn defects in the IL-12/ IFN-γ pathway, as well as individuals who raise anti- IFN-γ neutralization antibodies are extremely susceptible to mycobacterial infections [246]. They are especially susceptible to atypical commensal mycobacteria as well as to BCG. Green *et al.* showed that IFN-γ from CD4+ T cells is essential for the control of *M. tuberculosis* infection. By using a murine adoptive transfer model in which all cells
were capable of producing IFN-γ except CD4 T cells, they proved that IFN-γ secreted by CD4 T cells were required for host survival and long-term control of bacterial burden. Moreover, IFN-γ from CD4 T cells was necessary for a robust CD8 T cell response[247].

However, some evidence also indicates that while IFN-γ is required for bacterial control, increasing IFN-γ responses do not necessarily result in increased resistance to TB. Tsao et al. showed that TB patients with more severe pulmonary involvement, fever or body weight loss, had significantly higher bronchoalveolar lavage (BAL) fluid levels for IFN-γ and sIL-2Ra compared to those with a milder disease [248]. Cowley et al. found that CD4+ T cells controlled >90% of intracellular M. tuberculosis growth in the complete absence of IFN-γ activated macrophages through NO-dependent pathway[249]. Furthermore, BCG vaccinated IFN-γ-deficient mice exhibited significant protection against M. tuberculosis challenge that was lost upon depletion of CD4+ T cells, suggesting that CD4+ T cells possess IFN-γ independent mechanisms that are dominant in secondary responses to M. tuberculosis.

Furthermore, it was shown that M. tuberculosis antigen-specific effector T cells with defects in IFN-γ, TNF, Tbet, perforin or FAS could perform effectively control of M. tuberculosis infection after transferred into mice [250]. In summary, these findings demonstrate that CD4+ T cells also possess IFN-γ independent mechanisms that can limit the growth of M. tuberculosis.

The characteristic of the T cell elicited responses is critical for containment of M. tuberculosis. Several clinical studies have investigated whether trifunctional (secreting at least three cytokines) versus mono- and bifunctional (secreting 1 or 2 cytokines) CD4+ T cells are relatively increased in LTBI or in TB disease. Harari et al. showed that the frequency of TNF single-positive M. tuberculosis -specific CD4+ T cells was associated with active TB[251]. In addition, patients with active TB exhibited lower frequencies of trifunctional IFN-γ-, TNF- and IL-2-secreting M. tuberculosis-specific CD4+ T cells, compared with smear negative or latent infected individuals. Furthermore, upon anti-TB antibiotic treatment, specific IFN-γ+IL-2+TNF-α+ poly-functional CD4+ T cells increased, whereas TNF and IFN-γ-single positive T cells decreased [252, 253].
The ability of T cells to migrate from circulation to the lung parenchyma is critical for their protective capacity during \textit{M. tuberculosis} infection. Several murine studies have investigated the immune responses against infection mediated by \textit{M. tuberculosis} -specific Th1 subsets including PD-1+CXCR3+ T cells localized in lung parenchyma with poor IFN-\(\gamma\) expression capacity, and KLRG1+CXCR1+ T cells localized to the vasculature with high IFN-\(\gamma\) secreting capacity [254-256]. Surprisingly, mice adoptively transferred with PD1+ CD4 T cells showed much better control against \textit{M. tuberculosis} infection compared to the KLRG1+ (which labels effector T cells), suggesting that the control of \textit{M. tuberculosis} correlates with the ability of CD4+ T cells to efficiently enter the lung parenchyma rather than produce high levels of IFN-\(\gamma\)[256].

\subsection*{1.5.8.2 IL-17}

Over the past decade, a number of studies have analyzed the role of IL-17-producing cells (Th17 cells) in the control of \textit{M. tuberculosis} infection. Th17 cells development is initiated by IL-6 and TGF-\(\beta\), required for the expression of the orphan nuclear receptor ROR\(\gamma\)t via signal transducer and activator of transcription 3 (STAT3), which induces the expression of the receptor for IL-23. Then the binding of IL-23 stabilizes the Th17 phenotype[257]. The data referring to the role of Th17/IL-17 during primary \textit{M. tuberculosis} infection are conflicting. Several studies have described that Th17 cells are involved in immune protection against \textit{M. tuberculosis}, primarily due to the effect of Th17 signature cytokines in attracting and activating monocytes and Th1 cells [258]. An early study by Khader et al. reported that IL-23 had relatively minor effects in the infection control of \textit{M. tuberculosis} but was essential for generation of specific Th17 cells[259].

IL-23 was found to be required for long-term control of \textit{M. tuberculosis} infection through its ability to support the expression of CXCL13 in B cell follicles. This chemokine promotes the infiltration of T cells into the lesion[260]. By using mice deficient in the IL-17 receptor A subunit (IL-17RA\(-/-\) mice), Freches et al. showed that IL-17RA deficient mice were not to able to mediate long-term control of \textit{M. tuberculosis} infection, as demonstrated by a progressively increasing pulmonary bacterial burden and shortened survival times. Early but not late neutrophils recruitment was impaired in IL-17RA\(-/-\) mice, which indicated that neutrophil
recruitment in early infection phase was necessary for IL-17A-mediated long-term control[261]. It has been reported that mice with IL-17 deficient were not able to control infection by the hyper-virulent *M. tuberculosis* strain HN878, associated with CXCL13 induction, lymphoid follicle formation and macrophage activation [163]. Furthermore, Monin *et al.* showed that adoptively transferred *M. tuberculosis*-specific Th17 cells mediated the recall responses that displayed the protection at levels similar to vaccination strategies. They found that CXCR5 expression on adoptively transferred Th17 cells could regulate the localization of Th17 cells near macrophages within well-formed B cell follicles to mediate *M. tuberculosis* control in a IL-23/IL-17 dependent pathway, which identify new immune characteristics by targeting on Th17 recall responses for enhancing vaccine design against TB[262].

Murine studies reported that, early after infection, IL-17 was mainly produced by γδ T cells, which induce neutrophils infiltration and mature granuloma formation (180, 181). Furthermore, it was shown that IL-17 was required to induce optimal Th1/IFN-γ and delayed type hypersensitivity (DTH) responses against BCG infection[161, 263, 264].

Besides IL-17, IL-22 is also a cytokine secreted by Th17 cells. IL-22 can contribute to immune disease through the stimulation of inflammatory responses, S100s and defensins expressed by epithelial cells in the lung. The role of IL-22 in the control of *M. tuberculosis* infection has been investigated in several murine and human studies. A clinical investigation by Liu *et al.* found that the proportions of IL-22 T cells in patients with active TB were higher than those with the latent TB infection and the healthy control group[265]. In addition, Matthews *et al.* showed that IL-22 but not IL-17 was abundant in pleural or pericardial fluid from HIV negative TB patient, correlated positively MMP-9, a metalo peptidase known to degrade the pulmonary extracellular matrix [266]. Both of these studies indicated that IL-22 probably was involved in TB-induced pathology or the ensuing repair process. Besides Th1, Th17 and CD8 T cells, NK cells from TB patients produced IL-22[267], which activates macrophages. IL-22 enhanced phagolysosomal fusion and reduced *M. tuberculosis* growth in macrophages [268]. However, Behrends *et al.* have demonstrated that IFN-γ+ Th1 cells produce IL-22 early after infection of mice with *M. tuberculosis*, and that the absence of IL-22 does not affect the outcome of infection with *M. tuberculosis*, including the bacterial load in different organs, immune cells infiltration and cytokine expression [269].
Several studies suggested the involvement of Th17 cells in TB pathology. Th17/IL-17 induce the expression of chemokines and cytokines on many immune cell types, as well as the recruitment of neutrophils that promote local inflammatory responses and may lead to serious tissue damage[270]. Jurado et al. found that, compared with BCG-vaccinated healthy control, IFN-γ responses from T cells from TB patients were reduced significantly, and IL-17 were markedly augmented. The main source of IL-17 was CD4+IFN-γ+IL-17+ T cells, and the IL17-level in blood and pleural fluid from TB patients correlated positively with the disease severity [271]. Furthermore, Marin et al. have measured the frequencies of IL-17 and IFN-γ producing cells in patients with active TB, LTBI, and healthy control individuals. They found that the frequency of IFN-γ+ CD4 T cells was elevated in LTBI, and the percentage of IL-17+ CD4 T cells was higher in TB patients suggesting that active TB could modify the protective Th1 responses toward the pathological Th17 responses[272].

1.5.8.3 CD8+ T cells

A number of M. tuberculosis proteins, such as Ag85A, Ag85B, Ag85C, ESAT6 and CFP10, are recognized by human CD8+ T cells[273-277]. Several hypothesizes about the mechanisms for the recognition of M. tuberculosis antigens by CD8+ T cells have been presented in a number of studies after expression of antigen candidates in the cytosol of APC or screening the M. tuberculosis genomic sequence to identify peptide epitopes that are likely to bind to class I MHC proteins[278]. In infected APCs, Sec61, which mediates retrograde protein translocation, could translocate proteins secreted by M. tuberculosis from the phagosomal compartment across the phagosomal membrane into the cytosol where they could enter the class I MHC processing pathway. The cross-presentation of mycobacterial antigens by DCs could also lead to CD8+ T cells priming. Apoptotic vesicles from M. tuberculosis-infected macrophages could have been taken up by uninfected DCs, and peptides processed in the proteasome and presented on MHC-I molecules [278-281].

CD8+ T cells could mediate protection against M. tuberculosis infection by several mechanisms. CD8+ T cells from TB patients have been shown to secrete diverse pro-inflammatory cytokines, including IFN-γ, IL-17, TNF and IL-2 [282]. In addition, a hallmark of CD8+ T cells in many immune responses is the cellular cytotoxicity, which can be mediated by several mechanisms including the release of cytotoxic granules
containing perforin and granzymes and Fas/FasL dependent killing and TNF induced cell death[278]. Moreover, human CD8+ T cells and NK cells can produce granulysin, a microbicidal protein in cytotoxic granules, and can directly interact with microbial cell wall and/or membrane, leading to increased permeability and lysis[283]. Ernst et al. reported that granulysin increased the permeability of bacterial membranes based on its ability to allow access of cytosolic β-galactosidase to its impermanent substrate. They found that granulysin triggered fluid accumulation in the periplasm of *M. tuberculosis* using electron microscopy[284].

To investigate whether CD8+ T cells mediate a protective role during *M. tuberculosis* infection, many strategies such as CD8+ T cell depletion by antibodies, CD8 knockout mice, and adoptive transfer of CD8+ T cell have been used. β2m−/− mice, which lack CD8+ cells due to a defective presentation in the thymus, infected with virulent *M. tuberculosis* did not survive 6 weeks after infection, and showed uncontrolled bacterial burden and caseating necrosis in the lungs. This suggested that functional CD8 T cells and/or other T cells that require presentation via MHC-I for maturation, protect against *M. tuberculosis* infection[285]. Other knockout mouse models lacking CD8 T cells such as the *tap1−/−, cd8a−/−* and *KbDb−/−* mouse models showed that CD8+ T cells have a crucial role in immunity against *M. tuberculosis* infection[286-289]. Moreover, Tascon et al. showed that *M. tuberculosis* infected athymic nude mice, adoptively transferred with naïve wild type CD8+ T cells but not IFNγ−/− CD8+ T cells, could control the bacterial burden in lungs and spleen, indicating that IFN-γ-producing CD8 T cells contributed to the bacterial control[290]. In addition, Müller et al. showed that depletion of CD8+ T cells in thymectomized mice prior to *M. tuberculosis* infection (i.v.) displayed uncontrolled bacteria growth in the spleen 3 weeks after infection[291]. However, two other studies performed in a similar way found that depletion of CD8+ T cells did not affect the control of BCG replication, which probably indicates that CD8 T cells are involved in the control of virulent bacterial strain infection[291, 292]. Moreover, the increased susceptibility of β2m−/− mice over MHC-I−/− mice which actually do not show increased bacterial load compared to controls has been coupled to defective iron overload, which represents an exacerbating cofactor for TB.

The relative importance of CD8 and CD4 T cells in anti-*M. tuberculosis* immunity can be only performed on mice of the same age and sex by using the same infection dose
and time period in an aerosol infection chamber. A comparative study of the ability of MHC class I−/− and class II−/− mice to defend against *M. tuberculosis* infection showed that MHC class II-dependent immunity is much more important [293].

Several studies have demonstrated the presence of regulatory CD8+ T cells subpopulations. In CBA/J mice, Cyktor *et al.* found that clonally expanded (TCR Vβ8+ or 14+) CD8 T cells displayed an immunosuppressive phenotype (PD-1+, Tim-3+, CD122+) and were able to produce IL-10. However, depletion of Vβ8+CD8 T cells did not alter the outcome of infection with *M. tuberculosis* [294]. Furthermore, a clinical study by Silva *et al.* observed the presence of IL-10/TGF-β and low levels of granzyme-B expression in CD8+ T cells. This was correlated with increased bacterial load in sputum in active TB patients, providing a new insight into another role for CD8+ T cells in TB [282]. Mucosal-associated invariant T cells (MAIT), a MR1-restricted non-classical CD8 T cell subpopulation enriched in airway, can recognize *M. tuberculosis* antigen presented by airway epithelial cells and release several cytokines such as IFN-γ and TNF-α. The role of MAIT cells contribution to the host response against *M. tuberculosis* infection is not well known yet[295].
## 2 MATERIAL AND METHODS

The methods applied in our studies are described in detail in the published articles and in the manuscript. I want to give an overview about the genetically modified mice that have been used.

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>type of modification</th>
<th>effect</th>
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<tbody>
<tr>
<td>Stat3^{fl/fl} LysM cre*</td>
<td>conditional knockdown</td>
<td>excision of the Stat3 gene in myeloid cells</td>
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<tr>
<td>Socs3^{fl/fl} LysM cre*</td>
<td>conditional knockdown</td>
<td>excision of the Socs3 gene in myeloid cells</td>
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<tr>
<td>Socs3^{fl/fl} cd11c cre*</td>
<td>conditional knockdown</td>
<td>excision of the Socs3 gene in CD11c+ cells</td>
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<tr>
<td>Gp130^{mr}</td>
<td>knockin</td>
<td>mutation of the SOCS3 binding site in gp130 in all cells</td>
</tr>
<tr>
<td>p25-tg rag1^{+/-} ECFP</td>
<td>knockout</td>
<td>no mature CD8 T cells and B cells, only CD4 T cells that express ECFP and with transgenic TCR only recognizing P25 peptide from antigen 85B</td>
</tr>
<tr>
<td>p25-tg socs3^{fl/fl} lck cre*</td>
<td>Knockout and conditional knockdown</td>
<td>no mature CD8 T cells and B cells, only CD4 T cells that express ECFP and with transgenic TCR only recognizing P25 peptide from antigen 85B and excision of the Socs3 gene</td>
</tr>
<tr>
<td>p25-tg stat3^{fl/fl} lck cre*</td>
<td>Knockout and conditional knockdown</td>
<td>no mature CD8 T cells and B cells, only CD4 T cells that express ECFP and with transgenic TCR only recognizing P25 peptide from antigen 85B and excision of the Stat3 gene</td>
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<td>Mouse Line</td>
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<td>II12p40&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>knockout</td>
<td>disruption of the <em>Il12p40</em> gene in all cells no IL-12 and IL-23 expression</td>
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<td>Socs3&lt;sup&gt;fl/fl&lt;/sup&gt; actin creER</td>
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<td>excision of the <em>Socs3</em> gene all cells after tamoxifen treatment</td>
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<td>Socs3&lt;sup&gt;fl/fl&lt;/sup&gt; lck cre*</td>
<td>conditional knockdown</td>
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<tr>
<td>Rag1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>knockout</td>
<td>disruption of the Rag1 gene in all cells no mature B and T cells</td>
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</table>

*Conditional knockdown:

Mice were generated using the Cre-lox combination system, in which the Cre DNA recombinase is expressed under a cell-specific promoter. Although the target gene is flanked by LoxP sequences in all cells for recognition by the Cre enzyme, it will only be deleted specifically in Cre-expressing cells.
3 RESULTS AND DISCUSSION

PAPER I: ROLE OF SOCS3 IN T CELL DEVELOPMENT IN THYMUS

T cell development in thymus depends on the crosstalk between thymocytes and stromal cells such as thymic epithelial cells and dendritic cells. Such cellular communication takes place through cell contact and by cytokines [296].

Since SOCS3 is a major regulator of immune responses by regulating the responses to cytokines and growth factors, we hypothesized that SOCS3, may play a role in T cell development in thymus.

Deletion of SOCS3 in vivo has important effects on placental development, inflammation and insulin sensitivity. Genetic defect of SOCS3 leads to mid-gestational embryonic lethality due to increased STAT3 and MAP kinase activation [297]. Lack of suppression of LIF and fetal erythropoiesis signaling had been shown to account for the lethality of Socs3−/− mice [297]. LIF belongs to the IL-6 family and is involved in blastocyte implantation.

Thus, to study the effect of SOCS3 in T cell development in the thymus, we generated a tamoxifen-inducible conditional SOCS3 knockdown, using an actin promoter for the estrogen receptor activated Cre recombinase described in other studies [298]. Previous to this study, the expression of SOCS3 in thymocytes subpopulations has been shown (reference), while the effect of SOCS3 deletion in thymocytes differentiation using a OP9-δl1 system was relatively minor [299].

We found that the thymus from mice with SOCS3 deletion displayed an extraordinary atrophy, as well as reduction of cellularity. The frequency of DP cells among thymocytes was also diminished. Moreover, the percentage of activated (CD44high) CD4+ and CD8+ T cells in peripheral lymphoid organs were increased, which was probably because of the defective output of naïve T cells from thymus resulted in a lymphopenia-induced homeostatic proliferation of naïve T cells which in absence of antigenic stimulation will acquire T cell memory markers.

We performed many controls for these experiments. Controls included testing different doses of tamoxifen, the use of socs3fl/fl as well as actin cre ER mice to control for possible off targets effects of cre (as described below for lck cre), or for toxicity of Tamoxifen.

The tamoxifen-mediated activation of cre under actin promotor results in the deletion of socs3 gene in all cell populations [300]. Thus, we investigated whether SOCS3 expression in either bone marrow-derived vs or non-hematopoietic cells is involved in T cell development. For this purpose, we generated bone marrow radiation chimeras Δsocs3→socs3fl/fl and administrated them with Tm after reconstitution. We found that the SOCS3 deficient recipient mice adoptively transferred with bone marrow cells from wild type donors
displayed a defect T cell development similar to what was observed in the Δsocs3 mice, which strongly suggested that SOCS3 expression in thymic stromal cells but not in hematopoietic cells are required for T cell development in the thymus, as shown in Figure 6. The lack or minor participation of SOCS3 expression in hematopoietic cells was confirmed in socs3[0/+] cd4 cre and socs3[0/+] lck cre mice (deficient in SOCS3 in T cell populations at the DP stage and the DN2 respectively), in which T cell development in thymus was similar to non-mutant controls.

Figure 6: Representative dot plots of DN, DP, CD4 SP and CD8 SP thymocytes in bone marrow radiation chimeric □socs3 and socs3[0/+] mice (at least 5 per group) treated with Tm.

In the thymus diverse populations of thymic epithelial cells (TECs), including cortical and medullary TECs and their subpopulations, have distinct roles in coordinating the development and repertoire selection of functionally competent and self-tolerant T cells. These are characterized by a diversity of cell surface markers. We showed that that the thymic cortex and medulla of SOCS3 deficient mice was disorganized and the boundaries between them were not clear (Figure 7).

Both cTECs and mTECs are CD45- cells that express MHCII and EpCAM[301, 302]. They can be identified based on expression of specific proteins and surface markers. cTECs express keratin 8 (K8) and Ly51, whereas mTECs express keratin 5 (K5) and ulex europeus agglutinin-1 (UEA-1). cTECs can be divided into two subpopulations based on the expression of K5, a major population K8-K5+ and a minor population K8+K5+. On the other hand, mTECs can be separated in immature and mature subsets depending on the expression level of MHC II and CD80, as well as the autoimmune regulator (AIRE)[303, 304]. To identify the cTEC and mTEC in thymus, we performed the immunohistochemistry staining by using the anti-K8 and anti-K5 antibodies, which will characterize cTEC and mTEC, respectively. We found that the fraction of area with an overlapping staining was more
extense in SOCS3 deficient mice, as shown in Figure 7, which is accordance with the histological observation of a less defined interface.

Mature cTEC and mTEC mediate the positive and negative selection on DP and SP cell stage, respectively [305]. In our study, we also used several surface markers discussed above to define immature and mature TECs by flow cytometry and found a diminished frequency of mature cTECs (defined as for lower levels of MHCII) and mTECs, which probably indicates a defective role in positive and negative selection. A reduction of levels of IL-7 and the reduction of MHCII observed may contribute to this. Importantly, the numbers and frequencies of mTECs and cTECs in mutant and control mice were similar.

**Figure 7:** H&E staining (E) and keratin-5 and -8 staining (F) labelling the depicting the higher disorganization of thymi from Δsocs3 as compared to control mice.

Gp130, the common IL-6 receptor family, is expressed on thymocytes and TECs [306, 307] and has been shown to be required for proper thymic formation[308]. SOCS3 binds to gp130 and hampers STAT3 signaling altering the responses of macrophages and T cells to infection or to innate receptor agonists [309]. However, gp130<sup>F/F</sup> mice with a mutation that impairs SOCS3 binding, displayed normal thymus structure and thymocyte numbers and frequencies, which indicated that SOCS3 signaling via gp130 is redundant for thymopoiesis.

Several molecular mechanisms and signaling pathways in involved in TECs development have been reported. Transcription factor FOXN1[53] and Notch1/DLL4 mediated signaling pathways are required for cTEC differentiation cTEC development [51, 52]. The secretion of IL-7 is probably also mediated by Notch1 pathways and mediates β-selection. On the other hand, RANK/RANKL, CD40/CD40L, LTα/β mediated downstream signaling pathways including NFκB are considered critical for mTECs differentiation [63].

In order to identify molecular mechanisms that are altered in SOCS3 deficient TECs and that might regulate T cell development in thymus, we sorted the TECs and analyzed the transcripts by using microarray analysis. The assay identified 9392 transcripts expressed above threshold level in both groups and 703 transcripts were picked up because of differently expressed between groups, 367 higher and 336 lower in SOCS3 deficient than in WT TECs. A GO analysis indicated several pathways being altered including cytokine-
cytokine receptor interaction and epithelial-mesenchymal transition pathways. We found that the growth factor and receptor transcripts such as fgf9 and fgfr2 were significantly higher in WT TECs, which could be an explanation for the better TECs development in WT nice as shown above. IL-7 expressed by TECs is critical for the thymopoiesis in the early DN stages[310]. We found IL-7 mRNA level was reduced in SOCS3 defect TECs compared to controls. On the other hand, IL-7R expression level was increased in SP cells from SOCS3 deficient, probably as a response to low IL-7 levels.

Antigen presentation by TECs is critical for positive and negative selection. We found MHC II mRNA level was significant lower in SOCS3 deficient TECs, probably indicating a defect in position and negative selection for DP and CD4 SP cells. However, genes involved in mTEC development and central tolerance including Aire, CD40, CD80, several from the MHC-II locus and cathepsin M and L (involved in antigen presentation), chemokines and their receptors that mediate migration of thymocytes during thymic development, and members of the TNF-receptor family that mediate thymocyte development such as CD30 or rank were elevated in the SOCS3 deficient TECs as compared to the controls.

Many questions remain with regards to the role of SOCS3 in T cell maturation in the thymus. Among these, we believe the identification of the SOCS3 binding partner(s) in TECs that account for thymic atrophy and the defective T cell differentiation. Further, whether the alterations observed mainly affect positive or negative selection is at this time unknown. The fact that DN cells shows defective BrdU incorporation and show a relative accumulation in the Δsocs3 mice, as well as the blockade in SP differentiation, shows that a defect early in thymocytes in the thymic cortex is likely. The same is suggested by the reduced levels of IL-7 found in TECs mirrored by the abnormal frequency of γδ vs β TCR + cells.

Other questions as the role of SOCS3 in the generation of Treg in the thymus should also be addressed here.

Inflammation and the production of stress hormones cause thymus regression. SOCS3 deficient mice in hematopoietic cells show inflammatory responses during their adult life. The relative short time period from Tm administration to analysis and the lack of obvious sings of inflammation (increased size of lymph nodes or spleens) suggests that this is not probable cause.

Overall, we here show that SOCS3 plays a central role in maintenance of the thymic architecture, the maturation and morphology and tissue distribution of TECs and in the differentiation of T cells in the thymus. SOCS3 expression in T cells is central for their proper differentiation and function. Our results indicate that SOCS3 regulation in TECs provides proper niches for thymocytes maturation, and consequently is required for proper thymocytes development and naïve T cell export in the periphery.
As indicated in the introduction to the thesis STAT3 and SOCS3 are main controllers of immunity and inflammation.

Previous data showed that SOCS3 expression in myeloid cells is required for the control of *M. tuberculosis* infection since it modulates and IL-6/gp130/STAT3 signaling pathway to induce IL-12p40 expression in APCs and lead to timely T cell mediated protection [131]. Of importance, while it was obvious that SOCS3 in myeloid cells acted on T cell mediated immunity the defined mechanisms regulated are unknown. In this study, we investigated the role of STAT3 expression in myeloid cells in regulation of *M. tuberculosis* infection.

First, the role of STAT3 expression in myeloid cells in the control of infection with *M. tuberculosis* was examined using stat3<sup>fl/fl</sup> lymm cre mice. Mice with STAT3 deficiency in myeloid cells (stat3<sup>fl/fl</sup> lymm cre) showed significant lower bacteria burden in lungs and spleens after 4 and 8 weeks of infection, compared with the stat3<sup>fl/fl</sup> littermates. The area occupied by granuloma in the lung of stat3<sup>fl/fl</sup> lymm cre mice was reduced at 4 weeks after infection. Meanwhile, we found that the neutrophil density and the levels of neutrophil myeloperoxidase and elastase mRNAs were higher in lungs from stat3<sup>fl/fl</sup> lymm cre mice at 4 and 8 but not at 14 weeks after infection with *M. tuberculosis*-infected mice compared to controls. Neutrophil accumulation during late phase of infection have been associated with susceptibility to *M. tuberculosis* mediated by the excessive pro-inflammatory response and tissue damage, whereas early after infection neutrophils play a protective role and contribute to early priming of T cells in the draining lymph node[311, 312].

Several studies have reported that neutrophil mobilization during inflammation was mediated by G-CSF inducing CXCR2 up-regulation via STAT3 signaling pathway[313, 314]. In this case, we thought that the increased pulmonary neutrophils infiltration during *M. tuberculosis* infection was not a direct consequence of STAT3 deficiency in these cells.

IL-17 has been shown to stimulate the expression of chemokines required for neutrophil recruitment [315]. In agreement with this hypothesis, we found that the frequency of IL-17 secreting mycobacteria-specific CD4+ T cells, but not of γδ+ T cells, were elevated in the lungs from stat3<sup>fl/fl</sup> lymm cre mice compared to controls, as well as the il-6, il-23, il-17, and il-22 genes transcription level in lung tissue. While IL-6 and IL-23 are important for Th17 differentiation and maintenance, the increased IL-17 and IL-22 levels might participate in the elevated neutrophil levels in lungs from stat3<sup>fl/fl</sup> lymm cre *M. tuberculosis*-infected mice.

Next, we studied whether STAT3-deficient macrophages (BMMs) showed impaired intracellular bacterial growth. BMM from stat3<sup>fl/fl</sup> lymm cre mice expressed higher level TNF-α. Surprisingly, the intracellular bacteria levels in stat3<sup>fl/fl</sup> lymm cre and control BMMs
were similar, which indicated that the increased bacteria control in vivo was not generated by the macrophages mediated innate immune responses.

On the other hand, BMMSs or bone marrow derived dendritic cells (BMDC) from STAT3 deficient mice also showed increased il-6 and il-23 mRNA levels after mycobacterial stimulation. In addition, in a co-culture system, mycobacterial stimulated BMDC with STAT3 deficient promoted antigen-specific T cells to secrete more IL-17, as compared with WT controls. To our knowledge, this is the first report showing that STAT3 deficiency in myeloid cells promotes IL-17 secretion by antigen-specific T cells in vitro and in vivo, which was associated with the increased secretion of TH17 differentiation cytokines including IL-6 and IL-23 by STAT3-deficient APCs.

The increased expression of IL-6 and IL-23 in stat3<sup>fl/fl</sup> lym cre APCs was not restricted to the infection with attenuated or virulent mycobacterial since, it was observed after incubating mutant APCs with different TLR agonists or bacterial lysates, confirming previous data[316]. The opposite effect was observed using socs<sup>fl/fl</sup> lym cre BMM, which were poor inducers of IL-17 secretion by mycobacteria-specific T cells. In relation to this, DCs that secrete IL-12p40 (required for T cell differentiation into Th17 or Th1) in the lymph nodes of mycobacteria infected mice are primarily uninfected[317].

We showed that the improved M. tuberculosis control in stat3<sup>fl/fl</sup> lym cre mice is IL-17-mediated, since administration of neutralization anti-IL-17RA antibodies abrogated differences in bacterial burden between mutant and control mice, as well as the neutrophils infiltration shown by mpo transcript analysis. IL-17 might contribute to long-term protection, control of infection after vaccination or control of hyper-virulent strains of M. tuberculosis. IL-17 has been also shown to mediate CXCL13 induction in the lung, a chemokine that contributes to the localization of pro-inflammatory cytokine-producing CXCR5+ T cells within lymphoid structures, promoting those macrophage activation and mycobacterial control[162, 163, 318-320].

Finally, the role of STAT3 in myeloid versus T cells in regulation of antigen-specific IFN-γ and IL-17 T cell responses was compared. Contrary to the role of STAT3 in APCs, IL-17 secretion was hampered in mycobacteria-specific STAT3-deficient T cells. STAT3 is required for the responses to IL-6, IL-21 and IL-23 and for the expression of RORγt by T cells [134]. Instead, SOCS3-deficient antigen-specific T cells secreted higher IL-17 levels as previously reported in other systems[154], while IFN-γ responses were inhibited. Thus, while the role of STAT3 in T cells in the control of M. tuberculosis remains to be studied, these results illustrate the pleiotropic effect of STAT3 in regulation of infection-induced immune responses in different cell types.

Taken together, we here showed using SOCS3- and STAT3-deficient mice that STAT3 in myeloid cells is detrimental for the control of infection with M. tuberculosis. SOCS3 acts as a mirror of STAT3 in this model. Surprisingly, this occurs via impairing secretion of IL-17 by antigen-specific T cells, as shown in Figure 8.
Figure 8: Signaling pathways mediated by STAT3 and SOCS3 in APCs affect the outcome of *M. tuberculosis* infection
PAPER III. CISH IN THE CONTROL OF M. TUBERCULOSIS INFECTION

The “cytokine-inducible SRC homology 2 (SH2) domain protein” CISH is one of SOCS family members. Single nucleotide polymorphisms (SNPs) in CISH gene were associated with increased risk of TB [181-184]. We investigated the role of CISH in the outcome of M. tuberculosis infection using a CISH knockout mouse model.

First, we compared the bacteria burden in lungs and spleen from WT and cish\(^{-/-}\) mice at different time points after M. tuberculosis infection. Cish\(^{-/-}\) mice displayed increased bacterial load in lungs at 1.5 and 2.5 weeks. In spleens, cish\(^{-/-}\) mice contained higher bacterial loads at 2.5 weeks (bacterial load was under detection levels in the spleen at earlier time points) compared with WT controls. Instead, the bacteria load in lungs and spleens of WT and cish\(^{-/-}\) mice at 4 and 8 weeks after infection were comparable. According to the susceptibility of WT and cish\(^{-/-}\) mice with M. tuberculosis infection, we suggested that CISH regulate innate immune mechanisms of M. tuberculosis control.

In line with this, T cells from cish\(^{-/-}\) mice stimulated with CD3/CD28 expressed comparable levels of IFN-\(\gamma\) as WT T cells. Moreover, rag1\(^{-/-}\) mice adoptively transferred with WT or cish\(^{-/-}\) T cells and infected with M. tuberculosis showed similar bacteria load, indicating that CISH is required for innate immune responses but is redundant for T cell mediated protection against M. tuberculosis infection.

TNF-\(\alpha\), NO/iNOS, IL-6 and IL-1 can be induced in an innate manner and are relevant for M. tuberculosis infection control [321-323]. We found reduced transcript levels of tnf and inos (but not il-6 and il1b) in lungs of cish\(^{-/-}\) mice early, but not at late time points after infection. Thus CISH could positively regulate TNF-\(\alpha\) and iNOS expression early after infection. In the contrary, CISH was redundant in regulation of il6 or il1b mRNA levels in lungs.

We performed M. tuberculosis infections of BMM in vitro in macrophages and found that cish mRNA was increased after infection. However, we neither observed the reduction of tnf or inos mRNA levels observed inn vivo (despite these are stimulated by the infection in vitro), not a difference in the bacterial load of BMM.

During the writing of this thesis another manuscript addressing the role of CISH during infection with M. tuberculosis in mice has been published[324]. In this manuscript, contrary to ours, mice lacking CISH were more resistant to infection with M. tuberculosis and this phenomenon, which is observed at 3 and 6 weeks after infection, was linked to the binding of CISH to inhibit proton pump in macrophages. In this model CISH function seems not to be related to inhibition of JAK/ STAT pathway.

Neutrophils are the first wave of host defense to infection and have been shown to be involved in mediating protective immune responses during the early stage of M. tuberculosis infection [233, 325]. However, similar neutrophil frequencies were found in lungs from control and cish\(^{-/-}\) mice excluding a role for CISH in neutrophil recruitment.
In summary, and despite the precise molecular mechanisms need to be examined in further
detail, the CISH mediates control of *M. tuberculosis* in mice early infection via regulation of
innate immune mechanisms.
We have used extensively used in our studies gene conditional knockdown system mediated by DNA recombinase Cre (cre that recognizes a loxP sequence, both enzyme and target sequence derived from a bacteriophage). If cells that contain loxP sites in their genome express Cre, a recombination event can occur between these sites. The Cre recombination can be used to create deletions, insertions, translocations or inversions at certain sites in the genome. Many genes have been targeted in this manner and their function in different cell types, tissues and organs in the organism under specific promoters can be studied [326].

Despite this tool care must be considered when analyzing the effect of cre under the control of strong promoters since off targets effects of cre in the specific populations have been reported. Cre probably mediated gene recombination by binding to pseudo-loxP sites. For example, mice with the Cre expression controlled by insulin promoter displayed the insulin resistant regardless of any loxP-targeted gene[327].

The tyrosine kinase p56 termed Lck is critical for T lymphocyte development and function[328-330]. Many different kinds of transgenic mice generated by the Cre system controlled by lck promoter have been described as an important model to study the signaling pathways and molecular mechanisms in T cell development or function. Since we have been previously used lck cre transgene[131]. However, whether the Cre mediated toxicity for T cell development and function under lck promoter has not been well described in depth yet. Still, several previous studies have stated that Cre expression controlled by lck promoter might intervene with normal T cell development, such as the changes of the frequency of different stage of DN cells and the ratio of CD4/CD8 SP cells [331-336].

For this purpose, we bred a C57BL/6 mouse strain with Cre recombinase expression controlled by lck promoter (lck-cre⁺). We found that the thymic cellularity from lck-cre⁺ mice was reduced by 65%, which is contributed by reduced numbers of DP, CD4 SP and CD8 SP cells but not DN cells. Several studies have described the expression of Lck in different stages of T cell development in thymus by using the GFP transgenic mouse model. Lck is detected in all T cell development stages, especially in DP and CD4 and CD8 SP cells[337, 338]. Probably, the loss of DP, CD4 and CD8 SP cells in lck-cre⁺ mice was generated by the DNA-damage induced apoptosis. As expected, lck cre⁺ DP cells displayed higher frequency of Annexin V, a maker of apoptosis compared to controls.

The αβ and γδ T cell lineage commitment occurs in DN 2 and 3 stages during the b-selection[339]. We found that the number of γδ TCR positive cell population was higher in thymus, spleen and Lymph nodes in lck-cre⁺ mice than that in control group. A study by Saint-Ruf et al. reported that the expression level of Lck in γδ T cell lineage is lower than that in αβ T cell lineage[340]. IL-7 produced by thymic epithelial cells is involved in many biological events during T cell development including proliferation, differentiation and survival. IL-7Rαhigh DN2 cells are biased to be γδ T cell lineage[26].
Coincidentally, increased frequency of IL-7R$^+$ total thymocytes and γδ TCR$^+$ cells within DN cells was found in in $lck$-cre$^+$ mice.

All the results shown above indicated that Cre expression under the control of $lck$ promoter altered the T cell development in thymus. On the other hand, we did a meta analysis of the literature and found that studies using Cre under $lck$ promoter are biased depending in the control used. So care must be taken consideration to choose the correct control group (in this case a cre$^+$ control) when cre induced toxicity is obvious or even better, to avoid the use of strong promoters for cre-mediated genetic changes.
Since first used in human in 1921, Bacille Calmette-Guérin (BCG) is the only vaccine against *M. tuberculosis*[341]. The interaction between the antigen presenting cells and lymphocytes within draining lymph node (dLN) is the critical step to initiate the adaptive immune response and create the immune memory.

This study focused on DCs migration and CD4 T cell priming after BCG skin infection. In order to study the DC migration, 2 hours after BCG inoculation in the footpad, we injected Carboxyfluorescein succinimidyl ester (CFSE) to label all the skin cells. Thus we could define that DCs by using cell markers by flow cytometry in the draining lymph node. All skin DCs express MHC II and CD11c. Five different kinds of DCs in healthy mouse skin were defined by several surface markers including EpCAM (CD326), langerin (CD207), CD11b and CD103[342].

We found that MHC II$^+$CD11c$^{high}$EpCAM$^{low}$CD11b$^{high}$ DCs are the main DCs population that migrated from skin to the popliteal LN (pLN), which is the primary dLN of the footpad. Coincidentally, Bachy and co-authors also identified that the MHC II$^{high}$ CD11c+EpCAM-CD207- skin DCs subset dominated in HIV specific antigen presentation and inducing CD8 T cells priming[343]. In addition, Ochiai et al. reported that CD326$^{low}$CD11b$^{high}$ DC was highly responsive to BCG and *E.coli* inoculation[344].

In summary, the studies shown above together with our findings support the MHC II$^+$CD11c$^{high}$EpCAM$^{low}$CD11b$^{high}$ skin DCs with the capacity of mobilization from skin to dLN after BCG infection.
4 SUMMARY

In summary, in this thesis I present we have addressed the role of role of SOCS and STATs in T cells development as well as in the outcome of \textit{M.\textit{tuberculosis}} infection

Specifically we found that

1. SOCS3 is required for T cell development specifically by signaling in TECs.
2. SOCS3 is critical for cortex and medulla formation and organization in thymus
3. SOCS3 regulates many signaling pathways involved in thymic epithelial cells development

STAT3 in \textit{M.\textit{tuberculosis}} infection

1. STAT3 in myeloid cells hampers the bacteria control
2. STAT3 in myeloid cells inhibits TH17 responses
3. STAT3 in APCs inhibit the generation of IL-17 secreting mycobacteria-specific T cells

CISH in \textit{M.\textit{tuberculosis}} infection

1. CISH is critical for bacteria control in the early time points of \textit{M.\textit{tuberculosis}} infection
2. CISH is redundant for the regulation of T cells in \textit{M. \textit{tuberculosis}} infection
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