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The role of the STAT1β isoform in $CD4^+$ T cell differentiation

Bachelor thesis

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Submitted by

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Table of content

1. Introduction

1.1 T lymphocytes

T lymphocytes, also called T cells, are types of leukocytes that are essential for tissue homeostasis, immune responses and immunological memory (Kumar et al. 2018). T cells arise from hematopoietic stem cells in the bone marrow. Lymphoid progenitor cells migrate to the thymus where they develop into T cell precursors. At the cortico-medullary junction precursor cells enter the thymus to migrate to the outer cortex from where they return to the medulla (Figure 1) (Hale and Fink 2009). During this process, T cell precursors undergo several differentiation steps characterized by expression of cell surface proteins, such as cluster of differentiation (CD)4, CD8, CD44 and CD25. T cells start their journey as double negative T cells ($CD4^-CD8^-$), then become double positive ($CD4^+CD8^+$) and finally undergo lineage commitment into either $CD4^+$ or $CD8^+$ T cells (Zuniga-Pflucker 2004). T cells that have successfully passed the T cell selections complete their maturation in peripheral lymphoid organs (spleen and lymph nodes), where they exist as naïve T cells. Upon activation, $CD8^+$ T cells are able to differentiate into $CD8^+$ cytotoxic T cells and kill target cells. They represent an important defence against intracellular pathogens, especially viruses, and cancer cells. $CD4^+$ T cells can differentiate into several distinct subsets, each having specialized immune functions. $CD4^+$ T cell subsets can be defined by signature cytokines, which they secrete to fight a wide array of pathogens, and signature transcription factors (Seder and Ahmed 2003).

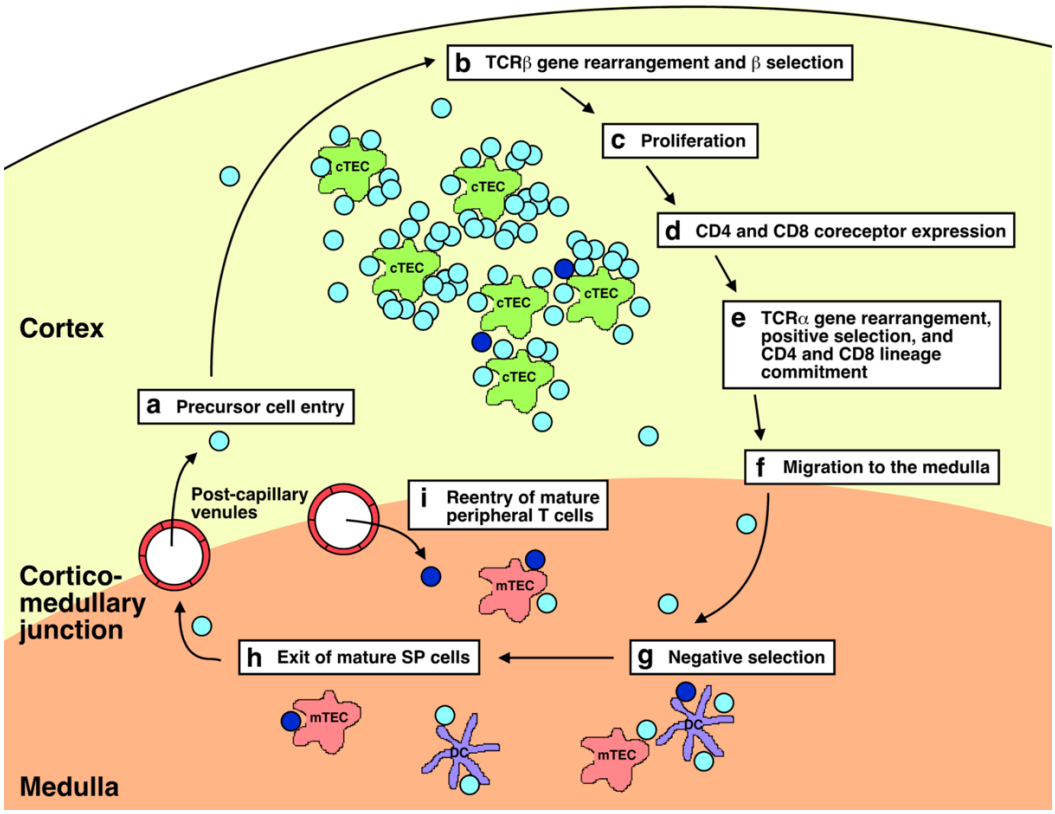

Fig. 1: Overview of T cell development in the thymus. cTEC – cortical thymic epithelial cell, mTEC – medullary thymic epithelial cell, DC – dendritic cell, SP – single positive T cells (Hale and Fink 2009).

1.1.1 Differentiation of naïve $CD4^+$ T cells into T helper cell subsets

Between 20 % and 40 % of total T cells within lymph nodes are composed of naïve T cells, which are able to maintain their functionality as individuals age (Thome et al. 2016). In humans, the life span of naïve T cells has been shown to range between four and six years, whereas in mice, naïve T cells only persist seven to eleven weeks (Vrisekoop et al. 2008, den Braber et al. 2012) . To maintain a steady population size, naïve T cells reside in secondary lymphoid organs, such as lymph nodes and spleen, which provide T cells with the essential survival factor interleukin (IL)-7 (Surh and Sprent 2002).

When encountering foreign antigen, naïve $CD4^+$ T cells change from being sub-mitogenic into active proliferation and differentiate into distinct T helper (T_H) cell subsets. Successful

activation of naïve $CD4^+$ T cells requires three signals: (i) binding of the T cell receptor (TCR) to a cognate antigen peptide presented by antigen presenting cells (APCs) via major histocompatibility complex (MHC) class II molecules, (ii) costimulatory interaction of CD28 on $CD4^+$ T cells with CD80 and CD86 on APCs and (iii) a specific cytokine milieu that determines the differentiation path (Boyman et al. 2009). Several types of T_H cells have been described, starting in 1986 when T_H1 and T_H2 cells were identified according to the production of characteristic cytokines. Interferon-γ (IFN-γ), IL-2 and tumor necrosis factor (TNF)-α were found to be produced by T_H1 cells, while T_H2 cells produce IL-4, IL-5 and IL-13. The cytokines necessary for undergoing T_H cell differentiation were identified as IL-12 for T_H1 and IL-4 for T_H2 cells (Mosmann et al. 1986, Saravia et al. 2019).

A third subset of T_H cells, namely T_H17 cells, was discovered two decades later. T_H17 cells are characterized by the production of IL-17A, IL-17F and IL-22 (Park et al. 2005). Distinct cytokines have been described to regulate different steps of T_H17 differentiation: (i) transforming growth factor (TGF)-β and IL-6 induce differentiation, (ii) IL-21 facilitates proliferation and (iii) IL-23 stabilizes T_H17 cells (Martinez et al. 2008). In the early 2000s other unconventional T_H cells have been defined: T_H9 cells, which are designated as IL-9 producers, and T_H22 cells, which produce exclusively IL-22. Additional $CD4^+$ T cell subsets are regulatory T cells (T_{regs}), which act immunosuppressive, and follicular T_H cells (T_{FH}), which promote humoral immunity within germinal centres by stimulating B cells (Saravia et al. 2019).

The differentiation of naïve $CD4^+$ T cells into distinct T_H cell subsets is mediated by master transcription factors, which regulate cell fate by either inducing the expression of lineage-specific genes or repressing the expression of genes associated with other lineages. In the last two decades master transcription factors for all T_H cell subsets have been identified (Figure 2): T-bet (T_H1), retinoic acid receptor-related orphan receptor-γt (RORγt) (T_H17), GATA-3 (T_H2), Bcl6 (T_{FH}) and Foxp3 (T_{regs}) (Saravia et al. 2019).

Upon elimination of the infection, the initial resting state of the immune system is restored as the majority of effector $CD4^+$ T cells dies. A small fraction of $CD4^+$ T cells survives to become memory cells. However, their role in protective responses and memory function is still not fully understood (Gasper et al. 2014).

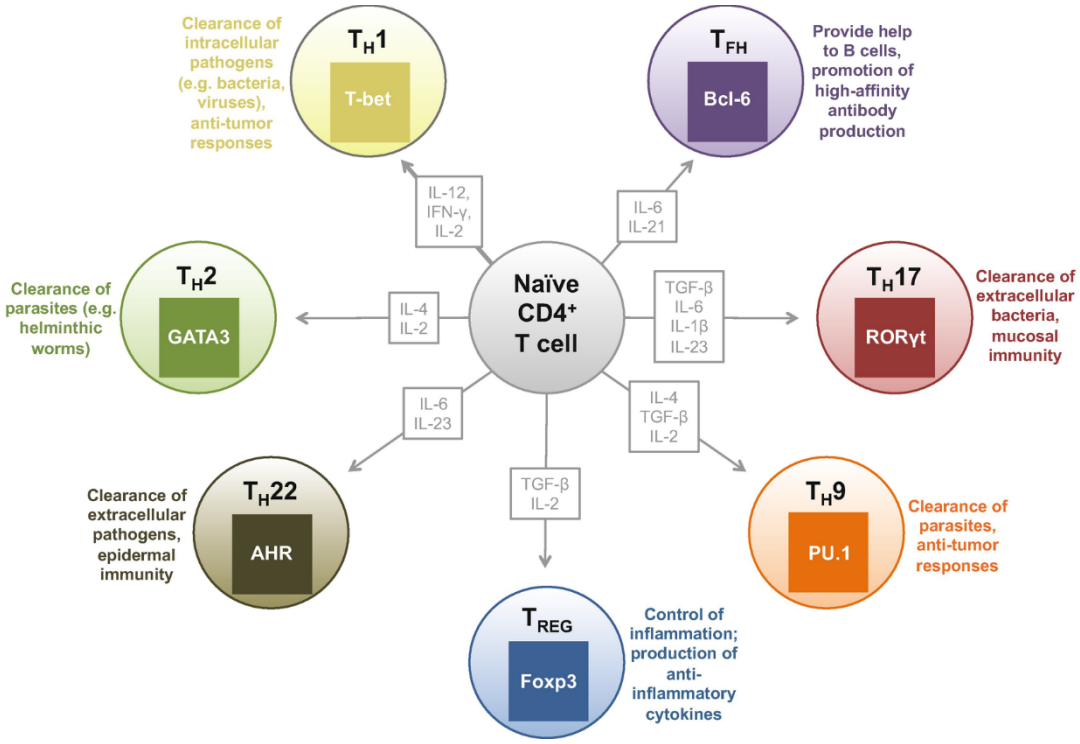

Fig.2: Schematic representation of T_H regulatory factors, lineage-defining transcription factors and responses (Read et al. 2019).

1.1.2 The role of T_H cell subsets in immunity

T_H1 cells mainly promote macrophage activation and clearance of intracellular pathogens by producing IFN-γ. Activated macrophages produce cytokines, such as IL-12 and IFN-γ, which favour the differentiation of T_H1 cells (Romagnani 1995, Zhu and Paul 2010). IFN-γ also affects non-leukocytes and induces the secretion of proinflammatory cytokines, such as TNF, and chemokines. In addition, T_H1 cells influence endothelial cells, by causing them to express adhesion molecules and induce retraction and vascular smooth-muscle relaxation, which leads to the cardinal signs of inflammation (Spellberg and Edwards 2001, Cosmi et al. 2014). Contrary to their protective function, T_H1 cells can promote immunopathologies, such as organ-specific autoimmunity, contact dermatitis and chronic inflammatory disorders (Cosmi et al. 2014).

The T_H17 subset differs substantially from T_H1 cells, as they display more plasticity in phenotype and function. They play a key role in the defense against pathogens of fungal or bacterial origin. In addition, T_H17 cells induce the production of T cell-dependent immunoglobulin (Ig)A antibodies by B-cells, providing mucosal immunity, particularly within Peyer´s patches in the gut. However, it has been documented that T_H17 cells also play a pathogenic role in inflammatory disorders, such as experimental autoimmune encephalomyelitis (EAE) and rheumatoid arthritis (Stockinger and Omenetti 2017).

T_H2 cell responses are linked to the defence against extracellular parasites and allergies. In addition, they affect the regulation of humoral immunity by activating B cells and promoting antibody production (Zhu 2018). Main functions of T_{regs} are to maintain tolerance to self-antigen and modulate the immune system in several scenarios, such as tumour immunity, autoimmunity, allergy and inflammation (Gershon and Kondo 1971, Barbi et al. 2014). T_{FH} are necessary for the formation and maintenance of germinal centres (GCs) and support the production of most memory B cells and plasma cells (Crotty 2011).

$CD4^+$ T cells can also display cytotoxic characteristics by secretion of cytotoxic granules containing granzyme B and perforin. $CD4^+$ cytotoxic T lymphocytes (CTLs) can develop from almost all T_H effector subsets (except T_{FH}), however most of CTL derive from T_H1. In humans and mice $CD4^+$ CTLs have been mostly observed during viral infections and contribute to an antiviral immune response (Zaunders et al. 2004, Juno et al. 2017, Takeuchi and Saito 2017).

1.2 Signal transducer and activator of transcription 1 (STAT1)

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway was discovered in the early 1990s (Darnell et al. 1994). In mammals, the JAK family consists of four members: JAK1-3 and tyrosine kinase (TYK) 2. The STAT family includes seven proteins: STAT1-4, STAT5A, STAT5B and STAT6. The highly evolutionary conserved JAK/STAT pathway is activated through extracellular signalling proteins, such as cytokines, growth factors (Schindler et al. 2007, Abroun et al. 2015) and hormones (Dehkhoda et al. 2018).

STAT1 was discovered in 1992 and is a key transcription factor downstream of all types of interferons (IFNs) (Schindler et al. 1992, Stark and Darnell 2012). IFNs can be divided into

type I IFNs (IFN-α subtypes, IFN-β, IFN-κ, IFN-ε, IFN-δ, IFN-τ), type II IFN (IFN-γ) and type III IFNs (IFN-λ subtypes) (Wang et al. 2017). STAT1 is also activated by other cytokines, such as interleukin-21 (IL-21), IL-27, IL-35 and IL-26 (only in humans) (Meissl et al. 2017, Hammaren et al. 2019). Each type of IFN binds to a specific type of cell surface receptor (Figure 3). This results in auto- and/or trans-phosphorylation of JAKs. STATs are then activated by phosphorylation and translocate to the nucleus as homo- or heterodimers. STAT1/STAT2 heterodimers associate with the IFN-regulatory factor 9 (IRF9) to form the IFN-stimulated gene factor 3 (ISGF3), which binds to consensus sequences in regulatory regions of target genes known as interferon-stimulated response elements (ISREs). STAT1 homodimers, which mainly get activated in response to type II IFN, bind to IFNγ-activation sites (GAS) (Levy and Darnell 2002, Stark et al. 2018). STAT1 regulates various different cellular activities, such as differentiation, apoptosis and proliferation (Mui 1999).

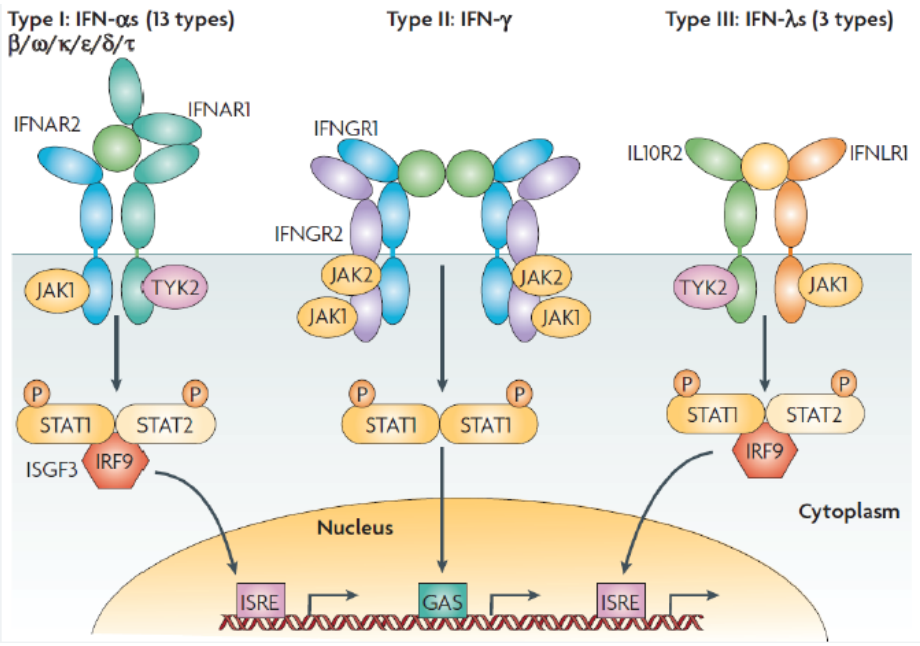

Fig. 3: Signalling pathway by different types of IFNs (Wang et al. 2017).

The *STAT1* gene has been identified to undergo both loss-of-function (LOF) and gain-of-function (GOF) mutations in humans, which both cause severe immune diseases (O'Shea et al. 2015). LOF of *STAT1* results in a higher susceptibility towards infections with mycobacteria

and viruses in humans and many bacterial and viral pathogens in mice, which reflects the impairment of type I and type II IFN- mediated immunity. In contrast, GOF mutations of *STAT1* in humans cause severe autoimmune diseases and high susceptibility to candidiasis, which has been linked to an increase in the responses to type I and type II IFNs, and possibly IL-21, and impaired IL-17-dependent immunity, respectively (Boisson-Dupuis et al. 2012).

STAT1 occurs in two isoforms, which are generated by alternative splicing. The full-length STAT1α (91kDa) and the truncated STAT1β (84kDa), which is missing the C-terminal transactivation domain (TAD) (Zakharova et al. 2003). For a long time, it was believed that STAT1β is transcriptionally inactive as a homodimer. Using mice that express only STAT1α or only STAT1β, our lab demonstrated that STAT1β is not an inactive isoform but is capable of inducing target genes in response to type II IFN and to confer antibacterial immunity *in vivo*, albeit to a considerably lower level than STAT1α. Mice expressing only STAT1α did not differ from wild-type mice with respect to type I and type II IFN responses of macrophages and innate immunity to bacterial and viral infections (Semper et al. 2014), raising the question about the physiologic significance of the STAT1β isoform.

2. Aim of the study

Previous studies in our lab established that the absence of STAT1β results in an increase in STAT1α protein levels in $CD4^+$ and $CD8^+$ T cells but not in macrophages, B cells and NK cells. In $CD4^+$ T cells, the increase in STAT1α levels correlated with an increase in STAT1-dependent gene induction compared to wild-type cells and an increase in IFN-γ production upon TCR-activation (A. Puga and K. Meissl, unpublished). This prompted the hypothesis that STAT1β may be needed to prevent excessive T_H1 differentiation and to enable a balanced T_H cell response. Aim of this study was to investigate the impact of STAT1β on T_H1 and T_H17 differentiation *in vitro*. The goal was to activate naïve $CD4^+$ T cells from mice that only express STAT1α (*Stat1*$^{\alpha/\alpha}$) and wild-type (*WT*) mice under T_H1/T_H17-polarizing or non-polarizing (T_H0) conditions and analyse the levels of signature cytokines (IFN-γ, IL-17A) and transcription factors (T-bet, RORγt) by flow cytometry. In addition, mRNA levels of other factors that are characteristic for specific T_H cell subsets should be analysed by RT-qPCR.

3. Material and Methods

3.1 Material

Table 1. Equipment		
Name	**Company**	**Identifier**
TC-Plate 48 well	Sarstedt	Cat. #83.3923
QuadroMACS separation unit	Miltenyi	Cat. #130-090-976
LS Columns	Miltenyi	Cat. #130-042-401
Pre-Separation filter 70 µl	Miltenyi	Cat. #130-095-823
MACS Smart strainer	Miltenyi	Cat. #130-098-463

Table 2. Buffers and media		
Name	**Company**	**Identifier**
Dulbecco´s Phosphate Buffered Saline (PBS)	Sigma-Aldrich	Cat. #D8537
RPMI-1640 Medium	Sigma-Aldrich	Cat. #R8758
autoMACS Running buffer	Miltenyi	Cat. #130-091-221
Fixation buffer	BioLegend	Cat. #420801
Permeabilization buffer	BioLegend	Cat. #421002

Table 3. Chemicals, reagents and commercial assays		
Name	**Company**	**Identifier**
Trypan Blue solution	Sigma-Aldrich	Cat. #T8154
Brefeldin A	Thermo Fisher	Cat. #00-4506-51
Red Blood Cell Lysing buffer Hybri-Max	Sigma-Aldrich	Cat. #R7757
Naïve $CD4^+$ T Cell Isolation Kit	Miltenyi	Cat. #130-104-453
Fetal Calf Serum (FCS)	Gibco	Cat. #10270-098
peqGOLD TriFast	VWR	Cat.#30-2010
Chloroform	Carl Roth	Cat. #3313.2
Isopropanol	Carl Roth	Cat. #6725.2
Ethanol	Scharlau	Cat. #ET00051000
Diethyl pyrocarbonate (DEPC)	Carl Roth	Cat. #K028

iSrcipt First Strand cDNA Synthesis Kit	Bio-Rad	Cat. #170-8891
$MgCl_2$ 25mM	Solis BioDyne	Cat. #05-11-00025
HOT FIREPol DNA Polymerase	Solis BioDyne	Cat. #01-02-01000
dNTP mix, 100 mM	Thermo Fisher	Cat. #R0182
10 x Reaction buffer B	Solis BioDyne	Cat. #01-02-01000
DNase I, RNase-free (1 U/µL)	Thermo Fisher	Cat. #EN0521
Cell Activation Cocktail (with Brefeldin A)	Biolegend	Cat. #423304
IL-2	PeproTech	Cat. #AF-200-02
Anti-IL-4 Ab	BD Biosciences	Cat. #554434
IL-6	Biolegend	Cat. #575702
IL-12	PeproTech	Cat. #210-12-A
TGFβ	R&D Systems	Cat. #240-B-002
Anti-CD3 Ab	BD Biosciences	Cat. # 553057
Anti-CD28 Ab	BD Biosciences	Cat. #553294

Table 4. Flow cytometry antibodies and viability dye

Name	**Fluorochrome**	**Clone**	**Company**	**Identifier**
Anti-T-bet Ab	APC	REA102	Miltenyi	Cat. #130-119-821
Anti-INFγ Ab	FITC	REA630	Miltenyi	Cat. #130-117-668
Anti-IL17A Ab	FITC	REA660	Miltenyi	Cat. #130-111-856
Anti-Rorγt Ab	APC	REA278	Miltenyi	Cat. #130-123-840
Anti-TCRβ Ab	PerCp	REA318	Miltenyi	Cat. #130-120-827
Anti-CD3ε Ab	PerCp	REA606	Miltenyi	Cat. #130-119-656
Anti-CD4 Ab	PB	REA604	Miltenyi	Cat. #130-118-568
Anti-CD44 Ab	PerCp	IM7	Thermo Fisher	Cat. #45-0441-82
Anti-CD62L Ab	PE	MEL-14	Thermo Fisher	Cat. #12-0621-82
Fixable viability dye	APC-Cy7	N/A	Thermo Fisher	Cat. #65-0865-18

Table 5. Mice

Strain	**Company/Source**	**Identifier**
WT	The Jacksons Laboratory	C57BL/6N
Stat1$^{\alpha/\alpha}$	N/A	(Semper et al. 2014)

Table 6. qPCR primers, assays and dye		
Name	**Company**	**Identifier**
Ube2d2-fwd 5'-AGG TCC TGT TGG AGA TGA TAT GTT-3'	Sigma Aldrich	N/A
Ube2d2-rev 5'-TTG GGA AAT GAA TTG TCA AGA AA-3'		N/A
Ube2d2-probe 5'-[6FAM]CCA AAT GAC AGC CCC TAT CAG GGT GG[BHQ1]-3'		N/A
Ifng-fwd 5'-TGA GTA TTG CCA AGT TTG AGG TCA-3'		N/A
Ifng-rev 5'-CGG CAA CAG CTG GTG GAC-3'		N/A
Ifng-probe 5'-[6FAM]CCA GCG CCA AGC ATT CAA TGA GCT[BHQ1]-3'		N/A
Stat1-fwd 5'-GAT CAG CTG CAA AGC TGG TTC-3'		N/A
Stat1-rev 5'-GCT TTT TAA GCT GCT GAC GGA-3'		N/A
Stat1-probe 5'-[6FAM]-CCA TTG TTG CAG AGA CC-3'		N/A
Mm_*Tbx21*_1_SG QuantiTect Primer Assay		QT00129822
Mm_*Eomes*_1_SG QuantiTect Primer Assay		QT01074332
Mm_*Gzmb*_1_SG QuantiTect Primer Assay	Qiagen	QT00114590
Mm_*Crtam*_1_SG QuantiTect Primer Assay		QT00119637
Mm_*Bcl6*_1_SG QuantiTect Primer Assay		QT01057196
Mm_*Prf1*_1_SG QuantiTect Primer Assay		QT00282002
EvaGreen Dye	Biotium	Cat. #31000

Table 7. Software		
Name	**Company**	**Identifier**
Prism 8.1.2	GraphPad Software	N/A
FlowJo	Becton, Dickinson & Company	N/A

3.2 Methods

3.2.1 Mice

Stat1$^{\alpha/\alpha}$ mice were described previously (Semper et al. 2014). Wild type (*WT*) mice were purchased from The Jacksons laboratory. All experiments were performed with age- and sex-matched (six-nine weeks old) mice on C57BL/6 background. Mice were bred and maintained at the University of Veterinary Medicine Vienna under specific pathogen-free conditions according to Federation of European Laboratory Animal Science Associations (FELASA) guidelines. Handling of mice was executed by trained personnel.

3.2.2 Experimental set up

Inguinal, axillary, brachial, mandibular, mesenteric, lumbar lymph nodes (LN) and spleen of *WT* and *Stat1*$^{\alpha/\alpha}$ mice were isolated after carbon dioxide (CO_2) euthanasia and cervical dislocation. Subsequently, $CD4^+$ T cells were isolated from the harvested organs. Naïve $CD4^+$ cells from two mice were pooled and T cells were activated *in vitro* under non-polarising conditions (T_H0) and polarising conditions (T_H1 and T_H17). Thereafter, cells were stimulated (3 h) for cytokine production using PMA (phorbol 12-myristate-13-acetate) and ionomycin in the presence of the protein transport inhibitor Brefeldin A (BFA). T_H cells were then either analysed by flow cytometry or used to isolate total RNA for subsequent RT-qPCR analysis.

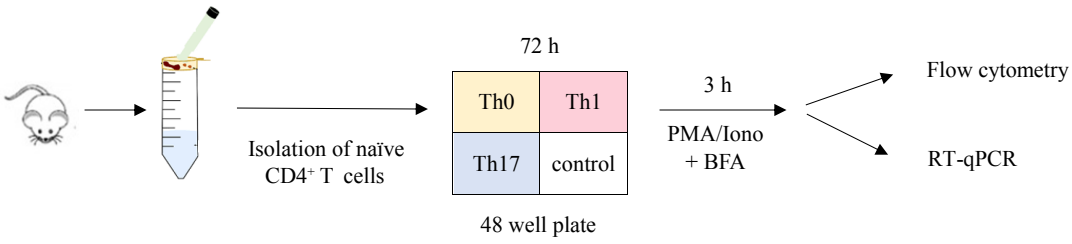

Fig.4: Schematic illustration of the experimental setup

3.2.3 *In vitro* T_H cell differentiation

3.2.3.1 Coating of plates

For *in vitro* polarisation into T_H0, T_H1 and T_H17 cells, a 48-well plate was coated with 250 µL/well of anti-CD3 (1 µg/mL) and anti-CD28 (3 µg/mL) antibody in PBS. After closing the plate with parafilm it was incubated overnight at 4 °C.

3.2.3.2 Isolation of naïve $CD4^+$ T cells

After isolation LN and spleen were stored in PBS on ice until further use. Subsequently, LNs and spleen were put in cell strainers on top of a 50 mL Falcon tube and mashed through a 100 µm strainer with the flat part of a syringe plunger. The cell strainer was rinsed with 20 mL PBS and cells were centrifuged at 300 x g for 5 min. This set up was kept the same for all centrifugation steps. The pellet was resuspended in 0.5 mL red blood cell lysis buffer and incubated 3 minutes at room temperature. Erythrolysis was stopped by adding 10 mL PBS. The cell suspension was filtered through a cell strainer into a 50 mL Falcon tube. Cells were centrifuged and resuspended in 10 mL PBS. A 20 µL aliquot was used for cell counting. The aliquot was mixed 1:1 with Trypan Blue and cells were counted on a TC20 automated cell counter (Bio-Rad).

$CD4^+$ T cell isolation was continued using a kit for naïve $CD4^+$ T cell isolation according to manufacturer's instructions (Miltenyi). Briefly, cells were centrifuged and resuspended in 40 µL MACS buffer per 10^7 cells. 10 µL of Biotin-antibody cocktail per 10^7 cells was added, mixed well and incubated at 4 °C for 5 min. 20 µL of MACS buffer per 10^7 cells was added, followed by 20 µL of anti-Biotin MicroBeads and 10 µL of anti-CD44 MicroBeads per 10^7 cells. The suspension was mixed and incubated for 5 min at 4 °C. After incubation, 2 mL of MACS buffer was added per sample. LS columns with pre-separation filters were placed on a MACS Separator and each column was primed with 3 mL MACS buffer. Samples were filtered through the column, which were washed twice with 3 mL MACS buffer. The flow-through containing naïve $CD4^+$ T cells was collected, centrifuged and resuspended in 1 mL of T-cell medium [RPMI containing 10 % foetal calf serum (FCS), penicillin (100 µg/mL), streptomycin (100 U/mL) and β-mercaptoethanol (50 µM)]. Cells were counted as previously described and

diluted in T cell medium. 0.25×10^6 cells per well were plated into the pre-coated plate in 500 µL of T cell medium.

3.2.3.3 T_H cell differentiation

In order to polarize cells into specific T_H cell subset, 500 µL of the polarization medium (containing T cell medium and cytokines, Table 8.) was added to the cells. Cells were incubated for three days at 37 °C, 5 % CO_2. Thereafter, stimulation with a cell activation cocktail containing PMA, Ionomycin and BFA, was performed according to manufacturer's instructions. Briefly, 500 µL of the cell supernatant was removed without touching the bottom of the well and then 500 µL of T cell medium containing cell activation cocktail (2X) was added. Cells were incubated for 3 h at 37 °C, 5 % CO_2 and then used for flow cytometry or isolation total RNA.

Table 8. Polarisation medium

T_H0	**Final concentration**
IL-2	2 ng/mL
T_H1	**Final concentration**
IL-2	2 ng/mL
Anti-IL-4 Ab	3 µg/mL
IL-12	5 ng/mL
T_H17	**Final concentration**
TGF-β	1 ng/mL
IL-6	20 ng/mL

3.2.4 Phenotypic characterisation of $CD4^+$ T helper cell subsets

3.2.4.1 Flow cytometry

After incubation, cells were resuspended inside the well and collected. Each well was additionally washed with 1 mL of PBS and suspension was centrifuged 5 min at 300 x g. For surface staining, 50 µL of antibody mix [anti-TCRβ Ab (1:50), anti-CD3ε Ab (1:50), anti-CD4 Ab (1:50) and viability dye (1:1000)] was added and the cells were incubated for 15 min at 4 °C (light protected). Cells were washed with 1 mL of PBS followed by a 5 min centrifugation step at 300 x g. The cells were then vortexed and 250 µL per sample of fixation buffer was added. Cells were incubated with fixation buffer for 15 min at 4 °C (protected from light). Thereafter, cells were permeabilized with 2 mL of 1X permeabilization buffer and centrifuged for 5 min at 350 x g. For intracellular staining, 100 µL antibody mix was added and cells were incubated for 30 min at room temperature (protected from light). For T_H1 cells, anti-T-bet Ab (1:50) and anti-INF-γ Ab (1:50) and for T_H17 anti-IL-17A Ab (1:50) and anti-Rorγt Ab (1:50) was used. Subsequently, 2 mL PBS was added and the cells were centrifuged for 5 min at 300 x g. The supernatant was removed and cells were immediately processed for flow cytometry analysis (Cytoflex, Beckman Coulter).

3.2.4.2 Gating strategy

After isolation of naïve $CD4^+$ T cells, the purity was analysed by FACS (Figure 5). First duplets and dead cells were gated out. Naïve $CD4^+$ T cells were characterized as $TCR\beta^+CD8^-CD4^+CD62L^+CD44^-$. Purity of naïve cells was typically ~85%.

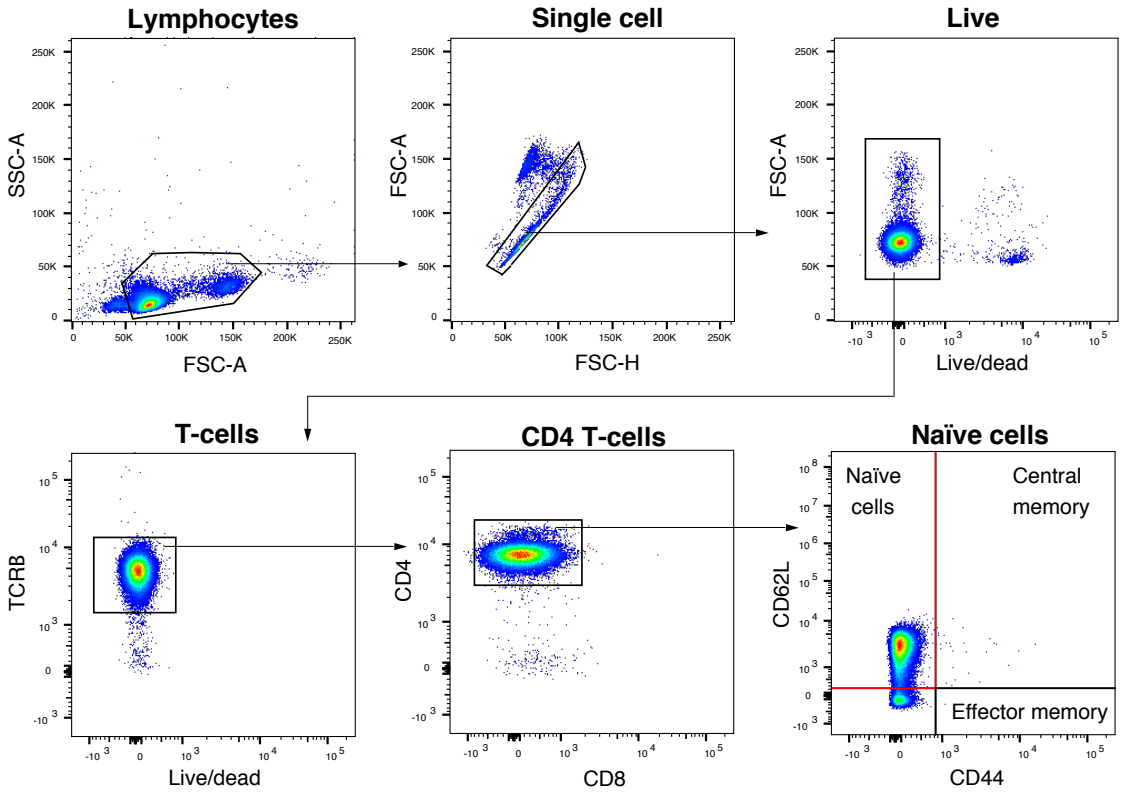

Fig.5: Purity of negative fraction after separation with LS columns was checked using flow cytometry.

FACS analysis (Cytoflex, Beckman Coulter) was performed with at least 0.25 x 10^6 cells. First duplets and dead cells were gated out. $CD4^+$ T cell were defined $TCR\beta^+CD3\varepsilon^+CD4^+$ cells. The MdFI of transcription factors (T-bet and RORγt) and production signature cytokines (IFN-γ and IL-17A) were quantified out of $CD4^+$ T cells (Figure 6).

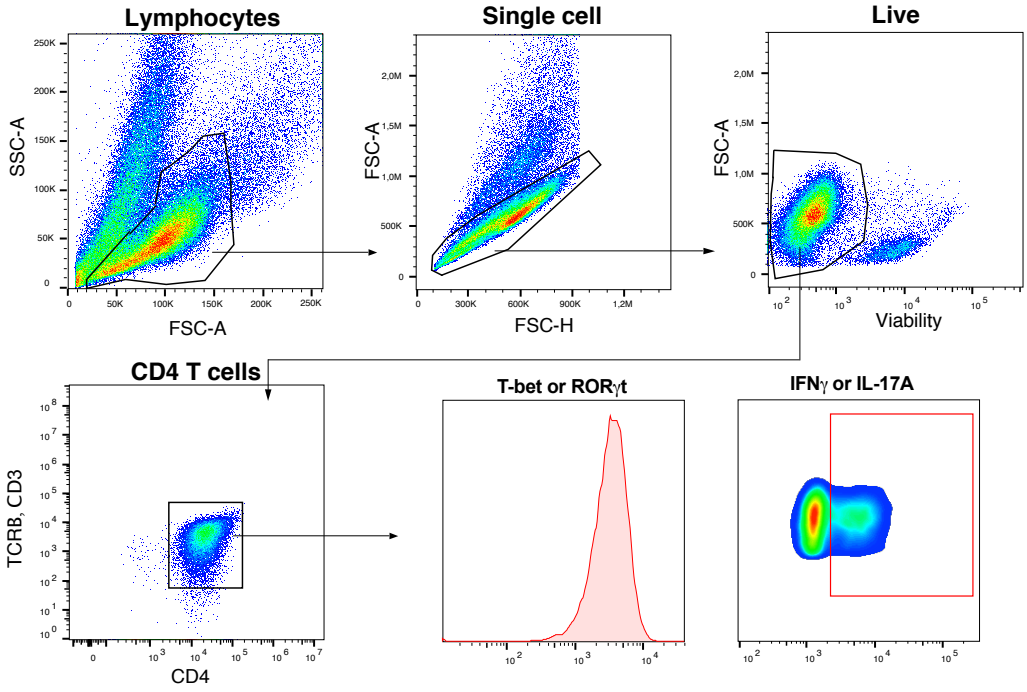

Fig.6: Gating strategy for $CD4^+$ T cells

3.2.4.3 RNA isolation and RT-qPCR

Total RNA was isolated using TriFast reagent according to the manufacturer´s instructions. A NanoDrop microvolume spectrophotometer was used to measure RNA quality and concentration. With 1 µg of RNA reverse, transcription was performed using the iScript First Strand cDNA Synthesis Kit. For the reverse transcriptase (RT-) negative control, 0.5 µg RNA were used. If required (*Prf1*), potential DNA contaminations were removed by digesting 1.5 µg total RNA per sample using Thermo Fisher DNase Treatment Kit. Samples were diluted 1:3 with RNase-free water (samples treated with DNase were used undiluted) and stored at -20 °C until further use. Quantitative PCR (qPCR) was performed to determine the expression of genes of interest (*Stat1, Tbx21, Ifng , Eomes, Crtam, Bcl6, Gzmb* and *Prf1*) (Table 6). To confirm that

genomic DNA is not amplified in RT- negative samples, a pre-run was performed for every gene. The qPCR mastermix consisted of 4 mM $MgCl_2$, 100 nM Evagreen® or FAM-labelled probe, 1 U/rxn Hotfire polymerase, 200 µM dNTP mix and 1x Hotfire B buffer. For genes analysed with Qiagen assays, the master mix contained 2.5 mM $MgCl_2$. 18 µL of RT-qPCR master mix and 2 µL cDNA or water (for no template controls; NTC) were used for each reaction and all samples were analysed in duplicates. The qPCR program consisted of 15 min 95 °C and 40 cycles of 95 °C for 20 sec followed by 60 °C for 1 min. For every gene, a standard curve was generated using a serial 4-fold dilution of the sample with the lowest C_T values (determined beforehand in a pre-run). The baseline threshold for each run was set at the relative fluorescence (RFU) of 300 on a logarithmic scale. Gene expression was calculated relative to the housekeeping gene (HKG) *Ube2d2*. Following criteria were set up as quality controls: differences of C_T values of duplicates ≤ 0.5, correlation coefficient (R^2) of the standard curve ≥ 0.99 and qPCR efficiencies (E) between 86 % and 118 %.

3.2.5 Statistical analysis

Statistical analysis (t-test) was performed using GraphPad Prism 8.1.2. Differences were interpreted as significant if a p-value ≤ 0.05 was reached (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

4. Results

To analyse how the absence of STAT1β influences the differentiation of T_H cells, naïve $CD4^+$ T cells were isolated from *WT* mice and mice lacking the STAT1β isoform (*Stat1*$^{\alpha/\alpha}$) and differentiated *in vitro* into T_H1 or T_H17 cells or activated under non-polarizing conditions (T_H0) for 3 days. Thereafter, cells were stimulated for 3 h with PMA/Ionomycin in the presence of BFA to enable the analysis of intracellular cytokine levels by flow cytometry. In addition, the abundance of signature transcription factors was analysed by flow cytometry. In a separate set of experiments, total RNA was isolated from T_H0 and T_H1 cells and the expression of T_H subset-specific genes (e.g. *Ifng, Tbx21, Eomes, Bcl6, Crtam*) was determined by RT-qPCR.

4.1. Absence of STAT1β results in increased IFN-γ production and *Prf1* expression upon T_H1 cell differentiation

Previous studies in the laboratory have shown that $CD4^+$ T cells from *Stat1*$^{\alpha/\alpha}$ mice produce more IFN-γ upon TCR-mediated activation than $CD4^+$ T cells from wild-type mice, irrespectively of whether they are activated in the presence or absence of T_H1-polarizing cytokines. We next wanted to test whether this correlates with an increased expression of the T_H1 master transcription factor T-bet. We thus isolated naive $CD4^+$ T cells from *Stat1*$^{\alpha/\alpha}$ and *WT* mice, activated them under T_H1-polarizing conditions and analysed intracellular levels of T-bet and IFN-γ by flow cytometry. Surprisingly, the median fluorescence intensity (MdFI) of T-bet was significantly decreased in *Stat1*$^{\alpha/\alpha}$ compared to *WT* cells (Figure 7A), whereas IFN-γ production was significantly increased (Figure 7B). To better understand how the absence of STAT1β affects T_H1 differentiation, we analysed the expression of *Stat1*, *Tbx21* (encodes T-bet) and *Ifng* by RT-qPCR. Since IFN-γ can be produced by $CD4^+$ cytotoxic T lymphocytes ($CD4^+$ CTLs) (Cheroutre and Husain 2013), we also analysed the expression of genes associated with $CD4^+$ CTLs, namely eomesodermin (*Eomes*), granzyme B (*GzmB*), perforin 1 (*Prf1*) and cytotoxic and regulatory T cell molecule (*Crtam*) (Takeuchi and Saito 2017, Preglej et al. 2020). B cell lymphoma 6 (*Bcl6*) was included as a marker for T_{FH} cells (Saravia et al. 2019). Expression of *Stat1*, *Tbx21*, *Ifng, Eomes, GzmB* and *Bcl6* was not significantly changed

in *Stat1*$^{\alpha/\alpha}$ compared to *WT* cells (Figure 7C). In contrast, *Prf1* was upregulated and *Crtam* downregulated in *Stat1*$^{\alpha/\alpha}$ compared to *WT* cells (Figure 7C). Taken together the results suggest that the absence of STAT1β increases the production of IFN-γ but not T-bet under T_H1-polarizing conditions and differentially affects the expression of the $CD4^+$ CTL-associated genes *Prf1* and *Crtam*.

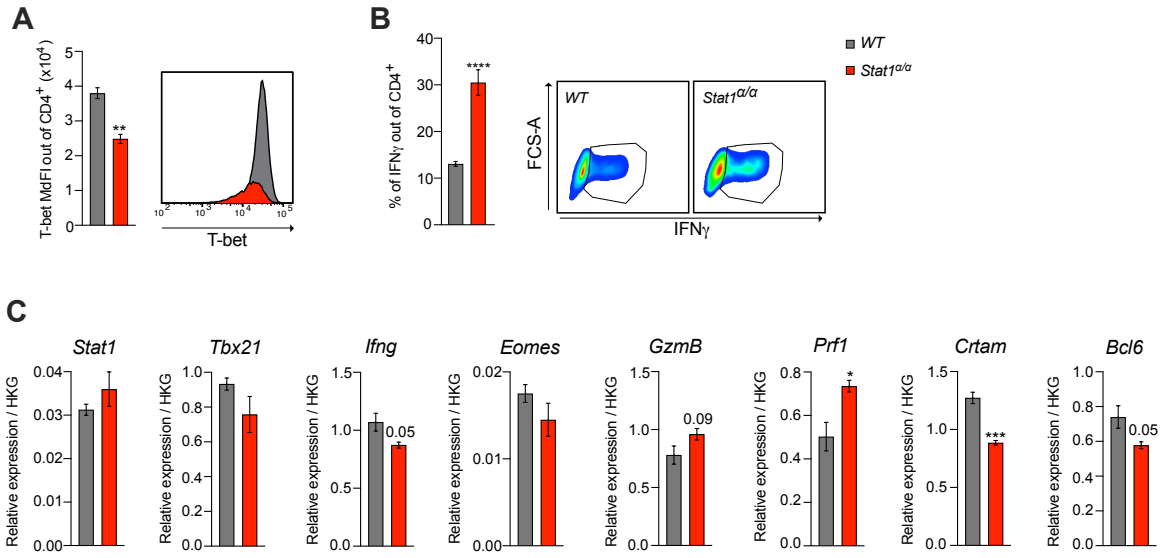

Fig.7: Stat1$^{\alpha/\alpha}$ *cells produce more IFN-γ upon stimulation. Naïve* $CD4^+$ *T cells were isolated from WT and Stat1*$^{\alpha/\alpha}$ *mice and differentiated in vitro into* T_H1 *cells. Cells were stimulated towards cytokine production for 3 h with PMA/ionomycin in the presence of BFA. MdFI of T-bet (**A**) and production of IFN-γ (**B**) in* T_H1 $CD4^+$ *cells. Gene expression (**C**) calculated relative to the HKG Ube2d2. (A-B) n=3, N=2; (C) n=4, N=1; Mean values ± SEM are given. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001 and **** p ≤ 0.0001; p values between 0.05 and 0.1 are indicated. HKG - housekeeping gene. n,replicates; N,experimental repetitions.*

4.2. Absence of STAT1β results in increased expression of T_H1 markers and $CD4^+$ CTL-associated genes upon activation under non-polarizing conditions

Antigenic stimulation in the presence of IL-2 (T_H0 condition) is sufficient to induce cytotoxic activity in $CD4^+$ T cells. Moreover, cytotoxicity under T_H0 condition is even more enhanced in comparison with T_H1-skewed conditions (Brown et al. 2009, Takeuchi and Saito 2017). Next, we activated $CD4^+$ T cells under T_H0 conditions and analysed the same parameters as in the previous experiments. The levels of T-bet and production of IFN-γ were significantly increased in *Stat1*$^{\alpha/\alpha}$ compared to *WT* cells (Figure 8A and 8B). In addition, we found increased mRNA level of *Stat1*, *Tbx21*, *Ifng, Eomes, GzmB* and *Bcl6* in *Stat1*$^{\alpha/\alpha}$ cells (Figure 8C). *Crtam* mRNA levels were not different between genotypes (Figure 8C). To determine whether the addition of PMA/Ionomycin affects mRNA expression of these genes, we also included cells that were not stimulated with PMA/Ionomycin (Figure 8D). Expression of *Stat1* and *Eomes* remained significantly increased, while mRNA levels of *Tbx21, Crtam and Bcl6* were similar in cells from mice of both genotypes (Figure 8D). Interestingly, level of *Ifng* were even lower in *Stat1*$^{\alpha/\alpha}$ than in *WT* cells (Figure 8D). Collectively, the results suggest that STAT1β not only suppressed T_H1 differentiation but also suppresses the cytotoxic program in $CD4^+$ cells activated under T_H0 conditions.

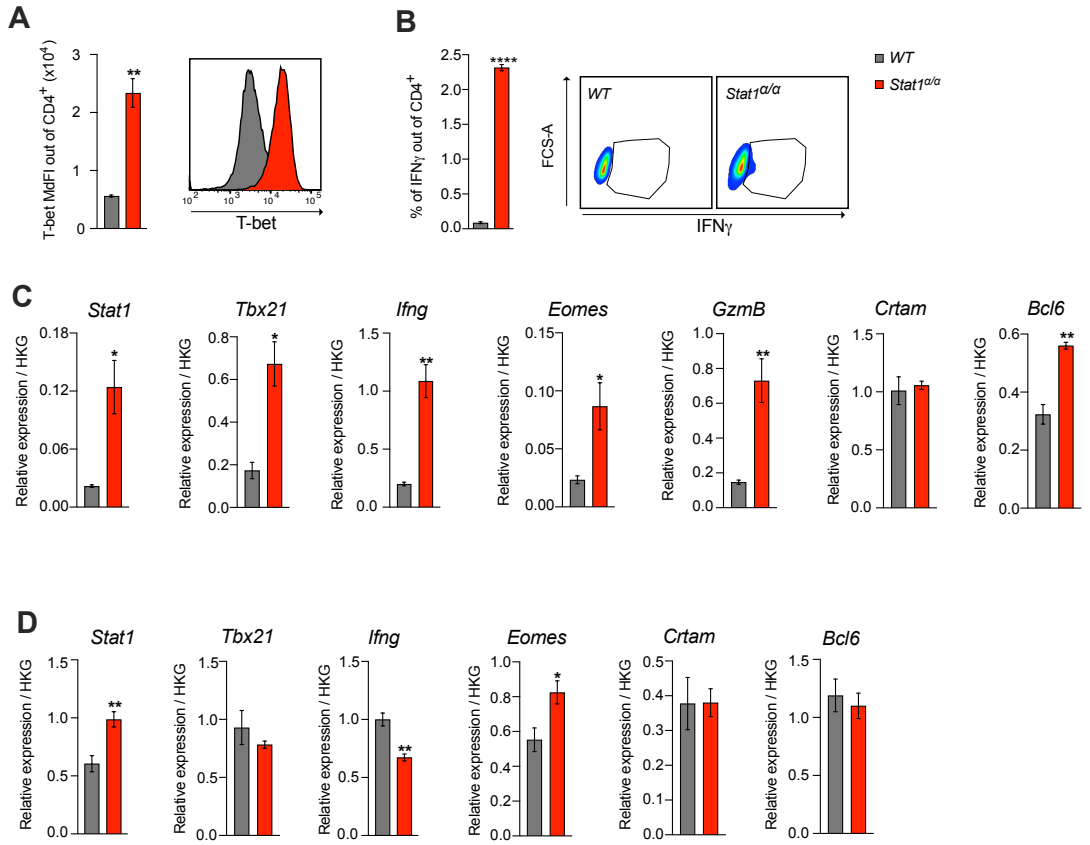

*Fig.8: Stat1$^{\alpha/\alpha}$ cells display an enhanced CTL program. Naïve CD4$^+$ T cells were isolated from WT mice and Stat1$^{\alpha/\alpha}$ mice and activated under non-polarising (T_H0 condition). Cells were stimulated towards cytokine production for 3 h with PMA/ionomycin in the presence of BFA (A-C) or kept in T cell medium without PMA/ionomycin and BFA (D). MdFI of T-bet (**A**) and production of IFN-γ (**B**) of CD4$^+$ T cells activated under T_H0 conditions. Gene expression (**C-D**) calculated relative to the HKG Ube2d2. (A-B) n=3, N=2; (C-D) n=3-4, N=1;Mean values ± SEM are given. * p ≤ 0.05, ** p ≤ 0.01, and **** p ≤ 0.0001. HKG - housekeeping gene. n,replicates; N,experimental repetitions.*

4.3. Absence of STAT1β results in decreased IL-17A production upon activation of CD4$^+$ T cells under T_H17-polarizing conditions.

Studies have shown an increase of IL-17A production in the absence of IFN-γ and *STAT1* GOF patients have an impaired T_H17 differentiation, which is associated with an impaired defence against *Candida* infections (Villarino et al. 2010, Boisson-Dupuis et al. 2012). We thus wanted to investigate the differentiation of naïve CD4$^+$ cells into T_H17 cells in the presence

or absence of STAT1β. The MdFI of the transcription factor RORγt showed no significant differences (Figure 9A), whereas the production of IL-17A was significantly lower in *Stat1*$^{\alpha/\alpha}$ than in *WT* cells (Figure 9B). These data suggest that the absence of STAT1β impairs IL-17A production without affecting the expression of the T_H17 lineage-defining transcription factor RORγt.

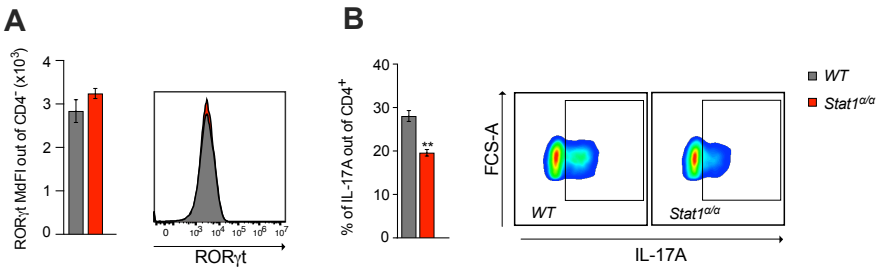

Fig. 9: Stat1$^{\alpha/\alpha}$ *cells have an impaired IL-17A production. Naïve CD4*$^{+}$ *T cells were isolated from WT mice and Stat1*$^{\alpha/\alpha}$ *mice and differentiated in vitro into* T_H17 *cells. Cells were stimulated towards cytokine production for 3 h with PMA/ionomycin in the presence of BFA. MdFI of RORγt (**A**) and production of IL-17A (**B**) in CD4*$^{+}$ *T cells in* T_H17 *condition. (A-B) n=3, N=2;Mean values ± SEM are given. ** p ≤ 0.01. n,replicates; N,experimental repetitions.*

5. Discussion

To better understand the consequences of STAT1β deficiency on $CD4^+$ T_H cell differentiation and cytokine production, we isolated naïve $CD4^+$ T cells from *WT* and *Stat1*$^{\alpha/\alpha}$ mice and differentiated them *in vitro* under T_H1- , T_H17- or non-polarizing (T_H0) conditions.

We show on mRNA and protein level that the absence of STAT1β results in an increased production of IFN-γ and an increase in T-bet levels in T_H0 cells, suggesting that STAT1β deficiency causes a bias towards T_H1 cell differentiation. STAT1 regulates its own expression and protein levels have an impact on cellular responses to cytokines (Gil et al. 2006, Regis et al. 2008, Gough et al. 2010). Previous studies have shown that the absence of STAT1β causes an increase in STAT1α levels, which correlates with an increase in STAT1-dependent transcriptional responses to IL-27 in $CD4^+$ T cells (A. Puga and K. Meissl, unpublished). Although STAT1 is required for optimal T_H1 differentiation (Knosp and Johnston 2012), overactivation results in enhanced T_H1 responses, as evidence by studies with $CD4^+$ T cells from patients harbouring *STAT1* GOF mutations (Marodi et al. 2012, Baris et al. 2016, Weinacht et al. 2017). Thus, it seems likely that STAT1β prevents excessive T_H1 responses by suppressing STAT1α protein levels.

In addition to the increased levels of IFN-γ and T-bet, we observed an upregulation of *Eomes*, which is a characteristic gene for cytotoxic T cells, in STAT1β deficient $CD4^+$ T cells upon activation in the absence or polarizing cytokines (i.e. T_H0 conditions) (Takeuchi and Saito 2017, Preglej et al. 2020). Induction of Eomes in $CD8^+$ T cells was found to be STAT1-dependent (Martinet et al. 2015) and it is possible that a similar regulation occurs in $CD4^+$ CTLs. However, the factors that regulate the differentiation of cytotoxic $CD4^+$ T cells are still incompletely understood (Brown et al. 2009, Takeuchi and Saito 2017). In line with an increased expression of *Eomes*, we also found an upregulation of *GzmB* mRNA in the absence of STAT1β, further supporting the notion that $CD4^+$ T cells show an enhanced differentiation into cytotoxic $CD4^+$ T cells in the absence of STAT1β. We did not observe upregulation of *Crtam*, which is another marker for cytotoxic $CD4^+$ T cells, in the absence of STAT1β. However, as the regulation of CRTAM may occur at the posttranscriptional level, it would be necessary to check the protein level via surface staining in future experiments (Takeuchi et al. 2016, Takeuchi and Saito 2017). Surprisingly, we only observed an increase in IFN-γ but not

in T-bet and *Eomes* in the absence of STAT1β when $CD4^+$ T cells were activated under T_H1 conditions. We speculate that this is most likely due to the maximal expression of these factors already in *WT* cells which may preclude further upregulation in the absence of STAT1β.

Interestingly, we also observed an increase in the expression of *Bcl6*, which is a marker for T_{FH} cells, in the absence of STAT1β upon activation under T_H0 conditions. Recent studies pointed out that GOF mutations of *STAT1* do not only lead to enhanced T_H1 responses, but also increase the amount of T_{FH} cells in the blood (Choi et al. 2013, Weinacht et al. 2017). *Bcl6* attenuates the differentiation of other $CD4^+$ T_H cell lineages by repressing the expression of other master transcription factors (Nakayamada et al. 2014). Lu and colleagues provide evidence for the existence of T_{FH}-like cells, as they showed that T_{FH} cells within germinal centres can express cytokines characteristic for other T_H lineages, such as IL-4, IL-17 and IFN-γ (Lu et al. 2011), indicating plasticity between T_{FH} and other T_H subsets. It remains to be investigated if the absence of STAT1β results in increased differentiation of T_H1 and T_{FH} cells, T_{FH}-like cells that express T-bet and IFN-γ or a combination thereof.

Previously published studies (Irmler et al. 2007, Villarino et al. 2010) showed that increased cytotoxicity and higher abundance of IFN-γ suppresses the cytokine production of T_H17 cells. Moreover, *STAT1* GOF mutations result in an impaired T_H17 differentiation in humans and are associated with an impaired defence against *Candida* infections (Marodi et al. 2012). In line with this, we found decreased IL-17A production in STAT1β deficient $CD4^+$ T cells when differentiated under T_H17-skewing conditions.

Collectively, our data suggests that STAT1β in $CD4^+$ T cells (i) increases T_H1 and suppresses T_H17 differentiation, (ii) upregulates a cytotoxic program in $CD4^+$ T cells upon activation and (iii) may affect T_H cell plasticity.

6. Summary

T lymphocytes are an important part of the adaptive immune system. After development and maturation in the thymus, naïve $CD4^+$ T cells migrate to the periphery to reside in secondary lymphoid organs, such as lymph nodes and spleen. Naïve $CD4^+$ T cells are activated via signals from antigen-presenting cells (APCs) and exposure to cytokines, which direct their differentiation into certain T helper (T_H) cell subsets. The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling pathway has a major role in the polarization of $CD4^+$ T cells. STAT1 acts as a key transcriptional factor downstream of interferon (IFN) signalling. In humans and mice, STAT1 occurs in two isoforms: the full-length STAT1α and the truncated STAT1β. Aim of this study was to investigate the impact of STAT1β on the differentiation of T_H cell subsets using mice that express only the STAT1α isoform (*Stat1*$^{\alpha/\alpha}$ mice) and wild-type (*WT*) mice. Naïve $CD4^+$ T cells were isolated from spleen and lymph nodes of *WT* and *Stat1*$^{\alpha/\alpha}$ mice and differentiated under T_H1-, T_H17- or non-polarizing (T_H0) conditions. The abundance of signature transcription factors (T-bet and RORγt) and cytokines (IFN-γ and IL-17A) was quantified using flow cytometry. In addition, total RNA was isolated from T_H0 and T_H1 cells and the expression of genes associated with T_H1 (*Stat1*, *Ifng, Tbx21)* T_{FH} (*Bcl6*) and cytotoxic $CD4^+$ T cells (*Gzmb, Prf1 and Crtam*) was determined by RT-qPCR. Our results show an increase in IFN-γ production in both T_H1 and T_H0 conditions in *Stat1*$^{\alpha/\alpha}$ compared to *WT* cells, indicating increased T_H1 differentiation in the absence of STAT1β. Moreover, we found an increased expression of genes related to a cytotoxic program upon activation of *Stat1*$^{\alpha/\alpha}$ $CD4^+$ T cells under T_H0 conditions, suggesting that STAT1β may also inhibit the differentiation of cytotoxic $CD4^+$ T cells. The absence of STAT1β also resulted in an impaired production of IL-17A upon activation under T_H17-polarising conditions. Collectively, our data suggest that the absence of STAT1β disbalances T_H cell differentiation towards an increase in T_H1 cells, $CD4^+$ CTLs and possibly T_{FH}, and a decrease in T_H17 cell programs.

7. Zusammenfassung

T-Lymphozyten sind ein wichtiger Bestandteil des adaptiven Immunsystems. Nach der Entwicklung und Reifung im Thymus wandern naive $CD4^+$ T-Zellen in die Peripherie, um sich in sekundären lymphoiden Organen wie Lymphknoten und Milz anzusiedeln. Naive $CD4^+$ T-Zellen werden über Signale von Antigen-präsentierenden Zellen und durch Zytokine, die die Differenzierung in bestimmte T-Helfer (T_H) Zell-Untergruppen steuern, aktiviert. Der Janus Kinase (JAK)/ „signal transducer and activator of transcription" (STAT) Signalweg spielt eine wichtige Rolle bei der Polarisierung von $CD4^+$ T-Zellen. STAT1 fungiert als Schlüsseltranskriptionsfaktor in der Interferon (IFN) - Signalübertragung. Bei Menschen und Mäusen existiert STAT1 in zwei Isoformen: STAT1α (volle Länge) und STAT1β (verkürzt). Ziel dieser Studie war es, den Einfluss von STAT1β auf die Differenzierung von T_H-Zell-Untergruppen unter Verwendung von STAT1α-Isoform (*Stat1*$^{\alpha/\alpha}$)- und Wildtyp (*WT*)-Mäusen zu untersuchen. Naive $CD4^+$ T-Zellen wurden aus Milz und Lymphknoten von *WT* und *Stat1*$^{\alpha/\alpha}$ Mäusen isoliert und unter T_H1-, T_H17- oder nicht polarisierenden (T_H0) Bedingungen differenziert. Die Menge an charakteristischen Transkriptionsfaktoren (T-bet und RORγt) und Zytokinen (IFN-γ und IL-17A) wurde mittels Durchflusszytometrie quantifiziert. Zusätzlich wurde totale RNA aus T_H0- und T_H1-Zellen isoliert und mittels RT-qPCR die Expression von Genen, die mit T_H1- (*Stat1*, *Ifng, Tbx21*) T_{FH}- (*Bcl-6*) oder cytotoxischen $CD4^+$ T-Zellen (*Gzmb, Prf1* und *Crtam*) assoziiert sind, bestimmt. Unsere Ergebnisse zeigen sowohl unter T_H1- als auch unter T_H0-Bedingungen einen Anstieg der IFN-γ-Produktion in *Stat1*$^{\alpha/\alpha}$ im Vergleich zu *WT*-Zellen, was auf eine erhöhte T_H1-Differenzierung in Abwesenheit von STAT1β hinweist. Darüber hinaus fanden wir bei Aktivierung von *Stat1*$^{\alpha/\alpha}$-$CD4^+$ T-Zellen unter T_H0-Bedingungen eine erhöhte Expression von Genen, die mit einem zytotoxischen Programm zusammenhängen, was darauf hindeutet, dass die Abwesenheit von STAT1β auch die Differenzierung von zytotoxischen $CD4^+$ T-Zellen verstärkt. Im Gegensatz dazu führte das Fehlen von STAT1β führte bei Aktivierung unter T_H17-polarisierenden Bedingungen auch zu einer beeinträchtigten Produktion von IL-17A. Zusammengefasst zeigen unsere Daten, dass das Fehlen von STAT1β das Gleichgewicht der Differenzierung von T_H-Zellen beeinflusst, wodurch eine Zunahme von T_H1-Zellen, $CD4^+$ CTLs und möglicherweise T_{FH} und eine Abnahme des T_H17-Zellprogramms entsteht.

Abbreviations

Ab … antiboby

APC … antigen-presenting cell

Bcl6 … B cell lymphoma 6

BFA … brefeldin A

CADM1 … cell adhesion molecule-1

CD … cluster of differentiation

CTL … cytotoxic T lymphocytes

CRTAM … MHC class-I related T cell-associated molecule

DEPC … diethyl pyrocarbonate

Eomes … eomesodermin

FCS … fetal calf serum

FELASA … Federation of European Laboratory Animal Science Associations

Fig … Figure

GAS … IFN-γ-activated sequences

GC … germinal centres

GOF … gain-of-function

Granzyme B … GzmB

HKG … house keeping gene

IFN … interferon

Ig … Immunoglobulin

IL … interleukin

IRF9 … IFN-regulatory factor 9

ISGF3 … IFN-stimulated gene factor 3

IRSE … interferon-stimulated response element

JAK … Janus kinase

LN … lymph node

LOF … loss-of-function

MdFI … median fluorescence intensity

MHC … major histocompatibility complex

NTC … no template control

PBS … phosphate buffered saline

Perforin 1 … Prf1

PMA … phorbol 12-myristate-13-acetate

qPCR … quantitative PCR

R^2 … Correlation coefficient

RFU … relative fluorescence units

RORγt … retinoic acid receptor-related orphan receptor-γt

RT … reverse transcriptase

STAT … signal transducer and activator of transcription

TAD … C-terminal transactivation domain

Tbx21 … T-box transcription factor 21, T-bet

TCR … T cell receptor

T_{FH} … follicular T helper cell

TGF … transforming growth factor

T_H cell … T helper cell

TNF … tumor necrosis factor

T_{reg} … regulatory T cell

TYK 2 … Tyrosine kinase 2

WT … wild type

Tables and Figures

Tables

Figures

Literature

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