IL-10 and the JAK-STAT Pathway in the Regulation of Metabolism and Mucosal Homeostasis

IL-10 and the JAK-STAT pathway in the regulation of metabolism and mucosal homeostasis

A dissertation presented by

Yu Hui Kang

to

the Division of Medical Sciences

in partial fulfillment on the requirements

for the degree of

Doctor of Philosophy

in the Subject of

Immunology

Harvard University

Cambridge, Massachusetts

August 2019

Dissertation Advisor: Scott Snapper Yu Hui Kang

IL-10 and the JAK-STAT pathway in the regulation of metabolism and mucosal homeostasis

Abstract

The JAK-STAT pathway integrates signals from multiple cytokines to elicit a specific output, and aberrations in this pathway contribute to the pathogenesis of multiple diseases such as inflammatory bowel disease (IBD). Interleukin 10 (IL-10), for example, utilizes STAT3 to suppress inflammation, and mutations in IL-10 or its receptor have been associated with IBD. However, it is currently unclear how the JAK-STAT pathway achieves specificity and how it contributes to IBD. Here, we investigate the roles of STAT1 in IBD and STAT3 in IL-10R signaling.

We first investigated how $CD4^+$ T cells use STAT1 to drive intestinal inflammation. In an adoptive transfer model, *Stat1*$^{-/-}$ T cells are unable to expand and induce colitis. This defect is only partially explained by the loss of Type I and II interferon signaling. Mechanistically, *Stat1*$^{-/-}$ T cells display reduced expression of *Nlrc5* and multiple MHC class I molecules, which are inhibitors of NK cell killing. Consequently, NK cell depletion significantly rescues the survival of *Stat1*$^{-/-}$ T cells and restores colitis induction. Interestingly, only *Stat1*$^{-/-}$ T cells that underwent spontaneous proliferation are eliminated, suggesting that NK cells specifically target microbially activated T cells. *Stat1*$^{-/-}$ mice have normal $CD4^+$ T cell numbers as innate STAT1 signaling is required for their elimination. Together, our data points to a critical role for STAT1 in shielding T cells from NK cell mediated cytotoxicity.

Next, we investigated how IL-10 utilizes STAT3 to achieve specificity, hypothesizing that IL-10-STAT3 might exhibit non-canonical functions outside of the nucleus. We show that, in addition to the nucleus, IL-10 also induces activated pSTAT3 in macrophage mitochondria. Upon acute IL-10 stimulation, STAT3 interacted with multiple mitochondrial proteins. This correlated with metabolic changes that indicate enhanced functions of these proteins. STAT3's DNA binding activity is not required

for IL-10 to inhibit pro-inflammatory gene expression, suggesting that non-transcriptional functions of STAT3 are sufficient for IL-10 mediated suppression. However, IL-10 requires STAT3 mediated transcription to enhance anti-inflammatory M2 macrophage polarization, suggesting that IL-10-dependent non-canonical STAT3 acts specifically to regulate the metabolic state during inflammation. Together, our data reveals a novel mechanism of IL-10R-STAT3 signaling that might explain its immunosuppressive effects.

Table of Contents **Page No.**

List of Figures **Page No.**

3. Chapter 3 – The role of mitochondrial STAT3 in IL-10R signaling

Acknowledgements

While this dissertation bears the name of an individual, none of the work presented here is possible without the support of several individuals and organizations. Behind the science is a journey, one of perseverance, joy and often frustration, which have shaped me into the person I've become. I am grateful for the fruits of this journey.

I would first like to thank my dissertation advisor, Dr. Scott Snapper. Not only did Scott teach me a great deal about immunology and scientific research, he also gave me valuable insight into the business of science. He was also constantly patient with me despite my flaws and showed genuine care for me, not just my work. He has also been a fearless advocate for me in the scientific community and is always willing to connect me with the best people where needed for my work. Scott also fostered a wonderful environment to work in, where everyone is always willing to help. All these have been integral to my development as a scientist, and I will always be grateful to Scott.

I would also like to thank my colleagues and mentors in the lab, whom I've been fortunate to work with and learn from throughout graduate school. I'd first like to thank Dr. Amlan Biswas, who taught me how to be a scientist of high integrity and rigor. Amlan has shaped much of my research and has been a constant source of support during the ups and downs of graduate school. I'd also like to thank Dr. Dror Shouval, who has been an excellent example of how basic and clinical research should be integrated. I'd also like to thank my bay mate Michael Field, who has been a great friend and valuable source of technical advice. Dr. Bruce Horwitz, Dr. Naresh Redhu and Evan Conaway and have provided helpful input into my work, and Michelle Dong has helped me greatly with my work. There are also several basic and clinical science colleagues who have been a constant source of inspiration for applied research.

Outside the lab, I'd to thank my group of friends who have provided a home away from home. My immunology classmates, especially Marty, Rong En, Guadalupe, Huiyuan and Deb, have walked this

journey together with me and have made graduate school fun and rewarding. I'm also grateful to Ruggles Baptist Church, who have been a constant source of support and have been integral in helping me remain faithful throughout this journey.

I'd also like to thank several organizations, without whom this would not be possible. I'd like to thank the Harvard Immunology PhD Program for taking me in and committing to providing high quality training. My colleagues at the Harvard Digestive Disease Center have also been provided valuable guidance and a highly collaborative work environment. Finally, I'd like to thank the Agency for Science, Technology and Research in Singapore, who provided financial support for much of my education. I am grateful for their commitment and I look forward to contributing to the Singapore research landscape.

Finally, I'd like to thank my family. I am grateful for my parents, who raised me and supported me all these years. They constantly supported my desire to pursue graduate education even it meant supporting from afar. Most importantly, I'd like to thank my wife Ella, who took the brave decision to marry me and move to Boston during my graduate education. Ella has been my best friend and closest confidante, and she has constantly encouraged me to strive for high quality work. She has seen at my best and at my worst, and much of what I am now can be credited to her. Thank you, and I love you all.

To Ella and my parents

"Great are the works of the LORD, studied by all who delight in them." Psalm 111:2

Chapter 1

Introduction

Chapter 1.1 – The JAK-STAT signaling pathway

The JAK (Janus Kinase) - STAT (Signal Transducers and Activators of Transcription) pathway is one of several major signaling pathways that regulate a wide range of biological processes such as development[1], cell division[2], and immunity[3], with abnormalities in this pathway being associated with a wide spectrum of diseases[4]. In the immune system, it is a major hub integrating various cytokine signals to achieve their desired outcomes.

The discovery of the JAK-STAT signaling pathway began with the discovery of Interferons (IFNs)[5] and the subsequent push to understand their mechanism of action. After identifying the DNA element that conferred responsiveness to IFN- the interferon-stimulated response element (ISRE) – gel-shift assays were performed using this element to identify interferon-stimulated gene factor 3 (ISGF3) as the IFN-induced complex. The ISGF3 complex was subsequently found to consist of STAT1, STAT2, and IRF9[6]. The activation of these STATs were then found to be dependent on tyrosine phosphorylation on the STATs by JAKs[7,8]. Other STAT family members were subsequently identified[9–13].

A large body of work has led up to the current paradigm of JAK-STAT signaling: Upon binding of a cytokine to its receptor, the receptor dimerizes, leading to the apposition of their associated JAKs. This causes the JAKs to phosphorylate and activate each other, as well as specific residues on the receptor tail that then recruit specific STATs. The recruitment of these STATs is followed by their activation by JAK-mediated phosphorylation. These activated STATs homo- or heterodimerize, leave the receptor and translocate to the nucleus where they initiate a specific transcriptional program[14].

The biological importance of JAK-STAT signaling is evident in its association with various diseases. Loss of function mutations in *JAK3*, *TYK2* and *STAT5* are associated with primary immunodeficiency, while loss of function mutations in *STAT1* and *STAT3* are associated with susceptibility to viral/mycobacterial infections and autosomal hyper IgE syndrome respectively[15]. Gain of function (GOF) mutations in the JAK-STAT pathway are also associated with several diseases, such as *STAT1* GOF with chronic mucocutaneous candidiasis and IPEX-like phenotypes[16,17], *STAT3* GOF with

lymphoproliferation and autoimmunity[18] and *JAK2* GOF with myeloproliferative neoplasms such as polycythemia vera[19,20].

Consequently, the JAK-STAT pathway is a major therapeutic target. Ruxolitinib, for example, is a JAK1-JAK2 inhibitor approved for treatment of myeloproliferative neoplasms[21]. Another example is tofacitinib, a JAK1-JAK3 (and to a lesser extent JAK2) inhibitor that is approved for treatment of arthritis and more recently ulcerative colitis[22,23]. Therapies targeting the STATs have so far remained elusive, with several in development[22].

Despite being a seemingly simple pathway, several questions remain unsolved regarding the precise mechanism of JAK-STAT signaling. For example, it is currently unclear how a small number of JAKs and STATs can integrate a wide variety of cytokine inputs to achieve distinct outcomes, as well as how STATs precisely execute their respective transcriptional program[24]. Moreover, studies have emerged describing functions of STATs outside of the classical paradigm, such as roles for unphosphorylated STATs[25] and non-nuclear functions of STATs[26]. Unraveling these complexities will go a long way towards designing effective therapeutic modalities.

Chapter 1.2 – Inflammatory Bowel Disease (IBD)

Inflammatory Bowel Diseases (IBD) represent a group of diseases characterized by chronic inflammation of the gastrointestinal (GI) tract, typically manifested as Crohn's disease (CD) or ulcerative colitis (UC)[27]. CD and UC have distinct presentations, with CD typically displaying discontinuous, transmural inflammation that can occur anywhere along the GI tract and UC typically showing continuous inflammation limited to the mucosa of the rectum and varying amounts of the more proximal colon[27–29]. The incidence of IBD rose steadily in the western world from the middle of the 20th century and it currently affects more than 0.3% of the population. Incidences of IBD in newly industrialized nations are beginning to rise, correlating with a transition towards a westernization of their societies[30].

IBD is the prototypical complex disease, with genetic, environmental, microbial and immune factors all contributing towards its pathogenesis[31]. Genetic analyses have provided much insight into the nature of these factors. These analyses first stemmed from the discovery of *NOD2* as being associated with CD[32,33]. NOD2 is an intracellular sensor of peptidoglycan and is expressed in phagocytes, epithelial cells, Paneth cells and endothelial cells, pointing to a role for innate immunity and microbial responses in CD[34]. Autophagy is also implicated in CD based on the association of SNPs in two autophagy genes *ATG16L1* and *IRGM*[35,36]. In adaptive immunity, a role for Th17 cells has been implicated in both CD and UC based on the association of multiple genes in the IL-23-Th17 axis (*IL23R, JAK2, STAT3,*)[37,38].

These genetic analyses are complemented by various mouse models of IBD, which provide mechanistic insight into how these genes drive disease[39]. For example, mice harboring a myeloid specific deletion of *Stat3* display spontaneous colitis. These myeloid cells produce excessive pro-inflammatory cytokines, suggesting an important role for these cell types in preventing colitis[40]. The colitis phenotype is absent when these *Stat3* mutant mice are also deficient for IL-12p40 or RAG2*,* pointing to a role for proinflammatory T cells in disease pathogenesis[41]. Deletion of the multiple drug resistance gene *mdr1a* also leads to spontaneous colitis, in a manner that is primarily associated with epithelial defects[42]. A role for effector T cells in mediating colitis has also been shown by several groups. These include the

observation that naïve T cells can induce colitis when transferred into lymphopenic mice[43], as well as the develop of spontaneous colitis in mice with defects in regulatory T cell (Treg) generation (*Il2*$^{-/-}$, *Cd25*$^{-/-}$) [44,45]. These models have also pointed us to the role of the gut microbiota, as many of them do not develop disease in germ free or antibiotic treatment conditions[39].

Together, these studies have advanced our current understanding of IBD. A clearer picture emerges where, in a healthy intestine, multiple systems (e.g. epithelium, innate immunity, adaptive immunity) cooperate to regulate the host immune response towards intestinal microbes. These systems break down in IBD, triggering a hyperinflammatory response and subsequent damage to the intestine[27,39].

Chapter 1.3 –IL-10 Receptor (IL-10R) signaling and IBD

IL-10 is a potent anti-inflammatory cytokine produced by several innate and adaptive immune cells as well as certain non-hematopoietic cells like fibroblasts and epithelial cells[46,47]. IL-10 was originally discovered as "Cytokine Synthesis Inhibitory Factor" for its inability to inhibit Th1 cell function[48]. Several key studies showed that this was achieved by inhibiting the function of antigen presenting cells[49–51]. Since then, IL-10 has been shown to elicit a broad range of effects on various immune cells[52]. Many of these effects are inhibitory, such as the downregulation of pro-inflammatory cytokine and chemokine production in macrophages[50,52], the inhibition of dendritic cell maturation[51] and the inhibition of pathological effector T cell generation[53]. However, IL-10 can promote the activity of $CD8^+$ T cells and NK cells[54–56], as well as the generation/function of regulatory T cells[57,58], suggesting cell-type specific regulation.

IL-10 signals via the IL-10R, which is composed of 2 subunits – IL-10Rα and IL-10Rβ. The IL-10Rα subunit (encoded by the *Il10ra* gene) is responsible for ligand binding while the IL-10Rβ subunit (encoded by the *Il10rb* gene) is responsible for recruiting signaling kinases[59,60]. The IL-10Rα subunit is specific to the IL-10R and is expressed by most hematopoietic cells as well as some nonhematopoietic cells like fibroblasts[46,52]. In certain cases, expression of *Il10ra* is induced upon activation[53,61]. In contrast, the IL-10Rβ subunit is ubiquitously expressed and is shared with other cytokine receptors, such as those for IL-22, IL-26 and IL-28[62]. Therefore, the specificity of the IL-10R for IL-10 is determined by the IL-10Rα subunit.

IL-10 engages its receptor sequentially, beginning with two molecules of IL-10 binding to 4 molecules of IL-10Rα with high affinity[63]. This then recruits IL-10Rβ chains to form an active heterotetrameric signaling complex per molecule of IL-10[60]. Engagement of the IL-10R leads to the activation of JAK1 and TYK2, which phosphorylate the IL-10Rα chain and recruit STAT3[64,65]. STAT3 is

phosphorylated at a specific residue (Y705), leading to its dimerization, activation and translocation to the nucleus to initiate gene transcription[66,67].

Multiple lines of evidence point to STAT3 as being the primary driver of IL-10R signaling. In STAT3 deficient macrophages, IL-10 no longer suppresses the induction of TNF or IL-6 by LPS[40]. Similar findings were reported in macrophages engineered to overexpress a dominant negative STAT3[67]. Moreover, expression of a constitutively active version of STAT3 can mimic the immunosuppressive functions of IL-10[68]. IL-10 has also been shown to activate STAT1 and in some cases STAT5, but these STATs are not believed to interact with the IL-10R[65,66]. Studies in *Stat1*$^{-/-}$ mice and cells expressing dominant negative STAT1 indicate that STAT1 is not required for IL-10 mediated effects[69,70]. However, increasing *Stat1* expression by pre-treatment with IFN can inhibit subsequent IL-10 mediated functions[71,72]. This suggests that the other STATs might play a role in modulating IL-10R signaling.

The mechanism by which IL-10 mediates its suppressive functions is complex and mostly studied in terms of transcriptional regulation. When activated by the IL-10R, STAT3 translocates to the nucleus to initiate the transcription of multiple genes, whose products then suppress the transcription of ~20% of LPS induced genes[73,74]. Multiple candidate IL-10 response genes have been suggested to mediate the anti-inflammatory response, such as *Bcl3*[75], *Hmox1*[76] and *Nfil3*[77], but none of these candidates alone can explain all the effects of the IL-10 mediated response[78]. Moreover, the genes induced by IL-10-STAT3 can differ from cell-type to cell-type[79]. It is also unclear why STAT3 can be anti-inflammatory in some cases (e.g. via the IL-10R) but pro-inflammatory in others (e.g. via the IL-6R and the IL-23R)[24], although some studies have pointed to a role for SOCS3[80]. A deeper understanding of these differences will significantly aid efforts to target this pathway for specific immune outcomes.

The importance of the IL-10-IL-10R axis in intestinal homeostasis was first evident in mouse models, where *Il10*$^{-/-}$ and *Il10rb*$^{-/-}$ mice develop spontaneous colonic inflammation in a genetically susceptible background[81,82]. This was strengthened by the subsequent discovery of patients with defects in *IL10, IL10RA* and *IL10RB* who present with IBD within the 1st year of life[83,84]. Allogeneic bone marrow

transplantation can ameliorate disease in *Il10*$^{-/-}$ mice and induce sustained remission in *IL10RB* deficient patients, indicating that the immune system plays an important role in the IBD phenotype[83,85]. The critical role of IL-10 in IBD was further supported by genome wide association studies implicating *IL10* as a risk allele for UC[86,87].

Several elegant studies using mouse models have provided valuable insight into how IL-10 or IL-10R deficiency causes IBD. Tregs are a key source of IL-10 for the gut, as mice harboring a deletion of the *Il10* gene in T cells or in Tregs develop colitis[88,89]. The disease in these mice is however not as severe as full *Il10*$^{-/-}$ mice, suggesting important roles for IL-10 produced by other cell types. Mice harboring a myeloid cell specific deletion in *Il10* do not develop spontaneous colitis[90], but *Il10*$^{-/-}$*Rag1*$^{-/-}$ mice develop more severe colitis than their *Rag1*$^{-/-}$ counterparts when reconstituted with naïve T cells, suggesting a role for innate derived IL-10[57]. Further roles for IL-10 produced by other cell types have been demonstrated in other induced models of colitis[91,92]. Tregs also need to respond to IL-10, as *Foxp3*^{Cre} *Il10ra*$^{fl/fl}$ mice develop spontaneous IBD[58]. *Il10rb*$^{-/-}$ Tregs also fail to suppress a T cell transfer model of colitis[57]. The IL-10R is also expressed on effector T cells, and this is important in suppressing their pathogenicity in colitis models[93–95]. In the innate immune compartment, macrophage expression of IL-10R is also shown to be important in preventing the colitic phenotype. Spontaneous colitis develops in mice lacking the IL-10R only in macrophages, and T cells induce more severe colitis in *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice and *Lyz2*^{Cre} *Il10ra*$^{fl/fl}$ *Rag1*$^{-/-}$ mice compared to control *Rag1*$^{-/-}$ mice[90,96–98]. The enhanced colitis in *Lyz2*^{Cre} *Il10ra*$^{fl/fl}$ *Rag1*$^{-/-}$ mice was not due to defective IL-10R signaling in dendritic cells or neutrophils, and transfer of WT anti-inflammatory macrophages can rescue disease in *Il10rb*$^{-/-}$ *Rag1*$^{-/-}$ mice, thus pointing to macrophages as an important IL-10 responding myeloid cell[96,97].

Due to the important role of IL-10 in maintaining intestinal homeostasis, there have been several attempts to utilize IL-10 as a therapeutic modality for IBD. While IL-10 has been shown to be effective in animal models[99–101], clinical trials utilizing recombinant IL-10 in CD failed to show significant benefit compared to placebo[102,103]. Several reasons have been proposed for this, such as variability in patient

responses to IL-10, inadequate dosing, and the possibility that IL-10 works to prevent but not treat disease[102]. However, further work will be required to investigate these mechanisms so as to improve the chances of developing a successful IL-10 supplementation therapy.

Chapter 1.4 – JAK-STAT signaling in CD4$^+$ T cell differentiation

CD4$^+$ T cells play a critical role in orchestrating the immune response. The existence of distinct subsets of CD4$^+$ T cells was first shown in mice with Th1 and Th2 cells[104]. These cell types were identified principally via their production of signature cytokines, IFNγ for Th1 cells and IL-4, IL-5 and IL-13 for Th2 cells. The Th1/Th2 classification remained the paradigm until the discovery of the Th17 subset characterized by the production of IL-17A, IL-17F and IL-22[105]. Tregs are also an important and distinct subset of T cells that suppress T cell responses, but there was initial controversy regarding the identity of this subset[106].

The characterization of these subsets was followed by efforts to differentiate them *in vitro* from naïve T cells. From these studies, we learned that T cell differentiation requires (1) T cell receptor (TCR) engagement, (2) co-stimulation and (3) specific cytokines that polarize the T cell towards a specific lineage[107]. These signals are critical in inducing master transcription factors (TF) that subsequently promote the transcription of genes to further drive cell differentiation[108]. The master TFs are T-bet for Th1 cells[109], GATA-3 for Th2 cells[110], RORγt for Th17[111] cells and Foxp3 for Tregs[112,113].

It is in the requirement for cytokine stimulation that the JAK-STAT pathway becomes important in T helper cell fate. Th1 differentiation, for instance, requires IL-12 (STAT4) and subsequently IFNγ (STAT1) for optimal T-bet expression. Hence, STAT4 and STAT1 deficient T cells display impaired Th1 differentiation *in vitro*[114–116]. Th2 differentiation requires IL-4 (STAT6), thus requiring STAT6 signaling. STAT3 signaling is important for Th17 differentiation, which could be explained by the requirement for IL-6, IL-21 and IL-23 in driving this cell fate. STAT5, which mediates IL-2R signaling, is important in promoting Th2 and Treg differentiation. These STATs promote T cell differentiation by inducing the master TFs.

The biological importance of these CD4$^+$ T cell subsets is evidenced by their contribution to various beneficial and pathological immune responses. Th1 cells are important for control of intracellular

pathogens, and patients with monogenic mutations that impair Th1 differentiation (*IFNGR1, IFNGR2, STAT1, IL12RB1, IL12B)* develop increased susceptibility to mycobacterial infections[117,118]. Th2 cells are important in clearing helminth infections, but elevated Th2 responses are involved in allergies[117]. Th17 cells are important in clearing extracellular bacteria and fungi[119,120]. Consequently, mutations in the Th17 pathway are associated with susceptibilities to *Candida* and *Staphylococcus* infections[121]. However, certain types of Th17 cells can also contribute to the pathogenesis of inflammatory diseases like multiple sclerosis[122,123] and inflammatory bowel disease[37,124]. Tregs are critical in suppressing autoreactive T cells, and mutations in *Foxp3* leads to autoimmunity and immunodeficiency, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome in patients[125,126]. Conversely, in the tumor microenvironment, Tregs play a key role in creating an immunosuppressive setting that inhibits anti-tumor responses[127]. Consequently, there are multiple therapeutic modalities in place to alter T helper cell differentiation in order to achieve a desired immune outcome, such as the blockade of cytokines that promote proinflammatory T cell responses in inflammatory diseases and checkpoint blockade therapy for generating favorable anti-tumor immunity[128,129].

Given the critical role of the JAK-STAT pathway in T cells and their ability to orchestrate inflammatory responses, there is relevant interest in understanding how this pathway is involved in inflammatory disorders like IBD. In Chapter 2 of this dissertation, I'll be exploring the role of one of the members of this pathway – STAT1 – in T cell mediated colitis.

Chapter 1.5 - IL-10 mediated regulation of macrophage function

Macrophages are a highly diverse set of phagocytic cells that populate various tissues and play key roles in almost every aspect of host biology. In normal physiology, these cells are involved in processes like bone remodeling, neuronal patterning, metabolic homeostasis and angiogenesis[130]. They also play a key role in host defense, being critical producers of inflammatory cytokines and reactive oxygen species that contribute to microbial killing, as well as regulators of the resolution process thereafter[130,131]. However, their widespread involvement in host physiology also leads to their contribution to various pathologies, such as cancer, arthritis and IBD[130].

Analogous to T cells, macrophages can also adopt various states. One commonly used method to classify these cells is the M1-M2 classification[132]. M1 macrophages represent classically activated macrophages and they produce inflammatory cytokines like TNF, IL-12 and IL-23. M2 macrophages on the other hand represent alternatively activated macrophages that are involved in parasite clearance and promote immunoregulatory and wound healing functions[133]. These macrophages can be identified by the upregulation of specific markers, such as *Il12a, Il12b, Il6* and *Tnf* for M1 macrophages and *Retnla/Fizz1*, *Arg1* and *Chil3* for M2 macrophages[134]. M1 and M2 macrophages have been largely studied *in vitro* where they can be polarized by LPS + IFNγ and IL-4/IL-13 respectively, mirroring Th1 and Th2 cells[131,133]. Although this classification is useful for understanding macrophage biology, there is no basis to suggest macrophages exist only in these states[134]. Rather, it is more likely that macrophages can adopt a wide spectrum of states depending on their context, with M1 and M2 being the extremes[135].

As described earlier, one of the key targets of IL-10 *in vivo* are macrophages, and the ability of IL-10 to suppress LPS induced inflammatory cytokines like TNF is well established[50]. In line with IL-10's immunosuppressive functions, macrophages lacking the IL-10R displayed an enhanced polarization towards the pro-inflammatory M1 state *in vitro*[96]. This is also seen *in vivo* in the colonic lamina propria[96,98]. Conversely, IL-10R deficient macrophages display defective generation of anti-inflammatory M2 macrophages *in vitro*, which might be due at least in part to IL-10's ability to upregulate IL-4R

surface expression[73]. The transfer of WT but not *Il10rb*$^{-/-}$ polarized M2 macrophages into *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice ameliorates T cell induced colitis, suggesting an important role for IL-10 in M2 macrophage function *in vivo*[96]. Finally, macrophages cultured from IL-10R deficient patients also display enhanced M1 and reduced M2 polarization *in vitro*, consistent with the murine data. Altogether, these point to a critical role for IL-10 in altering macrophage function in IBD.

This enhanced pro-inflammatory state of IL-10R deficient macrophages can be targeted for therapeutic intervention. An example of a target is the pro-inflammatory cytokine IL-1β, a M1 marker that we and others showed to be upregulated in IL-10R deficient murine and human macrophages[136,137]. IL-1β is produced via a 2-step process – priming and activation[138,139]. In the priming step, inflammatory triggers like LPS upregulate the transcription of pro-IL-1β and components of the inflammasome (e.g. NLRP3)[138]. In the activation step, these inflammasome components are assembled into a complex consisting of (1) a sensor of cytosolic pathogen associated molecular patterns (PAMPs) or danger signals, (2) an adaptor protein ASC and (3) pro-caspase 1. Pro-caspase 1 is cleaved to generate active caspase 1, which then cleaves pro-IL-1β to generate its mature form[140].

IL-10 inhibits both the priming and activation steps, reducing NLRP3 expression, enhancing the ubiquitination and degradation of NLRP3 and blocking the LPS induced production of mitochondrial reactive oxygen species (mtROS) that is important for inflammasome activation[136,137]. This inhibition of IL-1β is important *in vivo*. The degree of colitis in *Il10*$^{-/-}$ deficient mice is reduced when caspase 1 is deleted[137]. T cells lacking the IL-1 receptor also induce less colitis in *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice[136]. Moreover, in 2 patients presenting with very early onset IBD due to *IL10RA* mutations, we were able to achieve marked clinical, endoscopic and histological improvement using the IL-1 receptor antagonist anakinra. This suggests that blockade of the IL-1-IL-1R axis might be useful as a bridge for hematopoietic stem cell transplantation in these patients[136].

Chapter 1.6 – Metabolic regulation of macrophage function

Cells do not exist in isolation, and they are dependent on the extracellular milieu to provide nutrients for their survival and function. These nutrients are taken into the cell and processed via key metabolic pathways to generate substrates for various biochemical reactions. Immune cells are no exception, and it has become increasingly clear that these cells adapt their metabolic machinery for their effector functions – a process called metabolic reprogramming.

It is well known that cells undergo metabolic reprogramming in response to changes in oxygen levels. Under normoxic conditions, cells mainly utilize oxidative phosphorylation (OXPHOS) to generate ATP needed to fuel bioenergetic processes. OXPHOS involves the uptake of fuels like glucose, fatty acids and amino acids like glutamine, which are broken down into smaller units (e.g. acetyl-CoA) and fed into the tricarboxylic acid (TCA) cycle to generate the high energy intermediates NADH and $FADH_2$. These intermediates transfer their high energy electrons to the electron transport chain (ETC) of the mitochondria, and the ETC uses this energy to pump H^+ into the intermembrane space to generate a proton-motive force. Finally, this proton-motive force is used by ATP synthase to produce ATP from ADP, and the high energy electrons are used to reduce O_2 to make H_2O[141,142]. Under hypoxic conditions however, there is insufficient O_2 to drive OXPHOS. Therefore, in order to maintain ATP generation and recycle the high energy intermediates, cells primarily utilize glycolysis. Pyruvate, generated via the breakdown of glucose, is broken down to lactate instead of acetyl-CoA for the TCA cycle. There are important exceptions to this rule, such as during the "Warburg effect" or aerobic glycolysis – a term coined by Otto Warburg who discovered that tumor cells utilize glycolysis even in normoxic conditions[142].

The field of immunometabolism emerged from the discovery that upon activation[143–145], immune cells also switch their metabolic state from OXPHOS to glycolysis, utilizing aerobic glycolysis similar to the Warburg effect. Inflammatory cells generally utilize anabolic processes while anti-inflammatory cells utilize catabolic processes.

LPS stimulated macrophages like M1 macrophages undergo Warburg metabolism via multiple mechanisms[146]. For example, M1 macrophages upregulate iNOS, which utilizes extracellular arginine to produce nitric oxide (NO). NO potently inhibits OXPHOS by nitrosylating and inhibiting iron-sulfur proteins in the ETC[147]. LPS also induces the activation of mTOR[148], a key sensor of metabolic state that integrates extracellular and intracellular signals to regulate metabolism and growth[149]. One of the functions of mTOR activation is to increase HIF-1α expression, and HIF-1α induces the transcription of multiple glycolytic genes like GLUT1 to increase glucose uptake and pyruvate dehydrogenase kinase which inhibits pyruvate dehydrogenase of the TCA cycle[150]. LPS also increases the expression of u-PFK2, an isoform of PFK-2 that generates more fructose-2,6-bisphosphate (F-2,6-BP) which in turn activates the glycolytic enzyme PFK1[151]. These mechanisms collectively upregulate glycolysis in M1 macrophages. Moreover, LPS can also inhibit AMP kinase, a kinase that promotes OXPHOS and inhibits mTOR[152] (**Figure 1**).

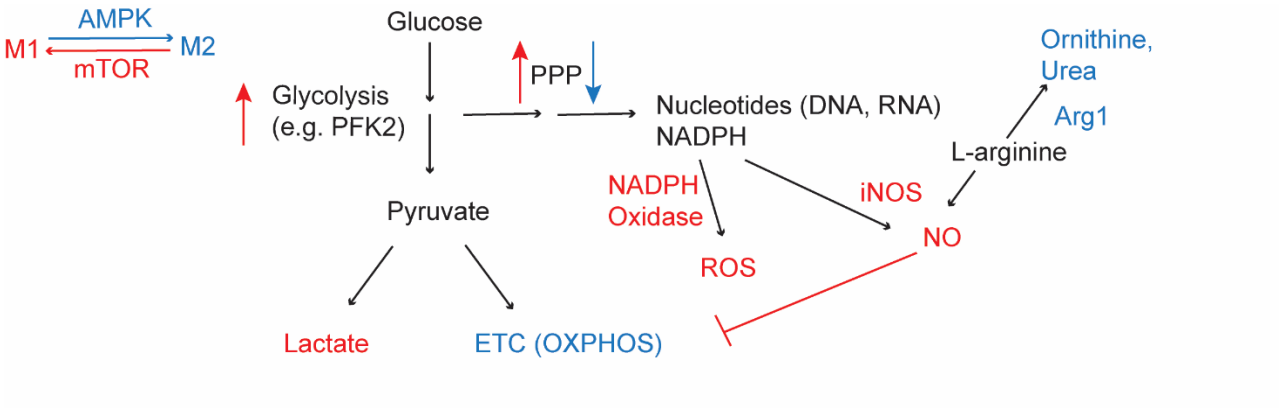


Figure 1. Schematic of metabolic processes that are upregulated in M1 (red) and M2 (blue) macrophages. In M1 macrophages, there is a metabolic shift towards aerobic glycolysis and reduction of OXPHOS, orchestrated by mTOR signaling. Glycolysis is upregulated by e.g. switching to the u-PFK2 isoform. The enhanced flux of glucose is diverted towards anabolic processes like the pentose phosphate pathway (PPP), which generates nucleotides and NADPH. NADPH is important for the production of ROS and NO, and NO contributes to the potent inhibition of OXPHOS. Pyruvate is diverted to lactate instead of acetyl-CoA for the TCA cycle and OXPHOS. In M2 macrophages, there is a reduction of PPP and enhancement of OXPHOS. Arginase 1 diverts arginine away from NO production towards ornithine production. AMPK is a key regulator of the M2 metabolic phenotype.

These metabolic changes are critical for inflammatory M1 macrophage function. For example, the glycolytic intermediates generated from increased glycolysis are diverted to the pentose phosphate pathway (PPP), which generates precursors for nucleotide synthesis as well as NADPH. NADPH can be used for the generation of ROS via the NADPH oxidase and NO from L-arginine via iNOS, which are both important for microbial killing[153]. A more recent study unveiled 2 breaks in the TCA cycle in M1 macrophages[154]. The first break occurs due to reduced expression of isocitrate dehydrogenase, which catalyzes the breakdown of isocitrate[154]. The accumulation of citrate as a result of this break is exported to the cytosol where it is broken down to acetyl-CoA to be used for fatty acid synthesis, a mechanism that may be helpful to package more cytokines for secretion[155] (**Figure 2**).

Citrate is also metabolized by IRG1 to generate itaconate[156]. Itaconate is a potent anti-inflammatory metabolite analogous to IL-10, suppressing inflammation by mechanisms such as the activation of NRF2 which protects against oxidative and electrophilic stress as well as the inhibition of succinate dehydrogenase (SDH)[157]. As SDH catalyzes the breakdown of succinate to fumarate, the inhibition of SDH by itaconate together with NO causes the second break in the TCA cycle and the subsequent accumulation of succinate[158,159]. This accumulation of succinate is derived from glutamine via anaplerosis through α-ketoglutarate and the GABA shunt instead of the TCA cycle due to the inhibition of OXPHOS in inflammatory macrophages[159]. Succinate accumulation due to SDH inhibition stabilizes HIF-1α through the inhibition of prolyl hydroxylase (PHD) enzymes, thus promoting glycolysis, inflammation and IL-1β production[159]. However, SDH also catalyzes the oxidation of succinate, which contributes to ROS generation via reverse electron transport (RET) through Complex I and subsequent IL-1β production[160]. Therefore, while inhibition of SDH by itaconate causes succinate accumulation, the attenuation of RET dampens succinate oxidation and IL-1β production at later time points, allowing itaconate to serve as a negative feedback mechanism[153] (**Figure 2)**.

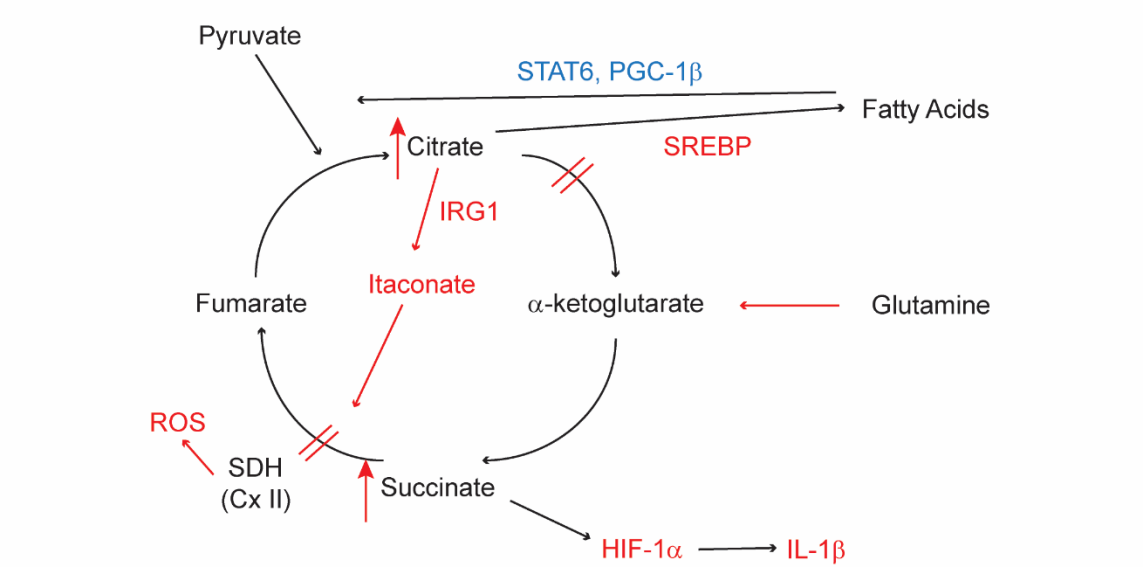


Figure 2. Rewiring of the TCA cycle in M1 (red) and M2 (blue) macrophages. In M1 macrophages there are 2 breaks in the TCA cycle, leading to the accumulation of citrate and succinate. Citrate is exported to the cytosol for processes like fatty acid synthesis, enhanced by SREBP. Succinate stabilizes HIF-1α, which promotes IL-1β production. Succinate can also be oxidized by SDH to generate ROS which is important for IL-1β activation. Citrate is also used to generate itaconate. Itaconate inhibits SDH, thereby reducing ROS and IL-1β production as a negative feedback mechanism. In M2 macrophages, STAT6 and PGC-1β are important in promoting fatty acid oxidation.

The mechanisms involved in LPS stimulation are generally opposed in M2 macrophages. M2 macrophages display reduced expression of iNOS and increased expression of the M2 marker arginase (Arg-1)[134], driving arginine metabolism away from NO production and towards the production of urea and ornithine[132]. M2 macrophages also display enhanced OXPHOS due to IL-4 and reduced glycolysis relative to M1 macrophages owing at least in part to the alternate isoform of PFK2 (PFKFB1) that breaks down F-2,6-BP more readily[153,161]. IL-4/IL-13 activate TSC1 and TSC2 which are inhibitors of mTOR[162], enhancing AMPK activity which increases fatty acid oxidation[163]. Activity through the PPP is limited by expression of CARKL[164]. Collectively, these processes boost catabolism in M2 macrophages and antagonize the generation of the M1 metabolic profile (**Figure 1, 2**).

IL-10 was recently shown to also alter macrophage metabolic state. IL-10 induces the transcription of *Ddit4*, an inhibitor of mTOR signaling. This opposes the LPS induced metabolic shift, suppressing glucose uptake and promoting OXPHOS. The inhibition of mTOR also leads to the upregulation of mitophagy, thus removing dysfunctional mitochondria that have low membrane potential and high ROS[137]. As mitochondrial ROS contributes to inflammasome activation[165], the induction of mitophagy by IL-10 reduces IL-1β production by macrophages[137].

As described earlier, IL-10 induces a complicated transcriptional program, with no one mediator that can explain all of IL-10 mediated immunosuppression[78]. Moreover, STAT3 has also been reported to have activities beyond canonical DNA binding and transcription, such as translocation to the mitochondria and the endoplasmic reticulum[26]. In Chapter 3 of this dissertation, I explore one of STAT3's non-canonical functions – its translocation to the mitochondria – in relation to IL-10R signaling and macrophage metabolism.

Chapter 1.7 - References

1. Zeidler, M. P. & Bausek, N. The Drosophila JAK-STAT pathway . *Jak-Stat* **2**, e25353 (2013).
2. Chin, Y. E. *et al.* Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21WAF1/CIP1 Mediated by STAT1. *Science.* **272**, 719–722 (1996).
3. O'Shea, J. J. & Murray, P. J. Cytokine signaling modules in inflammatory responses. *Immunity* **28**, 477–87 (2008).
4. Villarino, A. V., Kanno, Y. & O'Shea, J. J. Mechanisms and consequences of Jak-STAT signaling in the immune system. *Nat. Immunol.* **18**, 374–384 (2017).
5. Isaccs, A. & Lindenmann, J. Virus Interference. I. The Interferon. *Proc. R. Soc. London. Ser. B, Biol. Sci.* **147**, 258–267 (1957).
6. Stark, G. R. & Darnell, J. E. J. The JAK-STAT Pathway at Twenty. *Immunity* **36**, 503–514 (2012).
7. Velazquez, L., Fellous, M., Stark, G. R. & Pellegrini, S. A protein tyrosine kinase in the interferon alpha/beta signaling pathway. *Cell* **70**, 313–322 (1992).
8. Müller, M. *et al.* The protein tyrosine kinase JAK1 complements defects in interferon-α/β and -γ Signal transduction. *Nature* **366**, 129–135 (1993).
9. Zhong, Z., Wen, Z. & Jr, J. E. D. Stat3 : A STAT Family Member Activated by Tyrosine Phosphorylation in Response to Epidermal Growth Factor and Interleukin-6. *Science (80-.).* **264**, 95–98 (1994).
10. Zhong, Z., Wen, Z. & Darnell, J. E. Stat3 and Stat4: members of the family of signal transducers and activators of transcription. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 4806–4810 (1994).
11. Wakao, H., Gouilleux, F. & Groner, B. Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. *EMBO J.* **13**, 2182–2191 (1994).
12. Mui, A. L., Wakao, H., O'Farrell, A. M., Harada, N. & Miyajima, A. Interleukin-3, granulocyte-macrophage colony stimulating factor and interleukin-5 transduce signals through two STAT5 homologs. *EMBO J.* **14**, 1166–1175 (2018).
13. Liu, X., Robinson, G. W., Gouilleux, F., Groner, B. & Hennighausen, L. Cloning and expression of Stat5 and an additional homologue (Stat5b) involved in prolactin signal transduction in mouse mammary tissue. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 8831–5 (1995).
14. Shuai, K. & Liu, B. Regulation of JAK-STAT signalling in the immune system. *Nat. Rev. Immunol.* **3**, 900–11 (2003).
15. O'Shea, J. J., Holland, S. M. & Staudt, L. M. JAKs and STATs in Immunity, Immunodeficiency, and Cancer. *N. Engl. J. Med.* **368**, 161–170 (2013).
16. Liu, L. *et al.* Gain-of-function human STAT1 mutations impair IL-17 immunity and underlie chronic mucocutaneous candidiasis. *J. Exp. Med.* **208**, 1635–48 (2011).
17. Uzel, G. *et al.* Dominant gain-of-function STAT1 mutations in FOXP3 wild-type immune dysregulation–polyendocrinopathy–enteropathy–X-linked–like syndrome. *J. Allergy Clin. Immunol.* **131**, 1611-1623.e3 (2013).

18. Milner, J. D. *et al.* Early-onset lymphoproliferation and autoimmunity caused by germline STAT3 gain-of-function mutations. *Blood* **125**, 591–600 (2015).

19. Baxter, E. J. *et al.* Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet* **365**, 1054–1061 (2005).

20. James, C., Ugo, V., Couedic, J.-P. Le & Vainchenker, W. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature* **7037**, 1144–8 (2005).

21. Mascarenhas, J. & Hoffman, R. Ruxolitinib: The first FDA approved therapy for the treatment of myelofibrosis. *Clin. Cancer Res.* **18**, 3008–3014 (2012).

22. O'Shea, J. J. & Plenge, R. JAK and STAT signaling molecules in immunoregulation and immune-mediated disease. *Immunity* **36**, 542–50 (2012).

23. Sandborn, W. J. *et al.* Tofacitinib as Induction and Maintenance Therapy for Ulcerative Colitis. *N. Engl. J. Med.* **376**, 1723–1736 (2017).

24. Murray, P. J. The JAK-STAT Signaling Pathway: Input and Output Integration. *J. Immunol.* **178**, 2623–2629 (2007).

25. Yang, J. & Stark, G. R. Roles of unphosphorylated STATs in signaling. *Cell Res.* **18**, 443–451 (2008).

26. Avalle, L. & Poli, V. Nucleus, Mitochondrion, or Reticulum? STAT3 à La Carte. *Int. J. Mol. Sci.* **19**, 2820 (2018).

27. Abraham, C. & Cho, J. H. Inflammatory Bowel Disease. *N Engl J Med* **361**, 2066–78 (2009).

28. Danese, S. & Fiocchi, C. Ulcerative colitis. *N. Engl. J. Med.* **365**, 1713–25 (2011).

29. Baumgart, D. C. & Sandborn, W. J. Crohn's disease. *Lancet* **380**, 1590–1605 (2012).

30. Ng, S. C. *et al.* Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet* **390**, 2769–2778 (2017).

31. De Souza, H. S. P., Fiocchi, C. & Iliopoulos, D. The IBD interactome: An integrated view of aetiology, pathogenesis and therapy. *Nature Reviews Gastroenterology and Hepatology* **14**, 739–749 (2017).

32. Hugot, J.-P. *et al.* Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* **411**, 599–603 (2001).

33. Karaliuskas, R. *et al.* A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* **411**, 603–6 (2001).

34. Abraham, C. & Cho, J. H. Functional consequences of NOD2 (CARD15) mutations. *Inflamm. Bowel Dis.* **12**, 641–650 (2006).

35. Hampe, J. *et al.* A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat. Genet.* **39**, 207–211 (2007).

36. Parkes, M. *et al.* Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. *Nat. Genet.* **39**, 830–832 (2007).

37. Steinhart, A. H. *et al.* A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science (80-.).* **314**, 1461–3 (2006).

38. Barrett, J. C. *et al.* Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat. Genet.* **40**, 955–962 (2008).

39. Elson, C. O. *et al.* Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota. *Immunol. Rev.* **206**, 260–276 (2005).

40. Takeda, K. *et al.* Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of stat3 in macrophages and neutrophils. *Immunity* **10**, 39–49 (1999).

41. Kobayashi, M. *et al.* Toll-like receptor-dependent production of IL-12p40 causes chronic enterocolitis in myeloid cell-specific Stat3-deficient mice. *J. Clin. Invest.* **111**, 1297–1308 (2003).

42. Panwala, C. M., Jones, J. C., Viney, J. L. & Alerts, E. A Novel Model of Inflammatory Bowel Disease: Mice Deficient for the Multiple Drug Resistance Gene, mdr1a, Spontaneously Develop Colitis. *J Immunol* **161**, 5733–5744 (2012).

43. Powrie, F. *et al.* Inhibition of Thl responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. *Immunity* **1**, 553–562 (1994).

44. Sadlack, B. *et al.* Ulcerative Colitis-like Disease in Mice with a Disrupted Interleukin-2 Gene. *Cell* **75**, 253–261 (1993).

45. Willerford, D. M. *et al.* Interleukin-2 receptor α chain regulates the size and content of the peripheral lymphoid compartment. *Immunity* **3**, 521–530 (1995).

46. Shouval, D. S. *et al.* Interleukin 10 Receptor Signaling: Master Regulator of Intestinal Mucosal Homeostasis in Mice and Humans. in *Advances in Immunology* **122**, 177–210 (Elsevier Inc., 2014).

47. Colgan, S. P., Hershberg, R. M., Furuta, G. T. & Blumberg, R. S. Ligation of intestinal epithelial CD1d induces bioactive IL-10: Critical role of the cytoplasmic tail in autocrine signaling. *Proc. Natl. Acad. Sci.* **96**, 13938–13943 (2002).

48. Fiorentino, D. F., Bond, M. W. & Mosmann, T. R. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med.* **170**, 2081–2095 (1989).

49. Fiorentino, D. F. *et al.* IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J. Immunol.* **146**, 3444–51 (1991).

50. Fiorentino, D. F., Zlotnik, A., Mosmann, T. R., Howard, M. & O'Garra, A. IL-10 inhibits cytokine production by activated macrophages. *J. Immunol.* **147**, 3815–22 (1991).

51. Steinbrink, K., Wölfl, M., Jonuleit, H., Knop, J. & Enk, A. H. Induction of tolerance by IL-10-treated dendritic cells. *J. Immunol.* **159**, 4772–80 (1997).

52. Moore, K. W., Malefyt, R. D. W., Robert, L. & Garra, A. O. Interleukin-10 and the Interleukin-10 Receptor. *Annu. Rev. Immunol.* **1**, 683–765 (2001).

53. Huber, S. *et al.* Th17 Cells Express Interleukin-10 Receptor and Are Controlled by Foxp3- and Foxp3+ Regulatory CD4+ T Cells in an Interleukin-10-Dependent Manner. *Immunity* **34**, 554–565 (2011).

54. Cai, G., Kastelein, R. A. & Hunter, C. A. IL-10 enhances NK cell proliferation, cytotoxicity and production of IFN-gamma when combined with IL-18. *Eur. J. Immunol.* **29**, 2658–65 (1999).

55. Mocellin, S. *et al.* IL-10 stimulatory effects on human NK cells explored by gene profile analysis. *Genes Immun.* **5**, 621–630 (2004).

56. Groux, H., Bigler, M., de Vries, J. E. & Roncarolo, M. G. Inhibitory and stimulatory effects of IL-10 on human CD8+ T cells. *J. Immunol.* **160**, 3188–93 (1998).

57. Murai, M. *et al.* Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nat. Immunol.* **10**, 1178–84 (2009).

58. Chaudhry, A. *et al.* Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity* **34**, 566–78 (2011).

59. Ho, A. S. *et al.* A receptor for interleukin 10 is related to interferon receptors. *Proc. Natl. Acad. Sci.* **90**, 11267–11271 (2006).

60. Kotenko, S. V *et al.* Identification and functional characterization of a second chain of the interleukin-10 receptor complex cells, blocking their ability to secrete cytokines such as interferon-γ (IFN-γ) and IL-2 (Fiorentino et al. *EMBO J.* **16**, 5894–5903 (1997).

61. Weber-nordt, R. M., Meraz, M. A. & Schreiber, R. D. Lipopolysaccharide-Dependent Induction of IL-10 Receptor Expression on Murine Fibroblasts. *J. Immunol.* **153**, 3734 (1994).

62. Ouyang, W., Rutz, S., Crellin, N. K., Valdez, P. a & Hymowitz, S. G. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annu. Rev. Immunol.* **29**, 71–109 (2011).

63. Josephson, K., Logsdon, N. J. & Walter, M. R. Crystal structure of the IL-10/IL-10R1 complex reveals a shared receptor binding site. *Immunity* **15**, 35–46 (2001).

64. Finbloom, D. S. & Winestock, K. D. IL-10 Induces the Tyrosine Phosphorylation of tyk2 and Jak1 and the Differential Assembly of STAT1a and STAT3 Complexes in Human T cells and Monocytes. *J. Immunol.* **155**, 1079–1090 (1995).

65. Weber-nordt, R. M. *et al.* Stat3 Recruitment by Two Distinct Docking Sites in the Interleukin-10 Receptor Intracellular Domain. *J. Biol. Chem.* **271**, 27954–27961 (1996).

66. Wehinger, J., Gouilleux, F. & Groner, B. IL-10 induces DNA binding activity of three STAT proteins (Stat1, Stat3, and Stat5) and their distinct combinatorial assembly in the promoters of selected. *FEBS Lett.* **394**, 365–370 (1996).

67. Williams, L., Bradley, L., Smith, A. & Foxwell, B. Signal Transducer and Activator of Transcription 3 Is the Dominant Mediator of the Anti-Inflammatory Effects of IL-10 in Human Macrophages. *J. Immunol.* **172**, 567–576 (2003).

68. Williams, L. M. *et al.* Expression of constitutively active STAT3 can replicate the cytokine-suppressive activity of interleukin-10 in human primary macrophages. *J. Biol. Chem.* **282**, 6965–75 (2007).

69. Meraz, M. a *et al.* Targeted Disruption of the Stat1 Gene in Mice Reveals Unexpected Physiologic Specificity in the JAK–STAT Signaling Pathway. *Cell* **84**, 431–442 (1996).

70. Farrell, A. O., Liu, Y., Moore, K. W. & Mui, A. L. IL-10 inhibits macrophage activation and proliferation by distinct signaling mechanisms : evidence for Stat3-dependent and -independent pathways. **17**, 1006–1018 (1998).

71. Herrero, C. *et al.* Reprogramming of IL-10 Activity and Signaling by IFN-γ. *J. Immunol.* **171**,

5034–5041 (2003).

72. Sharif, M. N. *et al.* IFN-α Priming Results in a Gain of Proinflammatory Function by IL-10: Implications for Systemic Lupus Erythematosus Pathogenesis. *J. Immunol.* **172**, 6476–6481 (2004).

73. Lang, R., Patel, D., Morris, J. J., Rutschman, R. L. & Murray, P. J. Shaping Gene Expression in Activated and Resting Primary Macrophages by IL-10. *J. Immunol.* **169**, 2253–2263 (2002).

74. Murray, P. J. The primary mechanism of the IL-10-regulated antiinflammatory response is to selectively inhibit transcription. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 8686–91 (2005).

75. Kuwata, H. & Watanabe, Y. IL-10-inducible Bcl-3 negatively regulates LPS-induced TNF-α production in macrophages. *Blood* **102**, 4123–4130 (2003).

76. Lee, T. & Chau, L. Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. *Nat. Med.* **8**, 1–7 (2002).

77. Smith, A. M. *et al.* A distal enhancer in Il12b is the target of transcriptional repression by the STAT3 pathway and requires the basic leucine zipper (B-ZIP) protein NFIL3. *J. Biol. Chem.* **286**, 23582–90 (2011).

78. Hutchins, A. P., Diez, D. & Miranda-Saavedra, D. The IL-10/STAT3-mediated anti-inflammatory response: Recent developments and future challenges. *Brief. Funct. Genomics* **12**, 489–498 (2013).

79. Hutchins, A. P., Takahashi, Y. & Miranda-Saavedra, D. Genomic analysis of LPS-stimulated myeloid cells identifies a common pro-inflammatory response but divergent IL-10 anti-inflammatory responses. *Sci. Rep.* **5**, 9100 (2015).

80. Yasukawa, H. *et al.* IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. *Nat. Immunol.* **4**, 551–6 (2003).

81. Kühn, R., Löhler, J., Rennick, D., Rajewsky, K. & Müller, W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* **75**, 263–274 (1993).

82. Spencer, S. D. *et al.* The Orphan Receptor CRF2-4 Is an Essential Subunit of the Interleukin 10 Receptor. *J. Exp. Med.* **187**, 571–578 (1998).

83. Glocker, E. & Kotlarz, D. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med* **361**, 2033–2045 (2009).

84. Glocker, E.-O. *et al.* Infant colitis--it's in the genes. *Lancet* **376**, 1272 (2010).

85. Bamba, S. *et al.* Bone marrow transplantation ameliorates pathology in interleukin-10 knockout colitic mice. *J. Pathol.* **209**, 265–273 (2006).

86. Franke, A. *et al.* Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility. *Nat. Genet.* **40**, 1319–1323 (2008).

87. Jostins, L. *et al.* Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* **491**, 119–124 (2012).

88. Roers, A. *et al.* T cell-specific inactivation of the interleukin 10 gene in mice results in enhanced T cell responses but normal innate responses to lipopolysaccharide or skin irritation. *J. Exp. Med.* **200**, 1289–1297 (2004).

89. Rubtsov, Y. P. *et al.* Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* **28**, 546–58 (2008).

90. Zigmond, E., Bernshtein, B. & Friedlander, G. Macrophage-Restricted Interleukin-10 Receptor Deficiency , but Not IL-10 Deficiency , Causes Severe Spontaneous Colitis. *Immunity* **40**, 720–733 (2014).

91. Krause, P. *et al.* IL-10-producing intestinal macrophages prevent excessive antibacterial innate immunity by limiting IL-23 synthesis. *Nat. Commun.* **6**, 1–12 (2015).

92. Olszak, T. *et al.* Protective mucosal immunity mediated by epithelial CD1d and IL-10. *Nature* **509**, 497–502 (2014).

93. Huber, S. *et al.* Th17 cells express interleukin-10 receptor and are controlled by Foxp3^{-} and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner. *Immunity* **34**, 554–65 (2011).

94. Kamanaka, M. *et al.* Memory/effector (CD45RB lo) CD4 T cells are controlled directly by IL-10 and cause IL-22–dependent intestinal pathology . *J. Exp. Med.* **208**, 1027–1040 (2011).

95. Asseman, C. & Mauze, S. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J. Exp. Med.* **190**, 995–1003 (1999).

96. Shouval, D. S. *et al.* Interleukin-10 receptor signaling in innate immune cells regulates mucosal immune tolerance and anti-inflammatory macrophage function. *Immunity* **40**, 706–19 (2014).

97. Li, B. *et al.* IL-10 engages macrophages to shift Th17 cytokine dependency and pathogenicity during T-cell-mediated colitis. *Nat. Commun.* **6**, 1–13 (2015).

98. Redhu, N. S. *et al.* Macrophage dysfunction initiates colitis during weaning of infant mice lacking the interleukin-10 receptor. *Elife* **6**, 1–23 (2017).

99. Duchmann, R., Schmitt, E., Knolle, P., Zum Buschenfelde, K. H. M. & Neurath, M. Tolerance towards resident intestinal flora in mice is abrogated in experimental colitis and restored and by treatment with interleukin-10 or antibodies to interleukin-12. *Eur. J. Immunol.* **26**, 934–938 (1996).

100. Barbara, G., Xing, Z., Hogaboam, C. M., Gauldie, J. & Collins, S. M. Interleukin 10 gene transfer prevents experimental colitis in rats. *Gut* **46**, 344–349 (2000).

101. Steidler, L. *et al.* Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. *Science (80-.).* **289**, 1352–1355 (2000).

102. Buruiana, F. E., Solà, I. & Alonso-Coello, P. Recombinant human interleukin 10 for induction of remission in Crohn's disease. *Cochrane Database Syst. Rev.* (2010). doi:10.1002/14651858.cd005109.pub3

103. Marlow, G. J., van Gent, D. & Ferguson, L. R. Why interleukin-10 supplementation does not work in Crohn's disease patients. *World J. Gastroenterol.* **19**, 3931–3941 (2013).

104. Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. & Coffman, R. L. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* **136**, 2348–57 (1986).

105. Harrington, L. E. *et al.* Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* **6**, 1123–32 (2005).

106. Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). *J. Immunol.* **155**, 1151–1164 (1995).

107. Gutcher, I. & Becher, B. APC-derived cytokines and T cell polarization in autoimmune inflammation. *J. Clin. Invest.* **117**, 1119–1127 (2007).

108. Zhu, J., Yamane, H. & Paul, W. E. Differentiation of Effector CD4 T Cell Populations. *Annu. Rev. Immunol.* **28**, 445–489 (2010).

109. Szabo, S. J. *et al.* A Novel Transcription Factor, T-bet, Directs Th1 Lineage Commitment. *Cell* **100**, 655–669 (2000).

110. Zheng, W. & Flavell, R. A. The Transcription Factor GATA-3 Is Necessary and Sufficient for Th2 Cytokine Gene Expression in CD4 T Cells. *Cell* **89**, 587–596 (1997).

111. Ivanov, I. I. *et al.* The Orphan Nuclear Receptor RORγt Directs the Differentiation Program of Proinflammatory IL-17+ T Helper Cells. *Cell* **126**, 1121–1133 (2006).

112. Hori, S., Nomura, T. & Sakaguchi, S. Control of regulatory T cell development by the transcription factor Foxp3. *Science.* **299**, 1057–1061 (2003).

113. Fontenot, J. D., Gavin, M. A. & Rudensky, A. Y. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat. Immunol.* **4**, 330–336 (2003).

114. Afkarian, M. *et al.* T-bet is a STAT1-induced regulator for IL-12R expression in naïve CD4+T cells. *Nat. Immunol.* **3**, 549–557 (2002).

115. Kaplan, M. H., Sun, Y.-L., Hoey, T. & Grusby, M. J. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* **382**, 174–177 (1996).

116. Thierfelder, W. E. *et al.* Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* **382**, 171–174 (1996).

117. Zhu, J. & Paul, W. E. CD4 T cells: fates, functions, and faults. *Blood* **112**, 1557–1569 (2008).

118. Filipe-Santos, O. *et al.* Inborn errors of IL-12/23- and IFN-γ-mediated immunity: molecular, cellular, and clinical features. *Seminars in Immunology* **18**, 347–361 (2006).

119. Happel, K. I. *et al.* Divergent roles of IL-23 and IL-12 in host defense against Klebsiella pneumoniae . *J. Exp. Med.* **202**, 761–769 (2005).

120. Huang, W., Na, L., Fidel, P. L. & Schwarzenberger, P. Requirement of Interleukin-17A for Systemic Anti– Candida albicans Host Defense in Mice . *J. Infect. Dis.* **190**, 624–631 (2004).

121. Milner, J. D. *et al.* Impaired TH17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature* **452**, 773–776 (2008).

122. Cua, D. J. *et al.* Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* **421**, 744–748 (2003).

123. Langrish, C. L. *et al.* IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* **201**, 233–240 (2005).

124. Ahern, P. P. *et al.* Interleukin-23 drives intestinal inflammation through direct activity on T cells. *Immunity* **33**, 279–88 (2010).

125. Wildin, R. S. *et al.* X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome

is the human equivalent of mouse scurfy. *Nat. Genet.* **27**, 18–20 (2001).

126. Bennett, C. L. *et al.* The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat. Genet.* **27**, 20–21 (2001).

127. Nishikawa, H. & Sakaguchi, S. Regulatory T cells in cancer immunotherapy. *Current Opinion in Immunology* **27**, 1–7 (2014).

128. Leach, D. R., Krummel, M. F. & Allison, J. P. Enhancement of Antitumor Immunity by CTLA-4 Blockade. *Science (80-.).* **271**, 1734–1736 (1996).

129. Neurath, M. F. Cytokines in inflammatory bowel disease. *Nat. Rev. Immunol.* **14**, 329–42 (2014).

130. Wynn, T. A., Chawla, A. & Pollard, J. W. Macrophage biology in development, homeostasis and disease. *Nature* **496**, 445–55 (2013).

131. Sica, A. & Mantovani, A. Macrophage plasticity and polarization : in vivo veritas. *J. Clin. Invest.* **122**, 787–795 (2012).

132. Mills, C. D., Kincaid, K., Alt, J. M., Heilman, M. J. & Hill, A. M. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J. Immunol.* **164**, 6166–73 (2000).

133. Biswas, S. K. & Mantovani, A. Orchestration of metabolism by macrophages. *Cell Metab.* **15**, 432–437 (2012).

134. Murray, P. J. Macrophage Polarization. *Annu. Rev. Physiol.* **79**, 541–566 (2017).

135. Biswas, S. K. & Mantovani, A. Macrophage plasticity and interaction with lymphocyte subsets: Cancer as a paradigm. *Nat. Immunol.* **11**, 889–896 (2010).

136. Shouval, D. S. *et al.* Interleukin 1β Mediates Intestinal Inflammation in Mice and Patients With Interleukin 10 Receptor Deficiency. *Gastroenterology* **151**, 1100–1104 (2016).

137. Ip, W. K. E., Hoshi, N., Shouval, D. S., Snapper, S. & Medzhitov, R. Anti-inflammatory effect of IL-10 mediated by metabolic reprogramming of macrophages. *Science (80-.).* **356**, 513–519 (2017).

138. Malik, A. & Kanneganti, T.-D. Inflammasome activation and assembly at a glance. *J. Cell Sci.* **130**, 3955–3963 (2017).

139. Dinarello, C. A. Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunol. Rev.* **281**, 8–27 (2018).

140. Rathinam, V. A. K. & Fitzgerald, K. A. Inflammasome Complexes: Emerging Mechanisms and Effector Functions. *Cell* **165**, 792–800 (2016).

141. Meier, J. A. & Larner, A. C. Toward a new STATe: The role of STATs in mitochondrial function. *Semin. Immunol.* **26**, 20–28 (2014).

142. Vander Heiden, M. G., Cantley, L. & Thompson, C. B. Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. *Science (80-.).* **324**, 1029–1033 (2009).

143. Hard, G. C. Some biochemical aspects of the immune macrophage. *Br. J. Exp. Pathol.* **51**, 97–105 (1970).

144. Sbarra, A. J. & Karnovsky, M. L. The Biochemical Basis of Phagocytosis. *J. Biol. Chem.* **234**, 1355–1362 (1959).

145. Krawczyk, C. M. *et al.* Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. *Blood* **115**, 4742–9 (2010).

146. Kelly, B. & O'Neill, L. A. J. Metabolic reprogramming in macrophages and dendritic cells in innate immunity. *Cell Res.* **25**, 771–784 (2015).

147. Clementi, E., Brown, G. C., Feelisch, M. & Moncada, S. Persistent inhibition of cell respiration by nitric oxide: Crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. *Proc. Natl. Acad. Sci.* **95**, 7631–7636 (1998).

148. Byles, V. *et al.* The TSC-mTOR pathway regulates macrophage polarization. *Nat. Commun.* **4**, 1–11 (2013).

149. Jones, R. G. & Pearce, E. J. MenTORing Immunity: mTOR Signaling in the Development and Function of Tissue-Resident Immune Cells. *Immunity* **46**, 730–742 (2017).

150. Düvel, K. *et al.* Activation of a metabolic gene regulatory network downstream of mTOR complex 1. *Mol. Cell* **39**, 171–183 (2010).

151. Rodríguez-Prados, J.-C. *et al.* Substrate Fate in Activated Macrophages: A Comparison between Innate, Classic, and Alternative Activation. *J. Immunol.* **185**, 605–614 (2010).

152. Sag, D., Carling, D., Stout, R. D. & Suttles, J. Adenosine 5′-Monophosphate-Activated Protein Kinase Promotes Macrophage Polarization to an Anti-Inflammatory Functional Phenotype. *J. Immunol.* **181**, 8633–8641 (2008).

153. Van den Bossche, J., O'Neill, L. A. & Menon, D. Macrophage Immunometabolism: Where Are We (Going)? *Trends Immunol.* **38**, 395–406 (2017).

154. Jha, A. K. *et al.* Network Integration of Parallel Metabolic and Transcriptional Data Reveals Metabolic Modules that Regulate Macrophage Polarization. *Immunity* **42**, 419–430 (2015).

155. Feingold, K. R. *et al.* Mechanisms of triglyceride accumulation in activated macrophages. *J. Leukoc. Biol.* **92**, 829–839 (2012).

156. Michelucci, A. *et al.* Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production. *Proc. Natl. Acad. Sci.* **110**, 7820–7825 (2013).

157. O'Neill, L. A. J. & Artyomov, M. N. Itaconate: the poster child of metabolic reprogramming in macrophage function. *Nature Reviews Immunology* **19**, 273–281 (2019).

158. Lampropoulou, V. *et al.* Itaconate Links Inhibition of Succinate Dehydrogenase with Macrophage Metabolic Remodeling and Regulation of Inflammation. *Cell Metab.* **24**, 158–166 (2016).

159. Tannahill, G. M. *et al.* Succinate is an inflammatory signal that induces IL-1β through HIF-1α. *Nature* **496**, 238–42 (2013).

160. Mills, E. L. *et al.* Succinate Dehydrogenase Supports Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages. *Cell* **167**, 457-470.e13 (2016).

161. Mills, E. L. & O'Neill, L. A. Reprogramming mitochondrial metabolism in macrophages as an anti-inflammatory signal. *Eur. J. Immunol.* **46**, 13–21 (2016).

162. Zhu, L. *et al.* TSC1 controls macrophage polarization to prevent inflammatory disease. *Nat. Commun.* **5**, 1–13 (2014).

163. O'Neill, L. A. J., Kishton, R. J. & Rathmell, J. A guide to immunometabolism for immunologists.

Nat. Rev. Immunol. **16**, 553–65 (2016).

164. Haschemi, A. *et al.* The sedoheptulose kinase CARKL directs macrophage polarization through control of glucose metabolism. *Cell Metab.* **15**, 813–826 (2012).

165. Zhou, R., Yazdi, A. S., Menu, P. & Tschopp, J. A role for mitochondria in NLRP3 inflammasome activation. *Nature* **469**, 221–226 (2011).

Chapter 2

STAT1 signaling shields T cells from NK cell mediated cytotoxicity

Chapter 2.1 – Attributions

The work presented in this chapter are adapted from the following publication:

Kang YH, Biswas A, Field M and Snapper SB. STAT1 signaling shields T cells from NK cell-mediated cytotoxicity. *Nat. Commun.* **10** (912), 1-13 (2019).

Y.H.K. and S.B.S. conceived the study. Y.H.K., A.B., and S.B.S. designed the experiments. Y.H.K., M.F. and A.B. performed the experiments, acquired and analyzed the data. Y.H.K. and S.B.S. wrote the manuscript.

Chapter 2.2 – Introduction

The JAK-STAT signaling pathway is a critical signaling hub that processes information from various cytokines to achieve distinct transcriptional outcomes[1]. In T cells, JAK-STAT signaling pathway is mostly studied in terms of their regulation of T cell differentiation[2]. Among the seven mammalian STAT family members, STAT1 is known to be important for the induction of Th1 cells downstream of IFNγ due to its induction of the transcription factor T-bet[3,4]. This proinflammatory property of STAT1 is important for controlling infections, where patients with loss-of-function mutations in *STAT1* develop susceptibility to viral/ mycobacterial infections[5]. STAT1 has also been shown to suppress regulatory T cell differentiation *in vivo*, thus promoting inflammatory diseases like graft-vs-host-disease (GvHD)[6]. However, STAT1 can also suppress Th17 differentiation[7], and *Stat1*$^{-/-}$ mice develop aggravated Th17-mediated autoimmune diseases including experimental autoimmune encephalomyelitis (EAE)[8,9].

Inflammatory bowel diseases (IBD) are likely to arise from an aberrant immune response toward intestinal microbes in a genetically susceptible host, with T cells playing an important role[10]. Crohn's disease in particular is characterized by a skewing of the CD4$^+$ T cell profile toward proinflammatory Th1 and Th17 subsets, which are believed to be critical for disease pathogenesis[11]. Patients with Crohn's disease also display higher STAT1 expression, albeit only modestly in CD4$^+$ T cells[12]. However, the mechanism by which STAT1 modulates CD4$^+$ T cells in IBD is currently unclear and presumed to be through altering differentiation states[13].

IL-10 is a critical anti-inflammatory cytokine for maintaining intestinal immune homeostasis, as evidenced in mice and humans deficient in IL-10 or IL-10 receptor (IL-10R) that develop spontaneous IBD[14–16]. We and others recently described the importance of IL-10R signaling in macrophages in the prevention of colitis. Mice harboring a macrophage specific deletion of IL-10R develop spontaneous colitis, and *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice but not *Rag1*$^{-/-}$ mice develop colitis upon reconstitution with WT CD4$^+$ T cells[17–19]. Subsequent studies in our model and others pointed to a role for pathogenic Th17 cells in

driving the disease[20–25].As STAT1 is a critical regulator of Th1/Th17 differentiation, we further investigated its role in the ability of $CD4^+$ T cells to induce colitis.

Here, we describe a role for STAT1 in enabling T cells to induce colitis by protecting them from NK cell-mediated cytotoxicity. *Stat1*$^{-/-}$ T cells fail to expand and induce colitis *in vivo* unless NK cells are depleted. This is because STAT1 is required to maintain sufficient levels of *Nlrc5* and the inhibitory NK ligand MHC class I, thus enabling evasion of rejection by host NK cells. Surprisingly, this requirement for STAT1 is largely independent of both Type I and II IFN signaling, the classical activators of STAT1. Moreover, this mechanism is specific to *Stat1*$^{-/-}$ T cells that underwent spontaneous proliferation, suggesting that NK cells eliminate *Stat1*$^{-/-}$ T cells only when they are activated by the microbiota. *Stat1*$^{-/-}$ mice however display normal levels of $CD4^+$ T cells as *Stat1* expression in the innate compartment is required to eliminate the T cells. Altogether, our study reveals a critical role of STAT1 that is distinct from T cell differentiation and adds a new perspective to studies on T cell mediated inflammatory disease.

Chapter 2.3 – T cells require STAT1 to expand and induce colitis *in vivo*

To investigate the role of STAT1 signaling in T-cell driven colitis, we adoptively transferred unfractionated WT or *Stat1*$^{-/-}$ CD4^{+} T cells into *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice (**Figure 3a**). WT T cells induced severe colitis in *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ recipient mice as expected[18]. In contrast, mice transferred with *Stat1*$^{-/-}$ T cells displayed no signs of intestinal inflammation as evidenced by the lack of weight loss, colonic thickening and histological inflammation (**Figure 3a, b**). Flow cytometric analysis of the colonic lamina propria revealed a marked reduction of *Stat1*$^{-/-}$ T cells compared to WT T cells (**Figure 3c**). This was not due to aberrant homing of *Stat1*$^{-/-}$ T cells to the intestine, as a similar reduction of T cells was observed in the spleen (**Figure 3d**).

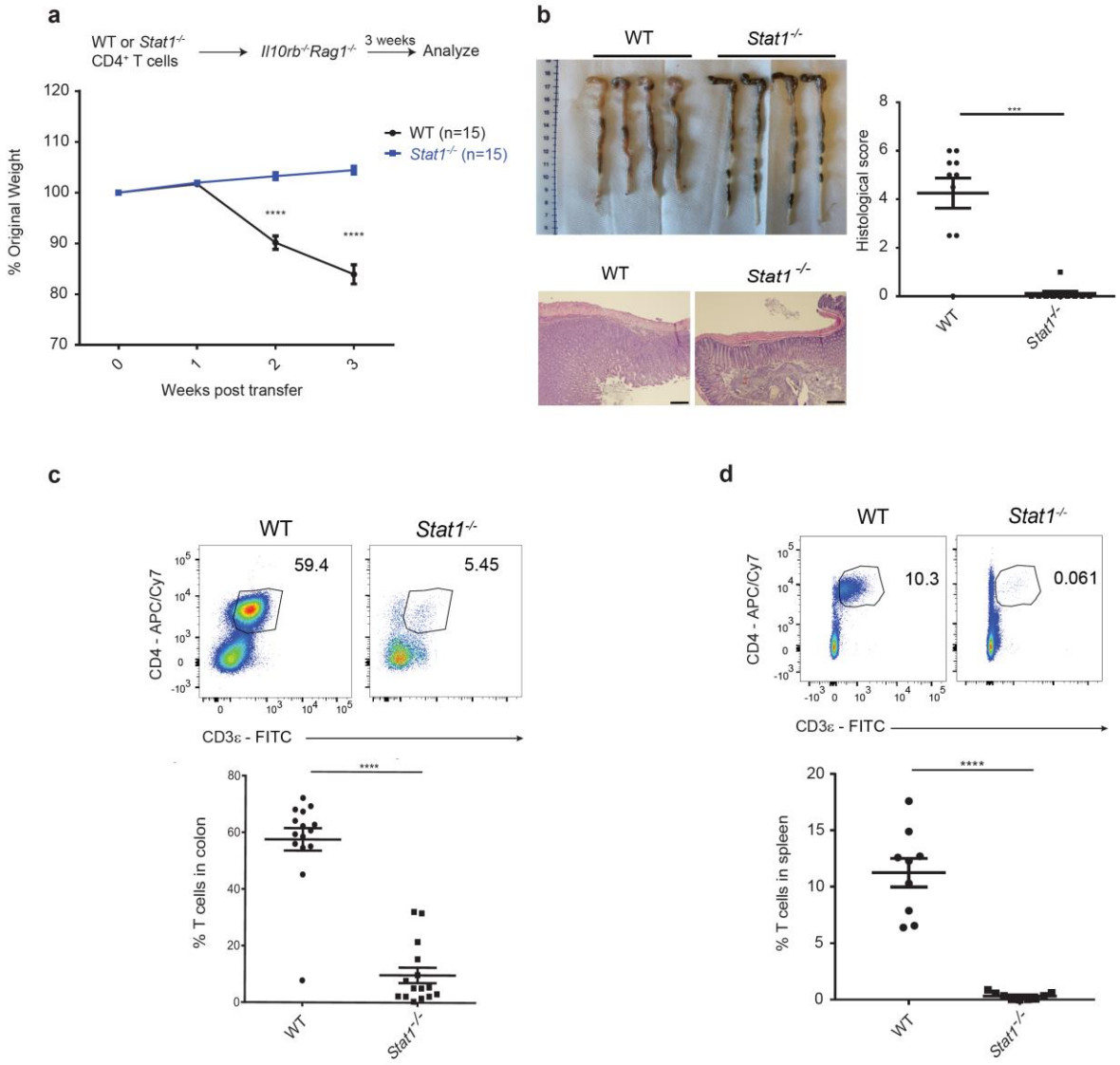


Figure 3. *Stat1*$^{-/-}$ T cells fail to induce colitis due to defective expansion. *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice were injected i.p. with 1 × 10^6 unfractionated WT or *Stat1*$^{-/-}$ CD4$^+$ T cells. (a) Mean % original body weights ± SEM following T cell transfer. (b) Representative images of colons, as well as representative H&E images of distal colon sections with mean histological scores ± SEM at 3 weeks post transfer. Scale bar represents 200 µm. (c, d) Representative flow cytometry plots of CD4$^+$ T cells (gated on live CD45$^+$ cells) in the (c) colon and (d) spleen followed by their mean frequencies ± SEM at 3 weeks post transfer. All data are pooled from two to three independent experiments, with each point representing an individual mouse. ****$p < 0.0001$, ***$p < 0.001$ by (a) two-way ANOVA with Bonferroni's correction or (b–d) two-tailed Mann–Whitney test.

Chapter 2.4 – STAT1 is required for T cell expansion independent of colonic inflammation

We next asked if the reduction of *Stat1*$^{-/-}$ T cells was dependent on colonic inflammation by transferring unfractionated WT or *Stat1*$^{-/-}$ CD4^{+} T cells into *Rag1*$^{-/-}$ mice. *Rag1*$^{-/-}$ mice develop colitis with naïve but not unfractionated WT T cells (**Figure 4a, b)**[18,26]. Similar to *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice, *Stat1*$^{-/-}$ T cells were markedly reduced in the colons and spleens of *Rag1*$^{-/-}$ mice, indicating that STAT1 is required for robust *in vivo* T cell expansion independent of colonic inflammation and innate IL-10R expression (**Figure 4c, d**).

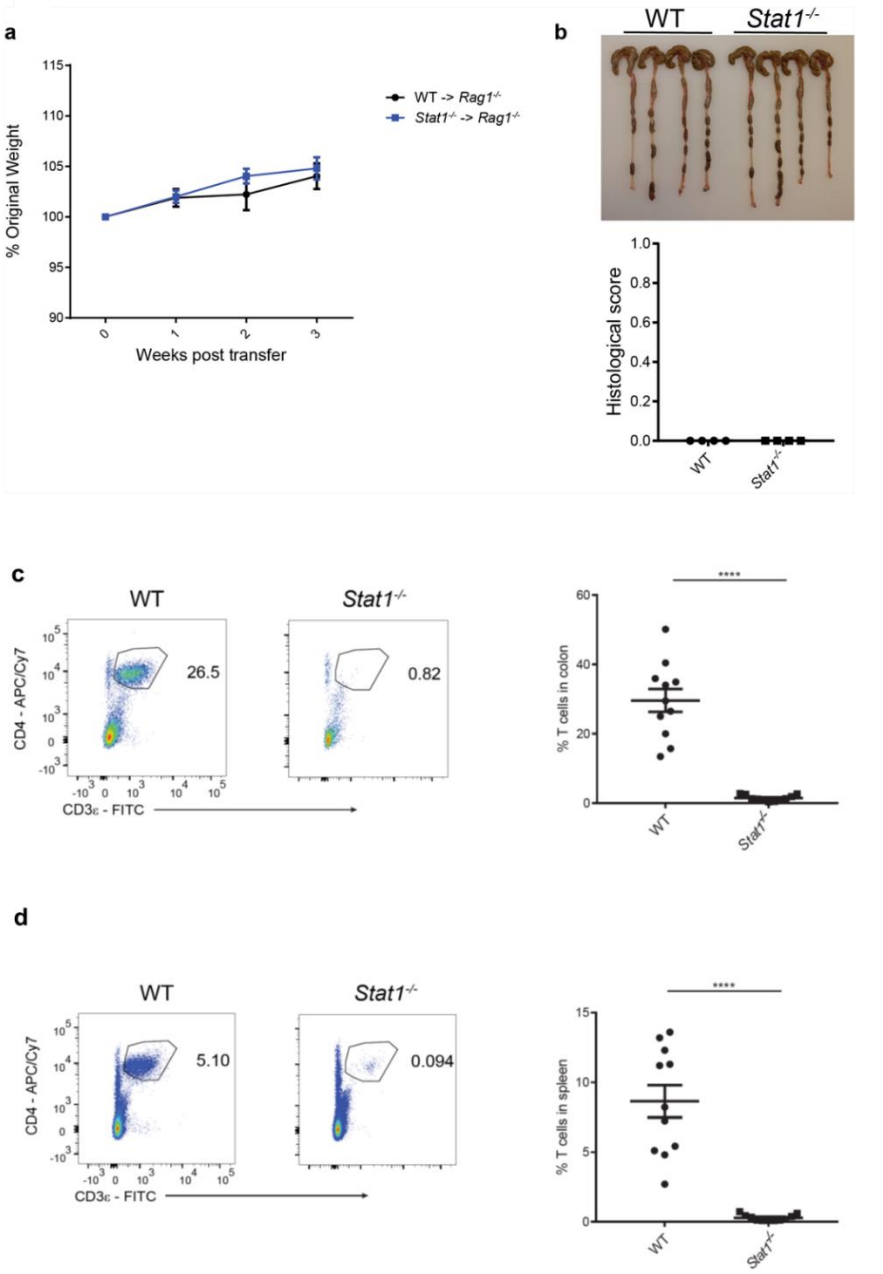


Figure 4. *Stat1*$^{-/-}$ T cells fail to expand in *Rag1*$^{-/-}$ mice. *Rag1*$^{-/-}$ mice were injected i.p. with 1 × 10^6 WT or *Stat1*$^{-/-}$ unfractionated CD4$^+$ T cells and analyzed 3 weeks post transfer. (a) Mean % original body weights ± SEM following T cell transfer. (b) Representative images of colons, as well as mean histological scores ± SEM of proximal/distal colon at 3 weeks post transfer. (c, d) Representative flow cytometry plots of CD4$^+$ T cells (gated on live CD45$^+$ cells) in the colon (c) and spleen (d) followed by their mean frequencies ± SEM. Data are pooled from (a, b) one or (c, d) three independent experiments, with each point representing an individual mouse. ****$p < 0.0001$ by two-tailed Mann–Whitney test.

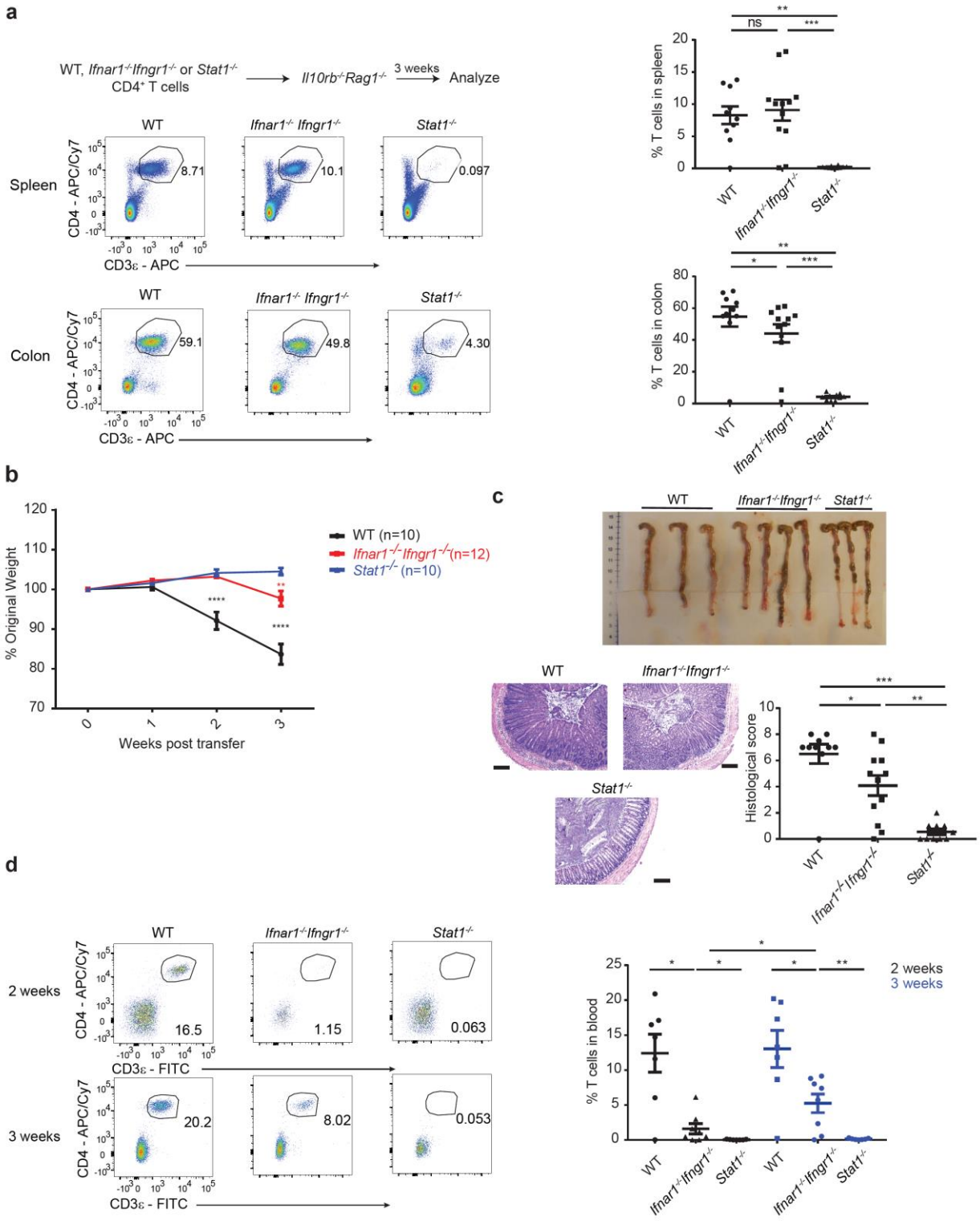
Chapter 2.5 – The defective expansion of *Stat1*$^{-/-}$ T cells is only partially dependent on Type I and II IFN signaling

IFNs are the classical inducers of STAT1 signaling with both Type I and Type II IFN individually reported to regulate T cell function[3,27–29]. We therefore sought to determine if the impaired expansion of *Stat1*$^{-/-}$ T cells was due to the lack of both type I and type II IFN signaling by transferring *Ifnar1*$^{-/-}$*Ifngr1*$^{-/-}$ CD4^{+} T cells into *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice (**Figure 5a**). Surprisingly, the abrogation of both Type I and Type II IFN receptors failed to recapitulate STAT1 deficiency, as *Ifnar1*$^{-/-}$*Ifngr1*$^{-/-}$ CD4^{+} T cells expanded to similar levels as WT T cells in the spleen and colon 3 weeks post transfer (**Figure 5a**).

Ifnar1$^{-/-}$*Ifngr1*$^{-/-}$ T cells were also able to induce colitis unlike *Stat1*$^{-/-}$ T cells (**Figure 5b, c**). However, the severity of colitis induced by *Ifnar1*$^{-/-}$*Ifngr1*$^{-/-}$ T cells was reduced compared to WT T cells (**Figure 5b, c**), which correlated with a reduced rate of expansion of *Ifnar1*$^{-/-}$*Ifngr1*$^{-/-}$ T cells in the blood (**Figure 5d**). As expected, *Stat1*$^{-/-}$ T cells did not expand in the blood (**Figure 5d**). These data suggest that while Type I + II IFN partially contribute to the STAT1-dependent signaling, the impaired expansion of *Stat1*$^{-/-}$ T cells is predominantly an IFN-independent process at later time points.

Figure 5. Type I + II IFN signaling do not explain the defective expansion of *Stat1*$^{-/-}$ T cells. *Il10rb*$^{-/-}$ *Rag1*$^{-/-}$ mice were injected i.p. with 1×10^6 WT, *Ifnar1*$^{-/-}$*Ifngr1*$^{-/-}$ or *Stat1*$^{-/-}$ CD4$^+$ T cells. (a) Representative flow cytometry plots of CD4$^+$ T cells (gated on live CD45$^+$ cells) in the spleen and colon followed by their mean frequencies ± SEM at 3 weeks post transfer. (b) Mean % initial body weights ± SEM following T-cell transfer. (c) Representative images of colons, as well as representative H&E images of distal colon sections with mean histological scores ± SEM at 3 weeks post transfer. Scale bar represents 200 μm. (d) Representative flow cytometry plots of CD4$^+$ T cells (gated on CD45$^+$ cells) in the blood followed by their mean frequencies ± SEM at 2 and 3 weeks post transfer. All data are pooled from 2-3 independent experiments, with each point representing an individual mouse. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ by (b) two-way ANOVA with Bonferroni's correction (WT compared to *Ifnar1*$^{-/-}$*Ifngr1*$^{-/-}$ or *Stat1*$^{-/-}$, *Ifnar1*$^{-/-}$*Ifngr1*$^{-/-}$ compared to *Stat1*$^{-/-}$) or by (a, c, d) two-tailed Mann–Whitney test.

Figure 5 (continued)



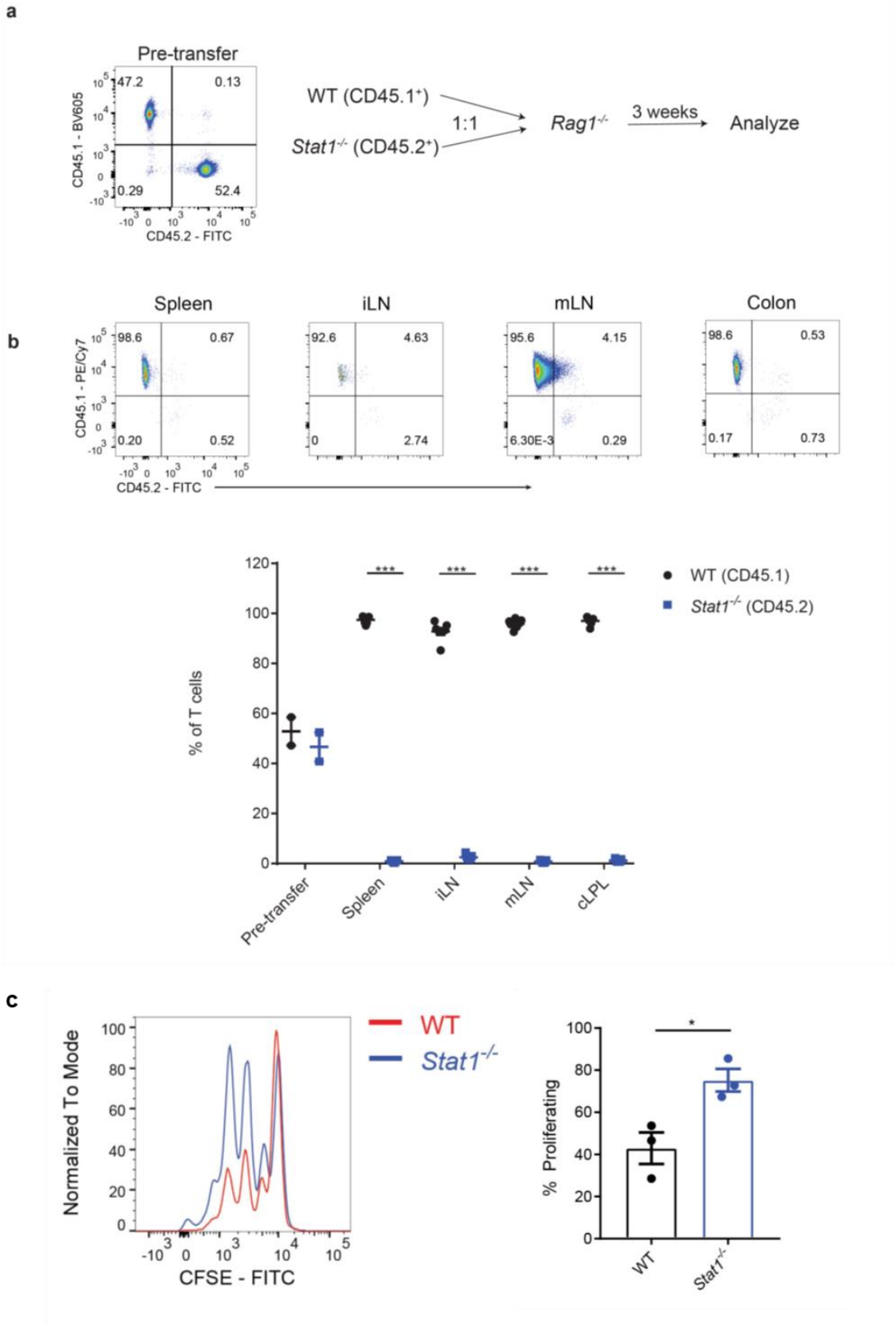
Chapter 2.6 - Cell-intrinsic role for STAT1 in *in vivo* T cell expansion

To understand the mechanisms linking STAT1 to T cell expansion *in vivo*, we first asked if the presence of WT T cells could rescue the defective expansion of *Stat1*$^{-/-}$ T cells by transferring equal ratios of congenically marked WT (CD45.1^{+}) and *Stat1*$^{-/-}$ (CD45.2^{+}) T cells into *Rag1*$^{-/-}$ mice (**Figure 6a**). Notably, all of the T cells identified three weeks post transfer were WT, indicating that the defective expansion of *Stat1*$^{-/-}$ T cells is cell-intrinsic (**Figure 6b**).

We also asked if the defective expansion of *Stat1*$^{-/-}$ T cells could be recapitulated *in vitro*. In contrast with the *in vivo* defect, *Stat1*$^{-/-}$ T cells displayed a hyperproliferative phenotype compared to WT T cells upon *in vitro* stimulation (**Figure 6c**), consistent with earlier reports[6,30]. This indicates that the expansion defect of *Stat1*$^{-/-}$ T cells is not cell autonomous and requires an *in vivo* environment.

Figure 6. The defective expansion of *Stat1*$^{-/-}$ T cells is cell-intrinsic and requires an *in vivo* environment. (a, b) WT (CD45.1^{+}) or *Stat1*$^{-/-}$ (CD45.2^{+}) unfractionated CD4^{+} T cells were injected i.p. at a 1:1 ratio (0.8–1× 10^{6}/type) into *Rag1*$^{-/-}$ mice. (a) Schematic of experiment and representative plot of cells injected. (b) Representative images of CD45.1^{+} vs CD45.2^{+} cells (gated on live CD45^{+} CD3ε^{+} CD4^{+} T cells) from various organs followed by their mean frequencies ± SEM. (c) WT or *Stat1*$^{-/-}$ unfractionated CD4^{+} T cells were stimulated *in vitro* with anti-CD3 (3μg/ml) and anti-CD28 (1μg/ml) for 3 days and cell proliferation analyzed. Representative flow cytometry plots are shown (gated on live CD4^{+} cells) with mean frequencies ± SEM. Data is pooled from (a,b) 2 or (c) 3 independent experiments, with each point representing (a,b) an individual mouse or (c) the average across technical replicates in each experiment. (b) ***$p < 0.001$ by two-tailed Mann–Whitney test or (c) *$p<0.05$ by unpaired two-tailed t-test.

Figure 6 (continued)



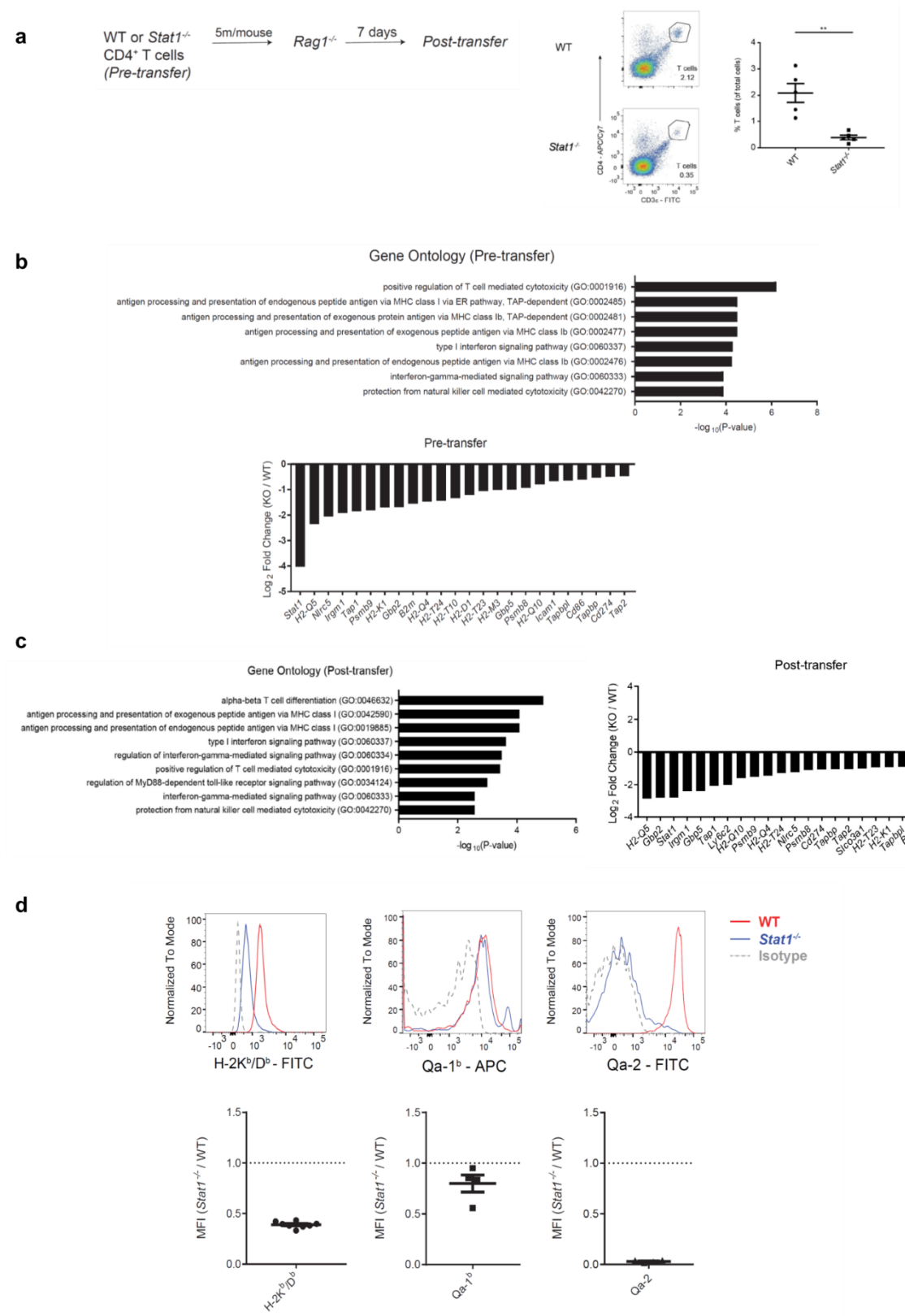
Chapter 2.7 - Downregulation of the MHC class I antigen presentation pathway in *Stat1*$^{-/-}$ T cells

To investigate whether a dysregulated transcriptional profile might account for the observed defect, we performed gene expression analysis on *Stat1*$^{-/-}$ T cells pre and post transfer into *Rag1*$^{-/-}$ mice by RNA-seq. As *Stat1*$^{-/-}$ T cells failed to expand to appreciable amounts *in vivo* after 3 weeks, we transferred a larger number of cells and analyzed their gene expression at 1 week post transfer, a time point where *Stat1*$^{-/-}$ T cells were beginning to decline (**Figure 7a**). Gene ontology (GO) analysis of genes differentially regulated between WT and *Stat1*$^{-/-}$ T cells revealed, as expected, categories related to Type I and II IFN signaling in both settings (**Figure 7b, c**). Interestingly, categories related to the MHC class I (MHC-I) antigen presentation pathway were significantly enriched (**Figure 7b, c**). Consistent with the GO analysis, *Stat1*$^{-/-}$ T cells had reduced expression of *Nlrc5*, MHC-I (*H2-K1, H2-D1, B2m, H2-T23*) and various genes involved in MHC-I antigen presentation (*Tap1, Tap2, Psmb8, Psmb9*) (**Figure 7b, c**).

NLRC5 is a critical transactivator of multiple MHC-I genes, and STAT1 is required to induce its expression by binding to the *Nlrc5* promoter in response to IFNγ[31–34]. Consistent with our RNA-seq data and with earlier reports[33,35], *Stat1*$^{-/-}$ T cells displayed reduced surface levels of the classical MHC-I molecules H-2K^{b}/H-2D^{b}. Interestingly, surface levels of the non-classical molecule Qa-1 was only mildly affected by STAT1 deficiency whereas levels of Qa-2 were severely reduced (**Figure 7d**).

Figure 7. Downregulation of the MHC class I antigen presentation machinery in *Stat1*$^{-/-}$ T cells. RNA-seq was performed on WT or *Stat1*$^{-/-}$ unfractionated CD4^{+} T cells pre- and post-transfer (5 X 10^{6} cells injected i.p.) into *Rag1*$^{-/-}$ mice. (a) Schematic of experimental setup and representative flow cytometry plots of CD4^{+} T cells (spleen + lymph nodes) post-transfer, along with their mean frequencies ± SEM. Each point represents an individual mouse. **p<0.01 by two-tailed Mann-Whitney test. (b) Selected Gene ontology terms (PANTHER) showing differential expression of the MHC class I pathway, as well as downregulation of specific genes involved in MHC class I antigen presentation in *Stat1*$^{-/-}$ T cells compared to WT T cells pre-transfer. (c) Similar analysis as in (b) but performed in T cells post-transfer (sorted as CD45^{+} CD3ε^{+} CD4^{+}). All genes displayed are significantly different (n = 3 biological replicates, $p < 0.05$ with correction for multiple testing by Benjamini-Hochberg procedure). (d) Representative flow cytometry plots showing surface expression of classical and non-classical MHC class I molecules on CD4^{+} T cells from the spleens of WT or *Stat1*$^{-/-}$ mice, followed by their cumulative enumeration expressed as a ratio of Median Fluorescence Intensity (*Stat1*$^{-/-}$ / WT) ± SEM. Data is pooled from three or more independent experiments, with each point representing an individual mouse. Similar numbers of WT and *Stat1*$^{-/-}$ mice were used for the comparison.

Figure 7 (continued)



Chapter 2.8 - Depletion of NK cells rescues *Stat1*$^{-/-}$ T cell expansion and colitis

The MHC-I molecule is the classic inhibitory ligand for NK cells, and cellular expression of MHC-I protects cells from NK mediated killing[36]. Tumors or virally infected cells can reduce MHC-I expression to evade CD8^{+} T cell recognition, but this renders them susceptible to NK mediated killing—a phenomenon described as missing self[36,37]. The reduced expression of MHC-I on *Stat1*$^{-/-}$ T cells led us to hypothesize that their defective expansion was due to elimination by NK cells, which are present and more active in *Rag1*$^{-/-}$ mice[38]. This hypothesis was supported by the GO analysis, which revealed the category: Protection from natural killer cell mediated cytotoxicity (**Figure 7b, c**).

To test the hypothesis that *Stat1*$^{-/-}$ T cells were eliminated *in vivo* by NK cells, we depleted NK cells in *Rag1*$^{-/-}$ and *Il10rb*$^{-/-}$ *Rag1*$^{-/-}$ mice at the time of T cell transfer by employing an anti-NK1.1 antibody. The anti-NK1.1 depletion regimen successfully eliminated the NK cells (**Figure 8a**). In *Rag1*$^{-/-}$ mice, the depletion of NK cells significantly rescued the survival of *Stat1*$^{-/-}$ T cells, thus supporting the hypothesis (**Figure 8b**).

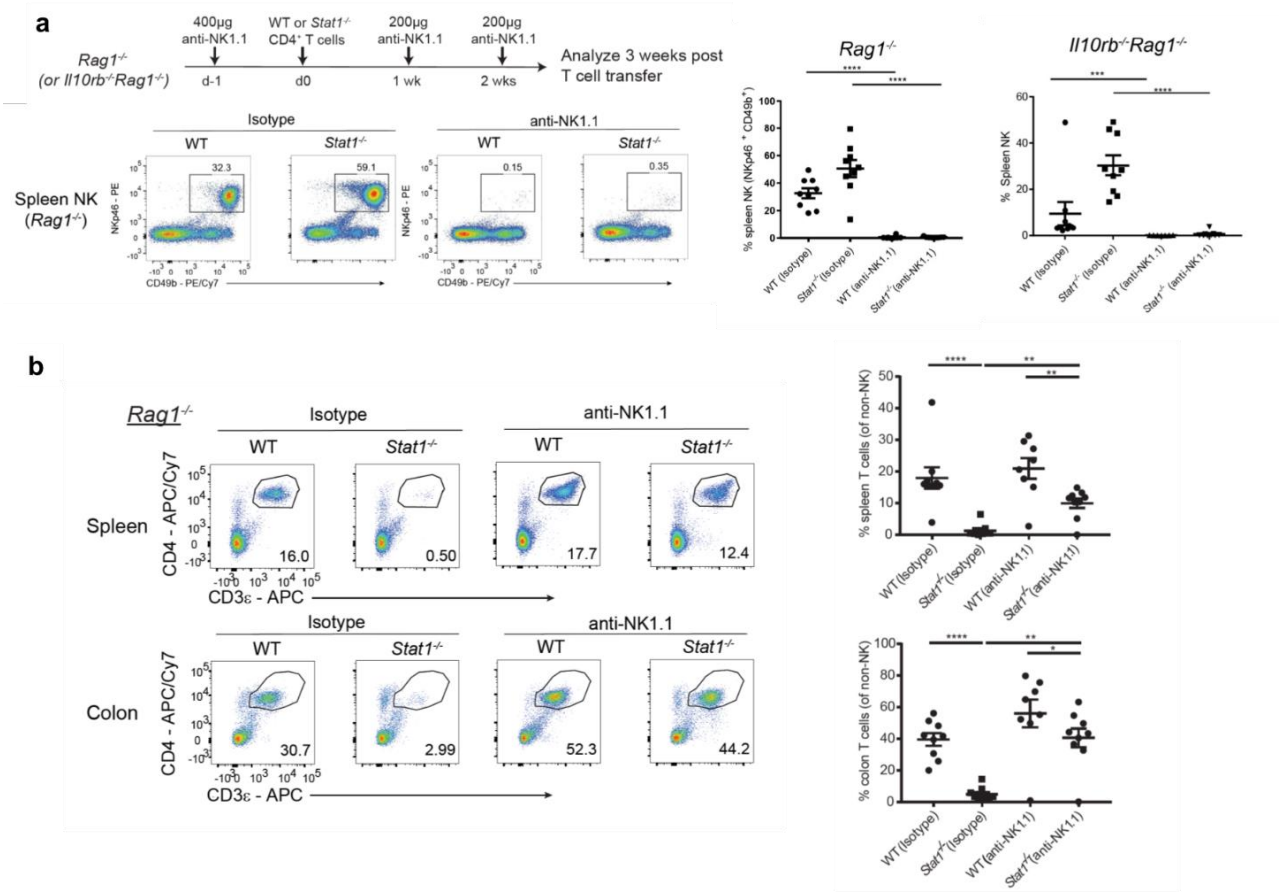


Figure 8. Depletion of NK cells restores *Stat1*$^{-/-}$ T cell expansion. 1× 10^6 WT or *Stat1*$^{-/-}$ CD4$^+$ T cells were injected i.p. into *Rag1*$^{-/-}$ mice that were treated with NK depleting antibody (or isotype control). (a) Schematic of experimental setup shown. Representative flow cytometry plots of splenic NK cells (NKp46$^+$ CD49b$^+$) in *Rag1*$^{-/-}$ mice injected with control or NK cell depleting antibody at 3 weeks post transfer are also shown with their mean frequencies ± SEM in both *Rag1*$^{-/-}$ and *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice. (b) Representative flow cytometry plots of CD4$^+$ T cells (gated on live CD45$^+$ non-NK cells) in the spleen and colon of *Rag1*$^{-/-}$ mice with their mean frequencies ± SEM at 3 weeks post transfer. Data pooled from three independent experiments, with each point representing an individual mouse. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by two-tailed Mann–Whitney test.

We next asked if *Stat1*$^{-/-}$ T cells were able to induce colitis in the absence of NK cells by transferring them into NK cell-depleted *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice. Strikingly, *Stat1*$^{-/-}$ T cells were able to induce disease in NK cell-depleted *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice unlike their control-treated NK cell-replete counterparts (**Figure 9a, b**). The induction of disease correlated with a restored expansion of *Stat1*$^{-/-}$ T cells in the spleen and the colon, suggesting that *Stat1*$^{-/-}$ T cells have the ability to cause colitis if they are allowed to expand (**Figure 9c**).

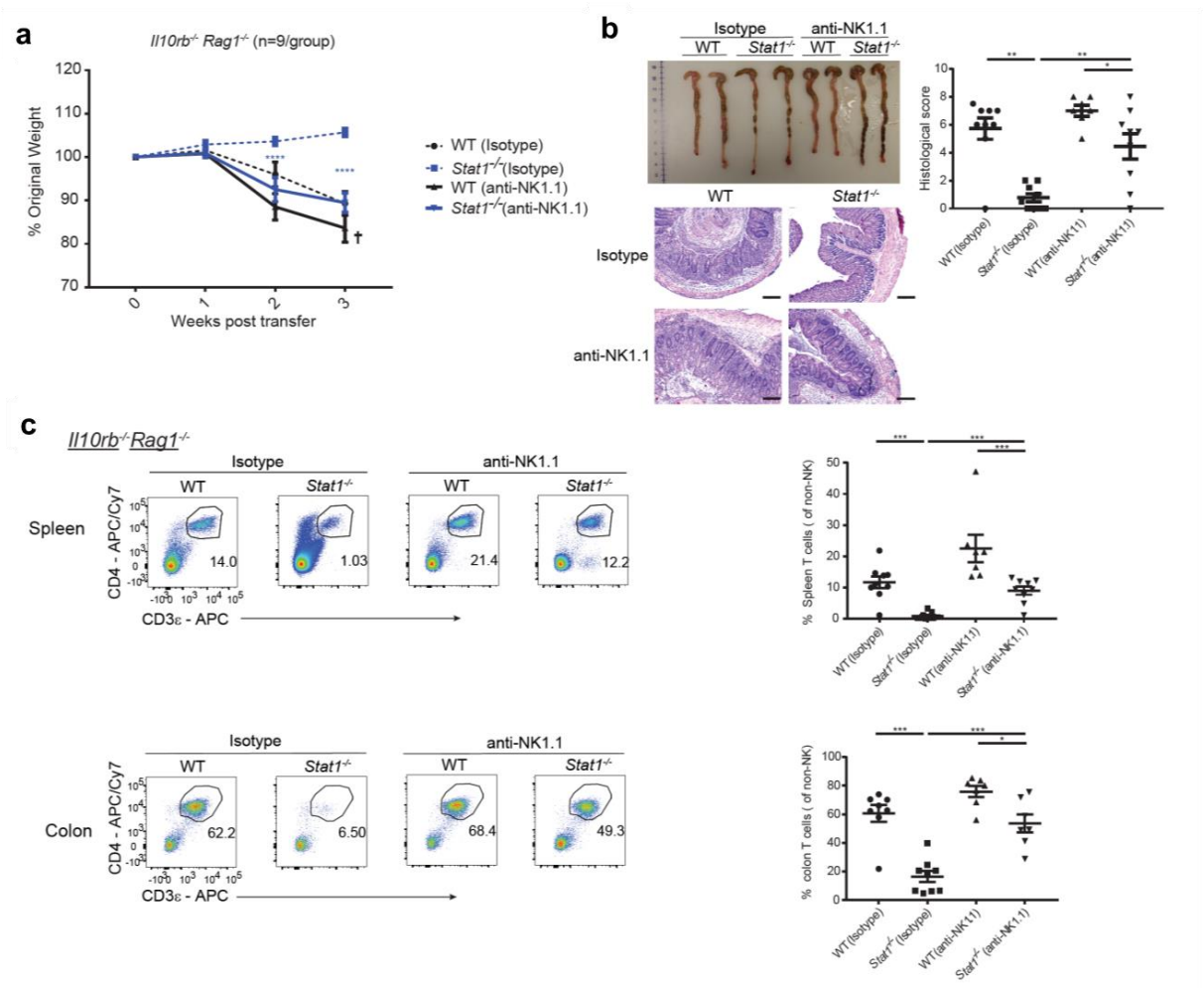


Figure 9. Depletion of NK cells restores *Stat1*$^{-/-}$ T cell induced colitis. 1× 10^6 WT or *Stat1*$^{-/-}$ CD4$^+$ T cells were injected i.p. into *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice that were treated with NK depleting antibody (or isotype control) as in Figure 8. (a) Mean % initial body weights ± SEM following T cell transfer. (b) Representative images of colons, as well as representative H&E images of distal colon sections with mean histological scores ± SEM at 3 weeks post transfer. (c) Representative flow cytometry plots of CD4$^+$ T cells (gated on live CD45$^+$ non-NK cells) in the spleen and colon with their mean frequencies ± SEM at 3 weeks post transfer. Scale bar represents 200 μm. Data pooled from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by (a) two-way ANOVA with Bonferroni's correction (*Stat1*$^{-/-}$ anti-NK1.1 compared to *Stat1*$^{-/-}$ Isotype) or by (b,c) two-tailed Mann–Whitney test. ✝ Two mice (WT anti-NK1.1) were sacrificed before the 3 week time point due to excessive weight loss thus their weights only apply till week 2.

Chapter 2.9 - Depletion of NK cells does not affect *Stat1*$^{-/-}$ T cell differentiation *in vivo*

We also analyzed the differentiation profile of *Stat1*$^{-/-}$ T cells *in vivo*. In agreement with previous reports[7], *Stat1*$^{-/-}$ T cells displayed enhanced Th17 differentiation. However, this differentiation profile was seen in both control and NK cell-depleted *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice, suggesting that the primary role of STAT1 in T cell driven colitis is to protect the T cells from NK mediated elimination, rather than to repress their intrinsic Th17 differentiation potential (**Figure 10**).

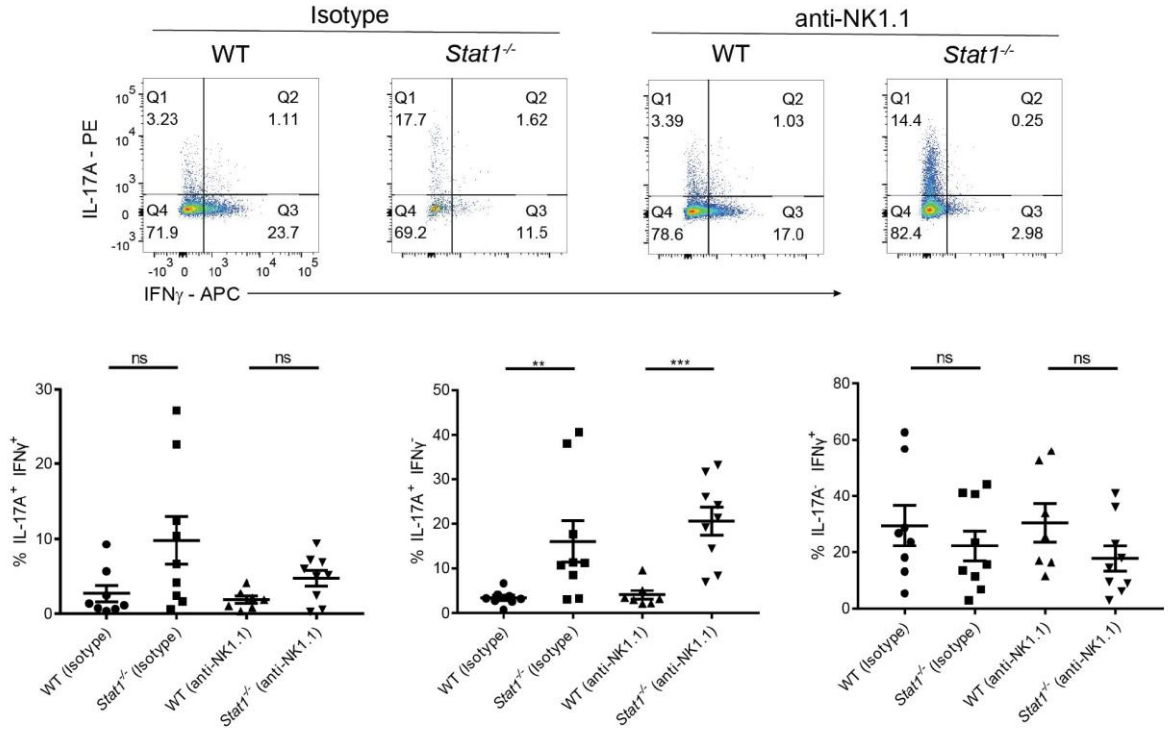


Figure 10. NK cell depletion does not affect *Stat1*$^{-/-}$ T cell differentiation. Colonic T cells (gated on live CD45^{+} TCRβ^{+} CD4^{+} T cells) from *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice at 3 weeks post transfer were analyzed for their differentiation profile by IL-17A and IFNγ staining. Representative plots are shown along with their mean frequencies ± SEM. Data pooled from 3 independent experiments, with each point representing an individual mouse. **p<0.01, ***p<0.001 by two-tailed Mann-Whitney test.

Chapter 2.10 - NK cells specifically target *Stat1*$^{-/-}$ T cells undergoing spontaneous proliferation

T cells undergo two distinct modes of proliferation upon transfer into chronically lymphopenic hosts (e.g., *Rag1*$^{-/-}$ mice)—slow, true homeostatic proliferation (HP) that is driven primarily by IL-7, as well as rapid, spontaneous proliferation (SP) that is driven by the microbiota and IL-6[39–43]. We asked whether the NK cell-mediated elimination of *Stat1*$^{-/-}$ T cells requires T cell proliferation by transferring CellTrace Violet (CTV) labeled WT or *Stat1*$^{-/-}$ CD4^{+} T cells into *Rag1*$^{-/-}$ mice. *Stat1*$^{-/-}$ T cells displayed a reduction in the SP population compared to WT T cells, with no difference in the HP population (**Figure 11a**). Importantly, the depletion of NK cells significantly rescued the *Stat1*$^{-/-}$ SP population, suggesting that NK cells specifically restrict *Stat1*$^{-/-}$ T cells undergoing SP (**Figure 11b**). Interestingly, this rescue was not complete as we also observed an increase in the SP of WT T cells upon NK cell depletion, which is consistent with the incomplete rescue of *Stat1*$^{-/-}$ T cell expansion as well as the degree of colitis induced in *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice (**Figure 8-9**).

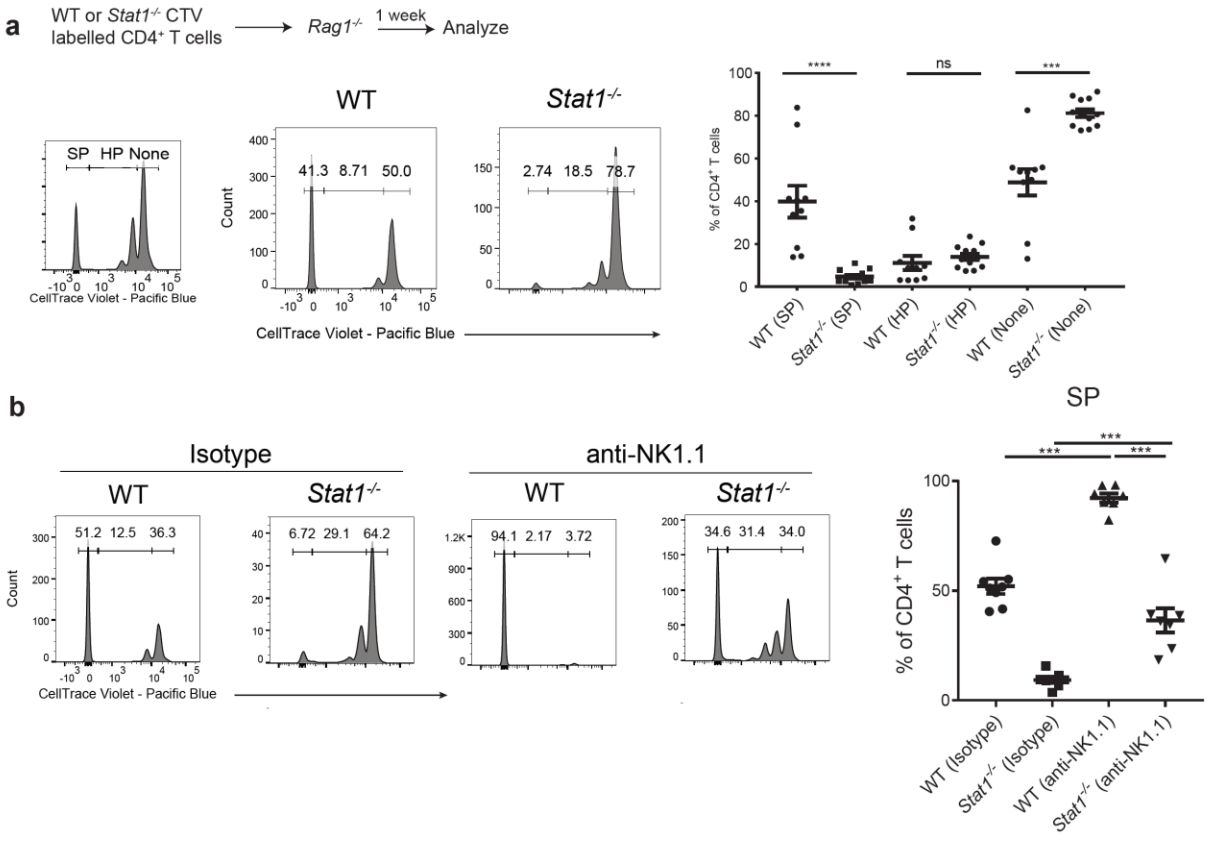


Figure 11. NK cells specifically restrict *Stat1*$^{-/-}$ T cells undergoing spontaneous proliferation. *Rag1*$^{-/-}$ mice were injected with equal numbers (3–4× 10^6) of WT or *Stat1*$^{-/-}$ CTV labeled unfractionated CD4$^+$ T cells and analyzed after 1 week. (a) Schematic of experiment, as well as representative flow cytometry plots of T cells in the spleen + lymph nodes (gated on live CD45$^+$ CD3ε$^+$ CD4$^+$ cells) followed by their mean frequencies ± SEM. (b) Similar to (a), but with 400μg anti-NK1.1 antibody or Isotype Control injected 1 day prior to T cell transfer. CTV profiles of the T cells are shown as well as the mean frequencies ± SEM of the SP population. Pooled from three to four independent experiments, with each point representing an individual mouse. ***p < 0.001, ***p < 0.0001 by two-tailed Mann–Whitney test.

We also assessed for cell death in these populations by staining for activated caspases using FAM-FLICA, a fluorescently conjugated pan-caspase inhibitor. Compared to WT T cells, *Stat1*$^{-/-}$ T cells displayed increased cell death specifically in the SP population, with no differences in the HP or non-proliferating populations (**Figure 12a**). Importantly, the increased cell death in *Stat1*$^{-/-}$ SP T cells was reversed by NK cell depletion (**Figure 12b**).

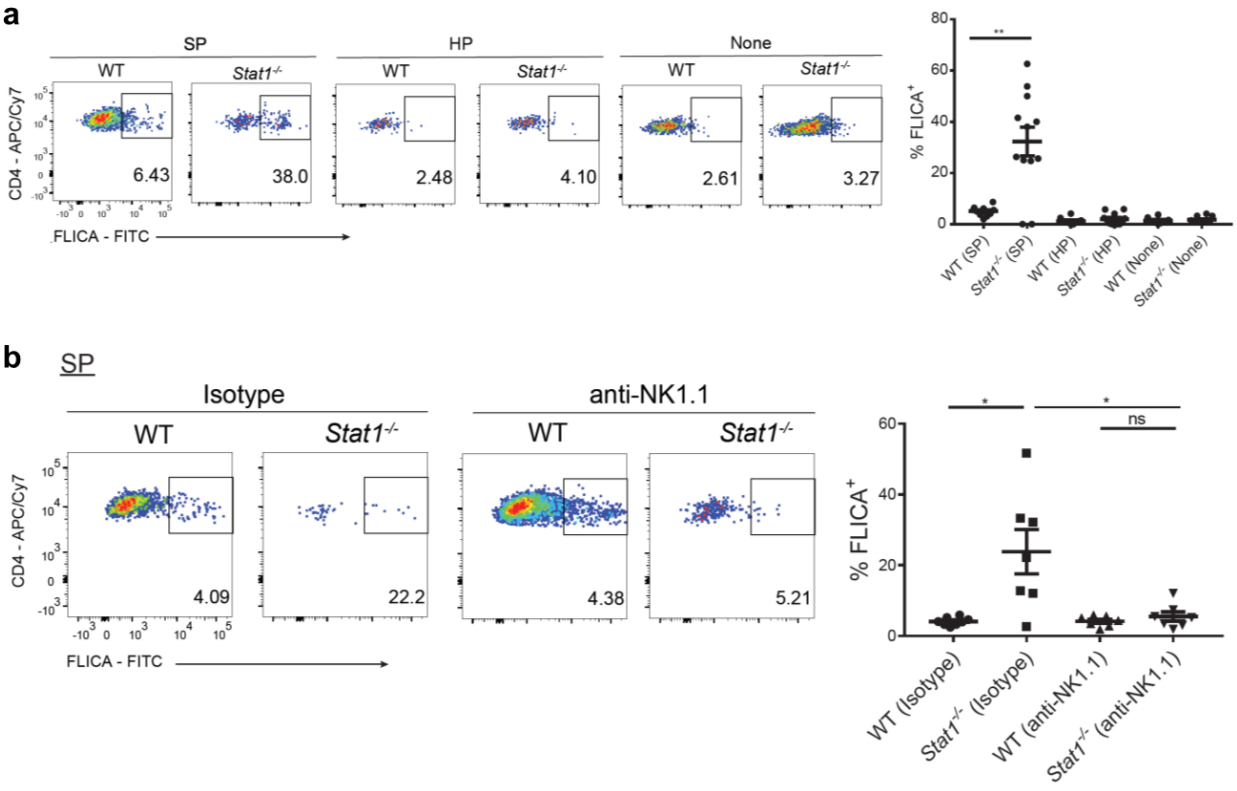


Figure 12. NK cells promote cell death in *Stat1*$^{-/-}$ SP T cells. *Rag1*$^{-/-}$ mice were injected with equal numbers (3–4× 10^6) of WT or *Stat1*$^{-/-}$ CTV labeled unfractionated CD4$^+$ T cells and analyzed after 1 week. (a) Representative images of FLICA staining from the T cell SP, HP and non-proliferating populations as in Figure 9a, as well as their mean frequencies ± SEM. (b) Similar to (a), but with 400µg anti-NK1.1 antibody or Isotype Control injected 1 day prior to T cell transfer. FLICA staining in the SP population shown with mean frequencies ± SEM. Pooled from three to four independent experiments, with each point representing an individual mouse. *$p < 0.05$, **$p < 0.01$ by two-tailed Mann–Whitney test.

We next asked whether lymphopenic expansion was required for the control of *Stat1*$^{-/-}$ T cells by transferring WT or *Stat1*$^{-/-}$ T cells into lymphoreplete WT CD45.1 mice. Consistent with earlier reports[44], there was reduced overall proliferation when T cells are transferred into lymphoreplete mice compared to lymphopenic mice. We did not observe a reduction in overall percentages when *Stat1*$^{-/-}$ T cells are transferred into lymphoreplete mice (**Figure 13a**). However, in the SP population, there is a mild but consistent decrease when T cells lack STAT1 (**Figure 13b**). Taken together, these data strongly suggest that NK cells eliminate *Stat1*$^{-/-}$ T cells only when they undergo SP.

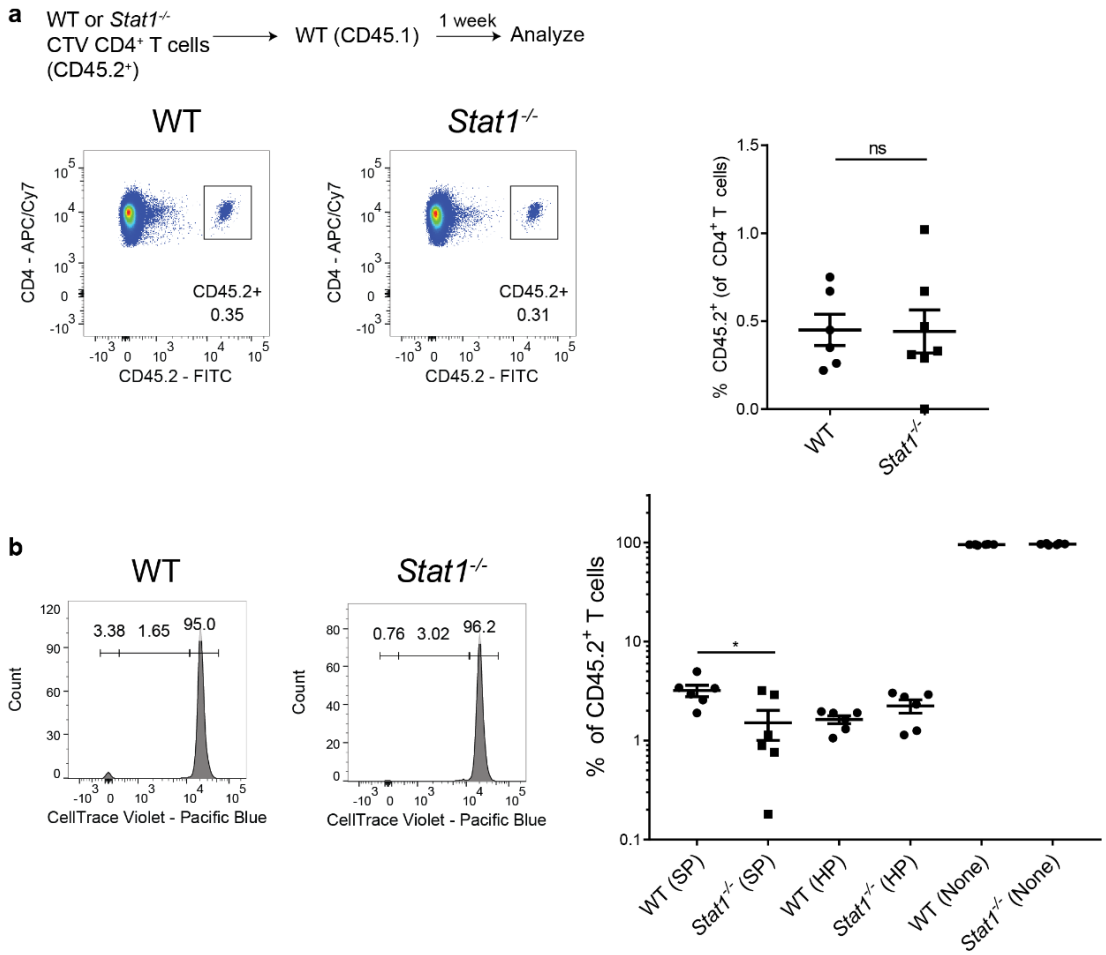


Figure 13. Reduced *Stat1*$^{-/-}$ SP T cells in lymphoreplete mice. WT CD45.1 mice were injected i.p. with 2 X 10^6 unfractionated WT or *Stat1*$^{-/-}$ CTV labelled CD4$^+$ T cells. (a) Representative flow cytometry plots of CD45.2$^+$ cells in the spleen + lymph nodes (gated on live CD45$^+$ CD3ε$^+$ CD4$^+$ T cells) and their mean frequencies ± SEM at 1 week post transfer. (b) Representative flow cytometry plots of CTV profiles in CD45.2$^+$ cells in the spleen + lymph nodes and the mean frequencies ± SEM of SP, HP and non-proliferating populations at 1 week post transfer. Data pooled from 2 independent experiments. *p<0.05 by two-tailed Mann-Whitney test.

Chapter 2.11 – CSF1R^{+} macrophages and ILCs do not restrict *Stat1*$^{-/-}$ T cell expansion

The incomplete rescue of *Stat1*$^{-/-}$ T cell expansion, spontaneous proliferation and induced colitis suggested that other cell types might be involved in restricting *Stat1*$^{-/-}$ T cells (**Figure 8b, 9, 11**). We thus performed preliminary experiments addressing this possibility, depleting several cell types in addition to NK cells and transferring CellTrace Violet (CTV) labelled *Stat1*$^{-/-}$ T cells. If these other cell types were involved in eliminating *Stat1*$^{-/-}$ T cells, depletion of these cell types will result in further enhancement of T cell expansion.

We first depleted macrophages in NK-depleted *Rag1*$^{-/-}$ mice by using an anti-CSF1R blocking antibody, as it was recently shown that macrophages can phagocytose *β2m*$^{-/-}$ tumors[45]. The antibody treatment effectively reduced splenic red pulp macrophages (**Figure 14a**). Compared to NK-depleted *Rag1*$^{-/-}$ mice that received the control antibody, the depletion of macrophages did not lead to an increase in T cell levels at 2 weeks post transfer (**Figure 14b**). We next explored the role of ILCs using *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice, which naturally also do not have NK cells. We did not observe an enhancement of *Stat1*$^{-/-}$ T cell expansion in this setting (**Figure 14b**). However, the CTV profile suggested that these T cells displayed reduced proliferation compared to the NK-depleted *Rag1*$^{-/-}$ mice (**Figure 14c**). While this suggests that ILCs might promote the expansion of *Stat1*$^{-/-}$ T cells, further work is needed to address other factors such as γc signaling on innate immune cells and stromal cells, as well as slight differences in the genetic background (*Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice are B6;B10 vs *Rag1*$^{-/-}$ which are B6).

Collectively, these data suggest that CSF1R-dependent macrophages and ILCs are not the reason behind the incomplete rescue of *Stat1*$^{-/-}$ T cells. It is possible that other cell types that we've not looked at mediate this difference. It is also possible that in addition to protecting T cells from NK cell killing, there might be other defects in *Stat1*$^{-/-}$ T cells, perhaps defective intrinsic proliferation *in vivo*, that might explain the incomplete rescue.

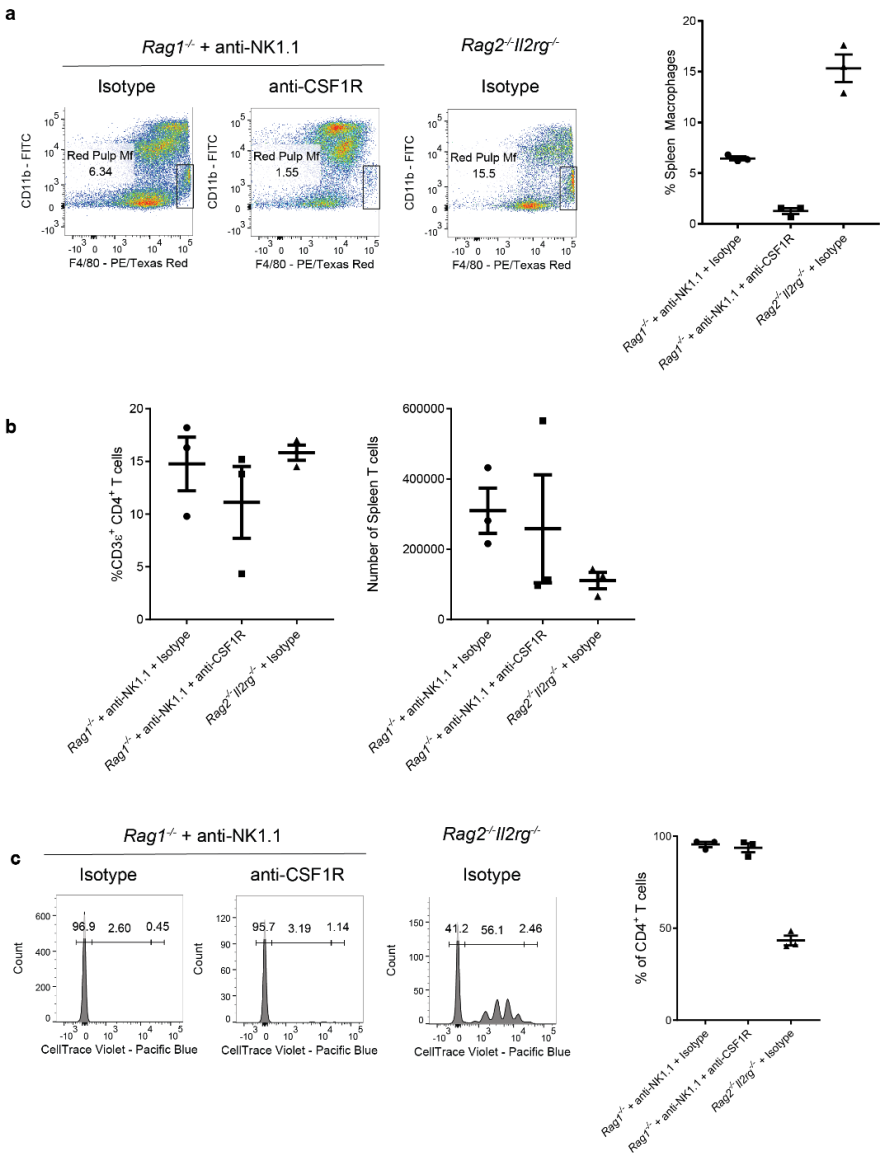


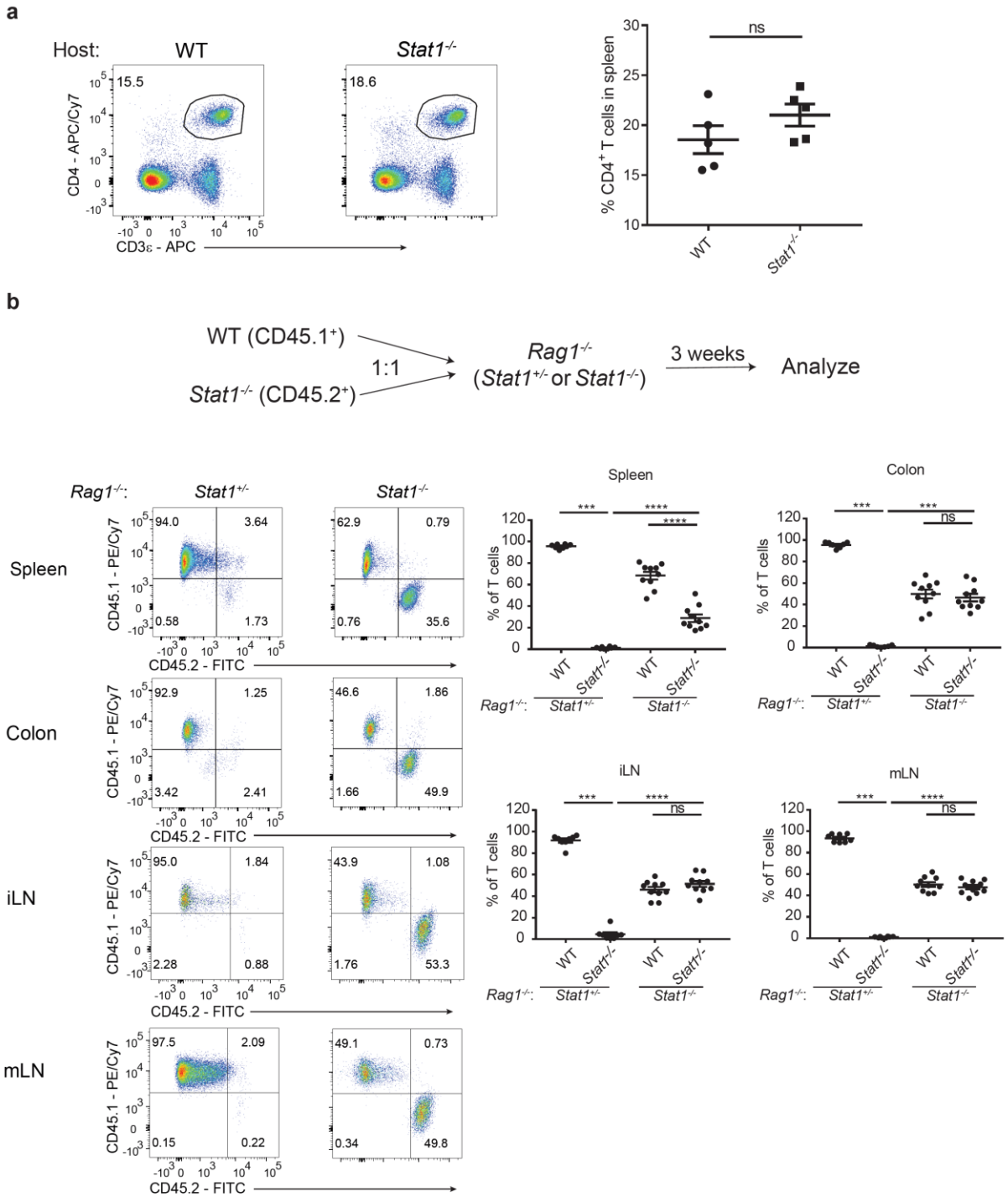
Figure 14. *Stat1*$^{-/-}$ T cell expansion upon additional depletion of macrophages or ILCs. *Stat1*$^{-/-}$ CellTrace Violet (CTV) labelled T cells were injected i.p. into NK-depleted *Rag1*$^{-/-}$ mice that were treated with control or anti-CSF1R antibody, or *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice treated with control antibody. (a) Representative flow cytometry plots of splenic red pulp macrophages (CD11b^{lo}F4-80^{+})[46] shown at 2 weeks post T cell transfer, with their mean frequencies ± SEM. (b) CD4^{+} T cell percentages and total numbers in the spleen (mean frequencies ± SEM) are shown 2 weeks post T cell transfer. (c) Representative CTV plots of T cells shown, along with the level of spontaneous proliferation (CFSE^{lo}) at 2 weeks post T cell transfer (mean frequencies ± SEM). *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice were cohoused for at least 2 weeks with *Rag1*$^{-/-}$ mice to control for the microbiota.

Chapter 2.12 – Innate *Stat1* expression is required to reject *Stat1$^{-/-}$ T cells*

Despite the potent elimination of *Stat1$^{-/-}$* T cells upon adoptive transfer into lymphopenic hosts, *Stat1$^{-/-}$* mice had normal levels of CD4^{+} T cells, suggesting additional mechanism(s) in place to prevent their elimination by NK cells (**Figure 15a**). As STAT1 is required for NK cells to achieve optimal cytotoxicity[47,48], we hypothesized that these T cells were not eliminated in *Stat1$^{-/-}$* mice due to a defect in killing by *Stat1$^{-/-}$* NK cells. To test this hypothesis, we deleted *Stat1* in the innate compartment by generating *Stat1$^{-/-}$Rag1$^{-/-}$* mice and transferred congenically marked WT (CD45.1^{+}) and *Stat1$^{-/-}$* (CD45.2^{+}) T cells into them. Whereas *Stat1$^{-/-}$* T cells were efficiently depleted in the *Stat1$^{+/-}$Rag1$^{-/-}$* littermate controls, deletion of *Stat1* in the innate compartment restored the expansion of *Stat1$^{-/-}$* T cells (**Figure 15b**). This indicates that the elimination of *Stat1$^{-/-}$* T cells is dependent on innate STAT1 signaling.

Figure 15. Innate *Stat1* expression is required to eliminate *Stat1*$^{-/-}$ T cells. (a) Representative flow cytometry plots of CD4^{+} T cells in the spleen of WT and *Stat1*$^{-/-}$ mice followed by their mean frequencies ± SEM. (b) WT (CD45.1^{+}) or *Stat1*$^{-/-}$ (CD45.2^{+}) CD4^{+} T cells were injected i.p. at a 1:1 ratio (1 × 10^{6}/ type) into *Stat1*$^{-/-}$*Rag1*$^{-/-}$ mice or their *Stat1*$^{+/-}$*Rag1*$^{-/-}$ littermate controls and analyzed after 3 weeks. Representative images of CD45.1^{+} vs CD45.2^{+} cells (gated on live CD45^{+} CD3ε^{+} CD4^{+} T cells) from various organs are shown followed by their mean frequencies ± SEM. Pooled from three independent experiments, with each point representing an individual mouse. ***$p < 0.001$, ****$p < 0.0001$ by two-tailed Mann–Whitney test.

Figure 15 (continued)



Chapter 2.13 – Discussion

In this study we have identified a critical role for STAT1 in T cell survival, where STAT1 signaling, through the upregulation of *Nlrc5* and MHC-I, protects T cells from NK cell-mediated elimination *in vivo*. We also show that this is important in the setting of T cell-mediated immunopathology, as *Stat1*$^{-/-}$ T cells can induce colitis if allowed to survive and expand in a NK-deficient environment **(Figure 16)**.

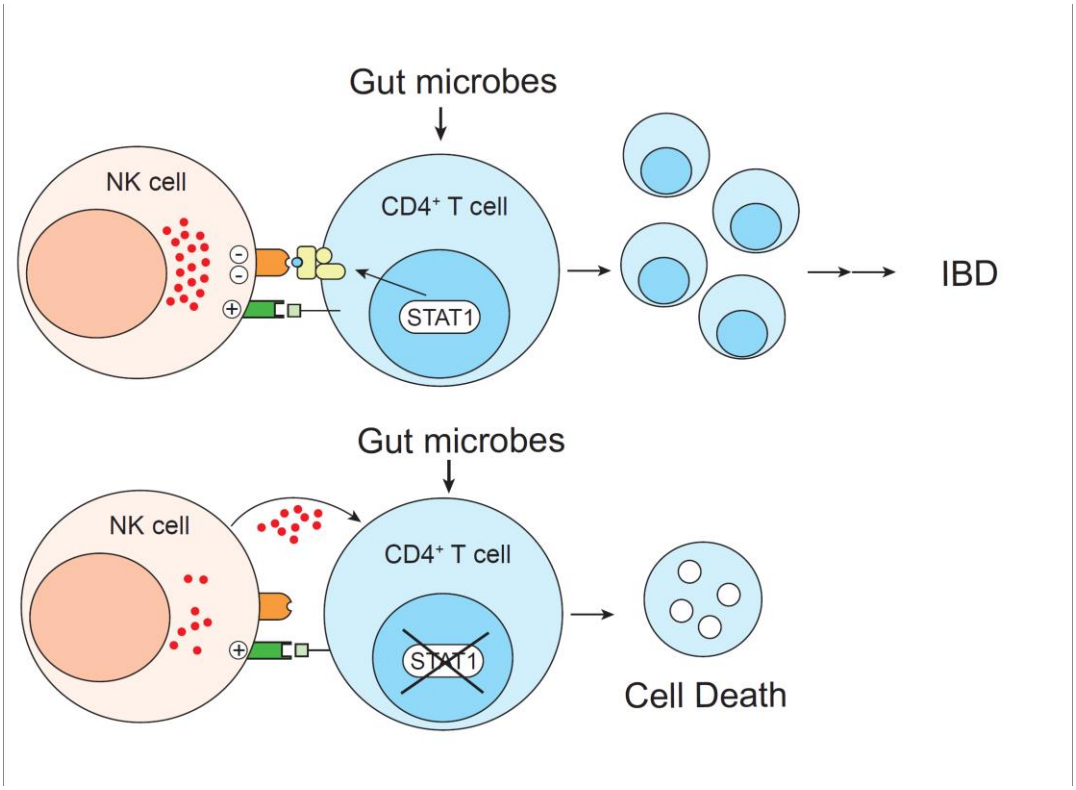


Figure 16. STAT1 signaling shields T cells from NK cell mediated cytotoxicity. (Top) When WT T cells are activated by the microbiota (Spontaneous Proliferation), they utilize STAT1 signaling to maintain sufficient expression of inhibitory MHC-I to evade rejection by NK cells. This allows the T cells to expand and, in susceptible hosts, cause colitis. (Bottom) T cells that lack *Stat1* fail to upregulate MHC-I when undergoing SP, causing them to be recognized by NK cells as "missing self". NK cells subsequently release their cytotoxic machinery to kill the *Stat1*$^{-/-}$ T cells.

In T cells, most studies on the JAK-STAT pathway have focused on its effects on T cell differentiation, with STAT1 promoting Th1 differentiation (through the induction of T-bet) and inhibiting Th17 differentiation[3,4,7]. In IBD, previous studies on STAT1 signaling in T cells focused on the STAT1-dependent transcription factor T-bet[13,24].While it was noted that *Stat1*$^{-/-}$ T cells were unable to cause colitis, the profile of *Stat1*$^{-/-}$ T cells *in vivo* was not analyzed and STAT1 was assumed to act in a similar fashion as T-bet[13]. We observe that in our model of colitis[18], STAT1 modulates the disease outcome primarily by promoting T cell survival rather than altering differentiation, as *Stat1*$^{-/-}$ T cells displayed similar differentiation profiles in both control and NK-depleted *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ hosts (**Figure 10**). It is notable that only *Stat1*$^{-/-}$ T cells undergoing SP are eliminated by NK cells. This suggests that NK cells restrict T cells only when they are activated (**Figure 11-13**), in agreement with earlier studies where *Ifnar1*$^{-/-}$ antiviral T cells are only eliminated by NK cells when the mice are virally infected[28,49]. We hypothesize that similar mechanisms might be used in the regulation of viral-driven and commensal-driven T cell responses.

The upstream signal(s) that activates the STAT1-NLRC5-MHC class I axis in T cells *in vivo* has not been fully elucidated. *In vitro*, *Nlrc5* expression in T cells is primarily triggered by autocrine IFNγ signaling[33]. *In vivo*, type I IFN has been reported to protect antiviral T cells and NK cells from NK mediated elimination during LCMV infection[28,49,50]. Our data stands in contrast with these studies, showing that deletion of both Type I and Type II IFN receptors fails to fully recapitulate the defective survival of *Stat1*$^{-/-}$ T cells in the setting of IBD (**Figure 5**). This is consistent with an earlier report showing that type I IFN signaling is not required for naïve T cells to induce colitis in *Rag*$^{-/-}$ hosts[51], but we further extend this observation to include Type II IFN signaling. What are the IFN-independent signals that might account for the discrepancy between *Ifnar1*$^{-/-}$*Ifngr1*$^{-/-}$ and *Stat1*$^{-/-}$ T cells? IL-7 has been proposed as a possible candidate, being able to induce STAT1 activation in T cells *in vitro* and *in vivo*[35,44] as well as MHC-I *in vitro*[35]. Therefore, in addition to the conventional STAT5-driven proliferative and pro-survival response[52], IL-7 might activate STAT1 signaling to induce MHC-I for protection from NK

cells. However, our finding that NK cells specifically eliminate *Stat1*$^{-/-}$ T cells undergoing SP and not the IL-7 driven HP argues against this hypothesis (**Figure 11-13**). IL-6, which has been reported to be important in driving SP, also activates STAT1[42,43,53]. However, a recent report showed that IL-6R deficient T cells only display defective expansion in *Rag1*$^{-/-}$ mice when there is colonic inflammation[54], which is in contrast to our observations with *Stat1*$^{-/-}$ T cells (**Figure 4**). It is possible that the STAT1-dependent signal is provided by multiple cytokines, including IL-6 and type I+II IFN. Alternatively, the maintenance of MHC-I levels might be driven by tonic STAT1 signaling that is independent of any upstream cytokine engagement.

We observe that *Stat1*$^{-/-}$ T cells fail to expand in both *Rag1*$^{-/-}$ and *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice (**Figure 3-4**), suggesting that IL-10Rβ signaling is not required for NK cells to eliminate *Stat1*$^{-/-}$ T cells. However, earlier work has shown roles for IL-10 and IFNλ/IL-28—both of which signal via the IL-10Rβ chain—in stimulating NK cells[55–57]. It is possible that *Il10rb*$^{-/-}$ NK cells might still have sufficient cytotoxic ability to eliminate *Stat1*$^{-/-}$ T cells, as IL-28R deficient NK cells are only partially defective[57]. Alternatively, there might be other mechanisms in *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice that compensate for this defect, such as IL-10R deficiency in macrophages which promotes a proinflammatory environment[18] and/or RAG1 deficiency which has been shown to lead to NK cell hyperresponsiveness[38].

In our study, we also show that innate STAT1 signaling is required to eliminate *Stat1*$^{-/-}$ T cells, which would explain why *Stat1*$^{-/-}$ mice have normal levels of T cells (**Figure 15**). We believe that this is likely due to the impaired cytotoxic capability of *Stat1*$^{-/-}$ NK cells as previously reported[47,48]. However, we do not exclude the possibility that this can also be due to altered NK education, where NK cells are educated to recognize the low level of MHC-I on *Stat1*$^{-/-}$ T cells as normal. Alternatively, STAT1 might be utilized by other innate cell types that subsequently modulate NK cell cytotoxicity. Further studies utilizing NK specific *Stat1* deletion and *in vitro* cytotoxicity assays against T cells will shed light into these possibilities.

In summary, we describe a critical role for STAT1 in promoting T cell survival by maintaining sufficient MHC class I expression to evade NK cell-mediated killing. This mechanism is largely IFN-independent and is critical in enabling T cells to induce intestinal inflammation. Our findings shed a new light on JAK-STAT signaling in T cells, adding critical functions for this pathway beyond T cell differentiation that have potential therapeutic implications for IBD and other T cell-mediated inflammatory disorders.

Chapter 2.14 – Materials and Methods

Mouse strains. C57BL/6J (Strain 000664), B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ (CD45.1, Strain 002014), B6.129S7-*Rag1*^{tm1Mom}/J (*Rag1*$^{-/-}$, Strain 002216), B6.129S(Cg)-*Stat1*^{tm1Dlv} (*Stat1*$^{-/-}$, Strain 012606), B6.Cg-*Ifngr1*^{tm1Agt}*Ifnar1*$^{tm1.2Ees}$/J (*Ifnar1*$^{-/-}$*Ifngr1*$^{-/-}$, Strain 029098) mice were purchased from Jackson Labs. B10;B6-*Rag2*^{tm1Fwa} *Il2rg*^{tm1Wjl} mice (*Rag2*$^{-/-}$*Il2rg*$^{-/-}$, Strain 4111) were purchased from Taconic. *Il10rb*$^{-/-}$ *Rag1*$^{-/-}$ mice were generated by crossing *Il10rb*$^{-/-}$ mice (a gift from Thaddeus Stappenbeck, Washington University) with *Rag1*$^{-/-}$ mice. *Stat1*$^{-/-}$*Rag1*$^{-/-}$ mice were generated by crossing *Stat1*$^{-/-}$ mice with *Rag1*$^{-/-}$ mice. All mice were on the B6 background and maintained in a specific pathogen-free animal facility in Boston Children's Hospital. All experiments were conducted after approval from the Animal Resources at Children's Hospital and according to regulations by the Institutional Animal Care and Use Committee (IACUC).

Adoptive T cell transfer and colitis induction. In T cell transfer experiments, unfractionated CD4^{+} T cells were isolated from the spleens and lymph nodes of donor mice (WT, CD45.1, *Stat1*$^{-/-}$, *Ifnar1*$^{-/-}$*Ifngr1*$^{-/-}$) by negative selection (Miltenyi Biotec CD4^{+} T-cell isolation Kit, Cat No. 130-104-454). 1 × 10^{6} T cells (92.7–98.6% pure) were then adoptively transferred into recipient mice (*Rag1*$^{-/-}$, *Il10rb*$^{-/-}$*Rag1*$^{-/-}$) by i.p. injection in PBS unless otherwise stated. In some experiments, CD45.1^{+} T cells and *Stat1*$^{-/-}$ T cells were mixed at a 1:1 ratio before being transferred into recipient mice (*Rag1*$^{-/-}$, *Stat1*$^{-/-}$*Rag1*$^{-/-}$). In some experiments, T cells were labeled with 5 μM CellTrace Violet (Thermo Fisher) in PBS + 0.1% FBS for 10 min at 37 °C prior to injection. All recipient mice were at least 6 weeks old and matched for sex, age, and

housing between groups. *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice were monitored weekly for body weight changes post T cell transfer. For NK depletion assays, each mouse was first injected with 400 μg anti-NK1.1 (or isotype control) 1 day prior to T cell transfer. For experiments lasting beyond 1 week, depletion of NK cells was maintained by injections of 200 μg anti-NK1.1 (or isotype control) weekly post transfer. For Macrophage depletion assays, mice were injected with 400 μg anti-CSF1R (or isotype control) twice weekly beginning 1 week before T cell transfer. All antibody injections were administered i.p. in InVivoPure pH 7.0 Dilution Buffer (BioXCell).

Histological scoring. To evaluate signs of histological inflammation, sections of distal colons were stained in haematoxylin and eosin and scored in a blinded fashion. Scoring was based on histological evidence of crypt hyperplasia (0–3), inflammatory cell infiltration (0–3) and presence of crypt abscesses (0–2), summed up to give the overall score (0–8). Representative images were acquired using an Olympus BX41 upright microscope with DP70 color CCD (Figure 3-4) or a Keyence automated epifluorescent microscope (Figure 5, 9).

Isolation of colonic lamina propria cells. Cells were isolated from the lamina propria as described[18]. Briefly, the large intestine (colon + cecum) was removed, cut open longitudinally and then into small sections before being incubated in Hank's balanced salt solution (HBSS) containing 0.5% fetal bovine serum (FBS), 10 mM EDTA, 1.5 mM dithiothreitol and 10mM HEPES at 37 °C for 35 min with agitation to remove the epithelial cell layer. After the removal of the epithelial cells, tissues were washed in PBS, finely diced and incubated in HBSS buffer (w Ca/Mg) containing 20% FBS, 10 mM HEPES, 1.5 mM CaCl2 and collagenase VIII (200 U/ ml) at 37 °C for 40 min with agitation. Tissues were then repeatedly flushed through a 10 ml syringe and further incubated for 15 min. Digested tissues were filtered, washed in PBS and used for flow cytometry.

***In vitro* T cell proliferation.** Unfractionated $CD4^+$ T cells were first labeled with 5 μM CFSE for 5 min at room temperature and washed repeatedly with PBS containing FBS. They were then cultured in 96-well flat-bottom plates containing plate-bound anti-CD3ε (3 μg/ml, eBioscience) and soluble anti-CD28

(1 μg/ml, eBioscience) for 3 days. T cells were cultured in DMEM containing 10% FBS, L-glutamine, pyruvate, non-essential amino acids, MEM vitamins, L-arginine, L-asparagine, folic acid, β-mercaptoethanol and pen/strep.

Reagents. For flow cytometric staining, antibodies against the following were used (Clone name, dilution, manufacturer and catalog number in brackets): CD3ε (145-2C11, 1:300–400, Biolegend #100312/100306), TCRβ (H57-597, 1:400, Biolegend #109222), CD4 (GK1.5, 1:300 Biolegend #100414), NKp46 (29A1.4, 1:50, Biolegend #137604), CD49b (HMα2, 1:200 Biolegend #103517), CD45 (30-F11, 1:500, Biolegend #103140), H2-K^b/D^b (28-8-6, 1:100, Biolegend #114606/114607), Qa-2 (695H1-9-9, 1:100, Biolegend #121709), Qa-1b (6A8.6F10.1A6, 1:10, Miltenyi Biotec #130-104-220), CD16/32 (93, 0.5 μg/106 cells, Biolegend #101302), IL-17A (TC11-18H10.1, 1:125, Biolegend 506904), IFNγ (XMG1.2, 1:200, Biolegend #505809/eBioscience #17-7311-82), Mouse IgG2a, κ Isotype Ctrl (MOPC-173, 1:100, Biolegend #400207/400211), Mouse IgG1, κ Isotype Ctrl (MOPC-21, 1:66.7, Biolegend #400119), CD45.1 (A20, 1:300, Biolegend #110729), CD45.2 (104, 1:300, Biolegend #109806). For T-cell stimulation, antibodies against CD3ε (145-2C11, 3 μg/ml, eBioscience #16-0031-86) and CD28 (37.51, 1 μg/ml, eBioscience #16-0281- 85) were used. For NK depletion assays, antibodies against NK1.1 (PK136, BioXCell #BP0036) or Isotype Control (C1.18.4, BioXCell #BP0085) were used. For macrophage depletion assays, antibodies against CSF1R (AFS98, BioXCell #BP0213) or Rat IgG2a isotype control, anti-trinitrophenol (2A3, BioXCell #BP0089) were used.

Flow cytometry. For flow cytometry and sorting experiments, cells were stained in flow cytometric staining buffer (2% FBS plus 0.1% NaN_3 in PBS) and MACS buffer (0.5% BSA and 2mM EDTA in PBS), respectively. For antibody staining of surface markers, cells were incubated with anti-CD16/32 (Biolegend) for 10 min at room temperature to block Fc receptors, before being incubated with antibodies for 20–30min at 4 °C. Cells were also incubated with Zombie Violet Fixable Viability Dye (1:400, Biolegend) or 7-AAD (1:20, BD Biosciences) according to the manufacturer's instructions to identify and exclude dead cells. For intracellular cytokine staining, cells were incubated with PMA (50 ng/ml),

ionomycin (500 ng/ml), and GolgiStop (1:1000, BD Biosciences) for 4 h at 37 °C. After staining for surface markers, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), followed by staining in Perm/Wash buffer (BD Biosciences) according to the manufacturer'sinstructions. For assessment of cell death, cells were stained with the FAM-FLICA Poly Caspase Kit (ImmunoChemistry Technologies) for 1 h at 37 °C in T-cell media prior to antibody surface staining. All samples were acquired with a BD Canto II or LSRFortessa Flow Cytometer (BD Biosciences) and analyzed with FlowJo (FlowJo, LLC).

RNA sequencing. In the post-transfer setting, WT or *Stat1*$^{-/-}$ T cells (gated as CD45^{+} CD3ε^{+} CD4^{+}) were FACS sorted from the spleen and lymph nodes of *Rag1*$^{-/-}$ mice post transfer directly into RLT lysis buffer (Qiagen) and RNA extracted using the RNeasy Micro kit (Qiagen). As *Stat1*$^{-/-}$ T cells showed reduced survival/expansion *in vivo*, it was not technically feasible to acquire sufficient cells for purity analysis by flow cytometry, hence purity was determined by confirming the downregulation of *Stat1* in the *Stat1*$^{-/-}$ T cells. Library preparation, RNA-seq and analysis were performed at the Molecular Biology Core Facility (MBCF) of Dana-Farber Cancer Institute, Boston, using the Clontech SMARTer v4 kit for mRNA library generation and the Illumina NextSeq 500 Platform (Single-end 75 bp) for sequencing. The data was analyzed using the VIPER algorithm[58], with reads aligned to the mouse mm9 genome using STAR, transcripts assembled with Cufflinks and differential analysis performed with DESeq2. Gene Ontology analysis was performed using the PANTHER Over-representation test (http://www.geneontology.org/). The raw and processed data for RNA sequencing are deposited in the NCBI GEO database under GSE116475.

Statistical analysis. Statistical analyses were performed with GraphPad Prism software using two-way ANOVA with Bonferroni's multiple comparisons test, two-tailed Mann–Whitney test or two-tailed t-test as indicated in the figure legends. Significance was defined as p-value < 0.05 using the following notations: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Chapter 2.15 – References

1. O'Shea, J. J. & Murray, P. J. Cytokine signaling modules in inflammatory responses. *Immunity* **28**, 477–87 (2008).
2. Delgoffe, G. M. & Vignali, D. A. A. STAT heterodimers in immunity A mixed message or a unique signal ? *JAK-STAT* **2**, e23060 (2013).
3. Afkarian, M. *et al.* T-bet is a STAT1-induced regulator for IL-12R expression in naïve CD4+T cells. *Nat. Immunol.* **3**, 549–557 (2002).
4. Szabo, S. J. *et al.* A Novel Transcription Factor, T-bet, Directs Th1 Lineage Commitment. *Cell* **100**, 655–669 (2000).
5. O'Shea, J. J., Holland, S. M. & Staudt, L. M. JAKs and STATs in Immunity, Immunodeficiency, and Cancer. *N. Engl. J. Med.* **368**, 161–170 (2013).
6. Ma, H. *et al.* Absence of Stat1 in donor CD4+ T cells promotes the expansion of Tregs and reduces graft-versus-host disease in mice. *J. Clin. Invest.* **121**, 2554–2569 (2011).
7. Harrington, L. E. *et al.* Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* **6**, 1123–32 (2005).
8. Bettelli, E. *et al.* Loss of T-bet, But Not STAT1, Prevents the Development of Experimental Autoimmune Encephalomyelitis. *J. Exp. Med* **200**, 79–87 (2004).
9. Villarino, A. V, Gallo, E. & Abbas, A. K. STAT1-activating cytokines limit Th17 responses through both T-bet-dependent and -independent mechanisms. *J Immunol* **185**, 6461–6471 (2010).
10. Neurath, M. F. Cytokines in inflammatory bowel disease. *Nat. Rev. Immunol.* **14**, 329–342 (2014).
11. Abraham, C. & Cho, J. H. Inflammatory Bowel Disease. *N Engl J Med* **361**, 2066–78 (2009).
12. Mudter, J. *et al.* Activation Pattern of Signal Transducers and Activators of Transcription (STAT) Factors in Inflammatory Bowel Diseases. *Am. J. Gastroenterol.* **100**, 64–72 (2005).
13. Neurath, M. F. *et al.* The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. *J. Exp. Med.* **195**, 1129–1143 (2002).
14. Kühn, R., Löhler, J., Rennick, D., Rajewsky, K. & Müller, W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* **75**, 263–274 (1993).
15. Spencer, S. D. *et al.* The Orphan Receptor CRF2-4 Is an Essential Subunit of the Interleukin 10 Receptor. *J. Exp. Med.* **187**, 571–578 (1998).
16. Glocker, E.-O., Kotlarz, D., Klein, C., Shah, N. & Grimbacher, B. IL-10 and IL-10 receptor defects in humans. *Ann. N. Y. Acad. Sci.* **1246**, 102–7 (2011).
17. Zigmond, E., Bernshtein, B. & Friedlander, G. Macrophage-Restricted Interleukin-10 Receptor Deficiency , but Not IL-10 Deficiency , Causes Severe Spontaneous Colitis. *Immunity* **40**, 720–733 (2014).
18. Shouval, D. S. *et al.* Interleukin-10 receptor signaling in innate immune cells regulates mucosal immune tolerance and anti-inflammatory macrophage function. *Immunity* **40**, 706–19 (2014).
19. Redhu, N. S. *et al.* Macrophage dysfunction initiates colitis during weaning of infant mice lacking the interleukin-10 receptor. *Elife* **6**, 1–23 (2017).

20. Shouval, D. S. *et al.* Interleukin 1β Mediates Intestinal Inflammation in Mice and Patients With Interleukin 10 Receptor Deficiency. *Gastroenterology* **151**, 1100–1104 (2016).

21. Coccia, M. *et al.* IL-1β mediates chronic intestinal inflammation by promoting the accumulation of IL-17A secreting innate lymphoid cells and CD4(+) Th17 cells. *J. Exp. Med.* **209**, 1595–609 (2012).

22. Li, B. *et al.* IL-10 engages macrophages to shift Th17 cytokine dependency and pathogenicity during T-cell-mediated colitis. *Nat. Commun.* **6**, 1–13 (2015).

23. Shouval, D. S. *et al.* Enhanced TH17 Responses in Patients with IL10 Receptor Deficiency and Infantile-onset IBD. *Inflamm. Bowel Dis.* **23**, 1950–1961 (2017).

24. Krausgruber, T. *et al.* T-bet is a key modulator of IL-23-driven pathogenic CD4+ T cell responses in the intestine. *Nat. Commun.* **7**, 11627 (2016).

25. Huber, S. *et al.* Th17 cells express interleukin-10 receptor and are controlled by Foxp3^{-} and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner. *Immunity* **34**, 554–65 (2011).

26. Powrie, F., Leach, M. W., Mauze, S., Caddle, L. B. & Coffman, R. L. Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. *Int. Immunol.* **5**, 1461–1471 (1993).

27. Srivastava, S., Koch, L. K. & Campbell, D. J. IFNαR Signaling in Effector but Not Regulatory T Cells Is Required for Immune Dysregulation during Type I IFN–Dependent Inflammatory Disease. *J. Immunol.* **193**, 2733–2742 (2014).

28. Crouse, J. *et al.* Type I Interferons Protect T Cells against NK Cell Attack Mediated by the Activating Receptor NCR1. *Immunity* **40**, 961–973 (2014).

29. Overacre-Delgoffe, A. E. *et al.* Interferon-γ Drives T reg Fragility to Promote Anti-tumor Immunity. *Cell* 1–12 (2017). doi:10.1016/j.cell.2017.05.005

30. Lee, C.-K., Smith, E., Gimeno, R., Gertner, R. & Levy, D. E. STAT1 Affects Lymphocyte Survival and Proliferation Partially Independent of Its Role Downstream of IFN-γ. *J. Immunol.* **164**, 1286–1292 (2000).

31. Meissner, T. B. *et al.* NLR family member NLRC5 is a transcriptional regulator of MHC class I genes. *Proc. Natl. Acad. Sci.* **107**, 13794–13799 (2010).

32. Kuenzel, S. *et al.* The Nucleotide-Binding Oligomerization Domain-Like Receptor NLRC5 Is Involved in IFN-Dependent Antiviral Immune Responses. *J. Immunol.* **184**, 1990–2000 (2010).

33. Staehli, F. *et al.* NLRC5 Deficiency Selectively Impairs MHC Class I- Dependent Lymphocyte Killing by Cytotoxic T Cells. *J. Immunol.* **188**, 3820–3828 (2012).

34. Tong, Y. *et al.* Enhanced TLR-induced NF-κB signaling and type I interferon responses in NLRC5 deficient mice. *Cell Res.* **22**, 822–835 (2012).

35. Lee, B. C. *et al.* Differential Regulation of Constitutive Major Histocompatibility Complex Class I Expression in T and B Lymphocytes. *J. Exp. Med.* **190**, 1451–1464 (1999).

36. Kärre, K., Ljunggren, H. G., Piontek, G. & Kiessling, R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* **319**, 675–678 (1986).

37. Yewdell, J. & Bennink, J. R. Mechanisms of viral interferance with MHC class I antigen

processing and presentation. *Annu Rev Cell Dev Biol* **15**, 579–606 (1999).

38. Karo, J. M., Schatz, D. G. & Sun, J. C. The RAG recombinase dictates functional heterogeneity and cellular fitness in natural killer cells. *Cell* **159**, 94–107 (2014).

39. Surh, C. D. & Sprent, J. Homeostasis of Naive and Memory T Cells. *Immunity* **29**, 848–862 (2008).

40. Booki Min, P., Yamane, H., Hu-Li, J., Min, B. & Paul, W. E. Spontaneous and Homeostatic Proliferation of CD4 T Cells Are Regulated by Different Mechanisms. *J Immunol* **174**, 6039–6044 (2005).

41. Kieper, W. C. W. *et al.* Cutting Edge: Recent Immune Status Determines the Source of Antigens That Drive Homeostatic T Cell Expansion. *J. Immunol.* **174**, 3158–3163 (2005).

42. Tajima, M. *et al.* IL-6 – dependent spontaneous proliferation is required for the induction of colitogenic IL-17 – producing CD8 + T cells. *J. Exp. Med.* **205**, 1019–1027 (2008).

43. Feng, T., Wang, L., Schoeb, T. R., Elson, C. O. & Cong, Y. Microbiota innate stimulation is a prerequisite for T cell spontaneous proliferation and induction of experimental colitis. *J. Exp. Med.* **207**, 1321–1332 (2010).

44. Le Saout, C. *et al.* IL-7–dependent STAT1 activation limits homeostatic CD4+ T cell expansion. *JCI Insight* **2**, 1–18 (2017).

45. Barkal, A. A. *et al.* Engagement of MHC class I by the inhibitory receptor LILRB1 suppresses macrophages and is a target of cancer immunotherapy. *Nat. Immunol.* **19**, 1–9 (2017).

46. Hashimoto, D. *et al.* Pretransplant CSF-1 therapy expands recipient macrophages and ameliorates GVHD after allogeneic hematopoietic cell transplantation. *J. Exp. Med.* **208**, 1069–1082 (2011).

47. Lee, C. K. *et al.* Distinct requirements for IFNs and STAT1 in NK cell function. *J. Immunol.* **165**, 3571–3577 (2000).

48. Kovacic, B. *et al.* STAT1 acts as a tumor promoter for leukemia development. *Cancer Cell* **10**, 77–87 (2006).

49. Xu, H. C. *et al.* Type I Interferon Protects Antiviral CD8+ T Cells from NK Cell Cytotoxicity. *Immunity* **40**, 949–960 (2014).

50. Madera, S. *et al.* Type I IFN promotes NK cell expansion during viral infection by protecting NK cells against fratricide. *J. Exp. Med.* **213**, 225–233 (2016).

51. Kole, A. *et al.* Type I IFNs regulate effector and regulatory T cell accumulation and anti-inflammatory cytokine production during T cell-mediated colitis. *J. Immunol.* **191**, 2771–9 (2013).

52. Carrette, F. & Surh, C. D. IL-7 signaling and CD127 receptor regulation in the control of T cell homeostasis. *Semin. Immunol.* **24**, 209–217 (2012).

53. Lütticken, C. *et al.* Association of transcription factor APRF and protein kinase Jak1 with the interleukin-6 signal transducer gp130. *Science (80-.).* **263**, 89–92 (1994).

54. Li, B., Jones, L. L. & Geiger, T. L. IL-6 Promotes T Cell Proliferation and Expansion under Inflammatory Conditions in Association with Low-Level RORγt Expression. *J. Immunol.* **201**, 2934–2946 (2018).

55. Cai, G., Kastelein, R. A. & Hunter, C. A. IL-10 enhances NK cell proliferation, cytotoxicity and

production of IFN-gamma when combined with IL-18. *Eur. J. Immunol.* **29**, 2658–65 (1999).

56. Mocellin, S. *et al.* IL-10 stimulatory effects on human NK cells explored by gene profile analysis. *Genes Immun.* **5**, 621–630 (2004).

57. Souza-Fonseca-Guimaraes, F. *et al.* NK cells require IL-28R for optimal in vivo activity. *Proc. Natl. Acad. Sci.* **112**, E2376–E2384 (2015).

58. Cornwell, M. I. *et al.* VIPER: Visualization Pipeline for RNA-seq, a Snakemake workflow for efficient and complete RNA-seq analysis. *BMC Bioinformatics* **19**, 1–14 (2018).

Chapter 3

The role of mitochondrial STAT3 in IL-10R signaling

Chapter 3.1 – Attributions

The work presented in this chapter is contributed by the following individuals: Yu Hui Kang, Michelle Dong, Xu Shi, Robert Gerszten, and Scott B. Snapper. We are also grateful to Ross Tomaino from the Taplin Mass Spectrometry Facility for assistance with IP-Mass Spectrometry experiments.

Y.H.K. and S.B.S. conceived the study. Y.H.K. and S.B.S. designed the experiments. Y.H.K. and M.D. performed the experiments, except for the metabolomics data which were acquired and analyzed by X.S. and R.G.

Chapter 3.2 – Introduction

IL-10 is a potent immunosuppressive cytokine that is best known for suppressing the generation of inflammatory macrophages *in vitro* and *in vivo*[1–4]. This is achieved through the induction of multiple effector genes, which inhibit the production of multiple proinflammatory cytokines and mediators such as IL-1β[5]. These mechanisms of inhibition have important functional consequences, as evidenced in studies by us and others showing a critical role for IL-1β in contributing to the colitis phenotype in IL-10/IL-10R deficiency[6,7].

However, it is now well established that underlying the transcriptional changes associated with inflammatory macrophage activation is a profound skewing of their metabolic profile. These changes include the upregulation of glycolysis, the dampening of OXPHOS and the breakage of the TCA cycle, all of which serve to divert metabolites towards anabolic processes[8]. This process of metabolic reprogramming is critically regulated by transcriptional factors like mTOR which regulate HIF-1α[9]. Whether IL-10 plays a role in regulating the metabolic reprogramming machinery was not clear until recently, when it was reported that IL-10 inhibits mTOR signaling[7]. Inhibition of mTOR by IL-10 promoted OXPHOS as well as mitophagy, a process that clears dysfunctional mitochondria thereby reducing mtROS. Mechanistically, IL-10 was shown to induce the mTOR inhibitor *Ddit4*. Genetic ablation of *Ddit4* abrogated the IL-10 driven metabolic response, indicating DDIT4 as an important downstream mediator of IL-10R signaling[7]. The IL-10 mediated induction of *Ddit4* was achieved via canonical STAT3 dependent transcriptional induction[7], putting *Ddit4* along with other genes important for IL-10 function such as *Bcl-3*, *Nfil3* and *Hmox1*[10–12].

While STAT3 has mainly been studied in terms of its regulation of gene transcription, recent reports have pointed to functions of STAT3 outside of the nucleus. In several cell types (pro-B cells, liver and the heart), STAT3 was shown to be present in the mitochondria where it binds to Complex I and II of the ETC. The presence of STAT3 in the mitochondria at steady state promoted the activity of Complex I

and II in a manner that was dependent on phosphorylation of a S727 residue on STAT3 instead of the Y705 residue activated by cytokine stimulation[13]. Mitochondrial STAT3 was also important in cancer cells, as Ras mediated transformation required mitochondrial and not nuclear STAT3 signaling[14].

Subsequent studies found additional roles for mitochondrial STAT3 at steady state and in the context of cytokine stimulation. In most of these studies, the presence of STAT3 in the mitochondria was associated with the preservation of mitochondrial function and the enhancement of oxidative metabolism[15]. Mitochondrial STAT3 promoted optimal ETC function, increased mitochondrial membrane potential and increased ATP production[16]. Although increased ETC activity is usually associated with increased ROS, mitochondrial STAT3 also reduces ROS by promoting the formation of respiratory chain super-complexes and enhancing the production of ROS scavengers like glutathione[17,18].

STAT3 has also been reported to bind and regulate mitochondrial proteins other than the ETC. For example, STAT3 can bind to cyclophilin D and inhibit the opening of the mitochondrial permeability transition pore (MPTP). Stress conditions can cause sustained opening of the MPTP, releasing cytochrome c and triggering the intrinsic apoptotic machinery. Therefore, mitochondrial STAT3 protects cells from apoptotic cell death[19]. STAT3 can also bind to the pyruvate dehydrogenase E1 complex in response to insulin, promoting pyruvate to acetyl-CoA conversion and increasing mitochondrial membrane potential[20]. Other reported binding targets of STAT3 include Prohibitin 1, Tom20 and GRIM-19. Prohibitin 1 is involved in modulating ETC activity[21], while Tom20 and GRIM-19 are thought to facilitate STAT3's entry into the mitochondria[19,22]. In addition to binding mitochondrial proteins, STAT3 has also been reported to bind to mitochondrial DNA and regulate mitochondrial transcription[23,24].

These mitochondrial functions of STAT3 have important effects on cellular and organismal function. In cancer cells, mitochondrial STAT3 promotes cellular transformation of MEF cells as well as growth and invasion of breast cancer cells[14,25]. In embryonic stem cells, mitochondrial STAT3 signaling downstream of LIF promotes cellular proliferation and metabolic reprogramming but does not contribute to the maintenance of the undifferentiated phenotype. In the heart, mitochondrial STAT3 is an important

regulator of cardiac function, providing protection from ischemic damage by reducing ROS production, promoting cell viability and enhancing Complex I function[26].

In immune cells, there have been only a handful of studies investigating the role of mitochondrial STAT3. IL-6, for example, was shown to activate the translocation of pY-STAT3 and total STAT3 into the mitochondria of activated T cells. There, IL-6 enhances the formation of respiratory chain supercomplexes, thus helping to maintain high mitochondrial membrane potential (MMP) while reducing ROS. Interestingly, the IL-6 induced MMP did not affect OXPHOS or glycolysis, but rather boosted Ca^{2+} flux into the mitochondria which is regulated by the MMP. The increased mitochondrial Ca^{2+} enhanced nuclear NFAT accumulation and sustained the expression of *Il21* and *Il4* late during activation[17]. In mast cells, IgE engagement led to mitochondrial pS-STAT3 activation, which facilitated mast cell degranulation through boosting OXPHOS and ATP production[27]. The contribution of mitochondrial STAT3 for other cytokines and cell types, as well as the relative importance of mitochondrial and nuclear STAT3 signaling, remain important but unclear.

These positive effects of mitochondrial STAT3 on mitochondrial respiration, as well as the protective effects of reducing mtROS and apoptosis, are reminiscent of the role of IL-10 in opposing LPS-induced Warburg metabolism and mitochondrial damage[7]. Therefore, we hypothesized that mitochondrial STAT3 might contribute to IL-10 mediated suppression in macrophages.

Chapter 3.3 – IL-10 induces mitochondrial pY-STAT3 translocation

It is well known that IL-10 induces the phosphorylation of STAT3 at the Y705 residue[28], but the canonical view is that pY-STAT3 only translocates to the nucleus[29]. We first asked if IL-10 might also induce pY-STAT3 in the mitochondria. WT murine bone marrow derived macrophages (BMDM) were stimulated with IL-10, separated into mitochondrial, nuclear and cytosolic fractions by differential detergent permeabilization and analyzed by Western Blotting. COX IV, Histone H3 and α-tubulin were used as loading controls and markers of purity for the mitochondrial, nuclear and cytosolic fractions respectively. In contrast to the prevailing view, pY-STAT3 was detected in all fractions upon stimulation with IL-10 (**Figure 17**).

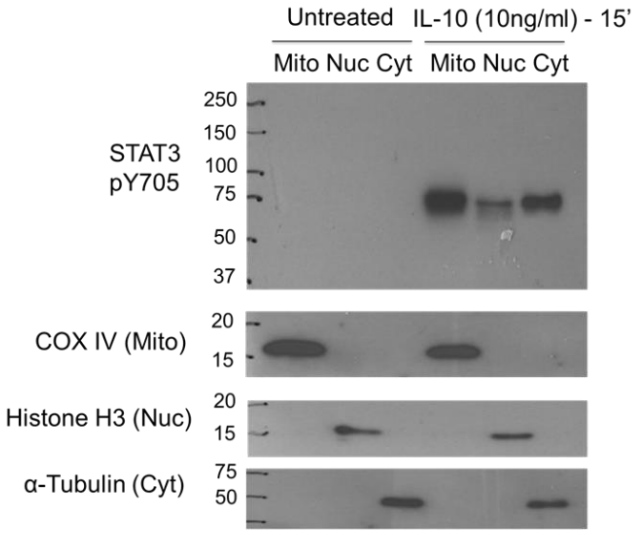


Figure 17. IL-10 induces translocation of STAT3 into the mitochondria. WT BMDM were stimulated with IL-10 for 15 min, separated into mitochondrial (Mito), nuclear (Nuc) and cytoplasmic (Cyt) lysate and analyzed by Western Blot. COX IV (Mito), Histone H3 (Nuc) and α-tubulin (Cyt) serve as loading controls and markers for their specific compartments. n = 3 independent experiments.

Chapter 3.4 – IL-10 also activates mitochondrial pY-STAT3 in the context of inflammation

IL-10 is primarily known for its ability to suppress inflammation, hence we asked if IL-10 can also induce mitochondrial STAT3 during inflammation. This was achieved by stimulating *Il10*$^{-/-}$ macrophages with LPS for 24h before adding IL-10 at the last 15 minutes, 1h and 6h. *Il10*$^{-/-}$ macrophages were used instead of WT to eliminate the effects of LPS-induced endogenous IL-10 that can mask the effect of exogenous IL-10 simulation[7,30]. Similar to WT macrophages, IL-10 induced pY-STAT3 translocation in all compartments (mitochondria, nucleus and cytosol) in LPS induced *Il10*$^{-/-}$ BMDM (**Figure 18a**). The temporal kinetics of IL-10 induced pY-STAT3 was similar across all compartments, being upregulated shortly after IL-10 stimulation and tapering off over time (**Figure 18a**). We did not find appreciable changes in total STAT3 levels upon IL-10 stimulation, except in the nucleus where there is a slight increase (**Figure 18b**). Together these suggest that IL-10 induces mitochondrial pY-STAT3 in both uninflamed and inflamed settings.

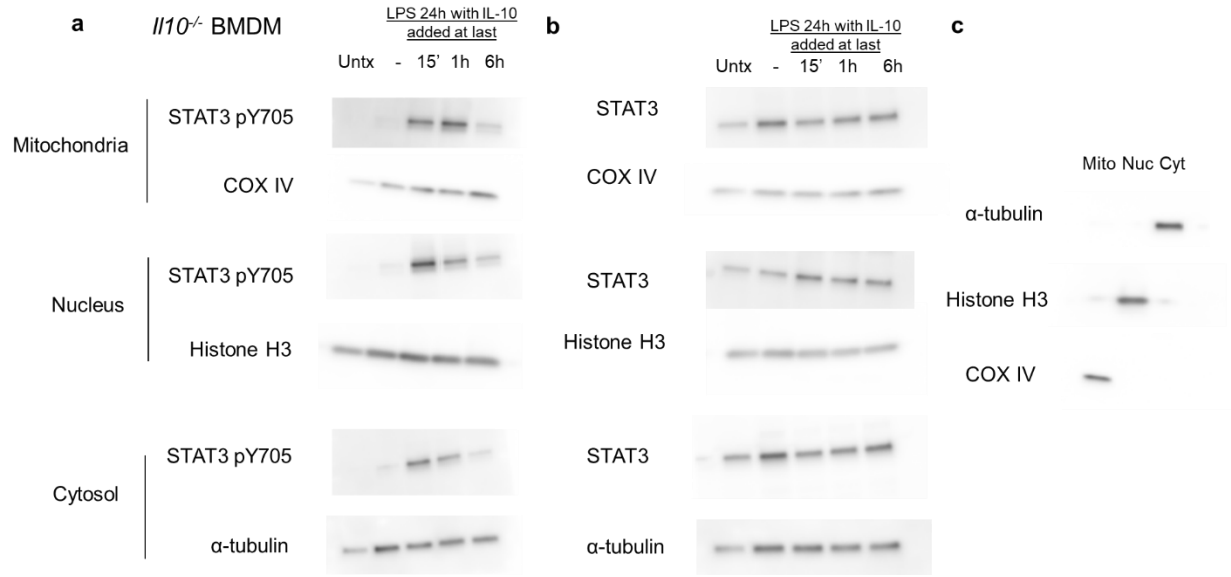


Figure 18. Similar IL-10 induced mitochondrial pY-STAT3 during inflammation. *Il10*$^{-/-}$ BMDM were stimulated with LPS for 24h, with IL-10 (10ng/ml) at various time points from the end of the LPS stimulation. The kinetics of (a) pY-STAT3 and (b) STAT3 translocation were assayed in various compartments as in Figure 1. COX IV (Mito), Histone H3 (Nuc) and α-tubulin (Cyt) serve as (a-b) loading controls and (c) purity markers for their specific compartments. Representative of 2 independent experiments.

Chapter 3.5 – IL-10 induces cellular but not mitochondrial pS-STAT3 activation

STAT3 phosphorylation at the serine 727 residue (pS-STAT3) has been reported to be critical for mitochondrial STAT3 function[13,14]. Therefore, we asked if IL-10 might also activate pS-STAT3 in WT BMDM. In whole cell lysates, we detected a basal level of pS-STAT3 that was further increased by IL-10 stimulation (**Figure 19a**), consistent with earlier reports[31]. However, this increase in pS-STAT3 was not reflected in the mitochondrial fraction (**Figure 19b**). This suggests that IL-10 induced pS-STAT3 might primarily serve to regulate non-mitochondrial functions of STAT3 such as nuclear transcription.

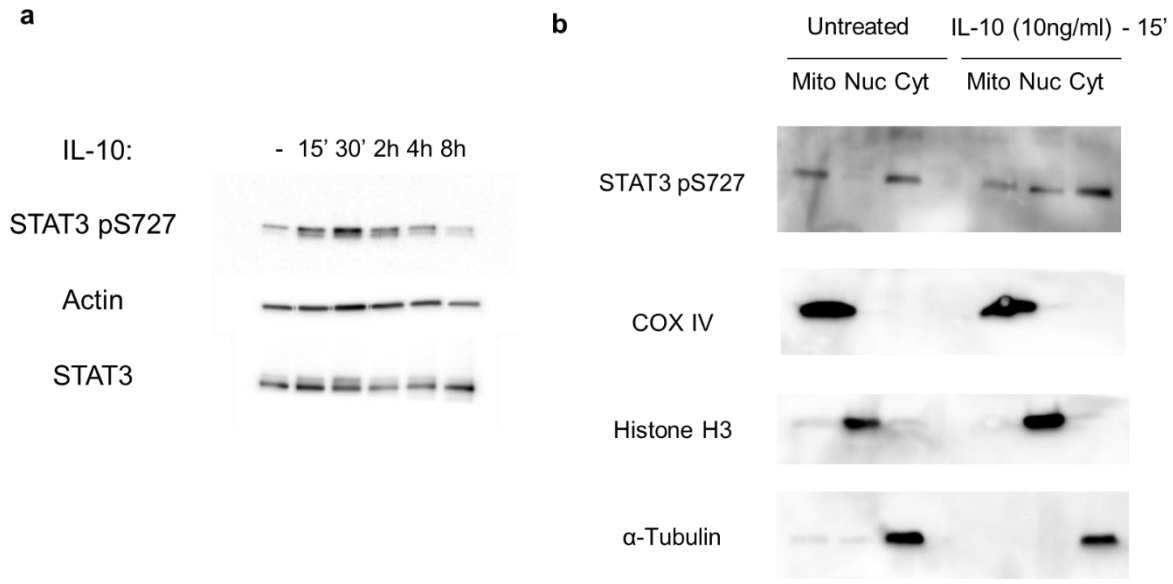


Figure 19. IL-10 induces cellular but not mitochondrial pS-STAT3 activation. WT BMDM were stimulated with IL-10 (10ng/ml) for the indicated time points. (a) Levels of pS-STAT3 in whole cell lysates were analyzed by western blot. (b) Macrophages were separated into mitochondrial (Mito), nuclear (Nuc) and cytoplasmic (Cyt) fractions and pS-STAT3 analyzed by Western Blot. COX IV (Mito), Histone H3 (Nuc) and α-tubulin (Cyt) serve as loading controls and markers for their specific compartments. Representative of (a) 1 or (b) 3 independent experiments.

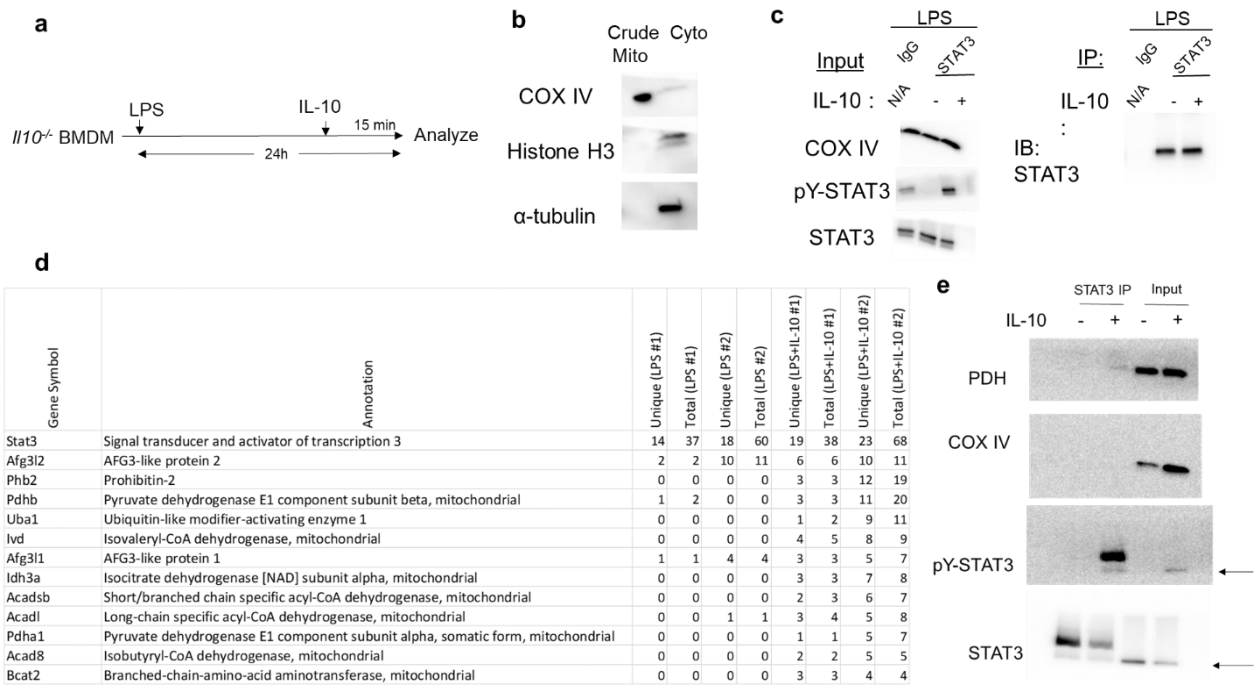
Chapter 3.6 – IL-10 induced mitochondrial STAT3 binding targets

STAT3 has been reported to modulate mitochondrial function by directly interacting with various mitochondrial proteins such as the ETC complexes, the MPTP (via cyclophilin D) and mitochondrial DNA[13,19,24]. We thus sought to determine whether STAT3 bound to different mitochondrial proteins upon IL-10 stimulation. LPS stimulated *Il10*$^{-/-}$ BMDM were stimulated with IL-10 for 15 min (**Figure 20a**) and mitochondria enriched by differential centrifugation, which we confirmed in a separate experiment to have good purity (**Figure 20b**). Equal amounts of mitochondrial lysates were immunoprecipitated with anti-total STAT3 (or isotype control) (**Figure 20c**) and subjected to mass spectrometric analysis. We narrowed down candidate IL-10 specific STAT3 binding partners using the following criteria: (1) Peptides that bound to the isotype control were excluded, (2) Peptides that did not display an increase of at least 2 peptides after IL-10 treatment in both duplicate experiments were excluded, unless there were multiple subunits of a known complex bound and (3) only proteins which are known to be localized to mitochondria were included (to exclude contaminating proteins).

We identified 13 mitochondrial proteins that specifically bound to STAT3 upon IL-10 treatment, STAT3 being the top hit as expected (**Figure 20d**). Some of these proteins have been reported to bind to STAT3 under other conditions, such as the pyruvate dehydrogenase (PDH) E1 complex subunits PDHA1, PDHB and the Prohibitin (PHB) complex subunit PHB2. PDH governs the entry of pyruvate to the TCA cycle, and acetylated STAT3 has been shown to interact with PDH E1 upon insulin stimulation. The insulin STAT3-PDH interaction correlated with increased PDH activity and sustained ETC function[20], suggesting that IL-10-STAT3 might also enhance OXPHOS using a similar mechanism. The PHB complex comprises of PHB1 and PHB2, and PHB1 was reported to protect against mitochondrial stress via binding to mitochondrial STAT3[21]. PHB2 was more recently reported to bind to LC3 and facilitate mitophagy upon outer mitochondrial membrane rupture[32]. It is thus possible that, in addition to inhibiting mTOR[7], IL-10 might also enhance mitophagy by directly enhancing PHB2 function.

In addition to known binding targets, the IP-MS data also revealed some novel binding targets (IVD, BCAT2, ACADSB, ACAD8) involved in the metabolism of the branched chain amino acids (BCAA) valine, leucine and isoleucine. BCAA metabolism is a multi-step process involving deamination (BCATs e.g. BCAT2), decarboxylation and further catabolism via a series of reactions (e.g. IVD for leucine, ACADSB for Isoleucine and ACAD8 for Valine). These reactions generate acetyl-CoA that feed into the TCA cycle[33]. As multiple targets were identified that metabolize all 3 BCAAs, this strongly suggests that IL-10-STAT3 might have a role in acutely regulating BCAA metabolism. Other interesting candidates included IDH3a, involved in the conversion of isocitrate to α-ketoglutarate in the TCA cycle, as well as the long chain specific acyl-CoA dehydrogenase (ACADL), which catalyzes the first step of the oxidation of long chain fatty acids[34,35].

In preliminary studies, we validated one of the IL-10 dependent interactions – the PDH complex – due to its known association with STAT3[20] and our identification of 2 subunits of this complex. Immunoprecipitating STAT3 in isolated mitochondria, we could observe by Western Blotting an interaction with PDH only upon IL-10 stimulation (**Figure 20e**).



Gene Symbol	Annotation	Unique (LPS #1)	Total (LPS #1)	Unique (LPS #2)	Total (LPS #2)	Unique (LPS+IL-10 #1)	Total (LPS+IL-10 #1)	Unique (LPS+IL-10 #2)	Total (LPS+IL-10 #2)
Stat3	Signal transducer and activator of transcription 3	14	37	18	60	19	38	23	68
Afg3l2	AFG3-like protein 2	2	2	10	11	6	6	10	11
Phb2	Prohibitin-2	0	0	0	0	3	3	12	19
Pdhb	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	1	2	0	0	3	3	11	20
Uba1	Ubiquitin-like modifier-activating enzyme 1	0	0	0	0	1	2	9	11
Ivd	Isovaleryl-CoA dehydrogenase, mitochondrial	0	0	0	0	4	5	8	9
Afg3l1	AFG3-like protein 1	1	1	4	4	3	3	5	7
Idh3a	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	0	0	0	0	3	3	7	8
Acadsb	Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial	0	0	0	0	2	3	6	7
Acadl	Long-chain specific acyl-CoA dehydrogenase, mitochondrial	0	0	1	1	3	4	5	8
Pdha1	Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial	0	0	0	0	1	1	5	7
Acad8	Isobutyryl-CoA dehydrogenase, mitochondrial	0	0	0	0	2	2	5	5
Bcat2	Branched-chain-amino-acid aminotransferase, mitochondrial	0	0	0	0	3	3	4	4

Figure 20. Mitochondrial proteins that bound to STAT3 upon IL-10 stimulation. (a) *Il10*$^{-/-}$ BMDM were stimulated with LPS for 24h, with IL-10 (10ng/ml) at the last 15 minutes, and crude mitochondria isolated by differential centrifugation. (b) Purity of the mitochondrial fraction analyzed using COX IV, Histone H3 and α-tubulin as in previous figures. (c) Representative loading controls shown for the input lysate, as well as verification of STAT3 pull down. (d) List of candidates of IL-10 induced mitochondrial STAT3 binding proteins shown. This experiment was done in duplicates, with lysates were pooled from 3 independent experiments. (e) Mitochondrial lysates from LPS or LPS+IL-10 stimulated BMDM were subjected to STAT3 IP and blotted for PDH. n=1. Arrows indicate the (pY)STAT3 band.

Chapter 3.7 - IL-10 induced temporal metabolic changes

To understand how IL-10 induced mitochondrial STAT3 might affect cellular metabolism, we performed metabolomic analyses on macrophages stimulated with IL-10 (in collaboration with the lab of Dr Rob Gerszten). *Il10*$^{-/-}$ macrophages were stimulated as described above with LPS for 24h followed by IL-10 at the last 15min, 1h, 4h and 24h (**Figure 21a**). Cellular lysates were then subjected to untargeted positive and negative ion metabolomics and metabolite levels normalized to untreated cells.

We focused on metabolites that were significantly changed by acute (15 min, 1h) IL-10 treatment relative to LPS (**Figure 21b**). Most of these metabolites were upregulated by LPS treatment and reduced upon IL-10 treatment, consistent with IL-10's effect in opposing LPS mediated metabolic reprogramming[7]. Several of the metabolites are part of pathways regulated by the putative STAT3 binding targets (**Figure 20d**). For example, several fatty acid-carnitine species (C14, C16, C18) were upregulated by LPS and downregulated by acute IL-10 treatment, suggesting that IL-10 enhances the breakdown of these fatty acids (**Figure 21b**). Acyl-CoA dehydrogenases such as ACADL catalyze the first step of fatty acid oxidation, and different members of these dehydrogenases recognize different lengths of fatty acids[34,36]. The STAT3 IP target ACADL, unlike other dehydrogenases (SCAD, MCAD), recognizes C14-18 species, suggesting that IL-10 might enhance fatty acid oxidation through STAT3 mediated modulation of ACADL[36]. IL-10 also reduced C4-carnitine levels, a by-product of the catabolism of the BCAA valine that is regulated by the IP target ACAD8 (**Figure 21b**)[33], suggesting that IL-10-STAT3 might also enhance ACAD8 activity. Other metabolites modulated by IL-10 included fumarate and possibly malate, which are TCA cycle intermediates (**Figure 21b**).

Not all metabolites however were inhibited by IL-10. Cyclic AMP for instance, was detected to be increased by LPS and further increased by acute IL-10 treatment in both positive and negative ion platforms, suggesting that IL-10R signaling might induce a cyclic AMP burst (**Figure 21b**).

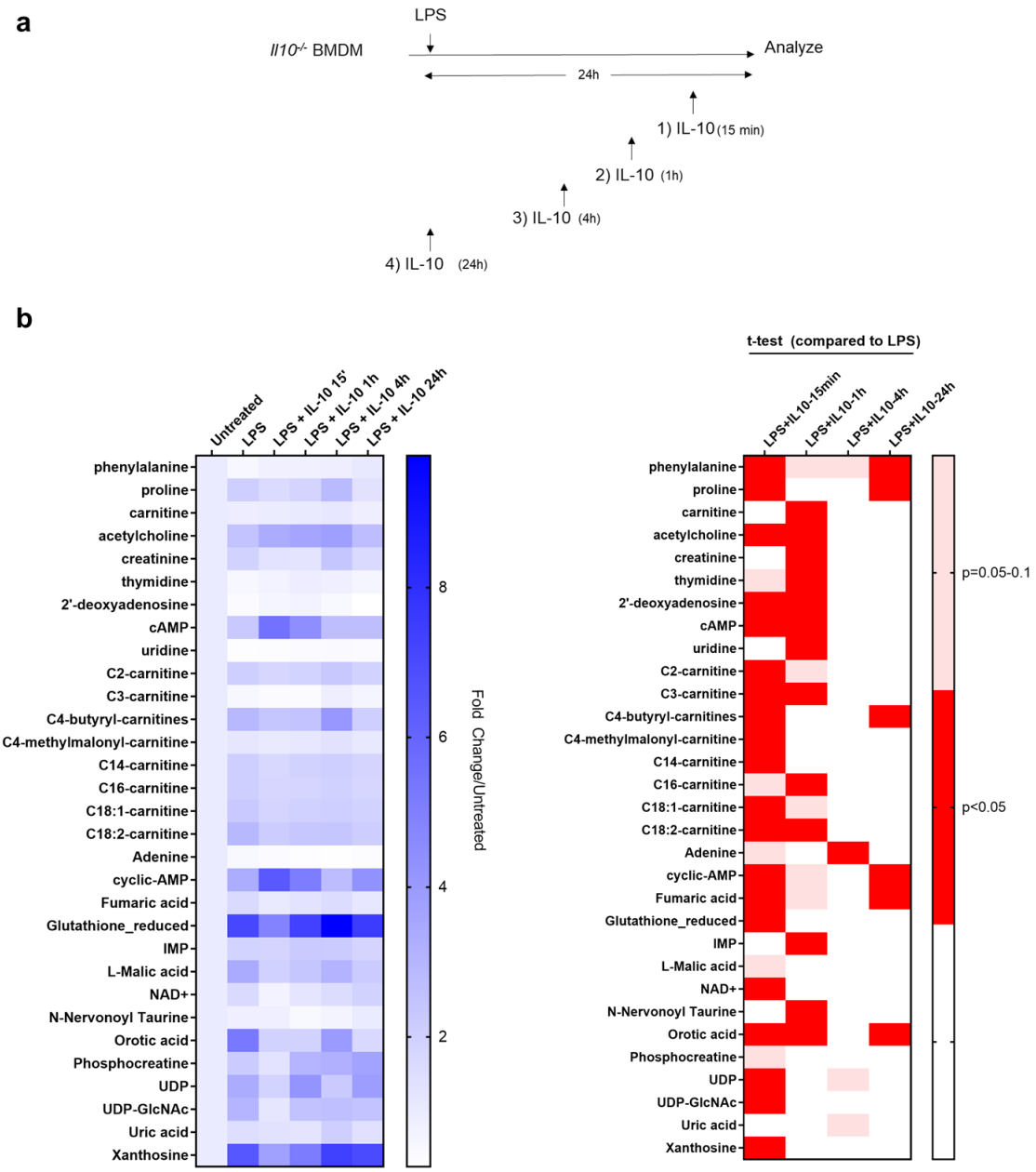


Figure 21. Metabolites acutely regulated by IL-10. (a) *Il10*$^{-/-}$ BMDM were stimulated with LPS for 24h, with IL-10 (10ng/ml) at the last 15 minutes, 1h, 4h and 24h and cell lysates analyzed for metabolomics. (b) Metabolites that are acutely regulated by IL-10 (15min, 1h) are shown, with the left graph displaying fold changes of metabolites relative to untreated cells and the right showing significance (p) values of IL-10 treated metabolites relative to LPS alone. Data are combined from both positive and negative ion metabolomic platforms. n=3 biological replicates, each replicate being cells from 1 mouse. Significance was assessed by t-test.

Chapter 3.8 – Defective IL-10 induced transcription in *Stat3*ΔV463 macrophages

The canonical view of IL-10R signaling is that IL-10 induces the transcription of selected genes that then suppress genes induced by inflammatory triggers like LPS[5]. We reasoned that if mitochondrial STAT3 had a functional impact on IL-10R signaling through protein-protein interaction, we would observe at least some effects of IL-10 in the absence of STAT3 driven transcription. To test this hypothesis, we utilized macrophages that expressed a mutation (ΔV463) in *Stat3*'s DNA binding domain (herein termed STAT3Tg), thereby abolishing STAT3's transcriptional ability[37].

We validated that this mouse was indeed defective in STAT3 mediated transcription by comparing the induction of known IL-10 induced genes (*Socs3, Bcl3, Il4ra)* with WT and STAT3KO ($LysM^{Cre}$ $STAT3^{fl/fl}$) macrophages. Consistent with previous reports, STAT3Tg macrophages displayed defective transcription of these genes upon stimulation with IL-10, although not as complete as their STAT3KO counterparts (**Figure 22a**). We also validated this defect on a protein level by measuring the IL-10 induced surface expression of IL-4Rα using flow cytometry (**Figure 22b**).

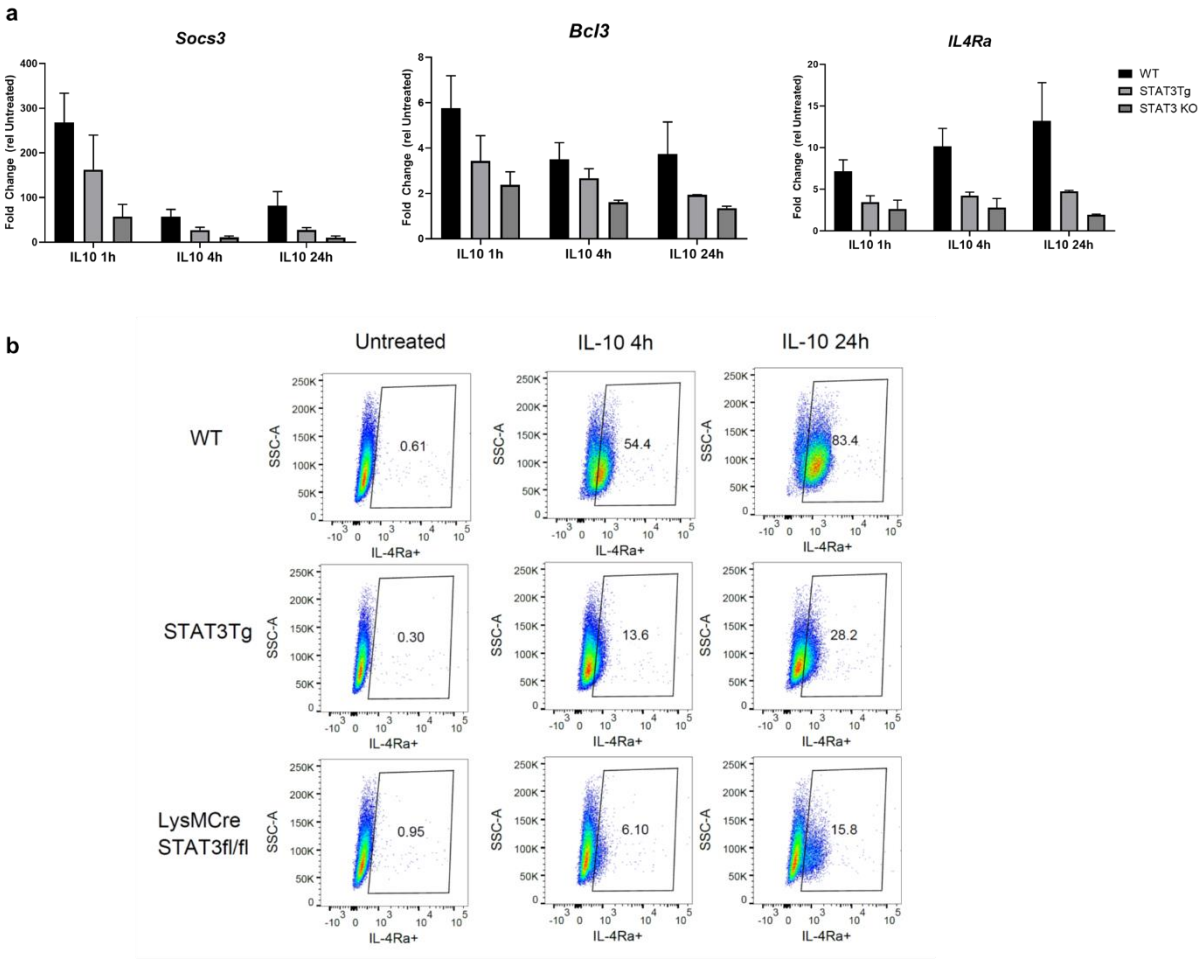


Figure 22. IL-10 induced transcription in STAT3Tg macrophages. WT, STAT3Tg or STAT3 KO BMDM were stimulated with IL-10 (20ng/ml) for various time points. (a) Gene expression of several known IL-10 induced genes were measured by qRT-PCR. Values depict the mean of 3 (1h and 4h time point) or 2 (24h time point) independent experiments, and the error bars depict the S.E.M. (b) Surface expression of IL-4Rα was measured by flow cytometry. Representative flow plots shown out of 3 independent experiments.

Chapter 3.9 - IL-10 mediated suppression does not depend only on STAT3 transcriptional activity

We then asked whether IL-10 can still exert its immunosuppressive effects in the absence of STAT3 mediated transcription by stimulating WT, STAT3Tg and STAT3KO macrophages with LPS or LPS + IL-10 for 24h. As expected, IL-10 was able to suppress the LPS induced transcription of *Tnf*, *Il12b* and *Nos2* in WT but not STAT3 KO macrophages. Strikingly, in STAT3Tg macrophages, the IL-10 mediated suppression of *Il12b* and *Nos2*, and to a lesser extent *Tnf*, were intact, suggesting that non-transcriptional STAT3 can mediate at least some aspects of IL-10 driven suppression (**Figure 23**). While mitochondrial STAT3 has been shown to bind to mtDNA and regulate mitochondrial transcription[23,24], our data suggests that mtDNA binding might not apply to IL-10R signaling.

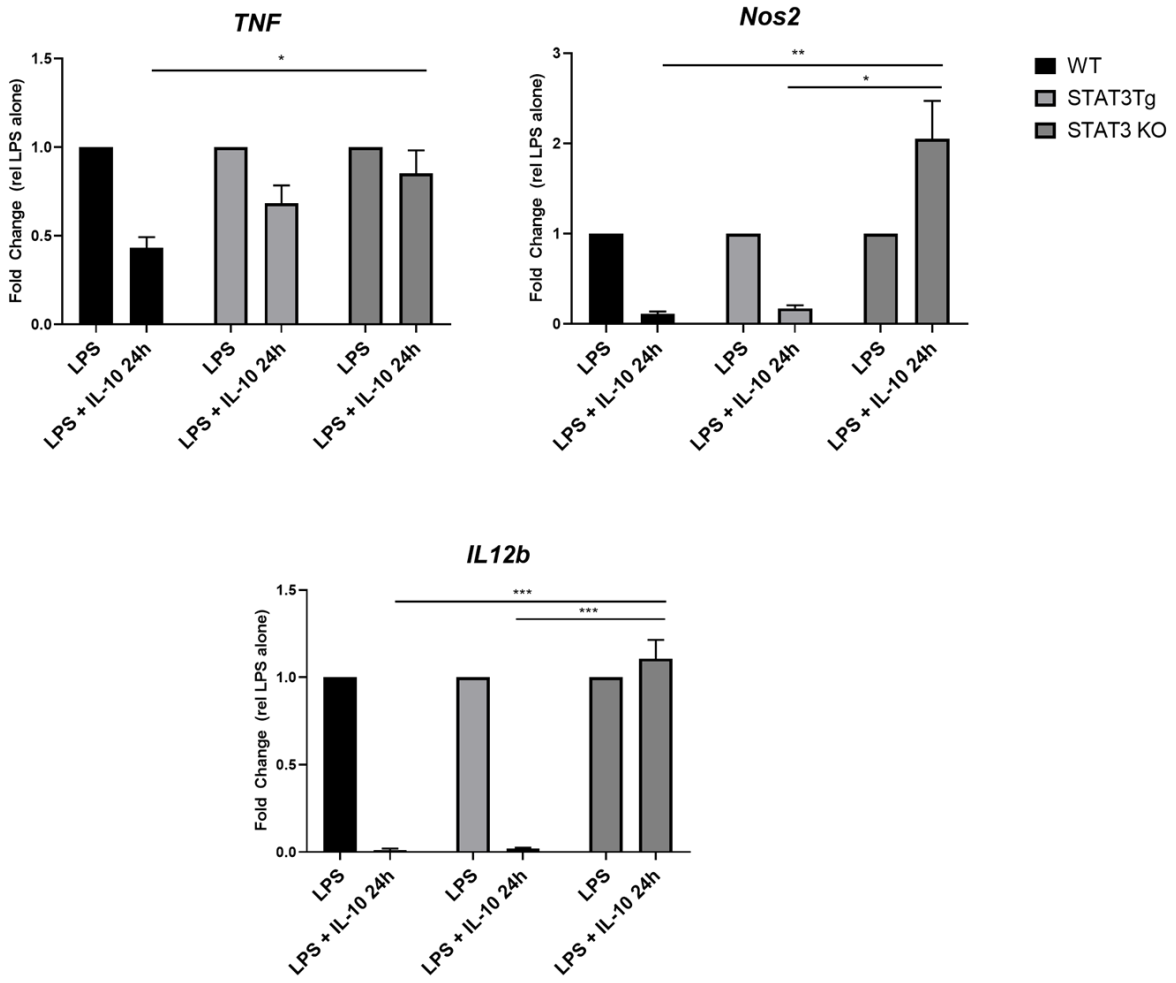


Figure 23. IL-10 mediated suppression in the absence of STAT3 driven transcription. WT, STAT3Tg or STAT3 KO BMDM were stimulated with LPS (100ng/ml) with or without IL-10 (20ng/ml) for 24h. Gene expression of several LPS induced pro-inflammatory genes were measured by qRT-PCR. Values depict the mean of 3 independent experiments, and the error bars depict the S.E.M. *$p<0.05$, ** $p<0.01$, ***$p<0.001$ by two-tailed unpaired t-test, corrected for multiple comparisons using the Holm-Šídák method.

Chapter 3.10 - IL-10 requires STAT3 mediated transcription to enhance M2r polarization

In addition to suppressing inflammatory responses, IL-10 can also drive the polarization of macrophages into a M2r phenotype along with IL-4 and TGFβ[4,38]. Of these M2r stimuli, only IL-10 uses STAT3 signaling. We thus used M2r polarization as a proxy for IL-10-STAT3 signaling to ask whether STAT3 mediated transcription was required for the IL-10 driven M2r polarization. As expected, WT but not STAT3 KO M2r macrophages expressed the M2 markers *Ym1, Fizz1* and *Arg1* (**Figure 24**). STAT3Tg macrophages however had reduced expression of the M2 markers *Fizz1*, *Arg1* and to a lesser extent *Ym1*, similar to STAT3 KO macrophages (**Figure 24**). This suggests that, unlike the suppression of pro-inflammatory responses, IL-10 requires STAT3 mediated transcription to enhance M2r polarization.

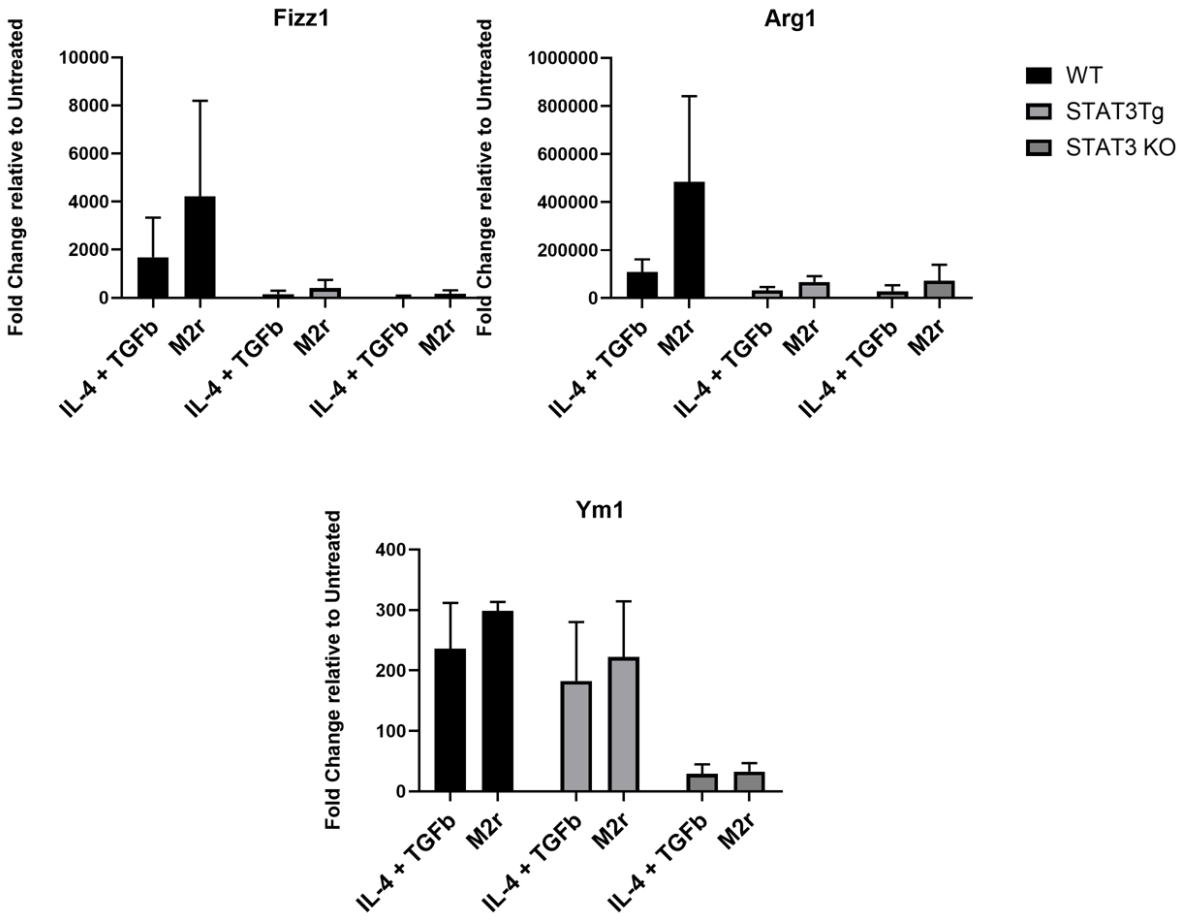


Figure 24. STAT3 mediated transcription is required for M2r polarization. WT, STAT3Tg or STAT3 KO BMDM were stimulated with IL-4 + TGFβ or M2r (IL-4 + TGFβ + IL-10) stimuli for 24h (all 20ng/ml). Gene expression of several M2 markers were measured by qRT-PCR. Values depict the mean of 2 independent experiments, and the error bars depict the S.E.M.

Chapter 3.11 – Discussion

STAT family members were originally discovered through their ability to bind DNA, hence subsequent studies into STAT function focused on its regulation of gene transcription[39]. This was the case for IL-10R signaling, where STAT3 was found to induce the transcription of a vast anti-inflammatory program consisting of multiple effector genes that subsequently inhibit inflammation[5]. However, several reports have challenged this paradigm, describing a role for STAT3 signaling in the mitochondria where it generally promotes mitochondrial function. This is achieved through STAT3 driven interactions with multiple mitochondrial proteins such as ETC complexes and the MPTP (via cyclophilin D), as well as with mtDNA[16,40].

In this study, we evaluated whether IL-10 might, in addition to nuclear STAT3 signaling, also exert its immunosuppressive effects through mitochondrial STAT3 signaling. We found that IL-10 activates pY-STAT3 but not pS-STAT3 in the mitochondria, despite increases in whole cell levels for both STAT species (**Figure 17-19**). This was somewhat unexpected given that earlier studies revealed a critical importance of S727 and not Y705 phosphorylation for mitochondrial STAT3 function[13,14]. However, several of these studies were conducted at steady state, and reports on cytokine mediated mitochondrial STAT3 signaling have not always described increases in pS-STAT3 levels[17,24]. One example of this is IL-6, which increases mitochondrial pY and total STAT3 in T cells[17]. It is possible that in the context of IL-10R signaling, IL-10 induced mitochondrial pY-STAT3 might be serine phosphorylated at later time points, or that pY-STAT3 might dimerize with existing pS-STAT3 for mitochondrial function. Recently, it was shown that acetylation of STAT3 is required for its translocation to the mitochondria in the context of insulin signaling[20]. Whether STAT3 acetylation plays a role in IL-10R signaling is unclear.

We also identified a few mitochondrial proteins that bound to STAT3 upon IL-10 stimulation, which correlated with acute changes in various metabolites (**Figure 20-21**). These included some previously reported mitochondrial proteins like PDH and PHB1/2[20,21], but not others like Complex I, II of

the ETC and cyclophilin D[13,19]. We also detected some novel binding proteins like ACADL which is involved in FAO, various enzymes involved in BCAA catabolism and other TCA cycle enzymes (**Figure 20**). Further studies would be needed to validate these hits and address how IL-10-STAT3 precisely modulates the functions of these targets. This could involve doing targeted metabolic flux analyses on pathways modulated by these enzymes after IL-10 stimulation using tools like labelled pyruvate (for PDH), palmitate (for ACADL in fatty acid oxidation) and BCAAs. Macrophages could also be starved *in vitro* of these metabolites and subsequently re-fed to test for changes in respiratory parameters. Finally, the role of acute IL-10 stimulation in mitophagy should also be assessed given that the target PHB2 has been reported to regulate mitophagy[32].

The identification of several interacting proteins suggests that IL-10-STAT3 might coordinately regulate multiple aspects of mitochondrial metabolism before transcription of anti-inflammatory response genes takes place. How might these altered metabolites contribute to IL-10 mediated suppression? One possibility is that these catabolic processes (FAO, BCAA catabolism) feed into the TCA cycle[33,34], promoting OXPHOS and suppressing the anabolic processes associated with aerobic glycolysis in inflammatory macrophages. Another possibility is that these metabolites enhance the generation of itaconate, a potent anti-inflammatory metabolite like IL-10 that is made from the TCA cycle via IRG1[41]. BCAT1, the cytosolic counterpart of BCAT2 that is involved in BCAA metabolism, was shown to promote IRG1 levels and itaconate production, although it also promoted proinflammatory macrophage activation[42]. This potential link between IL-10 and itaconate is an interesting angle to be addressed.

Finally, we observed using the STAT3Tg macrophages that STAT3 mediated transcription was required for M2r polarization but not for the suppression of LPS induced proinflammatory genes (**Figure 23-24**). Why would non-canonical STAT3 specifically affect inflammatory (M1) and not anti-inflammatory (M2) macrophages? This apparent contradiction can be explained by the observation that M1 and M2 macrophages have distinct metabolic profiles, with M1 macrophages having a broken TCA cycle, reduced OXPHOS and reduced fatty acid oxidation and M2 macrophages having an intact TCA

cycle[8]. Most studies into mitochondrial STAT3 have pointed to its protective functions in the mitochondria[16], hence it would be logical that IL-10-mitochondrial STAT3 might be relevant only during inflammation when mitochondrial function is reduced. Being non-transcriptional, the effects of mitochondrial STAT3 can be achieved more rapidly, allowing IL-10 to rapidly suppress inflammation before gene transcription takes place. This idea has some support in terms of the regulation of enhancer activation[43]. In the case of M2r polarization, OXPHOS is still intact, therefore mitochondrial STAT3 functions would not be necessary. One key mechanism by which IL-10 enhances M2r polarization is through IL-10's ability to upregulate IL-4R expression, thereby sensitizing macrophages to the master M2 stimulus IL-4. As IL-4R upregulation is mediated by STAT3 driven transcription, this would explain the dependence of M2 macrophages on STAT3 driven transcription (**Figure 22b**)[44].

While we have shown that IL-10 is still able to suppress pro-inflammatory gene expression in the absence of STAT3 driven transcription, we have not linked these effects to mitochondrial STAT3 function. How might metabolic changes driven by mitochondrial STAT3 affect the transcription of these genes? One possibility is that mitochondrial STAT3 promotes flux through the TCA cycle to regulate metabolites that affect transcription. In inflammatory macrophages, there is a break in the TCA cycle at SDH that leads to the accumulation of succinate. Succinate stabilizes HIF-1α which in turn induces the transcription of glycolytic genes as well as some immune genes like *Il1b* and *Il12b*[45,46]. To compensate for the break at SDH, macrophages also utilize the aspartate-arginosuccinate shunt (AASS) to replenish levels of fumarate, the metabolic product of SDH[47]. Interestingly, the AASS pathway was shown to be important for the upregulation of iNOS (*Nos2*)[48]. Therefore, it's possible that IL-10 might reduce the transcription of these genes by decreasing the levels of succinate and inhibiting the AASS pathway. It will be interesting to characterize the metabolic parameters of IL-10 stimulated STAT3Tg macrophages to assess the levels of these metabolites. It will also be helpful to generate STAT3Tg macrophages on an *Il10*$^{-/-}$ background to assess the effect of acute IL-10 treatment on these metabolites.

In conclusion, we have described a non-canonical role for STAT3 in regulating mitochondrial function and inflammatory gene expression. We show that, in additional to nuclear STAT3, IL-10 induces mitochondrial pSTAT3 translocation as well as STAT3 binding to various mitochondrial proteins. Canonical STAT3 mediated transcription is required for IL-10 mediated M2r polarization but not immunosuppression, suggesting that mitochondrial STAT3 might have critical functional consequences for subsequent inflammatory gene expression. Our data reveals novel perspectives behind IL-10R signaling and provides additional complexity behind the JAK-STAT pathway that might shed light into its precise mechanism of action.

Chapter 3.12 – Materials and Methods

Mice. C57BL/6J (Strain 000664), B6.129P2-Il10^{tm1Cgn} (*Il10*-/-, Strain 002251), C57BL/6-Tg(Stat3*)9199Alau (STAT3Tg, Strain 027952), B6.129P2-Lyz2$^{tm1(cre)Ifo}$ (LysM^{Cre}, Strain 004781) and B6.129S1-Stat3^{tm1Xyfu} (*Stat3$^{fl/fl}$*, Strain 016923) mice were purchased from Jackson Labs. LysM^{Cre} *Stat3$^{fl/fl}$* mice were generated by crossing LysM^{Cre} mice with *Stat3$^{fl/fl}$* mice. All mice were on the B6 background and maintained in a specific pathogen-free animal facility in Boston Children's Hospital. All experiments were conducted after approval from the Animal Resources at Children's Hospital and according to regulations by the Institutional Animal Care and Use Committee (IACUC).

Generation of BMDM. Bone marrow macrophages were generated as described[49]. Briefly, mouse femurs and tibiae were flushed with a $25\frac{5}{8}$G needle to collect bone marrow cells. These cells were then cultured in untreated tissue culture dishes in macrophage media (Dulbecco's modified Eagle's medium (DMEM) with 20% heat-inactivated fetal bovine serum (FBS), penicillin streptomycin and 30% L929-cell conditioned medium). L929 conditioned medium were generated by culturing L929 cells, which secrete macrophage colony-stimulating factor (M-CSF). Cells were fed with macrophage medium every 2-3 days and rested in DMEM at day 6-7. Mature macrophages were then cultured in various cytokines as indicated in the figure legends.

Reagents and antibodies. The following antibodies were used for the experiments. For Flow Cytometry, Anti-CD16/CD32 (Biolegend, Clone 93) and PE anti-IL-4Rα (Biolegend, Clone I015F8) were used. For Immunoprecipitation, anti-STAT3 (Thermo Scientific, Clone 9D8) or Isotype Control (Mouse IgG Isotype control, Thermo Scientific) were used. For immunoblotting, the following antibodies were used: Anti-STAT3 (Cell Signaling, Clone 79D7), Anti-STAT3 pY705 (Cell Signaling, Clone D3A7), Anti-STAT3 pS727 (Cell Signaling, Cat #9134), Anti-COX IV (Cell Signaling, Cat #4844), Anti-Histone H3 (Cell Signaling, Clone D1H2), Anti-α-tubulin (Cell Signaling, Clone DM1A), Anti-PDH (Cell Signaling, Clone C54G1), Anti-β-actin (Sigma, Clone AC-15).

Mitochondrial isolation. In some experiments, macrophages were separated into mitochondrial, nuclear and cytosolic fractions using the Cell Fractionation Kit – Standard (Abcam). To isolate mitochondria for IP-MS, differential centrifugation was used as described[13]. Briefly, macrophages were resuspended in ice cold Mitochondrial Buffer (20mM HEPES pH 7.4, 250mM sucrose, 1mM EDTA) with protease and phosphatase inhibitors and dounce homogenized (~12 strokes) using a Teflon-Elvejhem homogenizer. Prior to homogenization, macrophages were sometimes spun and the cell pellet snap frozen to facilitate homogenization. The cell suspension was then centrifuged at 500g for 5min at 4°C for 2-3 times to remove unbroken cells and nuclei. The supernatant was then spun at 7800g for 10min at 4°C to obtain a pellet which is the crude mitochondrial fraction. For IP-MS, the mitochondria were washed with Buffer A and spun again, before being lysed in Pierce IP Lysis Buffer (Thermo Scientific) with protease and phosphatase inhibitors.

Western Blotting. To generate whole cell lysates, macrophages were lysed in RIPA buffer for 30min on ice, followed by centrifugation at 13200rpm for 10-15 min on a bench-top centrifuge to remove the precipitated debris. Mitochondria were lysed in Pierce IP Lysis Buffer (Thermo Scientific) (Figure 20c-e) or in digitonin/dodecylmaltoside containing buffer (Figure 20b). Protein concentrations were determined using the DC protein assay (Bio-rad) according to the manufacturer's instructions. Protease and phosphatase inhibitors were included in all lysis buffers. A fixed amount of proteins were then resolved by 4-20% gradient SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were then blocked in 5% non-fat dry milk in TBS-T (0.1% Tween 20) for 1h at RT. Following some washes, the membranes were incubated with primary antibodies overnight at 4°C in TBS-T containing 5% milk or 5% BSA according to the manufacturer's instructions. The membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies in 5% milk in TBS-T for 1 h at RT. After secondary antibody incubation, the membranes were washed and developed using a SuperSignal West Pico Chemiluminescent kit (Thermo Scientific) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

Immunoprecipitation and Mass Spectrometry. For the STAT3 IP-MS, 10µg STAT3 antibody or IgG control was crosslinked to 25µl of Protein A/G magnetic beads using the Pierce™ Crosslink Magnetic IP/Co-IP Kit (Thermo Scientific) according to the manufacturer's instructions. 1mg of pre-cleared crude mitochondrial lysate was then incubated with the crosslinked beads overnight at 4ºC. After incubation, the beads were washed and eluted according to the manufacturer's instructions, but in 70µl Elution buffer and 11.25µl Neutralization buffer. 50µl of the IP eluate was resolved on an SDS-PAGE, stained with Coomassie Blue and sent for Mass Spectrometry at the Taplin Mass Spectrometry Facility at Harvard Medical School.

Excised gel bands were cut into approximately $1mm^3$ pieces. Gel pieces were then subjected to a modified in-gel trypsin digestion procedure[50]. Gel pieces were washed and dehydrated with acetonitrile for 10 min. followed by removal of acetonitrile. Pieces were then completely dried in a speed-vac. Rehydration of the gel pieces was with 50 mM ammonium bicarbonate solution containing 12.5 ng/µl modified sequencing-grade trypsin (Promega, Madison, WI) at 4ºC. After 45 min., the excess trypsin solution was removed and replaced with 50 mM ammonium bicarbonate solution to just cover the gel pieces. Samples were then placed in a 37ºC room overnight. Peptides were later extracted by removing the ammonium bicarbonate solution, followed by one wash with a solution containing 50% acetonitrile and 1% formic acid. The extracts were then dried in a speed-vac (~1 hr). The samples were then stored at 4ºC until analysis. On the day of analysis, the samples were reconstituted in 5 - 10 µl of HPLC solvent A (2.5% acetonitrile, 0.1% formic acid). A nano-scale reverse-phase HPLC capillary column was created by packing 2.6 µm C18 spherical silica beads into a fused silica capillary (100 µm inner diameter x ~30 cm length) with a flame-drawn tip[51]. After equilibrating the column each sample was loaded via a Famos auto sampler (LC Packings, San Francisco CA) onto the column. A gradient was formed and peptides were eluted with increasing concentrations of solvent B (97.5% acetonitrile, 0.1% formic acid). As peptides eluted, they were subjected to electrospray ionization and then entered into an LTQ Orbitrap Velos Pro ion-trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Peptides were detected,

isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Peptide sequences (and hence protein identity) were determined by matching protein databases with the acquired fragmentation pattern by the software program, Sequest (Thermo Fisher Scientific, Waltham, MA)[52]. All databases include a reversed version of all the sequences and the data was filtered to between a one and two percent peptide false discovery rate.

For the co-IP experiments, 150μg of pre-cleared mitochondrial lysate was incubated in 5μg STAT3 antibody overnight at 4ºC. The lysate was then incubated with Pierce Protein A/G Magnetic beads (Thermo Scientific) and eluted with SDS sample buffer according to the manufacturer's instructions for subsequent Western Blotting.

Quantitative RT-PCR. BMDM was collected for RNA using TRIzol reagent (Life Technologies). RNA was isolated with the Direct-zol RNA Miniprep kit (Zymo Research), and a fixed amount used for reverse transcription with the iScript select cDNA Synthesis Kit (Bio-Rad). qPCR was then performed on the cDNA using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-rad) on a CFX96 Real-Time System (Bio-Rad) according to the manufacturer's instructions. Gene expression was measured as fold changes using the ΔΔCt method, normalized against β-actin and then against untreated or LPS treated samples as indicated in the figure legends. The following primer sequences were used:

Gene	Forward Primer	Reverse Primer
b-actin	GCTGTATTCCCCTCCATCGT	GCCATGTTCAATGGGGTACT
Tnf	GACCCTCACACTCAGATCATCTTC	CTCCTCCACTTGGTGGTTTG
Socs3	GGGTGGCAAAGAAAAGGAG	GTTGAGCGTCAAGACCCAGT
Il4ra	TCTGCATCCCGTTGTTTTGC	GCACCTGTGCATCCTGAATG
Ym1	CTGAGAAGCTCATTGTGGGA	CTCAGTGGCTCCTTCATTCA
Fizz1	GTCCTGGAACCTTTCCTGAG	AGCTGGATTGGCAAGAAGTT
Bcl3	TCGGGTGGATGAGGATGGAGAC	AGTATTCGGTAGACAGCGGCTATG
Il12b	GGTGTAACCAGAAAGGTGCG	AAGGTGTCATGATGAACTTAGG
Nos2	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC
Arg1	CAGAAGAATGGAAGAGTCAG	CAGATATGCAGGGAGTCACC

Metabolomics. BMDM were seeded in 3 biological replicates, each replicate being 1 mouse. After treatment with LPS and IL-10, the cells were washed with PBS and harvested in cold LC-MS grade methanol. These samples were then stored at -80ºC for metabolomic analysis (in collaboration with the lab of Dr Robert Gerszten). Lysates were transferred to 1.5ml Eppendorf tubes and centrifuged at 14,000 rpm for 20min at 4°C. Two aliquots of supernatant were transferred to new Eppendorf tubes and dried-down using a SpeedVac. The extracted metabolites were re-dissolved with 100ul of Acetonitrile-Methanol (3:1, vol/vol) solution containing the isotope labeled Val-d8 and Phenolalanine-d8 for the analysis.

Profiling of amino acids, biogenic amines, nucleotides, neurotransmitters, vitamins and other polar metabolites was performed by LC-MS in positive ion mode. Profiling of TCA cycle intermediates, sugars and bile acids was performed by LC-MS in negative ion mode as previously described[53,54]. Briefly, 10 µl of reconstituted sample was loaded onto either a 150 x 2.1 mm Atlantis HILIC column (Waters, Milford, MA) for positive mode analysis or 5µl was loaded on a 100 x 2.1mm 3.5µm XBridge amide column (Waters) for negative mode analysis using an HTS PAL autosampler (Leap Technologies, Carrboro, NC) or Agilent 1290 Infinity autosampler. The metabolites were separated using an Agilent

1200 Series HPLC system (Agilent Technologies, Santa Clara, CA) coupled to a 4000-QTRAP mass spectrometer (AB SCIEX, Foster City, CA) in positive mode analysis; an Agilent 1290 infinity HPLC binary pump system (Agilent Technologies, Santa Clara, CA) coupled to a 6490-QQQ mass spectrometer (Agilent Technologies, Santa Clara, CA) in negative mode analysis MultiQuant software v2.1 (AB SCIEX, Foster City, CA) and MassHunter quantitative software were used for automated peak integration respectively and metabolite peaks were also assessed manually for quality of peak integration.

Flow Cytometry. BMDM were first stained with the viability dye Zombie Violet (1:400) in PBS for 15 min at RT. They were subsequently washed with FACS buffer (2% FBS + 0.1% NaN_3 in PBS) and incubated with anti-CD16/CD32 (Biolegend, 1μl/10^6 cells) for 10 min at RT to block Fc receptors. PE anti-IL-4Rα (Biolegend, 1:150 final) was subsequently added and the cells incubated at 4°C for 20 min before flow cytometric analysis on a FACS Canto II Flow Cytometer (BD).

Statistics. Data were analyzed for statistical significance via the two-tailed Student's t-test. A p-value of < 0.05 was considered statistically significant. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$).

Chapter 3.13 - References

1. Fiorentino, D. F., Zlotnik, A., Mosmann, T. R., Howard, M. & O'Garra, A. IL-10 inhibits cytokine production by activated macrophages. *J. Immunol.* **147**, 3815–22 (1991).

2. Zigmond, E., Bernshtein, B. & Friedlander, G. Macrophage-Restricted Interleukin-10 Receptor Deficiency , but Not IL-10 Deficiency , Causes Severe Spontaneous Colitis. *Immunity* **40**, 720–733 (2014).

3. Redhu, N. S. *et al.* Macrophage dysfunction initiates colitis during weaning of infant mice lacking the interleukin-10 receptor. *Elife* **6**, 1–23 (2017).

4. Shouval, D. S. *et al.* Interleukin-10 receptor signaling in innate immune cells regulates mucosal immune tolerance and anti-inflammatory macrophage function. *Immunity* **40**, 706–19 (2014).

5. Hutchins, A. P., Diez, D. & Miranda-Saavedra, D. The IL-10/STAT3-mediated anti-inflammatory response: Recent developments and future challenges. *Brief. Funct. Genomics* **12**, 489–498 (2013).

6. Shouval, D. S. *et al.* Interleukin 1β Mediates Intestinal Inflammation in Mice and Patients With Interleukin 10 Receptor Deficiency. *Gastroenterology* **151**, 1100–1104 (2016).

7. Ip, W. K. E., Hoshi, N., Shouval, D. S., Snapper, S. & Medzhitov, R. Anti-inflammatory effect of IL-10 mediated by metabolic reprogramming of macrophages. *Science (80-.).* **356**, 513–519 (2017).

8. Van den Bossche, J., O'Neill, L. A. & Menon, D. Macrophage Immunometabolism: Where Are We (Going)? *Trends Immunol.* **38**, 395–406 (2017).

9. Byles, V. *et al.* The TSC-mTOR pathway regulates macrophage polarization. *Nat. Commun.* **4**, 1–11 (2013).

10. Kuwata, H. & Watanabe, Y. IL-10-inducible Bcl-3 negatively regulates LPS-induced TNF-α production in macrophages. *Blood* **102**, 4123–4130 (2003).

11. Lee, T. & Chau, L. Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. *Nat. Med.* **8**, 1–7 (2002).

12. Smith, A. M. *et al.* A distal enhancer in Il12b is the target of transcriptional repression by the STAT3 pathway and requires the basic leucine zipper (B-ZIP) protein NFIL3. *J. Biol. Chem.* **286**, 23582–90 (2011).

13. Wegrzyn, J. *et al.* Function of Mitochondrial Stat3 in Cellular Respiration. *Science* **323**, 793–798 (2009).

14. Gough, D. J. *et al.* Mitochondrial STAT3 supports Ras-dependent oncogenic transformation. *Science* **324**, 1713–6 (2009).

15. Poli, V. & Camporeale, A. STAT3-mediated metabolic reprograming in cellular transformation and implications for drug resistance. *Front. Oncol.* **5**, 1–9 (2015).

16. Meier, J. A. & Larner, A. C. Toward a new STATe: The role of STATs in mitochondrial function. *Semin. Immunol.* **26**, 20–28 (2014).

17. Yang, R. *et al.* Mitochondrial Ca^{2+} and membrane potential, an alternative pathway for Interleukin 6 to regulate CD4 cell effector function. *Elife* **4**, 1–22 (2015).

18. Garama, D. J. *et al.* A Synthetic Lethal Interaction between Glutathione Synthesis and Mitochondrial Reactive Oxygen Species Provides a Tumor-Specific Vulnerability Dependent on STAT3. *Mol. Cell. Biol.* **35**, 3646–3656 (2015).

19. Boengler, K., Hilfiker-Kleiner, D., Heusch, G. & Schulz, R. Inhibition of permeability transition pore opening by mitochondrial STAT3 and its role in myocardial ischemia/reperfusion. *Basic Res. Cardiol.* **105**, 771–785 (2010).

20. Xu, Y. S. *et al.* STAT3 Undergoes Acetylation-dependent Mitochondrial Translocation to Regulate Pyruvate Metabolism. *Sci. Rep.* **6**, 1–16 (2016).

21. Han, J., Yu, C., Souza, R. F. & Theiss, A. L. Prohibitin 1 modulates mitochondrial function of Stat3. *Cell. Signal.* **26**, 2086–2095 (2014).

22. Tammineni, P. *et al.* The import of the transcription factor STAT3 into mitochondria depends on GRIM-19, a component of the electron transport chain. *J. Biol. Chem.* **288**, 4723–4732 (2013).

23. Macias, E., Rao, D., Carbajal, S., Kiguchi, K. & DiGiovanni, J. Stat3 Binds to mtDNA and Regulates Mitochondrial Gene Expression in Keratinocytes. *J. Invest. Dermatol.* **134**, 1971–1980 (2014).

24. Carbognin, E., Betto, R. M., Soriano, M. E., Smith, A. G. & Martello, G. Stat3 promotes mitochondrial transcription and oxidative respiration during maintenance and induction of naive pluripotency. *EMBO J.* **35**, 618–634 (2016).

25. Zhang, Q. *et al.* Mitochondrial localized Stat3 promotes breast cancer growth via phosphorylation of serine 727. *J. Biol. Chem.* **288**, 31280–31288 (2013).

26. Szczepanek, K. *et al.* Mitochondrial-targeted signal transducer and activator of transcription 3 (STAT3) protects against ischemia-induced changes in the electron transport chain and the generation of reactive oxygen species. *J. Biol. Chem.* **286**, 29610–29620 (2011).

27. Erlich, T. H. *et al.* Mitochondrial STAT3 plays a major role in IgE-antigen-mediated mast cell exocytosis. *J. Allergy Clin. Immunol.* **134**, 460-469.e10 (2014).

28. Finbloom, D. S. & Winestock, K. D. IL-10 Induces the Tyrosine Phosphorylation of tyk2 and Jak1 and the Differential Assembly of STAT1a and STAT3 Complexes in Human T cells and Monocytes. *J. Immunol.* **155**, 1079–1090 (1995).

29. Shuai, K. & Liu, B. Regulation of JAK-STAT signalling in the immune system. *Nat. Rev. Immunol.* **3**, 900–11 (2003).

30. Fiorentino, D., Zlotnik, A., Mosmann, T., Howard, M. & O'Garra, A. : IL-10 inhibits cytokine production by activated macrophages. *J Immunol* 3815–3822 (1991).

31. Zhu, Y. P., Brown, J. R., Sag, D., Zhang, L. & Suttles, J. Adenosine 5'-Monophosphate-Activated Protein Kinase Regulates IL-10-Mediated Anti-Inflammatory Signaling Pathways in Macrophages. *J. Immunol.* **194**, 584–594 (2015).

32. Wei, Y., Chiang, W. C., Sumpter, R., Mishra, P. & Levine, B. Prohibitin 2 Is an Inner Mitochondrial Membrane Mitophagy Receptor. *Cell* **168**, 224-238.e10 (2017).

33. Zhang, S., Zeng, X., Ren, M., Mao, X. & Qiao, S. Novel metabolic and physiological functions of branched chain amino acids: A review. *J. Anim. Sci. Biotechnol.* **8**, 4–15 (2017).

34. Adeva-Andany, M. M., Carneiro-Freire, N., Seco-Filgueira, M., Fernández-Fernández, C. & Mouriño-Bayolo, D. Mitochondrial β-oxidation of saturated fatty acids in humans. *Mitochondrion*

0–1 (2018). doi:10.1016/j.mito.2018.02.009

35. Yang, H., Ye, D., Guan, K. L. & Xiong, Y. IDH1 and IDH2 mutations in tumorigenesis: Mechanistic insights and clinical perspectives. *Clin. Cancer Res.* **18**, 5562–5571 (2012).

36. Ghisla, S. & Thorpe, C. Acyl-CoA dehydrogenases: A mechanistic overview. *Eur. J. Biochem.* **271**, 494–508 (2004).

37. Steward-Tharp, S. M. *et al.* A mouse model of HIES reveals pro- and anti-inflammatory functions of STAT3. *Blood* **123**, 2978–2987 (2014).

38. Parsa, R. *et al.* Adoptive transfer of immunomodulatory M2 macrophages prevents type 1 diabetes in NOD mice. *Diabetes* **61**, 2881–92 (2012).

39. Stark, G. R. & Darnell, J. E. J. The JAK-STAT Pathway at Twenty. *Immunity* **36**, 503–514 (2012).

40. Avalle, L. & Poli, V. Nucleus, Mitochondrion, or Reticulum? STAT3 à La Carte. *Int. J. Mol. Sci.* **19**, 2820 (2018).

41. O'Neill, L. A. J. & Artyomov, M. N. Itaconate: the poster child of metabolic reprogramming in macrophage function. *Nature Reviews Immunology* **19**, 273–281 (2019).

42. Papathanassiu, A. E. *et al.* BCAT1 controls metabolic reprogramming in activated human macrophages and is associated with inflammatory diseases. *Nat. Commun.* **8**, 16040 (2017).

43. Conaway, E. A., de Oliveira, D. C., McInnis, C. M., Snapper, S. B. & Horwitz, B. H. Inhibition of Inflammatory Gene Transcription by IL-10 Is Associated with Rapid Suppression of Lipopolysaccharide-Induced Enhancer Activation. *J. Immunol.* 1601781 (2017). doi:10.4049/jimmunol.1601781

44. Lang, R., Patel, D., Morris, J. J., Rutschman, R. L. & Murray, P. J. Shaping Gene Expression in Activated and Resting Primary Macrophages by IL-10. *J. Immunol.* **169**, 2253–2263 (2002).

45. Tannahill, G. M. *et al.* Succinate is an inflammatory signal that induces IL-1β through HIF-1α. *Nature* **496**, 238–42 (2013).

46. Marks, E. *et al.* Regulation of IL-12p40 by HIF controls Th1/Th17 responses to prevent mucosal inflammation. *Mucosal Immunol.* **10**, 1224–1236 (2017).

47. Mills, E. L., Kelly, B. & O'Neill, L. A. J. Mitochondria are the powerhouses of immunity. *Nat. Immunol.* **18**, 488–498 (2017).

48. Jha, A. K. *et al.* Network Integration of Parallel Metabolic and Transcriptional Data Reveals Metabolic Modules that Regulate Macrophage Polarization. *Immunity* **42**, 419–430 (2015).

49. Biswas, A. *et al.* WASP-mediated regulation of anti-inflammatory macrophages is IL-10 dependent and is critical for intestinal homeostasis. *Nat. Commun.* **9**, (2018).

50. Shevchenko, A., Wilm, M., Vorm, O. & Mann, M. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal. Chem.* **68**, 850–858 (1996).

51. Peng, J. & Gygi, S. P. Proteomics: The move to mixtures. *J. Mass Spectrom.* **36**, 1083–1091 (2001).

52. Eng, J. K., Mccormack, A. L. & Yates, J. R. An Approach to Correlate Tandem Mass Spectral Data of Peptides with Amino Acid Sequences in a Protein Database. *Am. Soc. mass Spectrom.* **5**, 976–989 (1994).

53. Kimberly, W. T. *et al.* Metabolite profiling identifies anandamide as a biomarker of nonalcoholic steatohepatitis. *JCI Insight* **2**, (2017).

54. Rhee, E. P. *et al.* A genome-wide association study of the human metabolome in a community-based cohort. *Cell Metab.* **18**, 130–143 (2013).

Chapter 4

Conclusions and Future Perspectives

Chapter 4.1 – Attributions

Part of the discussion presented in this chapter are adapted from the following publication:

Kang YH, Biswas A, Field M and Snapper SB. STAT1 signaling shields T cells from NK cell- mediated cytotoxicity. *Nat. Commun.* **10** (912), 1-13 (2019).

Chapter 4.2 - Regulation of other T cell inflammatory responses by STAT1

In Chapter 2 of this dissertation, I presented data showing a role for STAT1 signaling in protecting $CD4^+$ T cells from NK cell mediated cytotoxicity. This is relevant in the context of T cell induced IBD where, in a genetically susceptible host such as *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice, NK cells prevent the colitis induced by *Stat1*$^{-/-}$ T cells through "missing self" reactivity.

While we have focused mainly on IBD, we believe that the STAT1-mediated protection of cells from NK cell killing is relevant for controlling general immune responses. In cancer, it was reported that tumor cells utilize STAT1 expression to avoid rejection *in vivo*[1]. Moreover, *Stat1*$^{-/-}$ tumors eventually escape NK cell mediated control by re-expressing MHC-I through IRF-1 and β2-microglobulin upregulation, supporting the hypothesis that these tumors face a selective pressure on MHC-I expression[1]. This suggests that inhibitors against STAT1 can be used in tumors to drive their rejection. In the context of viral infection, type I IFN protects antiviral T cells from NK cell mediated elimination through the upregulation of inhibitory MHC-I or the downregulation of activating NK receptor ligands such as NCR1[2,3]. STAT1 induces MHC-I expression through the upregulation of *Nlrc5*[4–7], and NLRC5 deficient T cells are rejected by host NK cells during viral infection[8].

Several reports have also suggested that the STAT1 mediated protection of T cells might be important in driving other inflammatory disorders. In animal models of T cell induced autoimmune disease and GvHD, it was noted that *Stat1*$^{-/-}$ T cells displayed reduced expansion[9,10]. However, the mechanistic basis for this reduction was unclear, focusing mainly on the STAT1 mediated inhibition of Treg or Th17 differentiation[9,10]. We propose that NK cells might also play a prominent role in restraining the ability of *Stat1*$^{-/-}$ T cells to induce these inflammatory disorders.

We note however that the extent of STAT1 dependency might be different in different disease contexts. While *Stat1*$^{-/-}$ T cells were very efficiently eliminated in our study and others[10], the degree of reduction in *Stat1*$^{-/-}$ T cell expansion is not as pronounced in GvHD[9]. Moreover, in a recent report

employing a mouse model of multiple sclerosis, *Stat1*$^{-/-}$ T cells induced worse disease than WT T cells, although it is unclear whether they had an expansion defect[11]. It is possible that the inflammatory environment can provide STAT1-independent signals to the T cells to protect them from NK cells. It is also possible that the inflammatory environment can alter NK cell function towards reduced cytotoxicity, thereby impairing their ability to reject *Stat1*$^{-/-}$ T cells. It will thus be interesting to evaluate how these inflammatory signals alter the repertoire of NK cell receptors as well as the repertoire of NK ligands on T cells. In our study, we observed that *Stat1*$^{-/-}$ T cells were effectively eliminated in the colitis-susceptible *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice at 3 weeks post transfer. However, we do not exclude the possibility that at earlier time points, the inflammatory environment of *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice might have provided some STAT1-independent signals to protect the T cells. A deeper study into the effect of innate IL-10R deficiency in the rejection of *Stat1*$^{-/-}$ T cells will shed light into this possibility for IBD.

Our studies in lymphopenic mice may also be relevant for clinical cases of lymphopenia such as bone marrow transplantation and HIV infection, as it suggests that STAT1 might be important for immune cell reconstitution. An earlier report showed that HIV patients, who have CD4^{+} T cell lymphopenia, have increased expression of STAT1 in T cells[12]. In addition to driving MHC-I expression, STAT1 has an intrinsic ability to promote cell death and suppress proliferation by enhancing the expression of caspase 1/11 and p27^{kip1} (an inhibitor of cyclin dependent kinases) respectively[13]. T cells overexpressing STAT1 thus displayed reduced lymphopenic expansion[14], in agreement with our observation that *Stat1*$^{-/-}$ T cells hyper-proliferate *in vitro.* This led to the suggestion that inhibition of STAT1 might be a useful strategy to enhance T cell reconstitution. Our findings suggest that such an approach will need to be balanced with ensuring that there is sufficient MHC-I expression to prevent T cell rejection.

Chapter 4.3 – Targeting the STAT1-MHC-I pathway for immune modulation

Our observations raise the question of whether targeting of the STAT1-NLRC5-MHC-I pathway might be beneficial in suppressing T cell mediated inflammation. Several therapeutic modalities against the STATs have been developed, but these mostly target STAT3[15]. A few compounds such as fludarabine and pravastatin have been identified that demonstrate STAT1 inhibitory activity[16,17]. However, using STAT1 inhibitors to eliminate inflammatory T cells might not be trivial due to the confounding effects of STAT1 in suppressing intrinsic T cell proliferation, as described earlier. Moreover, inhibiting STAT1 signaling in innate cells can negatively affect NK cell mediated killing, hence any therapeutic options targeting STAT1 for T cell control will need to exhibit a degree of specificity for T cells.

Another possibility would be to target the NLRC5-MHC-I pathway. It was recently reported that inducible deletion of *β2m* on $CD4^+$ T cells could lead to their elimination[18], suggesting that transient inhibition of MHC-I might be sufficient to suppress $CD4^+$ T cell responses. Interestingly, the elimination of these T cells required inflammation through MCMV Δ157 infection, poly(I:C) stimulation or IL-15 administration, which the authors concluded was due to the need for NK cells to be activated[18]. While we did not investigate the phenotype of NK cells in this study, RAG1 deficiency has been reported to lead to NK cell hyperresponsiveness[19]. However, our study also revealed an additional requirement for T cells to undergo spontaneous proliferation before they can be rejected, consistent with earlier studies on viral infections[2,3]. The requirements for both NK cells and T cells to be activated suggest that targeting MHC-I might lead to the specific reduction of inflammatory T cell responses without broad T cell killing, which is beneficial for restoring homeostasis. However, care must be taken into applying the correct dosage of MHC-I inhibition, as NK cells no longer exhibit missing self reactivity when there is a large proportion of $MHC\text{-}I^-$ cells[18].

What might be the specific NK cell ligands that are important for T cell rejection? We observed that *Stat1*$^{-/-}$ T cells displayed reduced levels of some ($H2\text{-}K^b/D^b$, Qa-2) but not all (Qa-1) MHC-I molecules. The observation that Qa-1 was only mildly reduced suggests that the NKG2A-Qa-1-Qdm

interaction is unlikely to explain the rejection of *Stat1*$^{-/-}$ T cells[20]. Both classical MHC-I and Qa-2 are known inhibitors of NK cell killing, however the relative contribution of these ligands to T cell rejection is unclear[21,22]. Moreover, other NK receptor ligands (e.g. NCR1 ligands[2]) might be involved in the regulation of T cells that we did not explore. It will thus be interesting to use genetic or pharmacological approaches to assess which STAT1 regulated ligands confer T cell protection from NK cells, and whether these ligands might be distinct from ligands used to protect other cell types like cancer cells.

Chapter 4.4 – NK cell mediated control of IBD

Although the focus of this study has been on the role of STAT1 in T cells, we observed that NK cell depletion also enhanced the spontaneous proliferation of WT T cells, which correlated with a moderately enhanced induction of colitis. This enhanced WT T cell proliferation accounts for the incomplete rescue of *Stat1*$^{-/-}$ T cells upon NK cell depletion. This suggests that NK cells might also be important in controlling WT T cell responses, which is consistent with earlier reports in infection[23] and IBD[24,25].

NK cells are part of the innate lymphoid cell (ILC) family[26], and ILC3s were recently shown to utilize MHC-II to recognize and kill T cells that are activated by the commensal microbiota. In pediatric IBD patients, the expression of MHC-II on ILC3s is reduced, which correlated with enhanced intestinal Th17 cells[27]. The control of T cells by ILC3s is analogous to the NK cell mediated control of spontaneously proliferating (SP) T cells, which are known to be microbially driven[28–30]. Thus, it's possible that that ILCs might serve as a general line of defense, with different ILC subsets utilizing different strategies to prevent excessive intestinal T cell responses.

We note that the mechanistic basis for the NK cell mediated control of WT T cells is unclear. It is possible that NK cells restrict a specific subpopulation of WT T cells, perhaps the one(s) with the highest activation state. The NK ligands regulating WT T cell expansion might also be distinct from those regulating *Stat1*$^{-/-}$ T cells. Further studies profiling WT T cells with and without NK cell depletion will significantly aid our understanding of this difference and potentially reveal novel therapeutic targets for directing NK cells towards different target cell types.

Importantly, the NK cell mediated control of WT T cells and colitis suggest that, distinct from inhibiting STAT1, NK cells can be harnessed to control aberrant immune responses in IBD. Current evidence regarding the role of NK cells in IBD is complicated, depending on subset and context[31]. One study found that mucosal NK cells can be divided into IFNγ producing NKp46^{+} or IL-22 producing NKp44^{+} subsets. The NKp46^{+} subset dominates in IBD patients, suggesting that this subset might be

pathogenic[32]. The cytotoxic profile of these NK cell subsets in the intestine and the effects of these subsets on disease outcome warrant closer investigation.

It is possible that certain therapeutic approaches for IBD might have unexplored effects on NK cells. One example of this is low dose IL-2 treatment. Due to the expression of the high affinity IL-2 receptor subunit CD25, low dose IL-2 has been used to selectively expand Tregs to suppress inflammation in GvHD and IBD[33,34]. However, NK cells can express CD25, and it was subsequently found that this therapy also expanded a subset of NK cells[35,36]. It will thus be interesting to revisit these studies and elucidate whether expansion of this NK cell subset is associated with clinical response and a reduction in activated T cells.

Our study utilizes lymphopenia induced proliferation in *Rag1*$^{-/-}$ mice to study colitogenic T cell responses, a model that is widely used in IBD research[37]. However, a question remains as to the applicability of lymphopenic expansion to human IBD. We believe that the specificity of NK cells in targeting microbially activated, spontaneous proliferating T cells is of significant relevance, as the microbiota is believed to be critical in driving the inflammatory immune response in IBD patients[38,39]. Interestingly, an early study into lymphopenia induced expansion described the existence of this mechanism in neonatal mice, which are naturally lymphopenic. T cell expansion in these mice was independent of IL-7 but required MHC-II-TCR interactions, indicating spontaneous and not homeostatic proliferation[40]. The existence of neonatal T cell activation is corroborated by the finding that human fetal intestines contain memory-like CD4^{+} T cells, although the source of antigens for their activation is unclear[41].

The existence of spontaneous proliferation in early life, at least in mice, suggests that NK cells might be important in shaping initial intestinal T cell responses. Aberrations in NK cell function might thus contribute to IBD in early life. Among the various genes thought to drive IBD in young patients[42], several genes have been reported to promote NK cell function. These include *IL10*, which suppresses proinflammatory macrophage and T cell responses but enhances NK cell cytotoxicity[43–49], *WAS*[50],

DOCK8[51], *PLCG2*[52] and *STXBP2*[53]. While several of these genes regulate multiple immune cell types, it is possible that, in a subset of these patients, the disease might be driven by defective control of commensal-specific T cells by NK cells. The role of NK cells in very early/early onset IBD should be further explored.

Chapter 4.5 – STAT3, more than a transcription factor

It has become increasingly clear that multiple levels of complexity are required for the JAK-STAT pathway to discriminate different cytokine signals. For STAT3, these include the cooperation of STAT3 with other co-factors to confer cell-type transcriptional specificity[54], the existence of STAT3α and STAT3β isoforms with distinct functions[55], the ability of STAT3 to dimerize with itself or other STATs[56] and modifications of STAT3 at different residues like phosphorylation at the Y705 or S727 residues, acetylation and methylation[57]. However, studies into these functions have predominantly converged on their regulation of STAT3 mediated gene transcription.

More recently, evidence has emerged pointing to roles of STAT3 outside of the nucleus. As described earlier, several reports described a role for STAT3 in the mitochondria where it preserves mitochondrial function by binding to various mitochondrial proteins such as ETC components and the MPTP (via cyclophilin D)[58]. The role of STAT3 in the mitochondria was also the focus of our study into IL-10R signaling. However, STAT3 can also exhibit functions in other subcellular locations. One example of this is the endoplasmic reticulum (ER), where STAT3 interacts with the IP_3R, specifically IP_3R_3[59]. The ER is a major intracellular store of cellular Ca^{2+}. IP_3Rs regulate the release of ER Ca^{2+}, which can then be readily taken up by the mitochondria. While low level Ca^{2+} release is important for the function of certain TCA cycle enzymes like isocitrate- and pyruvate dehydrogenase, excessive Ca^{2+} influx into the mitochondria triggers the opening of the MPTP and the initiation of the intrinsic apoptotic machinery[60]. STAT3's interaction with IP_3R_3 promotes IP_3R_3 degradation, hence the presence of ER STAT3 limits Ca^{2+} influx into the mitochondria and confers resistance to apoptosis[59]. Another example of non-nuclear STAT3 localization was shown in ovarian cancer cells, where pY-STAT3 localizes in focal adhesions and interacts with phosphorylated paxillin and focal adhesion kinases. The function of pY-STAT3 in these focal adhesions was unclear[61]. The presence of STAT3 in these subcellular locations raises the possibility that, in addition to activating distinct gene transcription programs, different cytokines might also activate different non-nuclear functions of STAT3. In light of these findings, it will

be interesting to explore whether IL-10 causes STAT3 to translocate to other locations in the cell, and whether this correlates with binding to different proteins in those locations.

Mitochondrial STAT3 has been reported to interact with several mitochondrial proteins, most of which are associated with enhanced functions of these proteins[58,62]. In our study, we also found several candidate mitochondrial proteins that interact with STAT3 upon IL-10 stimulation. It will be important to address which of these targets are important for IL-10 mediated functions. This can be achieved via the genetic or pharmacological inhibition of the targets in STAT3 ΔV463 macrophages to assess which ones lead to the abrogation of IL-10-mediated suppression as in STAT3KO macrophages. Agonists of these targets, or the provision of the downstream metabolites that these targets produce, could also be used to see if these can bypass the requirement for STAT3 in IL-10 mediated suppression. IL-10 is believed to exert its functions mainly by inducing the transcription of anti-inflammatory response genes.[63,64] In light of our findings, it will be interesting to revisit this assumption and assess whether IL-10 can also induce novel anti-inflammatory response factors through direct STAT3 mediated interactions rather than transcription.

Although STAT3 has been shown to regulate the function of mitochondrial proteins by protein-protein interactions, the mechanism by which STAT3 does this is unclear. One possible mode of action is through facilitating the apposition of the different mitochondrial enzymes involved in a single metabolic pathway to enhance flux. For example, mitochondrial ETC complexes have the ability to interact with one another to form respiratory chain super-complexes (RCS), enhancing electron transfer down the ETC and reducing ROS[65]. IL-6 recruits STAT3 to the RCS and enhances RCS formation, suggesting that mitochondrial STAT3 might act to regulate the formation of this complex[66]. We found that IL-10 induced STAT3 to interact with multiple components of BCAA metabolism (IVD, BCAT2, ACAD8, ACADSB). It is possible that, analogous to RCS formation, IL-10-STAT3 might also facilitate the nucleation of these enzymes to form a BCAA enzyme complex. Future studies should thus look into the effect of IL-10 on the formation of this complex and BCAA metabolism.

Much of our understanding into the roles of mitochondrial STAT3 have been achieved through using various STAT3 mutants, such as those that have defective DNA binding, those fused to a mitochondrial targeting sequence thus forcing STAT3 to only exert mitochondrial functions, as well as phosphorylation defective mutants in the serine 727 and tyrosine 705 residues. Some of these STAT3 mutants result in notable phenotypes. For example, the *Stat3* ΔV463 mutation occurs in the DNA binding domain and, along with other mutations in the same domain, lead to the development of hyper-IgE syndrome (HIES) or Job's disease[67]. HIES patients develop various high serum IgE, recurrent staphylococcal abscesses, pulmonary infections and mucocutaneous candidiasis, due at least in part from defective Th17 responses which are important for bacterial and fungal clearance[68]. *Stat3*ΔV463 mice themselves display increased susceptibility to infection by *Citrobacter rodentium*[69]. However, these mice are viable and fertile unlike *Stat3*$^{-/-}$ mice which are embryonically lethal[70], indicating a possible role for non-transcriptional functions of STAT3[69].

We found that IL-10 was able to suppress several LPS-induced proinflammatory genes in *Stat3*ΔV463 but not STAT3 deficient macrophages, suggesting that non-transcriptional functions of STAT3 are sufficient at least in part to mediate IL-10 mediated immune suppression. In the initial characterization of HIES patients, IL-10 mediated inhibition of LPS induced TNFα secretion was defective, which led to the conclusion that IL-10 signaling was defective[67]. While we also observed a lesser degree of inhibition of *Tnf* in *Stat3*ΔV463 macrophages, IL-10-mediated suppression of *Nos2* and *Il12b* were intact, suggesting that mitochondrial STAT3 might regulate only part of the IL-10 mediated anti-inflammatory response. It will thus be important to comprehensively profile the degree of IL-10 mediated suppression in *Stat3*ΔV463 mice and HIES patients, to assess which aspects of IL-10R signaling are mediated by non-transcriptional functions. It will also be interesting to compare these patients with STAT3 gain of function patients[71] to see if there are discrepancies in their presentations that might indicate non-canonical STAT3 function.

It will also be interesting to evaluate the mitochondrial functions of STAT3 *in vivo*. As *Stat3*$^{-/-}$ mice are embryonically lethal[70], it is not possible to directly compare *Stat3*ΔV463 mice with *Stat3*$^{-/-}$ mice. One solution would be to generate chimeras using bone marrow (BM) from WT, *Stat3*ΔV463 or *Tie2*^{Cre}*Stat3*$^{fl/fl}$ mice. *Tie2*^{Cre}*Stat3*$^{fl/fl}$ mice harbor a deletion of *Stat3* in the BM and endothelial compartments[72]. Colitis can be induced in these models to assess if non-transcriptional STAT3 can ameliorate the disease phenotype.

One of the outstanding questions in the field concerns the residues and domains of STAT3 that are required for mitochondrial STAT3 function. While several studies have pointed to a role for Ser727 phosphorylation[73,74], this modification also enhances canonical STAT3 mediated transcription[75]. Moreover, Y705 phosphorylation, which is tied to canonical STAT3 function, has also been described in the mitochondria[66]. STAT3 does not have a clear mitochondrial localization sequence[58], leading to the possibility that STAT3-mediated translocation to the mitochondria might be facilitated by interactions with other proteins like GRIM-19 and TOM-20[76,77]. What are the residues that might mediate its translocation? One possible region is the N-terminus of STAT3, which has been shown to facilitate its interaction with cyclophilin D[78]. It will be interesting to use the STAT3Tg macrophages as a tool to answer this question, generating systematic deletions and mutations in STAT3 to assess which residues/domains impair IL-10 mediated suppression of e.g. *Il12b*. These mutations can then be followed up upon to assess which mutants impair mitochondrial translocation.

Finally, our study and others raise the question of whether, from a therapeutic perspective, different inhibitors of STAT3 might inhibit different aspects of STAT3 signaling. Many STAT3 inhibitors target the SH2 and DNA binding domains, but some have been developed that affect mitochondrial function such as phospho-valproic acid[79,80]. Further dissections of these effects might allow us to better tailor different drugs for different diseases, such as by using agonists of mitochondrial STAT3 function for reversing mitochondrial damage.

Chapter 4.6 – Mitochondrial functions of other STATs in relation to STAT3

In mammals, there are 7 members of the STAT family – STAT1, 2, 3, 4, 5a, 5b, 6 which have very similar structural domains[81]. The ability of STAT3 to translocate to non-nuclear locations like the mitochondria raises the question of whether other STATs might also exploit this function.

STAT1, together with STAT2, were the first identified STATs mediating IFN function[82]. In many cell types, STAT1 and STAT3 have opposing functions. For example, STAT1 activates cell death and blocks proliferation while STAT3 promotes proliferation and survival[83]. In immune cells, STAT1 signaling downstream of IFNs generate a pro-inflammatory response, but STAT3 signaling is critical for cytokines like IL-10 to exert its anti-inflammatory response[83,84]. The importance of STAT3 in preventing inflammation is evidenced in $LysM^{Cre}$ $Stat3^{fl/fl}$ mice which develop colitis[84]. STAT1 was also reported to be present in mouse and rat heart mitochondria[77,85]. In the murine setting, STAT1 was found to translocate to the mitochondria upon ischemia/reperfusion injury. $Stat1^{-/-}$ mice had increased mitochondria within double membrane structures indicating increased mitophagy. Moreover, STAT1 interacted with LC3b, together suggesting that mitochondrial STAT1 might inhibit mitophagy[85]. IL-10R-STAT3 signaling promotes mitophagy through the inhibition of mTOR[64], and one of our STAT3 candidate binding targets - PHB2 - is a regulator of mitophagy. It is thus possible that, just as nuclear STAT1 and STAT3 have opposing effects on gene transcription, mitochondrial STAT1 and STAT3 might have direct opposing effects on mitophagy.

The presence of STAT5 in the mitochondria was also documented in a cell line expressing constitutively active STAT5 as well as in cells stimulated with IL-2 or IL-3. Interestingly, STAT5 was able to interact with the PDH E2 complex, similar to our observations with IL-10-STAT3 and with earlier reports[62]. While the effect of STAT5 on PDH function was not studied, a characteristic feature of cancer cells is to undergo aerobic glycolysis metabolism and shift pyruvate away from the mitochondria and OXPHOS. Therefore, it is possible that STAT5 might inhibit PDH activity to promote aerobic glycolysis, in contrast to STAT3 which enhances PDH function[62]. It will be interesting to evaluate whether STAT5

and STAT3 have opposite effects on PDH function, analogous to their opposing effects on nuclear transcription of genes like *Il17* in T cells[86]. IL-3-STAT5 was also able to bind to a putative STAT5 binding site at the regulatory D-loop of mitochondrial DNA, but the consequence of this on mitochondrial transcription was not studied[87].

Mitochondrial translocation of STAT2 has been documented in the setting of viral infection, but the purpose of this translocation was for degradation at a degradation complex formed at the mitochondria. While STAT2 was present at a basal level in the mitochondria, the role of this STAT2 is unclear[88]. STAT6 was also reported to be in the mitochondria, but the functional significance of this was also not studied[89].

What are the implications of mitochondrial STAT signaling? Firstly, our study and others suggest that the current transcription-centric understanding of JAK-STAT signaling might be insufficient. Complementary approaches such as STAT localization studies and immunoprecipitations might be needed to better understand how this pathway is utilized by different cytokines in different cell types to generate specific responses. Secondly, they suggest that aberrations in both canonical and non-canonical STAT signaling might differentially contribute to different diseases. It will be interesting to revisit these diseases as well as current JAK-STAT focused therapies like jakinibs to assess whether certain aspects of non-canonical STAT signaling can better predict disease outcome or therapeutic response.

In summary, while there are only a handful of studies investigating the function of mitochondrial STAT3 signaling and even fewer on other STATs, it has become clear that STATs utilize multiple levels of complexity in different locations to achieve a particular output. Unraveling the rules governing the functions of mitochondrial STATs, as well as the relative contribution of mitochondrial vs nuclear STAT signaling to different cytokine receptors, will significantly aid efforts to modulate these proteins for various diseases.

Chapter 4.7 – References

1. Kovacic, B. *et al.* STAT1 acts as a tumor promoter for leukemia development. *Cancer Cell* **10**, 77–87 (2006).
2. Crouse, J. *et al.* Type I Interferons Protect T Cells against NK Cell Attack Mediated by the Activating Receptor NCR1. *Immunity* **40**, 961–973 (2014).
3. Xu, H. C. *et al.* Type I Interferon Protects Antiviral CD8+ T Cells from NK Cell Cytotoxicity. *Immunity* **40**, 949–960 (2014).
4. Meissner, T. B. *et al.* NLR family member NLRC5 is a transcriptional regulator of MHC class I genes. *Proc. Natl. Acad. Sci.* **107**, 13794–13799 (2010).
5. Kuenzel, S. *et al.* The Nucleotide-Binding Oligomerization Domain-Like Receptor NLRC5 Is Involved in IFN-Dependent Antiviral Immune Responses. *J. Immunol.* **184**, 1990–2000 (2010).
6. Staehli, F. *et al.* NLRC5 Deficiency Selectively Impairs MHC Class I- Dependent Lymphocyte Killing by Cytotoxic T Cells. *J. Immunol.* **188**, 3820–3828 (2012).
7. Tong, Y. *et al.* Enhanced TLR-induced NF-κB signaling and type I interferon responses in NLRC5 deficient mice. *Cell Res.* **22**, 822–835 (2012).
8. Ludigs, K. *et al.* NLRC5 shields T lymphocytes from NK-cell-mediated elimination under inflammatory conditions. *Nat. Commun.* **7**, (2016).
9. Ma, H. *et al.* Absence of Stat1 in donor CD4+ T cells promotes the expansion of Tregs and reduces graft-versus-host disease in mice. *J. Clin. Invest.* **121**, 2554–2569 (2011).
10. Villarino, A. V, Gallo, E. & Abbas, A. K. STAT1-activating cytokines limit Th17 responses through both T-bet-dependent and -independent mechanisms. *J Immunol* **185**, 6461–6471 (2010).
11. Meyer zu Horste, G. *et al.* Fas Promotes T Helper 17 Cell Differentiation and Inhibits T Helper 1 Cell Development by Binding and Sequestering Transcription Factor STAT1. *Immunity* **48**, 556-569.e7 (2018).
12. Le Saout, C. *et al.* Chronic Exposure to Type-I IFN under Lymphopenic Conditions Alters CD4 T Cell Homeostasis. *PLoS Pathog.* **10**, (2014).
13. Lee, C.-K., Smith, E., Gimeno, R., Gertner, R. & Levy, D. E. STAT1 Affects Lymphocyte Survival and Proliferation Partially Independent of Its Role Downstream of IFN-γ. *J. Immunol.* **164**, 1286–1292 (2000).
14. Le Saout, C. *et al.* IL-7–dependent STAT1 activation limits homeostatic CD4+ T cell expansion. *JCI Insight* **2**, 1–18 (2017).
15. Miklossy, G., Hilliard, T. S. & Turkson, J. Therapeutic modulators of STAT signalling for human diseases. *Nat. Rev. Drug Discov.* **12**, 611–29 (2013).
16. Frank, D. a, Mahajan, S. & Ritz, J. Fludarabine-induced immunosuppression is associated with inhibition of STAT1 signaling. *Nat. Med.* **5**, 444–447 (1999).
17. Zhou, X. X., Gao, P. J. & Sun, B. G. Pravastatin attenuates interferon-γ action via modulation of stat1 to prevent aortic atherosclerosis in apolipoprotein E-knockout mice. *Clin. Exp. Pharmacol. Physiol.* **36**, 373–379 (2009).

18. Bern, M. D. *et al.* Inducible down-regulation of MHC class I results in natural killer cell tolerance. *J. Exp. Med.* **216**, jem.20181076 (2018).

19. Karo, J. M., Schatz, D. G. & Sun, J. C. The RAG recombinase dictates functional heterogeneity and cellular fitness in natural killer cells. *Cell* **159**, 94–107 (2014).

20. Lu, L. *et al.* Regulation of Activated CD4+ T Cells by NK Cells via the Qa-1-NKG2A Inhibitory Pathway. *Immunity* **26**, 593–604 (2007).

21. Kärre, K., Ljunggren, H. G., Piontek, G. & Kiessling, R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* **319**, 675–678 (1986).

22. Chiang, E. Y., Henson, M. & Stroynowski, I. The Nonclassical Major Histocompatibility Complex Molecule Qa-2 Protects Tumor Cells from NK Cell- and Lymphokine-Activated Killer Cell-Mediated Cytolysis. *J. Immunol.* **168**, 2200–2211 (2002).

23. Waggoner, S. N., Cornberg, M., Selin, L. K. & Welsh, R. M. Natural killer cells act as rheostats modulating antiviral T cells. *Nature* **481**, 394–398 (2012).

24. Fort, M. M., Leach, M. W. & Rennick, D. M. A role for NK cells as regulators of CD4+ T cells in a transfer model of colitis. *J. Immunol.* **161**, 3256–3261 (1998).

25. Yamaji, O. *et al.* The Development of Colitogenic CD4+ T Cells Is Regulated by IL-7 in Collaboration with NK Cell Function in a Murine Model of Colitis. *J. Immunol.* **188**, 2524–2536 (2012).

26. Klose, C. S. N. & Artis, D. Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. *Nat. Immunol.* **17**, 765 (2016).

27. Hepworth, M. R. *et al.* Group 3 innate lymphoid cells mediate intestinal selection of commensal bacteria-specific CD4+ T cells. *Science (80-.).* **348**, 1031–1035 (2015).

28. Kieper, W. C. W. *et al.* Cutting Edge: Recent Immune Status Determines the Source of Antigens That Drive Homeostatic T Cell Expansion. *J. Immunol.* **174**, 3158–3163 (2005).

29. Tajima, M. *et al.* IL-6 – dependent spontaneous proliferation is required for the induction of colitogenic IL-17 – producing CD8 + T cells. *J. Exp. Med.* **205**, 1019–1027 (2008).

30. Feng, T., Wang, L., Schoeb, T. R., Elson, C. O. & Cong, Y. Microbiota innate stimulation is a prerequisite for T cell spontaneous proliferation and induction of experimental colitis. *J. Exp. Med.* **207**, 1321–1332 (2010).

31. Liu, Z., Yadav, P. K. & Chen, C. Potential role of NK cells in the pathogenesis of inflammatory bowel disease. *Journal of Biomedicine and Biotechnology* **2011**, (2011).

32. Takayama, T. *et al.* Imbalance of NKp44+NKp46- and NKp44 -NKp46+ natural killer cells in the intestinal mucosa of patients with Crohn's disease. *Gastroenterology* **139**, 882-892.e3 (2010).

33. Koreth, J. *et al.* Interleukin-2 and Regulatory T Cells in Graft-versus-Host Disease. *N. Engl. J. Med.* **365**, 2055–2066 (2011).

34. Goettel, J. A. *et al.* Low-Dose Interleukin-2 Ameliorates Colitis in a Preclinical Humanized Mouse Model. *Cell. Mol. Gastroenterol. Hepatol.* 4–6 (2019). doi:10.1016/j.jcmgh.2019.05.001

35. Hirakawa, M. *et al.* Low-dose IL-2 selectively activates subsets of CD4+ Tregs and NK cells. *JCI Insight* **1**, (2016).

36. Kobrinsky, N. *et al.* Phase I trial of low-dose interleukin 2 therapy in patients with Wiskott-Aldrich syndrome. *Clin. Immunol.* **179**, 47–53 (2017).

37. Ostanin, D. V *et al.* T cell transfer model of chronic colitis : concepts , considerations , and tricks of the trade. 135–146 (2009). doi:10.1152/ajpgi.90462.2008.

38. Shale, M., Schiering, C. & Powrie, F. CD4+ T-cell subsets in intestinal inflammation. *Immunol. Rev.* **252**, 164–182 (2013).

39. Abraham, C. & Cho, J. H. Inflammatory Bowel Disease. *N Engl J Med* **361**, 2066–78 (2009).

40. Min, B. *et al.* Neonates support lymphopenia-induced proliferation. *Immunity* **18**, 131–140 (2003).

41. Li, N. *et al.* Memory CD4+ T cells are generated in the human fetal intestine. *Nat. Immunol.* (2019). doi:10.1038/s41590-018-0294-9

42. Uhlig, H. H. *et al.* The diagnostic approach to monogenic very early onset inflammatory bowel disease. *Gastroenterology* **147**, 990-1007.e3 (2014).

43. Shouval, D. S. *et al.* Interleukin-10 receptor signaling in innate immune cells regulates mucosal immune tolerance and anti-inflammatory macrophage function. *Immunity* **40**, 706–19 (2014).

44. Zigmond, E., Bernshtein, B. & Friedlander, G. Macrophage-Restricted Interleukin-10 Receptor Deficiency , but Not IL-10 Deficiency , Causes Severe Spontaneous Colitis. *Immunity* **40**, 720–733 (2014).

45. Huber, S. *et al.* Th17 Cells Express Interleukin-10 Receptor and Are Controlled by Foxp3- and Foxp3+ Regulatory CD4+ T Cells in an Interleukin-10-Dependent Manner. *Immunity* **34**, 554–565 (2011).

46. Murai, M. *et al.* Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nat. Immunol.* **10**, 1178–84 (2009).

47. Chaudhry, A. *et al.* Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity* **34**, 566–78 (2011).

48. Mocellin, S. *et al.* IL-10 stimulatory effects on human NK cells explored by gene profile analysis. *Genes Immun.* **5**, 621–630 (2004).

49. Cai, G., Kastelein, R. A. & Hunter, C. A. IL-10 enhances NK cell proliferation, cytotoxicity and production of IFN-gamma when combined with IL-18. *Eur. J. Immunol.* **29**, 2658–65 (1999).

50. Orange, J. S. *et al.* Wiskott-Aldrich syndrome protein is required for NK cell cytotoxicity and colocalizes with actin to NK cell-activating immunologic synapses. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 11351–6 (2002).

51. Kearney, C. J. *et al.* DOCK8 Drives Src-Dependent NK Cell Effector Function. *J. Immunol.* **199**, 2118–2127 (2017).

52. Caraux, A. *et al.* Phospholipase C-g2 is essential for NK cell cytotoxicity and innate immunity to malignant and virally infected cells. *Blood* **107**, 994–1002 (2011).

53. Côte, M. *et al.* Munc18-2 deficiency causes familial hemophagocytic lymphohistiocytosis type 5 and impairs cytotoxic granule exocytosis in patient NK cells. *J. Clin. Invest.* **119**, 3765–3773 (2009).

54. Hutchins, A. P. *et al.* Distinct transcriptional regulatory modules underlie STAT3's cell type-independent and cell type-specific functions. *Nucleic Acids Res.* **41**, 2155–70 (2013).

55. Maritano, D. *et al.* The STAT3 isoforms α and β have unique and specific functions. *Nat. Immunol.* **5**, 401–409 (2004).

56. Delgoffe, G. M. & Vignali, D. A. A. STAT heterodimers in immunity A mixed message or a unique signal ? *JAK-STAT* **2**, e23060 (2013).

57. Avalle, L. & Poli, V. Nucleus, Mitochondrion, or Reticulum? STAT3 à La Carte. *Int. J. Mol. Sci.* **19**, 2820 (2018).

58. Meier, J. A. & Larner, A. C. Toward a new STATe: The role of STATs in mitochondrial function. *Semin. Immunol.* **26**, 20–28 (2014).

59. Avalle, L. *et al.* STAT3 localizes to the ER, acting as a gatekeeper for ER-mitochondrion Ca2+ fluxes and apoptotic responses. *Cell Death and Differentiation* (2018). doi:10.1038/s41418-018-0171-y

60. Bittremieux, M., Parys, J. B., Pinton, P. & Bultynck, G. ER functions of oncogenes and tumor suppressors: Modulators of intracellular Ca2+ signaling. *Biochim. Biophys. Acta - Mol. Cell Res.* **1863**, 1364–1378 (2016).

61. Silver, D. L., Naora, H., Liu, J., Cheng, W. & Montell, D. J. Activated Signal Transducer and Activator of Transcription (STAT) 3. *Cancer Res.* **64**, 3550–3558 (2004).

62. Xu, Y. S. *et al.* STAT3 Undergoes Acetylation-dependent Mitochondrial Translocation to Regulate Pyruvate Metabolism. *Sci. Rep.* **6**, 1–16 (2016).

63. Hutchins, A. P., Diez, D. & Miranda-Saavedra, D. The IL-10/STAT3-mediated anti-inflammatory response: Recent developments and future challenges. *Brief. Funct. Genomics* **12**, 489–498 (2013).

64. Ip, W. K. E., Hoshi, N., Shouval, D. S., Snapper, S. & Medzhitov, R. Anti-inflammatory effect of IL-10 mediated by metabolic reprogramming of macrophages. *Science (80-.).* **356**, 513–519 (2017).

65. Enríquez, J. A. Supramolecular Organization of Respiratory Complexes. *Annu. Rev. Physiol.* **78**, 533–561 (2016).

66. Yang, R. *et al.* Mitochondrial Ca^{2+} and membrane potential, an alternative pathway for Interleukin 6 to regulate CD4 cell effector function. *Elife* **4**, 1–22 (2015).

67. Minegishi, Y. *et al.* Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome. *Nature* **448**, 1058–62 (2007).

68. Milner, J. D. *et al.* Impaired TH17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature* **452**, 773–776 (2008).

69. Steward-Tharp, S. M. *et al.* A mouse model of HIES reveals pro- and anti-inflammatory functions of STAT3. *Blood* **123**, 2978–2987 (2014).

70. Takeda, K. *et al.* Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proc. Natl. Acad. Sci.* **94**, 3801–3804 (1997).

71. Milner, J. D. *et al.* Early-onset lymphoproliferation and autoimmunity caused by germline STAT3 gain-of-function mutations. *Blood* **125**, 591–600 (2015).

72. Welte, T. *et al.* STAT3 deletion during hematopoiesis causes Crohn's disease-like pathogenesis and lethality: A critical role of STAT3 in innate immunity. *Proc. Natl. Acad. Sci.* **100**, 1879–1884 (2003).

73. Wegrzyn, J. *et al.* Function of Mitochondrial Stat3 in Cellular Respiration. *Science* **323**, 793–798 (2009).

74. Gough, D. J. *et al.* Mitochondrial STAT3 supports Ras-dependent oncogenic transformation. *Science* **324**, 1713–6 (2009).

75. Wen, Z., Zhong, Z. & Darnell, J. E. Maximal activation of transcription by statl and stat3 requires both tyrosine and serine phosphorylation. *Cell* **82**, 241–250 (1995).

76. Tammineni, P. *et al.* The import of the transcription factor STAT3 into mitochondria depends on GRIM-19, a component of the electron transport chain. *J. Biol. Chem.* **288**, 4723–4732 (2013).

77. Boengler, K., Hilfiker-Kleiner, D., Heusch, G. & Schulz, R. Inhibition of permeability transition pore opening by mitochondrial STAT3 and its role in myocardial ischemia/reperfusion. *Basic Res. Cardiol.* **105**, 771–785 (2010).

78. Meier, J. A. *et al.* Stress-induced dynamic regulation of mitochondrial STAT3 and its association with cyclophilin D reduce mitochondrial ROS production. *Sci. Signal.* **10**, 1–15 (2017).

79. Yang, R. & Rincon, M. Mitochondrial Stat3, the Need for Design Thinking. *Int. J. Biol. Sci.* **12**, 532–544 (2016).

80. Vrankova, K. *et al.* Targeting Mitochondrial STAT3 with the Novel Phospho-Valproic Acid (MDC-1112) Inhibits Pancreatic Cancer Growth in Mice. *PLoS One* **8**, e61532 (2013).

81. Wang, Y. & Levy, D. E. Comparative evolutionary genomics of the STAT family of transcription factors. *Jak-Stat* **1**, 23–33 (2012).

82. Stark, G. R. & Darnell, J. E. J. The JAK-STAT Pathway at Twenty. *Immunity* **36**, 503–514 (2012).

83. González-Navajas, J. M., Lee, J., David, M. & Raz, E. Immunomodulatory functions of type i interferons. *Nat. Rev. Immunol.* **12**, 125–135 (2012).

84. Takeda, K. *et al.* Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of stat3 in macrophages and neutrophils. *Immunity* **10**, 39–49 (1999).

85. Bourke, L. T., Knight, R. A., Latchman, D. S., Stephanou, A. & McCormick, J. Signal transducer and activator of transcription-1 localizes to the mitochondria and modulates mitophagy. *JAK-STAT* **2**, e25666 (2013).

86. Laurence, A. *et al.* Interleukin-2 Signaling via STAT5 Constrains T Helper 17 Cell Generation. *Immunity* **26**, 371–381 (2007).

87. Chueh, F. Y., Leong, K. F. & Yu, C. L. Mitochondrial translocation of signal transducer and activator of transcription 5 (STAT5) in leukemic T cells and cytokine-stimulated cells. *Biochem. Biophys. Res. Commun.* **402**, 778–783 (2010).

88. Goswami, R. *et al.* Viral degradasome hijacks mitochondria to suppress innate immunity. *Cell Res.* **23**, 1025–1042 (2013).

89. Khan, R., Lee, J. E., Yang, Y. M., Liang, F. X. & Sehgal, P. B. Live-Cell Imaging of the Association of STAT6-GFP with Mitochondria. *PLoS One* **8**, 1–9 (2013).

Appendix

nature COMMUNICATIONS

ARTICLE

https://doi.org/10.1038/s41467-019-08743-8 OPEN

STAT1 signaling shields T cells from NK cell-mediated cytotoxicity

Yu Hui Kang[1,2], Amlan Biswas[1,2,3], Michael Field[1,2] & Scott B. Snapper[1,2]

The JAK-STAT pathway critically regulates T-cell differentiation, and STAT1 is postulated to regulate several immune-mediated diseases by inducing proinflammatory subsets. Here we show that STAT1 enables $CD4^+$ T-cell-mediated intestinal inflammation by protecting them from natural killer (NK) cell-mediated elimination. *Stat1*$^{-/-}$ T cells fail to expand and establish colitis in lymphopenic mice. This defect is not fully recapitulated by the combinatorial loss of type I and II IFN signaling. Mechanistically, *Stat1*$^{-/-}$ T cells have reduced expression of *Nlrc5* and multiple MHC class I molecules that serve to protect cells from NK cell-mediated killing. Consequently, the depletion of NK cells significantly rescues the survival and spontaneous proliferation of *Stat1*$^{-/-}$ T cells, and restores their ability to induce colitis in adoptive transfer mouse models. *Stat1*$^{-/-}$ mice however have normal $CD4^+$ T cell numbers as innate STAT1 signaling is required for their elimination. Overall, our findings reveal a critical perspective on JAK-STAT1 signaling that might apply to multiple inflammatory diseases.

[1] Division of Gastroenterology, Hepatology and Nutrition, Boston Children's Hospital, Boston, MA 02115, USA. [2] Harvard Medical School, Boston, MA 02115, USA. [3] Present address: Discovery Immunology, Abbvie, 200 Sidney Street, Cambridge, MA 02139, USA. Correspondence and requests for materials should be addressed to S.B.S. (email: Scott.Snapper@childrens.harvard.edu)

The JAK-STAT signaling pathway plays a critical role in transducing signals from various cytokines to achieve distinct transcriptional outcomes[1]. In T cells, this pathway has been well studied in terms of their regulation of T-cell differentiation[2]. Among the seven mammalian signal transducer and activator of transcription (STAT) family members, STAT1 is known to be important for the induction of Th1 cells downstream of IFNγ due to its induction of the transcription factor T-bet[3,4]. STAT1 has also been shown to suppress regulatory T-cell differentiation[5]. These proinflammatory properties of STAT1 are important for controlling infections, where patients with loss-of-function mutations in *Stat1* develop susceptibility to viral/mycobacterial infections[6]. They are also important for promoting inflammatory diseases like graft-vs-host-disease (GvHD)[5]. However, STAT1 also suppresses Th17 differentiation[7], and $Stat1^{-/-}$ mice develop aggravated Th17-mediated autoimmune diseases including experimental autoimmune encephalomyelitis (EAE)[8,9].

Inflammatory bowel diseases (IBD) likely arise from an aberrant immune response toward intestinal microbes in a genetically susceptible host[10]. Crohn's disease in particular is characterized by a skewing of the $CD4^+$ T cell profile toward the proinflammatory Th1 and Th17 subsets, which are believed to be critical for disease pathogenesis[11]. Patients with Crohn's disease display higher STAT1 expression, albeit only modestly in $CD4^+$ T cells[12]. However, the mechanism by which STAT1 modulates $CD4^+$ T cells in IBD is currently unclear and presumed to be through altering differentiation states[13].

IL-10 is a critical anti-inflammatory cytokine for maintaining intestinal immune homeostasis, as evidenced in mice and humans deficient in IL-10 or IL-10 receptor (IL-10R) that develop spontaneous colitis[14–16]. We and others recently described the importance of IL-10R signaling in macrophages in the prevention of colitis, with $Il10rb^{-/-}Rag1^{-/-}$ mice but not $Rag1^{-/-}$ mice developing colitis upon reconstitution with WT $CD4^+$ T cells[17,18]. Subsequent studies in our model and others pointed to a role for pathogenic Th17 cells in driving the disease[19–24]. As STAT1 is a critical regulator of Th1/Th17 differentiation, we further investigated its role in the ability of $CD4^+$ T cells to induce colitis.

Here we describe a role for STAT1 in enabling T cells to induce colitis by protecting them from NK cell-mediated cytotoxicity. $Stat1^{-/-}$ T cells fail to expand and induce colitis in vivo unless NK cells are depleted. This is because STAT1 is required to induce sufficient levels of *Nlrc5* and the inhibitory NK ligand MHC class I to enable evasion of rejection by host NK cells. Surprisingly, this requirement for STAT1 is largely independent of both Type I and II IFN signaling, the classical activators of STAT1. Moreover, this mechanism is specific to $Stat1^{-/-}$ T cells undergoing spontaneous proliferation and requires STAT1 expression in the innate compartment. Altogether, our study reveals a critical role of STAT1 that is distinct from T-cell differentiation and adds a new perspective to studies on T-cell-mediated inflammatory disease.

Results

T cells require STAT1 to expand and induce colitis in vivo. To investigate the role of STAT1 signaling in T-cell driven colitis, we adoptively transferred unfractionated WT or $Stat1^{-/-}$ $CD4^+$ T cells into $Il10rb^{-/-}Rag1^{-/-}$ mice (Fig. 1a). WT T cells induced severe colitis in $Il10rb^{-/-}Rag1^{-/-}$ recipient mice as expected[17]. In contrast, mice transferred with $Stat1^{-/-}$ T cells displayed no signs of intestinal inflammation as evidenced by the lack of weight loss, colonic thickening and histological inflammation (Fig. 1a, b). Flow cytometric analysis of the colonic lamina propria revealed a marked reduction of $Stat1^{-/-}$ T cells compared to WT T cells (Fig. 1c). This was not due to aberrant homing of $Stat1^{-/-}$ T cells to the intestine, as a similar reduction of T cells was observed in the spleen (Fig. 1d).

We next asked if the reduction of $Stat1^{-/-}$ T cells was dependent on colonic inflammation by transferring unfractionated WT or $Stat1^{-/-}$ $CD4^+$ T cells into $Rag1^{-/-}$ mice, a strain that does not develop colitis when reconstituted with unfractionated WT T cells[17]. Similar to $Il10rb^{-/-}Rag1^{-/-}$ mice, $Stat1^{-/-}$ T cells were markedly reduced in the colons and spleens of $Rag1^{-/-}$ mice, indicating that STAT1 is required for robust in vivo T-cell expansion independent of colonic inflammation and innate IL-10R expression (Fig. 2a, b).

Partial dependency of STAT1 on Type I + II IFN signaling. IFNs are the classical inducers of STAT1 signaling with both Type I and Type II IFN individually reported to regulate T cell function[3,25–27]. We therefore sought to determine if the impaired expansion of $Stat1^{-/-}$ T cells was due to the lack of both type I and type II IFN signaling by transferring $Ifnar1^{-/-}Ifngr1^{-/-}$ $CD4^+$ T cells into $Il10rb^{-/-}Rag1^{-/-}$ mice (Fig. 3a). Surprisingly, the abrogation of both Type I and Type II IFN receptors failed to recapitulate STAT1 deficiency, as $Ifnar1^{-/-}Ifngr1^{-/-}$ $CD4^+$ T cells expanded to similar levels as WT T cells in the spleen and colon 3 weeks post transfer (Fig. 3a).

$Ifnar1^{-/-}Ifngr1^{-/-}$ T cells were also able to induce colitis unlike $Stat1^{-/-}$ T cells (Fig. 3b, c). However, the severity of colitis induced by $Ifnar1^{-/-}Ifngr1^{-/-}$ T cells was reduced compared to WT T cells (Fig. 3b, c), which correlated with a reduced rate of expansion of $Ifnar1^{-/-}Ifngr1^{-/-}$ T cells in the blood (Fig. 3d). As expected, $Stat1^{-/-}$ T cells did not expand in the blood (Fig. 3d). These data suggest that while Type I+II IFN partially contribute to the STAT1-dependent signaling, the impaired expansion of $Stat1^{-/-}$ T cells is predominantly an IFN-independent process at later time points.

Cell-intrinsic role for STAT1 in in vivo T-cell expansion. To understand the mechanisms linking STAT1 to T-cell expansion in vivo, we first asked if the presence of WT T cells could rescue the defective expansion of $Stat1^{-/-}$ T cells by transferring equal ratios of congenically marked WT ($CD45.1^+$) and $Stat1^{-/-}$ ($CD45.2^+$) T cells into $Rag1^{-/-}$ mice (Fig. 4a). Notably, all of the T cells identified three weeks post transfer were WT, indicating that the defective expansion of $Stat1^{-/-}$ T cells is cell-intrinsic (Fig. 4b).

We next asked if the defective expansion of $Stat1^{-/-}$ T cells could be recapitulated in vitro. In contrast with the in vivo defect, $Stat1^{-/-}$ T cells displayed a hyperproliferative phenotype compared to WT T cells upon in vitro stimulation (Supplementary Fig. 1), consistent with earlier reports[5,28]. This indicates that the expansion defect of $Stat1^{-/-}$ T cells is not cell autonomous and requires an in vivo environment.

Reduced expression of the MHC-I pathway in $Stat1^{-/-}$ T cells. To investigate whether a dysregulated transcriptional profile might account for the observed defect, we performed gene expression analysis on $Stat1^{-/-}$ T cells pre and post transfer into $Rag1^{-/-}$ mice by RNA-seq. As $Stat1^{-/-}$ T cells failed to expand to appreciable amounts after 3 weeks, we transferred a larger number of cells and analyzed gene expression at 1 week post transfer, a time point where $Stat1^{-/-}$ T cells were beginning to decline (Fig. 5a and Supplementary Fig. 2a). Gene ontology (GO) analysis of genes differentially regulated between WT and $Stat1^{-/-}$ T cells revealed, as expected, categories related to Type I and II IFN signaling. Interestingly, categories related to the MHC class I (MHC-I) antigen presentation pathway were significantly

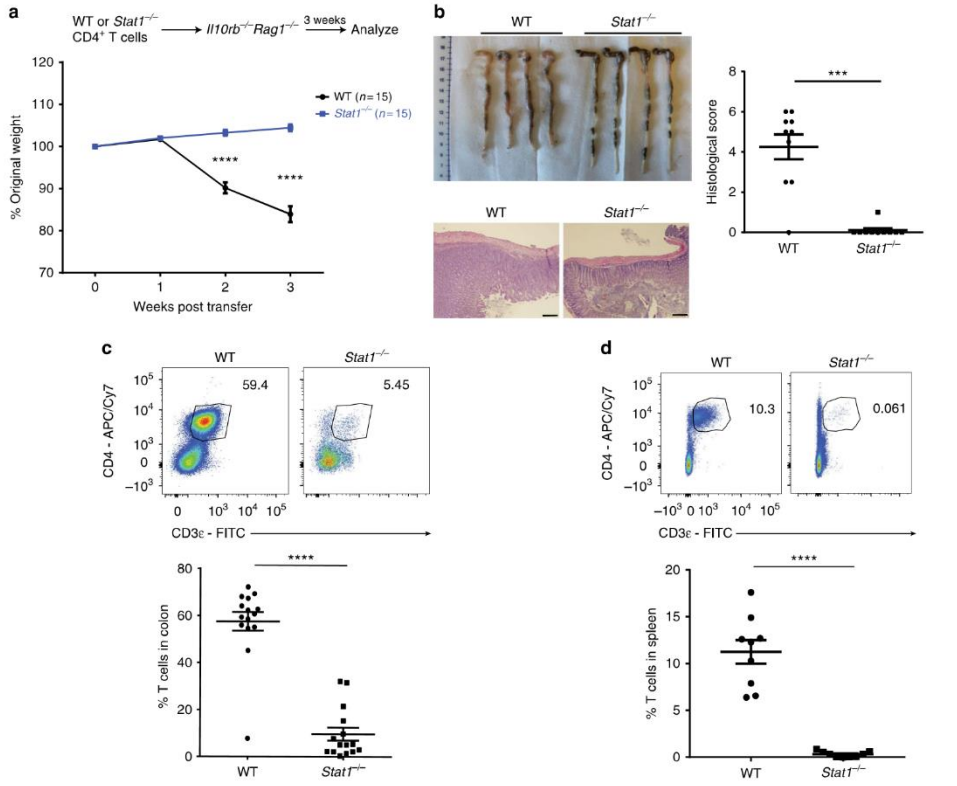


Fig. 1 *Stat1*$^{-/-}$ T cells fail to induce colitis due to defective expansion. *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice were injected i.p. with 1×10^6 unfractionated WT or *Stat1*$^{-/-}$ CD4$^+$ T cells. **a** Mean % original body weights ± SEM following T-cell transfer. Source data are provided as a Source Data file. **b** Representative images of colons, as well as representative H&E images of distal colon sections with mean histological scores ± SEM at 3 weeks post transfer. Scale bar represents 200 µm. **c**, **d** Representative flow cytometry plots of CD4$^+$ T cells (gated on live CD45$^+$ cells, Supplementary Fig. 4a) in the **c** colon and **d** spleen followed by their mean frequencies ± SEM at 3 weeks post transfer. All data are pooled from two to three independent experiments, with each point representing an individual mouse. $^{****}p < 0.0001$, $^{***}p < 0.001$ by **a** two-way ANOVA with Bonferroni's correction or **b**–**d** two-tailed Mann–Whitney test

enriched in both pre and post-transfer settings (Fig. 5a and Supplementary Fig. 2b). Consistent with the GO analysis, *Stat1*$^{-/-}$ T cells had reduced expression of *Nlrc5*, MHC-I (*H2-K1, H2-D1, B2m, H2-T23)* and various genes involved in MHC-I antigen presentation (*Tap1, Tap2, Psmb8, Psmb9)* (Fig. 5b and Supplementary Fig. 2c).

NLRC5 is a critical transactivator of multiple MHC-I genes, and STAT1 is required to induce its expression by binding to the *Nlrc5* promoter in response to IFNγ[29–32]. Consistent with our RNA-seq data and with earlier reports[31,33], *Stat1*$^{-/-}$ T cells displayed reduced surface levels of the classical MHC-I molecules H-2K^b/H-2D^b. Interestingly, surface levels of the non-classical molecule Qa-1 was only mildly affected by STAT1 deficiency whereas levels of Qa-2 were severely reduced (Fig. 5c).

NK depletion rescues *Stat1*$^{-/-}$ T-cell expansion and colitis. The MHC-I molecule is the classic inhibitory ligand for NK cells, and cellular expression of MHC-I protects cells from NK mediated killing[34]. Tumors or virally infected cells can reduce MHC-I expression to evade CD8$^+$ T-cell recognition, but this renders them susceptible to NK mediated killing—a phenomenon described as missing self[34,35]. The reduced expression of MHC-I on *Stat1*$^{-/-}$ T cells led us to hypothesize that their defective expansion was due to elimination by NK cells, which are present and more active in *Rag1*$^{-/-}$ mice[36]. This hypothesis was supported by the GO analysis, which revealed the category: Protection from natural killer mediated cytotoxicity (Fig. 5a and Supplementary Fig. 2b).

To test the hypothesis that *Stat1*$^{-/-}$ T cells were eliminated in vivo by NK cells, we depleted NK cells in *Rag1*$^{-/-}$ mice at the time of T-cell transfer by employing an anti-NK1.1 antibody (Fig. 6a and Supplementary Fig. 3a). In support of the hypothesis, the depletion of NK cells significantly rescued the survival of *Stat1*$^{-/-}$ T cells (Fig. 6a). We next asked if *Stat1*$^{-/-}$ T cells were able to induce colitis in the absence of NK cells by transferring

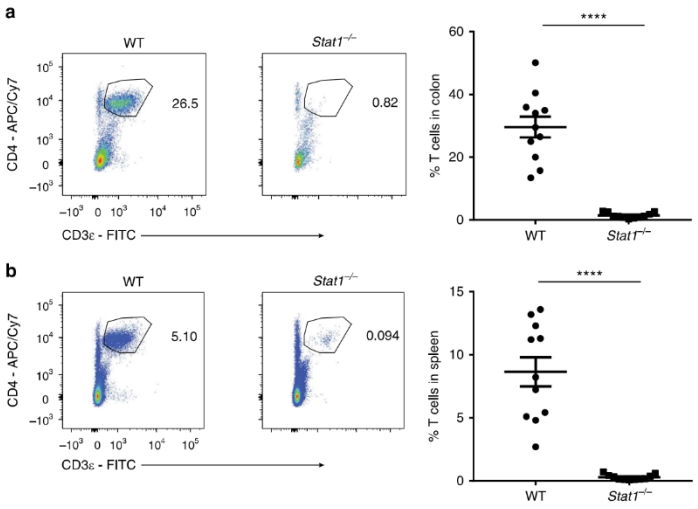


Fig. 2 *Stat1*$^{-/-}$ T cells fail to expand in *Rag1*$^{-/-}$ mice. *Rag1*$^{-/-}$ mice were injected i.p. with 1×10^6 WT or *Stat1*$^{-/-}$ unfractionated CD4$^+$ T cells and analyzed 3 weeks post transfer. **a**, **b** Representative flow cytometry plots of CD4$^+$ T cells (gated on live CD45$^+$ cells) in the colon (**a**) and spleen (**b**) followed by their mean frequencies ± SEM. Data are pooled from three independent experiments, with each point representing an individual mouse. ****$p <$ 0.0001 by two-tailed Mann–Whitney test

them into NK cell-depleted *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice. Strikingly, *Stat1*$^{-/-}$ T cells were able to induce disease in NK cell-depleted *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice unlike their control-treated NK cell-replete counterparts (Fig. 6b, c). The induction of disease correlated with a restored expansion of *Stat1*$^{-/-}$ T cells in the spleen and the colon (Fig. 6d). In agreement with previous reports[7], *Stat1*$^{-/-}$ T cells displayed an enhanced Th17 differentiation profile in vivo (Supplementary Fig. 3b). This differentiation profile was however seen in both control and NK cell-depleted *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice, suggesting that the primary role of STAT1 in T-cell-driven colitis is to protect the T cells from NK mediated elimination, rather than to repress their intrinsic Th17 differentiation potential.

NK cells target spontaneously proliferating *Stat1*$^{-/-}$ T cells. T cells undergo two distinct modes of proliferation upon transfer into chronically lymphopenic hosts (e.g., *Rag1*$^{-/-}$ mice)—slow, true homeostatic proliferation (HP) that is driven primarily by IL-7, as well as rapid, spontaneous proliferation (SP) that is driven by the microbiota and IL-6[37–41]. We asked whether the NK cell-mediated elimination of *Stat1*$^{-/-}$ T cells requires T-cell proliferation by transferring CellTrace Violet (CTV) labeled WT or *Stat1*$^{-/-}$ CD4$^+$ T cells into *Rag1*$^{-/-}$ mice. *Stat1*$^{-/-}$ T cells displayed a reduction in the SP population compared to WT T cells, with no difference in the HP population (Fig. 7a). Importantly, the depletion of NK cells significantly rescued the *Stat1*$^{-/-}$ SP population, suggesting that NK cells specifically restrict *Stat1*$^{-/-}$ T cells undergoing SP (Fig. 7b). Interestingly, this rescue was not complete as we also observed an increase in the SP of WT T cells upon NK cell depletion, which is consistent with the incomplete rescue of *Stat1*$^{-/-}$ T-cell expansion as well as the degree of colitis induced in *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice (Fig. 6).

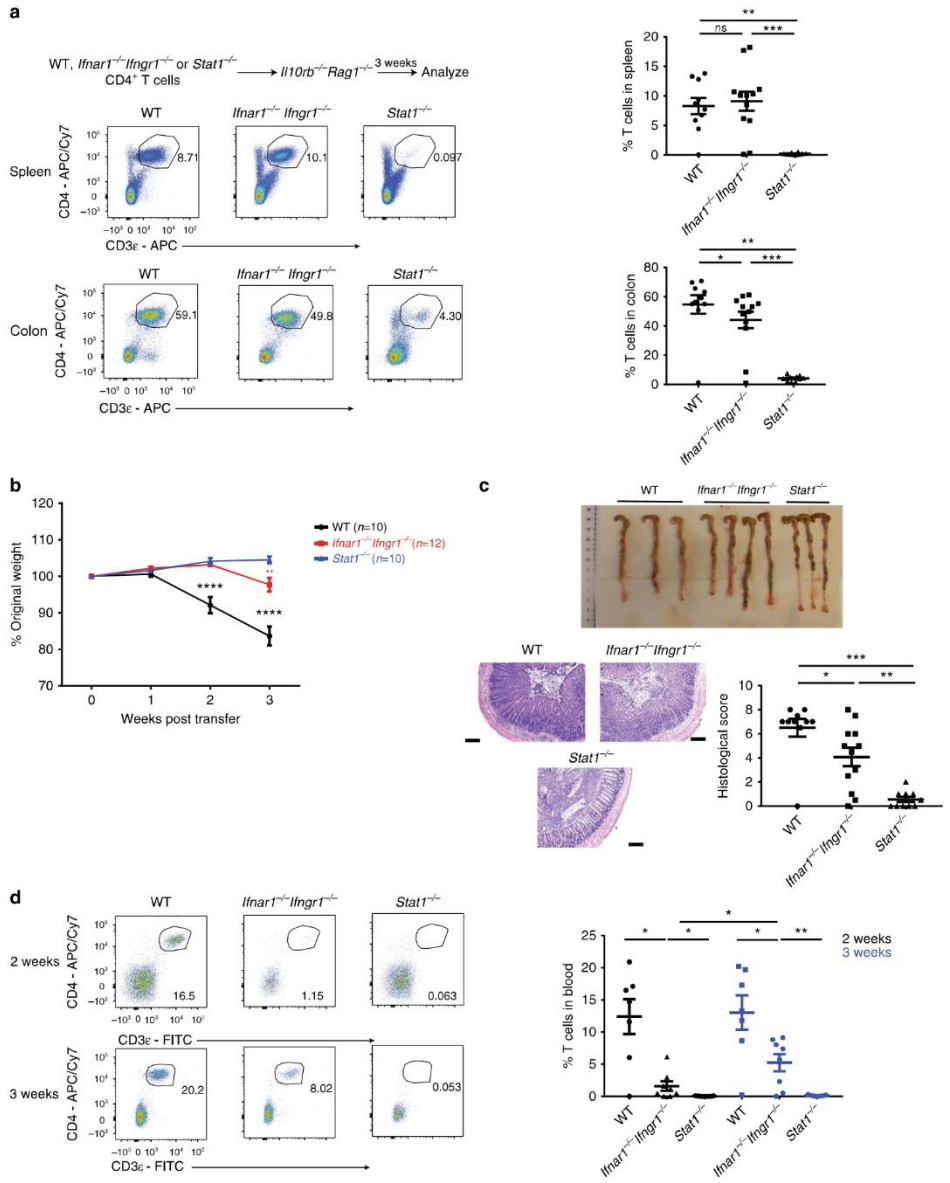
We also assessed for cell death in these populations by staining for activated caspases using FAM-FLICA, a fluorescently conjugated pan-caspase inhibitor. Compared to WT T cells, *Stat1*$^{-/-}$ T cells displayed increased cell death specifically in the SP population, with no differences in the HP or non-proliferating populations (Fig. 7c). Importantly, the increased cell death in *Stat1*$^{-/-}$ SP T cells was reversed by NK cell depletion (Fig. 7d). Taken together, these data strongly suggest that NK cells eliminate *Stat1*$^{-/-}$ T cells when they undergo SP.

Innate STAT1 expression is needed to reject *Stat1*$^{-/-}$ T cells. Despite the potent elimination of *Stat1*$^{-/-}$ T cells upon adoptive transfer into lymphopenic hosts, *Stat1*$^{-/-}$ mice had normal levels of CD4$^+$ T cells, suggesting additional mechanism(s) in place to prevent their elimination by NK cells (Fig. 8a). As STAT1 is required for NK cells to achieve optimal cytotoxicity[42,43], we hypothesized that these T cells were not eliminated in *Stat1*$^{-/-}$ mice due to a defect in killing by *Stat1*$^{-/-}$ NK cells. To test this hypothesis, we deleted *Stat1* in the innate compartment by generating *Stat1*$^{-/-}$*Rag1*$^{-/-}$ mice and transferred congenically marked WT (CD45.1$^+$) and *Stat1*$^{-/-}$ (CD45.2$^+$) T cells into them. Whereas *Stat1*$^{-/-}$ T cells were efficiently depleted in the *Stat1*$^{+/-}$ *Rag1*$^{-/-}$ littermate controls, deletion of *Stat1* in the innate compartment restored the expansion of *Stat1*$^{-/-}$ T cells (Fig. 8b). This indicates that the elimination of *Stat1*$^{-/-}$ T cells is dependent on innate STAT1 signaling.

Discussion

In this study we have identified a critical role for STAT1 in T-cell survival, where STAT1 signaling, through the upregulation of *Nlrc5* and MHC-I, protects T cells from NK cell-mediated elimination in vivo. We also show that this is important in the setting of T-cell-mediated immunopathology, as *Stat1*$^{-/-}$ T cells can induce colitis if allowed to survive and expand in a NK-deficient environment.

In T cells, most studies on the JAK-STAT pathway have focused on its effects on T-cell differentiation, with STAT1



promoting Th1 differentiation (through the induction of T-bet) and inhibiting Th17 differentiation[3,4,7]. In IBD, previous studies on STAT1 signaling in T cells focused on the STAT1-dependent transcription factor T-bet[13,23]. While it was noted that *Stat1*$^{-/-}$ T cells were unable to cause colitis, the profile of *Stat1*$^{-/-}$ T cells in vivo was not analyzed and STAT1 was assumed to act in a similar fashion as T-bet[13]. We observe that in our model of colitis[17], STAT1 modulates the disease outcome primarily by promoting T-cell survival rather than altering differentiation, as *Stat1*$^{-/-}$ T cells displayed similar

Fig. 3 Type I + II IFN signaling do not explain the defective expansion of *Stat1*$^{-/-}$ T cells. *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice were injected i.p. with 1×10^6 WT, *Ifnar1*$^{-/-}$*Ifngr1*$^{-/-}$ or *Stat1*$^{-/-}$ CD4$^+$ T cells. **a** Representative flow cytometry plots of CD4$^+$ T cells (gated on live CD45$^+$ cells, Supplementary Fig. 4b) in the spleen and colon followed by their mean frequencies ± SEM at 3 weeks post transfer. **b** Mean % initial body weights ± SEM following T-cell transfer. Source data are provided as a Source Data file. **c** Representative images of colons, as well as representative H&E images of distal colon sections with mean histological scores ± SEM at 3 weeks post transfer. Scale bar represents 200 µm. **d** Representative flow cytometry plots of CD4$^+$ T cells (gated on CD45$^+$ cells) in the blood followed by their mean frequencies ± SEM at 2 and 3 weeks post transfer. All data are pooled from two to three independent experiments, with each point representing an individual mouse. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ by **b** two-way ANOVA with Bonferroni's correction (WT compared to *Ifnar1*$^{-/-}$ *Ifngr1*$^{-/-}$ or *Stat1*$^{-/-}$, *Ifnar1*$^{-/-}$ *Ifngr1*$^{-/-}$ compared to *Stat1*$^{-/-}$) or by **a**, **c**, **d** two-tailed Mann–Whitney test

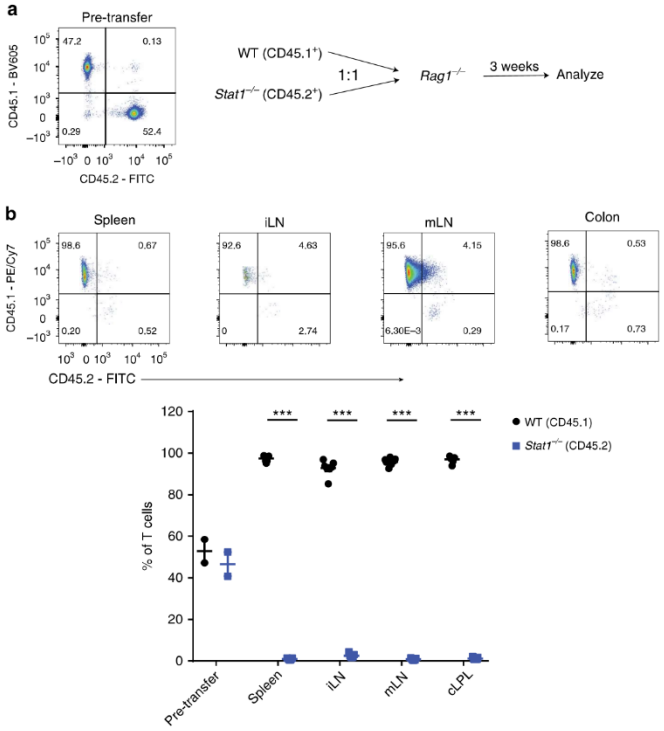


Fig. 4 The defective expansion of *Stat1*$^{-/-}$ T cells is cell-intrinsic. WT (CD45.1$^+$) or *Stat1*$^{-/-}$ (CD45.2$^+$) unfractionated CD4$^+$ T cells were injected i.p. at a 1:1 ratio ($0.8–1 \times 10^6$/type) into *Rag1*$^{-/-}$ mice. **a** Schematic of experiment and representative plot of cells injected. **b** Representative images of CD45.1$^+$ vs CD45.2$^+$ cells (gated on live CD45$^+$ CD3ε$^+$ CD4$^+$ T cells) from various organs followed by their mean frequencies ± SEM. Data is pooled from two independent experiments, with each point representing an individual mouse. ***$p < 0.001$ by two-tailed Mann–Whitney test. Accompanied by Supplementary Fig. 1

differentiation profiles in both control and NK-depleted *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ hosts (Supplementary Fig. 3b).

While we have focused mainly on IBD, we believe that the STAT1-mediated protection from NK cell killing is relevant for several T-cell-mediated inflammatory disorders. STAT1 induces MHC-I expression by inducing the transcription of *Nlrc5*[29–32], and deletion of *Nlrc5* in T cells to reduce MHC-I expression leads to their rejection by host NK cells during viral infection[44]. In the case of STAT1 deficiency, while several groups have reported a reduced expansion of *Stat1*$^{-/-}$ T cells in animal models of autoimmune disease and GvHD, the mechanistic basis for this reduction was unclear[5,9]. We propose that, similar to IBD, NK cells might also play a prominent role in restraining the ability of *Stat1*$^{-/-}$ T cells to induce these inflammatory disorders. This is analogous to studies in tumor biology, where tumor cells utilize STAT1 expression to avoid rejection in vivo[43]. Our finding that only *Stat1*$^{-/-}$ T cells undergoing SP are targeted by NK cells suggests that NK cells restrict T cells only when they are activated (Fig. 7). This is in agreement with earlier studies where *Ifnar1*$^{-/-}$ antiviral T cells are only eliminated by NK cells when the mice are virally infected[26,45] and suggests that a similar mechanism might be used to

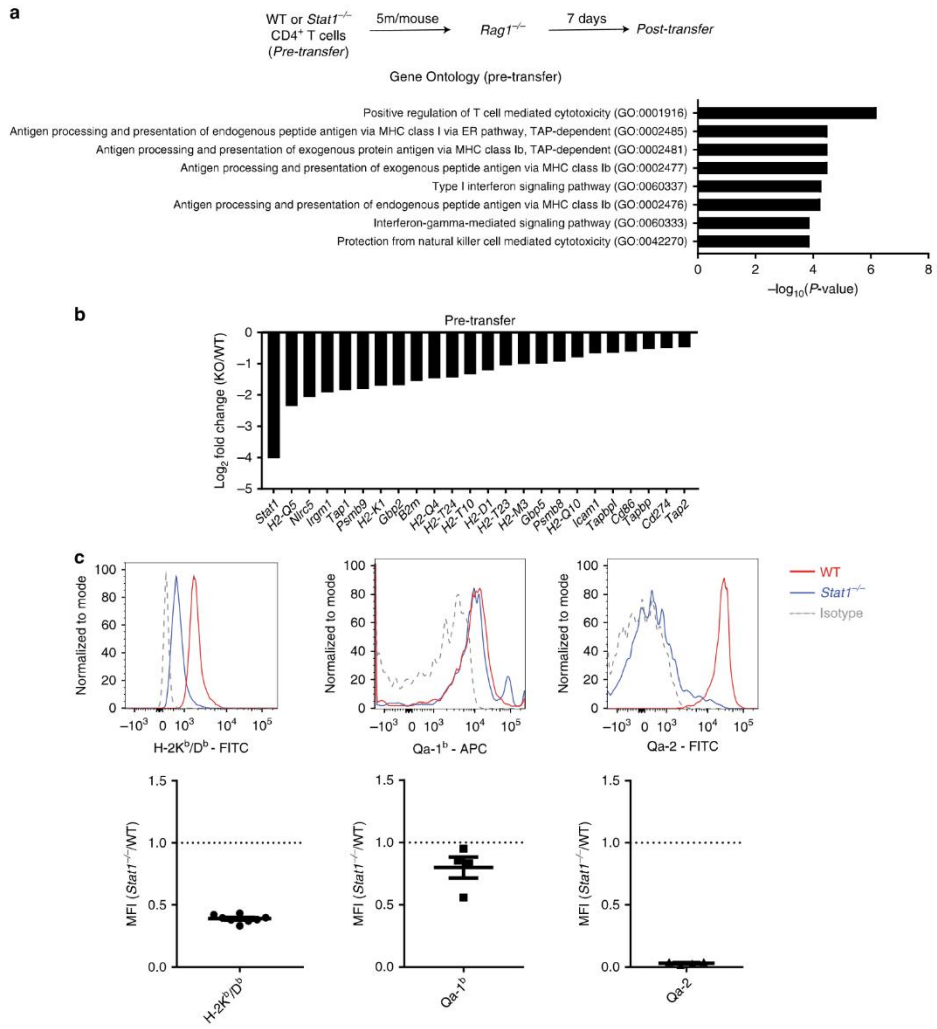
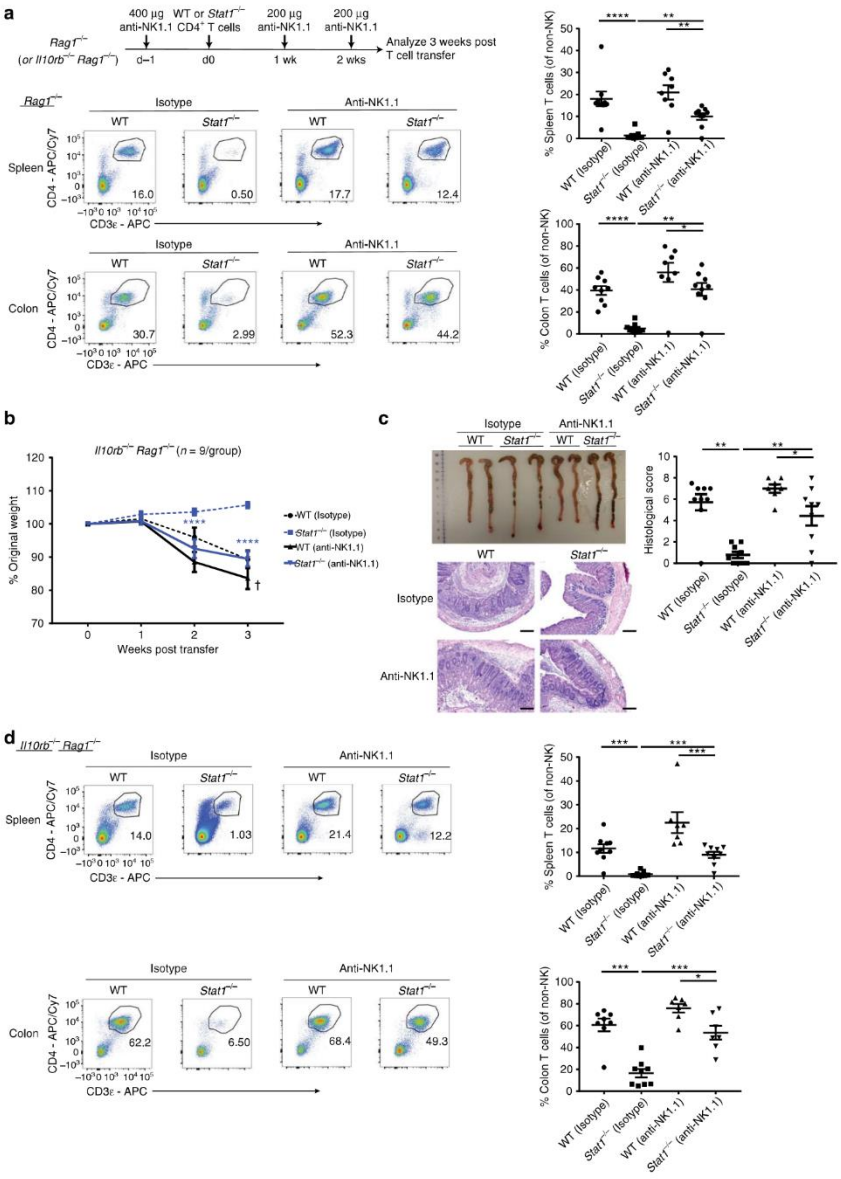


Fig. 5 Downregulation of the MHC class I antigen presentation machinery in *Stat1*$^{-/-}$ T cells. RNA-seq was performed on WT or *Stat1*$^{-/-}$ T cells pre-transfer. **a** Schematic of experimental setup and selected Gene ontology terms (PANTHER) showing differential expression of the MHC class I pathway in *Stat1*$^{-/-}$ T cells. **b** Downregulation of various genes involved in MHC class I antigen presentation in *Stat1*$^{-/-}$ T cells compared to WT T cells by RNA-seq. All genes displayed are significantly different between WT and *Stat1*$^{-/-}$ T cells ($n = 3$ biological replicates, $p < 0.05$ with correction for multiple testing by Benjamini-Hochberg procedure). Accompanied by Supplementary Fig. 2 where similar analyses were performed in T cells post transfer. **c** Representative flow cytometry plots showing surface expression of classical and non-classical MHC class I molecules on CD4+ T cells from the spleens of WT or *Stat1*$^{-/-}$ mice, followed by their cumulative enumeration expressed as a ratio of Median Fluorescence Intensity (*Stat1*$^{-/-}$ / WT) ± SEM. Data is pooled from three or more independent experiments, with each point representing an individual mouse. Similar numbers of WT and *Stat1*$^{-/-}$ mice were used for the comparison

regulate commensal-driven T-cell responses. Recently, it was reported that overexpression of STAT1 in T cells inhibits their expansion in lymphopenic mice, which led to the suggestion of targeting STAT1 to enhance T-cell numbers in clinical settings of lymphopenia like bone marrow transplantation and HIV infection[46]. Our findings suggest that this approach will have to be balanced with ensuring that there is sufficient MHC-I expression to protect T cells from being targeted by NK cells.



The upstream signal(s) that activates the STAT1-NLRC5-MHC class I axis in T cells in vivo has not been fully elucidated. In vitro, *Nlrc5* expression in T cells is primarily triggered by autocrine IFNγ signaling[31]. In vivo, type I IFN has been reported to protect antiviral T cells and NK cells from NK mediated elimination during LCMV infection[26,45,47]. Our data stands in contrast with these studies, showing that deletion of both Type I and Type II IFN receptors fails to fully recapitulate the defective survival of *Stat1*$^{-/-}$ T cells in the setting of IBD (Fig. 3). This is consistent with an earlier report showing that type I IFN signaling is not

Fig. 6 Depletion of NK cells restores *Stat1*$^{-/-}$ T-cell expansion and colitis. **a** 1 × 10^6 WT or *Stat1*$^{-/-}$ CD4$^+$ T cells were injected i.p. into *Rag1*$^{-/-}$ mice that were treated with NK depleting antibody (or isotype control). Schematic of experimental setup, as well as representative flow cytometry plots of CD4$^+$ T cells (gated on live CD45$^+$ non-NK cells, Supplementary Fig. 4c) in the spleen and colon of *Rag1*$^{-/-}$ mice with their mean frequencies ± SEM at 3 weeks post transfer. **b**–**d** Similar to **a**, but in *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice instead of *Rag1*$^{-/-}$ mice. **b** Mean % initial body weights ± SEM following T-cell transfer. Source data are provided as a Source Data file. **c** Representative images of colons, as well as representative H&E images of distal colon sections with mean histological scores ± SEM at 3 weeks post transfer. **d** Representative flow cytometry plots of CD4$^+$ T cells (gated on live CD45$^+$ non-NK cells, Supplementary Fig. 4c) in the spleen and colon followed by their mean frequencies ± SEM at 3 weeks post transfer. Scale bar represents 200 µm. Data pooled from three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ by **b** two-way ANOVA with Bonferroni's correction (*Stat1*$^{-/-}$ anti-NK1.1 compared to *Stat1*$^{-/-}$ Isotype) or by **a**, **c**, **d** two-tailed Mann–Whitney test. † Two mice (WT anti-NK1.1) were sacrificed before the 3-week time point due to excessive weight loss thus their weights only apply till week 2. Accompanied by Supplementary Fig. 3

required for naïve T cells to induce colitis in *Rag*$^{-/-}$ hosts[48], but we further extend this observation to include Type II IFN signaling. What are the IFN-independent signals that might account for the discrepancy between *Ifnar1*$^{-/-}$*Ifngr1*$^{-/-}$ and *Stat1*$^{-/-}$ T cells? IL-7 has been proposed as a possible candidate, being able to induce STAT1 activation in T cells in vitro and in vivo[33,46] as well as MHC-I in vitro[33]. Therefore, in addition to the conventional STAT5-driven proliferative and pro-survival response[49], IL-7 might activate STAT1 signaling to induce MHC-I for protection from NK cells. However, our finding that NK cells specifically eliminate *Stat1*$^{-/-}$ T cells undergoing SP and not the IL-7 driven HP argues against this hypothesis (Fig. 7). IL-6, which has been reported to be important in driving SP, also activates STAT1[40,41,50]. However, a recent report showed that IL-6R deficient T cells only display defective expansion in *Rag1*$^{-/-}$ mice when there is colonic inflammation[51], which is in contrast to our observations with *Stat1*$^{-/-}$ T cells (Fig. 2). It is possible that the STAT1-dependent signal is provided by multiple cytokines, including IL-6 and type I+II IFN. Alternatively, the maintenance of MHC-I levels might be driven by tonic STAT1 signaling that is independent of any upstream cytokine engagement.

The ability of NK cells to restrict T-cell expansion has been noted in mouse models of infection[52] and IBD[53,54], and our data and others[26,45] suggest that STAT1 signaling plays a critical role in this regulation. However, the extent of STAT1 dependency might be different in different disease contexts. While *Stat1*$^{-/-}$ T cells are very efficiently eliminated in our study and others[9], the degree of reduction in expansion of these T cells are not as pronounced in GvHD[5]. Moreover, in a recent report employing a mouse model of EAE, *Stat1*$^{-/-}$ T cells induced worse disease than WT T cells, although it is unclear whether they had an expansion defect[55]. It is thus possible that in certain disease contexts the inflammatory environment can provide STAT1-independent signals to the T cells to protect them from NK cells. Alternatively, the NK cells might be altered in these settings toward reduced cytotoxicity.

We observe that *Stat1*$^{-/-}$ T cells fail to expand in both *Rag1*$^{-/-}$ and *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice (Figs. 1, 2), suggesting that IL-10Rβ signaling is not required for NK cells to eliminate *Stat1*$^{-/-}$ T cells. However, earlier work has shown roles for IL-10 and IFNλ/IL-28—both of which signal via the IL-10Rβ chain—in stimulating NK cells[56–58]. It is possible that *Il10rb*$^{-/-}$ NK cells might still have sufficient cytotoxic ability to eliminate *Stat1*$^{-/-}$ T cells, as IL-28R deficient NK cells are only partially defective[58]. Alternatively, there might be other mechanisms in *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice that compensate for this defect, such as IL-10R deficiency in macrophages which promotes a proinflammatory environment[17] and/or RAG1 deficiency which has been shown to lead to NK cell hyperresponsiveness[36].

In our study, we also show that innate STAT1 signaling is required to eliminate *Stat1*$^{-/-}$ T cells, which would explain why *Stat1*$^{-/-}$ mice have normal levels of T cells (Fig. 8). We believe that this is likely due to the impaired cytotoxic capability of *Stat1*$^{-/-}$ NK cells as previously reported[42,43]. However, we do not exclude the possibility that this can also be due to altered NK education, where NK cells are educated to recognize the low level of MHC-I on *Stat1*$^{-/-}$ T cells as normal.

In summary, we describe a critical role for STAT1 in promoting T-cell survival by maintaining sufficient MHC class I expression to evade NK cell-mediated killing. This mechanism is largely IFN-independent and is critical in enabling T cells to induce intestinal inflammation. Our findings shed a new light on JAK-STAT signaling in T cells, adding critical functions for this pathway beyond T-cell differentiation that have potential therapeutic implications for IBD and other T-cell-mediated inflammatory disorders.

Methods

Mouse strains. C57BL/6J (Strain 000664), B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (CD45.1, Strain 002014), B6.129S7-*Rag1*^{tm1Mom}/J (*Rag1*$^{-/-}$, Strain 002216), B6.129S(Cg)-Stat1^{tm1Dlv} (*Stat1*$^{-/-}$, Strain 012606), B6.Cg-*Ifngr1*^{tm1Agt}*Ifnar1*$^{tm1.2Ees}$/J (*Ifnar1*$^{-/-}$*Ifngr1*$^{-/-}$, Strain 029098) mice were purchased from Jackson Labs. *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice were generated by crossing *Il10rb*$^{-/-}$ mice (a gift from Thaddeus Stappenbeck, Washington University) with *Rag1*$^{-/-}$ mice. *Stat1*$^{-/-}$*Rag1*$^{-/-}$ mice were generated by crossing *Stat1*$^{-/-}$ mice with *Rag1*$^{-/-}$ mice. All mice were on the B6 background and maintained in a specific pathogen-free animal facility in Boston Children's Hospital. All experiments were conducted after approval from the Animal Resources at Children's Hospital and according to regulations by the Institutional Animal Care and Use Committee (IACUC).

Adoptive T-cell transfer and colitis induction. In T-cell transfer experiments, unfractionated CD4$^+$ T cells were isolated from the spleens and lymph nodes of donor mice (WT, CD45.1, *Stat1*$^{-/-}$, *Ifnar1*$^{-/-}$*Ifngr1*$^{-/-}$) by negative selection (Miltenyi Biotec CD4$^+$ T-cell isolation Kit, Cat No. 130-104-454). 1 × 10^6 T cells (92.7–98.6% pure) were then adoptively transferred into recipient mice (*Rag1*$^{-/-}$, *Il10rb*$^{-/-}$*Rag1*$^{-/-}$) by i.p. injection in PBS unless otherwise stated. In some experiments, CD45.1$^+$ T cells and *Stat1*$^{-/-}$ T cells were mixed at a 1:1 ratio before being transferred into recipient mice (*Rag1*$^{-/-}$, *Stat1*$^{-/-}$*Rag1*$^{-/-}$). In some experiments, T cells were labeled with 5 µM CellTrace Violet (Thermo Fisher) in PBS + 0.1% FBS for 10 min at 37 °C prior to injection. All recipient mice were at least 6 weeks old and matched for sex, age, and housing between groups. *Il10rb*$^{-/-}$*Rag1*$^{-/-}$ mice were monitored weekly for body weight changes post T cell transfer. For NK depletion assays, each mouse was first injected with 400 µg anti-NK1.1 (or isotype control) 1 day prior to T-cell transfer. For experiments lasting beyond 1 week, depletion of NK cells was maintained by injections of 200 µg anti-NK1.1 (or isotype control) at 1 and 2 weeks post transfer. All antibody injections were administered i.p in InVivoPure pH 7.0 Dilution Buffer (BioXCell).

Histological scoring. To evaluate signs of histological inflammation, sections of distal colons were stained in haematoxylin and eosin and scored in a blinded fashion. Scoring was based on histological evidence of crypt hyperplasia (0–3), inflammatory cell infiltration (0–3) and presence of crypt abscesses (0–2), summed up to give the overall score (0–8). Representative images were acquired using an Olympus BX41 upright microscope with DP70 color CCD (Fig. 1) or a Keyence automated epifluorescent microscope (Figs. 3, 6).

Isolation of colonic lamina propria cells. Cells were isolated from the lamina propria as described[17]. Briefly, the large intestine (colon + cecum) was removed, cut open longitudinally and then into small sections before being incubated in Hank's balanced salt solution (HBSS) containing 0.5% fetal bovine serum (FBS), 10 mM EDTA, 1.5 mM dithiothreitol and 10 mM HEPES at 37 °C for 35 min with agitation to remove the epithelial cell layer. After the removal of the epithelial cells, tissues were washed in PBS, finely diced and incubated in HBSS buffer (w Ca/Mg)

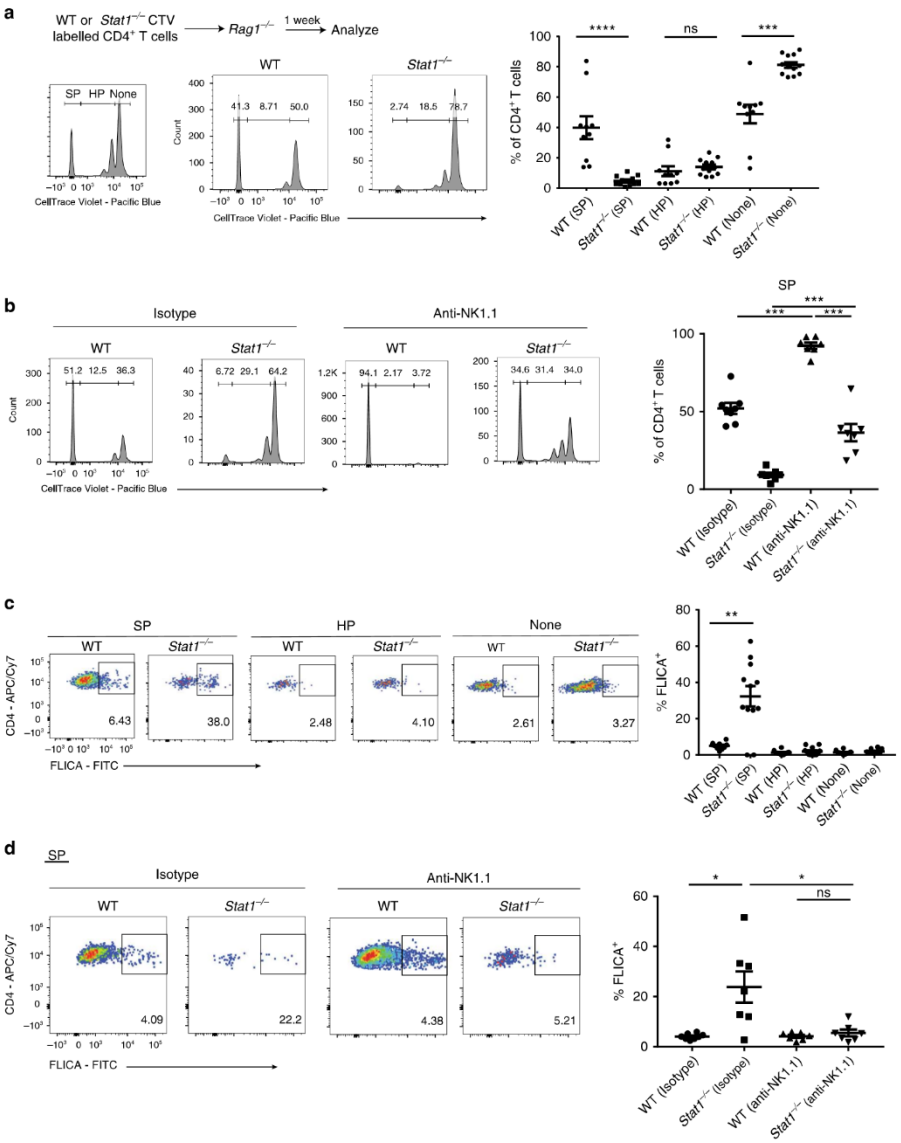


Fig. 7 NK cells specifically eliminate *Stat1*$^{-/-}$ T cells undergoing spontaneous proliferation. *Rag1*$^{-/-}$ mice were injected with equal numbers (3–4 × 10^6) of WT or *Stat1*$^{-/-}$ CTV labeled unfractionated CD4$^+$ T cells and analyzed after 1 week. **a** Schematic of experiment, as well as representative flow cytometry plots of T cells in the spleen + lymph nodes (gated on live CD45$^+$ CD3ε$^+$ CD4$^+$ cells, Supplementary Fig. 4d) followed by their mean frequencies ± SEM. **b**, **d** Similar to **a**, but with 400μg anti-NK1.1 antibody or Isotype Control injected 1 day prior to T-cell transfer. **b** CTV profiles of the T cells are shown as well as the mean frequencies ± SEM of the SP population. **c** Representative images of FLICA staining from the T cell SP, HP and non-proliferating populations in **a**, as well as their mean frequencies ± SEM. **d** FLICA staining in the SP population from **b** shown with mean frequencies ± SEM shown. Pooled from three to four independent experiments, with each point representing an individual mouse. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ***$p < 0.0001$ by two-tailed Mann–Whitney test

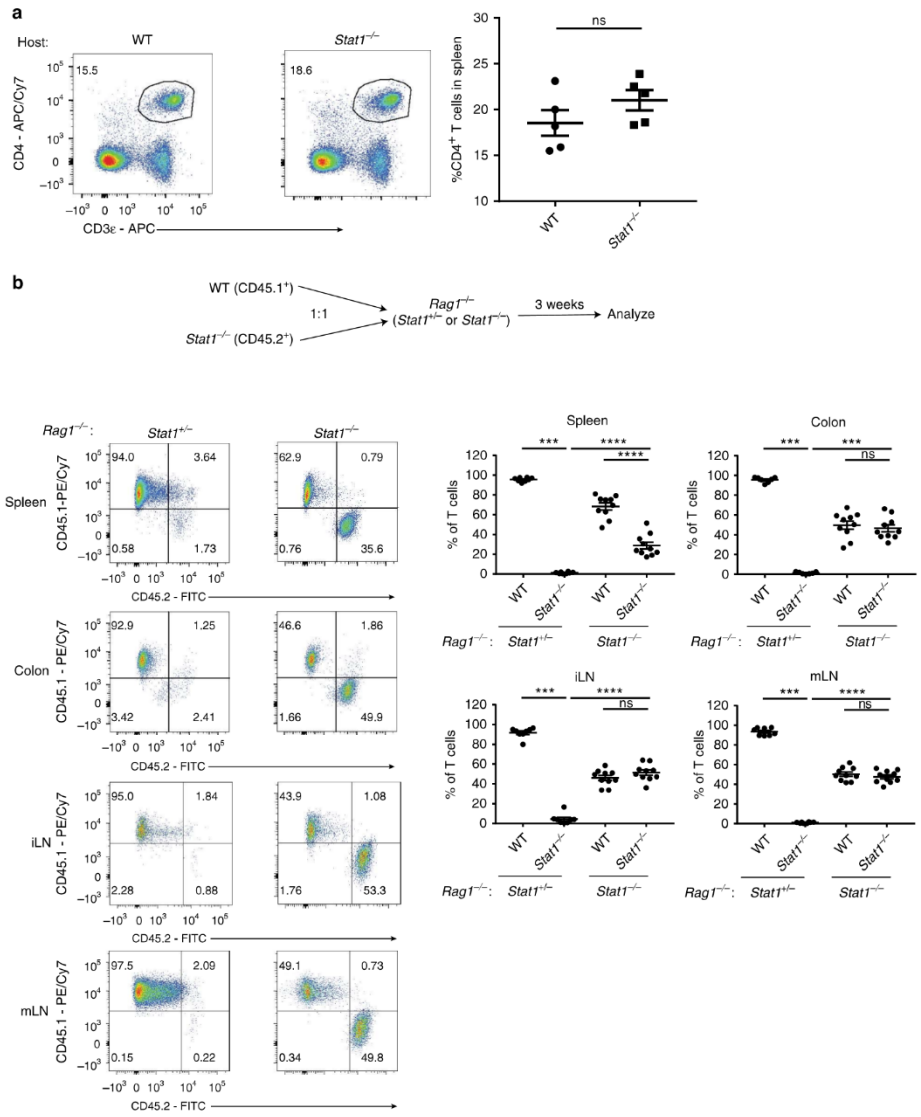


Fig. 8 Innate STAT1 expression is required to eliminate *Stat1*$^{-/-}$ T cells. **a** Representative flow cytometry plots of CD4$^+$ T cells in the spleen of WT and *Stat1*$^{-/-}$ mice followed by their mean frequencies ± SEM. **b** WT (CD45.1$^+$) or *Stat1*$^{-/-}$ (CD45.2$^+$) CD4$^+$ T cells were injected i.p. at a 1:1 ratio (1×10^6/type) into *Stat1*$^{-/-}$*Rag1*$^{-/-}$ mice or their *Stat1*$^{+/-}$*Rag1*$^{-/-}$ littermate controls and analyzed after 3 weeks. Representative images of CD45.1$^+$ vs CD45.2$^+$ cells (gated on live CD45$^+$ CD3ε$^+$ CD4$^+$ T cells) from various organs are shown followed by their mean frequencies ± SEM. Pooled from three independent experiments, with each point representing an individual mouse. ***$p < 0.001$, ****$p < 0.0001$ by two-tailed Mann–Whitney test

containing 20% FBS, 10 mM HEPES, 1.5 mM $CaCl_2$ and collagenase VIII (200 U/ml) at 37 °C for 40 min with agitation. Tissues were then repeatedly flushed through a 10 ml syringe and further incubated for 15 min. Digested tissues were filtered, washed in PBS and used for flow cytometry.

In vitro T-cell proliferation. Unfractionated $CD4^+$ T cells were first labeled with 5 µM CFSE for 5 min at room temperature and washed repeatedly with PBS containing FBS. They were then cultured in 96-well flat-bottom plates containing plate-bound anti-CD3ε (3 µg/ml, eBioscience) and soluble anti-CD28 (1 µg/ml, eBioscience) for 3 days. T cells were cultured in DMEM containing 10% FBS, L-glutamine, pyruvate, non-essential amino acids, MEM vitamins, L-arginine, L-asparagine, folic acid, β-mercaptoethanol and pen/strep.

Reagents. For flow cytometric staining, antibodies against the following were used (Clone name, dilution, manufacturer and catalog number in brackets): CD3ε (145-2C11, 1:300–400, Biolegend #100312/100306), TCRβ (H57-597, 1:400, Biolegend #109222), CD4 (GK1.5, 1:300 Biolegend #100414), NKp46 (29A1.4, 1:50, Biolegend #137604), CD49b (HMα2, 1:200 Biolegend #103517), CD45 (30-F11, 1:500, Biolegend #103140), H2-K^b/D^b (28-8-6, 1:100, Biolegend #114606/114607), Qa-2 (695H1-9-9, 1:100, Biolegend #121709), Qa-1b (6A8.6F10.1A6, 1:10, Miltenyi Biotec #130-104-220), CD16/32 (93, 0.5 µg/10^6 cells, Biolegend #101302), IL-17A (TC11-18H10.1, 1:125, Biolegend 506904), IFNγ (XMG1.2, 1:200, Biolegend #505809/eBioscience #17-7311-82), Mouse IgG2a, κ Isotype Ctrl (MOPC-173, 1:100, Biolegend #400207/400211), Mouse IgG1, κ Isotype Ctrl (MOPC-21, 1:66.7, Biolegend #400119), CD45.1 (A20, 1:300, Biolegend #110729), CD45.2 (104, 1:300, Biolegend #109806). For T-cell stimulation, antibodies against CD3ε (145-2C11, 3 µg/ml, eBioscience #16-0031-86) and CD28 (37.51, 1 µg/ml, eBioscience #16-0281-85) were used. For NK depletion assays, antibodies against NK1.1 (PK136, BioXCell #BP0036) or Isotype Control (C1.18.4, BioXCell #BP0085) were used.

Flow cytometry. For flow cytometry and sorting experiments, cells were stained in flow cytometric staining buffer (2% FBS plus 0.1% NaN_3 in PBS) and MACS buffer (0.5% BSA and 2 mM EDTA in PBS), respectively. For antibody staining of surface markers, cells were incubated with anti-CD16/32 (Biolegend) for 10 min at room temperature to block Fc receptors, before being incubated with antibodies for 20–30 min at 4 °C. Cells were also incubated with Zombie Violet Fixable Viability Dye (1:400, Biolegend) or 7-AAD (1:20, BD Biosciences) according to the manufacturer's instructions to identify and exclude dead cells. For intracellular cytokine staining, cells were incubated with PMA (50 ng/ml), ionomycin (500 ng/ml), and GolgiStop (1:1000, BD Biosciences) for 4 h at 37 °C. After staining for surface markers, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), followed by staining in Perm/Wash buffer (BD Biosciences) according to the manufacturer's instructions. For assessment of cell death, cells were stained with the FAM-FLICA Poly Caspase Kit (ImmunoChemistry Technologies) for 1 h at 37 °C in T-cell media prior to antibody surface staining. All samples were acquired with a BD Canto II or LSRFortessa Flow Cytometer (BD Biosciences) and analyzed with FlowJo (FlowJo, LLC).

RNA sequencing. In the post-transfer setting, WT or *Stat1*$^{-/-}$ T cells (gated as $CD45^+$ $CD3\varepsilon^+$ $CD4^+$, Supplementary Fig. 2d) were FACS sorted from the spleen and lymph nodes of *Rag1*$^{-/-}$ mice post transfer directly into RLT lysis buffer (Qiagen) and RNA extracted using the RNeasy Micro kit (Qiagen). As *Stat1*$^{-/-}$ T cells showed reduced survival/expansion in vivo, it was not technically feasible to acquire sufficient cells for purity analysis by flow cytometry, hence purity was determined by confirming the downregulation of *Stat1* in the *Stat1*$^{-/-}$ T cells. Library preparation, RNA-seq and analysis were performed at the Molecular Biology Core Facility (MBCF) of Dana-Farber Cancer Institute, Boston, using the Clontech SMARTer v4 kit for mRNA library generation and the Illumina NextSeq 500 Platform (Single-end 75 bp) for sequencing. The data was analyzed using the VIPER algorithm[39], with reads aligned to the mouse mm9 genome using STAR, transcripts assembled with Cufflinks and differential analysis performed with DESeq2. Gene Ontology analysis was performed using the PANTHER Over-representation test (http://www.geneontology.org/). The raw and processed data for RNA sequencing are deposited in the NCBI GEO database under GSE116475.

Statistical analysis. Statistical analyses were performed with GraphPad Prism software using two-way ANOVA with Bonferroni's multiple comparisons test, two-tailed Mann–Whitney test or two-tailed *t*-test as indicated in the figure legends. Significance was defined as *p*-value < 0.05 using the following notations: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data in this study are available from the corresponding author upon reasonable request. RNAseq data has been deposited in the GEO under GSE116475. A reporting summary for this Article is available as a Supplementary Information file, as well as a Source Data file with the source data underlying the weight curves in Figs. 1a, 3b, and 6b where individual data points are not displayed.

Received: 16 August 2018 Accepted: 14 January 2019
Published online: 22 February 2019

References

1. O'Shea, J. J. & Murray, P. J. Cytokine signaling modules in inflammatory responses. *Immunity* **28**, 477–487 (2008).
2. Delgoffe, G. M. & Vignali, D. A. A. STAT heterodimers in immunity A mixed message or a unique signal? *JAK-STAT* **2**, e23060 (2013).
3. Afkarian, M. et al. T-bet is a STAT1-induced regulator for IL-12R expression in naïve CD4+ T cells. *Nat. Immunol.* **3**, 549–557 (2002).
4. Szabo, S. J. et al. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* **100**, 655–669 (2000).
5. Ma, H. et al. Absence of Stat1 in donor CD4+ T cells promotes the expansion of Tregs and reduces graft-versus-host disease in mice. *J. Clin. Invest.* **121**, 2554–2569 (2011).
6. O'Shea, J. J., Holland, S. M. & Staudt, L. M. JAKs and STATs in immunity, immunodeficiency, and cancer. *N. Engl. J. Med.* **368**, 161–170 (2013).
7. Harrington, L. E. et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* **6**, 1123–1132 (2005).
8. Bettelli, E. et al. Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. *J. Exp. Med.* **200**, 79–87 (2004).
9. Villarino, A. V., Gallo, E. & Abbas, A. K. STAT1-activating cytokines limit Th17 responses through both T-bet-dependent and -independent mechanisms. *J. Immunol.* **185**, 6461–6471 (2010).
10. Neurath, M. F. Cytokines in inflammatory bowel disease. *Nat. Rev. Immunol.* **14**, 329–342 (2014).
11. Abraham, C. & Cho, J. H. Inflammatory bowel disease. *N. Engl. J. Med.* **361**, 2066–2078 (2009).
12. Mudter, J. et al. Activation pattern of signal transducers and activators of transcription (STAT) factors in inflammatory bowel diseases. *Am. J. Gastroenterol.* **100**, 64–72 (2005).
13. Neurath, M. F. et al. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. *J. Exp. Med.* **195**, 1129–1143 (2002).
14. Kühn, R., Löhler, J., Rennick, D., Rajewsky, K. & Müller, W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* **75**, 263–274 (1993).
15. Spencer, S. D. et al. The orphan receptor CRF2-4 is an essential subunit of the interleukin 10 receptor. *J. Exp. Med.* **187**, 571–578 (1998).
16. Glocker, E.-O., Kotlarz, D., Klein, C., Shah, N. & Grimbacher, B. IL-10 and IL-10 receptor defects in humans. *Ann. N. Y. Acad. Sci.* **1246**, 102–107 (2011).
17. Shouval, D. S. et al. Interleukin-10 receptor signaling in innate immune cells regulates mucosal immune tolerance and anti-inflammatory macrophage function. *Immunity* **40**, 706–719 (2014).
18. Zigmond, E., Bernshtein, B. & Friedlander, G. Macrophage-restricted interleukin-10 receptor deficiency, but not IL-10 deficiency, causes severe spontaneous colitis. *Immunity* **40**, 720–733 (2014).
19. Shouval, D. S. et al. Interleukin 1β mediates intestinal inflammation in mice and patients with interleukin 10 receptor deficiency. *Gastroenterology* **151**, 1100–1104 (2016).
20. Coccia, M. et al. IL-1β mediates chronic intestinal inflammation by promoting the accumulation of IL-17A secreting innate lymphoid cells and CD4(+) Th17 cells. *J. Exp. Med.* **209**, 1595–1609 (2012).
21. Li, B. et al. IL-10 engages macrophages to shift Th17 cytokine dependency and pathogenicity during T-cell-mediated colitis. *Nat. Commun.* **6**, 1–13 (2015).
22. Shouval, D. S. et al. Enhanced TH17 responses in patients with IL10 receptor deficiency and infantile-onset IBD. *Inflamm. Bowel Dis.* **23**, 1950–1961 (2017).
23. Krausgruber, T. et al. T-bet is a key modulator of IL-23-driven pathogenic CD4+ T cell responses in the intestine. *Nat. Commun.* **7**, 11627 (2016).
24. Huber, S. et al. Th17 cells express interleukin-10 receptor and are controlled by $Foxp3^-$ and $Foxp3^+$ regulatory $CD4^+$ T cells in an interleukin-10-dependent manner. *Immunity* **34**, 554–565 (2011).
25. Srivastava, S., Koch, L. K. & Campbell, D. J. IFNαR signaling in effector but not regulatory T cells is required for immune dysregulation during type I IFN-dependent inflammatory disease. *J. Immunol.* **193**, 2733–2742 (2014).
26. Crouse, J. et al. Type I interferons protect T cells against NK Cell Attack Mediated by the Activating Receptor NCR1. *Immunity* **40**, 961–973 (2014).
27. Overacre-Delgoffe, A. E. et al. Interferon-γ drives T_{reg} fragility to promote anti-tumor immunity. *Cell* **169**, 1130–1141 (2017).
28. Lee, C.-K., Smith, E., Gimeno, R., Gertner, R. & Levy, D. E. STAT1 affects lymphocyte survival and proliferation partially independent of its role downstream of IFN-γ. *J. Immunol.* **164**, 1286–1292 (2000).

29. Meissner, T. B. et al. NLR family member NLRC5 is a transcriptional regulator of MHC class I genes. *Proc. Natl Acad. Sci. USA* **107**, 13794–13799 (2010).
30. Kuenzel, S. et al. The nucleotide-binding oligomerization domain-like receptor NLRC5 is involved in IFN-dependent antiviral immune responses. *J. Immunol.* **184**, 1990–2000 (2010).
31. Staehli, F. et al. NLRC5 deficiency selectively impairs MHC class I-dependent lymphocyte killing by cytotoxic T cells. *J. Immunol.* **188**, 3820–3828 (2012).
32. Tong, Y. et al. Enhanced TLR-induced NF-κB signaling and type I interferon responses in NLRC5 deficient mice. *Cell Res.* **22**, 822–835 (2012).
33. Lee, B. C. et al. Differential regulation of constitutive major histocompatibility complex class I expression in T and B lymphocytes. *J. Exp. Med.* **190**, 1451–1464 (1999).
34. Kärre, K., Ljunggren, H. G., Piontek, G. & Kiessling, R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* **319**, 675–678 (1986).
35. Yewdell, J. & Bennink, J. R. Mechanisms of viral interference with MHC class I antigen processing and presentation. *Annu. Rev. Cell. Dev. Biol.* **15**, 579–606 (1999).
36. Karo, J. M., Schatz, D. G. & Sun, J. C. The RAG recombinase dictates functional heterogeneity and cellular fitness in natural killer cells. *Cell* **159**, 94–107 (2014).
37. Surh, C. D. & Sprent, J. Homeostasis of naive and memory T cells. *Immunity* **29**, 848–862 (2008).
38. Booki Min, P., Yamane, H., Hu-Li, J., Min, B. & Paul, W. E. Spontaneous and homeostatic proliferation of CD4 T cells are regulated by different mechanisms. *J. Immunol.* **174**, 6039–6044 (2005).
39. Kieper, W. C. W. et al. Cutting edge: recent immune status determines the source of antigens that drive homeostatic T cell expansion. *J. Immunol.* **174**, 3158–3163 (2005).
40. Tajima, M. et al. IL-6-dependent spontaneous proliferation is required for the induction of colitogenic IL-17-producing CD8+ T cells. *J. Exp. Med.* **205**, 1019–1027 (2008).
41. Feng, T., Wang, L., Schoeb, T. R., Elson, C. O. & Cong, Y. Microbiota innate stimulation is a prerequisite for T cell spontaneous proliferation and induction of experimental colitis. *J. Exp. Med.* **207**, 1321–1332 (2010).
42. Lee, C. K. et al. Distinct requirements for IFNs and STAT1 in NK cell function. *J. Immunol.* **165**, 3571–3577 (2000).
43. Kovacic, B. et al. STAT1 acts as a tumor promoter for leukemia development. *Cancer Cell.* **10**, 77–87 (2006).
44. Ludigs, K. et al. NLRC5 shields T lymphocytes from NK-cell-mediated elimination under inflammatory conditions. *Nat. Commun.* **7**, 10554 (2016).
45. Xu, H. C. et al. Type I interferon protects antiviral CD8+ T cells from NK cell cytotoxicity. *Immunity* **40**, 949–960 (2014).
46. Le Saout, C. et al. IL-7-dependent STAT1 activation limits homeostatic CD4+ T cell expansion. *JCI Insight* **2**, 1–18 (2017).
47. Madera, S. et al. Type I IFN promotes NK cell expansion during viral infection by protecting NK cells against fratricide. *J. Exp. Med.* **213**, 225–233 (2016).
48. Kole, A. et al. Type I IFNs regulate effector and regulatory T cell accumulation and anti-inflammatory cytokine production during T cell-mediated colitis. *J. Immunol.* **191**, 2771–2779 (2013).
49. Carrette, F. & Surh, C. D. IL-7 signaling and CD127 receptor regulation in the control of T cell homeostasis. *Semin. Immunol.* **24**, 209–217 (2012).
50. Lütticken, C. et al. Association of transcription factor APRF and protein kinase Jak1 with the interleukin-6 signal transducer gp130. *Science.* **263**, 89–92 (1994).
51. Li, B., Jones, L. L. & Geiger, T. L. IL-6 promotes T cell proliferation and expansion under inflammatory conditions in association with low-level RORγt expression. *J. Immunol.* **201**, 2934–2946 (2018).
52. Waggoner, S. N., Cornberg, M., Selin, L. K. & Welsh, R. M. Natural killer cells act as rheostats modulating antiviral T cells. *Nature* **481**, 394–398 (2012).
53. Fort, M. M., Leach, M. W. & Rennick, D. M. A role for NK cells as regulators of CD4+ T cells in a transfer model of colitis. *J. Immunol.* **161**, 3256–3261 (1998).
54. Yamaji, O. et al. The development of colitogenic CD4+ T cells is regulated by IL-7 in collaboration with NK cell function in a murine model of colitis. *J. Immunol.* **188**, 2524–2536 (2012).
55. Meyer zu Horste, G. et al. Fas promotes T helper 17 cell differentiation and inhibits T helper 1 cell development by binding and sequestering transcription factor STAT1. *Immunity* **48**, 556–569.e7 (2018).
56. Cai, G., Kastelein, R. A. & Hunter, C. A. IL-10 enhances NK cell proliferation, cytotoxicity and production of IFN-gamma when combined with IL-18. *Eur. J. Immunol.* **29**, 2658–2665 (1999).
57. Mocellin, S. et al. IL-10 stimulatory effects on human NK cells explored by gene profile analysis. *Genes Immun.* **5**, 621–630 (2004).
58. Souza-Fonseca-Guimaraes, F. et al. NK cells require IL-28R for optimal in vivo activity. *Proc. Natl Acad. Sci. USA* **112**, E2376–E2384 (2015).
59. Cornwell, M. I. et al. VIPER: visualization pipeline for RNA-seq, a Snakemake workflow for efficient and complete RNA-seq analysis. *BMC Bioinformatics.* **19**, 1–14 (2018).

Acknowledgements

We thank Jeremy Goettel for assistance in histological scoring, Ryan Kelly for technical assistance with the experiments and Jon Kagan, Ivan Zanoni and Bruce Horwitz for helpful discussions. We also thank the Dana-Farber Cancer Institute Flow Cytometry Core for cell sorting and the DFCI Molecular Biology Core Facilities - Genomics for RNA-seq and analysis. Y.H.K. is supported by an A*STAR National Science Scholarship, Singapore. A.B. is supported by the CCFA Career Development Award (327200) and a NIH KO1 award (K01DK109026). S.B.S. is supported by NIH Grants DK034854, and AI50950, the Helmsley Charitable Trust, and the Wolpow Family Chair in IBD Treatment and Research.

Author contributions

Y.H.K. and S.B.S. conceived the study. Y.H.K., A.B., and S.B.S. designed the experiments. Y.H.K., M.F. and A.B. performed the experiments, acquired and analyzed the data. Y.H.K. and S.B.S. wrote the manuscript.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-08743-8.

Competing interests: S.B.S. declares the following interests: Scientific advisory board participation for Pfizer, Janssen, Celgene, IFM therapeutics, and Pandion Inc. Grant support from Pfizer, Janssen, Merck. Consulting for Hoffman La Roche and Amgen. The remaining authors declare no competing interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Journal peer review information: *Nature Communications* thanks the anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.