

# Molecular analysis of pDC-mediated induction of IL-10 and IL-22 expression in human T cells

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Andrej Mantei

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1<sup>st</sup> Reviewer: Prof. Dr. Andreas Radbruch

2<sup>nd</sup> Reviewer: Prof. Dr. Rupert Mutzel

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**Do. Or do not. There is no try.**

(Yoda, 896 BBY – 4 ABY)



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## 2 Introduction

## 2.1 The immune system

The immune system is a complex network that comprises cells of the so-called innate and adaptive immune responses, both of which heavily interact with each other, executing in part similar, in part different functions. The mechanisms of the innate immune response constitute the first line of defense against pathogenic agents invading the organism. They recognize pathogens by pattern recognition receptors (PRR) that reside on their surface but also inside the cells. Phagocytic cells like macrophages and dendritic cells play a crucial role and serve as sentries of the immune system, mainly by engulfing and processing microbes and potentially harmful agents. Upon recognizing a pathogen pattern they become activated and start producing proinflammatory cytokines and chemokines to attract other innate immune cells, like monocytes, neutrophils, natural killer (NK) cells, eosinophils, and mast cells, thus initiating an inflammatory response.

In the wake of the innate immune response the adaptive immune system is activated, generating immune cells that specifically recognize pathogens, eradicate the invading organisms and build a long-term memory. The main effectors of the adaptive immune response are CD4 T helper cells, CD8 T killer cells and B cells, all of which normally reside in the lymphoid organs, but are recruited and activated by DCs or other antigen presenting cells (APC) in the scenario of an inflammation. Already during the phase of activation, DCs may determine the type of immune response through the antigens they present to the antigen-unexperienced cells, the specific profile of cytokines they produce and the expression of distinct co-stimulatory factors on their cell surface. Upon activation, CD4 T cells expand and differentiate into effector T<sub>H</sub> cell subsets with specific characteristics and functions, e.g. T<sub>H</sub>1, T<sub>H</sub>2 or T<sub>H</sub>17. Each subset has been characterized according to the production of certain hallmark cytokines (IFN- $\gamma$  for T<sub>H</sub>1; IL-4, IL-5, and IL-13 for T<sub>H</sub>2; and IL-17 for T<sub>H</sub>17) and is specialized on fighting different pathogens or to act in different types of inflammation<sup>1</sup>. For example, T<sub>H</sub>1 cells activate macrophages that eradicate intracellular pathogens, T<sub>H</sub>2 cells act against parasitic helminths and T<sub>H</sub>17 cells are mainly found to fight extracellular bacterial or fungal infections. DCs also activate CD8 T cells or cytotoxic T lymphocytes (CTL), which possess the capability of directly killing infected cells by use of perforins and granzymes.

Once pathogen-specific CD4 T cells and CD8 T cells have differentiated and expanded they migrate to the site of infection, where they aid the cells of the innate immune system.

Most effector functions of T helper cells rely on the expression of certain cytokines, which leads to further recruitment and activation of innate immune cells.

In contrast, B cells are activated by a combination of direct antigen recognition and interaction with dendritic cells and T cells. Mature B cells, so-called plasma cells, have acquired the capacity of massive production of pathogen-specific antibodies (immunoglobulins).

Acting together, these components of the innate and adaptive immune response in most cases manage to eradicate the pathogen.

### 2.1.1 Commitment of T helper cells to different T helper cell subsets

Guided by the microenvironment created by antigen-presenting cells (APC) activated naïve CD4 T cells differentiate into different types of mature CD4 T helper cells ( $T_H$  cells) with dedicated cytokine profiles <sup>2</sup>. Among these T helper cell subsets ( $T_H$  subsets) the  $T_H1$ ,  $T_H2$  and  $T_H17$  cells have been characterized best so far.

The signals that decide T cell fate are mostly conveyed through APC-expressed cytokines that – through the action of transcription factors of the STAT family (signal transducer and activator of transcription) – induce lineage-specific master regulators. Typically, specific STAT molecules are employed to activate specific master regulators. For example,  $T_H1$  differentiation is driven by IL-12 and the activation of STAT4 and the master regulator T-box transcription factor (TBET), which induces the  $T_H1$ -prototypical cytokine IFN- $\gamma$  <sup>3</sup>. Development of  $T_H2$  cells depends on IL-4, STAT6 and the master regulator GATA3 <sup>4</sup>, which induces the  $T_H2$  hallmark cytokines IL-4, IL-5 and IL-13 <sup>4</sup>.  $T_H17$  cells, which predominantly produce IL-17, are induced by transforming growth factor  $\beta$  (TGF- $\beta$ ) and IL-6, and – through the action of STAT3 – expression of the master regulator RAR-related orphan receptor gamma t (ROR $\gamma$ t) <sup>5,6</sup>.

However, recent research has shown that the regulation is much more complicated and involves several layers of transcription factors downstream of TCR and cytokine signaling pathways. According to current models, TCR signals induce pioneer factors that bind closed chromatin structures and in concert with the specific STAT molecules induce epigenetic patterns permissive of the generation of a range  $T_H$  subsets, while master regulators target genes either reinforce lineage commitment or inhibit alternative T cell fates <sup>7</sup>.

Despite those advances, the regulation of T helper cell differentiation in human is still poorly understood and further research is required to establish to what degree the regulatory network identified in mouse is also active in human T cells.

## 2.2 Interleukin-10 (IL-10)

The immune system, in addition to inflammation-driving mechanisms, also needs ways of repressing, limiting and terminating inflammatory responses. When it fails to do so, inflammation generally leads to damage to the host (immunopathology), inappropriate responses to non-pathogenic foreign particles such as pollen or commensal bacteria (e.g. allergy) and/or generation of inappropriate immune responses to the body itself (autoimmunity).

One ubiquitous mechanism of immune modulation is the production of anti-inflammatory interleukin-10 (IL-10). This cytokine has been demonstrated to be vital for the balance between protective and damaging immune responses<sup>8</sup>, thus playing an important role in the modulation of various types of infection, allergy and autoimmune conditions<sup>9</sup>.

### 2.2.1 Roles of IL-10 in the immune response

On the one hand, the action of IL-10 is of paramount importance in preventing or limiting excessive inflammatory responses and autoimmunity, thus protecting the organism from inflammation-related damage. On the other hand, the very same properties and functions can be exploited by pathogens to escape the immune response and establish or sustain an infection<sup>10</sup>.

#### 2.2.1.1 *Protection against excessive immune responses to pathogens*

IL-10 has been found to protect against excessive  $T_H1$  and  $T_H2$  reactions in diseases like Malaria ( $T_H1$ ), where high levels of inflammatory cytokines and excessive inflammation lead to potentially fatal complications<sup>11</sup>, and infection with *Schistosoma mansoni*, where  $T_H2$  contributes to formation of granulomas, which protect the eggs of *Schistosoma mansoni*, and can lead to severe fibrosis<sup>12</sup>. In fact, most severe fibrosis was found to be associated with locally low levels of IL-10 and IFN- $\gamma$ <sup>13</sup>, which otherwise would counteract the  $T_H2$ -related disease manifestation.

#### 2.2.1.2 *Suppression of protective immune responses against pathogens*

Owed to its powerful suppressive activity, expression of IL-10 can also result in suboptimal control and clearance of pathogens. There are many examples where pathogens exploit IL-10 to protect themselves from the host immune system.

Bacteria using the protection of IL-10 most often activate IL-10 sources of the host organism. For example, *Mycobacterium tuberculosis* (Mtb), which causes tuberculosis (TB), uses host-generated IL-10 to suppress the immune response and this way is often able to persist in the host for its entire lifetime, if not treated properly <sup>14</sup>. Here higher IFN- $\gamma$  and IL-10 levels that protect against fibrosis in *Schistosoma mansoni* infection are associated with active TB in tuberculosis patients <sup>15</sup>.

In contrast, certain viruses, like hepatitis C virus (HCV) and Epstein-Barr virus (EBV) promote their persistence by expressing viral IL-10, which they acquired from a host organism. Such viral IL-10 can show varying degrees of sequence identity and may be able to exert most or only a few functions of human IL-10 <sup>16</sup>.

#### 2.2.1.3 Allergic responses

Allergic responses, which are typically T<sub>H</sub>2-mediated and characterized by high levels of IgE, are thought to be alleviated by IL-10. Clinical studies have shown that the BAL of asthma patients contains lower levels of IL-10 <sup>17</sup> and that treatment of asthma with glucocorticoids induces IL-10 in human T cells <sup>18</sup>.

#### 2.2.1.4 Protection against autoimmunity

IL-10 plays a protective role in mouse models of multiple sclerosis (MS) (experimental autoimmune encephalomyelitis, EAE) as well as in the mouse model of rheumatoid arthritis (RA) <sup>19</sup>. In contrast, IL-10 is thought to be part of the pathogenic mechanism in systemic lupus erythematosus (SLE), where autoantibodies against nuclear antigens such as double-stranded DNA, result in immune complex deposition and multi-organ inflammation <sup>20</sup>.

#### 2.2.1.5 Cancer

The role of IL-10 in cancer is not clear. Some studies suggest that IL-10 contributes to suppression of antitumor immune responses, showing that IL-10-producing CD4 T cells suppressed the antitumor immune responses in some human cancers <sup>21</sup>, whereas different research indicated that stimulation of B cells and CD8 T cells by IL-10 may promote antitumor immune responses and suppress tumor angiogenesis, thus depriving tumors of nutrients <sup>22</sup>. Apparently, the exact role of IL-10 depends on location, source, timing, and level of IL-10 expression <sup>1</sup>.

## 2.2.2 Regulation of IL-10 expression

Although T<sub>H</sub>2 cells were the first cells to be described as producers of IL-10<sup>23</sup>, by now it is known that almost all immune cells can produce IL-10<sup>24</sup>.

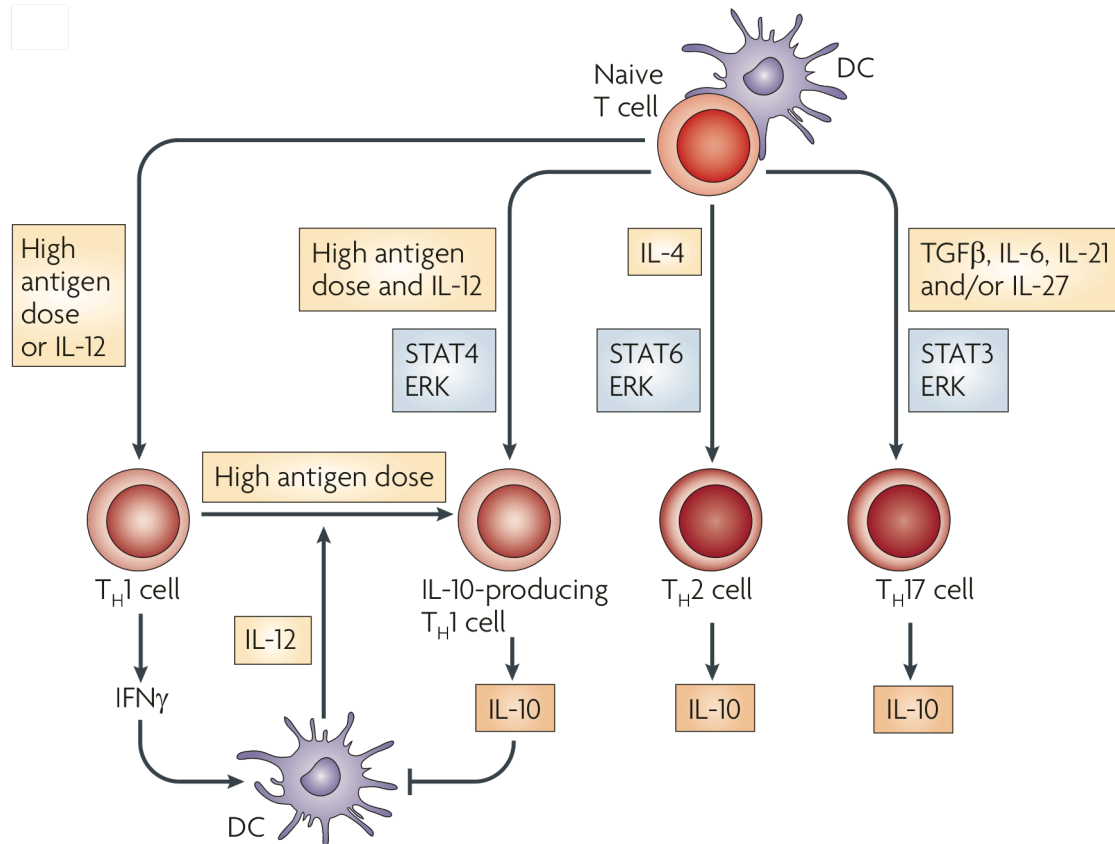
In fact, all types of effector CD4 T cells, like T<sub>H</sub>1, T<sub>H</sub>2, and T<sub>H</sub>17 cells, as well as Foxp3<sup>+</sup> CD4 regulatory T cells (T<sub>REG</sub>) have been demonstrated to secrete IL-10<sup>24</sup>.

The regulation of IL-10 expression involves various signals, including TCR activation, cytokine signaling and various environmental signals. Each affects several levels of intracellular regulation, from the accessibility of the *IL10* gene locus to the cooperative binding of transcription factors, which integrate the various signals for context specific transcription of the *IL10* gene. Nonetheless, many of these mechanisms as well as the exact conditions under which they become active have not yet been elucidated.

The *IL10* gene is located in the gene family cluster on chromosome 1<sup>25</sup> and is flanked upstream by IL19, IL20 and IL24 and downstream by the *MAPKAPK2* (MAP kinase-activated protein kinase 2) gene. Interestingly, there is high homology between human and mouse, indicating a strong evolutionary constraint, which likely is owed to the crucial role that plays IL-10 and its tight regulation in the mammalian immune system.

In contrast to cells of the innate immune system, which can directly produce IL-10, CD4 T cells need to differentiate into an effector T<sub>H</sub> cells before acquiring the capacity of IL-10 expression. Interestingly, IL-10 production by differentiated CD4 T cells appears to be tightly linked to their differentiation program<sup>24</sup>.

There have been many advances in recent years furthering the understanding of the regulation of IL-10 expression in the immune system. However, owed to the fact that most of analyses have been performed in mouse models these studies need to be repeated with human cells.



#### Overview of signals inducing IL-10 in different T helper cell subsets

Source: Saraiva, Margarida and Anne O'Garra. 2010. "The Regulation of IL-10 Production by Immune Cells." *Nature reviews. Immunology* 10(3):170–81. <sup>24</sup>

##### 2.2.2.1 Regulation by T cell receptor signaling

T cell activation through the T cell receptor (TCR) is indispensable for IL-10 induction. As an example, IL-10 has been found to be induced in T<sub>H</sub>1 cells by a combination of IL-12 signaling, which activates STAT4, and a strong TCR stimulus <sup>26</sup>. However, little is known about how the TCR signaling pathway interfaces with the pathways responsible for IL-10 expression.

Experiments have shown that the MAP kinase ERK is a positive regulator of IL-10 <sup>26</sup>. ERK activates the AP-1 complex, which binds in the *IL10* gene locus in T<sub>H</sub>2, but not T<sub>H</sub>1 cells <sup>27,28</sup>. Also, basic leucine zipper ATF-like transcription factor (BATF) has been shown to activate IL-10 expression in T<sub>H</sub>2 cells <sup>29</sup>. Another factor that interacts with AP-1 upon TCR activation is NFAT1. This transcription factor is known to partner with AP-1 to promote cytokine expression <sup>31</sup>, has been shown to bind in the *IL10* locus in T<sub>H</sub>2 and T<sub>H</sub>1 cell lines <sup>32</sup> and was demonstrated to enhance IL-10 expression in T<sub>H</sub>2 cells <sup>33</sup>.

In addition, IL-21-induced IL-10 production requires the transcriptional activator IRF4 <sup>30</sup>.

### 2.2.2.2 Regulation by cytokine signaling

Several experiments point towards a role for cytokine-activated STAT and SMAD proteins in the regulation of IL-10 expression in T<sub>H</sub> cells. Cytokines like IL-27, IL-21, IL-6 and IL-12 have been shown to induce IL-10 expression via specific STAT molecules (STAT1: IL-27<sup>34-36</sup>, STAT3: IL-27, IL-21 or IL-6<sup>34,37,38</sup>, STAT4: IL-12<sup>26</sup>, STAT6: IL-4<sup>26</sup>). In addition, IL-10 itself might employ STAT3 to enhance its own expression, as has been shown in macrophages<sup>39</sup>.

More direct evidence of the crucial role of STAT molecules is the finding that STAT4 in T<sub>H</sub>1 cells, STAT6 in T<sub>H</sub>2 cells<sup>40</sup> and STAT3 in T<sub>H</sub>17 cells<sup>30</sup> can bind in the *IL10* gene locus and increase its accessibility to other transcription factors. Although there are fewer experimental data on the involvement of SMADs, they have been found to regulate IL-10 production in T<sub>H</sub>1 and T<sub>H</sub>2 cells<sup>26,41</sup>.

#### IL-21

IL-21 has been demonstrated to induce *IL10* mRNA and IL10 protein expression by CD4 T cells under non-polarizing as well as under T<sub>H</sub>1 and T<sub>H</sub>17, but not under T<sub>H</sub>2 conditions. In fact, T<sub>H</sub>1 polarization in presence of IL-21 only slightly augmented IFN- $\gamma$  levels, but significantly increased IL-10 expression and led to an increase in the frequency of IFN- $\gamma$ <sup>+</sup>IL-10<sup>+</sup> T cells. Also the induction of IL-10 by IL-6 or IL-27 under T<sub>H</sub>17 conditions, either in part or fully, required IL-21 signaling<sup>38</sup>.

In addition, the IL-10 induction by IL-21 was shown to depend on STAT3 activation<sup>38</sup>.

#### IL-6

IL6 has been found to induce IL-10 under non-polarizing conditions and in combination with TGF- $\beta$  under T<sub>H</sub>17 conditions<sup>34</sup>. However, there appear to be differences concerning the signaling involved. While IL-10-induction in the T<sub>H</sub>17 setting is mediated by STAT3 alone, both STAT1 and STAT3 are required for IL-6-dependent IL-10 induction in non-polarized CD4 T cells<sup>34</sup>. In addition, IL-10 expression elicited by IL-6 under T<sub>H</sub>17 conditions was found to being mediated partly by IL-21<sup>38</sup>. Like IL-27, IL-6 employs Egr2 and possibly Blimp1 for the induction of IL-10<sup>42</sup>.

#### IL-27

IL-27 induces IL-10 in activated naïve T cells, non-polarized T cells, T<sub>H</sub>1 cells, FoxP3<sup>+</sup> T<sub>REG</sub> cells<sup>34</sup> and T<sub>H</sub>17 cells<sup>38</sup>. Although IL-27 promotes T<sub>H</sub>1 differentiation<sup>34,43,44</sup>, IL-27-dependent IL-10-induction does not require Tbet<sup>34</sup>. However, IL-27-dependent IL-10-induction is mediated by STAT3 and STAT1<sup>34,42</sup>, but also employs AhR, Maf, Egr2 and Blimp1<sup>42,45</sup>.

## IFN- $\alpha$

Although IFN- $\alpha$  has been early described as a factor that can induce IL-10 in T cells <sup>46</sup>, most data stems from *in vivo* research on IFN- $\alpha$  as therapeutic agent in a variety of diseases and disease models <sup>47</sup>. In different diseases caused by infectious agents like HCV, HIV and *Mycobacterium tuberculosis*, IFN- $\alpha$  expression is correlated with heightened levels of IL-10 <sup>47-51</sup>. Still, little is known about the mechanisms by which IFN- $\alpha$  induces IL-10 expression and most research in this regard focused on the IFN- $\alpha$ -mediated IL-10 expression by cells of the innate immune system <sup>47</sup>. However, IFN- $\alpha$ -stimulated IL-10 expression by innate immune cells is believed to depend at least partly on activation of STAT3 <sup>47</sup>. Very recent research also showed that IFN- $\alpha$  induces an upregulation of IL-10 production under T<sub>H</sub>1 and T<sub>H</sub>2 conditions <sup>52</sup>.

### 2.2.2.3 Regulation by T helper cell master regulators

To a large part, the role of the T<sub>H</sub> cell master regulators GATA3, TBET and ROR $\gamma$ t in IL-10 induction is not clear. However, there indications for the involvement of GATA3, which has been shown to facilitate remodeling of the *IL10* locus, but itself is incapable of transactivating the *IL10* promoter <sup>53,54</sup> and, moreover, is not required for IL-10 production in differentiated T<sub>H</sub>2 cells <sup>55</sup>. The exact role of GATA3 as well as of the other master regulators in the induction of IL-10 remains obscure.

### 2.2.2.4 Regulation by transcription factors Maf and Blimp1

Although transcription factor Maf is expressed in all T<sub>H</sub> subsets and has been shown to correlate with levels of IL-10 expression in many contexts <sup>26</sup>, usually, it is not able to transactivate the *IL10* gene promoter by itself <sup>56</sup>. Most likely, it transactivates *IL10* in concert with other transcription factors, like Nfat or AP-1, both of which have been shown to bind the *IL10* locus <sup>31,33</sup>

In T<sub>H</sub>1 cells, IL-12-driven Maf expression has been shown to correlate with IL-10 expression <sup>26</sup>, but direct evidence that Maf activates IL-10 is still missing <sup>2</sup>.

In contrast, in T<sub>R</sub>1 cells induced by IL-27 <sup>57</sup>, Maf in collaboration with AhR appears to positively regulate IL-10 production <sup>58</sup>, as knockdown of Maf or AhR correlated with a decrease in *IL10* mRNA expression and both transcription factors were found to bind and synergistically transactivate the *IL10* gene promoter <sup>58</sup>. In these cells, IL-21 is believed to regulate Maf expression <sup>59</sup>. Conversely, Maf has been shown to transactivate the *IL21* gene promoter <sup>60,61</sup>.

The PR domain zinc finger protein 1 (Blimp1) is encoded by the *Prdm1* gene. It has been found to be a transcriptional repressor of IFN- $\gamma$  and IL-2 downstream of the TCR <sup>62</sup> and to positively regulate IL-10 production in CD4 T cells <sup>63</sup>. Downstream of IL-27, Blimp1 has been shown to mediate IL-10 expression in CD4 T cells <sup>42</sup>, CD8 T cells <sup>64</sup> and CD4 T<sub>REG</sub> cells <sup>65</sup>. In the latter, this transcription factor acts in synergy with IRF4 in regulating histone acetylation at the *Il10* gene promoter <sup>65</sup>. Also in T<sub>H</sub>1 cells Blimp1 appears to be required for IL-10 expression <sup>66</sup>. However, the molecular mechanisms behind Blimp1-dependent regulation of IL-10 in T<sub>H</sub> cells are not yet completely understood.

#### 2.2.2.5 Regulation by Notch signaling

Notch signaling has been shown to be a costimulatory pathway, which – through ligation of DC- or macrophage-expressed Notch ligands to Notch receptors on CD4 T cells – is capable of regulating T<sub>H</sub> differentiation and IL-10 expression by CD4 T cells. Published data points towards Delta-like Notch ligands (DLL) as T<sub>H</sub>1-driving factors, whereas Jagged Notch ligands have been associated with T<sub>H</sub>2 differentiation <sup>67</sup>. In addition, Notch ligands DLL-1 and, in particular, DLL-4 have been shown to induce IL-10 in T<sub>H</sub>1 cells <sup>68</sup>. Interestingly, JAG-1, which had been identified as a IL-4 and T<sub>H</sub>2-driving factor, was found to ligate to CD46 on human T cells and induce IL-10 in IFN- $\gamma$  expressing CD4 T cells <sup>69</sup>.

Currently, the data on the role of Notch in T<sub>H</sub> differentiation and the induction of IL-10 in T<sub>H</sub> cells is rather limited.

## 2.3 Interleukin-22 (IL-22)

The cytokine IL-22 is a member of the IL-10 cytokine family, which comprises the cytokines IL-10, IL-19, IL-20, IL-24, IL-26, IL-28, IL-29<sup>70</sup>. IL-22 is essential for defense of mucosal surfaces against extracellular pathogens, like bacteria and yeast, but is also involved in the regeneration of epithelium, and wound healing. Consequently, its receptor, a heterodimer of the IL-10R2 and IL-22R1 subunits<sup>70</sup> is widely expressed on intestinal and respiratory epithelial cells, keratinocytes, and hepatocytes, but is not found on cells of hematopoietic origin. As part of its protective functions IL-22 is able of directly initiating inflammatory defense reactions of the epithelium. This and the fact that many cells of the innate and adaptive immune system are capable of expressing IL-22 necessitates a tight control of IL-22 expression. In general, dysregulation of IL-22 may lead to induction or enhancement of detrimental inflammatory responses accompanied by abnormal manifestations in the epithelium.

In addition to innate immune cells, like natural killer T cells (NKT), natural killer cells (NK) and lymphoid tissue inducer cells (LTi), cells of the adaptive immune system, like CD4 and CD8 T cells have been shown to produce IL-22<sup>70</sup>.

### 2.3.1 Functions of IL-22

In host defense against extracellular pathogens IL-22 acts (1) as proliferation-inducing factor that ensures integrity of the epithelial barrier, (2) proinflammatory factor that in concert with other cytokines like IL-17 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), induces the expression of antimicrobial cytokines and (3) as promoter of the secretion of other proinflammatory factors, like IL-6, G-CSF (granulocyte colony-stimulating factor) and IL-1b<sup>70</sup>.

All these functions serve the purpose of promoting barrier immunity and thus limiting bacterial replication and dissemination.

As mentioned before, IL-22 is an important player in wound healing and tissue regeneration in organs, like the intestine, the airways, the liver, and the thymus<sup>70</sup>, where it promotes epithelial proliferation through the induction of anti-apoptotic factors (e.g. Bcl2) and factors that are directly involved in proliferation and the cell cycle (e.g. cyclin D1 and CDK4)<sup>70</sup>.

IL-22 often acts in synergy with cytokines such as IL-17, IFN- $\alpha$ , IFN- $\gamma$ , and TNF- $\alpha$ <sup>71-74</sup>, which strongly enhance expression of the IL-22 receptor subunits, thus increasing the effect of IL-22<sup>75,76</sup> or, like IFN- $\alpha$ , promote the generation of IL-22-inducing DCs<sup>70</sup> or, like IL-17, enhance innate immune responses of tissue fibroblasts, epithelial cells and other stromal cells<sup>70</sup>. In particular IL-17 is an important co-player of IL-22 and essential for the host defense against certain infections. The importance of IL-17 becomes most evident by the fact that the presence of this cytokine can shift the function of IL-22 from protective to pathogenic<sup>70</sup>.

#### 2.3.1.1 *IL-22 in pathogenic conditions*

IL-22 induces many innate inflammatory and tissue-protective responses<sup>70</sup>. Although anti-inflammatory properties have been observed in some *in vivo* models, they are likely owed to the tissue-protective functions of IL-22. In contrast, its proinflammatory capacity combined with an uncontrolled expression has been found to lead to certain pathologic conditions. As an example, IL-22 is involved in psoriasis, where keratinocyte hyperproliferation and leukocyte infiltration and activation, can be tracked down to IL-22 overexpression, which induces keratinocyte proliferation and hyperplasia resulting in a thickened epidermis as well as abnormal differentiation of keratinocytes<sup>70</sup>. Moreover, IL-22 together with cytokines like IL-17 and IFN- $\gamma$ , is capable of amplifying inflammation through the induction of keratinocyte chemokines<sup>71</sup>. In addition to psoriasis, IL-22 has also been described as one factor driving rheumatoid arthritis<sup>70</sup>.

#### 2.3.2 Regulation of IL-22 expression in CD4 T helper cells

Significant differences have been found between the cellular sources of IL-22 in human and mouse. While IL-22 in mouse is mainly produced by T<sub>H</sub>17 cells, most of the IL-22-expressing cells in human peripheral blood are T<sub>H</sub>1 and T<sub>H</sub>22 cells.

In the human system, up to 35 % of IL-22-producing cells among peripheral blood mononuclear cells (PBMC) have been found to be T<sub>H</sub>1 cells<sup>77</sup>. An even larger population in the human blood (between 37 % and 65 %) is comprised of so-called T<sub>H</sub>22 cells<sup>72,77,78</sup>, IL-22 single producers that coexpress neither IFN- $\gamma$  nor IL-17<sup>77</sup>. In fact, in human, IL-22 expression correlates best with the expression of TBET (TBX21) and IFN- $\gamma$ <sup>79,80</sup>. This contrasts with data from mouse, where T<sub>H</sub>1-dependent expression of IL-22 is much less prominent<sup>71,81</sup> and cells reminiscent of human T<sub>H</sub>22, albeit found *in vivo*, are much rarer and have not been defined as separate T<sub>H</sub> subset<sup>71,81</sup>. Here, the major source of IL-22 are

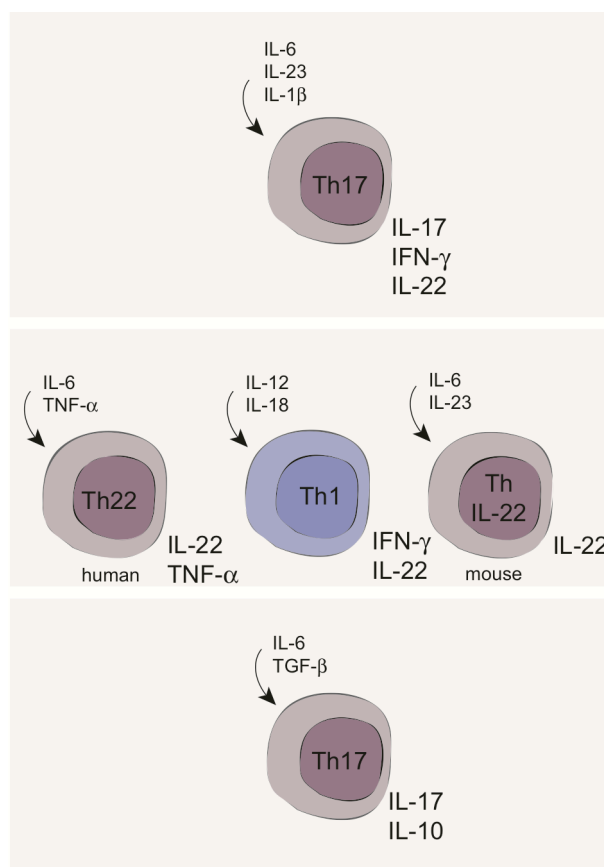
$T_H17$  cells<sup>71,81,82</sup>. In contrast, human IL-22 expression has not been found to be directly correlated to expression of IL-17 or the  $T_H17$  master regulator ROR $\gamma$ t<sup>79,80</sup>.

### 2.3.2.1 Cytokines inducing IL-22

Interestingly, in mouse like in human IL-6 is involved in the generation of IL-22-expressing cells in the context of  $T_H17$ . There it is required for expression of both IL-22 and IL-17. Depending on which other cytokines contribute to the  $T_H17$  differentiation diverse  $T_H17$  phenotypes can be observed. IL-6 and TGF- $\beta$  produce IL-17<sup>+</sup> cells that hardly coexpress IL-22. In contrast, the combination of IL-6, IL-23 and IL-1 $\beta$  drives generation of IL-17/IL-22 double-producers<sup>81</sup>. In human CD4 T cells, IL-6 alone or helped by TNF- $\alpha$  and/or vitamin D induces the  $T_H22$  phenotype<sup>81,83</sup>. The finding that  $T_H22$  cells can be coaxed to show typical  $T_H17$  cytokine expression patterns by stimulation with IL-1 $\beta$  or TGF- $\beta$ <sup>77</sup>, may indicate a close relationship between the  $T_H22$  and  $T_H17$  phenotypes.

IL-21, which is highly expressed in  $T_H17$  cells, was found to induce IL-22 expression comparable to IL-6<sup>84</sup> and is believed to act as a positive feedback loop enforcing  $T_H17$  differentiation<sup>84–86</sup>.

In addition, IL-23 alone or with IL-1 $\beta$  was shown to play a role in the induction of IL-22 in various types of innate and adaptive immune cells and in *in vivo* mouse models<sup>70</sup>.



**Fig. 2.1: Cytokines driving IL-22 expression in CD4 T cells**

Source: Rutz, Sascha, Céline Eidenschenk, and Wenjun Ouyang. 2013. "IL-22, Not Simply a Th17 Cytokine." *Immunological reviews* 252(1):116–32.<sup>70</sup>

Most cytokines that induce IL-22, like IL-6, IL-23 and IL-21, have been found to act via STAT3 activation. In fact, STAT3 proved to be crucial in certain settings. There, STAT3-deficiency strongly inhibited IL-22 production<sup>84,87</sup>, whereas overexpression of STAT3 in T cells led to significantly increased IL-22 levels<sup>87</sup>. The finding that, in mouse, STAT3 signaling led to strong IL-22 expression but failed to induce robust ROR $\gamma$ t expression and thus IL-17 expression<sup>60,81</sup> provides further evidence that induction of IL-22 is not strictly tied to the T<sub>H</sub>17 fate of T cells. Nonetheless, ROR $\gamma$ t is expressed in most innate and adaptive immune cells that produce IL-22, the most evident exceptions being the human T<sub>H</sub> cell subsets T<sub>H</sub>22 and T<sub>H</sub>1<sup>77,79</sup>. The data available so far suggests that ROR $\gamma$ t influences IL-22 expression indirectly by regulating IL-6 and IL-23 expression<sup>88</sup> but is not capable of independently regulating IL-22 production<sup>89</sup>.

### 2.3.2.2 *Transcription factor MAF*

TGF- $\beta$  was found to potently inhibit IL-22 expression in T<sub>H</sub>17 cells, which is evidenced by the fact that TGF- $\beta$  and IL-6 induce IL-17 single-producers whereas IL-6 with IL-23 and IL-1 $\beta$  induces IL-17/IL-22 coexpression<sup>60</sup>. The inhibitory effect of TGF- $\beta$  was not only observed in T<sub>H</sub>17 cells. Also addition of TGF- $\beta$  to human T<sub>H</sub>22<sup>77</sup>, Tc22<sup>70</sup>, and IL-17/IL-22-producing iNKT cells<sup>70</sup> significantly reduced IL-22 production. More in-depth analysis of this effect found that transcription factor MAF, which is induced downstream of TGF- $\beta$  and IL-6, acts as transcriptional repressor for IL-22<sup>60</sup>. MAF has also been described as IL-10 inducing factor, which explains the observation that IL-6 plus TGF- $\beta$  induce IL-17<sup>+</sup>IL-10<sup>+</sup> T<sub>H</sub>17 cells whereas T<sub>H</sub>17 differentiation by IL-6, IL-1 $\beta$  and IL-23 leads to IL-17<sup>+</sup>IL-22<sup>+</sup> cells.

### 2.3.2.3 *Transcription factor AhR*

Although the very close relationship between T<sub>H</sub>17 differentiation and IL-22 expression has been demonstrated in different contexts, several observations strongly suggest that additional pathways of IL-22 induction must exist. Here, the most telling observation is that in the human system T<sub>H</sub>1 cells largely contribute to the pool of IL-22-expressing cells.

Expression of AhR, a ligand-dependent transcription factor that senses environmental toxins, has been shown to correlate with IL-17 and IL-22 production across many cell types<sup>70</sup>. In addition, AhR was described as a crucial factor for IL-22 expression in T<sub>H</sub>17 cells<sup>90,91</sup>,  $\gamma\delta$  T cells<sup>70</sup>, and human T<sub>H</sub>22 cells<sup>77,78</sup>. In accordance with these findings addition of AhR ligands during an immune response increases IL-22 expression<sup>90</sup>.

Interestingly, AhR is not present in T cells that express IL-22 as a result of costimulation with IL-6 or IL-21 alone<sup>60</sup>. Correspondingly, AhR activation or blocking does not affect

IL-22 expression in these cells <sup>60</sup>. This indicates that AhR and STAT3 are part of two independent pathways that lead to IL-22 expression.

#### 2.3.2.4 *Notch*

Although some studies indicate an involvement of Notch in driving IL-22 expression, little is known about its role or mode of action in regard to IL-22 induction. Some studies have shown that Notch signaling is involved in T<sub>H</sub>17 development <sup>92,93</sup> and has a crucial role for the induction of IL-22 in T<sub>H</sub>17 cells <sup>94</sup>. Indeed, mouse CD4 T cells that lack one of the main mediators of Notch action, RBPJ, are strongly impaired in their IL-22 expression <sup>94</sup>. However, it appears that Notch does not directly induce IL-22 expression but rather acts as an enhancer of IL-22-promoting signals, like the STAT3- and the AhR-dependent pathways of IL-22 induction. In fact, Notch has been shown to promote the release of AhR ligands and is reported to stabilize STAT3 activation via HES1 <sup>95</sup>. Apart from these reports, data that elucidates the role of Notch in IL-22 induction are scarce, particularly in regard to the human system.

## 2.4 Plasmacytoid dendritic cells

Plasmacytoid dendritic cells (pDC) are key mediators of the innate immune response to viral infections. Sensing virus RNA and DNA via the pattern recognition receptors (PRR) Toll-like receptor (TLR) 7 and 9 they trigger manifold antiviral defense mechanisms, the most important one being the production of type I Interferon (IFN- $\alpha/\beta$ ), which aids in controlling viral replication <sup>96</sup>.

In fact, the importance of this mechanism in host defense against viral pathogens can be best estimated by the large number of ways viruses have developed to escape control by pDCs and type I IFN <sup>97</sup>

In addition to the control of viral infection, pDCs also play a role in immune suppression in several settings (reviewed by Karrich et al., 2014 <sup>98</sup>, and Bekerredjian-Ding et al., 2014 <sup>97</sup>).

### 2.4.1 Functions of plasmacytoid dendritic cells

Plasmacytoid dendritic cells are mainly activated through the binding of ligands to the pattern recognition receptors TLR7 and TLR9. Although Toll-like receptors (TLR) are expressed on many cells of the innate immune system, the expressed TLR types may vary substantially. It has been shown that TLR expression by human macrophages, monocytes and various subsets of dendritic cells is very variable <sup>99,100</sup>. Human pDCs have been found to exclusively and to a higher degree than other DC subsets express TLR7 and TLR9 <sup>99,100</sup>. In contrast, human monocytes more highly express TLR2 and TLR4 <sup>99</sup>, both of which are also found on myeloid DCs. Importantly, these differences between the various types of professional antigen presenting cells is less pronounced in the murine system where different DC subsets express comparable levels of mRNA for most TLRs (except TLR3/5 and 7) <sup>101</sup>.

The role of pDCs in antiviral defense is accentuated by the fact that pDCs express virus permissive receptors, like CD4, CXCR4, CCR5 (reviewed by Fitzgerald-Bocarsly and Jacobs, 2010 <sup>102</sup>, and Miller and Bhardwaj, 2013 <sup>103</sup>), and viral restriction factors <sup>104–106</sup>, which make pDCs virus “honeypots” that are equipped with potent intracellular defense mechanisms <sup>97</sup>.

#### 2.4.1.1 *Proinflammatory functions*

In the early activation phase, directly following TLR7/9 activation, pDCs dedicate a large part of their transcriptome to the expression of type I IFN genes (IFN- $\alpha$ , - $\beta$ , - $\omega$ ) and type III IFN genes (IFN- $\lambda$ 1, - $\lambda$ 2, - $\lambda$ 3) <sup>98</sup>. Type I IFNs as major effectors of pDCs are secreted in large amounts after activation. Their main role is the suppression of viral replication by arrest of cell proliferation and protein synthesis, promotion of T<sub>H</sub>1 responses <sup>107</sup> and enhancement of the formation of antibody secreting cells <sup>108</sup>. In addition to this proinflammatory mode of action pDC-derived type I IFNs have been shown to play also a role in immunosuppressive functions by inducing of T<sub>REG</sub> cells and suppressing T cells, B cells and innate immune cells <sup>97</sup>.

Interestingly, pDCs and type I IFNs, like IFN- $\alpha$ , have in part overlapping and in part antagonistic functions. This underlines the fact that pDC function strongly depends on the environment encountered by the dendritic cell <sup>97</sup>. For example, TGF- $\beta$  induces secretion of high levels of pDC-derived IL-6 and development of T<sub>H</sub>17 cells <sup>109</sup>, whereas soluble factors released from macrophages primed by apoptotic cells shift pDC function towards T<sub>REG</sub> induction <sup>110</sup>. Also factors like TNF, IL-10 and TGF- $\beta$  in Peyer's patches, tumor cells, or monocytes, activate different cytokine secretion patterns while decreasing IFN- $\alpha$  secretion <sup>97</sup>.

In addition to type I IFNs, pDCs produce proinflammatory cytokines like IL-6 and TNF- $\alpha$ , which add another layer to the modulation of T cell, B cell, NK cell and DC responses <sup>96</sup>. Plasmacytoid dendritic cells are also directly involved in T cell activation as they develop into potent antigen-presenting cells that show properties and a T-cell-stimulating capacity similar to conventional DCs <sup>111</sup>. An important part of pDC function is their cytotoxic action, which is mediated by TRAIL on the pDC surface. These factors induce cell death of TRAIL-sensitive infected cells and tumor cells <sup>98</sup>.

#### 2.4.1.2 *Tolerance*

Immature pDC are not well-equipped for T cell activation <sup>112</sup> and have been shown to contribute to T cell suppression through the induction of regulatory T cells (T<sub>REG</sub> cells) <sup>113</sup>. Release of IL-10 and expression of IDO, PD-L1, and ICOS-L are the main mechanisms employed for T<sub>REG</sub> induction by pDCs <sup>97</sup>. Consequently, pDCs have been reported to contribute to peripheral T cell tolerance in transplantation <sup>114</sup>, tumor escape <sup>115</sup>, oral tolerance <sup>116</sup>, and mucosal tolerance <sup>117</sup>. Some research suggests that tolerance mediated by pDCs may play a role in mouse gut and thymus <sup>98</sup>. Such tolerogenic pDCs are speculated to present the chemokine receptor CCR9, which is downregulated upon activation through TLRs,

which in turn is associated with diminished tolerogenic capacity <sup>118</sup>. In humans, such a tolerance-inducing pDC subset has not yet been described.

#### 2.4.1.3 *Autoimmune diseases*

Owed to their capacity of secreting large amounts of type I IFN and the ability to react to immune complexes comprising autologous nucleic acids <sup>97</sup>, pDCs have been a prime suspect in the induction of autoimmune diseases <sup>98</sup>. The ubiquitous presence of pDC increases the risk of systemic effects of IFN- $\alpha$ , a cytokine that enhances autoantibody production and drives inflammation and therefore can contribute to development and sustainment of autoimmune diseases <sup>119</sup>. In fact, IFN- $\alpha/\beta$ -secreting pDCs have been reported to be present in psoriatic skin lesions <sup>120,121</sup> and are thought to largely contribute to the high levels of type I IFN observed in SLE (systemic lupus erythematosus), Behcet's disease and Sjögren's syndrome <sup>122-124</sup>.

#### 2.4.1.4 *Induction of IL-22 expression in CD4 T helper cells*

Some data point towards a role of activated pDCs in the induction of IL-22 expression in T<sub>H</sub> cells. In murine models of wound healing, pDC depletion led to a deficiency in the expression of IL-17 and IL-22 but not IFN- $\gamma$  <sup>125</sup>. The same effect was observed in IFN- $\alpha$ R/ $\beta$ R-deficient mice <sup>125</sup>.

In addition, human pDCs stimulated with TLR9 agonist CpG B have been shown to induce T<sub>H</sub>22 cells in a IL-6- and TNF- $\alpha$ -dependent and an IFN- $\alpha$ -independent way <sup>77</sup>. In contrast, cultures with conventional dendritic cells (cDC) contained a soluble IL-22-inhibiting factor and expressed small amounts of IL-10 <sup>77</sup>.

## 2.5 Notch

Notch signaling is an evolutionarily conserved pathway of cell-to-cell communication that is mediated by Notch ligand–receptor interactions between adjacent cells.

Four mammal receptors (Notch 1–4) are known and are bound by five ligands of the Jagged and the Delta-like family (Jagged-1 and -2 [JAG-1 / JAG-2], Delta-like ligand 1, 3 and 4 [DLL-1, DLL-3, DLL-4] <sup>67</sup>.

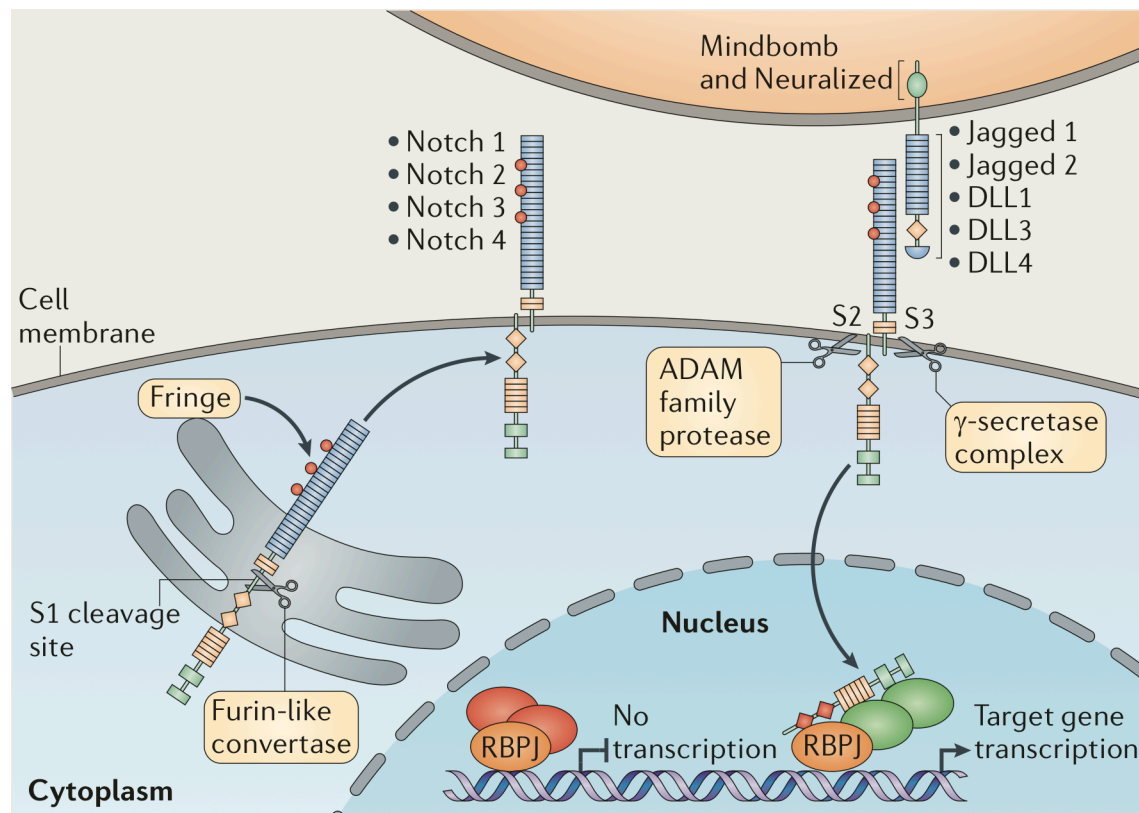
Genome-wide expression studies indicate that a large number of genes are regulated by Notch <sup>126,127</sup>. So far the best-characterized target genes of the Notch pathway are the basic helix-loop-helix (bHLH) transcriptional repressors of the hairy enhancer of split (HES) and hairy-related (HRT) protein families <sup>128</sup>.

### 2.5.1 Canonical Notch signaling

A signaling event is initiated by binding of a Notch ligand to heterodimeric Notch receptors expressed on the cell surface. This induces two successive proteolytic cleavages of the receptor. The first is mediated by an ADAM metalloprotease and leads to the shedding of the extracellular domain. The second cleavage is the rate-limiting step. It requires a  $\gamma$ -secretase and liberates the Notch intracellular domain (NICD). In experimental setups that involve inhibition of Notch signaling, this last cleavage step is often pharmacologically blocked using small-molecule  $\gamma$ -secretase inhibitors.

The NICD translocates to the nucleus, where it binds to the transcription factors of the recombination signal binding protein for immunoglobulin  $\kappa$  region (RBPJ) family (CSL in humans) and recruits additional co-activators, like the mastermind proteins (MAML1–3) and p300, thus inducing transcription of Notch target genes <sup>67</sup>.

In recent years evidence has amassed that the Notch signal can also be transmitted through non-canonical Notch pathways that do not require RBPJ and may even occur in absence of receptor cleavage or through crosstalk with other signaling pathways <sup>129–131</sup>.



### The canonical Notch signaling pathway

Co-repressors of RBPJ are shown in red, cofactors for RBPJ in green

Source: Radtke, Freddy, H. Robson MacDonald, and Fabienne Tacchini-Cottier. 2013. "Regulation of Innate and Adaptive Immunity by Notch." *Nature reviews. Immunology* 13(6):427–37.<sup>67</sup>

## 2.5.2 Notch and T helper cell differentiation

Several factors contribute to the signals driving differentiation and cell fate commitment of naive CD4 T cells. Accumulating data shows that Notch signaling might assume a prominent role among them, with Delta-like ligands and Jagged ligands exhibiting distinct  $T_H$ -driving capacities. However, the exact mechanisms are still far from being fully understood<sup>67,132–134</sup>.

Various studies suggest that  $T_H1$ -cell differentiation is mediated by a non-canonical Notch pathway. Although the pathway is yet unclear, Notch is thought to interact with one or more partners, including NF $\kappa$ B<sup>135,136</sup>.

So far most data support the notion that Notch signaling is rather involved in the control of  $T_H1$  cell effector functions, than in the differentiation of  $T_H1$  cells. In addition, Notch 1, 2 and 3 have each been individually shown to affect  $T_H1$  functions *in vivo*. So, it is conceivable that the different Notch receptors may be involved in different  $T_H1$  type contexts<sup>67</sup>.

## 2.6 Interferon- $\alpha$ (IFN- $\alpha$ )

The signaling pathways of type I IFN have been extensively reviewed by Ivashkiv and Donlin, 2014 <sup>47</sup>.

IFN- $\alpha$  belongs to the type I interferons (type I IFN), which are secreted by infected cells and have three major functions: (1) they induce antimicrobial states in infected and neighbouring cells to inhibit spread of infectious pathogens e.g. viruses; (2) they modulate the innate immune response, promote antigen presentation and stimulate natural killer cells, while at the same time limiting proinflammatory responses and cytokine production; (3) they activate the adaptive immune system.

Hematopoietic cells and in particular plasmacytoid dendritic cells are the predominant producers of IFN- $\alpha$ .

### 2.6.1 IFN- $\alpha$ signaling

#### 2.6.1.1 *The canonical type I IFN signaling pathway*

Upon binding of IFN- $\alpha$  to its receptor (IFNAR), the receptor-associated kinases JAK1 and TYK2 phosphorylate the transcription factors STAT1 and STAT2 <sup>47</sup>, which translocate to the nucleus as heterodimers and there form the ISGF3 complex together with IRF9. ISGF3 binds ISREs (IFN-stimulated response elements), which activate transcription of ISGs (interferon stimulated genes).

This canonical type I IFN signaling activates transcription of several hundred distinct ISGs causing infected cells to switch to an antiviral state <sup>47</sup>.

#### 2.6.1.2 *Basal type I IFN signaling*

In order to maintain rapid responsiveness to type I IFN signals, most cells constitutively express the components of the canonical type I IFN signaling pathway (IFNAR, JAK1, TYK2, STAT1, STAT2 and IRF9) and maintain the type I IFN signaling pathway in a responsive state by an autocrine loop involving constitutive expression of low-level IFN- $\beta$ , STAT1 and IRF9 <sup>47</sup>.

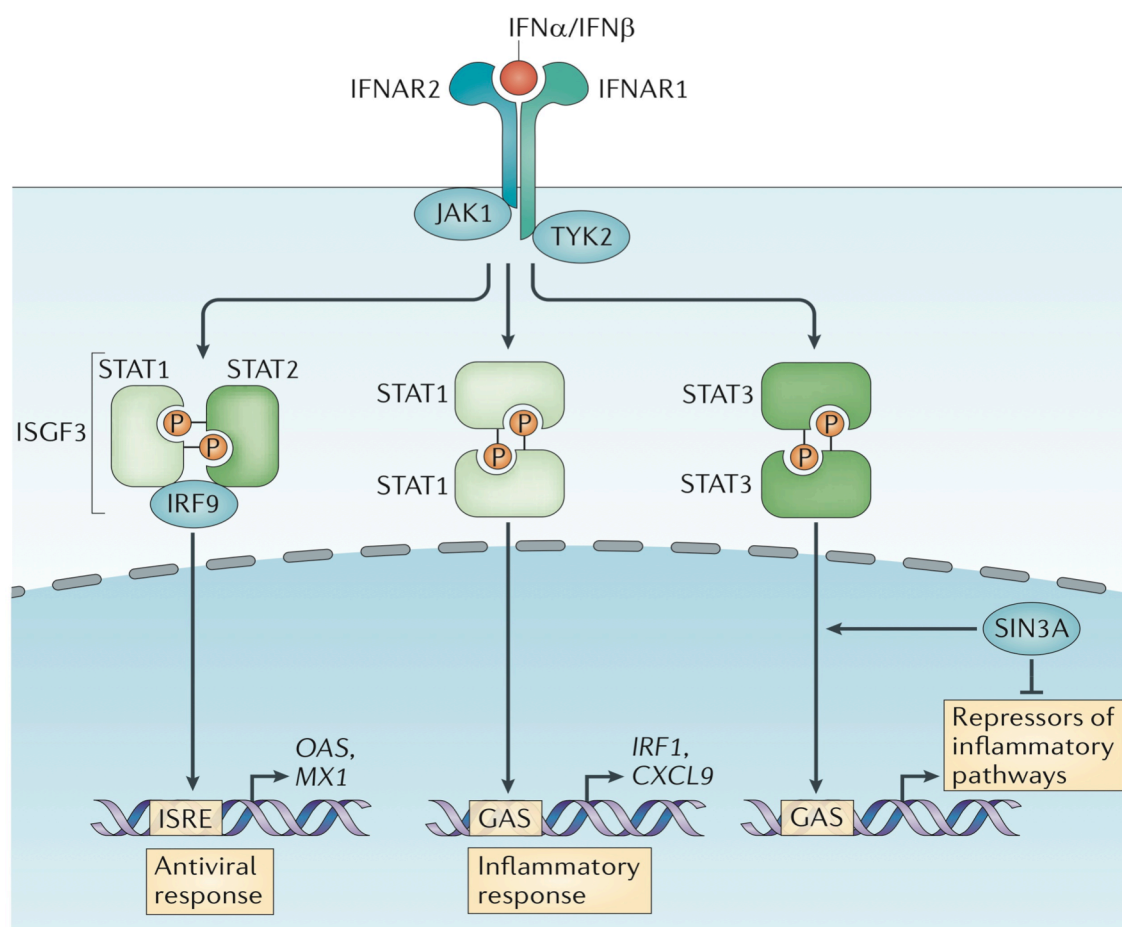
#### 2.6.1.3 *Modulation of type I IFN signals*

IFNAR signaling is regulated by several mechanisms downstream of many different types of receptors and ligands during immune responses.

One important mechanism of limiting type I IFN responses is downregulation of the IFNAR initiated by type I IFN itself <sup>137</sup>.

The level of STAT1 and IRF9 expression largely determines magnitude and outcome of type I IFN signals. Higher expression of STAT1 leads to increased STAT1 phosphorylation, which translates into enhanced formation of STAT1:STAT2 heterodimers, but also favors STAT1 homodimers, which activate expression of proinflammatory genes with GAS sequences<sup>47</sup>. This is counteracted by SOCS1, SOCS3 and USP18 (ubiquitin carboxy-terminal hydrolase 18), which compete with STAT1 or JAK2 for binding to the IFNAR.

Another important mechanism is the differential activation of STATs with distinct target genes and biological functions. Besides STAT1 and STAT2, the IFNAR activates STAT3, which can induce transcription of genes that suppress inflammatory responses<sup>47</sup>. In addition, STAT3 was found to sequester STAT1 in STAT1:STAT3 heterodimers, removing it from the pool of monomers available for the formation of STAT1 homodimers or STAT1:STAT2 heterodimers. Thus IFN-activated STAT3 serves to balance and limit the proinflammatory pathways induced by type I IFNs<sup>47</sup>.



**Fig. 2.2: The canonical type I interferon signaling pathway.**

IFNAR, type I IFN receptor; ISRE, IFN-stimulated response element; GAS, gamma-activated sequence

Source: Ivashkiv, Lionel B. and Laura T. Donlin. 2014. "Regulation of Type I Interferon Responses." *Nature reviews. Immunology* 14(1):36–49.<sup>47</sup>

## 3 Materials and Methods

### 3.1 Materials

#### 3.1.1 Media and buffers

<b>RPMI 1640 medium</b>		<b>Invitrogen/Life Technologies</b>
10 %	FCS	Sigma
100 U/mL	Penicillin	PAA Laboratories
100 g/mL	Streptomycin	PAA Laboratories
50 $\mu$ M	2-Mercaptoethanol	Invitrogen/Life Technologies

<b>DMEM medium</b>		<b>Invitrogen/Life Technologies</b>
10 %	FCS	Sigma
100 U/mL	Penicillin	PAA Laboratories
100 g/mL	Streptomycin	PAA Laboratories
50 $\mu$ M	2-Mercaptoethanol	Invitrogen/Life Technologies

<b>PBS (pH 7.2)</b>		
137 mM	NaCl	Merck
2.7 mM	KCl	Merck
1.5 mM	KH <sub>2</sub> PO <sub>4</sub>	Merck
8.0 mM	Na <sub>2</sub> HPO <sub>4</sub> $\times$ 2 H <sub>2</sub> O	Merck

<b>PBS/BSA (pH 7.2)</b>		
PBS		
0.5 m/v %	Bovine serum albumin frV	PAA Laboratories

<b>PBS/BSA/azide (pH 7.2)</b>		
PBS/BSA		
0.02 m/v %	NaN <sub>3</sub>	Merck

<b>2X HBS</b>		
50 mM	HEPES (pH 7.05)	Merck
10 mM	KCl	Merck
12 mM	Dextrose	Merck
280 mM	NaCl	Merck
1.5 mM	Na <sub>2</sub> HPO <sub>4</sub>	Merck
adjust pH to 7.05 $\pm$ 0.05		

<b>1.25 M CaCl<sub>2</sub> solution</b>		
1.25 M	CaCl <sub>2</sub>	Merck

<b>Saponin 5 m/v %</b>		
5 g	Saponin	Sigma
fill to 100 mL with PBS/BSA/azide		

<b>Saponin 0.5 m/v %</b>		
0.5 g	Saponin	Sigma
fill to 100 mL with PBS/BSA/azide		

<b>Formaldehyde 4 v/v %</b>		
54 mL	Formaldehyde 37 v/v %	Merck
fill to 500 mL with PBS		

### 3.1.2 Antibodies

Purchased antibodies were used as indicated by the manufacturer.

Specificity	Clone	Conjugate	Source
Stimulation of T cells			
CD3	UCHT1	–	BD Biosciences
CD28	CD28.2	–	BD Biosciences
CD2	LT2	Biotin	Miltenyi Biotec
CD3	BW264/56	Biotin	Miltenyi Biotec
CD28	15E8	Biotin	Miltenyi Biotec
Neutralizing antibodies			
IFN- $\alpha$	MMHA-11	–	R&D systems
IL-12	24910	–	R&D systems
IL-6	6708	–	R&D systems
TNF- $\alpha$	28401	–	R&D systems
ICOS-L	136726	–	R&D systems
DLL-1	HMD1-5	–	Elyaman et al., 2007 <sup>138</sup>
DLL-4		–	Ridgway et al., 2006 <sup>139</sup>
JAG-1	HMJ1-29	–	Elyaman et al., 2007 <sup>138</sup>
Immunofluorescent staining for flow cytometry			
IL-4	8D4-8	PE	BD Biosciences
IL-10	JES3-19F1	APC	BD Biosciences
IL-17	N49-653	Alexa Fluor® 647	BD Biosciences
Specificity	Clone	Conjugate	Source

IL-22	142928	PE	R&D systems
IFN- $\gamma$	25723.11	PE	BD Biosciences
IFN- $\gamma$	4S.B3	eFluor® 450	eBioscience/Affymetrix
CD4	RPA-T4	APC	BD Biosciences
CD45RO	UCHL1	PE	BD Biosciences
CD45RA	H100	FITC	BD Biosciences
CD304 (BDCA-4)	AD5-17F6	PE	Miltenyi Biotec
CD1 (BDCA-1)		FITC	Miltenyi Biotec
DLL-1	MHD1-314	PE	Miltenyi Biotec
DLL-4	MHD4-46	PE	Miltenyi Biotec
JAG-1/2	241002	PE	R&D systems
JAG-2	MHJ2-523	PE	Miltenyi Biotec

### 3.1.3 Recombinant proteins for functional assays

Protein	Concentration	Source
Cytokines		
rhIFN- $\alpha$ 2a	10 ng/mL	Miltenyi Biotec
rhTNF- $\alpha$	10 ng/mL	Miltenyi Biotec
rhIL-12	10 ng/mL	Miltenyi Biotec
rhIL-6	10 ng/mL	Miltenyi Biotec
rhIL-21	10 ng/mL	Miltenyi Biotec
rhIL-1 $\beta$	10 ng/mL	Miltenyi Biotec
rhIL-23	10 ng/mL	R&D systems
rhIL-27	10 ng/mL	R&D systems
Notch ligands		
rhDLL-1-his	coating with 1 $\mu$ g/mL	R&D systems
rhDLL-4-his	coating with 1 $\mu$ g/mL	R&D systems
rhJAG-1 Fc chimera	coating with 1 $\mu$ g/mL	R&D systems
rhJAG-2 Fc chimera	coating with 1 $\mu$ g/mL	R&D systems
Recombinant cytokine receptors for cytokine neutralization		
rhIL21 R Fc Chimera	10 $\mu$ g/mL	R&D systems

### 3.1.4 Cell-stimulating agents

Reagent	Concentration	Source
TLR agonists for stimulation of dendritic cells		
ODN 2006 (hCpG B)	0.5 $\mu$ M	TIB MolBiol
LPS ( <i>E. coli</i> )	1 $\mu$ g/mL	InvivoGen
Superantigen for T cell activation		
Staphylococcal enterotoxin B (SEB)	1 $\mu$ g/mL	Sigma-Aldrich

### 3.1.5 TLR agonists

Agonist	Concentration	Source
Cytokines		
ODN 2006 (hCpG B)	0.5 $\mu$ M	TIB MolBiol
LPS ( <i>E. coli</i> )	1 $\mu$ g/mL	InvivoGen

### 3.1.6 Real-Time PCR primers

Pre-designed primers for Real-Time PCR were ordered from Qiagen. The primers for the housekeeping gene, *UBE2D2* (ubiquitin-conjugating enzyme E2D 2), were designed in house.

Gene	Sequences
<i>UBE2D2</i>	F: GGCTTTGTTCCCAACACTTC R: ACACACGGATTTCATCAAA

Gene	QIAGEN Reference	Detected transcripts
<i>IL10</i>	Hs_IL10_1_SG QuantiTect Primer Assay	NM_000572
<i>IL22</i>	Hs_IL22_1_SG QuantiTect Primer Assay	NM_020525
<i>IFNG</i>	Hs_IFNG_1_SG QuantiTect Primer Assay	NM_000619
<i>MAF</i>	Hs_MAF_1_SG QuantiTect Primer Assay	NM_005360, XM_001134279
<i>PRDM1</i>	Hs_PRDM1_1_SG QuantiTect Primer Assay	NM_001198

### 3.1.7 Short interfering RNAs (siRNAs)

Short interfering RNAs (siRNA) against human *STAT1*, *STAT3*, *STAT4*, *PRDM1* and a non-binding control siRNA were designed. The siRNA against human *MAF* was based on the siRNA described by Moreaux et al., 2007<sup>140</sup>.

To increase knockdown efficiency and duration, siRNAs were ordered with chemical modifications according to the protocol described in Mantei et al., 2008<sup>141</sup>. The siRNAs were synthesized and supplied by IBA GmbH.

<b>siSTAT1</b>	
sense	5' ACA GAA AGA GCU UGA CAG TAA AG 3'
antisense	3' UAU CUU UCU CGA ACU GUC AUT 5'
<b>siSTAT3</b>	
sense	5' GCG GAG AAG CAU CGU GAG TGA GC 3'
antisense	3' CGC CUC UUC GUA GCA CUC ACT 5'
<b>siSTAT4</b>	
sense	5' AAA GAC AAA GCC UUC GGT AAA CA 3'
antisense	3' TTU CUG UUU CGG AAG CCA UUT 5'
<b>siPRDM1</b>	
sense	5' GAC GGC UUU AAU GAA GAG AAA AG 3'
antisense	3' CTG CCG AAA UUA CUU CUC UUT 5'
<b>siMAF</b>	
sense	5' AAA CGG CUC GAG CAG CGA CAA CC 3'
antisense	3' TTU GCC GAG CUC GUC GCU GUT 5'
<b>siCONTROL</b>	
sense	5' AAU UCU CCG AAC GUG UCA CGT TT 3'
antisense	3' TTA AGA GGC UUG CAC AGU GCA 5'

## 3.1.8 Materials for magnetic cell sorting (MACS)

Component	Source
Beads for magnetic cell sorting (MACS)	
CD4 MicroBeads, human	Miltenyi Biotec
CD45RO MicroBeads, human	Miltenyi Biotec
CD45RO MicroBeads, human	Miltenyi Biotec
CD19 MicroBeads, human	Miltenyi Biotec
anti-FITC MicroBeads, human	Miltenyi Biotec
anti-PE MicroBeads, human	Miltenyi Biotec

## 3.1.9 Other reagents

Component	Description/Use	Source
Ficoll Paque PLUS		Amersham Biosciences
CFDA-SE		Sigma-Aldrich
PMA		Sigma-Aldrich
Ionomycin		Sigma-Aldrich
Brefeldin A		Sigma-Aldrich
Polybren		Sigma-Aldrich
Insolution $\gamma$ -secretase inhibitor X	125 nM final concentration in culture	Calbiochem
AhR agonist (CH-223191)	1 nM final concentration in culture	Calbiochem
BD Phosflow Fix Buffer		BD Biosciences
BD Phosflow Perm Buffer III		BD Biosciences
LightCycler® FastStart DNA Master SYBR Green I		Roche Applied Science

## 3.1.10 Other materials

Component	Description/Use	Source
Anti-Biotin MACSiBead Particles, cell culture grade		Miltenyi Biotec
Nunc MaxiSorp® flat-bottom 96 well plate		eBioscience
LightCycler® 20 $\mu$ l capillaries		Roche Applied Science

## 3.1.11 Kits

<b>Kit</b>	<b>Source</b>
IL-10 Secretion Assay - Cell Enrichment and Detection Kit (PE), human	Miltenyi Biotec
FASER-Kit PE	Miltenyi Biotec
RNeasy Mini Kit	Qiagen
RNase-Free DNase Set	Qiagen
Amaxa® Human T Cell Nucleofector® Kit	Lonza

## 3.1.12 Instruments

<b>Instrument</b>	<b>Manufacturer</b>
Amaxa Nucleofector® II	Lonza
AutoMACS Pro	Miltenyi Biotec
Roche LightCycler 1.0	Roche Applied Science
BD FACS Calibur – Flow cytometer	BD Biosciences
BD LSR II – Flow cytometer	BD Biosciences

## 3.1.13 Software

<b>Software</b>	<b>Source</b>
FlowJo	Tree Star

## 3.2 Isolation of naïve T helper cells and dendritic cells

### 3.2.1 Density gradient centrifugation

Density gradient centrifugation can be used to separate leukocytes from whole blood. This method exploits the fact that leukocytes have a lower density than other blood cells like erythrocytes. A sugar-based medium with a density coefficient exactly between leukocytes and erythrocytes is used to separate those cell types. When a mixed suspension of erythrocytes and leukocytes is layered on top of such a medium the erythrocytes will sink to the bottom, while leukocytes will float on top of the medium. The separation process is accelerated by centrifugation.

#### 3.2.1.1 *Separation of PBMC from whole blood*

Falcon™ tubes (50 mL) with 15 mL of Ficoll-Paque PLUS were prepared. Whole blood was diluted with PBS in a 1:1 ratio and layered on top of the Ficoll solution. After centrifugation ( $800 \times g$ , 20 °C, 20 min, no brake) most of the uppermost layer was removed, leaving only approx. 10 mL to 15 mL on top of the interphase. The PBMC in the interphase were transferred into a separate tube by pipetting. The obtained PBMC were pelleted ( $300 \times g$ , 10 min, 4 °C), two times washed with PBS/BSA and after resuspension in PBS/BSA stored at 4 °C for further processing

### 3.2.2 Magnetic cell sorting

Magnetic cell sorting (MACS, Miltenyi Biotec) is based on the labeling of cells with small super-paramagnetic beads (microbeads), allowing isolation of the labeled cells from a cell suspension. The beads consist of dextran and iron oxide, have a size of 20 nm to 100 nm and are covalently bound to ligands or monoclonal antibodies specific for a cell surface marker. Cells that are bound by means of the ligand or antibody become magnetically labeled and can be separated from unlabeled cells by letting the cell suspension pass through a dedicated column placed in a strong magnetic field. Following several washing steps (e.g. with PBS/BSA/EDTA) that clear the column of unlabeled cells, the retained labeled cells can be eluted after removing the magnetic field. The result of this procedure are two fractions, a magnetically labeled fraction that is positive for the marker and a non-magnetic unlabeled fraction that is negative for the marker. Different types of columns use different matrices that have been either optimized to retain as little as possible unlabeled

cells or as many as possible labeled cells. This way magnetic cell sorting can be used to either highly enrich cells that are positive for a certain marker or deplete a cell suspension of a distinct cell population. Purity of the positive or negative fraction after enrichment or depletion, respectively, is usually in the range of 95 % to 99 %. In most cases, labeling cells with magnetic beads does not affect immunofluorescent staining and the behavior of sorted cells in cell culture is not altered by the labeling with magnetic beads. The sorting protocol can be performed manually or using an automated cell sorter (AutoMACS, Miltenyi Biotec).

#### 3.2.2.1 *Labeling of cells with magnetic beads*

A total  $1 \times 10^7$  cells were pelleted by centrifugation at  $300 \times g$  to  $400 \times g$ , resuspended in 100  $\mu$ L PBS/BSA solution containing the amount of marker-specific beads recommended by the manufacturer and incubated at 4 °C to 8 °C for 15 min. After incubation, the cells were washed two times with 1 mL of PBS/BSA and resuspended in 100  $\mu$ L RPMI medium or PBS/BSA buffer. For higher cell numbers this protocol was adapted accordingly.

#### 3.2.2.2 *Enrichment of naïve T helper cells*

Between  $1 \times 10^8$  to  $1 \times 10^9$  peripheral blood mononuclear cells (PBMC) were labeled with magnetic beads specific for CD19 and CD45RO and CD19- and CD45RO-positive cells were depleted with AutoMACS using the protocol “Deplete”. The CD19- and CD45RO-negative cell fraction was then labeled with beads specific for CD4 and sorted for CD4-positive cells with AutoMACS and the program for positive selection “Posseld2”. An aliquot of the positive cell fraction was stained for CD4, CD45RA and CD45RO and analyzed by flow cytometry. The purity of CD4<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup> cells was  $\geq 95$  %.

#### 3.2.2.3 *Enrichment of dendritic cells*

Between  $1 \times 10^8$  to  $1 \times 10^9$  peripheral blood mononuclear cells (PBMC) were labeled with magnetic beads specific for CD19 and CD14 and CD19- and CD14-positive cells were depleted with AutoMACS using the protocol “Deplete”. The CD19- and CD14-negative cell fraction was then stained with fluorescently labeled antibodies specific for the pDC marker CD304 (BDCA-4) and the mDC marker CD1 (BDCA-1), the stained cells were labeled with beads specific for the fluorochromes conjugated to the antibodies and sorted with AutoMACS and the program for positive selection “Posseld2”. Using flow cytometry-based cell sorting pDCs and mDCs were isolated from the cell fraction obtained from the MACS sort. The purity of BDCA-4<sup>+</sup>CD1<sup>-</sup>CD19<sup>-</sup>CD14<sup>-</sup> cells (pDCs) and of CD1<sup>+</sup>BDCA-4<sup>-</sup>CD19<sup>-</sup>CD14<sup>-</sup> cells (mDCs) was  $\geq 98$  %.

\* each washing step consisted of resuspending the cells in the indicated amount of buffer and pelleting by centrifugation at  $300 \times g$  to  $400 \times g$ .

### 3.3 Analysis of cell surface and intracellular markers

#### 3.3.1 Flow cytometry

Flow cytometry or fluorescence-activated cell sorting (FACS) is a method for analysis of cell populations for certain fluorescently labeled markers on a single-cell basis.

Fluorescently labeled cells are hydrodynamically focused and pass an array of lasers of different wavelengths. The laser light excites fluorochromes attached to cell-bound antibodies, which then fluoresce in a defined range of wavelengths. Laser light is also scattered in small angles (between  $3^\circ$  to  $10^\circ$ ) by the cells themselves (forward scatter, FSC) as well as by approx.  $90^\circ$  (side scatter, SSC). The forward scatter approximately correlates with the cell size, whereas the side scatter carries information about granularity and membrane folding. Different systems of band pass filters and detectors record the scattered light as well as the emitted fluorescent light. The detected fluorescent light and the scatter light are used to determine the properties of the analyzed cell population in regard to labeled markers and cell properties.

This data produced by the FACS machine is analyzed by specialized software that allows separation ("gating") of cell populations based on the measured properties and/or cell markers.

During standard FACS analysis, analyzed cells are discarded after measuring the cell properties. In a different approach the same principles can be used to specifically isolate cell populations for later use in experiments. In this scenario the cells are not discarded but separated according to their properties and the desired cell population is collected. This is done by computer-controlled modulation of an electric field that can redirect the electrically charged drops containing the cells, thus separating the cells of interest from cells that are to be discarded. Commonly, purity after cell sorting by flow cytometry is  $\geq 99\%$ .

The principles of flow cytometry have been extensively reviewed by Ibrahim and Van Den Engh, 2007<sup>142</sup>.

#### 3.3.2 Immunofluorescent staining of cellular markers

For an analysis by flow cytometry, the cell markers of interest need to be labeled with fluorochromes. This is usually done with fluorochromes conjugated to antibodies that are

specific for the desired cell marker. To analyze different markers in one experiment fluorochromes that emit at different wavelengths are used for distinct cell markers.

Different staining protocols exist, depending on whether cell surface markers or intracellular markers are to be examined.

Staining of cell surface markers can be performed on living cells, which, if desired, can be sorted by FACS and used for later experiments. In contrast, staining of intracellular markers requires permeabilization of the cell membrane in order for the marker-specific antibodies to reach their target. However, permeabilization must be preceded by fixation of the cells, which prevents loss of the cell contents. Both, intracellular staining and staining of cell surface markers can be combined.

#### 3.3.2.1 *Immunofluorescent staining of cell surface markers*

Between  $1 \times 10^5$  and  $1 \times 10^6$  cells were pelleted by centrifugation at  $300 \times g$  to  $400 \times g$  and resuspended in 100  $\mu\text{L}$  of staining solution. The staining solution contained fluorochrome-labeled antibodies diluted in PBS/BSA according to the indications of the manufacturer. The cell suspension was incubated at  $4^\circ\text{C}$  for 7 min to 10 min. After incubation, the cells were washed \* with 1 mL of PBS/BSA and, a second time, with 500  $\mu\text{L}$  PBS/BSA, then pelleted again and resuspended in 100  $\mu\text{L}$  of PBS/BSA and kept on ice or at  $4^\circ\text{C}$  until use.

#### 3.3.2.2 *Intracellular immunofluorescent staining of cytokines*

##### Fixation

A total of  $1 \times 10^6$  to  $1 \times 10^7$  cells were washed \* in 500  $\mu\text{L}$  of PBS and pelleted by centrifugation at  $300 \times g$  to  $400 \times g$ . The cell pellet was resuspended in 250  $\mu\text{L}$  of PBS and the same volume of 4 m/v % formaldehyde was added. The cells were incubated at  $4^\circ\text{C}$  for 20 min. The fixation was stopped by adding 500  $\mu\text{L}$  of PBS/BSA, pelleting the cells and washing \* again with 1 mL of PBS/BSA.

##### Permeabilization and intracellular staining

The fixated cells were resuspended in staining solution. The staining solution was based on 0.5 m/v % saponin and contained fluorescently labeled antibodies at the concentration recommended by the manufacturer. The cell suspension was incubated at RT for 20 min. After the staining, the cells were washed \* two times in 1 mL 0.5 m/v % saponin, followed by a washing step \* with 1 mL of PBS/BSA and resuspension in 100  $\mu\text{L}$  of PBS/BSA/azide. The stained cells were kept at  $4^\circ\text{C}$  until use.

### 3.3.2.3 *Immunofluorescent staining of phosphorylated STAT*

#### Fixation

A total of  $1 \times 10^6$  to  $1 \times 10^7$  cells were washed \* in 500  $\mu$ L of PBS and pelleted by centrifugation at  $300 \times g$  for 10 min. The cell pellet was resuspended in 1 mL of prewarmed ( $37^\circ\text{C}$ ) 1X BD™ Phosflow Fix Buffer (diluted with distilled  $\text{H}_2\text{O}$ ) and incubated at  $37^\circ\text{C}$  for 10 min. The fixation was stopped by pelleting the cells and washing \* with 1 mL of PBS/BSA.

#### Permeabilization

The fixated cells were resuspended in ice cold (or  $-20^\circ\text{C}$ ) BD™ Phosflow Perm Buffer III and incubated for 30 min on ice. After the incubation, the cells were pelleted and washed \* with 1 mL of PBS/BSA.

#### Intracellular staining

The pelleted cells were resuspended in 100  $\mu$ L of staining solution. The staining solution was based on PBS/BSA and contained fluorescently labeled antibodies at the concentration recommended by the manufacturer. The cell suspension was incubated at RT for 20 min. After the staining, the cells were washed \* two times in 1 mL PBS/BSA and resuspended in 100  $\mu$ L of PBS/BSA. The stained cells were kept at  $4^\circ\text{C}$  until use.

### 3.3.2.4 *Enhancement of Notch ligand staining*

The FASER kit (Miltenyi Biotec) allows enhancement of low immunofluorescence by iterative use of biotin-labeled anti-fluorochrome antibodies and fluorochrome-coupled anti-biotin antibodies. Since cell surface expression of Notch ligands is low compared to other cell surface markers, immunofluorescent staining of Notch ligands with PE-conjugated antibodies on the surface of T cells was enhanced by two staining cycles with the FASER Kit – PE (Miltenyi Biotec). The enhancement was performed according to the protocol of the manufacturer. Control stainings were performed following the same procedure but omitting the Notch-ligand-specific antibody.

\* each washing step consisted of resuspending the cells in the indicated amount of buffer and pelleting by centrifugation at  $300 \times g$  for 10 min.

### 3.4 T cell culture

#### 3.4.1 T cell stimulation in the pDC system

Sorted plasmacytoid dendritic cells (BDCA-4<sup>+</sup>CD1<sup>-</sup>CD19<sup>-</sup>CD14<sup>-</sup>) were incubated with sorted naïve T helper cells (CD4<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup>CD19<sup>-</sup>) in a ratio of 1:10 at a total cell number of  $1 \times 10^5$  to  $5 \times 10^5$  cells in round bottom 96-well plates in a volume of 100  $\mu$ L to 150  $\mu$ L of RPMI 1640 medium. For the activation of pDCs the culture medium contained 0.5  $\mu$ M ODN 2006 (human CpG B) and for activation of T cells 1  $\mu$ g/mL staphylococcal enterotoxin B (SEB). Soluble factors, like cytokines or antibodies, were added to the medium when needed and as described in the results, using the concentrations listed in the materials section. The cells were incubated for 5 to 7 days before cytokine analysis. Medium was replenished when necessary.

#### 3.4.2 T cell stimulation in mDC/T cell coculture

Sorted myeloid dendritic cells (CD1<sup>+</sup>BDCA-4<sup>-</sup>CD19<sup>-</sup>CD14<sup>-</sup>) were incubated with sorted naïve T helper cells (CD4<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup>CD19<sup>-</sup>) in a ratio of 1:10 at a total cell number of  $1 \times 10^5$  to  $5 \times 10^5$  cells in round bottom 96-well plates in a volume of 100  $\mu$ L to 150  $\mu$ L of RPMI 1640 medium. For the activation of mDCs the culture medium contained 1  $\mu$ g/mL LPS (*E.coli*) and for activation of T cells 1  $\mu$ g/mL staphylococcal enterotoxin B (SEB). Soluble factors, like cytokines or antibodies, were added to the medium when needed and as described in the results, using the concentrations listed in the materials section. The cells were incubated for 5 to 7 days before cytokine analysis. Medium was replenished when necessary.

### 3.4.3 T cell stimulation in the APC-free system

#### 3.4.3.1 *T cell stimulation with MACSiBeads*

Anti-biotin/anti-his MACSiBeads were coated with biotinylated activating anti-CD2, anti-CD3 and antiCD28 antibodies and, if needed, with his-tagged rhDLL-4.

Coating protocol:

- incubation at 4 °C for 12 h

5 × 10 <sup>7</sup> MACSibeads in 1 mL PBS
5 µg anti-CD2-biotin
5 µg anti-CD3-biotin
5 µg anti-CD28-biotin
10 µg rhDLL4 (if required)

- two times washing with 1 mL PBS/BSA
- resuspension in 1 mL PBS/BSA

Sorted naïve T helper cells (CD4<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup>CD19<sup>-</sup>) were incubated with MACSiBeads in a ratio of 1:10 at a total cell number of 1 × 10<sup>5</sup> to 5 × 10<sup>5</sup> cells in round bottom 96-well plates in a volume of 100 µL to 150 µL of RPMI 1640 medium. Soluble factors, like cytokines or antibodies, were added to the medium when needed and as described in the results, using the concentrations listed in the materials section. The cells were incubated for 5 to 7 days before cytokine analysis. Medium was replenished when necessary.

#### 3.4.3.2 *T cell stimulation in coated plates (test of different Notch ligands)*

High-binding 96-well flat bottom culture plates were coated with activating anti-CD3 antibodies and, if needed, with recombinant Notch ligands (DLL-1, DLL-4, JAG-1 or JAG-2).

Coating protocol:

- incubation at 4 °C for 6 h

100 µL PBS per well
0.1 µg anti-CD3
1 µg Notch ligand (if required)

- two times washing with 200 µL PBS/BSA per well
- fill with 200 µL PBS/BSA per well

A total cell number of  $1 \times 10^5$  to  $5 \times 10^5$  sorted naïve T helper cells (CD4<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup>CD19<sup>-</sup>) were incubated in coated 96-well plates in a volume of 100  $\mu$ L to 150  $\mu$ L of RPMI 1640 medium and 0.5  $\mu$ g/mL soluble activating anti-CD28. Soluble factors, like cytokines or antibodies, were added to the medium when needed and as described in the results, using the concentrations listed in the materials section. The cells were replated into non-coated 96-well plates after 48 h. In total, the cells were incubated for 5 to 7 days before cytokine analysis. Medium was replenished when necessary.

#### 3.4.4 Labeling of T cells with proliferation marker (CFDA-SE)

For the control of T cell proliferation, sorted T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) before cell culture.

Cells were washed in PBS, pelleted by centrifugation ( $300 \times g$ , 4 °C, 10 min) and resuspended to a density of  $1 \times 10^7$  cells/mL in PBS with 1  $\mu$ M CFDA-SE. After 3 min of incubation at RT the labeling was stopped by adding a surplus of medium (RPMI 1640 with 10 % FCS).

#### 3.4.5 Cytokine recall

For analysis of cytokine expression, a Ca<sup>2+</sup>-dependent cytokine recall (PMA, 50 ng/mL, plus ionomycin, 1  $\mu$ g/mL) was elicited in the presence of Brefeldin A, which blocks release of newly synthesized cytokines by inhibiting anterograde transport of proteins from the endoplasmic reticulum. After 4 h to 6 h, the cells were fixated and stored at 4 °C.

## 3.5 Analysis of mRNA expression

### 3.5.1 RNA extraction

RNA extraction from T cells was performed using the RNeasy Kit (Qiagen) and the RNase-Free DNase Set (Qiagen).

A maximum of  $1 \times 10^7$  cells was pelleted by centrifugation ( $300 \times g$  for 10 min) and 350  $\mu\text{L}$  of lysis buffer containing 2-mercaptoethanol were added. The solution with the lysed cells was either directly used for mRNA-extraction or stored at  $-80^\circ\text{C}$  for later use. All the following steps of mRNA extraction were performed at RT. The suspension was homogenized using syringes (1 mL, diameter of needle 0.7 mm). The following steps were performed according to the manufacturer's protocol (*RNeasy Mini Protocol for Isolation of Total RNA from Animal Cells, Spin protocol*). In addition, DNA on the RNeasy spin column was digested using the RNase-Free DNase Set and according to the manufacturer's protocol (*Optional On-Column DNase Digestion with the RNase-Free DNase Set*). The isolated mRNA was eluted in 30  $\mu\text{L}$  RNase-free water and stored at  $-80^\circ\text{C}$ .

### 3.5.2 Reverse Transcription

Reverse transcription of extracted mRNA was performed using the TaqMan® Reverse Transcription Reagents (Applied Biosystems). The following tables describe the reaction mix for one reaction and the thermal profile.

Reagent	Volume
TaqMan reaction buffer	2.0 $\mu\text{L}$
$\text{MgCl}_2$ (25 mM)	4.4 $\mu\text{L}$
dNTPs (2.5 mM)	4.0 $\mu\text{L}$
Random hexamer primers (50 $\mu\text{M}$ )	0.5 $\mu\text{L}$
Oligo (dT) <sub>16</sub> primers	0.5 $\mu\text{L}$
RNase inhibitor (20 U/ $\mu\text{L}$ )	0.4 $\mu\text{L}$
Reverse transcriptase (50 U/ $\mu\text{L}$ )	0.5 $\mu\text{L}$
mRNA extract	7.5 $\mu\text{L}$
<b>Total volume</b>	<b>20.0 <math>\mu\text{L}</math></b>

Reaction mix, reverse transcription

Time	Temperature
10 min	25 °C
40 min	48 °C
5 min	95 °C
hold	4 °C

Thermal profile, reverse transcription

### 3.5.3 Real-Time PCR

Reaction mix, SYBR Green and DNA polymerase were mixed immediately before the start of the PCR (ratio [enzyme]:[reaction mix + SYBR Green I] = 1:6.4).

*UBE2D2* (ubiquitin-conjugating enzyme E2D 2) was used as housekeeping gene for all targets<sup>143</sup>. The reactions were performed in a volume of 6 µL.

PCR mix for targets <i>IL10, IL22, IFNG, MAF, PRDM1</i> (2.5 mM MgCl <sub>2</sub> )	
Reagent	Volume
H <sub>2</sub> O	1.2 µl
MgCl <sub>2</sub> (25 mM)	0.6 µl
Reaction Mix + Enzyme	0.6 µl
Primer mix	0.6 µl
cDNA	3.0 µl

PCR mix for housekeeping gene <i>UBE2D2</i> (3 mM MgCl <sub>2</sub> )	
Reagent	Volume
H <sub>2</sub> O	1.08 µl
MgCl <sub>2</sub> (25 mM)	0.72 µl
Reaction Mix + Enzyme	0.60 µl
Primer F	0.30 µl
Primer R	0.30 µl
cDNA	3.00 µl

PCR mixes

	Temperature	Time	Ramping
Denaturation	95 °C	9 min	20 °C/s
Amplification (single fluorescence measurement at 72 °C)	95 °C	15 s	20 °C/s
	60 °C	15 s	20 °C/s
	72 °C	20 s ( <i>UBE2D2</i> : 30 s)	20 °C/s
Melting curve (continuous fluorescence measure- ment from 60 °C up to 95 °C)	95 °C	10 s	20 °C/s
	60 °C	20 s	20 °C/s
	95 °C	0 s	0.1 °C/s
Cool down	40 °C	30 s	20 °C/s

PCR program

### 3.6 *in vitro* suppression assay

#### 3.6.1 IL-10 secretion assay

Secretion assays (Miltenyi Biotec) are based on the coupling of a so-called affinity matrix to the cell surface of cytokine secreting cells. The affinity matrix has two specific binding sites, one attaches it to the cell surface (usually by binding the ubiquitous leukocyte surface marker CD45), the other binding site “catches” a specific cytokine secreted by the cell. A crucial factor is a sufficiently low cell density during the secretion phase, in order to avoid that cytokine from neighboring cells is caught by the matrix. The bound cytokines can be visualized by immunofluorescent staining. This method has the advantage that cytokine production can be analyzed on a single cell basis without killing the cell by fixation and permeabilization. This means the stained cells can also be sorted via flow cytometry and used in downstream experiments.

#### 3.6.2 Generation and isolation of IL-10-producing CD4 T cells

The *IL-10 Secretion Assay - Cell Enrichment and Detection Kit (APC)*, human, by Miltenyi Biotec was used to isolate IL-10-producing T cells from cell culture.

Naïve sorted CD4 T cells were stimulated either in the pDC system or in the APC-free system. After 7 days of culture the cells were stained with the catch matrix of the IL-10 secretion assay and a  $\text{Ca}^{2+}$ -dependent (PMA/Ionomycin) cytokine recall was elicited. In the following the secretion assay was performed according to the manufacturer’s protocol. After the secretion assay, IL-10-producing and non-IL-10-producing cells were isolated via flow cytometry.

#### 3.6.3 The *in vitro* suppression assay

Freshly sorted, naïve CD4 T cells ( $\text{CD4}^+\text{CD45RA}^+\text{CD45RO}^-$ ) were labeled with CFDA-SE and – as “indicator cells” – cocultivated in a 1:1-ratio with IL-10 producers or non-IL-10 producers. Sorted antigen-presenting cells (APC,  $\text{CD14}^+\text{CD19}^+$ ) were added to the T cells in a ratio of 1:5. T cells and APCs were stimulated with 0.5  $\mu\text{g/mL}$  SEB and 0.5  $\mu\text{g/mL}$  LPS (*E. coli*). After 4 days of culture the proliferation profile of the “indicator” cells was assessed by flow cytometry.

## 3.7 siRNA-mediated knockdown of transcriptions factors

### 3.7.1 RNA interference

RNA interference by short interfering RNAs (siRNA) is an evolutionary old process in eukaryotic cells for the targeted degradation of mRNA and inhibition of gene expression, playing diverse roles from defense against viruses to cell-internal gene regulation. The process of siRNA-based RNA interference starts from short double-stranded RNAs that, in the cell, are recognized by a specialized protein complex. One strand of the RNA duplex is incorporated into an mRNA-degrading protein complex (RISC complex), which confers sequence specificity to the mRNA-cleaving machinery, targeting it at any complementary mRNA strand. The following degradation of mRNA leads to a specific reduction of expression of this gene.

### 3.7.2 Transfection of primary T cells with siRNA

By introducing siRNAs into cells the RNAi machinery of these cells can be exploited to specifically inhibit gene expression (gene knockdown). A potent method of transferring siRNA into primary T cells is the Nucleofector™ technology, a specialized type of electroporation, which by a combination of specific solutions and patterns of electric pulses creates short-lived openings in the cell membrane allowing entry of small molecules, like siRNA, from the surrounding solution into the cell. Mantei et al., 2008 <sup>141</sup> had shown that, using this technology, siRNA can be transfected into primary T cells with close to 100 % transfection efficiency, resulting in a uniform knockdown of the expression of the targeted gene.

#### 3.7.2.1 *Transfection protocol*

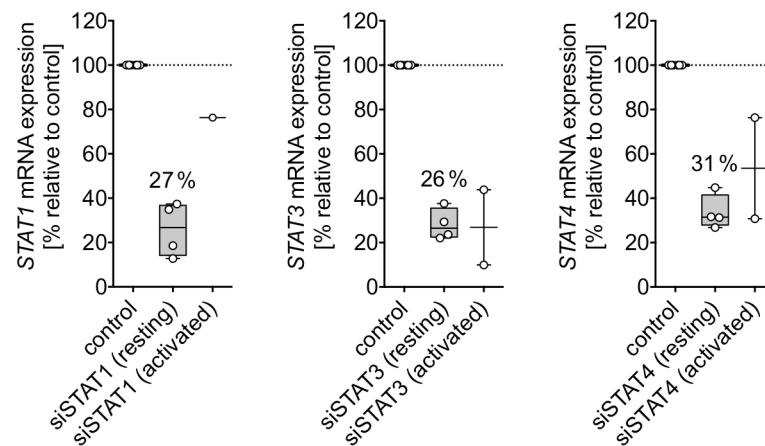
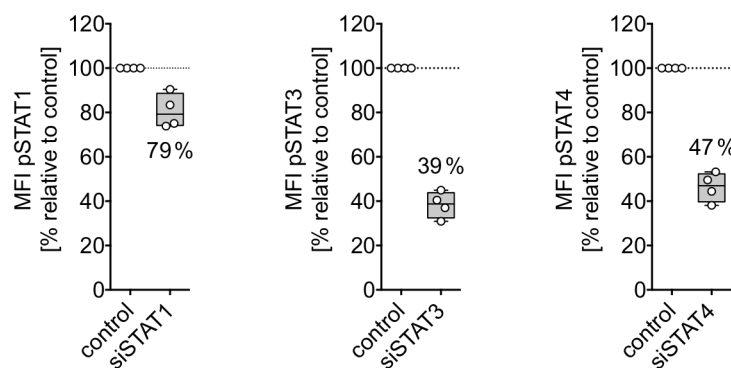
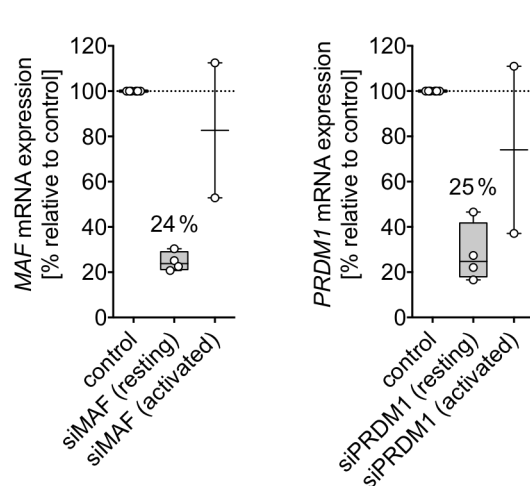
Up to  $5 \times 10^6$  human T helper cells per transfection were pelleted by centrifugation ( $300 \times g$ , 4 °C, 10 min) and resuspended in 100 µL of transfection medium provided with the transfection kit (*Human T Cell Nucleofector® Solution*). Up to 10 µL containing up to 1 nmol of siRNA were added, the cell suspension mixed by pipetting and transferred into a cuvette and the cells transfected in a Nucleofector II device using transfection program “X-001”. Directly after the program had finished, 500 µL of prewarmed medium (RPMI 1640, 10 % FCS, 37 °C) were added and the cells transferred into 24-well plates containing 1 mL of prewarmed medium (RPMI 1640, 10 % FCS, 37 °C). After a resting pe-

riod of 2 h (activated T cells) or 24 h (*ex vivo* T cells) the cells were pelleted by centrifugation ( $300 \times g$ , 4 °C, 10 min) and used in cell culture.

### 3.7.3 Knockdown efficiency

Short interfering RNAs (siRNA) against human *STAT1*, *STAT3*, *STAT4*, *MAF*, *PRDM1* and a non-binding control siRNA with stabilizing chemical modifications as described by Mantei et al., 2008<sup>141</sup>, were tested on sorted *ex vivo* naïve CD4 T cells. The T cells were transfected with 1 nmol of siRNA and then rested for one day. Transcription levels (mRNA) were determined either directly after the resting phase (resting T cells) or after polyclonal activation and two additional days of culture. STAT protein levels after knockdown were determined after the resting phase using IFN- $\alpha$  to induce phosphorylation and intracellular staining and flow cytometry analysis of phosphorylated STATs.

Average reduction of *STAT1*, *STAT3* and *STAT4* mRNA levels was 70 % to 75 % (Fig. 3.1 A). On the protein level average (median) reduction was approx. 20 %, 60 % and 55 % for *STAT1*, *STAT3* and *STAT4*, respectively (Fig. 3.1 B). Activation of the transfected T cells partially restored mRNA levels of *STAT1* and *STAT4* but not *STAT3* (Fig. 3.1 A).

**A | *STAT1*, *STAT3* and *STAT4* mRNA expression****B | *STAT1*, *STAT3* and *STAT4* protein expression****Fig. 3.1: Knockdown of *STAT1*, *STAT3* and *STAT4* in naïve human CD4 T cells****Fig. 3.2: Knockdown of *MAF* and *PRDM1* in human CD4 T cells**

The *MAF*- and *PRDM1*- specific siRNAs reduced the target mRNA levels in average by 75 %. T cell activation followed by two days of culture partially or completely restored mRNA levels. The speed at which mRNA levels were restored to normal differed between donors,

presumably owed to a varying capacity of the cells of different donors to proliferate at this early point in time.

#### 3.7.3.1 *siRNA transfection of T cells for cell culture*

If not stated otherwise the amount of siRNA used for knockdown experiments was 1 nmol of siRNA against human *STAT1*, *STAT4*, *MAF*, *PRDM1* or of control siRNA. Since the siRNA against *STAT3* was very potent and a too strong *STAT3* knockdown proved detrimental to cell survival, only 10 pmol of the siRNA against *STAT3* was used in transfections of cells for cell culture. Mantei et al. <sup>141</sup> had shown that a lower amount of siRNA only slightly affects knockdown efficiency, but rather impairs knockdown longevity.

## 3.8 Retroviral transduction

### 3.8.1 Generation of viral preparations

HEK293T cells were seeded in 10 cm dishes at a density of  $5 \times 10^6$ /mL in 10 mL of DMEM medium with 10 % FCS. The next day the cells were transfected using the  $\text{Ca}_2\text{PO}_4$  method. For the transfection, 5  $\mu\text{g}$  of the pseudotyping vector, 10  $\mu\text{g}$  of the envelope vector, and 15  $\mu\text{g}$  of the retroviral expression vector were mixed with 200  $\mu\text{L}$  of  $\text{CaCl}_2$  solution and 750  $\mu\text{L}$  of water in FACS tubes. Continuously vortexing the solution 1 mL of 2X HBS buffer was added very slowly. After incubating the solution for several minutes at RT it was added dropwise to the cells. After 4 h the cells were washed two times with PBS and incubated with fresh medium for 48 h. After incubation, the supernatant was removed, filtered (0.45  $\mu\text{m}$  pore width), supplemented with 10 mM HEPES as buffer and stored at 4 °C,

### 3.8.2 Infection of T cells

Naïve sorted T helper cells were stimulated with platebound anti-CD3 antibody and soluble anti-CD28. At approx. 48 h after stimulation the T cells were infected with virus. This was done by removing most of the supernatant and adding the viral preparation supplemented with polybren (8  $\mu\text{g}/\text{mL}$ ) to the cells (1 mL per well for a 24-well plate). The plate with the cells and the viral preparation was centrifuged at  $700 \times g$  for 75 min at 32 °C. Following centrifugation, the supernatant containing the virus was replaced by the cell culture supernatant that had been removed and stored before.

## 3.9 Statistics

Statistical significance (p-value) was determined using the Wilcoxon signed rank test (paired, two-tailed) for samples with more than 6 values (donors). Statistical significance (p-value) for samples with 4 to 6 values (donors) was estimated using Student's t-test (paired, two-tailed). p-values were calculated using GraphPad Prism 6.

## 4 Aims and objectives

The cytokines IL-10 and IL-22 are produced by a variety of immune cells and play important roles in the modulation of the immune reaction and the protection and maintenance of epithelial barriers, respectively. The importance of those two cytokines in the context of immune responses is underlined by the fact that both, IL-10 and IL-22, are produced by T helper cells, one of the central cell types orchestrating the immune response. Although considerable research has been done on the expression of IL-10 and IL-22 by T helper cells, the regulation of both cytokines in human is still poorly understood. This is not only owed to the finding that IL-10 is differently regulated in the various T helper cell subsets, but also to the fact that most data in regard to the regulation of IL-10 and IL-22 stem from mouse experiments.

This project aimed to further the understanding of the regulation of IL-10 and IL-22 expression in human by analyzing the factors and pathways governing expression of IL-10 and IL-22 in the interaction of human T helper cells and plasmacytoid dendritic cells (pDC), a subset of professional antigen-presenting cells that had been indicated to be a potent inducer of both cytokines in T cells.

A T cell/pDC model system was established and analyzed in regard to the expression of IL-10 and IL-22 as well as other T helper cell hallmark cytokines in order to define the ground state. This system was used to test whether cytokines and transcriptional modules known to play a role in mouse were also involved in driving IL-10 and IL-22 expression by human T helper cells.

The factors identified in the T cell/pDC model system were further investigated in a minimalistic culturing system that allowed analysis of the modulation of cytokine expression in response to different factors. Reproduction of the T cell phenotype observed in the T cell/pDC model in this minimalistic system would serve as a test of the validity of the findings.

By identifying the layers that govern regulation of key players of the immune response, namely IL-10 and IL-22, this study helps to advance the understanding of immune regulation in human, and may aid in translating basic research into therapeutic applications based on targeted immunomodulation.

## 5 Results

## 5.1 Induction of IL-10 and IL-22 by pDCs

In mouse, TLR9-activated plasmacytoid dendritic cells (pDC) had been shown to induce IL-10-expressing T<sub>H</sub>1 cells in a Notch- and DLL-4-dependent fashion <sup>144</sup>. Although tolerogenic pDCs had been described earlier, most reports ascribed this capacity to immature pDCs <sup>145</sup>. In contrast, the Notch-dependent induction of IL-10-producing T<sub>H</sub>1 cells by pDCs constituted a novel mechanism by which pDCs modulate the immune response.

This raised the question whether human pDCs employ the same mechanism and, if so, which regulation modules might be active in this context.

### 5.1.1 pDCs induce a mixed population of T cells expressing IFN- $\gamma$ , IL-10 and IL-22

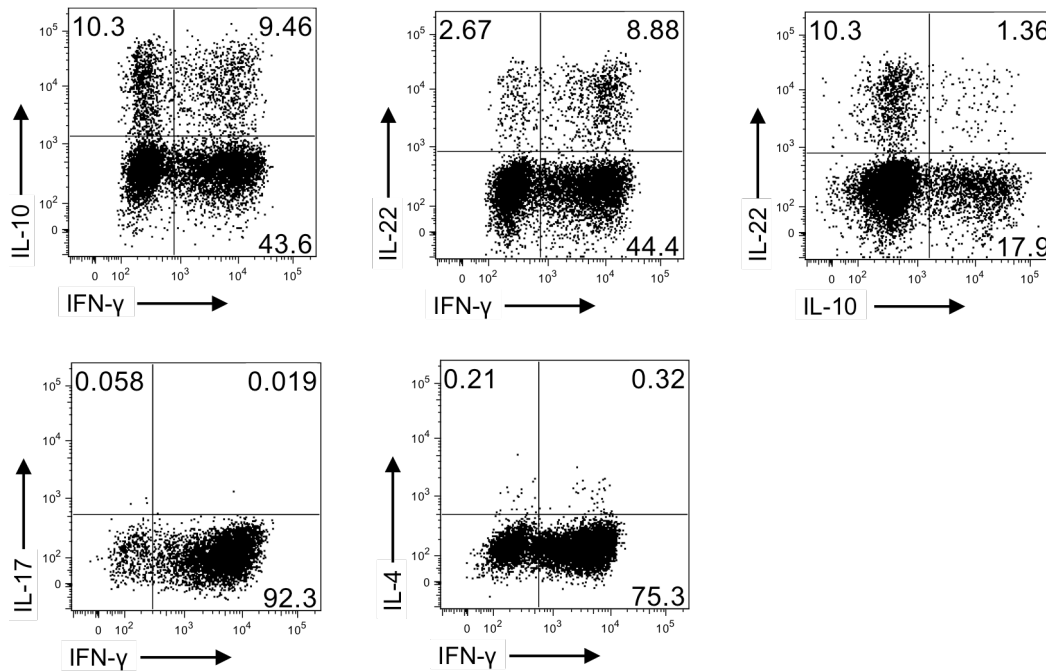
A coculture of pDCs and naïve human CD4 T cells was established as reference system ("pDC system"). In this system purified pDCs and sorted CD45RA<sup>+</sup>CD45RO<sup>-</sup>CD4<sup>+</sup> T cells (from here on referred to as "naïve CD4 T cells") isolated from peripheral human blood were cocultivated in the presence of superantigen SEB (staphylococcus B enterotoxin) and TLR9 agonist CpG B under non-polarizing conditions. After 5 to 7 days of culture cytokine expression in the T cells was measured by Ca<sup>2+</sup>-dependent (PMA/Ionomycin) cytokine recall ("cytokine recall") in the presence of a secretion blocker and analysis of intracellular cytokine expression by flow cytometry (FACS) ("intracellular cytokine analysis"). This pDC system was used for all following experiments involving cocultures of pDCs and T cells.

As had been described for the mouse system, pDCs induced a substantial population of IFN- $\gamma$ <sup>+</sup>IL-10<sup>+</sup> CD4 T cells. Surprisingly, differentiated T cells also contained a large population of IL-22-expressing cells, which only marginally overlapped with the subpopulation of IL-10-positive T cells.

Typically, naïve CD4 T cells stimulated in the presence of pDCs showed moderate to strong IFN- $\gamma$  expression and in most cases substantial populations of IL-10- and IL-22-producers that in part co-expressed IFN- $\gamma$  (see Fig. 5.1). IL-10 and IL-22 were independently expressed and produced no IL-17 and little or no IL-4.

For a better understanding of the expression profiles of IFN- $\gamma$ , IL-10 and IL-22, naïve CD4 T cells from a total of 51 different donors were analyzed for cytokine expression. All 51 donors were analyzed for IFN- $\gamma$  and IL-10 expression and 34 of these donors were ad-

ditionally analyzed for IL-22 expression. The obtained data allowed a more detailed definition of the cytokine expression in naïve CD4 T cells cocultivated with pDCs.



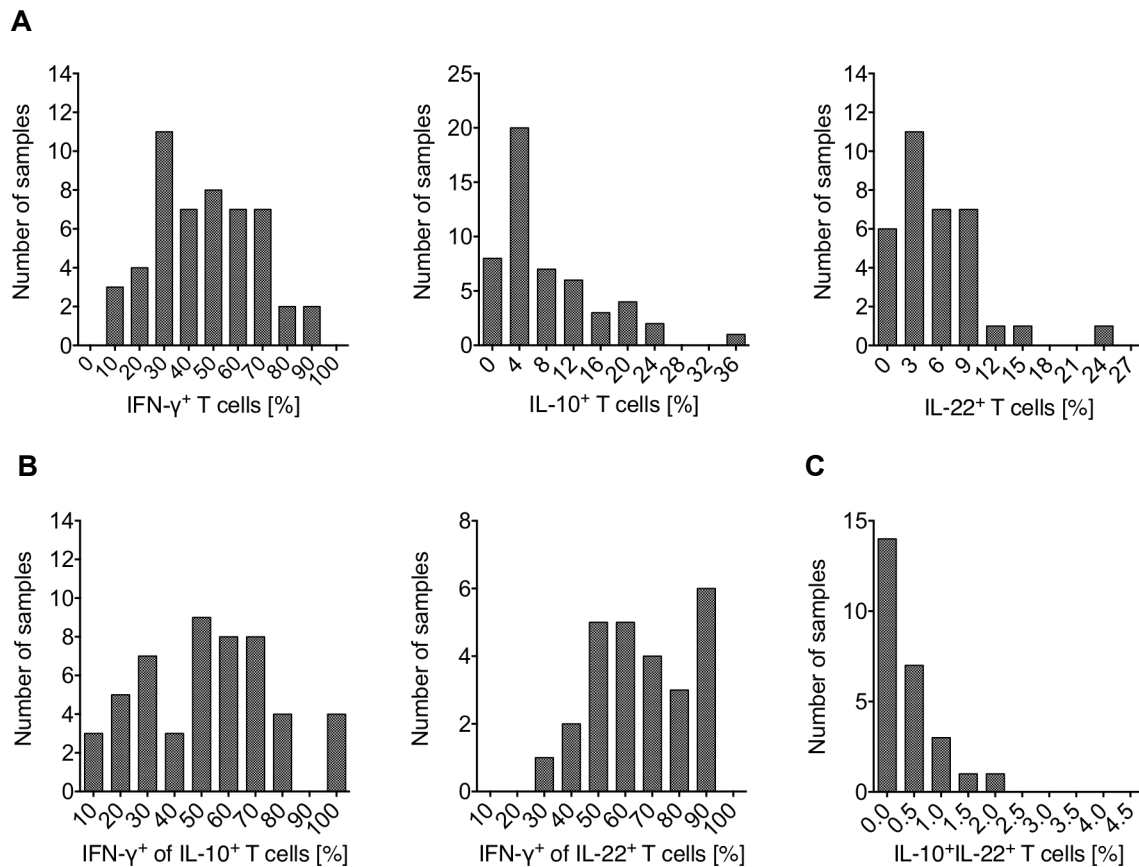
**Fig. 5.1: Plasmacytoid dendritic cells induce IFN- $\gamma$ , IL-10 and IL-22 in naïve human CD4 T cells but no IL-17 and no or little IL-4.**

Naïve CD4 T cells (CD4<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup>) were stimulated with SEB in the presence of TLR9-activated (CpG B) plasmacytoid dendritic cells. Intracellular cytokine expression was analyzed after 7 days by cytokine recall (PMA/ionomycin) and flow cytometry.

As illustrated in Fig. 5.2 A, T<sub>H</sub>1 commitment induced by pDCs varied largely, with frequencies of IFN- $\gamma$  expressing T cells ranging from 10 % to 90 % and the majority of samples containing between 30 % and 70 % IFN- $\gamma$ -positive cells.

Notable percentages of IL-10-expressing cells, ranging from 3 % to 25 % IL-10-expressing cells were found in most samples, while very few contained significantly less than 3 % of IL-10-producing cells. Frequencies of IL-22-secreting T cells ranged from approx. 2 % to 10 % of T cells for the majority of donors. Only in few donors less than 2 % or more than 10 % of IL-22 producing T cells were detected.

Coexpression of IL-10<sup>+</sup> and IFN- $\gamma$ <sup>+</sup> was observed in all samples. In contrast to mouse, where all IL-10-positive T cells also produced IFN- $\gamma$ <sup>144</sup>, this was only seen for a portion of IL-10-expressing cells in the human pDC culture. Nonetheless, all samples contained at least 10 % IFN- $\gamma$ -coexpressing cells in the IL-10<sup>+</sup> T cell population and more than half of the samples showed more than 50 % IFN- $\gamma$  coexpression within the IL-10<sup>+</sup> population (Fig. 5.2 B).



**Fig. 5.2: Plasmacytoid dendritic cells induce a mixed T cell population partially exhibiting T<sub>H</sub>1 properties as well as substantial IL-10 and IL-22 expression.**

Histograms showing the frequency distribution of **A** | IFN- $\gamma$ , IL-10 and IL-22 expression in T cells over all tested donors or **B** | IFN- $\gamma$  coexpression in the populations of IL-10 and IL-22 expressing T cells or **C** | IL-10/IL-22 double-producers.

Naïve CD4 T cells from different donors were stimulated in the pDC system (SEB + CpG B) and were analyzed for expression and coexpression of IFN- $\gamma$ , IL-10 and IL-22. The frequency distribution of cytokine expression over all donors was used to define the cytokine expression pattern of CD4 T cells stimulated by pDCs.

Double expression of IL-22 and IFN- $\gamma$  was more pronounced compared to IL-10. In all tested samples at least 30 % of IL-22 positive T cells were also positive for IFN- $\gamma$ , with the vast majority of samples showing 50 % or more IFN- $\gamma$ /IL-22 double-expressing cells in the IL-22<sup>+</sup> population (Fig. 5.2 B).

Analysis of IL-10/IL-22 coexpression showed that these two cytokines, although rarely produced by the same cells, were not negatively correlated but expressed independently (data not shown). Overall, the frequency of IL-10/IL-22-double-positive cells did not exceed 1.8 % and was 0.6 % or less for most samples.

In summary, cultivation of naïve CD4 T cells in the human pDC system generated a mixed T cell population expressing IFN- $\gamma$ , IL-10 and/or IL-22 and showing substantial coexpres-

sion of IFN- $\gamma$  with IL-10 or with IL-22, but also a significant proportion IL-10- and IL-22-single-expressors. Although IL-10/IL-22 double-expressing T cells were rare, the rate of coexpression was in the range expected for independent expression of IL-10 and IL-22.

### 5.1.2 IL-10- and IL-22-driving transcription modules in pDC-activated CD4 T cells

Several transcriptional modules that regulate IL-10 and IL-22 expression have been described in mouse. The transcription factor MAF (c-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog) is involved in IL-10 expression in T<sub>H</sub>17 and T<sub>R</sub>1 cells, AhR (aryl hydrocarbon receptor) was shown to drive both IL-10 and IL-22 expression in T<sub>H</sub>17 and T<sub>R</sub>1 cells, whereas the PR domain zinc finger protein 1, also known as BLIMP1, had been reported to be required for homeostasis and effector function, including IL-10 expression, of regulatory T cells.

Hypothesizing that these transcriptional modules might also govern IL-10 and IL-22 expression in pDC-activated CD4 T cells, their involvement was assessed through blocking experiments.

#### 5.1.2.1 *IL-10 induction depends on transcription factor MAF*

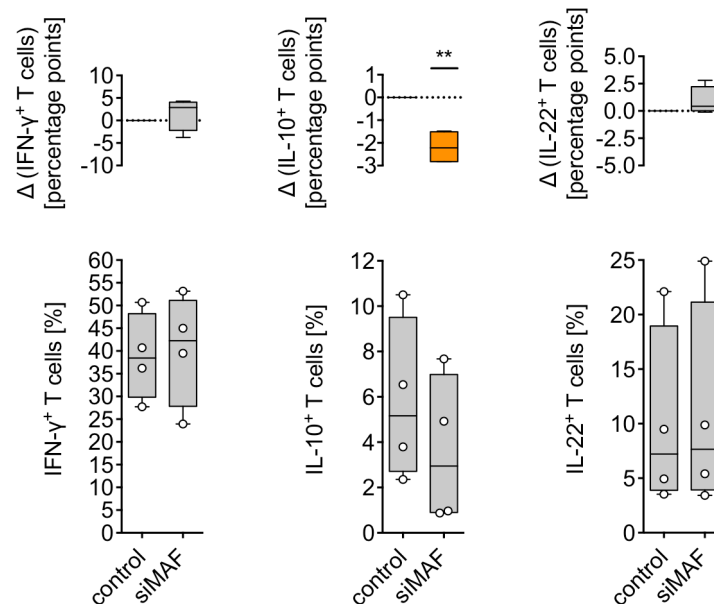
Transcription factor MAF had been reported to play a role for IL-10 expression in a variety of cell types in mouse, including macrophages<sup>146</sup>, T<sub>H</sub>1 and T<sub>H</sub>17 subsets<sup>37</sup> and in T<sub>R</sub>1 cells<sup>58</sup>. In addition it is believed to be a universal factor governing IL-10-expression in most immune cells (reviewed in Saraiva and O'Garra 2010<sup>24</sup>). Surmising MAF may also regulate IL-10-production in human T cells, the role of this transcription factor was assessed by specific siRNA-mediated knockdown.

Naïve CD4 T cells were activated in the pDC system and approx. 2 to 3 days after activation transfected with siRNA against *MAF* or with a non-specific scrambled control siRNA, and then returned to culture. After a total of 5 to 7 days, IFN- $\gamma$ , IL-10 and IL-22 expression were measured by cytokine recall and intracellular cytokine analysis.

The knockdown of *MAF* led to a pronounced reduction of IL-10 expression (average change: -47 %; range: -25 % to -75 %). Neither IFN- $\gamma$  nor IL-22 expression were significantly altered, although the knockdown slightly increased IL-22 levels (average change: +6 %; range: -3 % to +13 %) (see Fig. 5.3).

The results indicate that MAF promotes or is required for IL-10 expression similar to what had been reported in mouse. Interestingly, there was little effect of *MAF* knockdown on IL-22 expression, although Maf has been reported to act as transcriptional repressor of IL-22 in mouse T<sub>H</sub>17 cells<sup>60</sup>. This may be explained with the transient nature of siRNA-mediated MAF knockdown, which might not be sufficient to pronouncedly reduce the sup-

pression of IL-22. In fact, IL-22 expression was slightly, albeit not significantly, increased in cells transfected with siRNA against MAF.



**Fig. 5.3: Knockdown of *MAF* in T cells reduces pDC-induced IL-10-expression.**

Naïve CD4 T cells activated for 2 days in the pDC system (SEB + CpG B) were transfected with siRNA against *MAF* or control siRNA and returned to culture. After a total of 5 to 7 days the expression of IFN- $\gamma$ , IL-10 and IL-22 was assessed by cytokine recall and flow cytometry. | Bottom row: frequency of cytokine-expressing T cells in percent [%]. Each circle corresponds to a single donor. Top row: change in frequency of cytokine-expressing T cells compared to samples transfected with control siRNA (control) in percentage points. Circles signify outliers according to Tukey. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \*\*  $p < 0.005$

#### 5.1.2.2 *IL-22 expression is mediated by AhR*

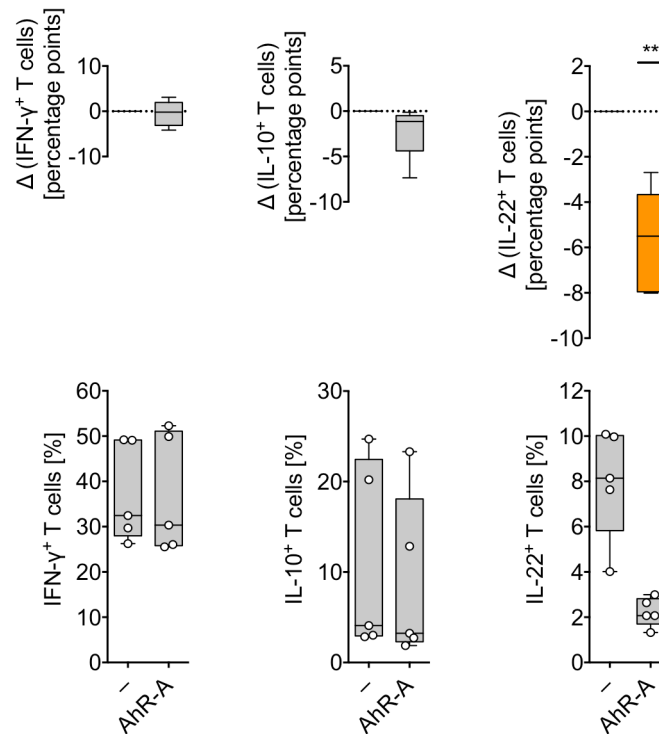
The aryl hydrocarbon receptor (Ahr) pathway was shown to act in concert with MAF in the induction of IL-10 expression in murine T<sub>R</sub>1 cells<sup>58</sup>, but also appears to be a key player in the generation of IL-22-expressing T cells<sup>94</sup>. Furthermore, Alam et al. had shown that Notch signaling stimulates release of AhR ligands by mouse CD4 T cells<sup>94</sup>.

To elucidate whether AhR is involved in the expression of IL-10 and/or IL-22 driven by pDCs, naïve CD4 T cells were activated in the pDC system with or without added AhR antagonist (AhR-A, CH-223191, 1 nM) and IFN- $\gamma$ , IL-10 and IL-22 expression was measured by cytokine recall and intracellular cytokine analysis after 5 to 7 days of culture. Fig. 5.4 shows the results of the experiments.

Blocking the AhR pathway strongly impaired IL-22 production (mean change: -71%; range: -61 % to -79 %). Although the number of IL-10-producing cells was also reduced by

application of AhR antagonist, this change was not statistically significant (mean change: -21 %; range: -5 % to -38 %).

The results suggest that pDC-mediated expression of IL-22 strongly depends on AhR signaling, but show no clear involvement of AhR in the induction of IL-10 expression. This is an interesting finding, since AhR has been demonstrated to induce IL-10 in cooperation with MAF.



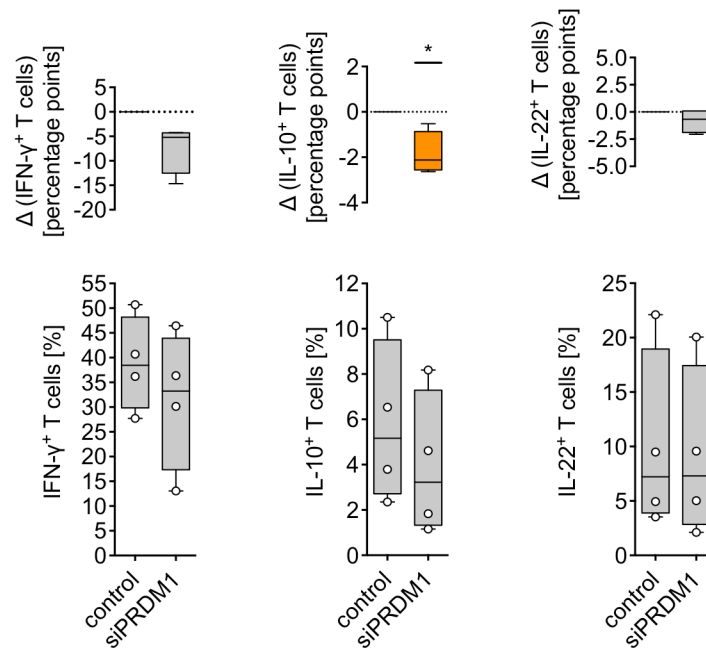
**Fig. 5.4: Blocking the AhR pathway strongly reduces pDC-induced IL-22 expression**

Naïve CD4 T cells were activated in the pDC system (SEB + CpG B) in presence or absence of AhR antagonist (CH-223191, 1 nM). After 5 to 7 days the expression of IFN- $\gamma$ , IL-10 and IL-22 was assessed by cytokine recall and flow cytometry. | Bottom row: frequency of cytokine-expressing T cells in percent [%]. Each circle corresponds to a single donor. Top row: change in frequency of cytokine-expressing T cells compared to samples without AhR antagonist in percentage points. Circles signify outliers according to Tukey. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \*\* p < 0.005

### 5.1.2.3 *BLIMP1 is required for IL-10 expression*

Several reports have discussed the involvement of BLIMP1 in the induction of IL-10 in CD4 T cells in general <sup>63</sup> and established a role for BLIMP1 in the IL-10 expression by effector-type regulatory T cells <sup>65</sup> and T<sub>R</sub>1-like T cells <sup>147</sup>.

To analyze the role of BLIMP1 in cytokine expression, naïve CD4 T cells were activated in the pDC system and approx. 2 to 3 days after activation transfected with siRNA against *PRDM1* (the gene coding for BLIMP1) or with a non-specific scrambled control siRNA, and then returned to culture. After a total of 5 to 7 days, IFN- $\gamma$ , IL-10 and IL-22 expression were measured by cytokine recall and intracellular cytokine analysis.



**Fig. 5.5: BLIMP1 knockdown significantly reduces pDC-induced IL-10 expression**

Naïve CD4 T cells activated for 2 days in the pDC system (SEB + CpG B) were transfected with siRNA against *PRDM1* or control siRNA and returned to culture. After a total of 5 to 7 days the expression of IFN- $\gamma$ , IL-10 and IL-22 was assessed by cytokine recall and flow cytometry. | Bottom row: frequency of cytokine-expressing T cells in percent [%]. Each circle corresponds to a single donor. Top row: change in frequency of cytokine-expressing T cells compared to samples transfected with control siRNA (control) in percentage points. Circles signify outliers according to Tukey. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \*  $p < 0.05$

Knockdown of *PRDM1* diminished IL-10 expression in a statistically significant manner (average change: -36%; range: -22 % to -70 %) and lead to a visible but statistically not significant decrease in IFN- $\gamma$  expression (average change: -22 %; range: -8 % to -53 %), whereas IL-22 expression was not significantly altered.

Although all three cytokines showed a more or less pronounced reduction this appeared not to be linked to a general decline in cell viability since no difference in T cell proliferation was observed between the controls and the cells transfected with siRNA against *PRDM1* (data not shown).

The reduction of IL-10 expression following *PRDM1* knockdown indicates BLIMP1 is required for the expression of IL-10 by T cells. This is backed by recent findings in mouse T<sub>H</sub>1 cells where BLIMP1 deficiency led to an abrogation of IL-10 expression by T cells <sup>66</sup>. Interestingly, these cells did not show an altered IFN- $\gamma$  expression, which contrasts with the reduction of IFN- $\gamma$ -producing cells observed here.

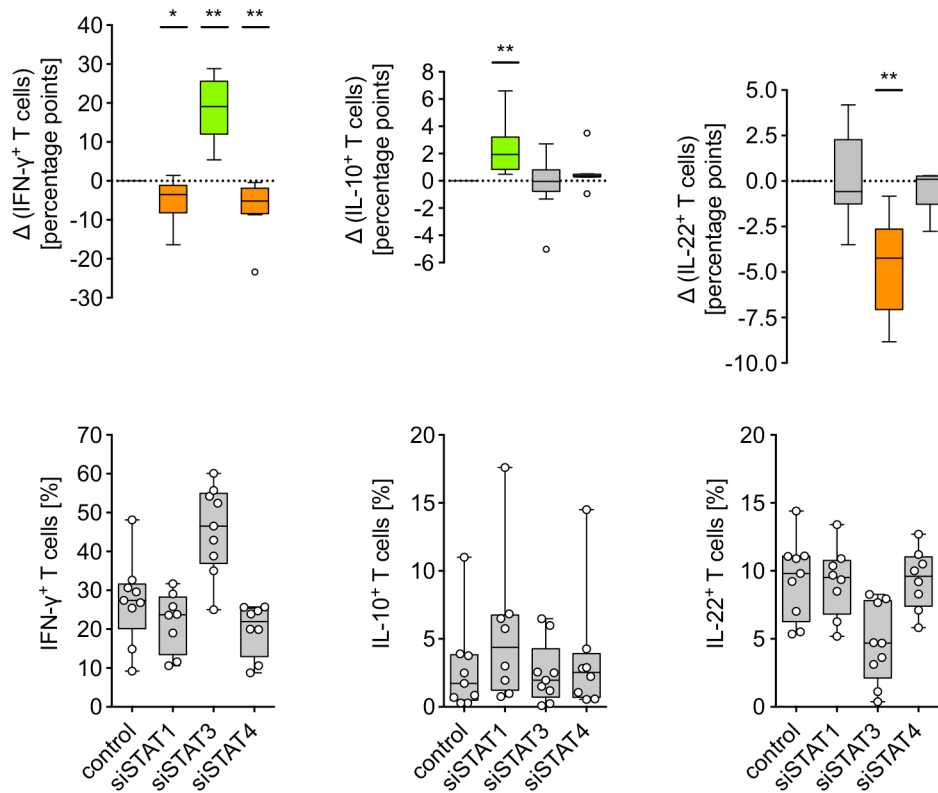
#### 5.1.2.4 *STAT1, STAT3 and STAT4 are involved in expression of IFN- $\gamma$ , IL-10 and IL-22*

The JAK/STAT pathway is a very important mechanism of transduction of cytokine signals. Many cytokine receptors convey the signal of a bound cytokine by activation of one and often several types of STAT molecules. Main effectors of pDCs and T<sub>H</sub>1 cells, like IFN- $\alpha$ , IL-6, TNF- $\alpha$ , IL-27, IL-12 and IFN- $\gamma$  act through phosphorylation of STAT1, STAT3 and/or STAT4.

All of these three STAT molecules have been reported to be involved in one or the other way in the IL-10 expression by T helper cells. For example, STAT4 activation in combination with a strong TCR signal mediated IL-10 induction in T<sub>H</sub>1 cells <sup>26,34,68</sup>. STAT3 was required for the generation of IL-10-expressing cells by IL-6 and TGF- $\beta$  in the T<sub>H</sub>17 context <sup>34</sup> and by IL-21 <sup>38</sup>, whereas induction of IL-10-expression by IL-6 independent of T<sub>H</sub>17 polarization required both STAT1 and STAT3 <sup>34</sup>.

In order to assess the influence of the individual STAT molecules on cytokine expression by T cells that were differentiated in the presence of pDCs, expression of each STAT molecule, STAT1, STAT3 and STAT4, was separately inhibited by siRNA-mediated knockdown. Since cytokines may be produced and act in early T cell priming as well as in later phases of cell differentiation, the STAT knockdown was induced in T cells either before (Fig. 5.6) or after (Fig. 5.7) activation.

For this purpose naïve CD4 T cells were either transfected with siRNA directly after isolation, rested for approx. 1 d and then activated in the pDC system (Fig. 5.6) or *ex vivo* naïve CD4 T cells were activated in the pDC system, after 2 or 3 days transfected with siRNA and returned to culture (Fig. 5.7). The transfected siRNA was directed against *STAT1*, *STAT3* or *STAT4*, or was a non-specific scrambled control siRNA. Cytokine expression was determined by cytokine recall and intracellular cytokine analysis after a total of 5 to 7 days in culture.



**Fig. 5.6: Knockdown of STAT1, STAT3 and STAT4 before T cell activation affects pDC-induced expression of IFN- $\gamma$ , IL-10 and IL-22 in different ways**

Naïve CD4 T cells were transfected with siRNA against *STAT1*, *STAT3*, *STAT4* or control siRNA, rested for 24 h and activated in the pDC system (SEB + CpG B). After 5 to 7 days the expression of IFN- $\gamma$ , IL-10 and IL-22 was assessed by cytokine recall and flow cytometry. | Bottom row: frequency of cytokine-expressing T cells in percent [%]. Each circle corresponds to a single donor. Top row: change in frequency of cytokine-expressing T cells compared to samples transfected with control siRNA (control) in percentage points. Circles signify outliers according to Tukey. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \* p < 0.05; \*\* p < 0.005

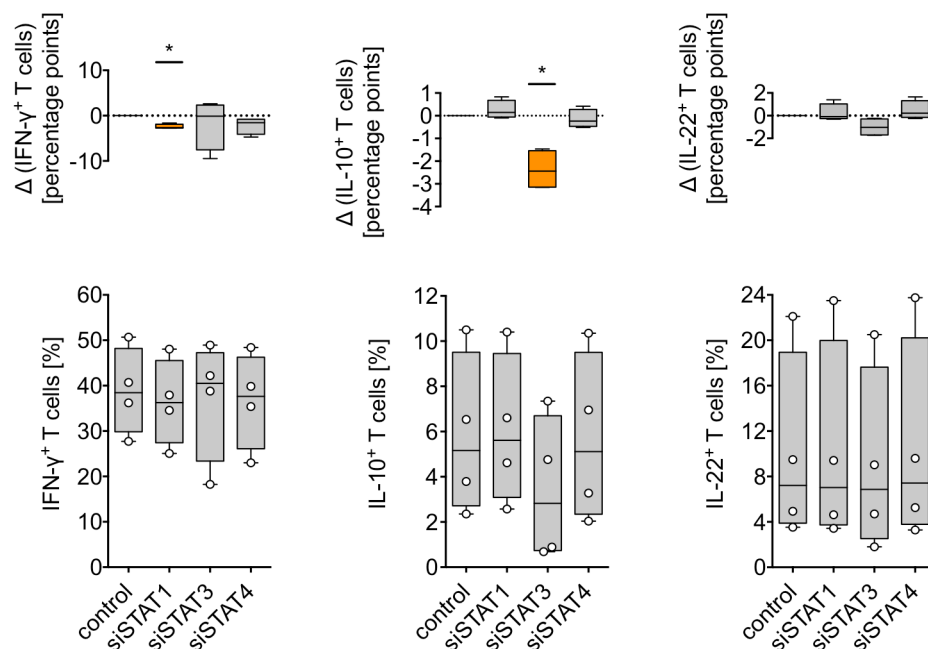
As Fig. 5.6 shows, there were significant differences regarding the effect of STAT inhibition on IFN- $\gamma$ , IL-10 and IL-22 expression for STAT1, STAT3 and STAT4.

Most notably, STAT1 and STAT4 knockdown and STAT3 knockdown had opposite effects on the cytokine IFN- $\gamma$ . While reduction of STAT1 and STAT4 signaling markedly impaired IFN- $\gamma$  expression, blocking the STAT3 signal led to a strong upregulation of IFN- $\gamma$  production. This indicated that STAT1- and STAT4-dependent pathways were driving IFN- $\gamma$  expression, whereas STAT3 appeared to convey an inhibitory signal.

In contrast, IL-22 expression was not significantly affected by STAT1 or STAT4 knockdown (Fig. 5.6), but strongly impaired by a reduced STAT3 expression, which suggested that expression of IL-22 was mediated by a STAT3-dependent mechanism.

Curiously, induction of IL-10 expression was not affected by an early knockdown of STAT3 (Fig. 5.6) but significantly reduced when STAT3 signaling was inhibited during the later phase of differentiation (Fig. 5.7). Here, STAT3 appeared to be involved in an IL-10-driving mechanism that is activated in an advanced stage of the differentiation process.

The fact that early reduction of STAT1 levels led to a significant increase of IL-10 expression (Fig. 5.6) suggested that the action of STAT1 in the early priming phase plays a role in negative regulation of IL-10 expression. STAT4 knockdown did not alter IL-10 expression.



**Fig. 5.7: STAT3 knockdown after T cell activation reduces pDC-induced IL-10 expression**

Naïve CD4 T cells activated for 2 days in the pDC system (SEB + CpG B) were transfected with siRNA against *STAT1*, *STAT3*, *STAT4* or control siRNA and returned to culture. After a total of 5 to 7 days the expression of IFN- $\gamma$ , IL-10 and IL-22 was assessed by cytokine recall and flow cytometry. | Bottom row: frequency of cytokine-expressing T cells in percent [%]. Each circle corresponds to a single donor. Top row: change in frequency of cytokine-expressing T cells compared to samples transfected with control siRNA (control) in percentage points. Circles signify outliers according to Tukey. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \*  $p < 0.05$

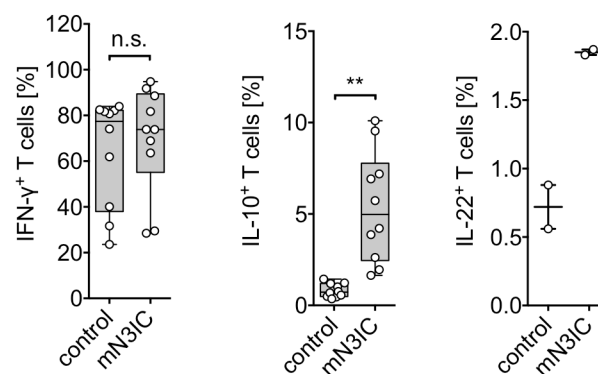
With the exception of the effect of STAT3 knockdown on IL-10 and IFN- $\gamma$  secretion, all knockdown-related effects that were observed in the early T cell priming phase (Fig. 5.6) were also discernible when knockdown was induced after T cell activation (Fig. 5.7). The fact that these effects were much less pronounced following late siRNA transfection indicated that most of the crucial STAT-related signaling events occur in the early priming phase.

Interestingly, these experiments showed that IL-10 as well as IL-22 expression required STAT3 signaling, which was observed as well in the context of murine T<sub>H</sub>17 polarization, but also independently of the T<sub>H</sub>17 subset and in many cases involving the cytokines IL-6 and/or IL-21<sup>34,38,84,87</sup>. It is important to stress that the cells generated in the experiments here were not T<sub>H</sub>17 cells as they did not express IL-17 (data not shown).

On the other hand, IFN- $\gamma$  expression has been linked to STAT4 and STAT1 signaling mainly in the T<sub>H</sub>1 setting. STAT4 is known to drive T<sub>H</sub>1 commitment in an IL-12-dependent fashion<sup>26</sup>, whereas IFN- $\gamma$ -activated STAT1 signaling appears to play a role in early T<sub>H</sub>1 polarizing events<sup>148–150</sup>. Also IL-27-mediated T<sub>H</sub>1 differentiation was shown to depend on STAT1 signals<sup>43,44</sup>

### 5.1.3 pDC-mediated IL-10 and IL-22 induction depends on Notch signaling and DLL-4 ligation

Several scientific reports had established a role for the Notch pathway and the activation of Notch by delta-like Notch ligands in the expression of IL-10 and IL-22 by murine CD4 T cells <sup>68,94</sup>. In addition, plasmacytoid dendritic cells were found to induce IL-10-expressing CD4 T cells by means of DLL-4 as well as being capable of driving IL-22 expression in T helper cells <sup>77,144</sup>.



**Fig. 5.8: Constitutively active Notch induces IL-10 and IL-22 expression in naïve CD4 T cells**

Polyclonally (anti-CD3/antiCD28) activated naïve CD4 T cells were infected with virus carrying an expression vector for constitutively active Notch (mN3IC) or an empty vector as control. After 6 days IFN- $\gamma$ , IL-10 and IL-22 expression was analyzed by cytokine recall and flow cytometry. Each circle corresponds to a single donor. | \*\*  $p < 0.005$ ; n.s., not significant

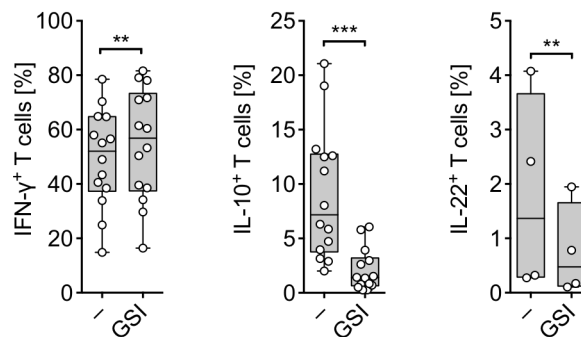
Rutz et al. and Alam et al. had demonstrated that overexpression of constitutively active Notch induces IL-10 expression <sup>68</sup> and IL-22 expression <sup>94</sup> in mouse CD4 T cells. In order to understand, if these findings can be replicated in human T cells, naïve human CD4 T cells were retrovirally transfected with an expression vector for constitutively active intracellular domain of Notch-3 (mN3IC) or a control vector and stimulated by platebound anti-CD3 and anti-CD28 in absence of dendritic cells. After five days cytokine expression was assessed by cytokine recall and intracellular cytokine analysis.

Fig. 5.8 shows that, although overexpression of mN3IC did not significantly affect IFN- $\gamma$  expression, it strongly increased the frequency of IL-10-expressing cells from an average of 0.7 % to 5.0 % (median values).

Also the frequency of IL-22-expressing cells was clearly increased among mN31C-transduced T cells (mean value: 1.9 %) compared to T cells transduced with the control vector (mean value: 0.7 %).

Having established that Notch is indeed capable of inducing both IL-10 and IL-22 a different approach was used to assess whether the Notch pathway is also responsible for induction of IL-10 and IL-22 expression in T cells stimulated by pDCs. Naïve CD4 T cells were activated in the pDC system in presence or absence of a  $\gamma$ -secretase inhibitor (GSI) and cytokine expression was assessed by cytokine recall and intracellular cytokine analysis after 5 to 7 days.

As Fig. 5.9 illustrates, blocking the Notch signal strongly reduced the number of both IL-10- and IL-22-expressing T cells, while the percentage of IFN- $\gamma$ <sup>+</sup> T cells was only slightly altered.



**Fig. 5.9: Blocking Notch signaling inhibits pDC-induced IL-10 and IL-22 expression**

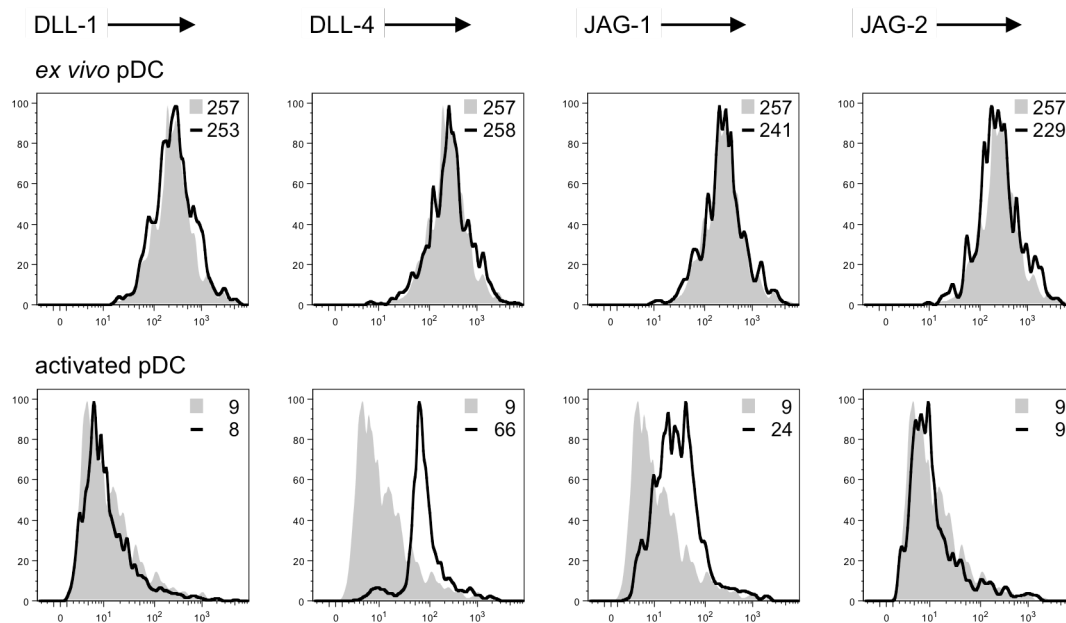
Naïve CD4 T cells were activated in the pDC system (SEB + CpG B) in presence or absence of a  $\gamma$ -secretase inhibitor (GSI). After 5 to 7 days the expression of IFN- $\gamma$ , IL-10 and IL-22 was assessed by cytokine recall and flow cytometry. | Median percentage of cytokine expressing cells: IFN- $\gamma$ : 55 % (“-”), 57 % (“GSI”); IL-10: 7.2 % (“-”), 1.4 % (“GSI”); IL-22: 1.4 % (“-”), 0.5 % (“GSI”) | Each circle corresponds to one donor | \*\*  $p < 0.005$ ; \*\*\*  $p < 0.0005$

In the mouse system, both IL-10 and IL-22 expression by CD4 T cells in T<sub>H</sub>1 or T<sub>H</sub>17 settings had been shown to be enhanced by costimulation with DLL-4 or DLL-1<sup>68,93,94</sup>. In addition, Kassner et al. had demonstrated that DLL-4 expression on pDCs was mandatory for the induction of IL-10 in CD4 T cells<sup>144</sup>.

Therefore, elucidating the role of Notch ligands in the pDC system appeared crucial to a better understanding of the capacity of pDCs to drive IL-10 and IL-22 expression. In a first step surface expression of Notch ligands on plasmacytoid dendritic cells was analyzed by flow cytometry. *Ex vivo* isolated pDCs were either directly analyzed for expression of the Notch ligands DLL-1, DLL-4, JAG-1, JAG-2 and the activation marker HLA-DR (not shown) or were stimulated for 3 hours with a combination of activating human anti-CD40 anti-

body and TLR9 ligand CpG B, and then analyzed for HLA-DR (not shown) and the Notch ligands.

While resting (*ex vivo*) plasmacytoid dendritic cells did not express detectable levels of any of the Notch ligands (Fig. 5.10) the stimulation induced robust upregulation of DLL-4 and modest expression of JAG-1.



**Fig. 5.10: pDCs express DLL-4 and JAG-1 upon stimulation with TLR9 agonist CpG B**

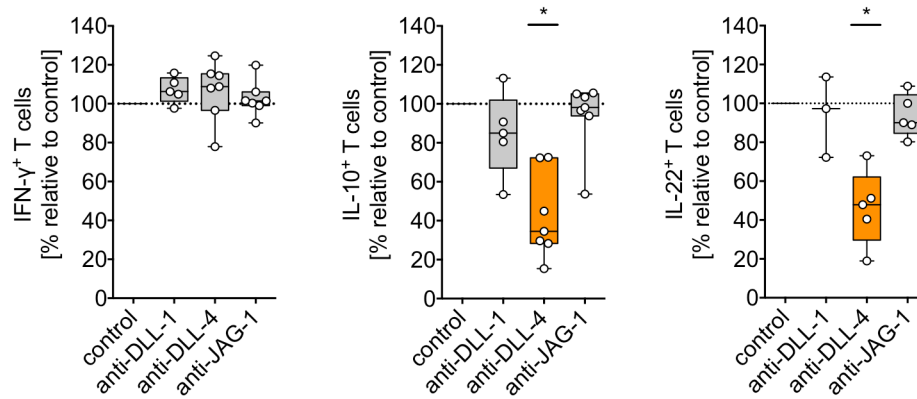
*Ex vivo* plasmacytoid dendritic cells and activated plasmacytoid dendritic cells (CpG B + anti-CD40 for 3 h) were stained for surface expression of Notch ligands DLL-1, DLL-4, JAG-1 and JAG-2 with immunofluorescent antibodies. Surface staining was enhanced using FASER Kit and the surface expression of Notch ligands analyzed by flow cytometry. | Filled light gray histograms: control staining without primary antibody; Histograms with black line: Notch ligand staining; Numbers in histogram: median of fluorescence intensity

In a second step the effect of a specific block of Notch ligands was assessed to understand which Notch ligand was involved in the induction of IL-10 and IL-22.

Naïve CD4 T cells were cultivated in the pDC system and specific blocking antibodies against JAG-1, DLL-1 or DLL-4 were added. After 5 to 7 days, expression of IFN- $\gamma$ , IL-10 and IL-22 was analyzed by cytokine recall and intracellular cytokine analysis.

As Fig. 5.11 shows, blocking DLL-4 lead to a strong reduction of both IL-10 expression and IL-22 expression. Although blocking DLL-1 decreased IL-10 expression as well, this effect was much less pronounced and no effect of the DLL-1 block on IL-22 expression was observed. JAG-1 had no effect whatsoever on IL-10, IL-22 and IFN- $\gamma$  expression was not significantly altered in any of the conditions.

In summary, the experiments showed that Notch signaling was both capable of driving IL-10 and IL-22 expression in human CD4 T cells and required for the induction of IL-10 and IL-22 producers by plasmacytoid dendritic cells. Furthermore, pDCs were shown to utilize Notch ligand DLL-4 for the induction of IL-10 and IL-22 expression in CD4 T cells.



**Fig. 5.11: DLL-4, but not DLL-1 or JAG-1 are required for IL-10 and IL-22 induction by pDCs**

Naïve CD4 T cells were activated in the pDC system (SEB + CpG B) in presence or absence of neutralizing antibodies specific for DLL-1, DLL-4 or JAG-1, or without blocking antibody. After 5 to 7 days the expression of IFN- $\gamma$ , IL-10 and IL-22 was assessed by cytokine recall and flow cytometry. | Frequencies of cytokine-expressing cells were normalized to samples without blocking antibody. Each dot corresponds to one donor. Green or orange color indicates frequency was increased or decreased, respectively, in statistically significant manner. | \*  $p < 0.05$

#### 5.1.4 pDC-derived cytokines influence IL-10 and IL-22 expression

Plasmacytoid dendritic cells are known to convey many of their functions through cytokines. The main effector cytokines produced by pDCs are IFN- $\alpha$ , IL-6, TNF- $\alpha$ , IL-27 and IL-12 <sup>96,98,151,152</sup>.

IFN- $\alpha$  was reported to be produced by pDCs <sup>96</sup> and be capable of inducing IL-10 expression in human T cells <sup>46</sup>, whereas IL-12 has been demonstrated to be involved in the induction of IL-10 in murine and human T<sub>H</sub>1 cells <sup>26,34,68,153</sup>. Several work groups had shown that the cytokines IL-21, IL-6, IL-27 play a role in the IL-10 induction in different settings or subsets <sup>34,38</sup>. ICOS was described as a key factor in the differentiation of T<sub>R</sub>1 cells <sup>57</sup>. In humans, the T<sub>H</sub>1 and T<sub>H</sub>22 subsets are known to express IL-22 <sup>77</sup> after treatment with IL-6 and TNF- $\alpha$  <sup>77</sup>, while IL-21 induces IL-22 in the context of murine T<sub>H</sub>17 responses <sup>84</sup>.

To assess which factors, in addition to DLL-4, may play a role in the induction of IL-10 and IL-22, the effect of adding exogenous IL-12, IFN- $\alpha$ , IL-21, IL-6, IL-27 and TNF- $\alpha$  and blocking of endogenous IL-12, IFN- $\alpha$ , IL-21, IL-6, TNF- $\alpha$  and ICOS ligand (ICOS-L) was analyzed in the pDC system.

For that purpose, naïve CD4 T cells were cultivated in the pDC system in presence of the effectors described above and IFN- $\gamma$ , IL-10 and IL-22 expression was assessed by cytokine recall and intracellular cytokine analysis after 5 to 7 days of cell culture.

##### 5.1.4.1 *IFN- $\alpha$ , IL-21, IL-6 and IL-27 positively regulate IL-10 expression*

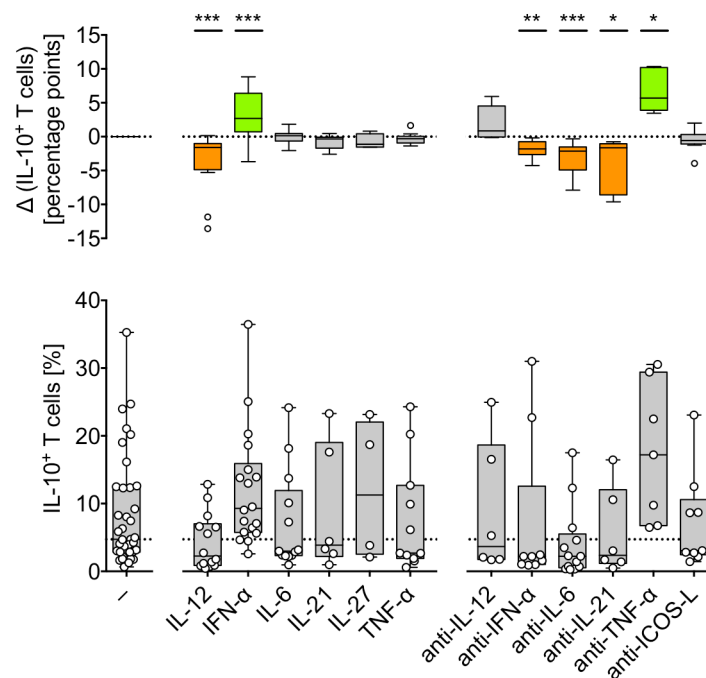
Adding IL-12 strongly reduced IL-10 induction by pDCs, while blocking IL-12 block did slightly but not significantly enhance IL-10 production in the pDC system (Fig. 5.12). This suggests that other than reported for T<sub>H</sub>1 polarization by abundant IL-12, IL-10 induction in this setting does not depend on IL-12.

In contrast, addition of IFN- $\alpha$  strongly increased the percentage of IL-10-expressing T cells whereas IFN- $\alpha$  block significantly reduced the number of IL-10<sup>+</sup> T cells, indicating that IFN- $\alpha$  secreted by pDCs was driving IL-10 expression while likely not being produced in saturating amounts.

Neither added IL-6, nor IL-21, IL-27 or TNF- $\alpha$  were capable of increasing IL-10 expression. In contrast, blocking IL-6 and IL-21 significantly reduced the IL-10 production, which suggests that both cytokines were present in the culture and involved in driving IL-10 expression. The addition of antibodies blocking ICOS ligands did not produce any effect. Surpris-

ingly, blocking TNF- $\alpha$  markedly augmented IL-10 expression, indicating that also TNF- $\alpha$  was part of the cytokine mix in the pDC culture and negatively regulated IL-10 expression. This finding is supported by a recent report that TNF- $\alpha$  inhibitors induce IL-10 in human CD4 T cells <sup>154</sup>.

In summary, the analysis of cytokines showed that IFN- $\alpha$ , IL-6 and IL-21 but not IL-12 contributed to the IL-10 induction in the pDC system, while TNF- $\alpha$  counteracted the factors driving IL-10 expression.



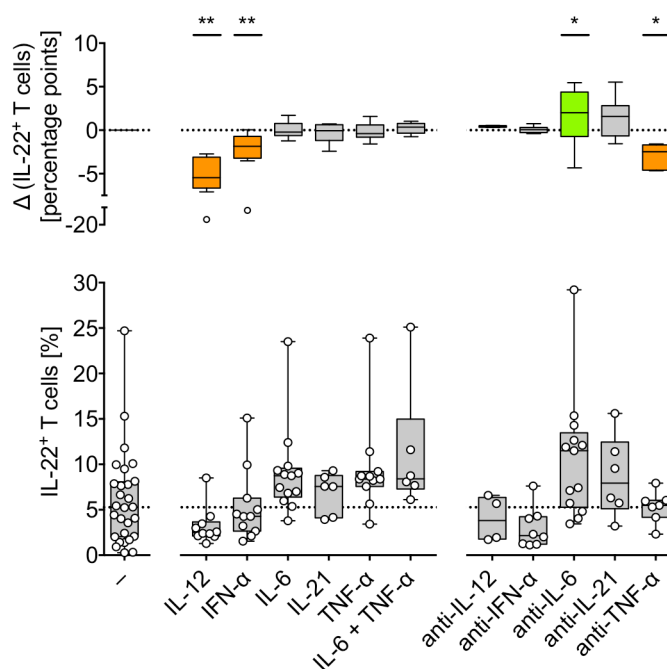
**Fig. 5.12: IFN- $\alpha$ , IL-21 and IL-6 play a role in the induction of IL-10 expression by pDCs**

Naïve CD4 T cells were activated in the pDC system (SEB + CpG B) with added cytokines or neutralizing antibodies, or without any of both. After 5 to 7 days expression of IL-10 was assessed by cytokine recall and flow cytometry. | Bottom row: frequency of cytokine-expressing T cells in percent [%]. Each circle corresponds to a single donor. Top row: change in frequency of cytokine-expressing T cells compared to control (“-”, sample without added cytokines or antibodies). Circles signify outliers according to Tukey. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \*  $p < 0.05$ ; \*\*  $p < 0.005$ ; \*\*\*  $p < 0.0005$

#### 5.1.4.2 pDC-derived and T<sub>H</sub>22-driving cytokines do not enhance IL-22 expression

The effect of cytokines on IL-22 expression (Fig. 5.13) was quite different compared to the results obtained in regard to IL-10 expression. None of the analyzed cytokines was found to enhance IL-22 expression. In contrast, addition of IL-12 and IFN- $\alpha$  significantly reduced the number of IL-22-expressing T cells. Interestingly, a IL-6 block increased the percentage of IL-22 producers while added IL-6 did not alter IL-22 levels. Likewise, the potent

T<sub>H</sub>22-driving combination of TNF- $\alpha$  and IL-6 failed to enhance IL-22 expression. The only indication that one of the tested cytokines might promote the generation of IL-22-expressing cells was the finding that blocking TNF- $\alpha$  reduced levels of IL-22-positive cells. But this did not translate into augmented IL-22 expression when additional TNF- $\alpha$  was introduced into the culture.



**Fig. 5.13: None of the tested cytokine enhances IL-22 expression**

Naïve CD4 T cells were activated in the pDC system (SEB + CpG B) with added cytokines or neutralizing antibodies, or without any of both. After 5 to 7 days expression of IL-22 was assessed by cytokine recall and flow cytometry. | Bottom row: frequency of cytokine-expressing T cells in percent [%]. Each circle corresponds to a single donor. Top row: change in frequency of cytokine-expressing T cells compared to control (“-”, sample without added cytokines or antibodies). Circles signify outliers according to Tukey. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \*  $p < 0.05$ ; \*\*  $p < 0.005$

#### 5.1.4.3 IFN- $\gamma$ expression is partly driven by IL-12 and TNF- $\alpha$ and is limited by IL-6

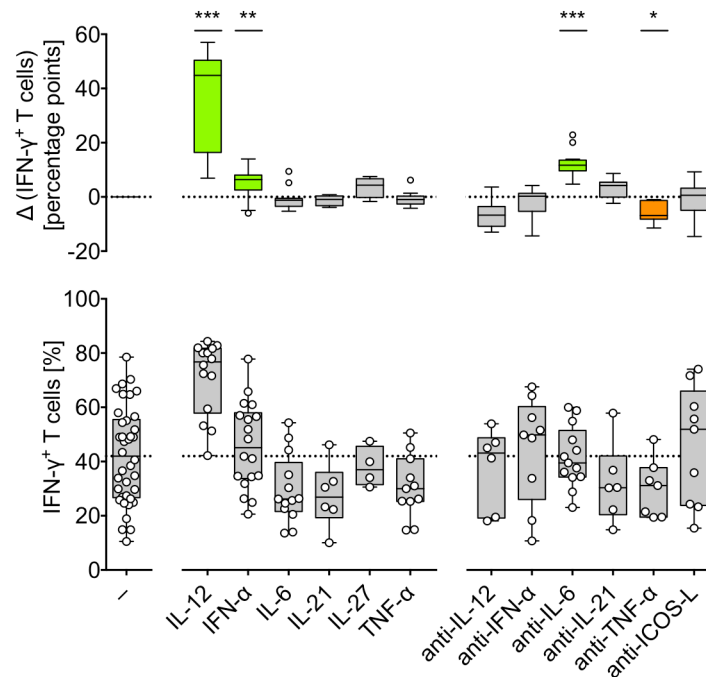
IL-12 as the prototypic T<sub>H</sub>1-driving cytokine induced a strong upregulation of IFN- $\gamma$ , and blocking of IL-12 led to a moderate reduction of IFN- $\gamma$  (Fig. 5.14), suggesting that small amounts of IL-12 were present in the pDC system and promoted T<sub>H</sub>1 commitment.

IFN- $\alpha$  only moderately enhanced IFN- $\gamma$  expression when added to the pDC system, but had no effect when blocked.

Addition of TNF- $\alpha$  did not alter IFN- $\gamma$  expression, but blocking the cytokine led to a modest reduction of IFN- $\gamma$ , indicating that TNF- $\alpha$  played a role in facilitating IFN- $\gamma$  expression. In-

terestingly, blocking IL-6 in the pDC system enhanced IFN- $\gamma$  expression, while added IL-6 did not alter the frequency of IFN- $\gamma^+$  T cells. This indicated that the IL-6 present in the pDC culture counteracted T<sub>H</sub>1 differentiation. IL-21 neither impaired nor promoted IFN- $\gamma$  expression and blocking IL-21 produced no significant changes. Lastly, adding IL-27 led to a small but not significant increase in IFN- $\gamma$  expression.

Together these results indicated, that moderate amounts of IL-12 were present in the pDC culture and together with TNF- $\alpha$  were at least in part responsible for T<sub>H</sub>1 polarization, whereas endogenously produced IL-6 suppressed T<sub>H</sub>1-commitment and IFN- $\alpha$  had no discernible effect. It cannot be excluded that additional factors contributed to T<sub>H</sub>1 commitment in this system.



**Fig. 5.14: The balance of IL-12, TNF- $\alpha$  and IL-6 defines IFN- $\gamma$  expression in the pDC system**

Naïve CD4 T cells were activated in the pDC system (SEB + CpG B) with added cytokines or neutralizing antibodies, or without any of both. After 5 to 7 days expression of IFN- $\gamma$  was assessed by cytokine recall and flow cytometry. | Bottom row: frequency of cytokine-expressing T cells in percent [%]. Each circle corresponds to a single donor. Top row: change in frequency of cytokine-expressing T cells compared to control ("—", sample without added cytokines or antibodies). Circles signify outliers according to Tukey. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \* p < 0.05; \*\* p < 0.005

### 5.1.5 IFN- $\alpha$ confers IL-10-inducing capacity to myeloid dendritic cells

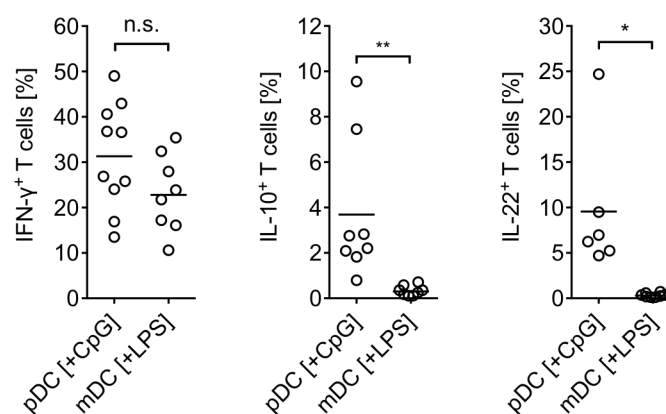
Experiments with antigen presenting cells (APC) ectopically expressing delta-like Notch ligands had shown that these APCs were capable of inducing IL-10 and IL-22 expression in CD4 T cells <sup>68,94</sup>. While there are contrasting reports on the expression of Notch ligands on myeloid or conventional DCs (mDC or cDC) in mouse <sup>144,155</sup>, Kassner et al. had shown that, unlike pDCs, they are not capable of inducing substantial numbers of IL-10-producing CD4 T cells <sup>144</sup>. In addition, human mDCs had been demonstrated to be inferior to pDCs in the induction of T<sub>H</sub>22 cells <sup>77</sup>.

In order to understand, whether the action of pDC-derived effectors can explain functional differences between pDCs and conventional DCs, a group of factors identified in the pDC culture were analyzed and tested in an mDC-T cell coculture.

#### 5.1.5.1 mDCs do not induce IL-10 and IL-22 expression in CD4 T cells

To gauge the capacity of mDCs of driving IL-10 and IL-22 expression, naïve CD4 T cells were cocultivated either with TLR9-stimulated (CpG B) pDCs or TLR4-stimulated (LPS) mDCs. IFN- $\gamma$ , IL-10 and IL-22 expression in T cells was assessed by cytokine recall and intracellular cytokine analysis after 6 to 7 days of culture.

Fig. 5.15 clearly shows that, in accordance with previous reports, mDCs failed to drive generation of IL-10- and IL-22-expressing T cells comparable to pDCs.



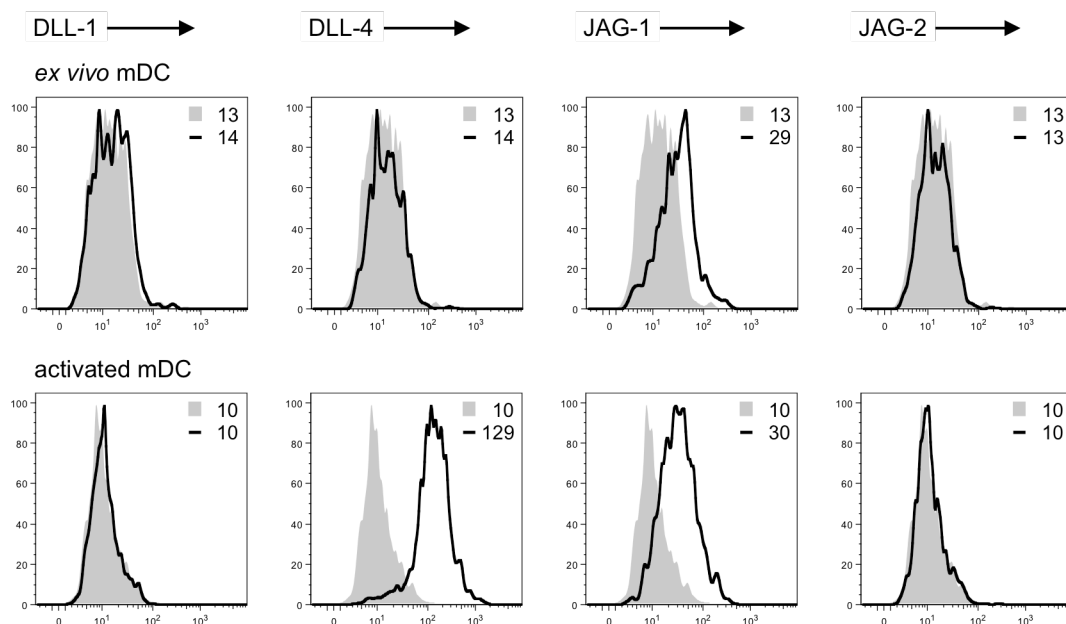
**Fig. 5.15: mDCs do not induce IL-10 or IL-22 expression in CD4 T cells**

Naïve CD4 T cells were activated in the presence of pDCs (SEB + CpG B) or mDCs (SEB + LPS). After 5 to 7 days expression of IFN- $\gamma$  was assessed by cytokine recall and flow cytometry. | Each circle corresponds to a single donor. | \*  $p < 0.05$ ; \*\*  $p < 0.005$ ; n.s., not significant

### 5.1.5.2 mDCs show expression patterns of Notch ligands similar to pDCs

The incapacity of mDCs to induce IL-10 and IL-22 expression prompted the question whether this might be owed to a difference in Notch ligand expression. To answer this question, the expression pattern of Notch ligands on mDCs was analyzed by surface staining of DLL-1, DLL-4, JAG-1 and JAG-2 and flow cytometry analysis and was compared to the pattern observed on pDCs.

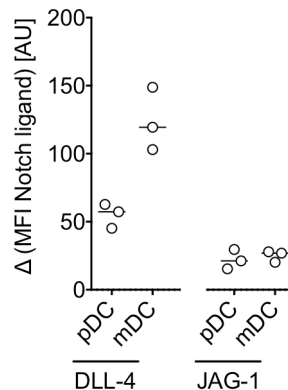
*Ex vivo* isolated mDCs were either directly analyzed for expression of the Notch ligands DLL-1, DLL-4, JAG-1, JAG-2 and the activation marker HLA-DR (not shown) or were stimulated for 3 hours with a combination of activating human anti-CD40 antibody and TLR4 ligand LPS, and then analyzed for HLA-DR (not shown) and the Notch ligands (Fig. 5.16).



**Fig. 5.16: mDCs express DLL-4 and JAG-1 upon stimulation with LPS**

*Ex vivo* myeloid dendritic cells or activated myeloid dendritic cells (LPS + anti-CD40 for 3 h) were stained for surface expression of Notch ligands DLL-1, DLL-4, JAG-1 and JAG-2 with immunofluorescent antibodies. Surface staining was enhanced using FASER Kit and the surface expression of Notch ligands analyzed by flow cytometry. | Filled light gray histograms: control staining without primary antibody; Histograms with black line: Notch ligand staining; Numbers in histogram: median of fluorescence intensity

While resting mDCs differed from pDCs by showing moderate surface expression of JAG-1, activated myeloid dendritic cells displayed very similar expression patterns, albeit with higher DLL-4 surface expression compared to pDCs (Fig. 5.17). These results suggested that a difference in Notch ligand expression probably was not the reason for the functional differences between mDCs and pDCs.



**Fig. 5.17: mDCs express significantly more DLL-4 than pDCs**

Activated plasmacytoid dendritic cells (CpG B + anti-CD40 for 3 h) and activated myeloid dendritic cells (LPS + anti-CD40 for 3 h) were stained for surface expression of Notch ligands DLL-4 and JAG-1 with immunofluorescent antibodies. Surface staining was enhanced using FASER Kit and the surface expression of Notch ligands analyzed by flow cytometry. | Each circle represents one donor.  $\Delta$  (MFI Notch ligand) was calculated as difference of Notch ligand staining and control staining.

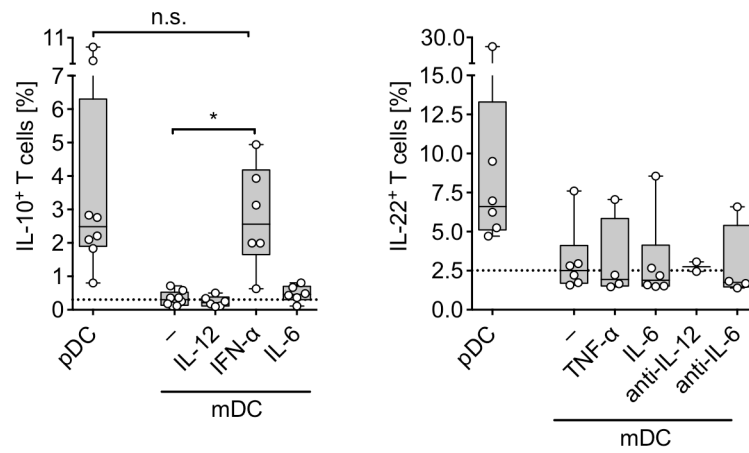
#### 5.1.5.3 *IFN- $\alpha$ renders mDCs capable of promoting IL-10 production in CD4 T cells*

In a second approach, cytokines, like IFN- $\alpha$ , IL-6, TNF- $\alpha$  or IL-12, were tested in order to determine whether they are able to confer the capability of inducing IL-10 and/or IL-22 expression to mDCs. Naïve CD4 T cells were activated in the pDC system or with TLR4-stimulated (LPS) mDCs in the presence of IFN- $\alpha$ , IL-12, IL-6 or TNF- $\alpha$  or without added cytokine. After 5 to 7 days, IL-10 cytokine expression was analyzed by cytokine recall and intracellular cytokine analysis.

The analysis demonstrated that IFN- $\alpha$ , but not IL-12 or IL-6, equipped mDCs with the ability to induce IL-10 production in T cells (Fig. 5.18). The frequencies of IL-10<sup>+</sup> T cells in both the pDC system and the mDC-T cell coculture supplemented with IFN- $\alpha$  were comparable (pDC: median = 2.5 %, range = 0.8 % to 9.6 %; mDC: median = 2.6 %, range = 0.6 % to 4.9 %). Interestingly, under all tested conditions mDCs failed to induce a pronounced IL-22 response.

Taken together these results demonstrated that pDC-derived functions like the induction of IL-10-producing T cells could be transferred to other systems, like an mDC coculture, by complementing them with critical components identified in the pDC system. In contrast, the capacity of pDCs to induce substantial populations of IL-22-producing T cells was not conferred to mDCs. It remained unclear whether mDCs still lacked the crucial component for IL-22 expression or produced an IL-22-inhibitory factor. A report by Duhon et al. sug-

gests that indeed mDCs secrete a soluble component that impairs IL-22-expression<sup>77</sup>. However, neither Duhén et al. nor the experiments performed here resolved the identity of this component.



**Fig. 5.18: IFN- $\alpha$  conferred to mDCs the capacity to induce IL-10 expression. IL-22 expression is not elicited by mDCs.**

Naïve CD4 T cells were activated in the presence of pDCs (SEB + CpG B) or mDCs (SEB + LPS) and with or without added cytokines or neutralizing antibodies. After 5 to 7 days expression of IL-10 and IL-22 was assessed by cytokine recall and flow cytometry. | Each circle corresponds to a single donor. | \*  $p < 0.05$ ;

\*  $p < 0.05$ ; n.s., not significant

## 5.2 Induction of IL-10- and IL-22-expressing CD4 T cells without pDCs

The experiments in the mDC-T cell coculture had demonstrated that using components of the pDC system functions of plasmacytoid dendritic cells could be transferred to other systems. This provided the basis for a detailed analysis of pDC functions in a more defined system. An APC-free system (“APC-free system”) was established that would allow a bottom-up approach of recreating the T cell phenotype observed in the pDC culture in a minimalistic system.

Not only would such a minimalistic system be a useful tool for the analysis of the pDC functions but could also serve as starting point for the generation of defined T cell populations for therapeutic use.

In this APC-free system, naïve T cells were stimulated by activating anti-CD3 antibodies coated onto latex beads or the well bottom of high-binding culture plates and by anti-CD28-antibodies added to the cell medium in soluble form. Any costimulatory agents to be tested could be added to this system either in a solution (e.g. cytokines) or by coating them onto the beads or the well bottom in parallel to the anti-CD3 antibodies (e.g. Notch ligands).

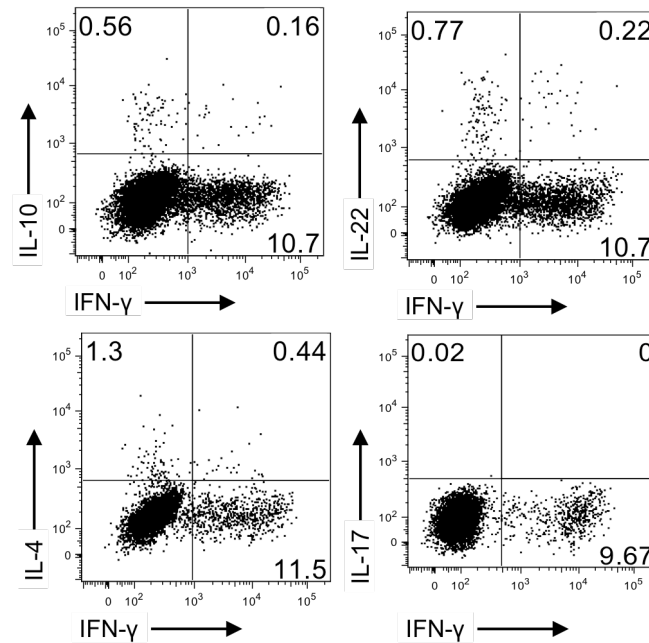
### 5.2.1 CD4 T cells activated in the base APC-free system express no or little IL-10 and IL-22

Naïve T cells from a total of 43 donors were tested in the APC-free system in absence of additional costimulatory agents, thus defining the “starting point” of the assay. The cells were stimulated as described above and cytokine expression was assessed after 5 to 7 days of culture by cytokine recall and intracellular cytokine analysis.

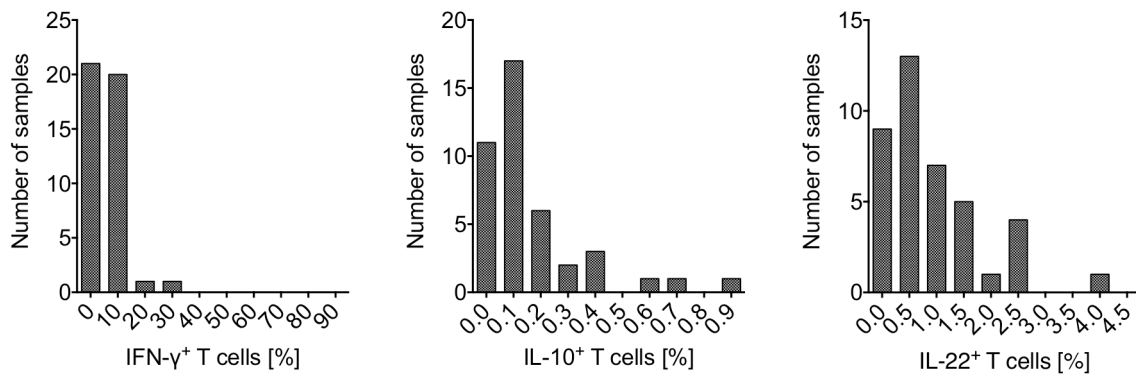
As Fig. 5.19 shows, only a low percentage of CD4 T cells cultivated in the base APC-free system produced IFN- $\gamma$ , the majority of samples contained between 2 % and 14 % IFN- $\gamma$ -expressing T cells. No significant IL-10 production was detected, with most samples showing frequencies of IL-10-positive T cells between 0.0 % and 0.4 % and no sample containing more than 0.9 % of IL-10-positive T cells. The percentage of IL-22-expressing T cells was slightly higher but nonetheless low and with one exception (4 %) did not exceed 2.5 %. Most samples showed frequencies of IL-22-expressing cells of 1.5 % or less.

All in all, cells that were cultured in the APC-free system without any additional costimulus showed a weak bias towards IFN- $\gamma$  expression, no significant IL-10 expression and low IL-22 expression.

**A | Expression of IFN- $\gamma$ , IL-10, IL-22, IL-4 and IL-17 in APC-free system**



**B | Baseline expression profile for IFN- $\gamma$ , IL-10 and IL-22 in APC-free system**



**Fig. 5.19: CD4 T cells cultivated in the base APC-free system express no IL-10, low amounts of IL-22 and moderate amounts of IFN- $\gamma$**

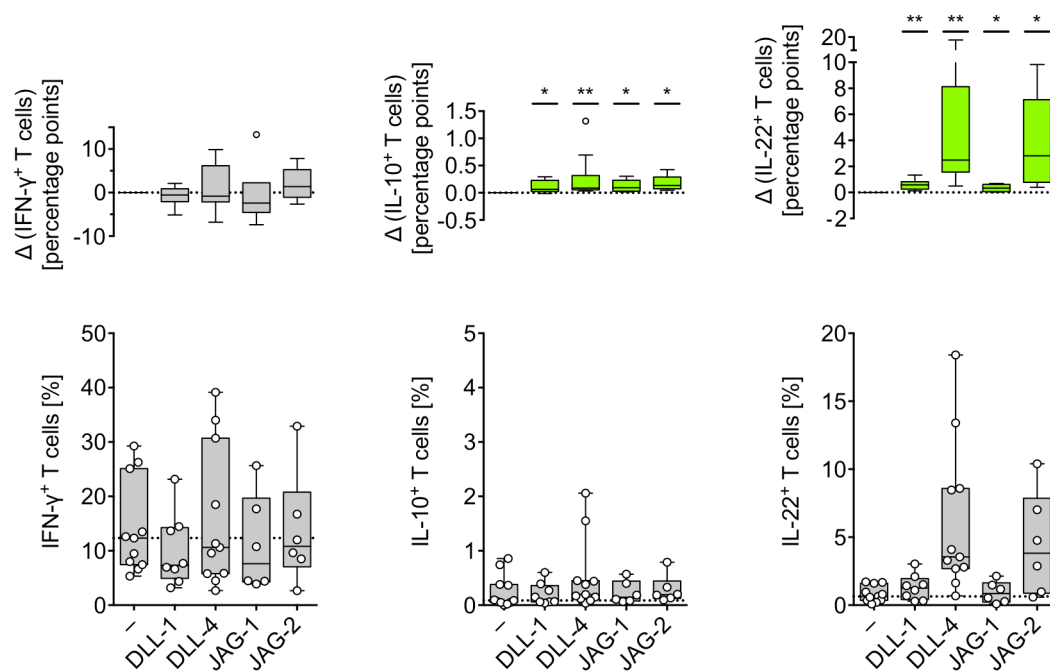
Naïve CD4 T cells were activated in the APC-free system (polyclonal stimulation: anti-CD3 + anti-CD28). After 5 to 7 days cytokine expression was assessed by cytokine recall and flow cytometry. **A |** Expression of IFN- $\gamma$ , IL-10, IL-22, IL-4 and IL-17 was analyzed. Shown data are representative of 4 experiments. **B |** A total of 43 donors was analyzed for IFN- $\gamma$ , IL-10 and IL-22 expression. The Frequency distribution of cytokine expression over all donors was used to define the cytokine expression pattern of CD4 T cells stimulated in the baseline APC-free system.

## 5.2.2 Notch ligand DLL-4 enhances expression of different cytokines

Not only had Notch ligand DLL-4 proved to be one of the crucial factors for the induction of IL-10 and IL-22 expression in the pDC system tested here, but also a host of scientific literature described the various Notch ligands as factors determining or altering cell fate of T helper cells (reviewed in Radtke, MacDonald, and Tacchini-Cottier, 2013<sup>67</sup>, and Yamane and Paul, 2013<sup>155</sup>). Therefore, the effect of costimulation by Notch ligands on the expression of IFN- $\gamma$ , IL-10 and IL-22 was assessed in the APC-free system.

### 5.2.2.1 DLL-4 and JAG-2 drive IL-22 expression without exogenous cytokines

The role of Notch ligands in IL-10 and IL-22 induction was examined by activating naïve T cells in the APC-free system in the presence of immobilized DLL-1, DLL-4, JAG-1 or JAG-2 or without costimulation by Notch ligands. After 5 to 7 days, cytokine expression was analyzed by cytokine recall and intracellular cytokine analysis.



**Fig. 5.20: DLL-4 and JAG-2 induce IL-22 expression**

Naïve CD4 T cells were activated in the APC-free system (anti-CD3 + anti-CD28) in presence or absence of platebound Notch ligands, DLL-1, DLL-4, JAG-1 or JAG-2. After 5 to 7 days IFN- $\gamma$ , IL-10 and IL-22 expression was assessed by cytokine recall and flow cytometry. | Bottom row: frequency of cytokine-expressing T cells in percent [%]. Each circle corresponds to a single donor. Top row: change in frequency of cytokine-expressing T cells compared to control (“-”, sample without Notch ligand). Circles signify outliers according to Tukey. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \*  $p < 0.05$ ; \*\*  $p < 0.005$

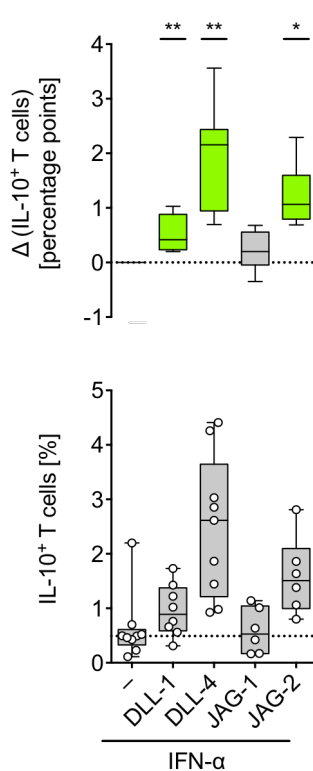
Fig. 5.20 shows that all four Notch ligands induced an increase in the percentage of IL-22-expressing T cells. Interestingly, this effect was only marginal for DLL-1 and JAG-1 (median: 1.3 % and 0.9 %, respectively) compared to T cells cultivated without Notch ligand (median: 0.6 %), whereas DLL-4 and JAG-2 promoted strong induction of IL-22-producing T cells (median: 3.6 % and 3.8 %).

The picture regarding IL-10 expression was quite different. Although all four Notch ligands slightly increased the percentage of IL-10-expressing T cells, none of the Notch ligands induced substantial populations of IL-10<sup>+</sup> T cells (median with Notch ligand: ≤ 0.2 %; median without Notch ligand: 0.09 %).

The frequency of IFN-γ-positive T cells was comparable to IFN-γ expression observed in the base APC-free system and not significantly altered by the presence of Notch ligands.

#### 5.2.2.2 *DLL-4 and JAG-2 require a second signal for IL-10 induction*

Since costimulation by Notch ligands alone failed to induce IL-10-expressing T cells, the same setting was tested in the presence of IFN-α (Fig. 5.21).



**Fig. 5.21: DLL-4 and JAG-2 potently induce IL-10 expression in the presence of IFN-α**

Naïve CD4 T cells were activated in the APC-free system (anti-CD3 + anti-CD28) in presence of IFN-α and with or without platebound Notch ligands, DLL-1, DLL-4, JAG-1 or JAG-2. After 5 to 7 days IL-10 expression was assessed by cytokine recall and flow cytometry. | Bottom row: frequency of cytokine-expressing T cells in percent [%]. Each circle corresponds to a single donor. Top row: change in frequency of cytokine-expressing T cells compared to control (“-”, sample without Notch ligand). Circles signify outliers according to Tukey. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \*  $p < 0.05$ ; \*\*  $p < 0.005$

Although IFN-α by itself promoted a modest increase in IL-10-producing T cells (median: without IFN-α: 0.09 %, with IFN-α: 0.5 %), Notch ligands DLL-1, DLL-4 and JAG-2 significantly enhanced IFN-α-mediated IL-10 induction, whereas JAG-1 failed to do so (median = 0.5 %). DLL-4 was most potent in driving IL-10 expression (median = 2.6 %), only ri-

valued by JAG-2 (median = 1.5 %). DLL-1 only modestly increased IL-10 production (median = 0.9 %).

These results demonstrate that DLL-4 in combination with CD3- and CD28-activation was sufficient for driving robust IL-22 expression.

The finding that DLL-4 in the absence of additional costimulatory signals failed to induce IL-10 expression comparable to that observed in the pDC system implied that IL-10 induction required another signal in addition to DLL-4 ligation. This was confirmed by the finding that DLL-4 together with IFN- $\alpha$  induced significant IL-10 expression. Interestingly, not only DLL-4, but also JAG-2 proved to be a potent enhancer of cytokine expression. Finally, the observation that all Notch ligands were capable of inducing at least a low increase in both IL-10 and IL-22 expression may suggest the existence of a baseline Notch signal or activity that any Notch ligand can trigger.

#### 5.2.2.3 *DLL-4 potentiates the capacity of cytokines to drive IL-10, IL-22 or IL-17 expression*

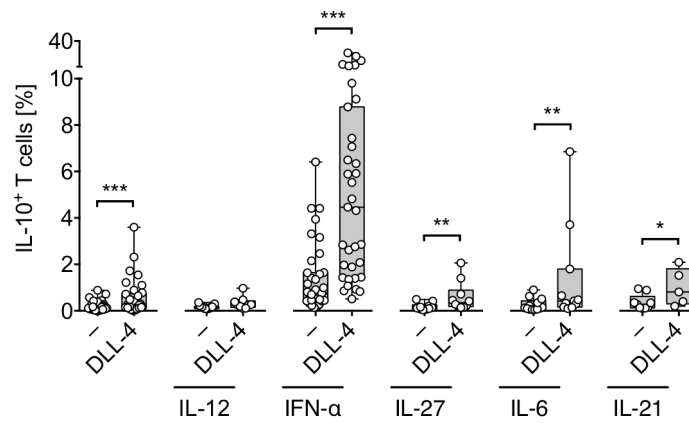
Notch ligand DLL-4 has been reported to enhance cytokine expression in various T<sub>H</sub> settings in mouse and human. It was shown to facilitate IL-10 production in mouse T<sub>H</sub>1 cells <sup>144</sup>, IL-22 expression in T<sub>H</sub>22 cells <sup>94</sup> and IL-17 and IL-22 expression in T<sub>H</sub>17 cells <sup>93</sup>.

In order to clarify whether DLL-4 costimulation can enhance IL-10 production induced by various cytokines that are known to be secreted by pDCs <sup>96,98,151,152</sup> and have been shown to play a role in IL-10 induction <sup>34,38,46,52,68</sup>, these cytokines were tested in combination with DLL-4 in the APC-free system.

Naïve CD4 T cells were activated in presence or absence of DLL-4 with IL-12, IFN- $\alpha$ , IL-27, IL-6, IL-21 or no added cytokine. After 5 to 7 days, IL-10 expression was assessed by cytokine recall and intracellular cytokine analysis.

Baseline IL-10 expression induced by the cytokines was different for each cytokine, but costimulation with DLL-4 significantly enhanced IL-10 induction by all tested cytokines, except IL-12 (Fig. 5.22). The resulting level of IL-10 expression was specific for each cytokine as was the relative increase mediated by DLL-4 (average increase based on median values: no cytokine: 2.3-fold; IL-12: 1.6-fold; IL-27: 2.8-fold; IL-6: 3.3-fold; IL-21: 2.9-fold; IFN- $\alpha$ : 5.5-fold). Interestingly, IFN- $\alpha$  stood out, not only in regard to the absolute level of IL-10 induction but also concerning the relative increase in IL-10 expression. For the cytokines IL-6, IL-21, IL-27 and the culture without added cytokine, the relative increase in the percentage of IL-10-expressing T cells lay in the range of 2.3-fold to 3.3-fold, whereas

IFN- $\alpha$ -treated cells showed a roughly 5.5-fold higher expression of IL-10 upon costimulation with DLL-4.



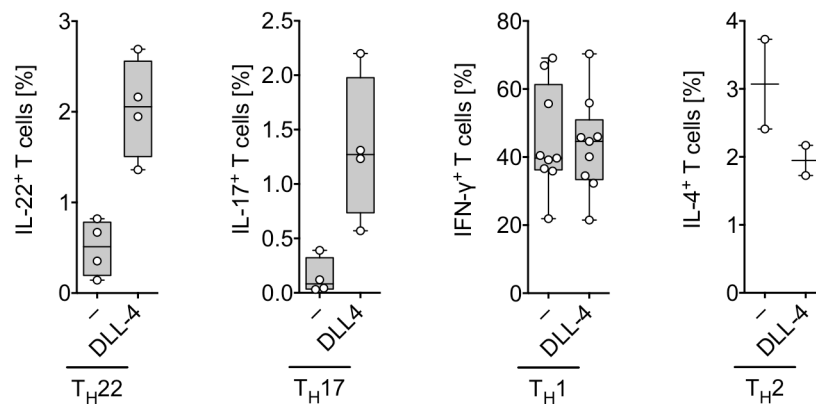
**Fig. 5.22: Cytokines like IFN- $\alpha$ , IL-6, IL-21 and IL-27 confer IL-10-inducing capacity to Notch ligand DLL-4 and vice versa**

Naïve CD4 T cells were activated in the APC-free system (anti-CD3 + anti-CD28) in presence or absence of DLL-4 and with or without added cytokines. After 5 to 7 days IL-10 expression was assessed by cytokine recall and flow cytometry. | Each circle corresponds to a single donor. | \*  $p < 0.05$ ; \*\*  $p < 0.005$ ; \*\*\*  $p < 0.0005$

As had been observed before, costimulation with DLL-4 without added cytokine already led to a small but significant increase in IL-10 expression. Interestingly, although not statistically significant, also the combination of DLL-4 and IL-12 slightly increased IL-10 expression compared to IL-12 alone.

This experiment clearly shows that DLL-4 enhances the IL-10-inducing capacities of cytokines like IFN- $\alpha$ , IL-6, IL-21 and IL-27 that are known to drive IL-10 expression in T cells, with IFN- $\alpha$  proving to be a particularly effective inducer of IL-10. In addition, DLL-4 appears to partially activate an IL-10-driving mechanism that requires supplementary signals – here provided by cytokines – to become fully activated.

In order to understand whether DLL-4-mediated enhancement is limited to IL-10 expression and IL-10-driving cytokines, prototypical cytokine expression was analyzed under the corresponding T<sub>H</sub> polarizing conditions.



**Fig. 5.23: DLL-4 enhances production of IL-22 and IL-17 under  $T_H22$  and  $T_H17$  polarizing conditions**

Naïve CD4 T cells were activated in the APC-free system (anti-CD3 + anti-CD28) in presence or absence of DLL-4 under conditions inducing  $T_H22$  (anti-IL-4 + anti-IFN- $\gamma$  + IL-6),  $T_H17$  (anti-IL-4 + anti-IFN- $\gamma$  + IL-6 + IL-1 $\beta$  + IL-23),  $T_H1$  (IL-12) or  $T_H2$  (anti-IFN- $\gamma$  + IL-4). After 5 to 7 days cytokine expression was assessed by cytokine recall and flow cytometry. | Each circle corresponds to a single donor.

Naïve CD4 T cells were activated in the APC-free system under  $T_H22$  conditions (anti-IL-4, anti-IFN- $\gamma$ , IL-6),  $T_H17$  conditions (anti-IL-4, anti-IFN- $\gamma$ , IL-1 $\beta$ , IL-6, IL-23, TGF- $\beta$ ),  $T_H1$  conditions (IL-12) and  $T_H2$  conditions (IL-4, anti-IFN- $\gamma$ ) in the presence and absence of DLL-4. After 5 to 7 days, cytokine expression was assessed by cytokine recall and intracellular cytokine analysis.

DLL-4 significantly enhanced IL-22 induction under  $T_H22$  skewing conditions and IL-17 expression under  $T_H17$  conditions but had no or inhibitory effect on IFN- $\gamma$  expression under  $T_H1$  and IL-4 expression under  $T_H2$  (Fig. 5.23).

These results indicated that DLL-4 may facilitate cytokine expression under some but not all conditions. Curiously, DLL-4 did not enhance IFN- $\gamma$  expression although being described as  $T_H1$ -promoting factor<sup>67</sup>. Of note, all cytokines and conditions that benefited from DLL-4 signaling have been described to act mainly through STAT3, whereas the two conditions that showed no enhancement of cytokine production are known to depend on STAT4 (IL-12) and STAT6 (IL-4), respectively.

### 5.2.3 IFN- $\alpha$ induces strong IL-10 expression in concert with IL-6 and IL-21

Although several of the cytokines that can be produced by pDCs facilitated IL-10 expression, the question which cytokine or cytokine combination was the main driver of IL-10 expression in the pDC system remained unanswered. To address this question a more detailed analysis was performed.

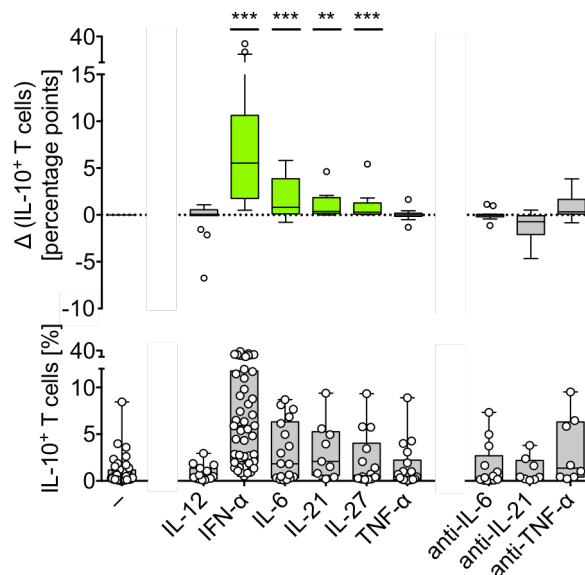
#### 5.2.3.1 *IFN- $\alpha$ induces highest IL-10 expression among pDC-derived cytokines*

Several typical pDC cytokines (IFN- $\alpha$ , IL-6, IL-21, TNF- $\alpha$ , IL-12) had been shown to be present in the pDC system and influence the outcome of T cell differentiation. To better understand the contribution of each of these single cytokines they were tested and compared in the APC-free system.

Naïve CD4 T cells were activated in presence of DLL-4 adding IL-12, IFN- $\alpha$ , IL-6, IL-21, IL-27 or TNF- $\alpha$ , or blocking IL-6, IL-21 or TNF- $\alpha$  by specific antibodies. Costimulation by DLL-4 in absence of added cytokine was used as reference point. After 5 to 7 days, IL-10 expression was assessed by cytokine recall and intracellular cytokine analysis (Fig. 5.24).

While adding IL-12 failed to enhance the number of IL-10-producing cells, IFN- $\alpha$  strongly increased the percentage of IL-10-expressing T cells. Although IFN- $\alpha$  showed by far the greatest capacity of enhancing IL-10 expression (median: 5.9%), also IL-6 and IL-21 significantly induced IL-10 (median: 1.8% and 2.1%, respectively), while IL-27 induced only very modest IL-10 expression (median: 0.6%). The block of IL-21, but not of IL-6, reduced baseline IL-10 expression, indicating that in contrast to IL-6, IL-21 is produced in the baseline culture. Neither adding nor blocking TNF- $\alpha$  produced an effect on IL-10 expression.

In agreement with the results from the pDC system, IL-12 did not induce IL-10 expression, while IFN- $\alpha$  most potently enhanced IL-10-production. In addition, the results show that IL-6 and IL-21 are not only involved in the IL-10 induction in the pDC system but in agreement with observations in mouse <sup>34,38</sup> are themselves capable of driving IL-10 expression.



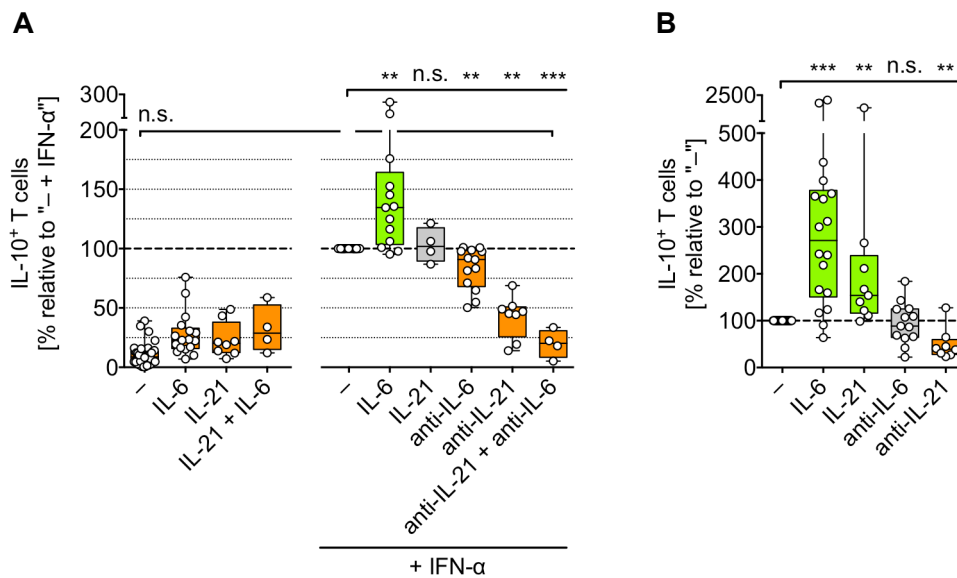
**Fig. 5.24: Cytokines like IFN-α, IL-21, IL-6 or IL-27 are required for induction of IL-10 by DLL-4**

Naïve CD4 T cells were activated in the APC-free system (anti-CD3 + anti-CD28) in presence of DLL-4 with added cytokines or neutralizing antibodies, or without any of both. After 5 to 7 days expression of IL-10 was assessed by cytokine recall and flow cytometry. | Bottom row: frequency of cytokine-expressing T cells in percent [%]. Each circle corresponds to a single donor. Top row: change in frequency of cytokine-expressing T cells compared to control (“-”, sample without added cytokines or antibodies). Circles signify outliers according to Tukey. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \*\* p < 0.005; \*\*\* p < 0.0005

#### 5.2.3.2 IFN-α promotes IL-10 expression via endogenous IL-6 and IL-21

The finding that IFN-α as well as IL-6 and IL-21 may be involved in the induction of IL-10 prompted the question whether these cytokines act independently or if their capacity of inducing IL-10 was interlinked. To address this question interdependence of IFN-α and IL-6 and IL-21 in activating IL-10 expression was analyzed. Since IFN-α had shown the strongest capacity of promoting IL-10 expression in T cells and is believed to be the main effector of pDCs, it was used as reference and was directly compared to IL-6, IL-21 or the combination of both. In addition, the effect of blocking of any endogenously produced IL-6 and/or IL-21 was examined.

Naïve CD4 T cells were activated in presence of DLL-4 adding IFN-α, IL-6, IL-21 or combinations of IFN-α and IL-6, IL-21 or IL-6/IL-21-blocking antibodies. Costimulation by DLL-4 and IFN-α (Fig. 5.25 A) or by DLL-4 alone (Fig. 5.25 B) was used as reference point. After 5 to 7 days, IL-10 expression was assessed by cytokine recall and intracellular cytokine analysis



**Fig. 5.25: IL-10 induction by IFN- $\alpha$  is partially mediated by IL-21 and IL-6**

Naïve CD4 T cells were activated in the APC-free system (anti-CD3 + anti-CD28) in presence of DLL-4 with added cytokines or neutralizing antibodies, or without any of both. After 5 to 7 days expression of IL-10 was assessed by cytokine recall and flow cytometry. **A** | Frequencies of IL-10-expressing cells were normalized to samples with IFN- $\alpha$  only ("- + IFN- $\alpha$ "). **B** | Frequencies of IL-10-expressing cells were normalized to samples without added cytokine or antibody ("-") | Each dot corresponds to one donor. Green or orange color indicates frequency was increased or decreased, respectively, in statistically significant manner. | \*\*  $p < 0.005$ ; \*\*\*  $p < 0.0005$ ; n.s., not significant

As Fig. 5.25 A clearly shows, neither IL-6 nor IL-21 or the combination of both was nearly as potent an IL-10-inducing factor as IFN- $\alpha$ . In average, addition of IFN- $\alpha$  induced two- to ten-fold more IL-10-positive T cells than these two cytokines. Interestingly, the percentage of IL-10-expressing T cells was significantly increased when IFN- $\alpha$  was supplemented with IL-6, whereas blocking IL-6 moderately reduced IFN- $\alpha$ -driven IL-10 expression.

Another interesting observation was that in the absence of IFN- $\alpha$  the IL-6 block had no effect on IL-10 expression (Fig. 5.25 B). This indicates that although endogenously produced IL-6 was involved in the induction of IL-10 it was only present in moderate amounts and likely only when IFN- $\alpha$  was added.

In contrast, exogenous IL-21 did not enhance IL-10-induction by IFN- $\alpha$  but led to a moderate increase of IL-10 producers in cultures without IFN- $\alpha$ . IL-21 block, on the other hand, strongly reduced IL-10-induction mediated by IFN- $\alpha$  (in average by approx. 50 % to 75 %) (Fig. 5.25 A), but also diminished the number of IL-10-producing T cells in the IFN- $\alpha$ -free culture (Fig. 5.25 B). When both, IL-6 and IL-21, were blocked in the presence of IFN- $\alpha$ , IL-10 expression was almost completely abrogated (reduction by 66 % to 95 %), bringing

it down to a level comparable to without added cytokine (average: 10 %, relative to culture with IFN- $\alpha$ ).

These results suggested that endogenously produced IL-6 and IL-21 largely mediated the IL-10 induction by IFN- $\alpha$ , but required the IFN- $\alpha$  signal for full activation of IL-10 expression. The observation that exogenous IL-21 enhanced IL-10 expression in IFN- $\alpha$ -free cultures but not in the presence of IFN- $\alpha$  indicated that IFN- $\alpha$  elicited IL-21 secretion by T cells. Most likely also endogenous IL-6 production was triggered by IFN- $\alpha$ , since blocking IL-6 in the presence of IFN- $\alpha$ , but not in culture without added IFN- $\alpha$ , significantly reduced IL-10 levels.

#### 5.2.4 Expression of IL-22 is not driven by exogenous cytokines

No cytokine could be discerned as IL-22-driving factor in the pDC system, whereas presence of Notch ligand DLL-4 had proven sufficient for pronounced IL-22 expression in the APC-free system. In an attempt to understand, if any of the classical pDC cytokines and typical IL-22-driving cytokines were capable of inducing IL-22 expression, IL-12, IFN- $\alpha$ , IL-6, IL-21, IL-1 $\beta$ , IL-23, TNF- $\alpha$ , the combination of IL-6 and TNF- $\alpha$  and blocking antibodies specific for IL-6, IL-21 and TNF- $\alpha$  were tested in the APC-free system.

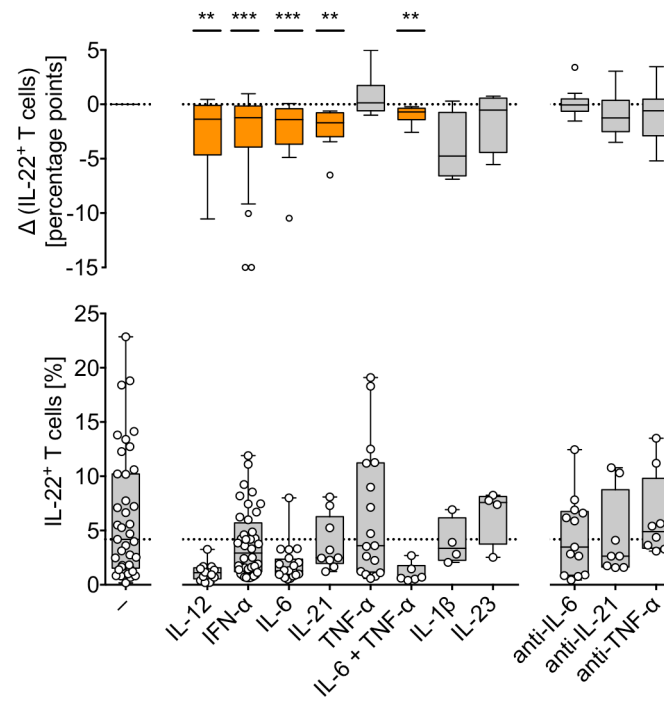
Most of the analyzed cytokines are known to induce or enhance IL-22 expression, i.e. IL-6 has been reported to drive IL-22 expression both in mouse (mainly T<sub>H</sub>17) and human (T<sub>H</sub>22) IL-6<sup>77,81</sup>, the cytokines IL-21, IL-1 $\beta$ , IL-23 have been described as IL-22 inducers in the T<sub>H</sub>17 context<sup>81,84</sup> and TNF- $\alpha$  has been found to enhance T<sub>H</sub>22 differentiation in human<sup>77</sup>. The combination of IL-6 and TNF- $\alpha$  has been described as potentially inducing T<sub>H</sub>22 polarization.

##### 5.2.4.1 *IL-22 expression requires only costimulation by DLL-4*

Naïve CD4 T cells were activated in the presence of DLL-4 alone or with IL-12, IFN- $\alpha$ , IL-6, IL-21, IL-1 $\beta$ , IL-23, TNF- $\alpha$ , the combination of IL-6 and TNF- $\alpha$  or blocking antibodies specific for IL-6, IL-21 and TNF- $\alpha$ . After 5 to 7 days, IL-22 expression was assessed by cytokine recall and intracellular cytokine analysis (Fig. 5.26).

Surprisingly, none of the added cytokines enhanced IL-22 expression. In contrast, all cytokines – with the exception of TNF- $\alpha$  – led to a more or less pronounced reduction of IL-22 expression. Blocking IL-6, IL-21 or TNF- $\alpha$  did not result in significant change of IL-22 expression, although blocking TNF- $\alpha$  and IL-21 slightly, but not in a statistically significant manner, reduced the number of IL-22-expressing T cells.

These results supported the hypothesis that, barring DLL-4, IL-22 induction is independent of exogenous factors in this setting.



**Fig. 5.26: No tested cytokine enhanced IL-22 expression**

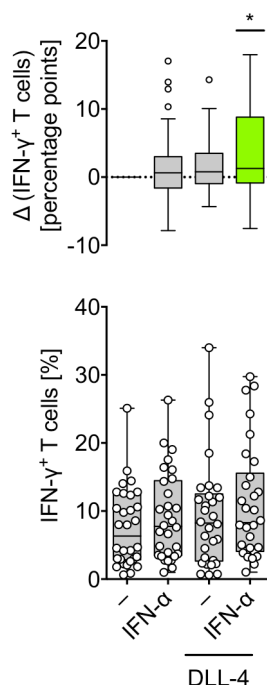
Naïve CD4 T cells were activated in the APC-free system (anti-CD3 + anti-CD28) in presence of DLL-4 with added cytokines or neutralizing antibodies, or without any of both. After 5 to 7 days expression of IL-22 was assessed by cytokine recall and flow cytometry. | Bottom row: frequency of cytokine-expressing T cells in percent [%]. Each circle corresponds to a single donor. Top row: change in frequency of cytokine-expressing T cells compared to control (“-”, sample without added cytokines or antibodies). Circles signify outliers according to Tukey. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \*\*  $p < 0.005$ ; \*\*\*  $p < 0.0005$

### 5.2.5 Pronounced $T_H1$ commitment is not required for induction of IL-22 or IL-10

The T cell populations generated in the pDC system were characterized by a pronounced IFN- $\gamma$  expression. Interestingly, immunoregulatory T cells of the IFN- $\gamma$ +IL-10 $^+$  phenotype are involved in various disease-related settings in human and in mouse <sup>1,13,15</sup>, and DLL-4 has been described to induce immunosuppressive IL-10-producing  $T_H1$  cells in mouse <sup>68</sup>. Thus, coexpression of IFN- $\gamma$  and IL-10 or IL-10-producing  $T_H1$  cells appear to be a hallmark of a certain kind of regulatory T cells.

To better understand the role of IFN- $\gamma$  and identify factors driving IFN- $\gamma$  expression under the conditions found to induce IL-10 and IL-22, expression patterns of IFN- $\gamma$  following stimulation under the various conditions were analyzed.

#### 5.2.5.1 Neither DLL-4, nor IFN- $\alpha$ drive $T_H1$ commitment



**Fig. 5.27: Neither DLL-4 nor IFN- $\alpha$  strongly enhance IFN- $\gamma$  expression**

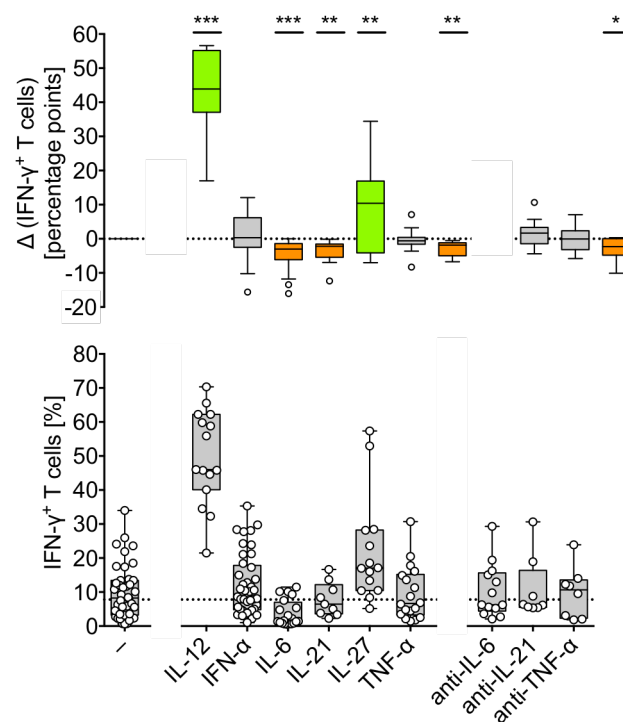
Naïve CD4 T cells were activated in the APC-free system (anti-CD3 + anti-CD28) in presence or absence of DLL-4 and/or IFN- $\alpha$ . After 6 days expression of IFN- $\gamma$  was assessed by cytokine recall and flow cytometry. | Bottom row: frequency of cytokine-expressing T cells in percent [%]. Each circle corresponds to a single donor. Top row: change in frequency of cytokine-expressing T cells compared to control (“–”, sample without added cytokines or antibodies). Circles signify outliers according to Tukey. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \*  $p < 0.05$

Published data on  $T_H1$ -skewing by IFN- $\alpha$  or DLL-4 is controversial. Although both have been described as  $T_H1$  promoting factors <sup>134,156,157</sup>, more recent data indicate that neither in mouse nor in human IFN- $\alpha$  is capable of sustaining  $T_H1$  commitment <sup>137,158</sup>. Also, DLL-4 is now assumed to rather support  $T_H1$  functions than induce  $T_H1$  polarization <sup>67</sup>.

IFN- $\gamma$  expression of naïve CD4 T cells activated in the APC-free system and analyzed after 6 days (Fig. 5.27) showed no significant difference in IFN- $\gamma$  expression between cells that

were cultivated in presence or absence of DLL-4 or IFN- $\alpha$ . Merely combined costimulation of DLL-4 and IFN- $\alpha$  lead to a slight increase in the frequency of IFN- $\gamma$ -expressing T cells. However, the degree of IFN- $\gamma$  expression remained far below the levels observed in the pDC system.

#### 5.2.5.2 Only IL-12 and IL-27 significantly enhance IFN- $\gamma$ expression



**Fig. 5.28: IFN- $\gamma$  expression is only enhanced by IL-12 or IL-27**

Naïve CD4 T cells were activated in the APC-free system (anti-CD3 + anti-CD28) in presence of DLL-4 with added cytokines or neutralizing antibodies, or without any of both. After 5 to 7 days expression of IFN- $\gamma$  was assessed by cytokine recall and flow cytometry. | Bottom row: frequency of cytokine-expressing T cells in percent [%]. Each circle corresponds to a single donor. Top row: change in frequency of cytokine-expressing T cells compared to control (“–”, sample without added cytokines or antibodies). Circles signify outliers according to Tukey. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \*  $p < 0.05$ ; \*\*  $p < 0.005$ ; \*\*\*  $p < 0.0005$

To understand, which of the cytokines secreted by pDCs might have been responsible for the pronounced IFN- $\gamma$  expression observed in the pDC system, the effect of exogenous IL-12, IFN- $\alpha$ , IL-21, IL-6, IL-27 and TNF- $\alpha$  was analyzed. In order to analyze whether cytokines like IL-6, IL-21 or TNF- $\alpha$  inhibit IFN- $\gamma$  expression the effect of blocking antibodies against these three cytokines was examined.

Naïve CD4 T cells were activated in the APC-free system in presence of DLL-4 and the described cytokines or antibodies. After 5 to 7 days of culture, expression of IFN- $\gamma$ , IL-10 and IL-22 was assessed by cytokine recall and intracellular cytokine analysis (Fig. 5.28).

IL-12 as the prototypic T<sub>H</sub>1 driving cytokine induced a strong upregulation of IFN- $\gamma$ , while, as shown before, IFN- $\alpha$  had no significant effect. Addition of TNF- $\alpha$  did not alter IFN- $\gamma$  expression, but blocking the cytokine led to a modest reduction of IFN- $\gamma$ . This indicates that endogenously produced TNF- $\alpha$  contributes to the induction of IFN- $\gamma$  expression. Interestingly, added IL-6 and IL-21 reduced IFN- $\gamma$  expression, while blocking these two cytokines had no effect. IL-27 was the only other cytokine that induced significant IFN- $\gamma$  expression, although IL-27-mediated IFN- $\gamma$  production was not comparable with the IFN- $\gamma$  response elicited by IL-12.

#### 5.2.5.3 *pDCs and DLL-4 with IFN- $\alpha$ induce comparable IL-10 and IL-22 expression in CD4 T cells*

The finding that the T cell populations generated in the pDC system and in the APC-free system differed significantly in the level of IFN- $\gamma$  expression raised the question whether this difference would affect the expression of IL-22 and IL-10, in other words, was IFN- $\gamma$  expression required for comparable IL-10 or IL-22 production?

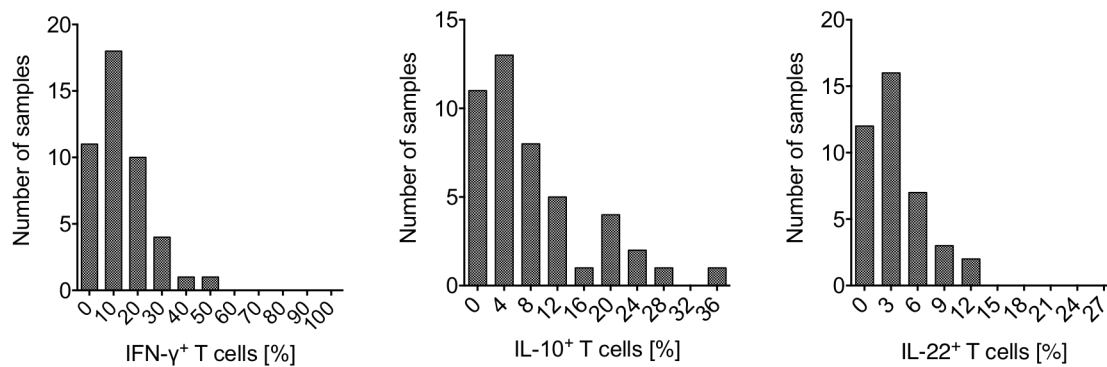
This question was addressed comparing the cohort of blood samples tested in the APC-free system in presence of DLL-4 and IFN- $\alpha$  with the cohort of blood samples tested in the *base pDC system* (without additional costimulus) in regard to the distribution of expression frequencies for IFN- $\gamma$ , IL-10 and IL-22 for each cohort. Whether the T cell populations generated in the APC-free system resembled the T cell populations generated by pDCs was assessed by comparing frequency distributions.

The pDC system (Fig. 5.29 B) and the APC-free system with DLL-4 and IFN- $\alpha$  (Fig. 5.29 A) show similar frequency distributions for both IL-10 and IL-22. In the APC-free system (Fig. 5.29 A) most samples (36/46 donors) contained 2 % to 18 % of IL-10 producers. Only a small portion of samples (4/46 donors) did not show IL-10 expression, whereas a slightly larger fraction (6/46) contained between 18 % and 36 % IL-10 producers. These values largely resembled the IL-10 frequency distribution found in the pDC system (Fig. 5.29 B) (3 % and 15 % in 41/51 donors;  $\leq 3$  % in 2/51 donors,  $\geq 15$  % in 8/51 donors).

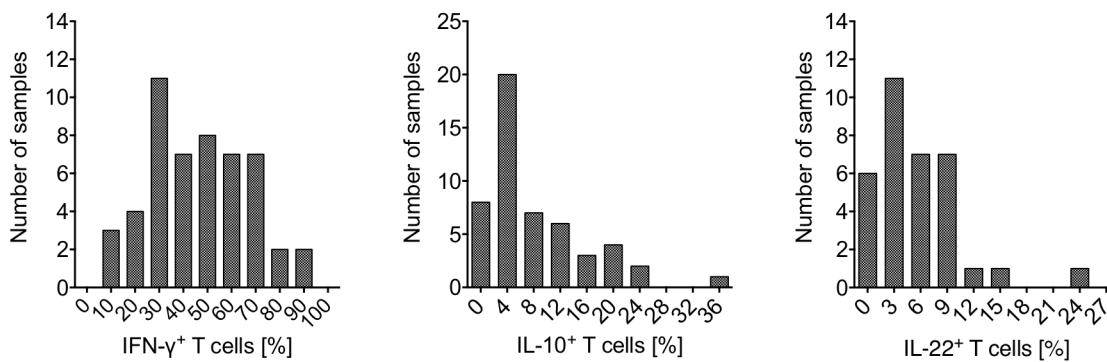
The frequencies of IL-22-positive T cells in the APC-free system (Fig. 5.29 A) lay between 1 % and 7 % in most samples (34/40 donors). The remaining samples (6/40) contained 7 % to 12 % IL-22-expressing T cells. Also IL-22 expression resembled the distribution found in the pDC system (Fig. 5.29 B) (2 % to 10 % in 28/34 donors;  $\leq 2$  % in 3/34 donors;  $\geq 10$  % in 3/34 donors).

In contrast, IFN- $\gamma$  expression was clearly different between the two systems. Significantly less cells that were stimulated in the APC-free system expressed IFN- $\gamma$  in comparison to cells cultured in the pDC system.

**A** | APC-free system with DLL-4 and IFN- $\alpha$ : Frequencies of IFN- $\gamma$ -, IL-10- and IL-22-expressing T cells



**B** | pDC system: Frequencies of IFN- $\gamma$ -, IL-10- and IL-22-expressing T cells

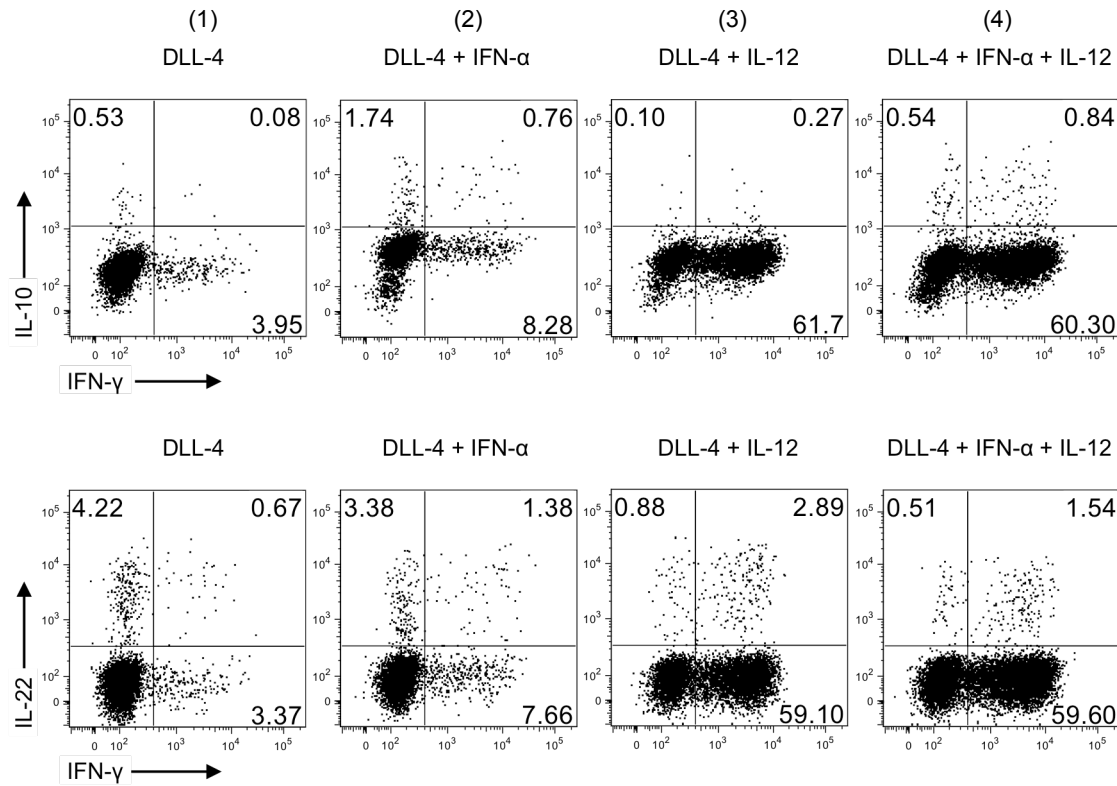


**Fig. 5.29: Cells differentiated in the presence of DLL-4 and IFN- $\alpha$  without APCs show IL-10 and IL-22 expression profiles similar to T cell populations generated by pDCs**

**A** | Expression profile of naïve CD4 T cells activated in the pDC system (see section 5.1.1 pDCs induce a mixed population of T cells expressing IFN- $\gamma$ , IL-10 and IL-22). **B** | A total number of 46 donors were activated in the APC-free system (anti-CD3 + anti-CD28), in presence of DLL-4 and IFN- $\alpha$ . Expression of IFN- $\gamma$ , IL-10 and IL-22 was analyzed by cytokine recall and flow cytometry after 5 to 7 days. The Frequency distribution of cytokine expression over all donors was used to define the cytokine expression pattern.

In summary, although T cells activated in the APC-free system show significantly lower expression of IFN- $\gamma$ , expression of IL-10 and IL-22 were similar, indicating that the difference in IFN- $\gamma$  expression did not directly translate into a difference in IL-10 and IL-22 expression.

#### 5.2.5.4 IL-22 and IL-10 expression is largely retained after $T_H1$ commitment



**Fig. 5.30: IL-10 and IL-22 expression is retained despite  $T_H1$  polarization**

Naïve CD4 T cells were activated in the APC-free system (anti-CD3 + anti-CD28) in presence of DLL-4. IFN-α, IL-12, IFN-α and IL-12 or no cytokine were added. Expression of IFN-γ, IL-10 and IL-22 was assessed by cytokine recall and flow cytometry after 7 days. | The shown data are representative of two independent experiments.

To further test the hypothesis that the degree of  $T_H1$  commitment and IL-10 and IL-22 expression were not directly linked, the effect of inducing strong  $T_H1$  polarization under culture conditions generating IL-22 and IL-10 expression was tested.

For this purpose, naïve CD4 T cells were activated in the APC-free system in presence of DLL-4 alone or adding IFN-α, IL-12 or the combination of IFN-α and IL-12. After 6 days of culture IFN-γ, IL-10 and IL-22 expression was analyzed by cytokine recall and intracellular cytokine analysis.

As has been demonstrated before, DLL-4 alone induced a substantial population of IL-22-producing cells ( $\approx 4.9\%$ ; Fig. 5.30-1) and introduction of IFN-α added a population of IL-10-expressors ( $\approx 2.5\%$ ; Fig. 5.30-2) while only marginally affecting IL-22 expression ( $\approx 4.8\%$ ). IFN-γ expression under both conditions was low ( $\approx 4\%$  and  $\approx 9\%$ , respectively).

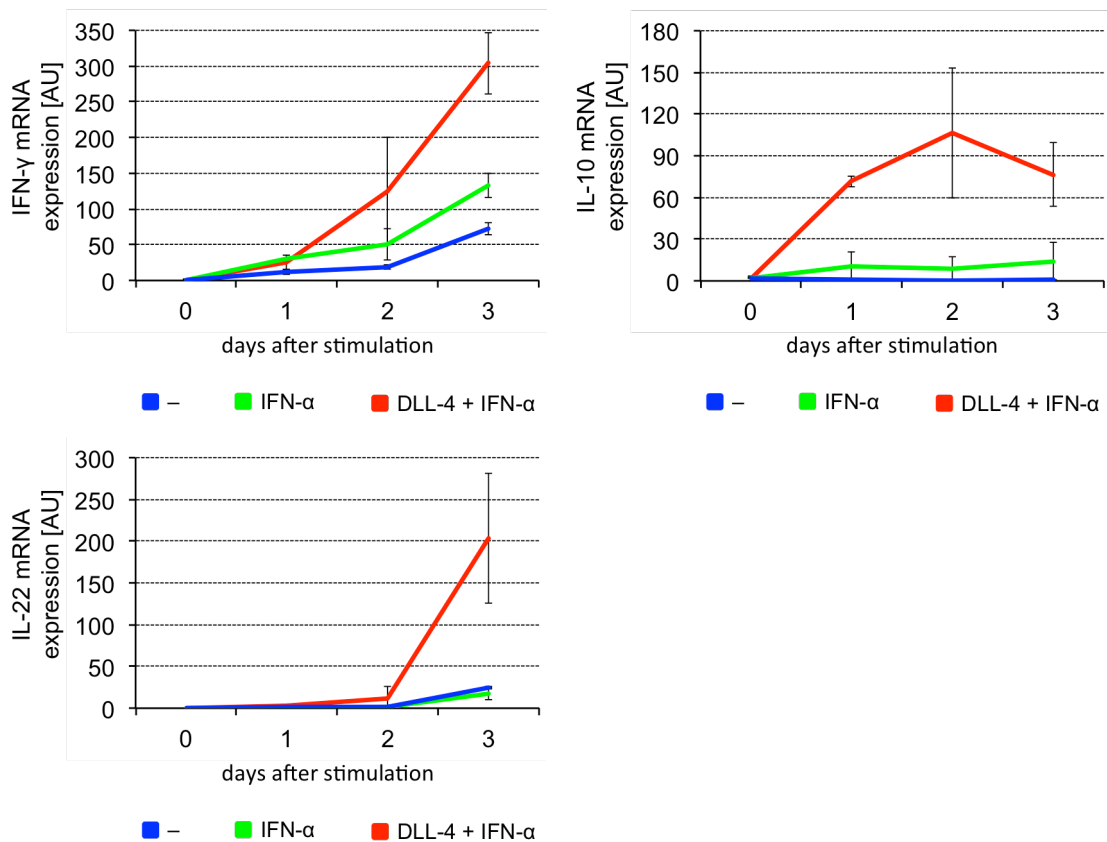
Inducing pronounced T<sub>H</sub>1 polarization through IL-12 strongly enhanced IFN- $\gamma$  expression in both conditions ( $\approx 62\%$  and  $\approx 61\%$ , respectively) while retaining a substantial part of IL-22 expression ( $\approx 3.8\%$  and  $\approx 2.1\%$ , respectively; Fig. 5.30-3 and Fig. 5.30-4) and IL-10 expression ( $\approx 1.4\%$ ; Fig. 5.30-4).

The results indicated that neither IL-22 nor IL-10 were directly linked to the level of T<sub>H</sub>1 polarization and that T<sub>H</sub>1 commitment could be added as an additional layer, “customizing” the populations generated in the APC-free system.

### 5.2.6 IFN- $\gamma$ , IL-10 and IL-22 show very distinct expression kinetics

Using the pDC system several transcriptional modules that appear to govern cytokine production by T cells activated by pDCs have been identified. In order to facilitate analysis of these modules in the APC-free system and establish a reference frame for the investigation of transcription factors, mRNA expression of IFN- $\gamma$ , IL-10 and IL-22 following stimulation in the APC-free system was assessed.

Naïve CD4 T cells were activated in the APC-free system in the presence of DLL-4 and IFN- $\alpha$ , IFN- $\alpha$  alone or without any additional costimulus (“–”). Levels of IFN- $\gamma$ , IL-10 and IL-22 mRNA in the T cells were determined by quantitative PCR before activation (day 0) and 1, 2 or 3 days after T cell activation.



**Fig. 5.31: mRNA Expression kinetics of IFN- $\gamma$ , IL-10 and IL-22**

Naïve CD4 T cells were activated in the APC-free system (anti-CD3 + anti-CD28) without DLL-4 and IFN- $\alpha$  (“–”), only with IFN- $\alpha$  (“IFN- $\alpha$ ”) or in presence of DLL-4 and IFN- $\alpha$  (“DLL-4+IFN- $\alpha$ ”). Transcript of *IFNG*, *IL10* and *IL22* was quantified before activation (0), and 1, 2 and 3 days after activation.

As Fig. 5.31 shows, T cells activated in the base APC-free system (no DLL-4, no IFN- $\alpha$ ) expressed no IL-10 mRNA and low levels of IFN- $\gamma$  and IL-22 mRNA until day 3. IFN- $\alpha$  by it-

self induced a modest increase in IFN- $\gamma$  and IL-10 mRNA levels but did not affect IL-22 mRNA expression. In contrast, combined costimulation of IFN- $\alpha$  and DLL-4 induced a strong increase in mRNA expression of all three cytokines, surpassing mRNA levels in the base APC-free system and of IFN- $\alpha$ -stimulated T cells by a factor of 2.5 to 10.

Of note, all three cytokines displayed very distinct expression kinetics. IL-10 mRNA was upregulated as soon as day 1, reaching a plateau between day 1 and 2. In contrast, IFN- $\gamma$  mRNA levels started to rise early, but increased gradually and appeared to not have reached the maximum at day 3. IL-22 mRNA levels remained very low until day 2 in all three settings and showed a significant increase only after 2 days of culture.

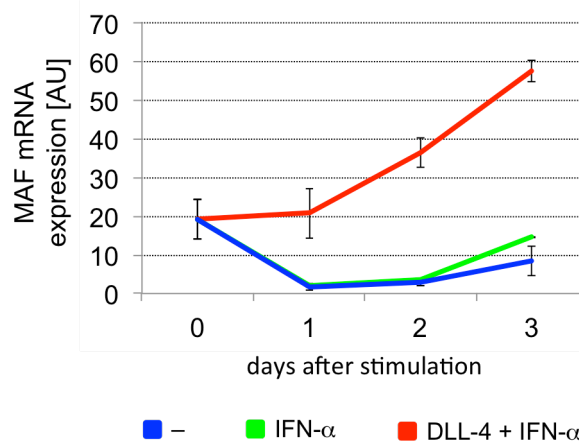
### 5.2.7 MAF, AhR and BLIMP1 contribute to IL-10 and IL-22 expression

The three transcriptional modules MAF, AhR and BLIMP1 had been shown to play a role in driving IL-10 or IL-22 expression in the pDC system. In order to establish, whether cells generated in the APC-free system resemble the populations found in the pDC system, and to gain a better understating of their role in the T cell differentiation process these modules were analyzed in the APC-free system.

#### 5.2.7.1 Sustained MAF expression is required for IL-10-expression

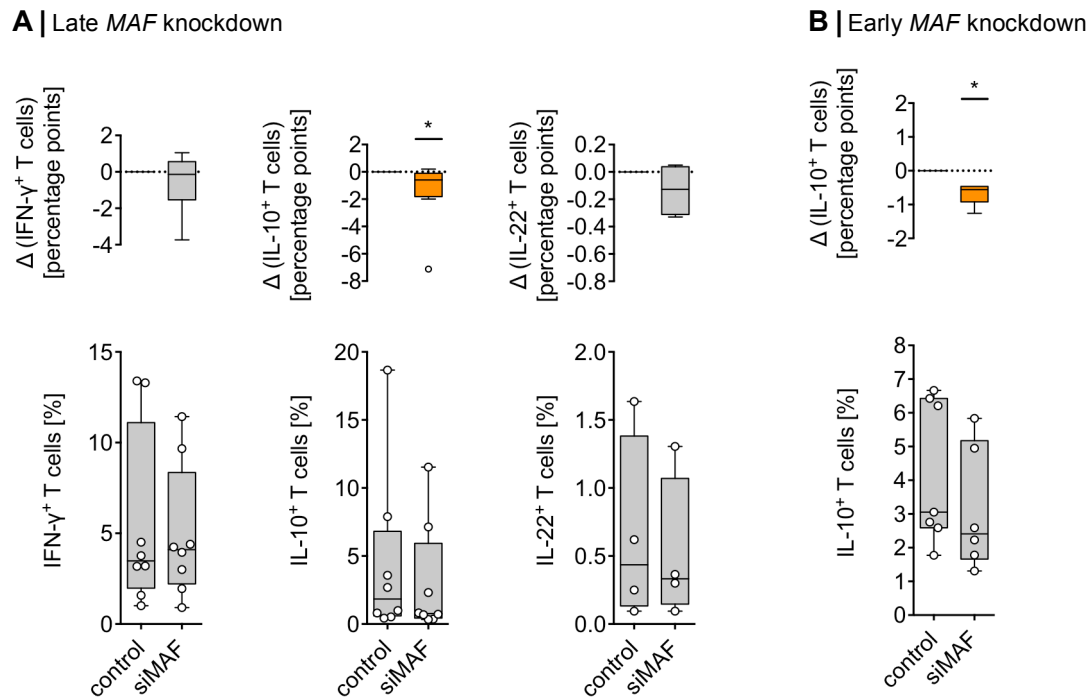
First, MAF expression under different stimulation conditions was examined. Naïve T cells were cultivated in the APC-free system in the presence of DLL-4 and IFN- $\alpha$ , IFN- $\alpha$  alone or without any additional costimulus ("–"). Levels of *MAF* mRNA were determined by quantitative PCR before activation (day 0) and 1, 2 or 3 days after T cell activation.

Fig. 5.32 shows that cells costimulated by both, IFN- $\alpha$  and DLL-4, started to upregulate *MAF* mRNA at least one day after stimulation and reached approx. threefold *MAF* levels at day three. In contrast, cells activated without costimulus or with IFN- $\alpha$  alone showed a much delayed and weaker rise in the levels of *MAF* transcript.



**Fig. 5.32: *MAF* transcription is strongly upregulated after stimulation with IFN- $\alpha$  and DLL-4**

Naïve CD4 T cells were activated in the APC-free system (anti-CD3 + anti-CD28) without DLL-4 and IFN- $\alpha$  ("–"), only with IFN- $\alpha$  ("IFN- $\alpha$ ") or in presence of DLL-4 and IFN- $\alpha$  ("DLL-4+IFN- $\alpha$ "). *MAF* transcript was quantified before activation (0), and 1, 2 and 3 days after activation.



**Fig. 5.33: MAF expression during early and late differentiation phases is required for the induction of IL-10 expression**

**A |** Naïve CD4 T cells activated for 2 days in the APC-free system were transfected with siRNA against *MAF* or control siRNA, returned to culture and expression of IFN- $\gamma$ , IL-10 and IL-22 assessed by cytokine recall and flow cytometry after 5 to 7 days. **B |** Naïve CD4 T cells were transfected with siRNA against *MAF* or control siRNA, rested for 24 hours and then activated in the APC-free system. Expression of IFN- $\gamma$ , IL-10 and IL-22 was assessed by cytokine recall and flow cytometry after 5 to 7 days. | Bottom row: frequency of cytokine-expressing T cells in percent [%]. Each circle corresponds to a single donor. Top row: change in frequency of cytokine-expressing T cells compared to samples transfected with control siRNA (control) in percentage points. Circles signify outliers according to Tukey. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \*  $p < 0.05$

The mRNA kinetics had shown that *MAF* was expressed very early during T cell activation in response to joint stimulation by DLL-4 and IFN- $\alpha$  and that expression was maintained and even moderately increased during a large part of T cell priming.

To access the function of *MAF* in this context, the influence on IL-10 expression of early and late siRNA-mediated *MAF* knockdown was analyzed.

Naïve CD4 T cells were either transfected with specific siRNA directly after isolation, rested for approx. 1 d and then activated in the APC-free system in presence of DLL-4 and IFN- $\alpha$  (Fig. 5.33 B) or *ex vivo* naïve CD4 T cells were directly activated in the presence of DLL-4 and IFN- $\alpha$ , transfected with siRNA after 2 or 3 days of culture and then returned to the culture (Fig. 5.33 A). The cells were transfected with siRNA against *MAF*, or non-

specific scrambled control siRNA. Cytokine expression was determined by cytokine recall and intracellular cytokine analysis after a total of 5 to 7 days in culture.

The knockdown of *MAF* during T cell differentiation (Fig. 5.33 A) produced a pronounced inhibition of IL-10 expression (average reduction: 35 %) without significantly altering IFN- $\gamma$  or IL-22 expression. Also knockdown of *MAF* before T cell activation (Fig. 5.33 B) led to a moderate reduction of IL-10 expression (average reduction: 20 %), indicating that *MAF* is required already in the early priming phase.

The finding that the combination of DLL-4 and IFN- $\alpha$ , but not T cell activation without any of the costimuli or with IFN- $\alpha$  alone, induced an upregulation of *MAF* and the loss of IL-10 producers upon *MAF* knockdown strongly suggested that *MAF* played a role in the induction of IL-10 expression during the early as well as the advanced T cell priming phase. The comparatively slow but continuous rise of *MAF* levels may indicate that *MAF* exerted its main function in later stages of T cell differentiation.

#### 5.2.7.2 *AhR signaling is required for both IL-10 and IL-22 expression*

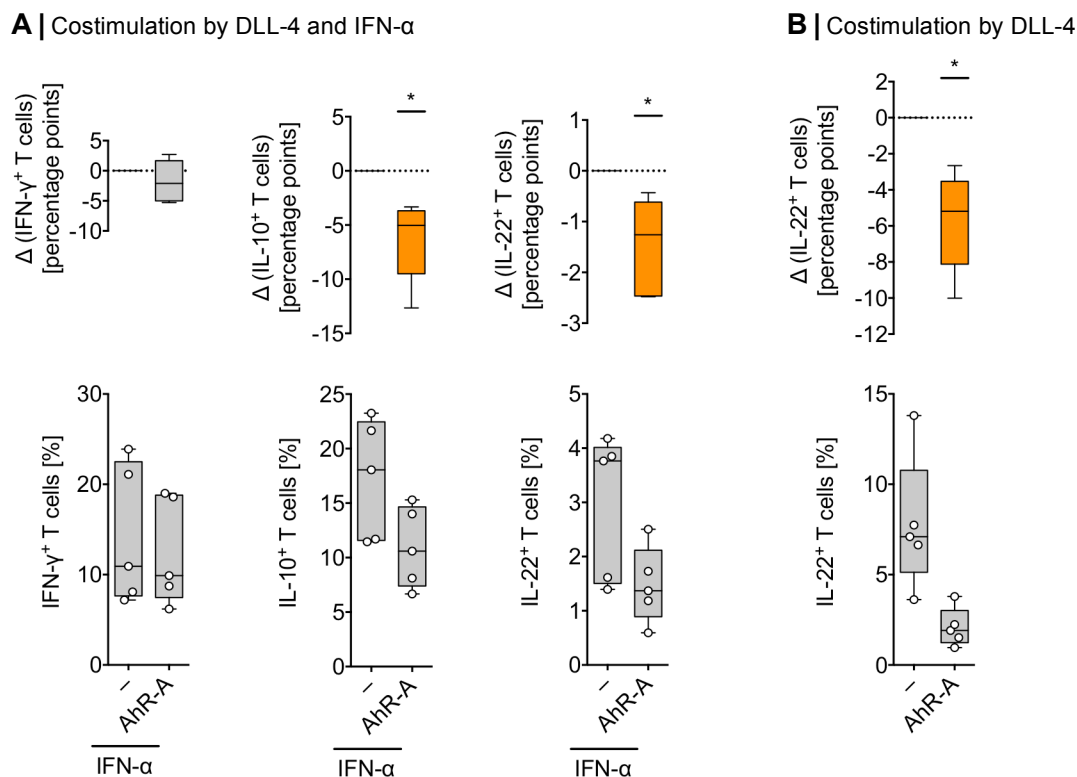
In the pDC system, activation of AhR (aryl hydrocarbon receptor) had been shown to be required for IL-22 expression. However, both induction of IL-10- as well as IL-22-producing cells had been described in literature to be mediated by AhR in certain settings, e.g. by murine T<sub>R</sub>1 cells<sup>58</sup> or T<sub>H</sub>17 cells<sup>90</sup>.

To analyze the role of AhR in the APC-free system, naïve CD4 T cells were activated in the presence of DLL-4 and in presence or absence of IFN- $\alpha$  and with or without added AhR antagonist (AhR-A). The expression of IFN- $\gamma$ , IL-10 and IL-22 was determined by cytokine recall and intracellular cytokine analysis after a total of 5 to 7 days in culture.

Interestingly, blocking the AhR pathway in this setting strongly impaired both IL-10 and IL-22 production in the presence of IFN- $\alpha$  while IFN- $\gamma$  expression was not significantly altered (Fig. 5.34 A). Also in absence of IFN- $\alpha$  expression of IL-22 was strongly impaired when AhR activity was inhibited (Fig. 5.34 A).

These results strongly suggested that the AhR complex plays an important role not only in the induction of IL-22 but also in the IL-10-expression mediated by DLL-4 and IFN- $\alpha$ . Such a clear involvement of AhR in the regulation of IL-10 was not observed in the pDC system, although blocking AhR had also slightly reduced pDC-induced IL-10 production. This may indicate that in regard to IL-10 expression the balance between the different transcriptionally active modules may be shifted towards the AhR complex in the APC-free system. Possibly, this was owed to a higher concentration of AhR ligand in the artificial system. In fact, DLL-4-Notch ligation on T cells had been demonstrated to induce release of AhR lig-

and <sup>94</sup> and flow cytometry analysis of the DLL-4-coated beads used in the experiments here had shown that amount and density of DLL-4 on these beads is by magnitudes higher than on pDCs (data not shown). Consequently, an abundance of T cell-produced AhR ligands in the APC-free culture might have tipped the balance towards pathways governed by AhR signaling.



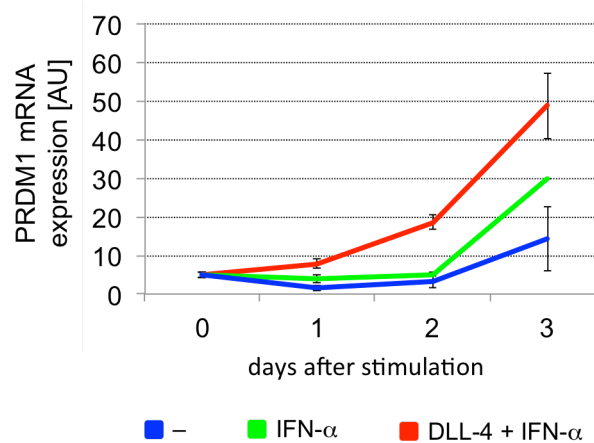
**Fig. 5.34: AhR signaling is required for induction of IL-22 and IL-10**

Naïve CD4 T cells were activated in the APC-free system in presence of DLL-4 and IFN-α (A) or DLL-4 alone (B) and in presence or absence of AhR antagonist (CH-223191, 1 nM). Expression of IFN-γ, IL-10 and IL-22 was assessed by cytokine recall and flow cytometry after 5 to 7 days. | Bottom row: frequency of cytokine-expressing T cells in percent [%]. Each circle corresponds to a single donor. Top row: change in frequency of cytokine-expressing T cells compared to samples without AhR antagonist in percentage points. Circles signify outliers according to Tukey. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \*  $p < 0.005$

#### 5.2.7.3 *BLIMP1 is required for IL-10 expression in late stages of T cell differentiation*

BLIMP1 has been found to positively regulate IL-10 expression in the pDC system and mounting evidence supports the notion that BLIMP1 is a critical for the expression of IL-10 in certain T<sub>H</sub> subsets, like T<sub>H</sub>1 <sup>66</sup>.

To answer the question whether BLIMP1 is also involved in driving IL-10 expression in the APC-free system, *PRDM1* transcript levels (the gene encoding BLIMP1) were analyzed. Naïve CD4 T cells were activated in the presence of DLL-4 and IFN- $\alpha$ , IFN- $\alpha$  alone or without any additional costimulus. Expression of *PRDM1* mRNA was determined by quantitative PCR before activation (day 0) and 1, 2 and 3 days after T cell activation.

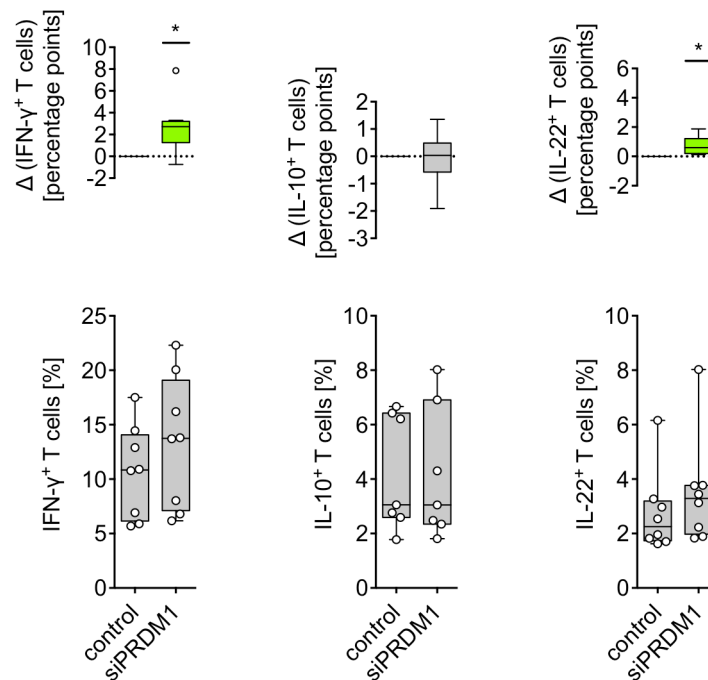


**Fig. 5.35: mRNA expression kinetics of *PRDM1***

Naïve CD4 T cells were activated in the APC-free system (anti-CD3 + anti-CD28) without DLL-4 and IFN- $\alpha$  (“–”), only with IFN- $\alpha$  (“IFN- $\alpha$ ”) or in presence of DLL-4 and IFN- $\alpha$  (“DLL-4+IFN- $\alpha$ ”). *PRDM1* transcript was quantified before activation (0), and 1, 2 and 3 days after activation.

Although a gradual increase in *PRDM1* transcript was observed for all three conditions (Fig. 5.35), *PRDM1* mRNA levels in cells activated in the presence of IFN- $\alpha$  and DLL-4 increased significantly faster. On day 3 after stimulation, *PRDM1* mRNA levels in cells stimulated with IFN- $\alpha$  and DLL-4 had risen approx. 10-fold, in contrast to a 3- and 6-fold increase in cells grown without additional costimulus or with IFN- $\alpha$  alone, respectively.

In order to assess a functional involvement of BLIMP1 in IL-10 expression the impact of siRNA-mediated reduction of BLIMP1 levels on cytokine expression was analyzed. Since BLIMP1 has been reported to fulfill different roles during T cell activation and in effector T cells, the effect of early (Fig. 5.36) and late *PRDM1* knockdown was examined. Naïve CD4 T cells were either transfected directly after isolation, rested for approx. 1 d and then activated in the APC-free system in presence of DLL-4 and IFN- $\alpha$  (Fig. 5.36) or *ex vivo* naïve CD4 T cells were directly activated in presence of DLL-4 and IFN- $\alpha$ , transfected after 2 or 3 days of culture and then returned to culture (Fig. 5.37). Cells were transfected with siRNA against *PRDM1*, or non-specific scrambled control siRNA. Cytokine expression was determined by cytokine recall and intracellular cytokine analysis after a total of 5 to 7 days of cell culture.



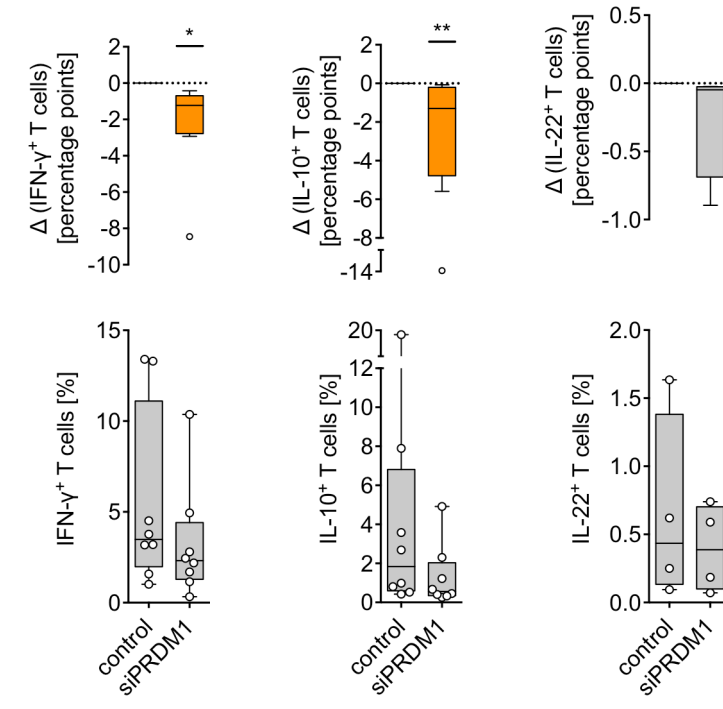
**Fig. 5.36: BLIMP1 (*PRDM1*) knockdown before T cell activation leads to increased IFN- $\gamma$  expression**

Naïve CD4 T cells were transfected with siRNA against *PRDM1* or control siRNA, rested for 24 hours and then activated in the APC-free system. Expression of IFN- $\gamma$ , IL-10 and IL-22 was assessed by cytokine recall and flow cytometry after 5 to 7 days. | Bottom row: frequency of cytokine-expressing T cells in percent [%]. Each circle corresponds to a single donor. Top row: change in frequency of cytokine-expressing T cells compared to samples transfected with control siRNA (control) in percentage points. Circles signify outliers according to Tukey. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \*  $p < 0.05$

Surprisingly, early and late knockdown of BLIMP1 led to very different results regarding the expression of IFN- $\gamma$ , IL-10 and IL-22. While early inhibition of BLIMP1 increased IFN- $\gamma$  and IL-22 production, but did not alter IL-10 expression (Fig. 5.36), late knockdown of BLIMP1 strongly impaired both IFN- $\gamma$  and IL-10 expression and slightly, albeit not in a statistically significant manner, reduced the number of IL-22-producing cells (Fig. 5.37). A general decline of cytokine expression as a result of reduced T cell viability after late transfection was excluded since no difference in proliferation between control and cells with reduced BLIMP1 levels was observed (data not shown).

BLIMP1 is known as a direct repressor of IFN- $\gamma$  and IL-2, and an important component of the IL-2 negative feedback loop during T cell activation<sup>62</sup>. On the other hand, several reports have discussed the involvement of BLIMP1 in the induction of IL-10 in CD4 T cells in general<sup>63</sup> and established a role of murine BLIMP1 in the IL-10 expression by effector-type regulatory T cells<sup>65</sup>, T<sub>R</sub>1-like T cells<sup>147</sup> and T<sub>H</sub>1 cells<sup>66</sup>. These reports explain both,

the observed increase of IFN- $\gamma$  expression and decrease of IL-10 expression, following knockdown of BLIMP1.



**Fig. 5.37: Late BLIMP1 (*PRDM1*) knockdown reduces IL-10 and IFN- $\gamma$  expression**

Naïve CD4 T cells activated for 2 days in the APC-free system were transfected with siRNA against *PRDM1* or control siRNA, returned to culture and expression of IFN- $\gamma$ , IL-10 and IL-22 assessed by cytokine recall and flow cytometry after 5 to 7 days. | Bottom row: frequency of cytokine-expressing T cells in percent [%]. Each circle corresponds to a single donor. Top row: change in frequency of cytokine-expressing T cells compared to samples transfected with control siRNA (control) in percentage points. Circles signify outliers according to Tukey. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \*  $p < 0.05$ ; \*\*  $p < 0.005$

Interestingly, these effects were not observed together but resulted from BLIMP1 inhibition at an early or advanced stage of T cell differentiation, respectively, suggesting that BLIMP1 fulfilled distinct functions at different stages of the differentiation process or the T cell life cycle.

In fact, there are reports, which indicate that BLIMP1 is a general regulator of T cell effector function, including cytokine expression, in fully or terminally differentiated T cells (reviewed by Crotty, Johnston and Schoenberger, 2010<sup>159</sup>).

### 5.2.8 STAT1, STAT3 and STAT4 differentially regulate expression of IFN- $\gamma$ , IL-10 and IL-22

STAT1, STAT4 and in particular STAT3 have been shown in the pDC system to play prominent roles in the regulation of IFN- $\gamma$ , IL-10 and IL-22. Consequently, analyzing their involvement in the differentiation of T cells in the APC-free system was of paramount importance.

Since T cells that were activated in the presence of DLL-4 alone and cells costimulated by both DLL-4 and IFN- $\alpha$  displayed very different IL-22 and IL-10 expression, STAT function under both conditions was analyzed.

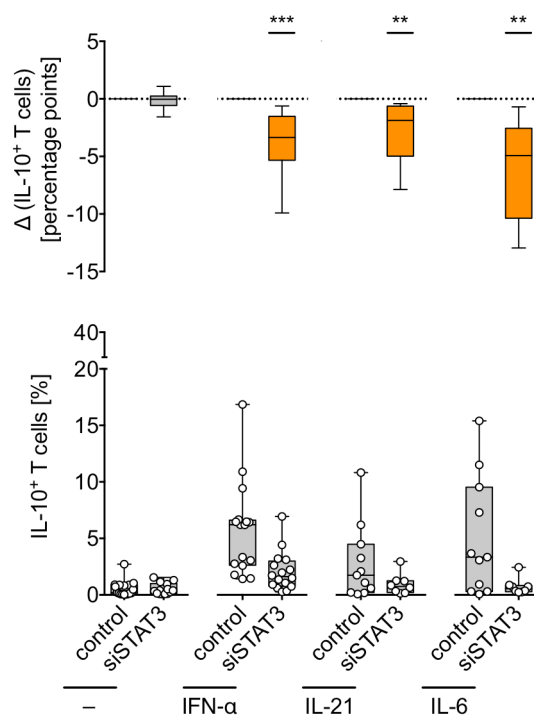
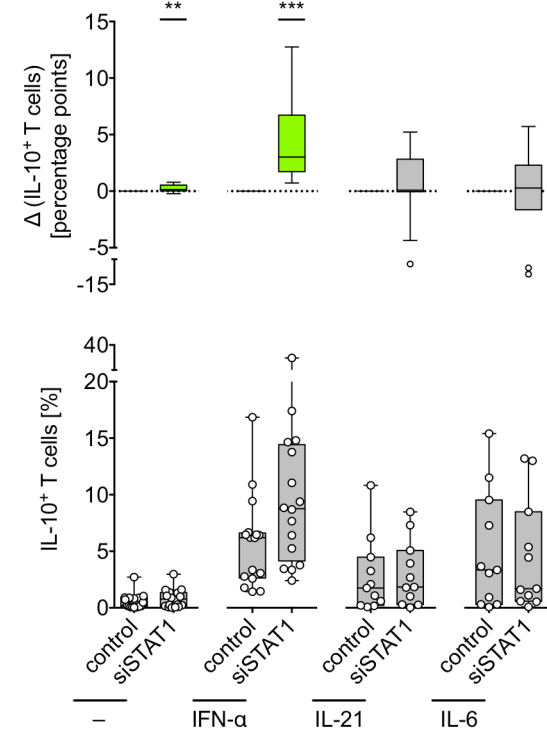
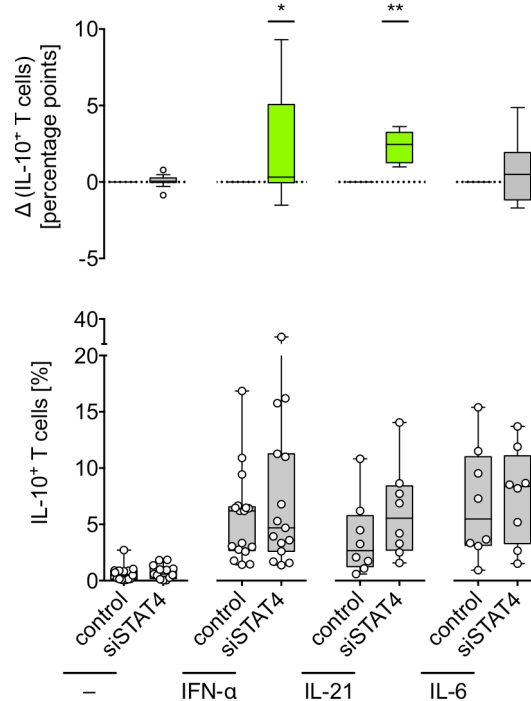
Naïve CD4 T cells were transfected with siRNA directly after isolation, rested for approx. 1 d and then activated in the APC-free system in presence of DLL-4 and cytokines like IFN- $\alpha$ , IL-6 or IL-21, or without added cytokine. The cells were transfected with siRNA against *STAT1*, *STAT3*, *STAT4* or non-specific scrambled control siRNA. Cytokine expression was determined by cytokine recall and intracellular cytokine analysis after 5 to 7 days in cell culture.

#### 5.2.8.1 *IL-10 expression is mediated by STAT3, but inhibited by STAT1*

IFN- $\alpha$ , IL-6 and IL-21 have been found to act in concert in inducing a substantial IL-10 expression in combination with DLL-4, and the knockdown experiments in the pDC system have shown that IL-10 expression depended on the action of STAT3 at a later phase of differentiation, whereas STAT1 inhibited the IL-10-driving pathway.

To analyze the involvement of STAT signaling more in detail, the effect of STAT1, STAT3 and STAT4 knockdown on IL-10-expression driven by IFN- $\alpha$ , IL-6 and IL-21 was assessed.

STAT knockdown in the APC-free system affected IL-10 expression in a similar way as was observed in the pDC system. Most prominently, STAT3 knockdown significantly impaired IL-10 expression induced by IFN- $\alpha$ , IL-6 or IL-21, indicating that all three cytokines directly or indirectly activated STAT3-dependent processes that led to the production of IL-10.

**A | IL-10: Effect of STAT3 knockdown****B | IL-10: Effect of STAT1 knockdown****C | IL-10: Effect of STAT4 knockdown****Fig. 5.38: IL-10 expression depends on STAT3 signaling and can be inhibited by STAT1**

Naïve CD4 T cells were transfected with siRNA against *STAT1*, *STAT3*, *STAT4* or control siRNA, rested for 24 hours and then activated in the APC-free system in the presence of DLL-4 and with or without added cytokines. Expression of IL-10 was assessed by cytokine recall and flow cytometry after 5 to 7 days. | Bottom row: frequency of cytokine-expressing T cells in percent [%]. Each circle corresponds to a single donor. Top row: change in frequency of cytokine-expressing T cells compared to samples transfected with control siRNA (control) in percentage points. Circles signify outliers according to Tukey. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \*  $p < 0.05$ ; \*\*  $p < 0.005$ ; \*\*\*  $p < 0.0005$

In the pDC system STAT1 knockdown had increased IL-10 expression. Interestingly, a comparable effect was observed in cells stimulated by IFN- $\alpha$ , but not IL-6 or IL-21. STAT1 has been described as repressor of IL-10-transactivation by STAT3<sup>160</sup>. The observation

that in the presence of IFN- $\alpha$  STAT1 inhibited IL-10 expression may be linked to the fact that – in contrast to the signaling pathways employed by IL-21 or IL-6 – STAT1 is an integral part of the signaling and transcription regulating machinery downstream of the IFN- $\alpha$  receptor (IFNAR) and not only becomes strongly activated, but its expression also massively upregulated following receptor binding of IFN- $\alpha$  <sup>47</sup>. The resulting abundance of STAT1 would explain the strong impact of STAT1 knockdown on STAT3-mediated IL-10 expression in IFN- $\alpha$ -stimulated T cells.

Curiously, STAT4 knockdown led to a modest to moderate increase in the number IL-10-producing T cells, when IFN- $\alpha$  or IL-21 were present. Considering the finding that IFN- $\alpha$  promoted IL-21 expression, it is conceivable that IL-21 was responsible for the increased IL-10 expression under both culture conditions. Although the mechanism behind this phenomenon remained unclear, several possible explanations present themselves. Firstly, STAT4-dependent gene expression or expression products may have interfered with STAT3-activated processes that induce IL-10. Alternatively, in IL-21-stimulated cells the competition for phosphorylation sites between STAT3 and STAT4 was skewed towards the activation of STAT3 by reduced STAT4 levels. In fact, interaction between STAT3 and STAT4, STAT3:STAT4 heterodimers and a competition of STATs for phosphorylation sites was reported before <sup>161</sup>.

Interestingly, no effect of STAT4 knockdown was seen in pDC-stimulated T cells. This may be explained by the finding that all STAT4-dependent processes in the pDC system appeared to take place in an early phase of T cell activation whereas STAT3-related processes driving IL-10 seemed to occur at a later point in time.

Taken together, these experiments supported the finding that IL-10 expression driven by DLL-4 and cytokines like IFN- $\alpha$ , IL-6 or IL-21 was mediated by STAT3. IFN- $\alpha$ -related activation of STAT1 appeared to be capable of counteracting this pathway. In addition, knockdown of STAT4 under certain conditions enhanced IL-10 expression, although the mechanism leading to this effect remained unclear.

#### 5.2.8.2 *STAT3-dependent and -independent pathways govern IL-22 expression*

The initial experiments had revealed that IL-22-expression was strongest when T cells were activated in the presence of DLL-4 but the absence of exogenous cytokines, like IFN- $\alpha$ , IL-6 and IL-21, which in fact were found to impair IL-22 expression. In addition, knockdown experiments showed that IL-22 expression in the pDC system required STAT3 signaling but was independent of STAT1 and STAT4 activation. In order to understand which STAT molecule affected IL-22 expression in the different settings and whether the

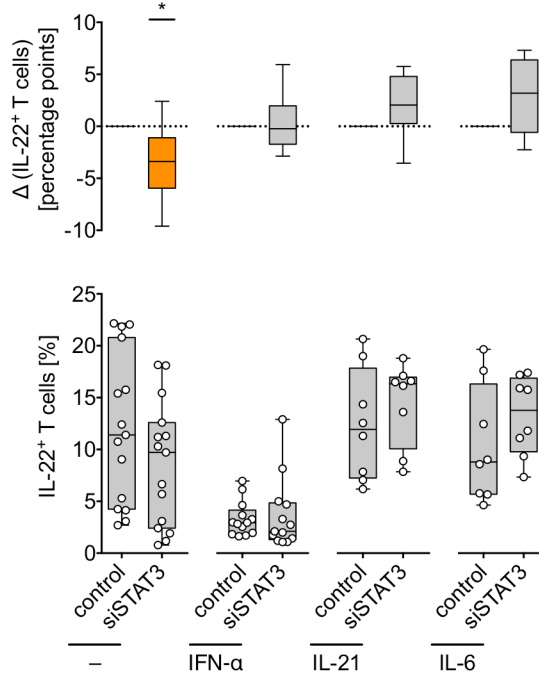
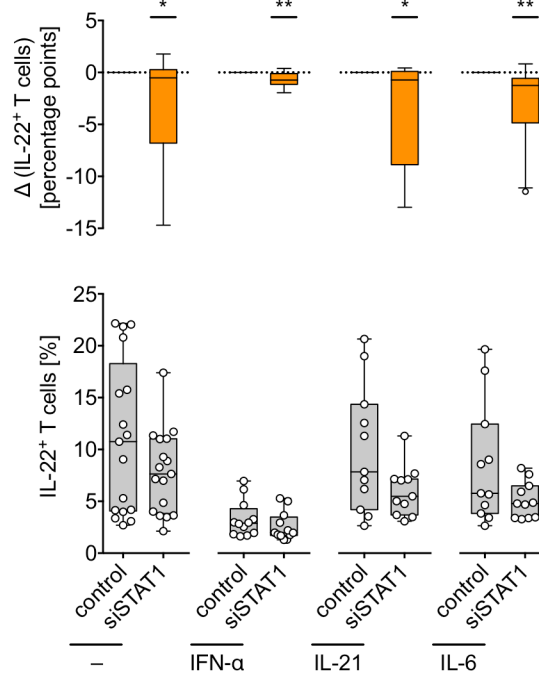
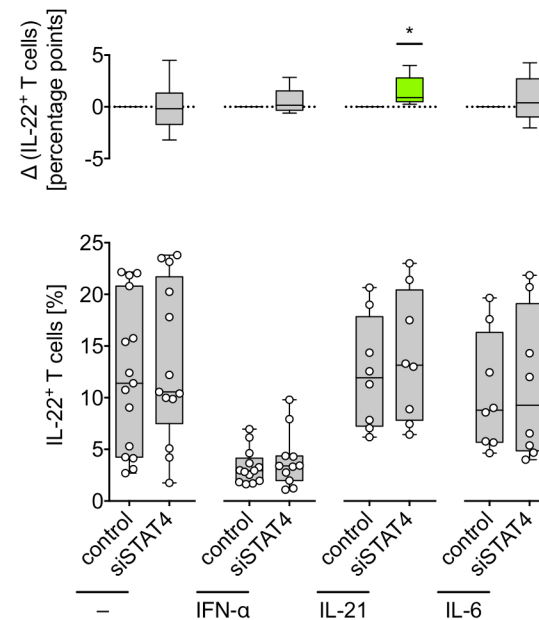
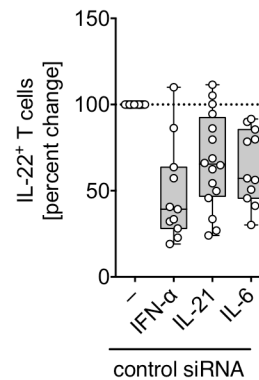
deleterious effect of IFN- $\alpha$ , IL-6 and IL-21 was connected to a particular STAT-signal, the effect of STAT knockdown in the presence of IFN- $\alpha$ , IL-6 or IL-21 or in absence of exogenous cytokine was examined.

Interestingly, STAT3 knockdown affected IL-22 expression only in the absence of additional cytokines (Fig. 5.39 A). When IFN- $\alpha$ , IL-21 or IL-6 were present, total IL-22 expression was decreased (Fig. 5.39 D) and STAT3 knockdown did no longer lead to a reduction in the number of IL-22 expressing cells (Fig. 5.39 A). Nonetheless, substantial IL-22 expression was also induced in the presence of the cytokines. These observations indicated that STAT3-dependent as well as STAT3-independent pathways governed IL-22 expression in DLL-4-stimulated T cells. In addition, cytokines that employ STAT3 signaling appeared to inhibit or interfere with the STAT3-dependent branch of IL-22-induction.

Knockdown of STAT1 modestly reduced IL-22 expression, but, in contrast to the STAT3 knockdown, affected IL-22 production under all tested conditions. However, from the data could not be concluded whether STAT1 and STAT3 were involved in the same pathway or STAT1-signaling was part of a STAT3-independent mechanism. Interestingly, AhR has been reported to form a transcriptionally active complex with STAT1 <sup>162</sup>. As a result the STAT1 knockdown may have affected the AhR-driven pathway of IL-22 induction.

As (Fig. 5.39 D) illustrates, STAT4 knockdown did not significantly influence IL-22 expression, except when IL-21 was added to the culture. A similar effect was observed on IL-10 expression (see preceding paragraph). It is possible that the same mechanisms described there might also augment STAT3-dependent IL-22 expression.

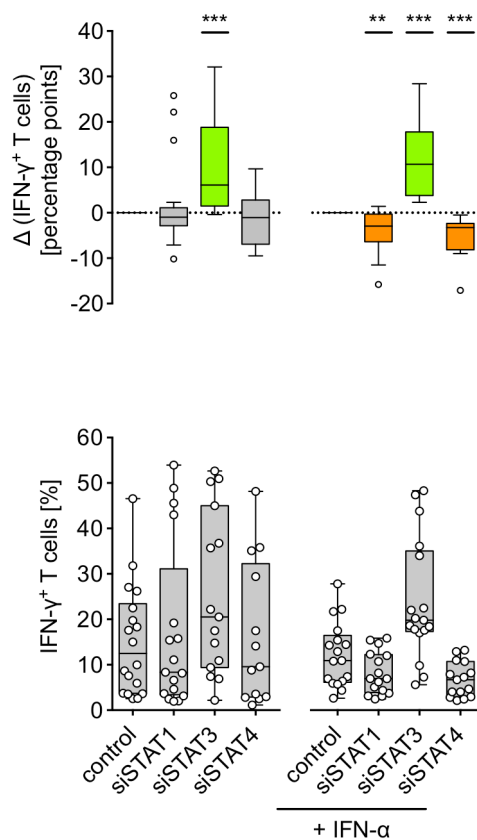
In summary, the data suggest that IL-22 expression was induced via STAT3-independent and -dependent mechanisms and that cytokines, which act through STAT3, blocked STAT3-related IL-22-induction. Furthermore, STAT1 was directly or indirectly involved in driving IL-22 expression. Since STAT1 and AhR have been reported to form a transcriptionally active complex <sup>162</sup>, it is conceivable that AhR and STAT1 together regulate IL-22 expression. Finally, STAT4 appeared to not be directly involved in IL-22 expression.

**A | IL-22: Effect of STAT3 knockdown****B | IL-22: Effect of STAT1 knockdown****C | IL-22: Effect of STAT4 knockdown****D | IL-22: Effect of cytokines****Fig. 5.39: IL-22 expression depends on STAT3 and STAT1**

Naïve CD4 T cells were transfected with siRNA against *STAT1*, *STAT3*, *STAT4* or control siRNA, rested for 24 hours and then activated in the APC-free system in presence of DLL-4 and with or without added cytokines. Expression of IL-22 was assessed by cytokine recall and flow cytometry after 5 to 7 days. | Bottom row: frequency of cytokine-expressing T cells in percent [%]. Each circle corresponds to a single donor. Top row: change in frequency of cytokine-expressing T cells compared to samples transfected with control siRNA (control) in percentage points. Circles signify outliers according to Tukey. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \*  $p < 0.05$ ; \*\*  $p < 0.005$

### 5.2.8.3 *STAT1/STAT4 and STAT3 reciprocally regulate IFN- $\gamma$ expression*

STAT knockdown in the pDC system has shown that both STAT1 and STAT4 signaling was involved in driving IFN- $\gamma$  expression, whereas STAT3 appeared to convey inhibitory functions. Analysis of STAT inhibition in cells cultivated in the presence of IFN- $\alpha$  produced similar results: STAT1 and STAT4 knockdown diminished IFN- $\gamma$  expression whereas the reduction of STAT3 levels augmented IFN- $\gamma$  production. Interestingly, cells activated without added cytokine were only affected by STAT3 knockdown, which increased IFN- $\gamma$  expression. Neither inhibition of STAT1 nor STAT4 signaling produced an effect in regard to IFN- $\gamma$  production, indicating that no endogenously produced cytokine activated STAT1- or STAT4-dependent IFN- $\gamma$  expression.



**Fig. 5.40: Effect of STAT knockdown on IFN- $\gamma$  expression**

Naïve CD4 T cells were transfected with siRNA against *STAT1*, *STAT3*, *STAT4* or control siRNA, rested for 24 hours and then activated in the APC-free system in presence of DLL-4 and with or without added IFN- $\alpha$ . Expression of IFN- $\gamma$  was assessed by cytokine recall and flow cytometry after 5 to 7 days. | Bottom row: frequency of cytokine-expressing T cells in percent [%]. Each circle corresponds to a single donor. Top row: change in frequency of cytokine-expressing T cells compared to samples transfected with control siRNA (control) in percentage points. Circles signify outliers according to Tukey. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \*\* p < 0.005; \*\*\* p < 0.0005

Addition of IFN- $\alpha$  to the cell culture shifted IFN- $\gamma$ -driving processes from STAT1- and STAT4-independent mechanisms to pathways that require STAT1 and STAT4 signaling but did not induce a sustained T<sub>H</sub>1 commitment. In fact, IFN- $\alpha$  is known to drive a strong but highly transient IFN- $\gamma$  expression through the action of STAT4<sup>137</sup>. In addition, STAT1, which is also a key factor of the main signaling pathway of type I IFN<sup>47</sup>, has been shown to

activate the T<sub>H</sub>1 master regulator TBET and to be required for T<sub>H</sub>1 commitment by T<sub>H</sub>1-driving cytokines like IFN- $\gamma$ <sup>148-150</sup> and IL-27<sup>34,43,44</sup>.

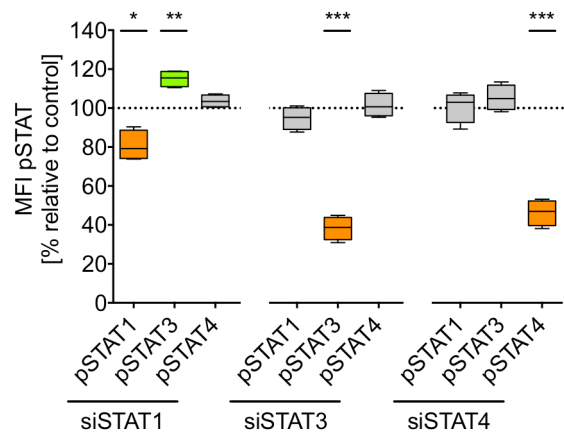
The mechanism behind STAT3-mediated inhibition of IFN- $\gamma$  expression remained obscure. STAT3 has been reported to elicit anti-inflammatory responses in general<sup>47</sup>. In addition, IL-21, which exerts its function through STAT3 activation, has been shown in the preceding experiments to be produced by T cells in presence and absence of IFN- $\alpha$  and to reduce IFN- $\gamma$  expression. Although IL-10 also acts through STAT3, it was not or only to a negligible degree induced in the absence of exogenous cytokines and therefore would likely not contribute to STAT3-dependent suppression of IFN- $\gamma$  expression in that setting. Lastly, IFN- $\gamma$  itself activates STAT3<sup>163,164</sup> and has been described to convey anti-inflammatory signals through STAT3<sup>165</sup>.

In summary, the baseline IFN- $\gamma$  expression in absence of exogenous cytokine appeared to be driven by STAT1- and STAT4-independent processes, whereas both STAT1 and STAT4 played a role in the induction of IFN- $\gamma$ -expressing cells when IFN- $\alpha$  was present. In contrast, STAT3 seemingly activated or was part of a pathway that limited IFN- $\gamma$  production and was active both in presence and absence of exogenous cytokines.

#### 5.2.8.4 *IFN- $\alpha$ -activated STAT1 limits STAT3 phosphorylation*

The surprising finding that knocking down STAT1 or STAT4 enhanced expression of IL-10 or IL-22 raised the question whether the knockdown of a certain STAT-molecules might affect activation of the other STATs. To test this hypothesis, naïve CD4 T cells were transfected with siRNA against *STAT1*, *STAT3* or *STAT4* or scrambled control siRNA, rested for one day and then treated with IFN- $\alpha$  and analyzed for STAT phosphorylation by intracellular staining and flow cytometry.

Although neither STAT3 nor STAT4 knockdown altered phosphorylation of the other two STATs, STAT1 knockdown significantly increased the phosphorylation of STAT3 (Fig. 5.41). In the preceding knockdown experiments STAT3 had been shown to be required for IL-10-expression. Therefore, a boost in STAT3 phosphorylation could explain the enhancing effect STAT1 knockdown had on IL-10 production.



**Fig. 5.41: Knockdown of STAT1 enhances phosphorylation of STAT3**

Naïve CD4 T cells were transfected with siRNA against *STAT1*, *STAT3*, *STAT4* or control siRNA, rested for 24 hours. STAT phosphorylation was elicited by incubation with 10 ng/mL IFN- $\alpha$  for 20 min. The phosphorylation state of STAT1, STAT3 and STAT4 was assessed by intracellular staining of phosphorylated STAT and flow cytometry. | MFI values (mean fluorescence intensity) shown have been normalized to the samples transfected with control siRNA. Green or orange color indicates frequency was increased or decreased, respectively, in a statistically significant manner. | \*  $p < 0.05$ ; \*\*  $p < 0.005$ ; \*\*\*  $p < 0.0005$

## 5.2.9 DLL-4 signaling directly affects STAT phosphorylation

### 5.2.9.1 *DLL-4-Notch ligation enhances cytokine-induced STAT phosphorylation*

The preceding experiments showed that STAT signals were critical for the induction of IL-10 and IL-22 and that DLL-4 enhanced the effect of cytokines that act through STAT3. In addition, several analyses had demonstrated that both cytokine and DLL-4 signaling have to occur in the early T cell priming phase (data not shown). This raised the question whether the Notch pathway and cytokine signal transduction might interface at the level of STAT activation. Indeed, several researchers have demonstrated that HES1, a classical Notch target gene, stabilizes the JAK-STAT phosphorylation complex and facilitates STAT3 phosphorylation <sup>95,166</sup>.

To answer the question whether DLL-4 ligation does alter cytokine-induced STAT activation, naïve CD4 T cells were activated in the APC-free system in presence or absence of DLL-4. T cells were stimulated for 6 h to 7 h in order to allow for Notch-dependent gene expression. After this activation phase, IFN- $\alpha$ , IL-6 or IL-21 were added to induce STAT activation and, following a short incubation, STAT phosphorylation was measured by intracellular phospho-STAT staining and flow cytometry. Phosphorylation levels at STAT tyrosine and serine residues were compared between cells activated with or without DLL-4.

Fig. 5.42 illustrates the results of the analysis of tyrosine phosphorylation sites in STAT1 (Y701), STAT3 (Y705) and STAT4 (Y693).

Interestingly, small but statistically significant increases in the phosphorylation at tyrosine residues were observed for all three STAT molecules, but not always with the same cytokine.

Phosphorylation at STAT1 Y701 was strongly induced by both, IFN- $\alpha$  and IL-6, whereas stimulation by IL-21 only led to a modest increase in phosphorylation levels (Fig. 5.42 A). Interestingly, cells stimulated in the presence of DLL-4 showed a small, but significant increase of IFN- $\alpha$ -induced STAT1 tyrosine phosphorylation compared to cells stimulated without DLL-4 (Fig. 5.42 A). Although phosphorylation by IL-6 was also slightly augmented by DLL-4, this effect was slightly less pronounced and not statistically significant. In contrast, DLL-4 did not affect STAT1 Y701 phosphorylation induced by IL-21.

Phosphorylation of STAT3 tyrosine residue 705 was strongly induced following the application of each of the three cytokines and was further increased by costimulation with

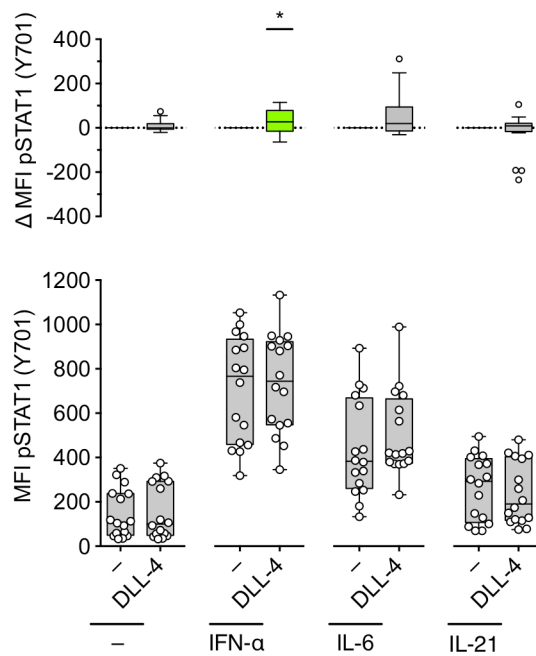
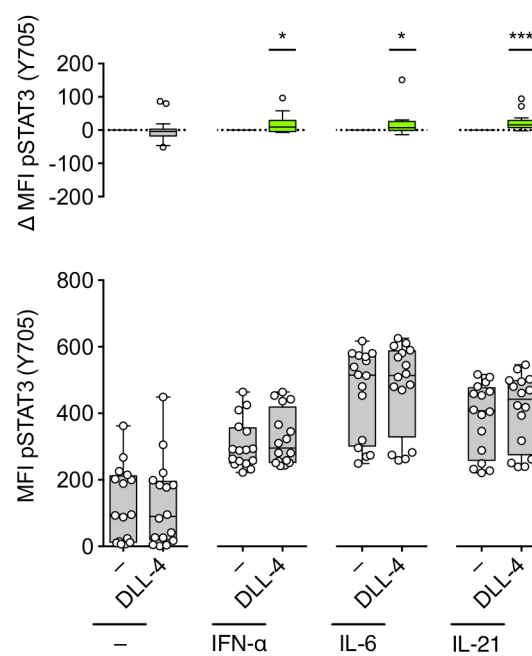
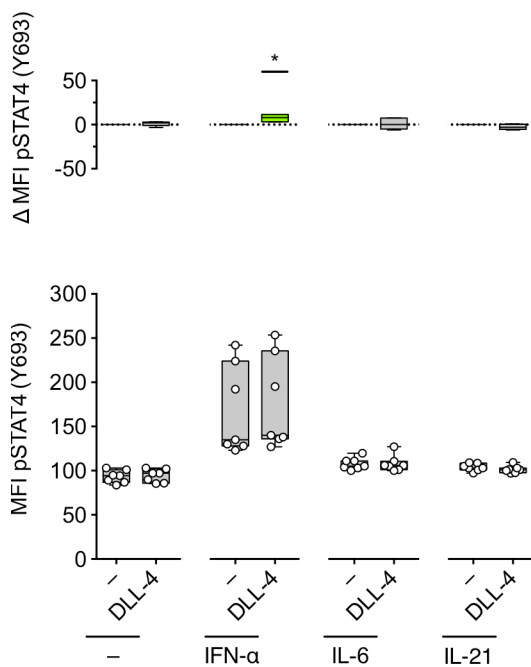
DLL-4 under all three conditions (Fig. 5.42 B). In contrast, increased phosphorylation at STAT4 Y693 was only observed after addition of IFN- $\alpha$  (Fig. 5.42 C). However, IFN- $\alpha$ -induced STAT4-Y693 phosphorylation was significantly higher in cells that had been activated in the presence of DLL-4, although the absolute difference was small.

Of note, DLL-4 did not enhance phosphorylation at the STAT tyrosine residues in absence of cytokine-mediated STAT-activation.

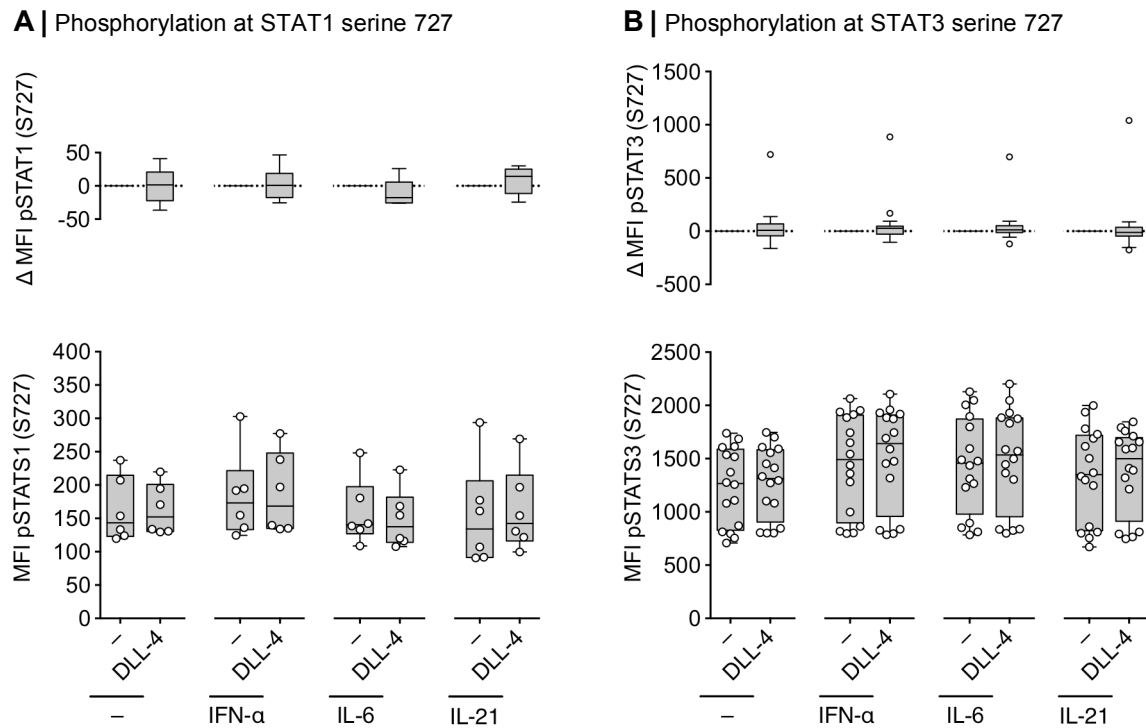
None of the tested cytokines induced significant phosphorylation of serine 727 of STAT1. In contrast, STAT3 serine 727 underwent modest activation by all three cytokines but did not show any difference in the activation state when comparing cells cultivated in presence or absence of DLL-4 (Fig. 5.43).

In summary, these experiments indicated that early cytokine and Notch signaling interlinked at the STAT level, where a DLL-4-dependent signal led to augmented activation of certain STATs. The exact pattern of DLL-4-mediated enhancement of STAT phosphorylation appeared to depend on the individual cytokine signal. Interestingly, DLL-4 signaling was not only capable of enhancing STAT3 phosphorylation but also affected the activation state of STAT1 and STAT4.

In most cases the observed absolute change in phosphorylation was small, raising the question whether such a small alteration would have an effect on cell fate. Here has to be taken into account that the duration of cytokine signals was very limited (approx. 20 min) in this experimental setting, whereas in cell culture exposure to cytokines occurs over a much longer period, potentially allowing Notch signals to more profoundly influence STAT signaling.

**A | Phosphorylation at STAT1 tyrosine 701****B | Phosphorylation at STAT3 tyrosine 705****C | Phosphorylation at STAT4 tyrosine 693****Fig. 5.42: DLL-4 ligation enhances cytokine-dependent phosphorylation at STAT tyrosine residues**

Naïve CD4 T cells were polyclonally (anti-CD3 + anti-CD28) activated in presence or absence of DLL-4. After 6 h of stimulation, STAT phosphorylation was elicited by addition of 10 ng/mL IFN- $\alpha$ , IL-6 or IL-21 and incubation for 20 min. Control cells were incubated without added cytokine. The phosphorylation state of STAT1, STAT3 and STAT4 tyrosine residues was assessed by intracellular staining of phosphorylated STAT tyrosine and flow cytometry. | Bottom row: MFI (mean fluorescence intensity) values [AU]. | Top row: change in MFI compared to control samples [AU]. Circles signify outliers according to Tukey. Green or orange color indicates MFI was increased or decreased, respectively, in a statistically significant manner. | \*  $p < 0.05$ ; \*\*\*  $p < 0.0005$



**Fig. 5.43: DLL-4 ligation does not affect phosphorylation at STAT serine residues**

Naïve CD4 T cells were polyclonally (anti-CD3 + anti-CD28) activated in presence or absence of DLL-4. After 6 h of stimulation, STAT phosphorylation was elicited by addition of 10 ng/mL IFN- $\alpha$ , IL-6 or IL-21 and incubation for 20 min. Control cells were incubated without added cytokine. The phosphorylation state of STAT1, STAT3 and STAT4 serine residues was assessed by intracellular staining of phosphorylated STAT serine residues and flow cytometry. | Bottom row: MFI (mean fluorescence intensity) values [AU]. E Each circle corresponds to a single donor. | Top row: change in MFI compared to control samples [AU]. Circles signify outliers according to Tukey. Green or orange color indicates MFI was increased or decreased, respectively, in a statistically significant manner.

### 5.2.10 APC-free T cell differentiation can be modulated to confer desired properties to CD4 T cells

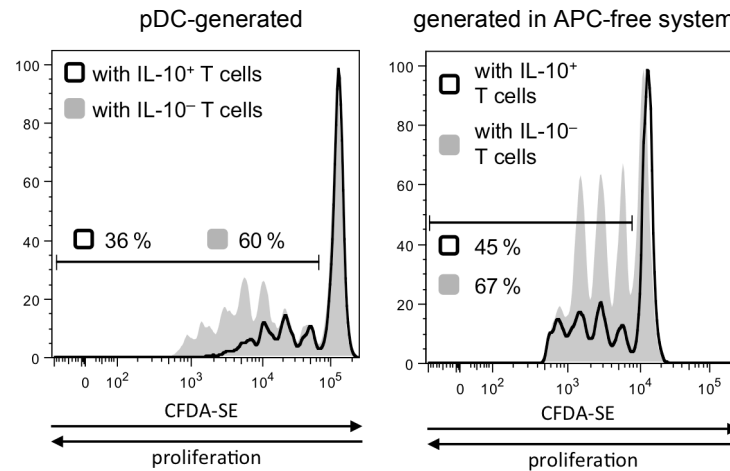
The Notch ligand DLL-4 and the cytokine IFN- $\alpha$  have been identified in the preceding experiments as main costimulatory factors employed by pDCs to drive expression of IL-10 and IL-22 in activated naïve CD4 T cells. In addition, T<sub>H</sub>1 commitment was found to add a separate functional layer instead of substantially interfering with the IL-10 and IL-22-inducing modules. This raised the question, whether the APC-free system with knowledge of the IL-10 and IL-22-driving modules can be utilized for specific generation of T cell populations with therapeutic potential from naïve CD4 T cells.

#### 5.2.10.1 *Immunosuppressive T cells can be generated in absence of pDCs*

In the mouse system Rutz et al. have shown that IFN- $\gamma$ +IL-10<sup>+</sup> CD4 T cells exhibit immunosuppressive activity <sup>68</sup>, whereas Kassner et al. demonstrated in mouse that these double producers can also be induced by coculture with pDCs <sup>144</sup>. Hypothesizing that the human IL-10<sup>+</sup> CD4 T cells that were induced in the pDC system and in the APC-free system here would have similar immunoregulatory capacities, the effect of these cells on the proliferation of effector cells was assessed in an *in vitro* suppression assay. For this purpose, using an IL-10 secretion assay and flow cytometry, IL-10 producers (IL-10<sup>+</sup>) and non-producers (IL-10<sup>-</sup>) were isolated from CD4 T cells that had been generated from naïve CD4 T cells in the pDC system or in the APC-free system.

In the suppression assay, naïve (CD45RA+CD45RO<sup>-</sup>) CD4 T cells were used as “indicator” cells and cocultivated either with the purified IL-10<sup>+</sup> or IL-10<sup>-</sup> T cells generated in the pDC system or the APC-free system as well as antigen-presenting cells (APC). After 4 days of culture, proliferation of the “indicator” cells was assessed. As Fig. 5.44 shows, the presence of IL-10 producers significantly diminished proliferation of the indicator cells. While the indicator cells divided normally in the presence of IL-10-negative T cells, the presence of IL-10<sup>+</sup> T cells significantly delayed proliferation.

This demonstrates that the APC-free system in combination with DLL-4 and selected cytokine signals can be a useful tool for the generation of different T cell phenotypes with potential therapeutic application.



**Fig. 5.44: IL-10-producing CD4 T cells generated in the pDC system and in the APC-free system suppress proliferation of activated T cells**

CFDA-SE-labeled naïve CD4 T cells were cocultivated with IL-10-producing T cells or non-IL-10-producers and sorted APCs. The cells were activated with SEB and LPS. Cell proliferation was assessed by flow cytometry after 4 days. Both, IL-10-producers and non-producers, had been generated in the pDC system or the APC-free system (with DLL-4 and IFN- $\alpha$ ) and sorted according to IL-10 expression. | Filled light gray histograms: proliferation profile of cells cocultivated with non-IL-10 producers. Histograms with black line: proliferation profile of cells cocultivated with IL-10-producing cells



## 6 Discussion

Much research has been done on the regulation of the immunosuppressive cytokine IL-10 in T helper cells. Also the regulation of IL-22, a cytokine that has been described only relatively recently, has been extensively investigated. Nonetheless, the picture of how IL-10 and IL-22 are regulated is still fragmented. In regard to IL-10, this is owed to the fact that it can be expressed by different types of T helper cells ( $T_H$  cells) and is differently regulated in each T helper cell subset. Understanding the regulation of IL-22, although this cytokine is less ubiquitously expressed in the T cell compartment than IL-10, also presents a challenge as there appear to be large differences in regulation and expression in mouse and human. In fact, most data concerning the regulation of IL-10 and IL-22 stem from mouse experiments and many were generated in *in vivo* models that, while much better reflecting the normal physiological situation, have limited precision due to the complexity of signals present in the living organism.

In view of the limited knowledge in regard to the regulation of those two cytokines in the human immune response, this study aimed to provide an overview of the regulatory network governing IL-10 and IL-22 expression by human T helper cells using the interaction of plasmacytoid dendritic cells (pDC) and T helper cells as model system. In fact, several reports on pDCs in mouse and human had indicated that pDCs are potent inducers of both IL-10 and IL-22 in CD4 T cells.

For this purpose the signals and transcriptional modules involved in IL-10 and IL-22 expression were analyzed in a pDC/T cell coculture system as well as a minimalistic APC-free system, using the regulation described in mouse as blueprint and trying to understand whether these factors known from mouse play the same role in the context of pDC-induced IL-10 and IL-22 expression by human T helper cells.

In mouse, IL-10 has been shown to be induced in all  $T_H$  subsets by different cytokines and STATs <sup>24</sup>. However, plasmacytoid dendritic cells and in particular their main effector cytokine, IFN- $\alpha$ , are mainly reported to promote  $T_H1$ ,  $T_H9$ ,  $T_{REG}$  and, in part,  $T_H17$  differentiation at the expense of  $T_H2$  <sup>52,97,107,109,110,167</sup>.

IL-10 expression in murine  $T_H1$  cells can be driven by the cytokine IL-12 and signaling through STAT4 <sup>68</sup>. Also the cytokine IL-27, which activates STAT1 and STAT3, is known to promote generation of IL-10-expressing  $T_H1$  cells <sup>34,43,44</sup>. In the  $T_H17$  lineage, IL-10 is induced by IL-6 and TGF- $\beta$ , which utilize STAT3 as the critical transcription factor <sup>34,38</sup>.

In contrast, IL-22 has not been largely described for murine  $T_H1$  cells. It is considered a  $T_H17$  cytokine, owed to the fact that the main source of IL-22 in mouse are  $T_H17$  cells <sup>70</sup>. IL-6, IL-21 and IL-23 have been found to be the cytokines and STAT3 the transcription fac-

tor responsible for the induction of IL-22 in murine T<sub>H</sub>17 cells<sup>70,81</sup>. However, data available on IL-22 production by human T helper cells shows that human IL-22 is not as strictly linked to T<sub>H</sub>17. Here it is T<sub>H</sub>1 cells and a specialized T<sub>H</sub> subset that has not yet been described in mouse, the T<sub>H</sub>22 cells, that produce most of the IL-22 in peripheral blood<sup>77</sup>. Little is known about human T<sub>H</sub>1-derived IL-22, but the differentiation to T<sub>H</sub>22 cells has been reported to be induced by the cytokines IL-6 and TNF- $\alpha$ <sup>77</sup>.

Several transcription factors were described to regulate IL-10 or IL-22 expression in mouse. Among those are MAF (c-Maf)<sup>60</sup>, AhR (aryl hydrocarbon receptor)<sup>94</sup> and Blimp1<sup>42,65,66</sup>. In addition, delta-like Notch ligands were shown to drive IL-10 and IL-22 expression in various settings<sup>68,94,144</sup>.

Using these pathways as a guide, induction of IL-10 and IL-22 expression in human T helper cells through pDCs or pDC-derived factors was analyzed in this study.

Human plasmacytoid dendritic cells induced a mixed T<sub>H</sub>1-like population with substantial IL-10 and IL-22 expression.

Induction of both, IL-10 and IL-22, required Notch signaling elicited by Notch ligand DLL-4. Interestingly, IL-22 expression could be induced in absence of exogenous cytokines by DLL-4 ligation alone. In contrast, IL-10 induction required an additional cytokine stimulus.

IFN- $\alpha$  was capable of potently inducing IL-10 expression in combination with DLL-4, but depended on IL-6 and IL-21 signaling to do so. Also the cytokines IL-6, IL-21 and IL-27 by themselves were found to promote IL-10 expression, albeit to a much lesser degree.

STAT3 proved to be the transcription factor that all IL-10-inducing cytokines activated and required for induction of IL-10. Interestingly, although also IL-22 was induced by a STAT3-dependent pathway, the endogenous factor responsible for the IL-22-driving STAT3 activation was not identified. In addition, there was strong evidence for a STAT3-independent pathway of IL-22 induction.

DLL-4-activated Notch signaling was shown to potentiate the effect of IL-10- and IL-22-driving cytokines by enhancing cytokine-dependent activation, i.e. phosphorylation, of STAT molecules. The transcription factors MAF, AhR and BLIMP1 were shown to be positive regulators of IL-10 expression. Also IL-22 expression was promoted by AhR, but not by MAF or BLIMP1.

The experiments suggested that, while IL-10 and IL-22 were in part reciprocally and in part independently regulated, their expression was largely independent from T<sub>H</sub>1 commitment and IFN- $\gamma$  expression.

Finally, this study demonstrated that, knowing the critical signals and the layers of regulation that are addressed by these signals, IFN- $\gamma$ , IL-10 and IL-22 expression by human

T helper cells can be modulated and T cell populations can be generated that possess defined characteristics in regard to the expression of these cytokines. Thus T cell populations can be equipped with specific capacities, like immunosuppressive activity.

## 6.1 Notch ligands DLL-4 and JAG-2 enhance expression of IL-10 and IL-22

Literature concerning the function of the Notch ligands in  $T_H$  differentiation is controversial. However, DLL ligands and Jagged ligands are generally thought to have opposing functions: DLL ligands are thought to drive  $T_H1$  or play a role in  $T_H1$  effector functions<sup>67,134,157</sup>, in  $T_H17$  differentiation<sup>93</sup> and in general are assumed to facilitate proinflammatory functions<sup>168</sup>, whereas Jagged ligands are thought to have a  $T_H2$ -skewing effect<sup>169</sup> and to play a role in the generation of  $T_{REG}$  cells<sup>168,170</sup>.

Although the individual types of Notch ligands in the APC-free system showed large differences in regard to cytokine expression, a surprising finding was that the differences were not greatest between Delta-like and Jagged ligands, but between DLL-4 and JAG-2 on the one side and DLL-1 and JAG-1 on the other side. Both, DLL-4 and JAG-2, showed a pronounced capacity of inducing or enhancing IL-22 and IL-10 expression, whereas DLL-1 and Jagged-1 had little or no effect.

This partly agrees and partly contrasts with data from murine models. On the one hand, DLL-1 and DLL-4 have been shown to induce IL-10-production in  $T_H1$  cells in an IL-12-dependent fashion<sup>68</sup> and mouse pDCs to utilize DLL-4 as IL-10-driving factor<sup>144</sup>. On the other hand, JAG-1, but not JAG-2, has been described as inducing IL-10 expression<sup>69,168</sup>.

In regard to IL-22 expression, DLL-1 and DLL-4 have been demonstrated to strongly enhance IL-22 expression in the major  $T_H$  settings ( $T_H0$ ,  $T_H1$ ,  $T_H2$  and  $T_H17$ ) in mouse<sup>93,94</sup> whereas JAG-1 does not directly affect IL-22 expression in mouse  $T_H17$ <sup>171</sup>. JAG-2 has not been described in the context of IL-22.

Interestingly, there was no significant effect of any of the Notch ligands on IFN- $\gamma$  expression, contradicting the paradigm that Delta-like ligands drive  $T_H1$  and Jagged ligands promote  $T_H2$ . In fact, a recent review by Radtke, MacDonald, and Tacchini-Cottier<sup>67</sup> suggests that DLL-4 does not induce  $T_H1$  differentiation but rather facilitates  $T_H1$  functions. Furthermore, Yamane and Paul<sup>155</sup> found that under certain conditions DLL-4 can enhance  $T_H2$  differentiation.

In summary, the data generated here confirm that DLL-4 is a main driver of IL-10 and IL-22 expression not only in mouse, but also in human.

In contrast, DLL-1 did not induce IL-22 expression here and was largely inferior to DLL-4 in promoting IL-10 expression. The surprising finding that JAG-2 in the settings tested here acts in a way very similar to DLL-4 has not been described yet and warrants further investigation.

Contrary to the widespread notion that certain Notch ligands facilitate differentiation of distinct T<sub>H</sub> subsets, none of the Notch ligands visibly skewed T cell differentiation towards or against T<sub>H</sub>1 commitment.

## 6.2 IFN- $\alpha$ , acting in concert with IL-6 and IL-21, drives robust IL-10 expression

Analysis of the influence of cytokines showed that different pDC-derived cytokines in combination with DLL-4 were able to induce IL-10 expression in T cells. Most prominently, IFN- $\alpha$ , IL-6 and IL-27, which are known to be produced by pDCs <sup>96,151</sup>, were capable of driving IL-10 expression. In contrast, TNF- $\alpha$ , another typical pDC cytokine <sup>96</sup>, did not induce IL-10. Interestingly, IL-21, which is usually not produced in large amounts by pDCs, not only induced substantial amounts of IL-10 by itself, but appeared to play an important role in pDC-mediated induction of IL-10 in T helper cells.

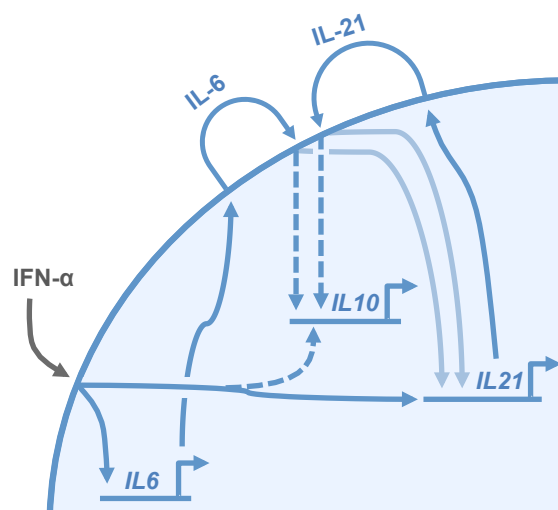
Among tested cytokines, IFN- $\alpha$  was the most potent IL-10 driving factor. Further analysis revealed that the effect of IFN- $\alpha$  strongly depended on the action of T cell-derived IL-6 and IL-21. In fact, IFN- $\alpha$  appeared to induce expression of IL-6 and IL-21, which then, in concert with IFN- $\alpha$ , induced IL-10. The finding that IL-21 and IL-6 by themselves failed to elicit comparable degrees of IL-10 expression suggested that promoting the release of IL-6 and IL-21 was not the sole function of IFN- $\alpha$ . Full induction of IL-10 expression appeared to require signaling by all three factors.

Interestingly, both, IFN- $\alpha$  and IL-6, are known to induce IL-21 expression in human T cells <sup>52,167,172</sup>, while IFN- $\alpha$  elicits downregulation of its own receptor <sup>137</sup>. Therefore, it appears likely that one role for T cell- as well as pDC-derived IL-6 is to aide IFN- $\alpha$  by continuing to stimulate the release of IL-21 after IFN- $\alpha$  has ceased to influence T cell differentiation.

It is not quite clear whether an equivalent mechanism exists in mouse, but circumstantial evidence suggests that this might be the case: IFN- $\alpha$  has been shown to induce IL-10 in murine *in vivo* models and data from *in vivo* skin injury models indicate that IFN- $\alpha$  is capa-

ble of and required for expression of IL-6<sup>125</sup>; IL-6 in combination with TGF- $\beta$  induces IL-10 and IL-21 in the murine T<sub>H</sub>17 subset<sup>38</sup> and this IL-10-driving effect in part depends on the action of IL-21<sup>38</sup>.

Interestingly, mouse IL-6 was shown to induce modest IL-10 production under non-polarizing, but not under T<sub>H</sub>1 conditions<sup>34</sup>. In contrast, IL-21, which is induced under non-polarizing, T<sub>H</sub>1 and T<sub>H</sub>17 conditions in mouse<sup>84–86</sup> and human<sup>172</sup>, is capable of stimulating its own release<sup>167,172</sup> and has been reported to induce IL-10 expression in non-polarizing, T<sub>H</sub>1 and T<sub>H</sub>17 in mouse<sup>38</sup>. This indicates that IL-21 may also here have been the main factor responsible for driving IL-10 expression. Fig. 6.1 illustrates the proposed mechanism of IFN- $\alpha$ -mediated induction of IL-10 expression aided by IL-6 and IL-21.



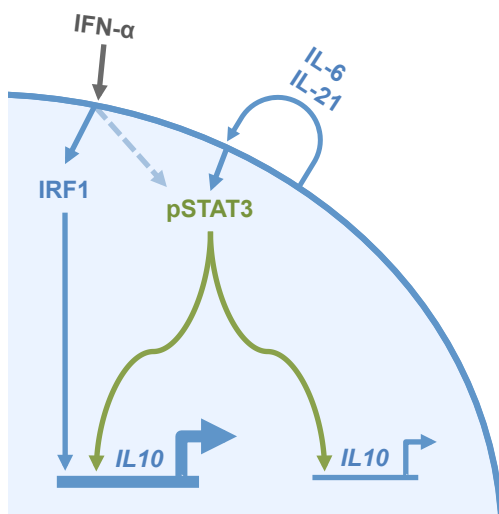
**Fig. 6.1: Proposed model for IFN- $\alpha$ -dependent IL-10 induction (1)**

IFN- $\alpha$  induces expression of IL-6 and IL-21. Exogenous IL-6, endogenous IL-6 and IL-21 induce further IL-21 secretion. Concerted action of all three cytokines (IL-21, IL-6 and IFN- $\alpha$ ) is required for full IL-10 expression.

According to the model developed above, IL-21 is a main effector of IL-10 induction but requires a second signal by IFN- $\alpha$  for full IL-10 expression. IL-21, and to some degree IL-6, are known to act mainly through STAT3<sup>34,38,42</sup>. This coincides with the observation that STAT3 was required for IL-10 induction in both, the pDC culture and the APC-free system. On the other hand, IFN- $\alpha$ -dependent gene expression is mainly mediated through the transcription complexes/factors ISG3 and IRF1<sup>47,173</sup>. In fact, IRF1 has been shown to enhance STAT3 transactivation of the IL-10 promoter two- to five-fold<sup>160</sup>.

Although IFN- $\alpha$  also strongly activates STAT3, it is assumed that IFN- $\alpha$  does not act directly through this STAT species, as its transcriptional activity is blocked by binding of IFN- $\alpha$ -induced SIN3<sup>47</sup>. This could explain the finding, that IFN- $\alpha$  required the action of STAT3-activating cytokines IL-6 and IL-21. This hypothesis is supported by the finding that late but not early STAT3-knockdown affected IL-10 expression in the pDC culture, which indicates that factors that are induced after the early T cell priming activate IL-10 expression through STAT3.

Curiously, early STAT3 knockdown sufficed for IL-10 inhibition in the APC-free system and IL-10 mRNA was expressed during early priming with DLL-4 and IFN- $\alpha$ . Although it cannot be excluded that STAT3-independent mechanisms contribute to early IL-10 induction, it appears more likely that the process leading to IL-10 expression was accelerated, owed to the fact that both DLL-4 and IFN- $\alpha$  were immediately available in the APC-free system whereas pDCs needed to upregulate expression of both factors before these could act on the T cells. It is also conceivable that – due to the much stronger IFN- $\alpha$  stimulus in the APC-free system – interferon-activated STAT3 is not completely inactivated by SIN3 and contributes to the IL-10 transactivation. In general the pDC cultures appeared to contain only moderate amounts of IFN- $\alpha$ , as adding IFN- $\alpha$  to those cultures led to a strong enhancement of IL-10 expression, which indicates that the microenvironment created by the pDCs was far from saturated with IFN- $\alpha$ . In fact, pDCs activated by type B CpG – which also has been used in the experiments here – are known to secrete only moderate amounts of IFN- $\alpha$ , in contrast to pDCs stimulated by type A CpG <sup>174</sup>.



**Fig. 6.2: Proposed model for IFN- $\alpha$ -dependent IL-10 induction**

The second function of IFN- $\alpha$  in the induction of IL-10 is the activation of IRF1, which enhances STAT3-dependent *IL10* transactivation initiated by IL-6 and IL-21 signaling.

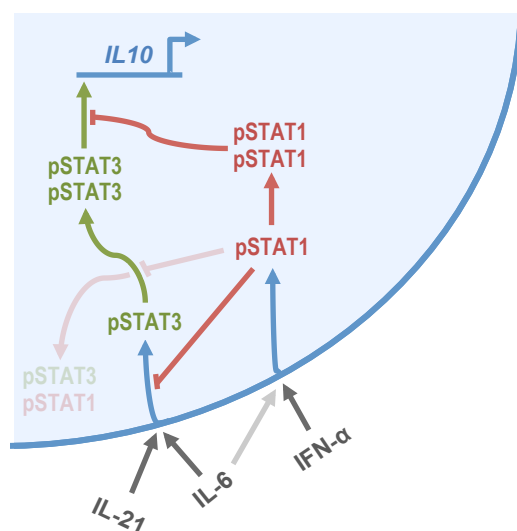
Taken together these results indicate that the main function of IFN- $\alpha$  is to elicit secretion of IL-21 and IL-6 (see Fig. 6.1) and the activation of a factor like IRF1, which enhances STAT3-dependent IL-10-transactivation induced by IL-6 and IL-21 (Fig. 6.2).

### 6.3 STAT1 and STAT3 reciprocally regulate expression of IL-10

STAT3 knockdown significantly reduced IL-10 expression under all tested conditions, strongly indicating that IL-10 expression depended on STAT3 signaling.

In fact, STAT3-dependent IL-10-induction has been reported for various settings, e.g. T<sub>H</sub>17 cells generated with IL-6 and TGF- $\beta$ <sup>34</sup> and T<sub>R</sub>1 cells generated by IL-27<sup>34,35,175</sup>. Other than has been shown in mouse, where DLL-4-mediated IL-10 expression in T<sub>H</sub>1 cells required IL-12 and STAT4 signaling<sup>68</sup>, neither of both factors appeared to contribute to the induction IL-10 in the human CD4 T cell cultures analyzed here. In contrast, addition of IL-12 to the pDC culture significantly reduced IL-10 expression and STAT4 was found to counteract IL-10-driving mechanisms in the presence of IFN- $\alpha$  or IL-21.

Interestingly, IFN- $\alpha$ -activated STAT1 showed a strong antagonizing effect on IL-10 expression, which was evidenced by the finding that STAT1 knockdown in T cells stimulated with IFN- $\alpha$  led to a pronounced increase of IL-10 expression. However, this effect was limited to cultures containing IFN- $\alpha$ , whereas in its absence STAT1 knockdown had no effect on IL-10 expression. This coincides with the role that STAT1 plays in the IFNAR signaling pathway. STAT1 is one of the main components of this pathway and becomes strongly activated and its expression significantly upregulated upon binding of IFN- $\alpha$ <sup>47</sup>. In contrast, STAT1-knockdown in cultures treated with IL-6 had no effect, despite the fact that IL-6 is also capable of inducing strong STAT1 phosphorylation. This may indicate that STAT1 needs to be highly activated and abundant to gain IL-10-inhibiting properties.



**Fig. 6.3: Proposed model for STAT1-mediated inhibition of STAT3-dependent IL-10 induction**

STAT1 may repress *IL10* transactivation by STAT3 (1) through competition for a common receptor binding site, (2) by sequestering STAT3 in non-activating STAT1:STAT3 heterodimers or (3) by blocking the STAT3 binding site in the *IL10* promoter.

However, more than one mechanism is conceivable by which high levels of STAT1 may counteract STAT3-dependent IL-10 expression.

Several workgroups have shown that different STAT species can compete for a receptor binding site and therefore limit each other's phosphorylation. This leads to increased activation of one species, if abundance of the other is reduced. Such competition has been demonstrated for STAT1 and STAT3<sup>163,176</sup> and STAT1 and STAT4<sup>158</sup>. In fact, investigating the effect of STAT1 knockdown on STAT3 phosphorylation showed that reducing STAT1 levels via siRNA led to a modest increase in STAT3 phosphorylation by IFN- $\alpha$ .

Another process limiting STAT3 activity can be the formation STAT1:STAT3 heterodimers. Research has shown formation of such heterodimers to be a mechanism of limiting STAT1 signal transduction in the IFN- $\alpha$  signal pathway<sup>47,177</sup>. Conversely, STAT1 may have counteracted STAT3-dependent IL-10 expression by removing STAT3 from the pool of monomers available for the formation of transcriptionally active STAT3 homodimers. This is supported by a publication showing that STAT1:STAT3 heterodimers fail to transactivate the *IL10* promoter<sup>160</sup>.

However, one would expect for both these mechanism to boost STAT3-dependent IL-22 expression. The fact that this was not observed suggests an IL-10-specific mechanism. In fact, Ziegler-Heitbrock et al.<sup>160</sup> have shown in human B cells that STAT1 homodimers compete with STAT3 homodimers for the STAT binding site in the *IL10* promoter while being incapable of transactivating *IL10* and thus specifically counteracting STAT3-mediated IL-10 expression.

Although it appears likely that all three processes are active in T cells, the results suggest that competition for the *IL10* binding site is the predominant mechanism behind the observed STAT1-mediated IL-10-suppression. This is supported by reports that increased formation of STAT1 homodimers downstream of the IFNAR leads to a switch of IFN- $\alpha$ -induced gene expression to inflammatory genes through binding of STAT1 homodimers to GAS elements, which cannot be activated by canonical IFNAR signaling (ISGF3 formed by STAT:STAT2 and IRF9)<sup>47</sup>.

One may speculate that STAT1-mediated inhibition of STAT3-related IL-10 induction is a mechanism of counteracting anti-inflammatory pathways in situations when proinflammatory stimuli enhance IFN- $\alpha$ -related STAT1 activation.

## 6.4 IL-22 is induced through STAT3 in absence of exogenous cytokines

IL-22 in mouse is mainly described as a T<sub>H</sub>17 cytokine <sup>70</sup>, which is induced by the cytokines IL-6, IL-23 and IL-21 <sup>81,84</sup>. In contrast, IL-22 in the human system is mostly expressed by T<sub>H</sub>1 and T<sub>H</sub>22 cells <sup>70,77</sup>, the latter of which have been shown to be generated in the presence of IL-6 and TNF- $\alpha$ .

In the experiments described here, production of IL-22 was entirely driven by the presence of DLL-4 and was not enhanced by any of the classical IL-22-inducing cytokines, like IL-6, IL-21 or TNF- $\alpha$ . Curiously, addition of IL-6 and IL-21 even reduced DLL-4-elicited IL-22 expression.

Knockdown of STAT3 significantly reduced the expression of IL-22 in both, the pDC system and the APC-free system, indicating the presence of a STAT3-dependent mechanism. This agrees with data from mouse, which shows that Delta-like Notch ligands induce IL-22 in a STAT3-dependent manner <sup>94</sup>. However, this raises the question, which endogenous factor might be responsible for activating the STAT-dependent pathway of IL-22 expression. Several reports point towards IL-21 as a likely candidate: It is expressed by T cells under non-polarizing and T<sub>H</sub>1 conditions <sup>172</sup>; it drives IL-22 under T<sub>H</sub>17 conditions in mouse <sup>84</sup>; and it acts through STAT1 and STAT3 as demonstrated by experiments here and in other workgroups <sup>38</sup>. However, under the conditions tested here IL-21 reduced IL-22 expression and abrogated the STAT3-dependent pathway.

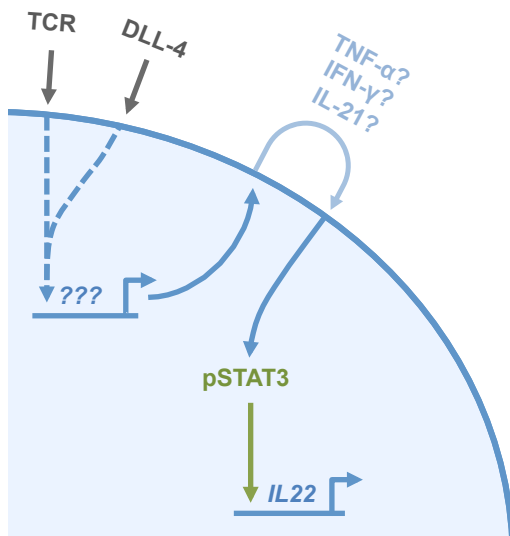
IL-22 expression has also been described as being correlated to IFN- $\gamma$  expression <sup>77</sup>. Several publications provide evidence that IFN- $\gamma$  activates STAT3 <sup>163</sup>, using STAT3-dependent pathways to exert functions different from those elicited through STAT1 in human and mouse <sup>164, 165</sup>. These data, together with the observation that low numbers of IFN- $\gamma$ -producing T cells were always present in the culture under non-polarizing conditions and without added cytokine, would render IFN- $\gamma$  a possible candidate for driving IL-22. However, the data generated in this project suggested that IL-22 expression was largely independent of IFN- $\gamma$  expression and T<sub>H</sub>1 commitment.

The cytokine TNF- $\alpha$  – although it did not significantly affect IL-22 expression in the systems tested here – is a third potential candidate for the IL-22 induction in absence of exogenous cytokines. In fact, the experiments indicated that T cell-derived TNF- $\alpha$  was present in the cultures. Moreover, TNF- $\alpha$  has been described as a strong inducer of T<sub>H</sub>22 differentiation when acting in concert with IL-6 <sup>77</sup>. In addition it is known to signal through

both, STAT1 and STAT3<sup>178</sup>, and as is one of the earliest endogenous soluble factors expressed upon activation of the T cell receptor<sup>179</sup>.

Curiously, also STAT1 knockdown in the APC-free system led to a significant, albeit modest, reduction of IL-22 expression, which indicates that also STAT1 is involved in the regulation of IL-22. However, it remained unclear whether STAT1 and STAT3 are part of the same IL-22-inducing mechanism or act separately.

Unfortunately, the experiments performed here did not provide conclusive evidence as to the identity of the endogenous factor that promotes IL-22 expression through STAT3 or STAT1.



**Fig. 6.4: Proposed model for STAT3-dependent IL-22 expression**

In absence of exogenous cytokines T cell activation and DLL-4 signaling induce an endogenous factor that activates STAT3-mediated IL-22 expression.

In summary, IL-22 expression did not require activation by an exogenous cytokine but could be driven by a STAT3-dependent pathway and an unknown endogenously produced factor that activates STAT3.

## 6.5 IFN- $\alpha$ and DLL-4 do not drive T<sub>H</sub>1 commitment

There have been contrasting reports regarding the capacity of IFN- $\alpha$  to drive T<sub>H</sub>1 commitment in mouse and human. However, recent data indicate that IFN- $\alpha$  alone is not capable of sustaining a full T<sub>H</sub>1 response. In fact, both in mouse and human, IFN- $\alpha$  has been shown to elicit a strong but also transient IFN- $\gamma$  response<sup>137,158</sup>. This is assumed to be caused by IFN- $\alpha$ -induced downregulation of the IFN- $\alpha$  receptor (IFNAR), which leads to a

mere transient STAT4 activation and the failure to produce sustained IFN- $\gamma$  expression, a prerequisite for committing T cells to the T<sub>H</sub>1 lineage<sup>137</sup>. In line with these reports, the experiments performed in this study showed that IFN- $\alpha$  was not capable of significantly augmenting IFN- $\gamma$  expression in absence of plasmacytoid dendritic cells.

Also the Notch ligand DLL-4 has been described as promoting T<sub>H</sub>1 commitment in mouse<sup>134,157</sup>. However, it did not enhance IFN- $\gamma$  expression in the experiments performed here. In fact, more recent data indicate that, contrary to the current paradigm, Delta-like Notch ligands do not drive T<sub>H</sub>1 polarization but rather support the functions of T<sub>H</sub>1 cells<sup>67</sup>.

Interestingly, although there was no significant difference in IFN- $\gamma$  expression between cultures with and without added IFN- $\alpha$ , the presence of this cytokine appeared to affect the pathways inducing IFN- $\gamma$  in the APC-free system. STAT knockdown experiments in absence of exogenous cytokine did not provide conclusive results regarding the role of STAT1 and STAT4 signaling in absence of exogenous cytokines. However, with added IFN- $\alpha$ , the expression of IFN- $\gamma$  was clearly governed by pathways that required STAT1 and STAT4 signaling. Although this showed some resemblance to the situation observed in the pDC culture, one has to keep in mind, that pDCs express a multitude of factors. In fact, also IL-12, which was shown to be present in the pDC culture, as well as IFN- $\gamma$  itself promote T<sub>H</sub>1 commitment through STAT4 and STAT1, respectively.

In contrast, STAT3 appeared to convey an IFN- $\gamma$ -suppressing signal in all settings. This was evidenced by the fact that STAT3 knockdown significantly augmented IFN- $\gamma$  expression. The data did not reveal the factor behind this effect. But several cytokines that act through STAT3 also reduced IFN- $\gamma$  expression when added to the culture. It is conceivable that this effect was to a large part owed to the action of IL-21. This cytokine is known to be produced under T<sub>H</sub>1 as well as non-polarizing conditions<sup>172</sup>, the cytokine blocking experiments suggested that endogenous IL-21 was present in the cultures and exogenous IL-21 reduced IFN- $\gamma$  expression.

Taken together, the experiments show that in the settings analyzed here, neither IFN- $\alpha$  nor DLL-4 promoted T<sub>H</sub>1 commitment. However, STAT3 signaling appeared to counteract IFN- $\gamma$ -driving signals.

## 6.6 Strong T<sub>H</sub>1 commitment is not required for expression of IL-10 and IL-22

Despite the fact that DLL-4 and IFN- $\alpha$  in the APC-free system failed to induce a comparable frequency of IFN- $\gamma$ -producers, expression levels and distribution of IL-10 and IL-22 closely resembled those observed in the pDC culture. In addition, analysis of IL-10 and IL-22 expression by cells that were activated in the presence of DLL-4 with or without IFN- $\alpha$  and/or IL-12 showed that T<sub>H</sub>1 commitment could be achieved while retaining a large part of IL-10 and/or IL-22 expression. This indicates that the induction of IL-10 and IL-22 by DLL-4 and IFN- $\alpha$  is not directly linked to T<sub>H</sub>1 commitment but rather depends on a distinct layer of regulation. However, different factors and pathways are likely to govern IL-10 and IL-22 induction in other T<sub>H</sub> subsets. Therefore, it cannot be excluded that a baseline T<sub>H</sub>1 bias is advantageous or even required for IL-10 and IL-22 induction by the mechanisms described here, as it might counteract commitment to alternative T cell fates.

In summary, the experiments demonstrated that strong IFN- $\gamma$  expression and pronounced T<sub>H</sub>1 polarization appeared to be not required for expression of IL-10 or IL-22.

## 6.7 DLL-4 enhances STAT3 signaling

The observation that DLL-4 enhanced IL-10, IL-22 and IL-17 induction by cytokines employing STAT1 and STAT3 and the finding that IL-22 and IL-10 expression were mediated by STAT3 raised the question, whether DLL-4-induced Notch signaling directly interfaces at the level of cytokine-induced STAT signaling.

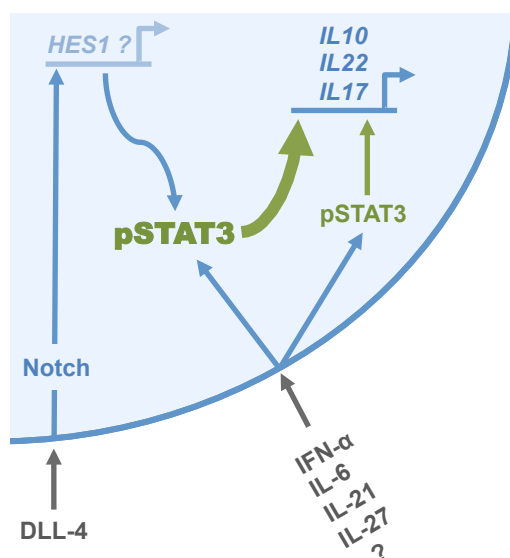
In literature, there are contrasting reports on this matter. While Mukherjee et al. showed that DLL-4 signaling did not alter STAT3 phosphorylation in mouse T<sub>H</sub>17 cells <sup>93</sup>, Kamakura et al. demonstrated in mammalian neural cells that Notch-induced proteins of the HES family bind to STAT3, recruit JAK2 kinase and stabilize the JAK2-STAT3 complex, which leads to phosphorylation of STAT3 <sup>95</sup>. In addition, Lee et al. reported that expression of Notch and HES1 in the human HeLa cell line induces STAT3 phosphorylation at tyrosine 705 by the SRC kinase <sup>166</sup>.

In the study at hand, the APC-free system was utilized to assess, whether DLL-4 costimulation alters phosphorylation of STAT1, STAT3 or STAT4 following stimulation with cytokines that have been found to promote IL-10 expression (IFN- $\alpha$ , IL-6 and IL-21).

The analysis demonstrated that upon cytokine challenge in the early priming phase phosphorylation at STAT3 tyrosine 705 was enhanced in activated T cells costimulated with DLL-4. In fact, increased phosphorylation at STAT3 tyrosine 705 was observed with all three tested cytokines. Interestingly, although there was no difference in the level of phosphorylation at the tyrosin residues of STAT1 (Y701) and STAT4 (Y693) when activated by IL-21 and IL-6, both STATs showed augmented phosphorylation after activation by IFN- $\alpha$ . In contrast, no alteration of the secondary STAT1 and STAT3 phosphorylation sites (serine 727) was observed. All these data indicated that DLL-4 affects STAT phosphorylation.

However, despite the fact that the enhancement of phosphorylation was statistically significant, the average change was comparatively small in relation to the total increase in phosphorylation induced by the cytokines. The question that needs to be asked here is: can such small alterations lead to the profound upregulation of IL-10 and IL-22 as seen in the experiments?

It is indeed unlikely that a change of 2 % or 5 % would significantly alter the quality of the STAT signal. But here has to be taken into account that in contrast to the experiment used to assess STAT phosphorylation cytokine signals in a cell culture persist much longer and therefore STAT activation may be altered more profoundly. In addition, Kamakura et al. had shown that Notch-induced HES1 stabilizes the phosphorylation-promoting complex of STAT3 and JAK2 <sup>95</sup>. It is conceivable that the result of this stabilization is not a large increase in the level of STAT phosphorylation but rather an increase in the duration of phosphorylation and thus activation. Since the assay was designed to identify changes in signal strength and not in signal duration, such an effect would be less evident and could lead to results like they were seen here.



**Fig. 6.5: Proposed model for Notch-enhanced STAT phosphorylation**

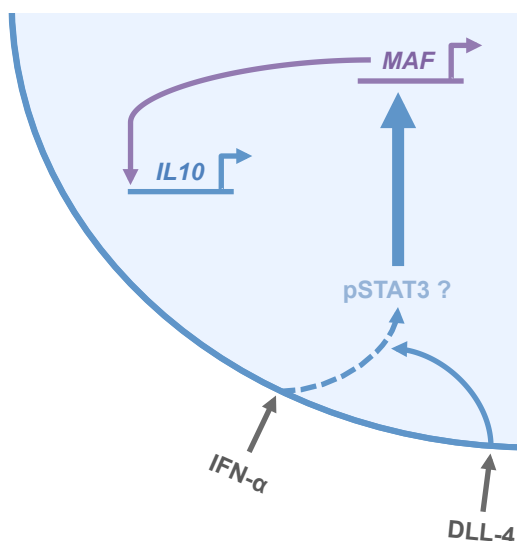
DLL-4 ligation leads to Notch-dependent expression and/or activation of HES proteins, which stabilize the complex of STAT molecules and kinases leading to augmented or prolonged phosphorylation and thus activation of the STAT. Stronger activation of STAT in turn enhances STAT-dependent cytokine expression.

In summary, the data support the hypothesis that DLL-4 modulates cytokine-driven expression of effector cytokines like IL-10, IL-22 or IL-17 by enhancing STAT phosphorylation in a Notch-dependent manner. In particular, activation of STAT3 benefitted from Notch signaling in the systems described here.

## 6.8 DLL-4, by enhancing MAF expression, facilitates IL-10 production

MAF has been shown to be capable of inducing IL-10 in different cell types like macrophages<sup>146</sup> and T cells as well as in various murine T<sub>H</sub> cell subsets, like T<sub>H</sub>1, T<sub>H</sub>17 and T<sub>R</sub>1 cells<sup>37,58</sup>. Owing to the fact that it is involved in IL-10 expression in most immune cells, it is believed to be a universal IL-10 transcription factor<sup>24</sup>.

The knockdown experiments performed here, confirmed that MAF was required for IL-10 expression induced in pDC culture as well as the APC-free system. In addition, analysis of *MAF* mRNA levels showed that DLL-4 strongly enhanced IFN- $\alpha$ -induced MAF expression in activated naïve CD4 T cells. Although, at this point, the mechanism behind this effect is not clear, IL-6 has been described as promoting MAF expression through the action of STAT3<sup>180</sup>, suggesting that DLL-4 might potentiate STAT3-dependent induction of MAF either through the enhancement of STAT3 phosphorylation or by promoting and/or accelerating IFN- $\alpha$ -dependent induction of cytokines like IL-6 that activate MAF expression. Both mechanisms may also act synergistically.



**Fig. 6.6: Proposed model for role of MAF in the induction of IL-10**

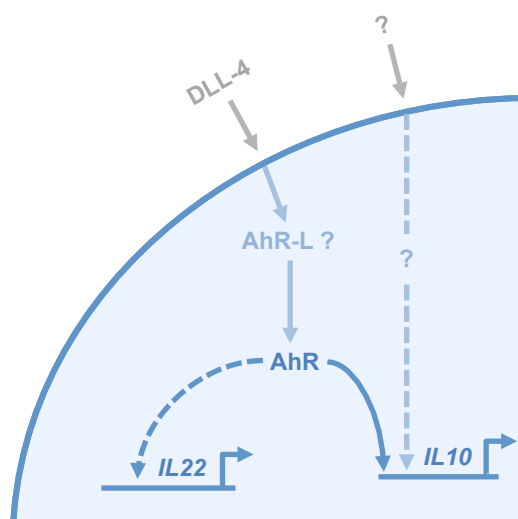
IFN- $\alpha$  and DLL-4 act together in inducing MAF through a putative STAT3-dependent mechanism. MAF transactivates *IL10*.

## 6.9 AhR promotes expression of IL-22 and IL-10

Several research groups have demonstrated that the aryl hydrocarbon receptor drives IL-22 expression in a STAT3-independent manner in mouse T<sub>H</sub>17 cells<sup>60,94</sup> and is required for T<sub>H</sub>22 differentiation<sup>77,78</sup>. In addition, Notch signaling in T<sub>H</sub>17 cells has been shown to induce AhR agonists, which in turn promote expression of both IL-17 and IL-22<sup>90</sup>.

In agreement with these results, blocking AhR activity in human CD4 T cells significantly reduced IL-22 expression. Interestingly, the knockdown experiments performed here indicated that IL-22 was induced in human CD4 T cells by STAT3-dependent as well as STAT3-independent pathways. This resembles the situation in the murine T<sub>H</sub>17 subset<sup>60,94</sup>, where Notch-induced AhR activation and cytokine-mediated STAT3 activation were found to independently regulate IL-22 expression.

In addition, to STAT3 also STAT1 appeared to be required for IL-22 expression. Although STAT1 may participate in the STAT3-dependent as well as the STAT3-independent mechanism, reports that AhR forms a transcriptionally active complex with STAT1<sup>162</sup> suggest that STAT1 and AhR might cooperatively promote IL-22 expression and the observed impairment of IL-22 expression by STAT1 inhibition might be owed to a reduced formation of transcription complexes by AhR and STAT1. However, the idea of a cooperative mechanism of AhR and STAT1 remains highly speculative.



**Fig. 6.7: Proposed model for a role of AhR in the induction of IL-10 and IL-22**

DLL-4 induces release of AhR ligand by T cells, which leads to enhanced AhR activation. Activated AhR promotes expression of IL-22 and, through cooperation with another factor, of IL-10.

The picture in regard to the role of AhR-signaling in the expression of IL-10 was less clear. On the one hand, the experiments with the AhR antagonist showed clearly that AhR signaling was also involved in driving IL-10 expression. On the other hand, although AhR signaling appeared to be active in T cells stimulated with DLL-4, AhR failed to promote IL-10 ex-

pression in absence of cytokines like IFN- $\alpha$ . This indicates that additional factors are required for the induction of IL-10.

Interestingly, AhR has been reported to be crucial for IL-10 expression in murine T<sub>R</sub>1 cells<sup>58</sup>, where it transactivates the *IL10* promoter in concert with Maf<sup>37,58</sup>.

In summary, the findings suggest that a) AhR positively regulates IL-22 and may be part of a STAT3-independent mechanism that drives IL-22 expression and b) AhR plays a role in the induction of IL-10 but is not sufficient to elicit IL-10 expression.

## 6.10 Late BLIMP1 regulates expression of IL-10

BLIMP1, which is encoded by the *PRDM1* gene, has been described in different IL-10-related contexts. In particular, recent research links this transcription factor to the induction of IL-10-producing cells. BLIMP1 has been shown to be required for IL-10 expression in mouse T<sub>REG</sub> cells<sup>65</sup>, where it transactivates the *IL10* promoter in concert with IRF4 (interferon regulatory factor 4) and a very recent paper demonstrated that BLIMP1 is absolutely required for IL-12- and STAT4-mediated IL-10 expression in mouse T<sub>H</sub>1 cells, where it transactivates the *IL10* promoter together with MAF<sup>66</sup>. Interestingly, in other contexts BLIMP1 has been described as a factor that correlates with terminal differentiation of CD8 and CD4 effector T cells and is required for effector functions like granzyme production and cytokine secretion.

In the systems analyzed here, IL-10 expression in part depended on the presence of BLIMP1 as was evidenced by knockdown experiments. In line with this finding, costimulation by DLL-4 and IFN- $\alpha$  led to the strongest upregulation of *PRDM1* mRNA. However, only BLIMP1 suppression after initial T cell priming had an effect on IL-10 expression, whereas knockdown of BLIMP1 before T cell activation did not alter IL-10 production, but enhanced IFN- $\gamma$  expression. In fact, BLIMP1 has been described as repressor of IFN- $\gamma$  and IL-2 and as part of the negative feedback loop of IL-2 during T cell priming<sup>62</sup>, which explains the effect of early loss of BLIMP1 on IFN- $\gamma$  expression.

More surprising was the finding that the reduction of BLIMP1 levels during the late stage of T cell differentiation appeared to impair IL-10 expression as well as the expression of IFN- $\gamma$ .

These observations strongly suggest that the role of BLIMP1 changes depending on the stage of T cell differentiation. This is of particular interest since most published data on BLIMP1 stems from experiments with Blimp1-knockout mice or T cells and would naturally overlook differential functions of BLIMP1 in distinct stages of T cell life.

In fact, these findings are in accordance with the abovementioned reports that BLIMP1 plays a role in the effector function of terminally differentiated T cells <sup>62,159</sup> and is required for the expression of IL-10 by T<sub>REG</sub> cells but not for the differentiation of these cells <sup>65</sup>.

Taken together the data indicate that BLIMP1 is required for the expression of IL-10 by human T helper cells, but probably does not play a direct role in the generation of IL-10-producing T cells.

## 6.11 AhR, STAT3 and MAF, central modules ruling expression of IL-10 and IL-22

One defining feature of the T cell population generated in the pDC culture as well as in the APC-free system with DLL-4 and IFN- $\alpha$  was the parallel expression of IL-10 and IL-22 and the reciprocal regulation of IL-10 and IL-22 by IL-10-promoting cytokines like IFN- $\alpha$ , IL-6, IL-21 and IL-27. This finding raised the question whether these cytokines might induce a common factor that acts as IL-10-promoter and IL-22-repressor.

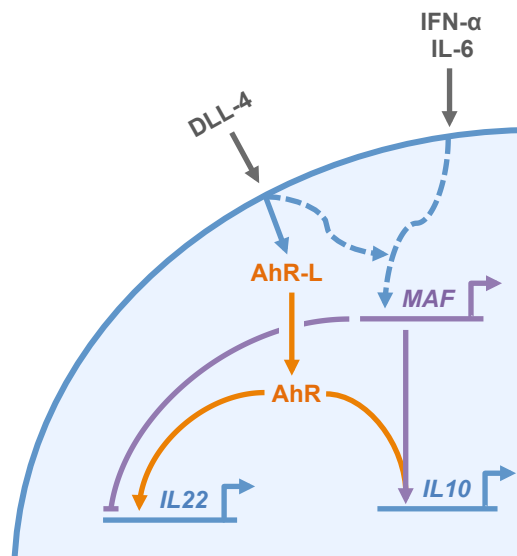
MAF (*v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog*) has been described both as direct transcriptional repressor of IL-22 in mouse T<sub>H</sub>17 cells <sup>60</sup> and as direct transcriptional activator of IL-10, in murine T<sub>H</sub>17, T<sub>H</sub>1 and T<sub>REG</sub> cells <sup>37,57,58</sup>. Even more, it has been suggested that Maf acts as a switch between IL-22 and IL-10 expressing T<sub>H</sub>17 cells in mouse <sup>60</sup>.

Some observations indicated that MAF might play a similar role for IL-10- and IL-22-expressing T cells induced by pDCs or by IFN- $\alpha$  and DLL-4. Firstly, MAF expression was strongly upregulated and IL-22-production strongly reduced in T cells cultivated in the presence of IFN- $\alpha$  and DLL-4. For example, IL-6, which in mouse T cells has been shown to induce MAF <sup>180</sup>, significantly reduced IL-22 and enhanced IL-10 expression. Secondly, IL-10 expression, but not IL-22 expression, was reduced by siRNA-mediated knockdown of MAF, which indicates that IL-10 but not IL-22 requires MAF for its expression.

Curiously, MAF knockdown did not significantly enhance IL-22 expression, despite the fact that MAF has been described as IL-22 repressor in mouse. Although this contradicts the hypothesis of MAF as reciprocal regulator of IL-10 and IL-22, it may simply be owed to the transient nature of siRNA-mediated MAF knockdown, which may not be sufficient to abrogate the inhibitory effect of MAF on IL-22. In fact, the experiments using MAF knockdown were designed to suppress MAF activity before or during the early T cell priming phase. However, IL-22 expression was found to start comparatively late, suggesting that a direct

IL-22 repressor like MAF would need to act in later stages of T cell differentiation or after the differentiation process has been concluded, i.e. in a phase that possibly was not accessed by the siRNA-mediated MAF-knockdown.

In the model proposed here, DLL-4-related Notch signaling would, through activation of AhR, induce IL-22 expression as a “first choice” cell fate. MAF expression induced by cytokines like IFN- $\alpha$  or IL-6 would divert cell fate towards IL-10-producers by repressing IL-22 and recruiting AhR as cofactor for transactivation of *IL10*. In some cells, abundance of AhR activation may even overcome the repressing effect of MAF and therefore induce IL-22 in addition to IL-10, creating double producers. A similar scenario was described for murine T<sub>H</sub>17 cells induced by IL-6 and TGF- $\beta$ . The tumor growth factor induces expression of Maf, which represses IL-22 and in concert with AhR transactivates *IL-10*<sup>60</sup>. However, IL-22 expression could be rescued by high levels of AhR<sup>90</sup>.



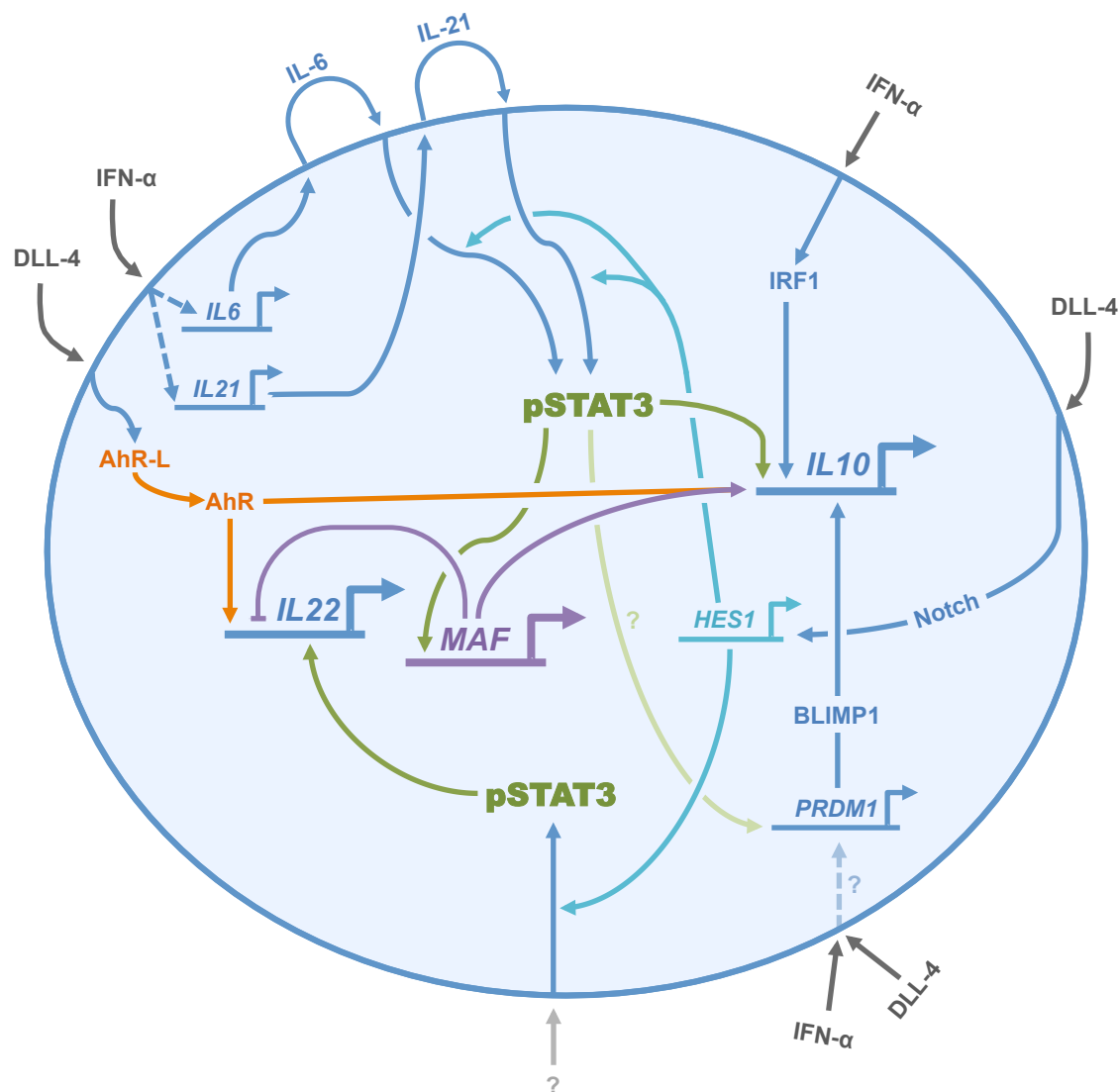
**Fig. 6.8: Proposed model for the interaction of AhR and MAF in the regulation of IL-10 and IL-22 expression**

DLL-4 is involved in both, the activation of AhR – through stimulation of the release of AhR ligands – and the expression of MAF – by concerted action with cytokines like IFN- $\alpha$  and IL-6. In absence of cytokines like IL-6 and IFN- $\alpha$ , AhR elicits IL-22 expression but fails to induce IL-10. When MAF is expressed, AhR and MAF together transactivate *IL10*, while MAF represses *IL22* expression.

STAT3 signaling plays a prominent role in the induction of IL-10, but also of IL-22. This is not only supported by the findings here, but by a host of published data that underline the importance of STAT3 signaling for IL-10 and IL-22 expression in mouse T<sub>H</sub>17 cells<sup>34,38,84,87,94</sup> and for IL-10 production by induced regulatory T cells<sup>34,42,45</sup>. The data generated in the experiments described here shows that expression of both, IL-10 and IL-22, are to a certain degree mediated by STAT3-signaling, which is potentiated by the DLL-4/Notch-mediated enhancement of STAT activation.

Importantly, also the other transcriptional modules described here, i.e. MAF, AhR and BLIMP1, have been reported to depend on STAT3 in certain contexts. For example: in mouse, IL-6 has been shown to induce MAF expression via STAT3-mediated transactivation of the MAF promoter<sup>180</sup>; a STAT3-governed pathway was reported to induce BLIMP1

in mouse B cells<sup>181</sup> and STAT3 downstream of IL-21 signaling was demonstrated to render the IL-22 locus accessible to AhR transactivation by controlling its epigenetic status<sup>182</sup>.



**Fig. 6.9: Proposed network governing IL-10 and IL-22 expression in human T helper cells differentiated in presence of DLL-4 and IFN-α**

The central transcriptional modules in this network are STAT3 and MAF, which directly or indirectly regulate IL-10 and IL-22 expression and themselves are regulated by other factors.

AhR, aryl hydrocarbon receptor; AhR-L, AhR ligand; DLL-4, Delta-like Notch ligand 4; HES1, hes family bHLH transcription factor 1; IFN-α, Interferon alpha; IRF1, Interferon regulatory factor 1; MAF, c-Maf; pSTAT3, phosphorylated STAT3; *PRDM1*, gene encoding BLIMP1

The results of the experiments described here indicate that, like in mouse, STAT3 signaling is a central mechanism driving differentiation of human CD4 T cells to IL-10 and IL-22 producers, whereas the single transcription modules, like MAF, AhR and Notch, are part of

the STAT3 pathway, modulating it and/or being modulated themselves and thus influencing the outcome of the STAT3-dependent differentiation process.

Fig. 6.9 illustrates the proposed model of IL-10 and IL-22 regulation in human CD4 T cells activated in the presence of DLL-4 and IFN- $\alpha$ , based on the experiments described here and on related published data. However, this model can only be rudimentary, showing a small part of the network governing IL-10 and IL-22 expression in human T helper cells in this setting.

## 6.12 Knowing the critical modules, T cell populations can be “custom-made”

As has been discussed in the preceding paragraphs, different modules interact in CD4 T cells to induce IL-10, IL-22 and IFN- $\gamma$  expression. The experiments showed that knowing these modules they can be specifically addressed in the APC-free system through costimulation factors like DLL-4, IFN- $\alpha$ , IL-6 and IL-12. More importantly, the data show that knowledge of the modules allows rational combination of costimuli with the aim of inducing a desired phenotype, be it IFN- $\gamma$ +IL-22<sup>+</sup> CD4 T cells, IFN- $\gamma$ +IL-10<sup>+</sup> CD4 T cells, populations of with IL-10-single producers or mixed populations of IL-10- and IL-22-expressing cells. This may open the way for creating specific T cell populations for specific therapeutic applications.

Both IL-10- and IL-22-producing CD4 T cells, owing to the specific functions of those two cytokines, could be used to target different conditions. It is conceivable that IL-10<sup>+</sup> T cells would find application in the treatment of autoimmune diseases as well as targeting chronic diseases like schistosomiasis, where IL-10-producing T<sub>H</sub>1 cells have been shown to counteract T<sub>H</sub>2-mediated fibrosis and pathogen persistence. IL-22, on the other hand, plays an important role in the defense of mucosal epithelia as well as in wound healing. Here, varying the ratio of IL-22- and IL-10-producing T cells may allow balancing pro- and anti-inflammatory properties of the T cell population, according to the specific needs of the application.

Indeed, the finding that IL-10-producing CD4 T cells generated in the APC-free system had a immunosuppressive capacity comparable to IL-10-producing CD4 T cells induced by plasmacytoid dendritic cells demonstrated that such an approach may one day become a viable option in the clinical context.

## 6.13 Outlook

This project was designed to provide an overview over factors that regulate the expression of the cytokines IL-10 and IL-22 in context of the interaction of human T helper cells and plasmacytoid dendritic cells. Various factors and their connections being involved in the regulation of IL-10 and IL-22 have been identified. However, although overall picture is still far from complete, the results presented here provide good starting points for further analyses.

As an example, the modulation of cytokine-derived signals through interaction of the Notch pathway with STAT molecules warrants further analysis, particularly in the light of recent reports that question the current paradigm that delta-like Notch ligands promote T<sub>H</sub>1 responses and jagged ligands induce T<sub>H</sub>2 differentiation. Understanding the mechanism by which Notch influences cytokine-mediated activation of STAT molecules would help to elucidate a currently still poorly understood layer of T cell regulation. In particular, comparison of the effect of different Notch ligands and Notch receptors on the phosphorylation of STATs would provide valuable insight and possibly answer the question of how different Notch ligands produce different outcomes.

Another important problem is the role of BLIMP1 in the induction of IL-10 and other effector cytokines. The data generated here indicated that BLIMP1 may fulfill different roles in different stages of the T cell life, a possibility that so far appears to have been largely neglected, owed to the fact that many experiments were done on or with BLIMP1 knockout animals or cells, which makes it hard to address differential functions of BLIMP1 in distinct stages of T cell differentiation. In fact, utilizing siRNA-mediated knockdown, as in the project described here, could help assessing temporally distinct roles of BLIMP1.

In regard to the expression of IL-22 questions remained unanswered. The exact identity as well as the interplay of STAT3-dependent and -independent pathways would have to be elucidated. Here, it would of great interest to understand, which endogenous factor drives STAT3-dependent expression, but also the putative connection between AhR, the STAT3-independent pathway and STAT1 would need to be examined or established in order to better define the mechanisms determining expression of IL-22.

In order to complete the greater picture, the interaction between the different modules that were involved in the regulation of IL-10, like BLIMP1, MAF, AhR and STAT3, would need further analysis as data from literature indicate that BLIMP1 and MAF themselves may be induced through STAT3, whereas BLIMP1 and AhR have been suggested to promote IL-10 expression in reaction to different triggering signals.

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Finally, but no less important, the stability of the signals driving IL-10 and IL-22 expression and the role of the described network in the regulation of IL-10 and IL-22 in memory T helper cells would need to be addressed in order to facilitate translation of the knowledge of regulatory pathways into therapeutic application.

## 7 Abstract

The cytokines IL-10 and IL-22 belong to the same family of cytokines but play very different, albeit important, roles in the immune response. IL-10 is one of the main mediators of immunosuppression and involved in regulating, limiting or suppressing inflammatory immune responses, thus preventing excessive and harmful immune reactions. IL-22 plays an important role in maintaining and defending the epithelial barriers that separate the organism from the outer environment and in tissue regeneration in a variety of organs. The importance of those two cytokines is underlined by the fact that T helper cells, ( $T_H$  cells) which orchestrate the adaptive immune response, produce IL-10 in various contexts, but are also the main source of IL-22 in peripheral blood.

Although considerable research has been done on the generation of IL-10- and IL-22-expressing T helper cells, the regulation of the cytokines IL-10 and IL-22 in human is still poorly understood. This is mainly owed to the fact that most data stem from experiments in mouse model systems.

In addition, IL-10 is differently regulated in each T helper cell subset:  $T_H1$ -related IL-10 is induced by IL-12 and STAT4; in  $T_H2$  cells, IL-10 is elicited by IL-4 and STAT6; and IL-6 and TGF- $\beta$  induce IL-10-expressing  $T_H17$  cells through STAT3. IL-22, on the other hand, has been described as  $T_H17$  cytokine, which requires the cytokines IL-6, IL-21 or IL23 and STAT3 signaling. However, it appears to be differently regulated in human, where beside  $T_H17$  cells,  $T_H1$  and  $T_H22$  cells are the main sources of IL-22 in peripheral blood. Furthermore, other costimulatory signals like the Notch pathway have been found to influence expression of IL-10 and IL-22. Lately, several transcription factors were described to regulate IL-10 or IL-22 expression in mouse, including c-Maf, the aryl hydrocarbon receptor (AhR) and Blimp1.

In view of the limited knowledge in regard to the regulation of those two cytokines in the human immune response, this study aimed to analyze signals and transcriptional modules governing expression of IL-10 and IL-22 by human T helper cells utilizing the interaction of T cells and plasmacytoid dendritic cells (pDC) as model system.

Using the regulation described in mouse as a guide, the signals and transcriptional modules involved in IL-10 and IL-22 expression were investigated first in a pDC/T cell coculture system and later in a minimalistic APC-free system that allowed analysis in a more defined environment.

Human plasmacytoid dendritic cells were found to induce a mixed  $T_H1$ -like population with substantial expression of IL-10 and IL-22. Analysis showed that induction of both cytokines depended on intracellular Notch signaling elicited by the Notch ligand DLL-4. While IL-22 expression was induced by DLL-4 and TCR-dependent T cell stimulation

alone, IL-10 induction required an additional stimulus. This second costimulus could be provided by certain STAT3-activating cytokines like IFN- $\alpha$ , IL-6, IL-21 or IL-27. IFN- $\alpha$ , the main effector cytokine of plasmacytoid dendritic cells, most potently induced IL-10 expression. Interestingly, IFN- $\alpha$ -driven IL-10 production heavily relied on IL-6 and IL-21, but all three cytokines were required for maximum expression of IL-10. In contrast to findings in mouse, IL-12 and STAT4 signaling were not involved in the induction of IL-10. In addition, neither IFN- $\gamma$  expression nor a pronounced T<sub>H</sub>1 commitment was required for expression of IL-10 or IL-22.

The analysis of transcription factors showed that different transcriptional modules were involved in the regulation of the two cytokines. DLL-4-activated Notch signaling was shown to potentiate the effect of IL-10- and IL-22-driving cytokines by enhancing cytokine-dependent activation, i.e. phosphorylation, of STAT molecules. STAT1 and STAT3 reciprocally regulated IL-10 expression, whereas the transcription factors MAF, AhR and BLIMP1 were required for production of IL-10. The data and literature indicated that these pathways may not act independently but complement each other or play a role in different phases of T cell differentiation.

IL-22 expression required AhR signaling, but also relied on STAT3- and STAT1-dependent pathways. The experiments showed strong evidence that IL-22 was induced by pathways recruiting STAT3 as well as by STAT3-independent processes. While the endogenous factors activating STAT3-dependent IL-22 expression remained unknown, literature suggests that STAT3-independent induction may be mediated by AhR, possibly acting in concert with STAT1.

In sum, the data show that regulation of IL-10 and IL-22 expression in murine and human T helper cells employs common as well as distinct signals and mechanisms. Here, an intricate network was identified in human T helper cells, which uses STAT3, AhR and MAF as central transcriptional modules, but also includes the transcription factors STAT1 and BLIMP1. Mainly cytokine-dependent STAT activation was found to trigger this network, whereas the Notch pathway modulates the activation of these STAT molecules.

Elucidating signals and transcriptional modules that govern expression of IL-10 and IL-22 in human T cells, this project helped advancing the understanding of the molecular regulation of these cytokines in human, which is an important pre-requisite for the identification of potential disease- and patient-specific alterations in T cell-mediated immunopathologies and to delineate tailor-made therapeutic strategies.



## 8 Kurzfassung

IL-10 und IL-22 gehören zu der gleichen Zytokinfamilie, spielen jedoch unterschiedliche und wichtige Rollen in der Immunantwort. Während IL-10 als einer der wichtigsten immunsuppressiven Faktoren in der Regulation, Begrenzung und Unterdrückung von inflammatorischen Immunantworten an der Verhinderung exzessiver und schädlicher Immunreaktionen beteiligt ist, spielt IL-22 sowohl eine wichtige Rolle in der Aufrechterhaltung und Verteidigung des Epithels, welches den Organismus von der Umgebung trennt, als auch in der Geweberegeneration in verschiedenen Organen. Die Bedeutung dieser beiden Zytokine im Rahmen der Immunantwort wird dadurch deutlich, dass T-Helferzellen ( $T_H$ -Zellen) – welche selbst eine zentrale Rolle bei der Regulierung des adaptiven Immunsystems spielen – IL-10 in verschiedenen Situationen exprimieren und die Hauptquelle für IL-22 in peripherem Blut sind.

Zwar ist und war die Regulation von IL-10 und IL-22 bereits das Ziel zahlreicher Untersuchungen, jedoch sind die Mechanismen, die der Expression dieser beiden Zytokine im Menschen zugrunde liegen, bisher nur wenig verstanden. Dies ist vor allem darauf zurückzuführen, dass der Großteil der Daten in Modellsystemen in der Maus generiert wurde.

Hinzu kommt, dass IL-10 in unterschiedlichen  $T_H$ -Zellpopulationen unterschiedlich reguliert ist. So wird es in  $T_H1$ -Zellen durch IL-12 und STAT4-Aktivierung induziert, in  $T_H2$ -Zellen durch IL-4 über STAT6 generiert und in  $T_H17$ -Zellen durch IL-6 und TGF- $\beta$  in Abhängigkeit von STAT3-Signalen erzeugt. IL-22 ist zwar als  $T_H17$ -Zytokin beschrieben, welches durch IL-6, IL-21 oder IL-23 über STAT3 induziert wird, Untersuchungen mit humanen T-Zellen deuten jedoch darauf hin, dass im Menschen  $T_H1$ - und  $T_H22$ -Zellen, neben  $T_H17$ -Zellen, die Hauptproduzenten von IL-22 in peripherem Blut sind. Darüber hinaus werden zunehmend weitere kostimulatorische Signale beschrieben – so zum Beispiel der Notch-Signalweg – die Einfluss auf die IL-10- und IL-22-Expression ausüben. Kürzlich hinzugekommen sind Transkriptionsfaktoren wie c-Maf (MAF), der Aryl-Hydrocarbon-Rezeptor (AhR) und Blimp1, die für die Regulation von IL-10 und IL-22 in murinen T-Zellen eine Rolle zu spielen scheinen.

Angesichts der bisher begrenzten Wissenslage in Bezug auf die Regulation von IL-10 und IL-22 im humanen Immunsystem wurde diese Studie mit dem Ziel durchgeführt, die Signale und Transkriptionsmodule zu analysieren, die die Expression von IL-10 und IL-22 in humanen T-Helferzellen im Rahmen der Interaktion zwischen plasmazytoiden dendritischen Zellen (pDZ) und T-Zellen bestimmen.

Ausgehend von den in der Maus beschriebenen Signalwegen wurden Stimuli und Transkriptionsfaktoren zunächst in einer pDZ-T-Zell-Kokultur als Modellsystem untersucht.

Die hier gewonnen Erkenntnisse wurden in der zweiten Phase der Studie in einem exakt definierten Minimalsystem, welches keine Stimulation durch pDZ erforderte, vertieft.

Die Experimente zeigten, dass humane plasmazytoide dendritische Zellen eine gemischte T<sub>H</sub>1-artige T-Zellpopulation induzierten, die sich durch ausgeprägte Expression von IL-10 und IL-22 auszeichnete. Weitere Analysen demonstrierten, dass die Induktion beider Zytokine die Aktivierung des Notch-Signalweges durch den Notch-Liganden DLL-4 erforderte. Während die IL-22-Produktion durch DLL-4 und T-Zell-Rezeptor-abhängige T-Zell-Stimulation in Abwesenheit weiterer Faktoren ausgelöst werden konnte, erforderte die Induktion von IL-10 einen zusätzlichen Stimulus. Diesen zweiten Kostimulus lieferten bestimmte STAT3-aktivierende Zytokine, wie IFN- $\alpha$ , IL-6, IL-21 oder IL-27.

Wie sich zeigte, induzierte IFN- $\alpha$ , das Haupt-Effektorzytokin plasmazytoider dendritischer Zellen, die stärkste IL-10-Produktion. Interessanterweise wurde die IFN- $\alpha$ -abhängige IL-10-Expression durch IL-6 und IL-21 vermittelt. Für eine maximale IL-10-Induktion wurden aber sowohl IL-6 und IL-21 als auch IFN- $\alpha$  benötigt. Im Gegensatz zum Maussystem erzeugten weder IL-12 noch STAT4-vermittelte Signale IL-10-produzierende T-Zellen. Auch die Expression von IFN- $\gamma$  oder eine ausgeprägte T<sub>H</sub>1-Antwort waren nicht notwendig für die Induktion von IL-10 oder IL-22.

Die Analyse der Transkriptionsfaktoren ergab, dass verschiedene Module der Transkription in der Regulation von IL-10 und IL-22 eine Rolle spielten. Es konnte gezeigt werden, dass DLL-4-aktiviertes Notch den Effekt von IL-10- und IL-22-induzierenden Zytokinen potenziert, indem es die zytokinabhängige Phosphorylierung – und damit Aktivierung – von STAT-Molekülen verstärkt.

Die IL-10-Produktion wurde reziprok durch STAT1 und STAT3 reguliert. Gleichzeitig waren sowohl MAF und AhR als auch BLIMP1 für die IL-10-Expression erforderlich. Sowohl die Ergebnisse der Experimente als auch Daten aus der Literatur lassen vermuten, dass diese Faktoren nicht unabhängig agieren, sondern sich gegenseitig ergänzen bzw. in unterschiedlichen Phasen der T-Zell-Differenzierung eine Rolle spielen.

Für die IL-22-Expression waren sowohl der Aryl-Hydrocarbon-Rezeptor als auch STAT3- und STAT1-abhängige Signalwege erforderlich. Die Experimente lieferten starke Hinweise darauf, dass IL-22 sowohl über STAT3-vermittelte als auch über STAT3-unabhängige Signalwege induziert werden kann. Zwar konnte der endogene STAT3-aktivierende Faktor nicht ermittelt werden, jedoch lassen andere Studien vermuten, dass der STAT3-unabhängige Signalweg über AhR und eine AhR-STAT1-Wechselwirkung verlaufen könnte.

Zusammengenommen zeigen die Daten, dass die Regulation von IL-10 und IL-22 in Maus und Mensch zum Teil ähnliche und zum Teil sehr unterschiedliche Signalwege beinhaltet. Zudem wurde ein komplexes Netzwerk identifiziert, in dessen Zentrum die Transkriptionsfaktoren STAT3, AhR und MAF stehen sowie STAT1 und BLIMP1 eine Rolle spielen. Die Prozesse dieses Netzwerkes werden durch zytokinabhängige STAT-Aktivierung in Gang gesetzt, welche wiederum durch den Notch-Signalweg moduliert wird.

In dieser Studie wurden Signale und Transkriptionsmodule identifiziert, welche maßgeblich an der Regulation der IL-10- und IL-22-Expression durch humane T-Zellen beteiligt sind. Dies ist eine wichtige Voraussetzung für die Erkennung krankheits- und patientenspezifischer Veränderungen bei T-Zell-abhängigen Immunerkrankungen und die Entwicklung maßgeschneiderter Therapien.

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## 10 Appendix

## 10.1 Publications

**Mantei, A.**<sup>1</sup>, Rutz, S.<sup>1</sup>, Janke, M., Kirchhoff, D., Jung, U., Patzel, V., Andreou I., Weber M., Scheffold, A. (2008). siRNA stabilization prolongs gene knockdown in primary T lymphocytes. *European Journal of Immunology*, 38(9), 2616–25.  
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<sup>1</sup> shared first authorship

## 10.2 Curriculum vitae

*For reasons of data protection, the curriculum vitae is not published in the electronic version.*

### 10.3 Acronyms

AhR	Aryl hydrocarbon receptor
APC	Antigen presenting cell
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFDA-SE	Carboxyfluorescein diacetate succinimidyl ester
CpG A	Cytosine-guanine dinucleotide type A
CpG B	Cytosine-guanine dinucleotide type B
CSL	CBF1/RBP-suppressor of hairless, Lag-1
DLL	Delta-like
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
EGF	Epidermal growth factor
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
FITC	Fluorescein Isothiocyanate
GSI	$\gamma$ -secretase inhibitor
HEK	Human embryonic kidney
HES	Hairy/Enhancer of Split
IFN	Interferon
IFNAR	Type I IFN receptor
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
MACS	Magnetic cell sorting
MAF	c-Maf musculoaponeurotic fibrosarcoma oncogene homolog
mRNA	Messenger RNA
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin

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PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptors
R	Receptor
r	Recombinant
rh	Recombinant human
RNA	ribonucleic acid
RNAi	RNA interference
RPMI	Rosewell Park Memorial Institute Medium
RT	Room temperature
siRNA	Short interfering RNA
STAT	Signal transducer and activator of transcription
T <sub>H</sub> cell	T helper cell

## 10.4 Erklärung

Hiermit erkläre ich, Andrej Mantei, geb. am [REDACTED] in L'viv (Ukraine), die vorliegende Dissertation selbstständig und ohne unerlaubte Hilfe angefertigt zu haben, und alle verwendeten Hilfsmittel und Inhalte aus anderen Quellen als solche kenntlich gemacht zu haben. Des weiteren versichere ich, dass die vorliegende Arbeit nie in dieser oder anderer Form Gegenstand eines früheren Promotionsverfahrens war. Die dem angestrebten Promotionsverfahren am Fachbereich Biologie, Chemie, Pharmazie der Freien Universität Berlin zugrunde liegende Promotionsordnung ist mir bekannt.

Berlin, im Februar 2015

Andrej Mantei

*For reasons of data protection, the birth date has been blackened in the electronic version.*