Cover: IL-10 protein structure.
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IL-10 IN THE PATHOGENESIS OF SYSTEMIC JUVENILE IDIOPATHIC ARTHRITIS AND REGULATION OF ITS PRODUCTION BY IFN-γ IN TLR-TRIGGERED INFLAMMATION

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Table of Contents

List of Abbreviations

AHR	Aryl hydrocarbon receptor
AKT	Protein kinase B
AP	Activator protein
APC	Antigen-presenting cells
APRIL	A proliferation-inducing ligand
ATF	Activating transcription factor
BAFF	B cell activating factor
BAFF-R	B cell activating factor receptor
BCMA	B cell maturation antigen
BCR	B cell receptor
B_{reg}	Regulatory B cell
CFA	Complete Freund's Adjuvant
CFSE	Carboxyfluorescein succinimidyl ester
CIA	Collagen-induced arthritis
CpG	Cytosine-phosphate-guanine
CREB	cAMP response element-binding protein
CRP	C-reactive protein
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DUSP	Dual-specificity phosphatase
EAE	Experimental autoimmune encephalomyelitis
ER	Endoplasmic reticulum

ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FO	Follicular
G-CSF	Granulocyte colony-stimulating factor
GAS	γ-interferon-activated site
GATA3	GATA-binding protein 3
GSK3	Glycogen-synthase kinase 3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HC	Healthy control
HSS	Hypersensitivity sites
ICOS	Inducible costimulatory molecule
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IFN-γR	IFN-γ receptor
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor 1
IL	Interleukin
IL-1Ra	IL-1 receptor antagonist
IL-10R	IL-10 receptor
IL-18BP	IL-18 binding protein
ILAR	International league of associations for rheumatology
IRF	Interferon regulatory factor
JAK	Janus kinase

JIA	Juvenile idiopathic arthritis
JNK	c-Jun N-terminal kinase
KO	Knockout/deficient
LPS	Lipopolysaccharides
M-CSF	Macrophage colony-stimulating factor
MAPK	Mitogen-activated protein kinase
MAS	Macrophage activation syndrome
MIF	Macrophage migration inhibitory factor
MKP	MAPK phosphatase
MMP	Matrix metalloprotease
MS	Multiple sclerosis
MSK	Mitogen- and stress-activated kinase
MZ	Marginal zone
MZP	Marginal zone precursor
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor κB
NK	Natural killer
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drugs
NSE	Neurospecific enolase
ODN	Oligodeoxynucleotides
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
pDC	Plasmacytoid dendritic cell

PGN	Peptidoglycan
PI3K	Phosphoinositide 3-kinase
PIC	Polyinosinic-polycytidylic acid
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
RANKL	Receptor activator of NF-κB
RISC	RNA-induced silencing complex
sJIA	Systemic juvenile idiopathic arthritis
SLE	Systemic lupus erythematosus
SOCS	Suppressor of cytokine signalling
SPF	Specific pathogen-free
STAT	Signal transducer and activator of transcription
TACI	Transmembrane activator and calcium modulator and cyclophilin ligand interactor
TCR	T cell receptor
TGF-β	Transforming growth factor β
Th	T helper
TIR	Toll/IL-1R
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAP	Tartrate-resistant acid phosphatase
T_{reg}	Regulatory T cell
TRIF	TIR-domain-containing adaptor-inducing IFN-β
TTP	Tristetrapolin

WT	Wild type

CHAPTER 1

GENERAL INTRODUCTION

1. Systemic juvenile idiopathic arthritis

1.1 Definition and classification

Juvenile idiopathic arthritis (JIA) is a heterogeneous group of arthritic diseases of unknown origin with onset before the age of 16 that persist for more than six weeks. It is the most common rheumatic syndrome in childhood, with a prevalence rate varying from 3.8 to 400 cases per 100.000 children. JIA can be subdivided in seven different subtypes, depending on the number of joints affected and the presence of psoriasis, enthesitis or systemic features. One of these subtypes is systemic JIA (sJIA), previously known as Still's disease, which accounts for roughly 10% of JIA cases in Europe and North America, with a higher proportion in Japan and India (54% and 24% respectively) and a disease-associated mortality rate between 0.5 and 1%. In contrast to other JIA subtypes, there is no sex bias, and the typical age of onset is between 1 and 5 years of age, with a peak incidence around 2 years, although it can occur in children of any age[1–9]. sJIA is unique among JIA subtypes in presenting severe systemic symptoms that often overshadow joint inflammation. It is defined according to the international league of associations for rheumatology (ILAR) as "arthritis with or preceded by fever of at least 2 weeks' duration that is documented to be daily for at least 3 days, and accompanied by an evanescent erythematous rash, enlargement of the lymph nodes, spleen or liver and/or serositis"[2].

1.2 Clinical manifestations and laboratory features

One of the most common clinical features is the high spiking quotidian fever, with a peak of at least 39°C, that occurs one or twice a day. The fever is typically accompanied by a salmon-coloured evanescent rash. Arthritis is the second most common feature and predominantly affects the knees, wrists and ankles, although any joint can be involved. In one third of the patients, only arthralgia is present at disease onset and arthritis develops later on, typically within a few months. Other symptoms include lymphadenopathy, hepatomegaly and splenomegaly. Serositis, apart from pericarditis, is less common and involvement of the central nervous system is rare. Abdominal pain may be present, and the child appears very ill and is often anaemic[4–6]. Next to anaemia, laboratory features include

high C-reactive protein (CRP) and cytokine levels, elevated erythrocyte sedimentation rate, increased white blood cell count with neutrophilia and thrombocytosis. High ferritin levels are seen in the majority of patients. Elevated liver enzymes (alanine and aspartate transaminases) and D-dimers are commonly observed[4–6,9]. The clinical course of sJIA can be highly variable. In a 5-year follow-up study, approximately 40% was classified as having a monocyclic course, 7% had a polycyclic course and over 50% had a persistent disease course[10].

1.3 Aetiology

The aetiology of sJIA is largely unknown[4,5,7,11]. Although infectious agents are triggering suspects, no single agent can be identified as the culprit, which could point to multiple agents being able to initiate the disease[4,8]. Consistent with this hypothesis, some studies observed a seasonal variation in sJIA onset[12,13]. However, it is important to note that sJIA is not an infectious disease, because the diagnosis comprises a negative screening for infections[4]. Likely, a combination of both environmental and genetic factors contributes to disease development. Accordingly, one hypothesis is that infectious agents typically encountered during childhood initiate sJIA in genetically susceptible children. Various disease susceptibility alleles have been described, which may contribute to an excessive inflammatory reaction once the disease is initiated. Polymorphisms in the promotor region and genes encoding tumour necrosis factor α (TNF-α), the interleukin (IL)-1 family (i.e. *IL1R2*, *IL1A*, *IL1F10* and *IL1RN*), IL-6, IL-10, IL-20 and macrophage migration inhibitory factor (MIF) are reported[14–21]. Furthermore, a monogenic form of sJIA was observed in five consanguineous families from Saudi Arabia, with a homoallelic missense mutation in LACC1, which encodes the enzyme laccase (multicopper oxidoreductase) domain–containing 1[22]. Until recently, strong associations with MHC class II alleles were not described in sJIA, in contrast to other JIA subtypes. Together with the absence of high-titre autoantibodies and autoreactive T cells, the absence of any sex bias and the prominent innate inflammation with fever and rash, this led to the consideration of sJIA as an autoinflammatory disease, rather than an autoimmune disease[8,11,23]. However, Ombrello *et al.* recently reported the MHC class II allele *HLA-DRB1*11* as a strong risk factor for sJIA in 9 different cohort populations, and identified a specific amino acid,

glutamate 68, as the most important risk carrier[24]. This might implicate a role for adaptive immunity in sJIA pathogenesis as well, although the authors also hypothesise that the MHC may act as an activator of MHC class II presenting cells, promoting pro-inflammatory cytokine production and preserving sJIA as a pure autoinflammatory disease[23,24].

1.4 Differential diagnosis

Until now, no specific biomarkers have been described for sJIA, although some candidates have been identified[25], and its diagnosis is mainly based on exclusions. Many illnesses can mimic sJIA, such as systemic autoimmune syndromes (e.g. systemic lupus erythematosus), other autoinflammatory disorders (e.g. Familial Mediterranean fever, cryopyrin-associated autoinflammatory syndrome (CAPS)), infections (e.g. viral infections, sepsis, mycoplasma infection), Kawasaki disease, systemic vasculitis and malignancies (e.g. leukemia), making diagnosis challenging, especially when arthritis is not yet present. In addition, other subtypes of JIA have to be excluded as well (Table 1). However, the typical fever pattern of sJIA, especially when accompanied by an evanescent rash, may eliminate some of the differential diagnoses and point to sJIA[4,5,9].

1.5 Treatment

Since sJIA symptoms can be detrimental, hospitalisation of patients is often required at presentation. Non-steroidal anti-inflammatory drugs (NSAIDs) are used as initial treatment to temper systemic inflammation and joint pain. In more severe cases, corticosteroid treatment is mostly prescribed, although long-term treatment should be avoided to minimize side effects. Treatment with disease-modifying anti-rheumatic drugs (e.g. methotrexate) or TNF inhibitors is less effective in sJIA as compared to other JIA subtypes. Other treatments have been tried with limited success, such as cyclosporine A and intravenous immunoglobulins. More recently, biological drugs blocking IL-1 or IL-6 have proven to be very effective[26–29]. The recombinant IL-1 receptor antagonist anakinra was the first to revolutionise sJIA treatment and leads to rapid resolution of systemic features and arthritis, also when given as a first-line treatment. Other IL-1 inhibitors, i.e. canakinumab, a human monoclonal antibody directed against IL-1β, and rilonacept, which inhibits IL-1 signalling, have also

been proven effective in sJIA. The IL-6 inhibitor tocilizumab, an anti-IL-6 receptor antibody, was also shown to be highly effective in sJIA and both canakinumab and tocilizumab have been approved in Europe and the USA for treatment of active sJIA. In patients with severe sJIA who did not respond to conventional therapy, autologous stem cell transplantation has been used, with about 50% of patients showing complete remission. However, because of the high associated mortality, this should only be used as a last option[4–6,11,30].

Table 1. sJIA diagnostic criteria and exclusions, according to the ILAR.

Diagnosis of sJIA (ILAR criteria)	
Definition	Arthritis in one or more joints with or preceded by fever of at least 2 weeks' duration that is documented to be daily ("quotidian") for at least 3 days, and accompanied by one or more of the following: 1. Evanescent (non-fixed) erythematous rash 2. Generalized lymph node enlargement 3. Hepatomegaly and/or splenomegaly 4. Serositis
Exclusions	a. Psoriasis or a history of psoriasis in the patient or first-degree relative b. Arthritis in an HLA-B27 positive male beginning after the 6th birthday c. Ankylosing spondylitis, enthesitis related arthritis, sacroiliitis with inflammatory bowel disease, Reiter's syndrome, or acute anterior uveitis, or a history of one of these disorders in a first-degree relative d. The presence of IgM rheumatoid factor on at least 2 occasions at least 3 months apart

From Petty et al. (2004).

1.6 Complications

sJIA has the highest mortality rate among all JIA subtypes, which is partly due to its association with several life-threatening complications, i.e. macrophage activation syndrome (MAS) and amyloidosis. MAS is the most severe sJIA complication, which develops in 10 – 15% of patients

(with subclinical MAS in 40-50% of the patients) and has a mortality rate ranging between 8 and 20%[4–6,30]. Patients with MAS experience a severe hyperinflammation and present with persistent high fever, hepatosplenomegaly, lymphadenopathy, pancytopenia, liver dysfunction, hyperferritinaemia, coagulopathy and central nervous system dysfunction[5,6]. Amyloidosis, or the deposition of amyloid A, was a major cause of death in sJIA patients during 1960-1970, but is now much less frequent[4]. It may affect various organs, including kidney, gastrointestinal tract, heart and liver, and may result in renal failure and organ dysfunction[4,5]. Furthermore, the combination of chronic inflammation and corticoid therapy results in osteoporosis and growth failure, which are both major problems in sJIA patients[4,5]. Corticoid treatment-associated infections also contribute to mortality in sJIA[9].

1.7 Pathogenesis

The precise aetiology of sJIA is still unknown, but progress has been made into the understanding of mechanisms underlying disease pathology and phenotype. One of the characteristics of sJIA is a cytokine storm, with highly elevated levels of pro-inflammatory cytokines that contribute to the ongoing inflammation. In addition, several cell types, predominantly of the innate immune system, might be involved in sJIA pathogenesis. In this section, key cytokines and cell types and their role in sJIA are discussed in more detail.

1.7.1 Cytokines

1.7.1.1 IL-1β

IL-1β is a pyrogenic cytokine with powerful pro-inflammatory functions, that stimulates both local and systemic responses. It is mainly produced by monocytes and macrophages in response to activation of Toll-like receptors (TLR). IL-1β promotes the recruitment of inflammatory cells at the site of inflammation and induces the expression of several enzymes, leading to the release of the inflammatory mediators prostaglandin E2 and nitric oxide. It has a global negative impact on articular cartilage and contributes to the development of bone erosions in arthritis. Furthermore, it has numerous systemic effects, such as fever, neutrophilia, thrombocytosis and increased production of acute-phase proteins. In

addition, it can induce the differentiation of T helper (Th) 17 cells and IL-17 production. Accordingly, several sJIA symptoms may be explained by IL-1β overexpression. Endogenous inhibitors, such as IL-1 receptor antagonist (IL-1Ra) and membrane-bound and soluble IL-1 receptor type II, can control the pro-inflammatory activities of IL-1β[31]. The importance of IL-1β in sJIA became clear from successful clinical trials in which IL-1β was neutralised by an anti-IL-1R antagonist (anakinra) or anti-IL-1β monoclonal antibodies (canakinumab)[26,32–34]. However, in plasma of sJIA patients, no increase in IL-1β could be detected, although it was increased on a transcriptional level in peripheral blood mononuclear cells (PBMCs)[26,35,36]. In addition, a distinct sJIA gene expression profile partially reversible by IL-1R antagonist (anakinra) has been reported[37]. Also in whole blood cultures or monocytes from sJIA patients stimulated with lipopolysaccharides (LPS), no increased IL-1β production could be observed compared to healthy controls[34,38], but an increased production was observed after stimulation of PBMCs with phorbol myristate acetate (PMA) and ionomycin[26]. In addition, serum from sJIA patients induced IL-1β transcription in PBMCs from healthy controls[26] and upregulation of TLR/IL-1R signalling-related genes was observed in sJIA PBMCs[39]. Interestingly, increased levels of IL-1Ra were detected in serum and PBMCs from sJIA patients, although not to the degree observed in polyarticular JIA, possibly suggesting an insufficient endogenous inhibition of IL-1β[8,26,35]. Although an excess of IL-1β could not be detected in plasma or blood cells from sJIA patients, the positive clinical response to IL-1 inhibitors suggests that local microenvironments, other cell types or different triggers or pathways may be involved in increased IL-1 expression[8].

1.7.1.2 IL-6

IL-6 is a pleiotropic cytokine that promotes expansion and activation of T cells, differentiation of B cells and regulates the acute-phase response. It can affect the vascular system, mitochondrial activities, insulin resistance, lipid metabolism, the neuroendocrine system and neuropsychological behaviour. IL-6 is produced by stromal cells and cells of the immune system in response to stimulation with IL-1β, TNF-α or TLR ligands, next to prostaglandins and other cytokines and stress responses[40]. Increased levels of IL-6 have been reported in the plasma and synovial fluid of sJIA patients, as well as an increased expression in sJIA monocytes[36,41,42]. In

addition, PBMCs from sJIA patients spontaneously produced higher levels of IL-6 than control cells, whereas no differences were seen after stimulation of PBMCs and whole blood cultures[38,43]. Several symptoms of sJIA can be attributed to the high IL-6 levels, such as fever, increased acute-phase proteins, thrombocytosis, microcytic anaemia, growth retardation and osteoporosis[8,44]. As with IL-1β, the importance of IL-6 in sJIA was highlighted by the successful treatment of patients with tocilizumab, a monoclonal anti-IL-6 receptor antibody[45].

1.7.1.3 IL-10

IL-10 is a well-known cytokine with numerous anti-inflammatory features. For example, IL-10 downregulates antigen presentation and T cell activation and inhibits the production of several pro-inflammatory cytokines and chemokines. IL-10 can be produced by a variety of cell types, such as T cells, monocytes, macrophages, eosinophils, neutrophils, dendritic cells (DC), natural killer (NK) cells and B cells[46–49]. In sJIA, contradictory results regarding IL-10 are reported. IL-10 levels in plasma are increased in sJIA patients compared to healthy controls, but are lower compared to polyarticular JIA patients[36]. Expression levels of IL-10 in PBMCs and purified monocytes are higher in patients than in healthy controls[42,50]. Also after *in vitro* stimulation, PBMCs from patients produced more IL-10 than healthy donor cells[51]. In contrast, when whole blood cells were stimulated *in vitro*, a decreased IL-10 production was observed in sJIA patients when compared to healthy controls[38]. Intriguingly, when PBMCs of sJIA patients were stimulated with LPS, addition of recombinant IL-10 inhibited the production of IL-1 and TNF-α, but had only minor effects on the production of IL-6[51]. This was confirmed in a second study, where it was reported that the required dose of IL-10 to inhibit LPS-induced IL-6 production was significantly higher in sJIA patients compared to healthy controls[43]. In conclusion, insufficient IL-10 production or signalling can, together with other cytokine-related abnormalities, contribute to the pathogenesis of sJIA. The production and regulation of this cytokine will be discussed in more detail in paragraph 2.1.

1.7.1.4 IL-17

IL-17 is a cytokine that plays an important role in orchestrating innate immune function and is secreted by Th17 cells, γδ T cells, CD8 T cells and

NK cells. IL-17 can upregulate IL-1β, IL-6 and TNF-α production and can drive chronic arthritis. On the other hand, high levels of IL-1β and IL-6 may favour the development of IL-17-producing cells[1,52–54]. Inconsistent results are reported regarding IL-17 levels in plasma or serum of sJIA patients, with normal or increased IL-17 production compared to controls[36,52]. γδ T cells of sJIA patients showed an increased IL-17 expression compared to control cells, and increased numbers of IL-17-producing $CD4^+$ and $CD4^-$ T cells are reported in sJIA PBMCs[52,55]. A positive correlation was observed between serum IL-17 levels and the erythropoiesis signature in sJIA, indicating a possible role for IL-17 in the anaemia characteristic of the disease[56]. Furthermore, it has been proposed that IL-17 plays a pathogenic role in the chronic arthritic phase of sJIA[30].

1.7.1.5 IL-18

IL-18 was originally described as "IFN-γ-inducing factor". It is a member of the IL-1 family and uses parallel signal transduction pathways. IL-18 is constitutively expressed in blood monocytes, keratinocytes and almost all epithelial cells[57,58]. IL-18 can contribute to the inflammatory process by increasing leukocyte recruitment via increased cell adhesion molecules and chemokine production and by increasing joint inflammation via inhibiting proteoglycan synthesis in chondrocytes, which is necessary for healthy cartilage[58]. In sJIA, excessive IL-18 levels are reported in plasma of active patients. In inactive patients, moderately increased levels were observed[34,36,41,59,60]. Shimizu *et al.* suggested that sJIA patients can be divided in two subgroups, depending on the amount of IL-18 or IL-6 they produce. The IL-6-dominant subgroup would suffer from more severe arthritis, while the IL-18-dominant group would be more prone to develop MAS[59]. The amount of biologically active IL-18 is regulated by the availability of its endogenous inhibitor IL-18 binding protein (IL-18BP)[57]. IL-18BP production is mainly induced by IFN-γ. In this way, IL-18-induced IFN-γ provides a negative feedback loop for free IL-18 levels by increasing IL-18BP production[57,58]. Interestingly, despite the very high IL-18 levels, IL-18BP levels were only moderately increased in sJIA patients, resulting in an imbalance and increased levels of free IL-18[60].

1.7.1.6 IFN-γ

Interferons (IFN) were first described as inhibitors of viral replication. IFN-γ is a type II interferon, although its antiviral actions may be less

important relative to its significant effects on leukocytes. IFN-γ is mainly produced by T cells and NK cells in response to stimulation with IL-12 and IL-18, or via triggering of the T cell receptor (TCR) or NK-activating receptors[61,62]. Traditionally, IFN-γ is considered a pro-inflammatory cytokine, stimulating the immune response by activating innate immune cells and Th1 cells and increasing antigen presentation. However, many immune-regulatory functions have been described nowadays, such as inhibition of neutrophil-specific chemokines, stimulation of regulatory T cell function and inhibition of IL-17 production[63]. The role of IFN-γ in sJIA is still controversial. Moderately increased levels of IFN-γ were found in plasma of sJIA patients, although levels were low compared to the high IL-18 levels[34,36,41,60]. Consistently, an increased proportion of IFN-γ-producing T cells was found in patients PBMCs[55]. In contrast, IFN-γ production was decreased after *in vitro* stimulation of PBMCs compared to healthy controls, and no IFN-γ gene signature could be detected in patient PBMCs, probably resulting from a limited exposure to IFN-γ[39,42,50,51,60,64]. Because of its importance in the immune response and its controversial role in sJIA, we will elaborate more on the functions of IFN-γ in paragraph 2.2.

1.7.1.7 TNF-α

TNF-α is a pro-inflammatory cytokine produced by monocytes, macrophages and activated T cells which is known to play a role in the development of arthritic symptoms[65]. In sJIA, plasma levels of TNF-α are rather low[36,41,66]. In addition, no difference in TNF-α production is observed in stimulated whole blood cultures, when compared to healthy controls[38]. Together with the poor results of anti-TNF-α treatment in sJIA patients, which is highly effective in other JIA subtypes and rheumatoid arthritis[65], this indicates that the role of TNF-α in sJIA pathogenesis is limited.

1.7.1.8 MIF

Migration inhibitory factor (MIF) was originally described as a molecule produced by T cells that inhibits the random migration of macrophages in culture. Later on, it was shown that also monocytes, macrophages, dendritic cells, B cells, neutrophils, eosinophils, mast cells and basophils can produce MIF and that MIF has several immune stimulatory activities, such as promoting the production of a broad range of pro-inflammatory

molecules, including cytokines (e.g. IL-1β, TNF-α, IL-6 and IL-8) and matrix metalloproteases (MMPs). Furthermore, high levels of MIF are associated with bone erosion and can contribute to arthritis[67–69]. Increased levels of MIF are found in plasma and serum of sJIA patients, and serum levels of MIF correlated with the persistence of systemic features and the number of active arthritic joints[36,69].

1.7.1.9 Overview

An overview of the expression and production of the abovementioned cytokines in sJIA patients compared to healthy controls is given in Table 2.

Table 2. Overview of cytokines in sJIA compared to healthy controls

	Plasma	PBMCs (RNA)[1]	PBMCs (protein)[2]	Stim. PBMCS[2]	Stim. Blood[2]	Ref.
IL-1β	=	↑		=/↑	=	26,34–38
IL-6	↑↑	↑	↑	=	=	36,38,41–43
IL-10	↑	↑		↑	↓	36,38,42,50,51
IL-17	=/↑		↑			36,52,55
IL-18	↑↑					34,36,41,59,60
IFN-γ	↑	=	↓			34,36,41,51,60
TNF-α	=				=	36,38,41,66
MIF	↑					36,69

[1] Cytokine expression or cytokine gene signature on mRNA level in unstimulated PBMCs; [2] Cytokine production in the supernatant of unstimulated PBMCs, stimulated PBMCs or whole blood cultures; =, ↑, ↓: unaltered, increased or decreased in sJIA patients compared to healthy controls. Ref = references.

1.7.2 Cell types

1.7.2.1 Monocytes/macrophages

Monocytes and macrophages are very diverse and plastic cells that can undergo different forms of polarized activation. Considering the elevated number of monocytes in sJIA, the therapeutic efficacy of inhibition of monocyte-derived cytokines and the association of sJIA with MAS, these cells are probably involved in sJIA pathogenesis[8,70].

Activated macrophages can be subdivided in a M1 or M2 phenotype, although these are likely two extremes of the spectrum of monocyte/macrophage activation states[70–72]. M1 macrophages are considered as classically activated macrophages with highly pro-inflammatory effects, secreting IL-1β, IL-6 and reactive oxygen and nitrogen intermediates, while alternative activated M2 macrophages have an anti-inflammatory phenotype with regulatory functions, and are involved in tissue repair and immunosuppression[71,73]. Typically, M1 macrophages are induced by IFN-γ, pathogen-derived products (TLR activation) and granulocyte-macrophage colony-stimulating factor (GM-CSF), while differentiation to the M2 phenotype is promoted by IL-4, IL-13, IL-10 and steroids[8,71,72].

In sJIA, the number of monocytes and their expression of general surface markers is increased (i.e. CD14 and CD16), indicating activation of these cells[74]. The cytokine environment in sJIA is highly pro-inflammatory, pointing to a M1 phenotype, but also M2-associated markers are upregulated in sJIA PBMCs or serum. For example, expression of MS4A4A and CD163 is increased in sJIA monocytes, and elevated serum levels of soluble CD163 and heme oxygenase 1 are reported in active sJIA patients[8,39,70,75]. Of note, this M2 phenotype was also observed in untreated patients[39]. Also in inactive patients, a M2 phenotype was observed, which might be the result of a compensatory mechanism to downregulate the ongoing subclinical inflammation[70,75]. Interestingly, sJIA monocytes have been reported to express a mixed M1/M2 phenotype during active disease, not resulting from a mixture of M1 and M2 macrophages, but from cells co-expressing markers of both subtypes, a phenotype that has been associated with regulation of immune responses[70].

Unrelated to M1/M2 phenotypes, another study reported an impaired response of sJIA monocytes to IFNs, potentially contributing to disease pathogenesis by dysregulating immune responses to infectious agents[73]. Furthermore, sJIA monocytes showed a lower expression of the aryl hydrocarbon receptor (AHR) when compared to healthy controls. Since AHR downregulation skews monocytes to macrophage differentiation, one may hypothesize that patients with a low AHR expression may be more prone to develop MAS. In addition, AHR promotes immune

tolerance and inhibits IL-1β secretion, resulting in a more pro-inflammatory phenotype of low-expressing AHR macrophages[37].

1.7.2.2 Neutrophils

Neutrophils are the most abundant leukocytes in human blood. They are relatively short-lived polymorphonuclear cells, and are typically the first leukocytes attracted to the site of inflammation. They can eliminate bacterial and fungal pathogens in multiple ways, but also release cytokines and contribute to the immune-inflammatory process[76,77].

In sJIA, increased neutrophil numbers are reported, and are associated with a peripheral expansion of immature $CD34^+$ $CD33^+$ myelomonocytic precursor cells[8,78,79]. This neutrophilia is typically seen during active disease, and rapidly normalizes in inactive patients and after treatment with anakinra[80]. Next to increased numbers, also an increase in neutrophil-related proteins (i.e. S100A8/A9, S100A12), proteins of neutrophil granules (MMP8, MMP9, elastase) and proteins involved in adhesion and chemotaxis of neutrophils are observed in patients with active sJIA[80,81]. In addition, more immature neutrophils and neutrophils with a primed phenotype (i.e. with an enhanced responsiveness and increased degranulation) are observed during active disease, supporting a role for neutrophils in sJIA pathogenesis. Interestingly, neutrophil counts were only elevated during the early acute phase of the disease and treatment with anakinra in this early phase seems to result in a better clinical response when compared to anakinra treatment at later time points, suggesting that the therapeutic efficacy of anakinra could be partially attributed to its effects on neutrophils[80]. Similarly, another study in patients with adult onset Still's disease showed a more pronounced upregulation of canakinumab-responsive genes in patients with strongly elevated neutrophil counts[82].

1.7.2.3 NK cells

NK cells are granular lymphocytes that can rapidly kill pathogen-infected cells, stressed cells, allogeneic cells and tumour cells via the release of cytotoxic molecules containing perforin and granzymes. NK cells are major producers of IFN-γ, but also produce other cytokines (such as TNF-α, GM-CSF and IL-10) and many chemokines. Their activation depends on a balance of activating receptors, which recognise stress-induced

ligands, and inhibitory receptors, which recognise MHC class I allotypes. To achieve their full effector potential, NK cells require priming with cytokines, such as IL-12, IL-15 or IL-18, or other immune cells, such as DCs[1,83,84].

Data regarding NK cell numbers in sJIA are not unanimous, with studies reporting normal or decreased NK cell numbers[38,41,74,85,86]. In some patients, a defective NK cell function was reported[87]. Perforin expression by NK cells was found to be defective or normal, and although granzyme A and B are normally expressed, a defective granzyme K expression was reported, which might influence the killing of activated autologous immune cells by NK cells[41,88]. In addition, IL-18-induced IFN-γ production was defective in NK cells of sJIA patients, an observation that might be attributed to a defective IL-18 receptor signalling[41,86]. Together, dysfunctional NK cells seem to play a role in sJIA pathogenesis.

1.7.2.4 T cells

The majority of T cells expressing αβ T cell receptor (TCR) chains, circulate through secondary lymphoid organs and are involved in adaptive immune responses. These cells are subdivided in $CD4^+$ T helper and regulatory cells, and $CD8^+$ cytotoxic T cells. A minority of T cells express the γδ TCR chains, and are part of the innate T cell response[89].

T cells have not been extensively studied in sJIA. Total T cells are described to be decreased or unaltered in active sJIA patients, and no increase in activation or differentiation markers was reported[74,85]. Numbers of CD4 T cells are unaltered in sJIA patients, but an increased proportion of IL-17-producing and IFN-γ-producing CD4 T cells has been reported[52,55,74,85]. On the other hand, regulatory CD4 T cells (T_{reg}) were found to be decreased in active sJIA patients compared to patients with inactive disease but were comparable to levels in healthy controls, suggesting that T_{reg} cells are important for controlling disease activity during quiescence[74]. In a number of studies, levels of γδ T cells were decreased in active sJIA patients[52,74,85]. However, despite this decrease in number, γδ T cells of sJIA patients were shown to overexpress IL-17, supporting a pathogenic role of these cells[52].

1.7.2.5 B cells

B cells are antibody-producing cells of the adaptive immune system, but can also present antigens to T cells and produce several cytokines. Some B cell-derived cytokines, such as lymphotoxin, are important in the homeostasis and activation of secondary lymphoid organs. Others, such as IL-6, IFN-γ and TNF-α influence the development of $CD4^+$ T cells, and B cell-derived IL-10 and IL-35 negatively regulate immune responses[90].

In sJIA, similar to T cells, not much attention has been given to B cells. Autoantibodies are typically absent in sJIA patients. However, in the later, chronic phase of the disease, when systemic features disappeared, adaptive immune activation has been observed, at times with increased antinuclear antibodies and rheumatoid factor[9,91].

Decreased or unaltered B cell numbers have been reported in sJIA. Serum levels of immunoglobulin G (IgG) were increased in sJIA serum, pointing to B cell hyperactivity[74,85]. Correspondingly, the ratio of the kappa and lambda chain of the B cell receptor was altered in sJIA patients compared to healthy controls, reflecting increased B cell activity in sJIA patients[92]. In addition, sJIA B cells were reported to have an increased IL-6 gene expression, and PBMCs from patients responding to tocilizumab show an altered B cell receptor signalling expression pattern[42,93].

At present, it remains unclear whether B cells play a pathogenic or protective role in sJIA disease development, since both sJIA development in the absence of B cells as well as successful treatment of sJIA by B cell depletion has been described in case reports[94,95].

1.8 Animal models

To study the pathogenesis of sJIA, some animal models have been developed. De Benedetti *et al.* were the first to describe an animal model that reflects the growth impairment seen in sJIA patients, using IL-6 transgenic mice. These mice overexpress IL-6 in neuronal tissue under the control of the rat neurospecific enolase (NSE) promotor. In some lines of these NSE/IL-6 transgenic mice, the IL-6 expression is leaky and not confined to the central nervous system. Here, IL-6 expression is also detected in other organs and is associated with high circulating IL-6 levels, resulting in decreased levels of insulin-like growth factor-I (IGF-I) and

stunted growth. Also in sJIA patients, increased IL-6 levels are accompanied by decreased IGF-I levels. No other sJIA symptoms are observed in this mouse model, but it provides insight in the mechanisms behind growth impairment associated with chronic inflammation in sJIA[96,97]. Interestingly, injection of IL-6 transgenic mice with LPS resulted in haematological and biochemical abnormalities that are reminiscent of MAS, a complication of sJIA[98].

Another animal model, described by Kawane *et al.*, mimics the arthritis seen in sJIA patients by using DNase II null mice. DNase II is an enzyme located in lysosomes that digests DNA from apoptotic cells and erythroid precursor cells after they have been engulfed by macrophages. The accumulation of undigested DNA in macrophages of DNase II null mice activates them to produce TNF-α and results in inflammatory cytokine production and the development of chronic polyarthritis. This arthritis was dependent on the expression of IL-1β, IL-6 and TNF-α, and affected joints showed an influx of neutrophils, macrophages and lymphocytes. The latter played a protective role in this model, since a lack of lymphocytes resulted in accelerated arthritis. Both in the mouse model and in sJIA patients, a good response to IL-1β and IL-6 blockade and high serum IL-18 levels are observed, and the authors conclude that DNase II null mice represent a good model to study arthritis in sJIA[99,100].

The first animal model that covered almost all sJIA symptoms was described by Avau *et al.* in IFN-γ deficient (KO) BALB/c mice subcutaneously injected with complete Freund's Adjuvant (CFA)[101]. When wild type (WT) mice were injected with CFA, they developed splenomegaly, lymphadenopathy, neutrophilia, thrombocytosis and increased cytokine expression. However, in the absence of IFN-γ, the severity of these symptoms was greatly increased, and CFA-injected IFN-γ KO mice additionally showed weight loss, arthritis, skin rash, increased numbers of immature RBC and neutrophils, anaemia, hemophagocytosis and increased IL-6 and IL-17 production, all symptoms reminiscent of sJIA. Thus, chronic immune stimulation of IFN-γ KO BALB/c mice with CFA results in a systemic inflammatory syndrome highly reminiscent of the clinical and immunologic features seen in sJIA[101].

2. Interleukin-10 and interferon-γ

The cytokines IL-10 and IFN-γ both exert a key role in maintaining the balance of the immune response. IFN-γ is considered a pro-inflammatory cytokine, stimulating the immune response against pathogens and malignant cells, while IL-10 is vital to avoid inappropriate hyperinflammation and organ damage.

In line with their opposing functions, IL-10 and IFN-γ can inhibit each other's production and signalling transduction, and are therefore traditionally considered as each other's antagonists[102]. Indeed, many reports describe the negative regulation of IL-10 production by IFN-γ[103–107]. Given their interesting mutual relation, the function and regulation of IL-10 and the function of IFN-γ will be described in more detail in the next parts.

2.1 Interleukin-10

2.1.1 Definition and function

IL-10 was discovered in 1989 as an immune mediator produced by Th2 cells that inhibits the synthesis of IL-2 and IFN-γ by Th1 clones, and was originally known as cytokine-synthesis inhibitory factor. The human IL-10 gene is located on chromosome 1 and encodes a non-glycosylated protein of 178 amino acids long, which is secreted after cleavage of the 18 amino acids-comprising signal peptide. The human IL-10 protein is a homodimer that is composed of two non-covalently bound monomers of 18,6 kDa. Based on structural similarity, the cytokines IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28 and IL-29 are combined in the "IL-10 family", but their biologic activities are diverse[108–110].

IL-10 is an anti-inflammatory cytokine that affects various cell populations, including T cells, B cells, NK cells, mast cells, neutrophils, eosinophils and monocytes and macrophages, with the latter being considered as its main target cells[48,108,111,112].

In monocytes and macrophages, IL-10 inhibits the release of pro-inflammatory mediators, such as TNF-α, IL-1β, IL-6, IL-8, IL-12, IL-18, IL-23, granulocyte colony-stimulating factor (G-CSF), GM-CSF and stimulates the release of anti-inflammatory molecules, such as IL-1Ra,

soluble TNF-α receptors and IL-27. In addition, the expression of IL-1RI and IL-1RII on activated monocytes is inhibited. Next to inhibition of pro-inflammatory cytokines, IL-10 also inhibits the production of chemokines by monocytes, resulting in an impaired recruitment of monocytes, DCs, neutrophils and T cells. Another important feature of IL-10 is the inhibition of antigen presentation, which is the result of a reduced expression of MHC II by inhibition of transport of mature MHC II to the plasma membrane and of a decreased expression of costimulatory (e.g. CD80/CD86) and adhesion (e.g. CD54) molecules. IL-10 enhances phagocytosis by macrophages by increasing the receptors responsible for the uptake of opsonised and non-opsonised microorganisms (i.e. CD64, CD32, CD16, CD14), but it reduces the ability of the cells to kill the engulfed organisms by decreasing the generation of superoxide anion (O2−) and nitric oxide (NO). Furthermore, the production of prostaglandin E2 is decreased by downregulation of cyclooxygenase 2 expression. IL-10 increases the expression of the CD163 scavenger receptor on macrophages, and skews them to a M2 phenotype. IL-10 enhances the differentiation of monocytes into macrophages and inhibits the development of myeloid dendritic cells. Together, the effects of IL-10 on monocytes and macrophages result in a downregulation of ongoing immune responses and inflammation[48,108,109,111,112].

In addition to monocytes and macrophages, IL-10 also affects T cells, both indirect and directly. Direct effects on T cells include the inhibition of proliferation and cytokine production of $CD4^+$ T cells. By reducing the production of IL-12 and IL-23 by monocytes and macrophages, IL-10 indirectly hampers the development of respectively Th1 and Th17 cells. On the contrary, IL-10 enhances the differentiation of T_{reg} cells and increases their suppressive capacity. Furthermore, the recruitment, proliferation and cytotoxic activity of $CD8^+$ T cells is increased by IL-10[102,108,111–113].

The biological effects of IL-10 on neutrophils and eosinophils are similar to the effects on monocytes and macrophages. Here, IL-10 decreases the production of pro-inflammatory mediators, such as TNF-α, IL-1β and GM-CSF, enhances IL-1Ra production and inhibits the secretion of neutrophil-attracting chemokines (e.g. CXCL8). Also the synthesis of

cyclooxygenase 2 and the resulting prostaglandin E2 production is inhibited[108,111].

Together with IL-4, IL-10 inhibits mast cell development induced by IL-3 and stem cell factor. It inhibits TNF-α, GM-CSF and NO production by these cells and decreases the expression of IgE receptor and its signalling molecules[108]. In NK cells, similar to CD8 T cells, IL-10 stimulates NK cytotoxic activity. Additionally, IL-10 increases IL-2-induced cytokine production (e.g. IFN-γ, TNF-α) by NK cells and increases IL-2-induced proliferation of CD56^{bright} NK cells[102,108,109].

In B cells, IL-10 enhances survival by increasing the expression of the anti-apoptotic protein bcl-2 and can act as a cofactor to increase B cell proliferation. IL-10 increases B cell differentiation into plasma cells and influences immunoglobulin class switching[108,109,111].

To conclude, IL-10 has important effects on the development of the immune response by inhibiting or activating specific immune cells and their function.

2.1.2 Mode of action

IL-10 elicits its biological effects via binding with the IL-10 receptor (IL-10R), a transmembrane glycoprotein composed of two IL-10R1 and two IL-10R2 subunits, of which IL-10R1 is the ligand-binding subunit. It is expressed by most hematopoietic cells, with the highest expression on monocytes and macrophages, but can also be induced on some non-immune cells. Binding of IL-10 with IL-10R1 induces a conformational change, followed by the recruitment of IL-10R2. IL-10R2 alone cannot bind IL-10, is constitutively expressed in most cells and is shared with other cytokines of the IL-10 family. Thus, the IL-10R1 expression levels correlate with the cellular response to IL-10. After binding of IL-10 with its receptor, two members of the Janus kinase family are activated: JAK1, which is associated with IL-10R1, and Tyk2, associated with IL-10R2. Activation of JAK1 and Tyk2 results in phosphorylation of two tyrosine residues of the IL-10R1 and subsequent activation of the transcription factor signal transducer and activator of transcription (STAT) 3. While STAT1 and STAT5 can also be activated, only STAT3 appears to be essential for all anti-inflammatory effects of IL-10. The activated transcription factors migrate into the nucleus and induce the expression of several target genes,

which subsequently inhibit the transcription of pro-inflammatory genes[108,111,114,115]. Suppressor of cytokine signalling 3 (SOCS3) inhibits the activity of Janus kinases and blocks the IL-6-mediated pro-inflammatory response. Another STAT3 target gene is *Bcl3*, which impairs nuclear factor (NF)-κB's DNA binding ability and suppresses TNF-α production. In addition, several cell-type-specific target genes have been identified. Together, STAT3 is able to bind both a relatively small core of conserved target genes in all cell types and a wide range of distinct cell-type-dependent targets to execute the IL-10 anti-inflammatory response[114].

2.1.3 IL-10 production and regulation of transcription

Almost all innate and adaptive immune cells can produce IL-10, including monocytes, macrophages, myeloid dendritic cells, granulocytes, NK cells, several T cell subsets ($CD4^+$ and $CD8^+$) and B cells. In addition, some non-haematopoietic cells are capable of producing IL-10, such as keratinocytes, epithelial cells and tumour cells. Which of these cell types is responsible for IL-10 production in a specific situation, depends on the stimulus, tissue and time point in the immune response[102,108,109]. In this part, the most important IL-10 producers and the signalling pathways behind their IL-10 production are described in more detail.

2.1.3.1 Monocytes, macrophages and dendritic cells

Monocytes, macrophages and DCs produce IL-10 mainly after stimulation of pattern recognition receptors (PRR). The best-known PRRs that induce IL-10 production are TLRs, but also C-type lectin receptors (such as Dectin-1 and DC-SIGN), NOD-like receptors and RIG-1-like receptors are reported to produce IL-10. In addition, ligation of CD40 and Fc receptors can induce IL-10 production[47,48].

TLRs are transmembrane glycoproteins that consist of an extracellular domain with leucine-rich repeats and a Toll/IL-1R (TIR) cytoplasmic tail required for signal transduction. They can be expressed on the plasma membrane or intracellularly and are activated by specific molecular patterns from microbial agents. In humans, 10 functional TLRs are described; TLR1-9 are conserved between mice and humans, the ligand for human TLR10 is unknown. TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the plasma membrane and recognize, among others, lipopeptides, peptidoglycan, LPS, flagellin and zymosan. On the other

hand, TLR3, TLR7, TLR8 and TLR9 recognize nucleic-acid-like structures and are expressed in intracellular vesicles. For example, TLR9 is expressed in the endoplasmic reticulum (ER) and recruited into endosomes or lysosomes after stimulation[116–118]. Activation of TLRs results in the recruitment of distinct adaptor proteins and triggers a specific biological response. All TLRs, except for TLR3, use the TIR-domain containing adaptor molecule MyD88. Recruitment of MyD88 results in the activation of mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)-protein kinase B (AKT) and NF-κB signalling pathways and induces cytokine production. TLR3 uses the TIR-domain-containing adapter-inducing interferon-β (TRIF), and TLR4 can use both MyD88 and TRIF. Recruitment of TRIF to TLR3 or TLR4 results in activation of the transcription factors interferon regulatory factor (IRF) 3 and NF-κB and subsequent type I IFN and cytokine production[47,116,117].

All TLRs are capable of inducing IL-10 production in monocytes, macrophages and dendritic cells, although not to the same extent in every cell type[47,119,120]. Several factors regulate the transcription of IL-10 in these cells (Figure 1). The activation of the MAPK extracellular signal-regulated kinase (ERK) is critical for IL-10 production, and the amount of ERK activation correlates with the levels of IL-10 produced. Next to ERK, the MAPK p38 is important for TLR-induced IL-10 production, and inhibition of either ERK, p38 or their downstream activated kinases mitogen- and stress-activated kinase (MSK) 1 and 2 results in a reduced IL-10 production. Downstream of MAPK, the transcription factors cAMP response element-binding protein (CREB), activator protein 1 (AP-1) and activating transcription factor (ATF) family members are activated and induce the production of IL-10. In addition, several other transcription factors, such as NF-κB p50 and NF-κB p65, c-MAF, Sp1 and Sp3 have been reported to bind the IL-10 promotor and regulate IL-10 production[47,112,117,121].

Furthermore, several autocrine and paracrine cytokines can modulate TLR-induced IL-10 production. IL-10 itself can have both a positive and negative effect on its production, by upregulating TPL2, which is upstream of ERK, or by inducing dual-specificity phosphatase-1 (DUSP1), which negatively regulates p38, respectively. Type I IFNs positively regulate IL-10 production. Although the mechanisms behind this positive

regulation may differ depending on the cell type, it has been reported that type I IFN activate the PI3K-AKT pathway, which inhibits the release of glycogen-synthase kinase 3 (GSK3). As GSK3 normally decreases TLR-induced IL-10 by suppressing the binding of CREB and AP1 to the IL-10 promotor, inhibition of GSK3 increases IL-10 production. In contrast to type I IFN, the type II IFN-γ is a well-known inhibitor of TLR-induced IL-10 in monocytes, macrophages and dendritic cells. IFN-γ inhibits ERK and p38 activation, hereby directly inhibiting IL-10 production. In addition, IFN-γ indirectly inhibits IL-10 by interfering with the PI3K pathway, increasing the release of GSK3 and the subsequent inhibition of CREB and AP1 activity[47,48,102,103]. Next to regulation by cytokines, TLR-induced IL-10 production can be enhanced by co-stimulation with CD40L or Fc receptor ligation[47,111].

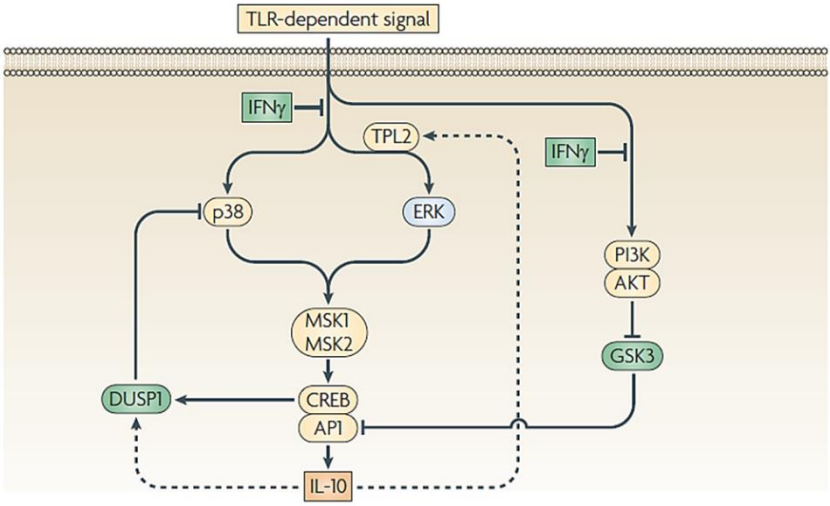

Figure 1: IL-10 induction by TLR-signalling in monocytes and macrophages and regulation by IFN-γ. *Dotted lines show autocrine regulation of IL-10 production via stimulation of TPL2 and DUSP1, which respectively increases and decreases IL-10 production. Adapted from Saraiva et al. (2010).*

2.1.3.2 T cells

Although it was initially thought that only Th2 cells produce IL-10, it is now known that all T cell subsets are potential IL-10-producers. The signal pathways resulting in IL-10 production have been less thoroughly investigated in T cells than in monocytes, macrophages and dendritic cells. However, it has been shown that IL-10 production by Th cells is

tightly linked with their differentiation program. For example, Th1 cells produce IL-10 after strong TCR signalling together with IL-12, and this IL-10 induction is STAT4 and ERK dependent. IL-12 increases PI3K-AKT signalling leading to inactivation of GSK3 and increased IL-10 production. In Th2 cells, IL-10 production requires TCR signalling in the presence of IL-4, which activates the transcription factors STAT6 and GATA-binding protein 3 (GATA3). In TCR-triggered Th17 cells, IL-6 and transforming growth factor (TGF)-β activate STAT3 and can induce IL-10 production. Thus, TCR signalling together with distinct cytokine combinations can induce IL-10 production in the different Th cell subsets. Upon TCR activation, nuclear factor of activated T cells (NFAT) translocates into the nucleus and interacts with AP1 to induce IL-10 expression[47,48,102,108,122]. Other transcription factors that can modulate IL-10 expression in T cells are IRF4, E4 promoter–binding protein 4, basic leucine zipper ATF-like transcription factor and c-MAF, and expression of c-MAF correlates with IL-10 secretion[47,108,123].

IL-10 production by T_{reg} cells also requires stimulation. Here, IL-2 is important for both T_{reg} maintenance and IL-10 production, via STAT5. In addition, TGF-β is required for IL-10 production by T_{reg} *in vivo.* Next to the traditional T_{reg} cells, characterised by the expression of the transcription factor Foxp3, regulatory Foxp3-negative IL-10-producing T cells have been described. These cells produce IL-10, but not IL-2, IL-4 or IFN-γ. They can be induced *in vitro* with various stimuli, such as cytokine cocktails (TGF-β, IL-10, IFN-α, IL-27) and immunosuppressive drugs (e.g. vitamin D3, dexamethasone) and *in vivo* by repeated stimulation with soluble antigen[48,108,124].

Furthermore, IL-27 increases IL-10 production by Th1 cells, and induces IL-10 in Th17 cells together with IL-23. Also IL-21 has been shown to induce IL-10 in Th cells[108,122]. In addition, dendritic cells can instruct T cells to produce IL-10. For example, plasmacytoid DCs (pDCs) can promote IL-10 secretion from CD4 and CD8 T cells through inducible costimulatory molecule (ICOS) ligand expression, or via delta-like-4 ligand on pDCs that activates Notch signalling in Th cells[122,125,126].

2.1.3.3 B cells

Although B cells are traditionally seen as antibody-producing cells, they can express a variety of cytokines, including IL-10. These IL-10-producing

B cells have been designated as B10 cells or regulatory B cells[127]. The main signalling pathways that result in IL-10 production by B cells are TLR signalling, CD40 signalling and B cell receptor (BCR) signalling[127,128].

TLR-induced IL-10 production in B cells has been mainly described after activation of TLR4, TLR7/8 and TLR9. However, little is known about the exact intracellular pathways that control IL-10 production[129–131]. TLR7/8 and TLR9-induced IL-10 has been reported to be dependent on ERK and STAT3[131], p38[130] or all three MAPK ERK, p38 and c-Jun N-terminal kinases (JNK)[132], while TLR4-induced IL-10 was shown to be dependent on p38[130], ERK and p90 ribosomal S6 kinases[129]. In contrast to macrophages, TLR4-induced IL-10 production in murine B cells was independent of MSK1, MSK2 and CREB, indicating that B cells and macrophages produce IL-10 by distinct mechanisms in response to TLR stimulation[129].

The production of TLR-induced IL-10 in B cells can be regulated by several factors. For example, IFN-α enhances TLR7/8- but not TLR9-induced IL-10 production in human B cells by increasing ERK and STAT3 activation[131]. In TLR4-stimulated murine B cells, IFN-γ and IL-12 upregulate and IL-21 and TGF-β downregulate IL-10 production[133]. Two members of the TNF family, a proliferation-inducing ligand (APRIL) and B cell activating factor (BAFF) are known to modulate TLR-induced IL-10 production in B cells via their receptor transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI)[132]. Hua *et al.* (2016) showed that APRIL, but not BAFF, increases TLR9-induced IL-10 in B cells partially via increased STAT3 and ERK activation. In contrast, Yehudai *et al.* (2012) and Saulep-Easton *et al.* (2016) showed that BAFF increases TLR9-induced IL-10, and Saulep-Easton *et al.* (2016) additionally reported that BAFF itself can also induce IL-10 production in B cells[132,134,135]. The precise mechanism by which BAFF upregulates TLR9-induced IL-10 remains to be investigated, but it has been shown that TLR9 stimulation increases TACI expression[135]. In addition, other factors, such as retinoic acid, can increase TLR9-induced IL-10 in B cells[136] and pDC/B cell contact increases TLR7- and TLR9-induced IL-10, potentially via CD27-CD70 interaction[137].

In addition to TLR signalling, stimulation of the BCR contributes to IL-10 production[48,127,128]. BCR signalling results in the activation of the proximal kinases of the Src family (Lyn, Blk, and Fyn) as well as the Syk and Btk tyrosine kinases and subsequent activation of the adaptor protein BLNK

and signalling enzyme phospholipase C-γ2 (PLCγ2). Activated PLCγ2 yields inositol 1,4,5-triphosphate (IP3), which releases Ca^{2+} from ER stores into the cytoplasm. The decreased Ca^{2+} in ER lumen then triggers the entry of Ca^{2+} via Ca^{2+} channels across the plasma membrane, a process that is dependent on the Ca^{2+} sensors STIM1 and STIM2. Several factors involved in the regulation of IL-10 production depend on Ca^{2+} signalling, such as CREB, NF-κB, ERK, p38, JNK and NFAT. However, in the absence of STIM proteins, only the activation of NFAT1 is impaired and a decreased IL-10 production is observed. NFAT1 translocates into the nucleus after dephosphorylation by the Ca^{2+}-activated phosphatase calcineurin, where it becomes transcriptionally active. Inhibition of calcineurin suppressed IL-10 secretion, suggesting that IL-10 production after BCR activation is regulated by the STIM-calcineurin/NFAT pathway. IL-10 production after BCR signalling occurs mainly when co-stimulated with CD40 and/or TLR agonists. Several transcription factors downstream of TLR signalling, such as AP1, may also co-operate with NFAT1 to induce the expression of IL-10. In addition, the transcription factor IRF4 may serve as a partner for NFAT1 to produce IL-10, since the absence of IRF4 in B cells results in decreased IL-10 expression after BCR signalling[127,128].

The third main signalling pathway resulting in IL-10 production by B cells is CD40 signalling. Ligation of CD40 on B cells results in activation of STAT3, NF-κB and ERK and enhances IL-10 production[128,138,139]. CD40 ligation also increases TLR-induced IL-10 production[127]. Furthermore, CD40 ligation in combination with IL-4 increases IL-10 via 1,25-dihydroxyvitamin D3, by recruiting the vitamin D receptor to the promoter of IL-10, and by modulation of calcium-dependent signalling. In addition, also exogenous 1,25-dihydroxyvitamin D3 enhances IL-10 production[140].

2.1.3.4 Additional IL-10-producing cell types

Many other cell types can produce IL-10 upon specific stimulation, but the exact mechanisms resulting in IL-10 production are less well described. For example, CD8 T cells produce IL-10 after activation of the TCR or interaction with CD40L. In NK cells, IL-10 expression is induced by stimulation with IL-2 and IL-12 and is dependent on STAT4. Furthermore, neutrophils produce IL-10 in response to TLR and C-type lectin 1 activation via MyD88 and Syk[48,112].

2.1.4 Epigenetic and post-transcriptional regulation of IL-10 production

Several studies have suggested that IL-10 is epigenetically regulated by changes in the chromatin structure at the *IL10* locus. These changes include the so-called DNase I hypersensitivity sites (HSS), specific regions were chromatin has lost its condensed structure, making it more accessible to transcription factors. Several common and cell-specific DNase I HSS have been found in the *IL10* locus[48,102,112]. In addition, epigenetic imprinting of the *IL10* locus modulates IL-10 expression. Acetylation of histones indicates an active transcription and is correlated with increased IL-10 expression in Th cells and macrophages. Methylation of cytosine-phosphate-guanine (CpG) DNA sequences is a heritable epigenetic mark and is associated with transcriptional silencing. Accordingly, a negative correlation between *IL10* promotor methylation and IL-10 expression in PBMCs has been observed[48,102,112]. In macrophages, phosphorylation of histone H3 correlates with transcription initiation of IL-10. In Th2 cells, GATA3 regulates IL-10 transcription by increasing histone H3 and H4 acetylation at the IL-10 promotor[47,48]. Conversely, histone deacetylase 11 negatively regulates the expression of IL-10 in antigen-presenting cells[102,112]. Overall, epigenetic imprinting regulates IL-10 in a cell type-dependent manner.

Post-transcriptional regulation is an essential mechanism to control cytokine production. One of the major mechanisms of post-transcriptional regulation is modulation of transcript stability[47]. The 3′ untranslated region of the *IL10* mRNA contains clusters of adenosine- and uridine-rich elements (AREs), making it susceptible to ARE-mediated mRNA decay mechanisms[47,48,141]. Tristetrapolin (TTP) is a zinc finger binding protein that mediates mRNA decay by linking transcripts to mRNA decay machinery, such as mRNA de-capping proteins, exosome endonuclease activity and RNA-induced silencing complex (RISC) members. TTP activity is negatively regulated by the p38 MAPK, which shows that p38 MAPK not only induces IL-10 transcription but also increases its mRNA stability. On the other hand, TTP is positively regulated by IL-10 itself, potentially reflecting a negative feedback mechanism. Next to TTP, the RNA-binding protein ARE/poly(U) binding degradation factor 1 can also bind to the 3′ UTR and initiate IL-10 mRNA degradation[47,48].

Furthermore, IL-10 transcript stability can be modulated by several microRNAs. miR-106a, let7, miR-98 and miR-1423p/5p have been shown to directly target the 3′ UTR of *IL10*, leading to mRNA degradation via the RISC complex. In contrast, miRNA-466L extends the IL-10 mRNA half-life by competing with TTP binding sites[142].

Together, epigenetic and post-transcriptional regulation may partially explain the differential IL-10 production by distinct cell types and contributes to the fine-tuning of IL-10 expression.

2.2 Interferon-γ

2.2.1 Definition and function

The interferons (IFNs) are a group of cytokines that interfere with viral replication, and can be divided in type I IFNs (i.e. IFN-α, IFN-β, IFN-ω, IFN-ε and IFN-κ), type II IFNs (i.e. IFN-γ) and type III IFNs (i.e. IFN-λ1-4). IFN-γ, the only type II IFN, was discovered almost 60 years ago and differs from the other IFNs in the fact that its antiviral properties are less important relative to its other immunomodulatory activities[62,143–145].

IFN-γ is predominantly produced by T cells and NK cells, but also NKT cells and professional antigen-presenting cells (APCs) have been described to produce IFN-γ. The most important positive regulators of IFN-γ production are the cytokines IL-12 and IL-18, while IL-4, IL-10, TGF-β and glucocorticoids are negative regulators[143–146].

IFN-γ was traditionally considered as a pro-inflammatory cytokine, since it activates macrophages and innate immune pathways, and induces Th1 polarization. However, IFN-γ also exerts several regulatory activities, such as the modulation of T_{reg} cell differentiation and limitation of inflammation-associated tissue damage[143,144,146].

Macrophage activation can be enhanced by IFN-γ in several ways: IFN-γ increases pinocytosis, receptor-mediated phagocytosis and killing of pathogens and malignant cells by enhancing the production of reactive oxygen and nitrogen species[144]. Furthermore, IFN-γ upregulates MHC class II expression on monocytes/macrophages and other professional APCs, such as dendritic cells and B cells, and induces MHC II expression on non-professional APCs, thereby promoting peptide-specific activation

of $CD4^+$ T cells. Next to MHC II, also the surface expression of MHC class I is upregulated by IFN-γ, resulting in increased $CD8^+$ T cell immune surveillance at the site of inflammation[143,144,146].

IFN-γ also modulates T helper and T_{reg} cell differentiation. In line with the fact that it is a major effector cytokine of Th1 cells, IFN-γ skews differentiation of naïve T cells towards a Th1 phenotype, either directly or by inducing the production of IL-12 by phagocytes. Conversely, IFN-γ inhibits the differentiation of Th2 cells by downregulating the IL-4-STAT6 pathway and by inhibiting the expression of the Th2 transcription factor GATA3. Furthermore, IFN-γ inhibits Th17 cell development and its effector functions[144,146]. Next to inhibition of Th2 and Th17 cells, it has been shown that IFN-γ enhances T_{reg} cell development and function in certain conditions, thereby contributing to the regulation of T cell activation and homeostasis[63,146]. Another regulatory function of IFN-γ related to T cell biology is the inhibition of T cell proliferation and induction of apoptosis via indoleamine 2,3-dioxygenase (IDO), an enzyme that converts tryptophan to kynurenine. Increased IDO expression results in a reduced access to free tryptophan and downstream metabolites, thereby affecting T cell proliferation[63].

As mentioned before, IFN-γ increases MHC II expression on B cells. Furthermore, IFN-γ mediates B cell maturation and isotype switching, increasing the production of IgG2a, while inhibiting IgG1 and IgE production[63,144]. The homing of immature B cells to the lymph nodes is inhibited by autocrine IFN-γ secretion, via downregulation of their adhesion to the extracellular matrix protein fibronectin[147]. In addition, IFN-γ can stimulate or inhibit B cell proliferation, depending on the B cell-activating stimulus used[148–150].

Although IFN-γ induces the expression of mononuclear cell-specific chemokines (e.g. IP-10, MIG) and cell adhesion molecules (e.g. ICAM-1, V-CAM-1), it inhibits the expression of neutrophil-specific chemokines (e.g. CXCL8) and the mobilisation of neutrophils. IFN-γ further limits inflammation-induced tissue damage by inhibiting the expression of tissue-destructive factors (e.g. matrix metalloproteases, serine proteases, complement factors) and inhibiting bone resorption (via inhibition of osteoclastogenesis)[63,144,146]. IFN-γ suppresses mycobacteria-induced myelopoiesis and macrophage colony-stimulating factor (M-CSF)-

dependent growth of bone-marrow-derived macrophages[144,151,152]. As previously mentioned, IFN-γ also regulates the levels of active IL-18 by inducing IL-18BP[57].

Taken together, while originally described as a pro-inflammatory cytokine, IFN-γ exerts anti-inflammatory activities as well. The importance of these anti-inflammatory effects became especially evident in an autoimmune and autoinflammatory context, by using mice that are deficient in IFN-γ or its receptor. Indeed, these mice are much more prone to develop experimental autoimmune encephalomyelitis (EAE) and autoimmune (i.e. collagen-induced arthritis (CIA)) and autoinflammatory arthritis (i.e. sJIA)[101,153,154]. In Table 3, an overview of the pro- and anti-inflammatory properties of IFN-γ is given.

Table 3: overview of pro- and anti-inflammatory properties of IFN-γ

Pro-inflammatory effects	**Anti-inflammatory effects**
*Macrophage activation *Increased antigen presentation *Th1 cell polarization *B cell maturation and isotype switching *Induction of mononuclear cell-specific chemokines *Induction of cell adhesion	*Inhibition of Th17 polarization *Stimulation of T_{reg} cell functions *Inhibition of T cell proliferation *Inhibition of neutrophil-specific chemokines *Inhibition of inflammation-induced tissue damage

Abbreviations: T_{reg} = regulatory T cell, Th = T helper

2.2.2 Mode of action

To exert its biological effects, IFN-γ binds with the IFN-γR to induce downstream signalling events. The IFN-γR is composed of two homodimers of the IFN-γR1, which is the high affinity chain, and IFN-γR2, which is the low affinity chain. IFN-γ binds to its receptor as a homodimer, and thereby induces a conformational change, allowing the recruitment of JAK1 and JAK2. Activation of JAK1/2 induces a second conformational change, resulting in STAT1 recruitment and subsequent phosphorylation of STAT1 by JAK1/2. The phosphorylated STAT1 forms a homodimer and translocates to the nucleus to bind with the γ-interferon-activated-site (GAS) DNA sequences and initiate transcription[145,146,155]. However, this canonical signalling pathway is not the only pathway by which IFN-γ exerts its functions. For example, when STAT1 is not present,

IFN-γ signals through STAT3 and in this way activates GAS DNA sequences[145,155]. Furthermore, signalling can take place through several MAPK, such as PyK2, ERK1/2 and JNK, adaptor proteins, such as small G protein Rap1, or glycogen synthase kinase 3 (GSK3) which all activate different transcription factors[145]. Of note, most non-canonical signalling pathways are typically activated later on, in the presence of chronically high IFN-γ levels[145]. Thus, the overall effect of IFN-γ is dependent on canonical (STAT1-dependent) and non-canonical (STAT1-independent) signalling pathways, that are activated sequentially.

2.3 Synopsis on IL-10 and IFN-γ in sJIA

sJIA is an autoinflammatory disease that is possibly caused by a defect in the regulation of the immune response. Although acute infections exclude the diagnosis of sJIA, it is thought that infectious agents may trigger the immune response. Support for this concept comes from the cytokine storm that is typical seen in sJIA and from the upregulation of TLRs seen in expression studies performed on PMBCs from sJIA patients[8]. The described polymorphisms in sJIA patients are indicative for a defective production of IL-10, which is a potent cytokine known to downregulate immune responses in different ways. Contradictory results are reported regarding the production of IL-10 in sJIA patients, which may be explained by the use of different procedures or inappropriate controls. Moreover, the regulation of IL-10 production is complex and cell-specific, making it essential to study IL-10 production at the cellular level. IFN-γ on the other hand, is traditionally considered a pro-inflammatory cytokine and inhibitor of IL-10 production. In contrast, IFN-γ plays an anti-inflammatory role in several autoimmune and autoinflammatory diseases, an observation that also became evident from the development of sJIA in IFN-γ KO mice. Until now, it is unknown whether the absence of IFN-γ affects IL-10 levels in the sJIA mouse model, and it remains unclear whether sJIA patients have a defective IL-10 production, indicating the need for further studies to answer these questions.

CHAPTER 2

RESEARCH OBJECTIVES

sJIA is a rare childhood immune disorder with a high morbidity. The disease is considered as an autoinflammatory syndrome, but its pathogenesis remains not fully understood. Furthermore, although treatment of sJIA patients has been greatly improved by the availability of IL-1 and IL-6 antagonists, part of the patients do not respond to these treatments, or escape after an initial good response. Therefore, a better understanding of the disease pathogenesis remains essential in the search for new treatment strategies.

IL-10 is one of the most important anti-inflammatory cytokines, and IL-10 polymorphisms resulting in a lower IL-10 production are believed to be associated with sJIA disease development[18,19]. Several studies reported on the production of IL-10 in sJIA patients, with conflicting results. For example, IL-10 levels in plasma of sJIA patients were increased compared to healthy controls, but were lower compared to polyJIA patients[36]. Furthermore, IL-10 levels were increased in patients compared to healthy controls after *in vitro* stimulation of PBMCs, but were decreased after stimulation of whole blood cells[38,51]. Given the fact that anti-inflammatory pathways seem unable to resolve the inflammation in sJIA, these data might be indicative for a defective IL-10 production in sJIA patients[8].

In **a first objective**, we aimed to provide evidence for the hypothesis that a relative defect in IL-10 production underlies sJIA pathogenesis, i.e. a defect resulting in an inadequate production of IL-10 that is not sufficient to dampen the ongoing immune response elicited by a harmless trigger. To this end, we analysed IL-10 levels in a recently developed mouse model for sJIA and determined the cell types responsible for IL-10 production in different organs. The role of IL-10 in sJIA pathogenesis was further evaluated by treating the mice with IL-10R-neutralising antibodies. Furthermore, we searched for potential relative defects in IL-10 production in a cohort of sJIA patients by comparing the levels of IL-10 in plasma with the production of classical inflammatory markers (e.g. CRP) and inflammatory cytokines (e.g. IL-6). In addition, cell-specific production of IL-10 was analysed *ex vivo* and after stimulating cells of sJIA patients with several triggers *in vitro*. Both in the mouse model and in sJIA patients, we demonstrated a defective IL-10 production.

Surprisingly, the defective IL-10 production in the sJIA mouse model was observed in the absence of IFN-γ, which was a very intriguing observation

considering the fact that IL-10 and IFN-γ are traditionally seen as each other's antagonists[102,103]. The sJIA mouse model is induced by injecting IFN-γ KO mice with CFA, an adjuvant that contains several TLR ligands, which are potent inducers of IL-10 production. Although almost all immune cells express TLRs, TLR-induced IL-10 production has predominantly been studied in monocytes, macrophages and dendritic cells. In these cell types, IFN-γ negatively regulates TLR-induced IL-10 production. However, in other cell types, the production and regulation of TLR-induced IL-10 is still incompletely understood. Therefore, the **second objective** of this thesis was to study the role of IFN-γ on the production of IL-10 in a comprehensive way, by using several TLR triggers as well as different cell populations from naïve mice and human healthy donors. In line with the data obtained in the sJIA mouse model, we observed that the traditional dogma of IL-10 and IFN-γ as antagonists is not valid in all situations, and demonstrated that IFN-γ can stimulate TLR9-induced IL-10 production. We further investigated the differential effect of IFN-γ on TLR-induced IL-10 in more detail and explored several mechanisms that might underlie the observed differences. Since IL-10, IFN-γ and TLR-triggering are all important players in the induction and regulation of immune responses, a better understanding of their interactions may contribute to an improved understanding of immune regulation in general.

CHAPTER 3

MATERIALS AND METHODS

Mice, sJIA mouse model, *in vivo* injections and antibody treatment.

IFN-γ KO and WT BALB/c mice were bred under specific pathogen-free (SPF) conditions in the Experimental Animal Centre of KU Leuven. DBA/1 mice were bred in the conventional animal facility. C57BL/6 IFNAR1 KO mice and WT littermates were bred under SPF conditions in the Animal Centre of VIB-UGent Center for Inflammation Research, Belgium. The sJIA mouse model was induced in WT and IFN-γ KO BALB/c mice of 6-8 weeks old, by subcutaneous injection of CFA (Difco) with added heat-killed Mycobacterium (1.5 mg/ml) as previously described[101]. Mice were euthanized when overt signs of inflammation occurred, between 16 and 21 days post CFA-injection. Age- and sex-matched non-injected littermates were included as controls. Monoclonal antibodies against the IL-10R (clone 1B1.2) were purified from hybridoma culture (kindly provided by Prof. Dr. O. Leo, ULB, Gosselies, Belgium). Mice were treated intraperitoneally with 200 μg of anti-IL-10R antibody (αIL10R) or phosphate-buffered saline (PBS) twice a week, starting 1 day prior to CFA challenge (day -1). CpG oligodeoxynucleotides (ODN) were injected intraperitoneally (ODN 1826, 200 μg/mouse, IDT). All experiments were approved by the Ethics Committee of KU Leuven (P182/2014).

Human samples.

A total of 29 patients (18 active sJIA and 11 inactive sJIA), and 21 age-matched healthy controls were recruited from the University Hospital of Leuven (Patient characteristics in Chapter 4, Supplementary Table 1). Informed consent was given according to the Declaration of Helsinki. The study was approved by the Ethics Committee of KU Leuven (S58814). sJIA patients met the classification criteria of the International League of Associations for Rheumatology[2] and were divided according to their disease state into active and inactive patients. Samples from active patients were collected at the occurrence of disease before therapeutic intervention, or at disease flares. Inactive patients were defined by the absence of fever, rash, arthritis and inflammatory parameters. For experiments using only human healthy donors, PBMCs were obtained from adult healthy donors via Red Cross Flanders.

Blood analysis, cell and plasma isolation.

Murine blood samples were obtained by cardiac puncture with heparin (LEO). Complete blood cell analysis was performed with a Cell-Dyn 3700 apparatus (Abbott Diagnostics). Peritoneal cells were isolated by lavage of the peritoneal cavity with PBS and 2% fetal bovine serum (FBS) and re-aspiration after 30s incubation. Spleen and lymph nodes were fragmented and passed through a cell-strainer to obtain single-cell suspensions. Plasma from patient samples was separated within 2h after withdrawal of EDTA-anticoagulated blood. Human PBMCs were obtained by Lymphoprep density centrifugation and frozen in liquid nitrogen in cryopreservation medium (90% FBS, 10 % DMSO).

In vitro stimulation, neutralisation and inhibition.

Cells were freshly isolated (mouse) or thawed from liquid nitrogen (human) and resuspended in RPMI-1640 medium containing 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml) and 50 µM 2-mercaptoethanol. Cells (0.25 - 1x10^6/ml) were stimulated at 37°C and 5% CO_2 with anti-CD3/CD28-antibody-coated beads (aCD3/CD28; 10 µl/ml; Gibco), peptidoglycan (PGN; 1 µg/ml; Sigma), polyinosinic-polycytidylic acid (PIC; 100 µg/ml; Sigma), LPS (200 ng/ml; Sigma), resiquimod (R848; 1 µg/ml; Sigma), CpG ODN1826 (class B CpG, mouse, 200 ng/ml, IDT), CpG ODN2006 (class B CpG, human, 1 µg/ml, IDT), IFN-γ (mouse: 150 ng/ml & 300 ng/ml; human: 15 ng/ml; Peprotech), recombinant human BAFF (50 ng/ml, Peprotech) or recombinant human IL-2 (100 ng/ml; PeproTech) and IL-12 (10 ng/ml; PeproTech). Neutralisation of IFN-β, BAFF and TACI was realised by anti-IFN-β-antibodies (10 µg/ml and 25 µg/ml; clone MIB-5E9.1; Biolegend), anti-BAFF-antibodies (0.1 µg/ml and 1 µg/ml; polyclonal; R&D Systems) and anti-TACI-antibodies (1 µg/ml; polyclonal; R&D systems). Activity of ERK1/2, p38 and JNK was suppressed by chemical inhibition with PD184352 (for MEK1/2; Sigma), SB203580 (Sigma) and SP600125 (R&D systems) respectively. Specificity of these inhibitors was previously tested by Davies *et al.* (2000) and Bain *et al.* (2007)[156,157]. As a control, isotype- or vehicle (DMSO)-treatment was used. Cells were counted manually via Trypan Blue or via IncuCyte S3 Live-Cell Analysis System and analysed by IncuCyte S3 2017 Software (Stemcell Technologies).

mRNA and protein quantification.

RNA was extracted from tissues using the PureLink RNA mini kit (Invitrogen) or from cell cultures using the RNeasy Micro kit (Qiagen). cDNA was obtained by reverse transcription using Superscript II Reverse Transcriptase and random primers (Invitrogen), according to the manufacturer's protocol. mRNA levels were analysed by qPCR using pre-designed TaqMan Gene Expression Assays (Applied Biosystems) and a 7500 Real-Time PCR System Apparatus. Expression levels of IL-10 (Mm.PT.58.13531087; Hs.PT.58.2807216), MKP1 (Hs.PT.58.39287533.g) and MKP2 (Hs.PT.58.1640850) were normalized for GAPDH (Mm99999915_g1; Hs.PT.39a.22214836) mRNA expression by the $2^{-\Delta\Delta CT}$ method. Expression levels of BAFF (Hs00198106_m1) and TACI (Hs00963364_m1) were analysed by Droplet digital PCR (Bio-Rad), using 50 ng cDNA, with pre-designed TaqMan Gene Expression Assays (Applied Biosystems) and housekeeping gene *POLR2A* (Hs00172187_m1), according to the manufacturer's instructions. Relative quantity of the target genes was measured with QuantaSoft v1.4 (Bio-Rad). IL-6, IL-10, IL-17 and BAFF protein levels were measured by ELISA (Duoset or Quantikine, R&D systems), IFN-α and IFN-β levels by Procartaplex (ThermoFisher), according to the manufacturer's protocol. IgM and IgG protein levels were measured by an in-house-developed sandwich ELISA (antibodies from Jackson Immuno research). In patient samples, C-reactive protein (CRP), IL-6, IL-10 and IL-18 levels were measured using Meso Scale Discovery (MSD; Meso Scale Diagnostics, LLC), according to the manufacturer's protocol. mRNA stability was measured by adding the transcription inhibitor Actinomycin D, which inhibits *de novo* mRNA synthesis, followed by analysis of the remaining amount of mRNA.

In vitro osteoclast generation.

For stimulation of osteoclast generation, splenocytes were resuspended in α-minimal medium (α-MEM) containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). $5x10^5$ cells in a total volume of 400 μl were seeded in chamberslides (ThermoFisher) and incubated for 3 days with the osteoclast-differentiating factor receptor activator of NF-κB ligand (RANKL; 100 ng/ml; R&D Systems) and M-CSF (20 ng/ml, R&D Systems). After 3 days, medium and stimuli were replaced and cells were stimulated for 3 more days, followed by staining of the cells for tartrate-resistant acid

phosphatase (TRAP) as previously described[158]. Cells staining red were considered to contain TRAP, TRAP-positive cells with 3 or more nuclei were defined as osteoclasts.

Flow cytometry and cell sorting.

Flow cytometry and fluorescence-activated cell sorting (FACS) was performed according to the "guidelines for the use of flow cytometry and cell sorting in immunological studies"[159]. Cells were isolated, incubated with FcR-block (Miltenyi Biotec) and stained with the following monoclonal antibodies: anti-mouse CD3 (clone 145-2C11; BioLegend), CD4 (GK1.5; BD Biosciences), CD8 (53-6.7; BD Biosciences), CD11b (M1/70; BD Biosciences), CD115 (clone AFS98; BioLegend), Ly6G (1A8; ThermoFisher), CD19 (1D3; ThermoFisher or 6D5; BioLegend), CD122 (TM-b1; ThermoFisher), CD49b (DX5; BD Biosciences), TCRγδ (eBioGL3; ThermoFisher), Foxp3 (REA788; Miltenyi Biotec), TER119 (Ter-119; ThermoFisher), CD71 (C2; BD Biosciences), CD34 (RAM34; BD Biosciences), F4/80 (BM8; ThermoFisher), CD23 (B3B4; ThermoFisher), CD24 (M1/60; BioLegend), IgM (II41; ThermoFisher), IgD (11-26c.2a; BD Biosciences), CD21/35 (7G6; BD Biosciences), CD1d (1B1; ThermoFisher), CD5 (53-7.3, BioLegend), B220 (RA3-6B2; BD Biosciences), TLR9 (26C593.2, ThermoFisher) and IFN-γRb (REA381; Miltenyi Biotec); anti-human CD3 (SK7, HIT3a; ThermoFisher), CD4 (RPA-T4, OKT4; ThermoFisher), CD14 (61D3; ThermoFisher), CD19 (HIB19; BD Biosciences), CD56 (NCAM 16.2, HCD56; BD Biosciences, BioLegend), CD24 (HL5; BD Biosciences), CD38 (HB7; ThermoFisher), BAFF (1D6; ThermoFisher), BAFF-R (8A7; ThermoFisher), TACI (1A1-K21-M22; BD Biosciences) and CD119 (IFN-γR1; GIR-208; ThermoFisher). Dead cells were excluded using Zombie Aqua516 (BioLegend), DAPI (Sigma) or Fixable Viability Dye eFluor780 (ThermoFisher). For intracellular staining, cells were stimulated for 4h in RPMI+10%FBS containing ionomycin (750ng/ml, Biotechne), phorbol 12,13-dibutyrate (500ng/ml, Biotechne) or PMA (100 ng/ml; Sigma) and brefeldin A (2μg/ml, Biotechne). After stimulation, cells were stained for surface markers and viability. Cells were fixed and permeabilized according to the manufacturer's protocol (BD Biosciences) and stained intracellularly with antibodies against murine IL-10 (JES5-16E3; BD Biosciences) or human IL-10 (JES3-19F1; BD Biosciences). Unstimulated cells and cells stimulated in the absence of

brefeldin A were used as negative controls for cytokine staining. Phosphorylation staining for p-ERK (MILAN8R; ThermoFisher), p-p38 (4NIT4KK; ThermoFisher) and p-JNK (N9-66; BD Biosciences) was performed according to the Phosflow protocol (BD Biosciences) after 15 min, 30 min, 1h, 6h, 14h, 20h and 38h of stimulation. Flow cytometry was performed on a BD LSR Fortessa X20 or BD FACSymphony, FACS was performed on BD FACS Aria III, all equipped with DIVA software. Results were analysed with FlowJo (version 10, LLC).

Cell enrichment.

For enrichment of B cells and $CD4^+$ T cells, MACS cell separation (Miltenyi Biotec) with mouse CD19 or CD4 microbeads (positive selection), EasySep human B cell enrichment kit (negative selection) or EasySep human CD4 positive selection kit II (Stemcell Technologies) were used according to the manufacturer's protocols.

CD4 T cell proliferation.

Enriched B cells were either used *ex vivo* or stimulated for 48h *in vitro* with IFN-γ, CpG or CpG+IFN-γ. Enriched $CD4^+$ T cells of the same mice/donors were then labelled with carboxyfluorescein succinimidyl ester (CFSE; 0.14 μM; ThermoFisher) according to the manufacturer's protocol. $2.5x10^5$ CFSE-labelled CD4 T cells were stimulated with aCD3/CD28 (Gibco) and co-cultured with $2.5x10^5$ B cells (1:1) (either isolated *ex vivo* or stimulated *in vitro* for 48h) or supernatant of stimulated B cells (40%) for 72h. After 72h, cells were isolated and stained for the surface markers CD3, CD4 and CD19. Cells were analysed by flow cytometry, dilution of the CFSE label shows the amount of CD4 T cell proliferation.

Statistics.

Data were analysed using GraphPad Prism 7 software. For comparison of two different groups, the two-tailed non-parametric Mann-Whitney U test was used for unpaired data. The Wilcoxon matched-pairs signed rank test was used for paired data. When three or more groups were included, data were first checked for statistical difference with the non-parametric Kruskal-Wallis test (unpaired data) or Friedman test (paired data). For analysis of grouped data, Two-Way ANOVA followed by Tukey's multiple comparisons test was used.

CHAPTER 4

Insufficient interleukin-10 production as a mechanism underlying the pathogenesis of systemic juvenile idiopathic arthritis

RESEARCH ARTICLE

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Abstract

Systemic juvenile idiopathic arthritis (sJIA) is a childhood immune-inflammatory disorder of unknown aetiology. One of the concepts is that the disease results from an inappropriate control of immune responses to an initially harmless trigger. In the current study, we investigated whether sJIA may be caused by defects in IL-10, a key cytokine in controlling inflammation. We used a translational approach, with a sJIA-like mouse model and sJIA patient samples. The sJIA mouse model relies on injection of Complete Freund's Adjuvant (CFA) in IFN-γ deficient BALB/c mice, corresponding wild type (WT) mice only develop a subtle and transient inflammatory reaction. Diseased IFN-γ deficient mice showed a defective IL-10 production in $CD4^+$ T_{reg} cells, $CD19^+$ B cells and $CD3^-CD122^+CD49b^+$ NK cells, with B cells as the major source of IL-10. In addition, neutralisation of IL-10 in WT mice resulted in a chronic immune-inflammatory disorder clinically and haematologically reminiscent of sJIA. In sJIA patients, IL-10 plasma levels were strikingly low as compared to pro-inflammatory mediators. Furthermore, $CD19^+$ B cells from sJIA patients showed a decreased IL-10 production, both *ex vivo* and after *in vitro* stimulation. In conclusion, IL-10 neutralisation in CFA-challenged WT mice converts a transient inflammatory reaction into a chronic disease and represents an alternative model for sJIA in IFN-γ competent mice. Cell-specific IL-10 defects were observed in sJIA mice and patients, together with an insufficient IL-10 production to counterbalance their pro-inflammatory cytokines. Our data indicate that a defective IL-10 production contributes to the pathogenesis of sJIA.

1. Introduction

Systemic juvenile idiopathic arthritis (sJIA) is a childhood-onset immune disorder, characterized by arthritis and systemic inflammatory features, including fever, rash and lymphadenopathy. sJIA is a subtype of JIA, the most common chronic rheumatic disease in childhood. sJIA represents roughly 10% of all forms of JIA and is unique in presenting severe systemic symptoms that often overshadow joint inflammation[2,4,8,11,160]. Several findings have led to the consideration of sJIA as a polygenic autoinflammatory disease, driven by pro-inflammatory cytokines related to the innate immune system, such as IL-1β, IL-6 and IL-18[1,3,4,8,26,45]. A striking feature of sJIA is its association with macrophage activation syndrome (MAS), a potentially life-threatening complication of several systemic inflammatory disorders, which is characterized by excessive activation of T cells and macrophages and massive inflammatory responses[60,160,161].

The aetiology of sJIA is largely unknown. One concept is that sJIA results from a relatively harmless trigger in children who cannot adequately suppress immune responses due to genetic or acquired defects[8]. Increased susceptibility for sJIA has been linked to polymorphisms in the IL-10 and IL-10 promotor genes associated with lower expression rates of the cytokine[18,19]. Since IL-10 is an anti-inflammatory cytokine with a crucial role in preventing excessive immune responses and autoimmune pathologies (reviewed in[49]), insufficient IL-10 production may co-operate with other cytokine-related abnormalities in the onset of sJIA. In literature, contradictory results are reported regarding IL-10 in sJIA. A decreased IL-10 production in sJIA patients, as compared to healthy controls, was shown when whole blood cells were stimulated with lipopolysaccharides (LPS) or phytohemagglutinin[38]. In contrast, when PBMCs of sJIA patients were left unstimulated or were stimulated with anti-CD3-antibodies, anti-CD28-antibodies or PMA, these produced more IL-10 than cells from healthy donors[51]. Quantification of IL-10 mRNA in PBMCs and purified monocytes, revealed higher IL-10 mRNA levels in sJIA patients than in healthy controls[42,50]. Lastly, IL-10 plasma levels in sJIA patients were increased compared to healthy controls, but were lower compared to levels found in patients with polyarticular JIA[36].

In the present study, we explored the hypothesis that defects in IL-10 may underlie the pathogenesis of sJIA. We took advantage of our recently developed mouse model for sJIA[101] and analysed IL-10 production in a cohort of patients. The mouse model relies on injection of wild type (WT) and IFN-γ deficient (IFN-γ KO) mice with Complete Freund's Adjuvant (CFA), containing heat-killed mycobacteria. While IFN-γ KO mice develop sJIA-like symptoms, WT mice only experience a subtle and transient inflammatory reaction[101]. We found lower IL-10 levels in tissues and cells of CFA-injected IFN-γ KO mice, and therefore investigated whether a sJIA-like syndrome would develop in WT mice injected with anti-IL-10-receptor (αIL10R) neutralizing antibodies. In sJIA patients, we explored possible defects in IL-10 production by analysing plasma levels in parallel with pro-inflammatory parameters, and by examining cell-specific IL-10 production both *ex vivo* and after *in vitro* stimulation.

2. Results

2.1 Defective IL–10 production in a mouse model for sJIA

As previously described, CFA induces a sJIA-like disease in IFN-γ KO mice, while WT mice only develop a more subtle and transient inflammation. Pro-inflammatory cytokines are induced by CFA and are often higher (i.e. IL-6 and IL-17) in the diseased IFN-γ KO mice[101]. In the current work, the production of IL-10, an anti-inflammatory cytokine, was comprehensively studied.

In the sJIA mouse model, IL-10 production was analysed by different approaches. When mRNA levels of IL-10 were determined in spleen, lymph nodes, liver and lung tissue, lower levels were found in the diseased IFN-γ KO mice as compared to WT mice and the differences reached statistical significance in spleen and liver tissue (data not shown). To verify IL-10 production at the protein level, we first performed ELISA on plasma of the mice but failed to detect IL-10. However, when splenocytes and lymph node cells were stimulated *in vitro* with anti-CD3/CD28-antibody-coated beads (aCD3/CD28), an approach by which we previously demonstrated increased production of pro-inflammatory cytokines in the diseased IFN-γ KO mice[101], we found a clear-cut lower

production of IL-10 in CFA-challenged IFN-γ KO mice versus WT counterparts (Figure 1A). We subsequently focused on spleen tissue and analysed IL-10 protein production at the cellular level by intracellular flow cytometry in $CD11b^+$ cells (which comprises monocytes, macrophages, neutrophils and dendritic cells), $CD4^+$ effector and regulatory T cells ($CD4^+$ T_{eff} and T_{reg} cells), $CD8^+$ T cells, γδ T cells, B cells and natural killer (NK) cells. We observed a significant decrease in IL-10 production in diseased IFN-γ KO CFA mice compared to WT counterparts in $CD4^+$ T_{reg} cells, B cells and NK cells (Figure 1B). In the other cell types analysed, no differences between diseased and control mice were observed (Supplementary Figure 1A). When $IL\text{-}10^+$ cell types were plotted with respect to all live cells, B cells were found to be the most abundant IL-10-producers in this mouse model, followed by $CD11b^+$ cells and $CD4^+$ T cells (Figure 1C). Analysis of the phenotype of these IL-10-producing B cells revealed that they cannot be assigned to one specific B cell subset, but are mainly divided across the follicular B cells, marginal zone B cells and transitional B cells type 1 (data not shown).

Next, we evaluated whether IFN-γ affects the amount of IL-10 produced per $IL\text{-}10^+$ cell, by taking the median fluorescence intensity (MFI) of IL-10 in $IL\text{-}10^+$ cells (Supplementary Figure 1B). In $IL\text{-}10^+$ B cells, the MFI of IL-10 was significantly lower in IFN-γ KO mice compared to WT mice, both in naïve conditions and after CFA injection. Also in $CD4^+$ T_{reg} cells, the amount of IL-10 produced by CFA-injected IFN-γ KO mice was significantly lower compared to their WT counterparts. In the other cell types analysed, no differences between diseased and control mice could be observed. Thus, in the absence of IFN-γ, B cells and $CD4^+$ T_{reg} cells of CFA-injected mice produce less IL-10 per $IL\text{-}10^+$ cell.

To further clarify the link between the absence of IFN-γ and a decreased IL-10 production in the CFA-injected mice, we stimulated splenocytes with aCD3/CD28 in the presence of two concentrations of IFN-γ (150 ng/ml and 300 ng/ml) (Supplementary Figure 1C). However, no effect on IL-10 production could be observed, suggesting that the defective IL-10 production is not a direct result of the absence of IFN-γ. In addition, we stimulated enriched splenic $CD4^+$ T cells with aCD3/CD28 in the absence and presence of IFN-γ (Supplementary Figure 1D). Here, only very small differences could be observed between CFA-injected WT and IFN-γ KO

mice, with no effect of exogenous IFN-γ addition. Together, these data show that the impaired IL-10 production by aCD3/CD28-stimulated splenocytes (Figure 1C) cannot be rescued by the addition of IFN-γ and allow us to hypothesize that not $CD4^+$ T cells, but other cell types (such as B cells) might be responsible for the observed IL-10 defect in total splenocytes.

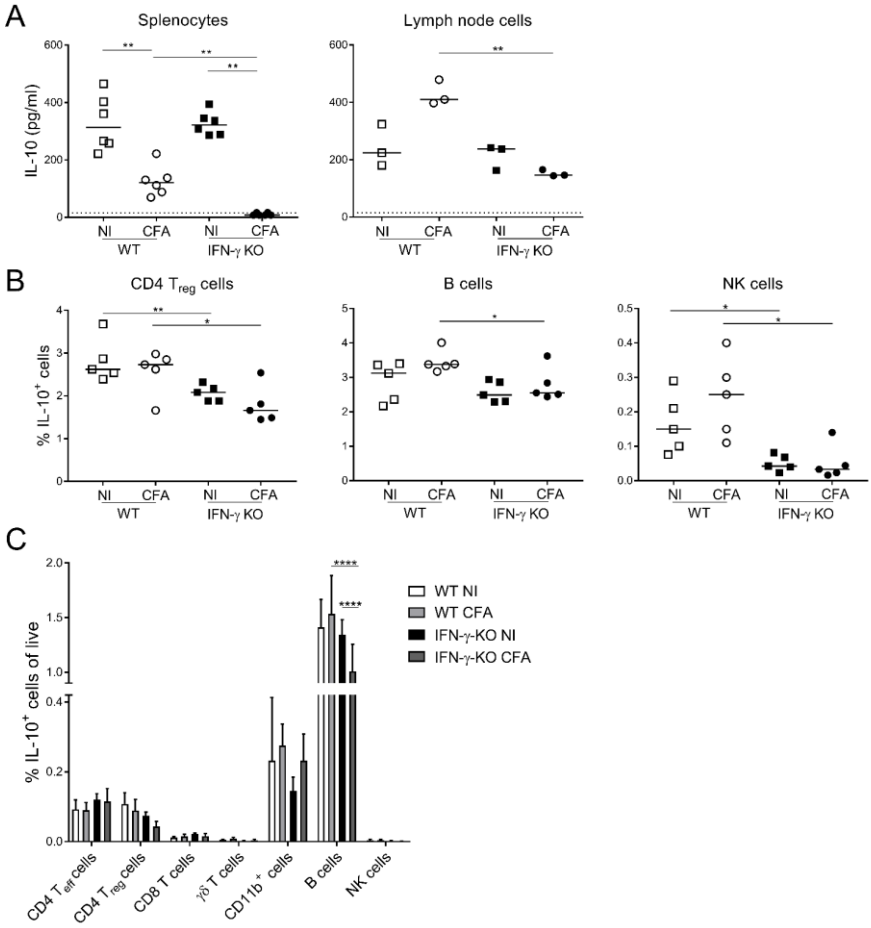


Figure 1: IL-10 production in a sJIA mouse model. *(A) IL-10 levels in supernatants of splenocytes (left) and lymph node cells (right) obtained from CFA-challenged WT mice (open circles) and IFN-γ KO mice (black circles) at day 21 post immunisation, and naïve (NI) littermates (squares) stimulated for 72 hours with anti-CD3/CD28-antibodies. IL-10 was measured in the supernatant by ELISA, dotted line*

*represents the lowest detection limit. (B) IL-10 protein production measured in the spleen by intracellular flow cytometry in CD4$^+$ T_{reg} cells (CD3$^+$ CD4$^+$ Foxp3$^+$), B cells (CD3$^-$ CD19$^+$) and NK cells (CD3$^-$ CD122$^+$ CD49b$^+$) from NI and CFA-challenged WT and IFN-γ KO mice. Data show the percentage IL-10$^+$ cells in the specific cell populations. Representative flow cytometry dot plots are depicted in Supplementary Figure 2. (B) IL-10 protein production measured in the spleen by intracellular flow cytometry in CD4$^+$ T_{eff} cells (CD3$^+$ CD4$^+$ Foxp3$^-$), CD4$^+$ T_{reg} cells (CD3$^+$ CD4$^+$ Foxp3$^+$), CD8$^+$ T cells (CD3$^+$CD8$^+$), γδ T cells (CD3$^+$ Foxp3$^-$ γδTCR$^+$), CD11b$^+$ cells, B cells (CD3$^-$ CD19$^+$) and NK cells (CD3$^-$ CD122$^+$ CD49b$^+$). Data show the percentage IL-10$^+$ cells with respect to all live cells. Dots represent individual mice, with median. Bars show median with range (n=5). Results are representative of 2 (B, C) or 3 (A, lymph node cells) different experiments or are from 2 independent experiments (A, splenocytes). * $p < 0.05$, ** $p < 0.01$; Kruskal-Wallis followed by Mann-Whitney U test. **** $p < 0.0001$; Two-Way ANOVA followed by Tukey's multiple comparisons test.*

2.2 IL-10 neutralisation in CFA-injected WT mice results in sJIA-like symptoms

Our results provide evidence for a defective IL-10 production in diseased CFA-injected IFN-γ KO mice as compared to their WT counterparts. In a next set of experiments, we investigated whether the lower IL-10 production may underlie sJIA disease development in this mouse model, by injecting CFA-challenged WT mice with αIL10R neutralising antibodies. sJIA-like symptoms and laboratory parameters were recorded and are shown in Figure 2.

First of all, αIL10R injection resulted in weight loss, with an earlier onset than seen in IFN-γ KO mice (Figure 2A). Furthermore, a significant splenomegaly and lymphadenopathy was seen in CFA-challenged αIL10R-treated WT mice, similar to CFA-challenged IFN-γ KO mice (Figure 2B). Two mice of the αIL10R group with a highly increased spleen weight (Figure 2B, grey triangles) also developed anaemia (Figure 2C, grey triangles), which was clinically evident by their pale ears, an sJIA feature usually not observed in WT mice[101]. Other haematological abnormalities typical of sJIA, i.e. thrombocytosis and neutrophilia, were demonstrated in αIL10R-injected mice (Figure 2C). In part of the mice, the immature character of red and white blood cells was analysed (Figure 2D).

CFA-injected IFN-γ KO mice showed an increase in immature TER119^{+}CD71^{+} RBC (Figure 2D & Avau *et al.* 2014[101]), which was also visible in the anaemic αIL10R-treated WT mouse (Figure 2D). The percentage of immature CD34^{+} WBC, determined in the spleen, was equally increased in IFN-γ KO CFA and WT CFA mice treated with αIL10R.

To further determine the parallels between αIL10R-treated WT mice and IFN-γ KO mice subjected to CFA, a flow cytometric analysis was performed on splenocytes. Most CFA-induced changes, i.e. decreased B cells, T cells and NK cells and increased neutrophils, were observed in WT mice after CFA challenge, and were more pronounced in αIL10R-treated WT mice (Figure 2E). An in-depth analysis of disease mechanisms was performed in part of the mice, by measuring IL-6 levels in the plasma and IL-17-producing capacity of lymph node cell cultures stimulated with aCD3/CD28. Although the inflammatory cytokines were elevated in αIL10R-treated mice, levels did not reach those of IFN-γ KO mice after CFA (Figure 2F).

Since the effect of αIL10R treatment was abolished after 2-3 weeks of injections, arthritis, which is a late feature that typically occurs after day 25 post injection, could not be observed. As an alternative, we determined the percentage of osteoclast precursor cells in the spleen, which can be considered as a parameter to predict a possible development of arthritis[162,163]. To this end, mice were euthanized at day 16 and osteoclast precursor cells (defined as CD3^{-}B220^{-}CD11b^{+}CD115^{+} cells[162,163]) were studied by flow cytometry (Figure 3A). In the CFA-injected IFN-γ KO mice and part of the αIL10R-treated mice, the percentage of osteoclast precursor cells was increased compared to CFA-injected WT mice (not significant for αIL10R treatment). In addition, induction of osteoclast generation via stimulation with receptor activator of nuclear factor κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) was analysed to confirm this observation (Figure 3B). In line with the increase in osteoclast precursors, a trend towards increased numbers of multinucleated osteoclasts was observed in CFA-injected αIL10R-treated mice and IFN-γ KO mice, indicating that these mice are more prone to develop arthritis (representative image of osteoclasts in Figure 3C).

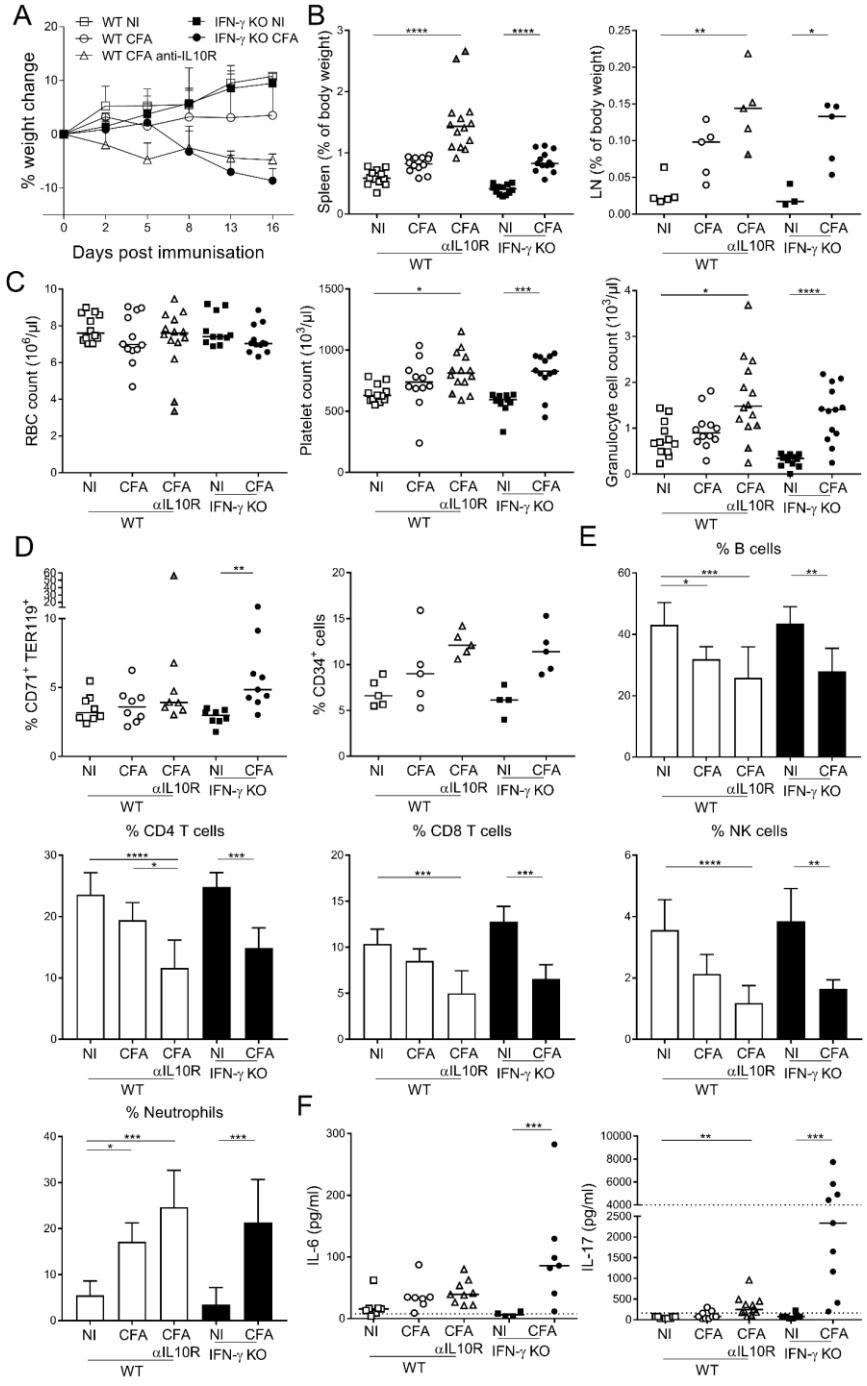

A
WT NI
WT CFA
WT CFA anti-IL10R
IFN-γ KO NI
IFN-γ KO CFA
% weight change
Days post immunisation
B
Spleen (% of body weight)
LN (% of body weight)
C
RBC count (10^6/μl)
Platelet count (10^3/μl)
Granulocyte cell count (10^3/μl)
D
% CD71+ TER119+
% CD34+ cells
E
% B cells
% CD4 T cells
% CD8 T cells
% NK cells
% Neutrophils
F
IL-6 (pg/ml)
IL-17 (pg/ml)
NI
CFA
αIL10R
WT
IFN-γ KO

Figure 2: Effect of anti-IL-10R-antibodies in CFA-challenged WT mice. *WT mice were injected with CFA together with PBS or antibodies against the IL-10R (αIL10R; 200 µg/mouse). CFA-challenged IFN-γ KO mice and naïve (NI) mice were included. (A) Mean ± SD percentage change in body weight (WT, n=3; IFN-γ KO, n=4). (B) Weight of spleen and lymph nodes expressed as a percentage of total body weight. (C) Red blood cell, platelet and granulocyte counts in blood samples. (D) Flow cytometric analysis of immature RBC (TER119⁺CD71⁺) in blood and immature WBC (CD34⁺) in spleen. (E) Flow cytometric profile of splenocytes: the percentage of B cells (CD3⁻CD19⁺), CD4⁺ and CD8⁺ T cells (CD3⁺CD4⁺; CD3⁺CD8⁺), NK cells (CD3⁻CD122⁺CD49b⁺) and neutrophils (CD11b⁺Ly6G⁺) is shown relative to single viable cells (WT CFA αIL10R, n=11; other subgroups, n=9). (F) IL-6 levels (pg/ml) in plasma and IL-17 levels (pg/ml) in supernatants from anti-CD3/CD28-stimulated lymph node cells, cultured for 72 hours, measured by ELISA (dotted lines represent the detection limits). Results are representative of 3 experiments (A) or are from 1 (B-right, D-right, F-left), 2 (D-left, E, F-right) or 3 (B-left, C) independent experiments in which mice were euthanized between days 16 and 19 p.i. Each symbol represents an individual mouse; with median. Bars represent median with range. Grey triangle: clinical anaemic mouse of the αIL10R-treated group. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001; Kruskal-Wallis followed by Mann-Whitney U test.*

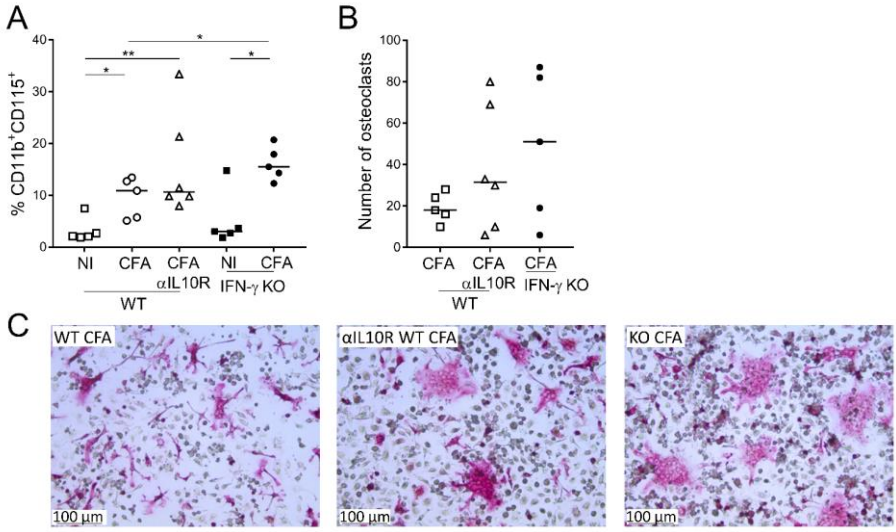


Figure 3: Analysis of arthritis in αIL10R-treated mice. *(A) The percentage of osteoclast precursor cells was determined in the spleen by flow cytometry as CD3⁻B220⁻CD11b⁺CD115⁺ cells. (B) The number of RANKL/M-CSF-induced TRAP⁺ multinucleated osteoclasts is shown. (C) Representative photographs of TRAP⁺*

*multinucleated cells (pink) of a CFA-injected WT mouse, αIL10R-treated CFA-injected WT mouse and a CFA-injected IFN-γ KO mouse. Results are from 1 experiment in which mice were euthanized between days 16 and 19 p.i. Each symbol represents an individual mouse; with median. * $p < 0.05$, ** $p < 0.01$; Kruskal-Wallis followed by Mann-Whitney U test.*

To summarize, neutralisation of IL-10 signalling in WT mice resulted in more pronounced sJIA-characteristics, with striking parallels between CFA-challenged IFN-γ KO mice and αIL10R-treated WT mice, indicating that endogenous IL-10 plays a protective role in sJIA disease development in this mouse model.

2.3 Insufficient IL-10 production in plasma of sJIA patients relative to their inflammatory status

In literature, higher IL-10 plasma levels in sJIA patients as compared to healthy controls have been reported[36,41]. However, increased IL-10 levels in sJIA patients versus healthy controls may be part of a homeostatic response and do not rule out a potential imbalance of IL-10 relative to pro-inflammatory cytokines. With this in mind, we used a different strategy to analyse the potential imbalance in IL-10, by analysing plasma IL-10 levels in relation to known markers of inflammation in sJIA. Levels of IL-10 and the inflammatory molecules CRP, IL-6 and IL-18 were analysed in plasma of healthy controls (HC), active sJIA patients and corresponding inactive sJIA patients after treatment (Figure 4A and B). In the active sJIA group, only 2 out of 18 sJIA patients showed an increased IL-10 production, while all other patients (active and inactive) had levels comparable to those in HC (Figure 4A). On the other hand, we observed very high levels of CRP and IL-6, general markers for systemic inflammation[164–166], in patients with active sJIA. We also confirmed that IL-18, a cytokine very prominent in sJIA[60], was strongly increased in patients with active disease (Figure 4B). Of note, although inactive sJIA patients had very low CRP levels, comparable to HC, IL-6 and IL-18 levels were still moderately increased (Figure 4B).

Together, these data show an increased production of pro-inflammatory cytokines in active sJIA patients, while IL-10 production is not increased and comparable to levels in HC. The data are indicative for an imbalance

between pro- and anti-inflammatory mediators in sJIA patients, which is visualized in Figure 4C showing the ratios of IL-10 to the respective inflammatory mediators.

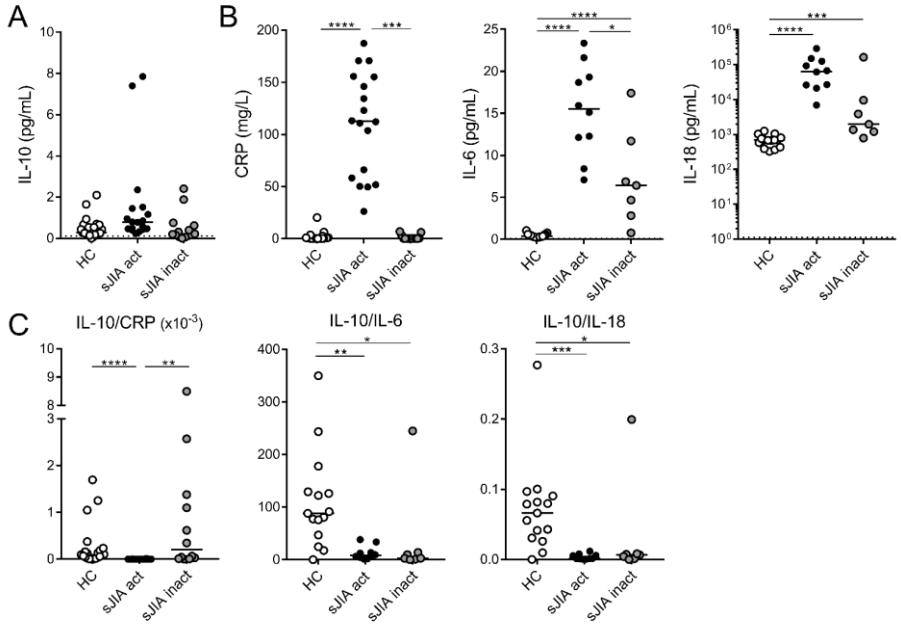


Figure 4: Levels of IL-10, CRP, IL-6 and IL-18 in plasma of healthy controls (HC) and patients. *Levels of IL-10 (A) and of CRP, IL-6 and IL-18 (B) in plasma of HC (n=15; for IL-10 and CRP: n=21; open circles) and patients with active (n=10; for IL-10 and CRP: n=18; black circles) and inactive (n=8; for IL-10 and CRP: n=11; grey circles) sJIA. (C) The ratio of IL-10/CRP, IL-10/IL-6 and IL-10/IL-18 is shown for the different groups. Proteins in plasma were measured with MSD multi-array technology, dotted lines show the lowest detection limits. Symbols represent individual subjects, with median. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001; Kruskal-Wallis followed by Mann-Whitney U test or Wilcoxon matched-pairs signed rank test. HC = age-matched healthy controls, act = active, inact = inactive.*

2.4 Cell-specific defects in IL-10 production in active and inactive sJIA patients

We searched for cell-specific defects in IL-10 production by quantifying IL-10 *ex vivo* in PBMCs from healthy controls, active sJIA patients and

corresponding inactive, treated sJIA patients using intracellular flow cytometry (Figure 5). We investigated IL-10 production by T cells ($CD3^+$), NK cells ($CD3^-CD56^+$), monocytes ($CD14^+$) and B cells ($CD19^+$). Within the T cells, NK cells and monocytes, we could not detect any differences in IL-10 production between the different groups (Figure 5). Also in the rest fraction ($CD3^-CD19^-CD14^-CD56^-$), no profound differences could be observed (data not shown). In contrast, in B cells, we observed a non-significant decrease in IL-10 production by sJIA patients. Blair *et al.* (2010) showed that within the B cell fraction of healthy donors, $CD19^+CD24^{hi}CD38^{hi}$ cells possess regulatory capacities and are the most important IL-10-producers[139]. Also other studies point to the regulatory and IL-10-producing capacities of this B cell subset[167–171]. Interestingly, when we looked in more detail at IL-10 production by this specific B cell subtype, a significantly impaired IL-10 production was seen in sJIA patients as compared to HC (Figure 5).

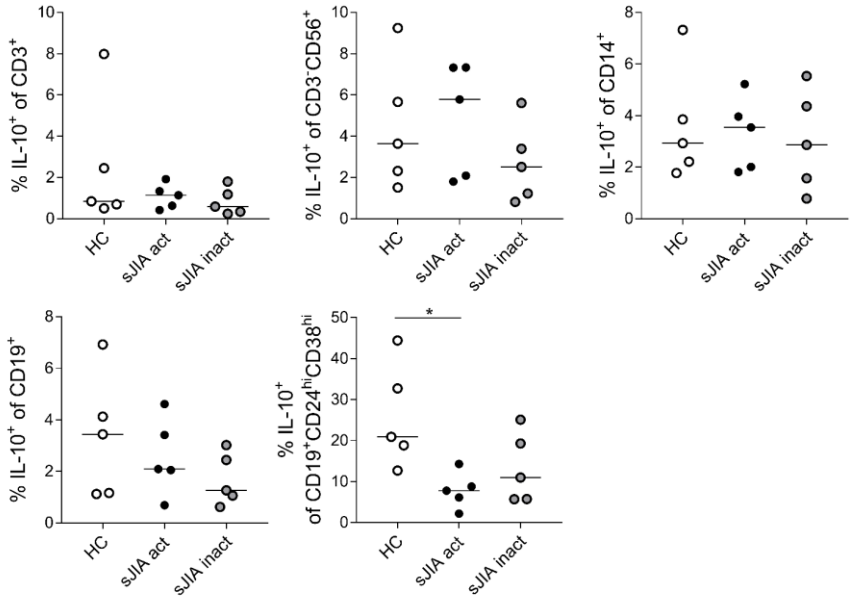


Figure 5: Ex vivo IL-10 production by PBMCs of healthy controls (HC), active and inactive sJIA patients. *IL-10 production was determined ex vivo in PBMCs of HC, active and corresponding inactive sJIA patients with intracellular flow cytometry, in T cells ($CD3^+$), NK cells ($CD3^-CD56^+$), monocytes ($CD14^+$), B cells ($CD19^+$) and regulatory B cells ($CD19^+CD24^+CD38^+$), representative flow cytometry*

*dot plots are depicted in Supplementary Figure 3. * p < 0.05; Kruskal-Wallis followed by Mann-Whitney U test. HC = age-matched healthy controls, act = active, inact = inactive.*

2.5 Defective IL-10 production in B cells from sJIA patients after *in vitro* stimulation

We also studied the potential of PBMCs from healthy controls, active and inactive sJIA patients to produce IL-10 after stimulation with CpG oligodeoxynucleotides (CpG), LPS, aCD3/CD28 and IL-2+IL-12, triggers known to induce IL-10 production by B cells, monocytes, T cells and NK cells respectively[172–175]. After 48h, we measured the amount of IL-10 produced in the supernatant of the stimulated PBMCs (Figure 6A). Stimulation with CpG, a known inducer of IL-10 in B cells[172], elicited less IL-10 production in sJIA patients when compared to HC, yet only significant in the active sJIA patients. IL-10 production induced by the other triggers was unaltered between the different groups. To verify the cell type responsible for this lower IL-10 production after CpG stimulation, we performed intracellular flow cytometry of CpG-stimulated PBMCs. Here, the data revealed that – despite the high variation – B cells from both active and inactive sJIA patients produced significantly less IL-10 than HC cells after stimulation with CpG (Figure 6B). To confirm the defective IL-10 production by B cells, PBMCs were sorted in B cells, $CD4^+$ T cells, monocytes and NK cells and stimulated with CpG, aCD3/CD28, LPS and IL-2+IL-12, respectively (Figure 6C). We observed a trend towards a lower IL-10 production by CpG-stimulated B cells of sJIA patients compared to healthy controls, which was only minimally seen in other cell types analysed.

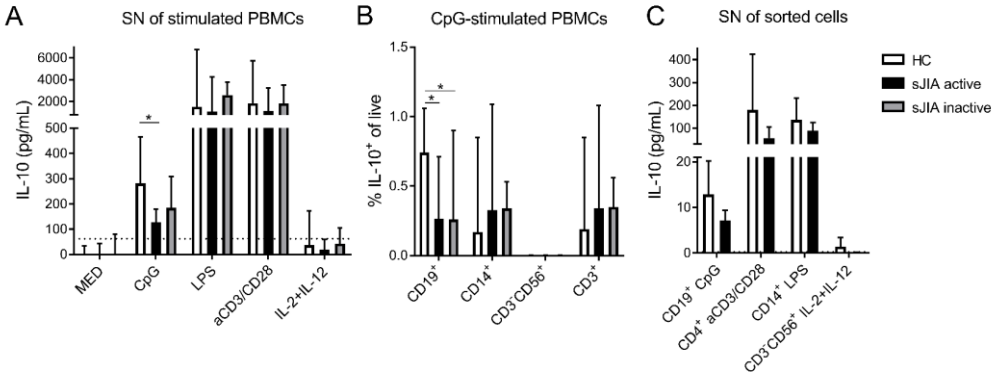


Figure 6: IL-10 production in supernatant and in CpG-stimulated PBMCs. *(A) IL-10 production in the supernatant (SN) of PBMCs from HC (n=5, white bars), active sJIA patients (n=4, black bars) and inactive sJIA patients (n=5, grey bars) stimulated with CpG, LPS, anti-CD3/CD28-antibodies or IL-2+IL-12 for 44h. (B) IL-10 was determined in B cells (CD19$^+$), monocytes (CD14$^+$), NK cells (CD3$^-$CD56$^+$) and T cells (CD3$^+$) by intracellular flow cytometry of PBMCs of HC (n=5), active (n=4) and inactive (n=5) sJIA patients stimulated with CpG for 48h. (C) IL-10 production in the supernatant of sorted B cells (CD19$^+$), CD4$^+$ T cells (CD3$^+$CD4$^+$), monocytes (CD14$^+$) and NK cells (CD3$^-$CD56$^+$) from HC (n=5) and active sJIA patients (n=3), stimulated with CpG, anti-CD3/CD28-antibodies, LPS and IL-2+IL-12 respectively. IL-10 was detected by ELISA (A) or MSD multi-array technology (C), dotted lines show the lowest detection limit. Bars show median with range. * $p < 0.05$; Two-Way ANOVA followed by Tukey's multiple comparisons test. HC = age-matched healthy controls, act = active, inact = inactive.*

3. Discussion

sJIA is thought to be caused by an excessive inflammatory immune reaction to a generally harmless trigger in predisposed children. We previously demonstrated that injection of IFN-γ KO mice with adjuvant containing heat-killed mycobacteria, leads to a chronic immune syndrome with clinical and pathological features reminiscent of sJIA, including a storm of pro-inflammatory cytokines[101]. Although the model is well recognized as a clinically relevant animal model of sJIA[52,176,177], its applicability may be disputed in view of the absence of documented IFN-γ mutations in sJIA patients. However, in the present study we demonstrated that IFN-γ KO mice, upon adjuvant challenge, acquire cell-

specific defects in IL-10, a key cytokine in anti-inflammatory immune responses. The adjuvant-induced IL-10 defects in IFN-γ KO mice are not merely a consequence of the disease but rather underlie disease pathogenesis as neutralisation of IL-10 in WT mice, which normally present with a transient syndrome, leads to a sJIA-like disease. In fact, as shown in Table 1, there is a striking similarity between disease development in CFA-injected IFN-γ KO mice and CFA-challenged WT mice in which IL-10 is neutralised. The data indicate that the susceptibility of IFN-γ KO mice to develop sJIA may – at least partially – be explained by acquired defects in IL-10 production. The development of sJIA in IFN-γ competent mice with neutralised IL-10 is in accordance with previous reports of low expression IL-10 polymorphisms in sJIA patients[18,19], and can therefore be considered as an alternative clinically relevant model.

The defective IL-10 production in CFA-injected IFN-γ KO mice was a rather unexpected observation, since IFN-γ is generally considered as an inhibitor of IL-10[47,102,144]. However, IFN-γ has been found to stimulate IL-10 production in Th1 cells and neutrophils, under certain *in vivo* conditions[178,179]. Furthermore, we recently observed that IFN-γ can increase IL-10 production upon stimulation of mouse and human B cells with the Toll-like receptor (TLR) 9 ligand CpG, an effect that could not be seen in other cell types or after stimulation with other TLR triggers (Imbrechts *et al.*, accepted manuscript[180]). Since CFA is an oil adjuvant with heat-killed mycobacteria, containing the TLR9 ligand CpG[181], it is intriguing to speculate that the defective IL-10 production by B cells in CFA-injected IFN-γ KO mice – as opposed to WT mice – may be conditioned by CpG. In addition to IL-10 defects in B cells and T_{reg} cells, we also observed an almost complete loss of IL-10 production by NK cells of IFN-γ KO mice (Figure 1), which may be in line with the general NK cell defects in the absence of IFN-γ (as originally described by Dalton *et al.*[182] and confirmed by Avau *et al.*[101]).

Table 1: Comparison of findings in CFA-challenged WT and IFN-γ KO BALB/c mice and WT mice treated with αIL10R

	CFA-injected mice		
	WT	**IFN-γ KO**	**WT αIL10R**
Clinical features			
Fever	-	-	-
Weight loss	±	+	+
Arthritis	±	+°	ND•
Skin rash	-	+°	+°
Splenomegaly	+	+	+
Lymphadenopathy	+	+	+
Hematologic features			
Neutrophilia	+	+	+
Thrombocytosis	+	+	+
Lymphopenia	±	+	+
Anaemia	-	+°	+°
Decreased haemoglobin	-	+°	+°
Immature RBC	-	+	±
Immature WBC	+	+	+
Immune/inflammatory features			
Increased IL-6	±	+	±
Increased IL-17	±	+	+*
Decreased NK cell numbers	±	+	+

*Features were graded as follows: + = present, ± = transient (weight loss), rarely present (arthritis), or only slightly different from non-injected mice, and - = absent. *not to the same extent as seen in IFN-γ KO mice; °in subsets of mice;• αIL10R-treated WT mice were euthanized between day 17 and day 19 p.i.. Since arthritis typically occurs later in the disease course (i.e. after day 25), it could not be determined in these experiments. Part of the table (WT versus IFN-γ KO) is adapted from Avau et al. 2014. For WT and IFN-γ KO mice, a summary of more than 10 experiments was used to compose this table.*

In previous studies, IL-10 levels in plasma of sJIA patients were analysed compared to healthy controls, showing an increased IL-10 production in sJIA patients[36,41]. However, since IL-10 is an important anti-inflammatory cytokine that serves to downregulate inflammatory processes, it seems reasonable that IL-10 production increases together with pro-inflammatory mediators in sJIA patients. Therefore, it might be more

informative to analyse IL-10 levels compared to pro-inflammatory markers in these patients. In contrast to previous publications, we did not observe an increased IL-10 production in plasma of sJIA patients when compared to healthy controls[36,41]. Levels of CRP, IL-6 and IL-18 on the other hand, were increased in patients with active sJIA, as reported previously[1,36,41,60]. The fact that the increase in pro-inflammatory cytokine production is not accompanied by an increased IL-10 production, points to an imbalance between pro- and anti-inflammatory mediators in sJIA patients and suggests that sJIA patients are not capable of producing sufficient IL-10 to adequately regulate the amount of inflammation they experience.

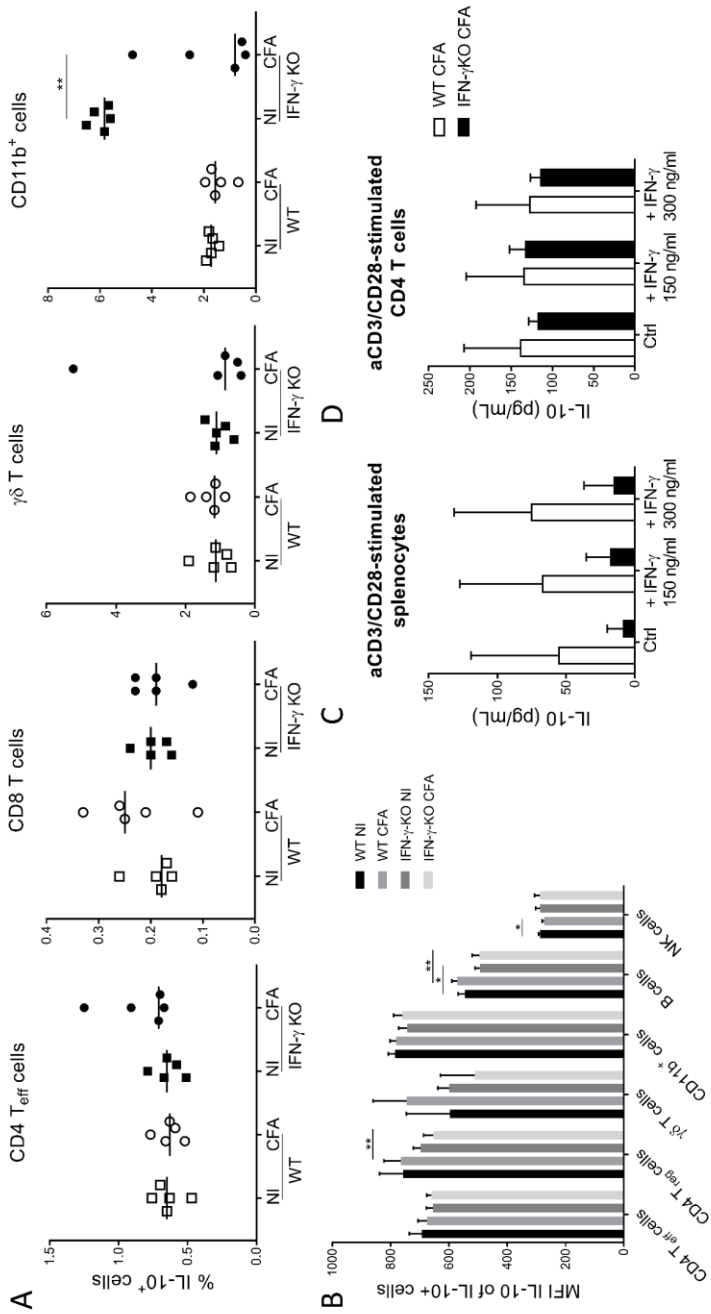
Human peripheral B cells can be divided in different subsets, mainly depending on their surface expression of CD19, CD24, CD38 and CD27. According to these markers, three main B cell subsets can be identified: immature transitional B cells ($CD19^{+}CD24^{hi}CD38^{hi}$), naïve B cells ($CD19^{+}CD24^{int}CD38^{int}$) and memory B cells ($CD19^{+}CD27^{+}$). To date, human B cells with regulatory functions have been essentially described within the $CD24^{hi}CD38^{hi}$ B cell subset, which is therefore seen as the regulatory B (B_{reg}) cell subset[170,183,184]. $CD24^{hi}CD38^{hi}$ B cells are able to produce IL-10 and thereby suppress $CD4^{+}CD25^{-}$ T cell proliferation as well as the release of IFN-γ and TNF-α by these cells. In addition, they inhibit naïve T cell differentiation into Th1 and Th17 cells and stimulate the conversion of $CD4^{+}CD25^{-}$ T cells into regulatory T cells, partially through the production of IL-10[171]. In active sJIA patients, we observed a significantly lower amount of IL-10-producing B_{reg} cells compared to healthy controls, which is in line with our observation of a defective IL-10 production by B cells in the mouse model. A defective IL-10 production by $CD24^{hi}CD38^{hi}$ B cells in sJIA patients may account for the enrichment of both Th1 and Th17 cell populations in the blood of sJIA patients, which was observed both in active and inactive sJIA patients[55]. Correspondingly, we also observed a lower number of IL-10-producing $CD24^{hi}CD38^{hi}$ B cells in PBMCs of inactive sJIA patients, although not significantly. A defective IL-10 production by $CD24^{hi}CD38^{hi}$ B cells has previously been reported in patients with systemic lupus erythematosus (SLE) and psoriasis, after stimulation with CD40L or CpG and CD40L respectively. The total number of $CD24^{hi}CD38^{hi}$ B cells was unaltered in SLE patients and even increased in patients with psoriasis[139,183]. In sJIA patients, we observed

similar numbers of $CD24^{hi}CD38^{hi}$ B cells compared to healthy controls (data not shown). Also the total percentage of B cells was similar in all groups (data not shown), conform to the study of Fall *et al.*, but in contrast to Macaubas *et al.*, who reported decreased B cell numbers in sJIA[39,74].

In line with the observations *ex vivo*, B cells from active and inactive sJIA patients produced less IL-10 compared to controls after *in vitro* stimulation with CpG. Unmethylated CpG motifs are prevalent in bacterial and viral genomic DNA, and trigger an immune response by activating TLR9[172]. The reduced IL-10-producing response of sJIA B cells after triggering of TLR9 with CpG might be partially responsible for the inability of sJIA patients to dampen the ongoing inflammation. Indeed, it is suggested that sJIA is caused by an excessive inflammatory reaction to a generally harmless trigger, which often contains CpG motifs, and that inadequate downregulation of immune activation is central to sJIA disease development[8].

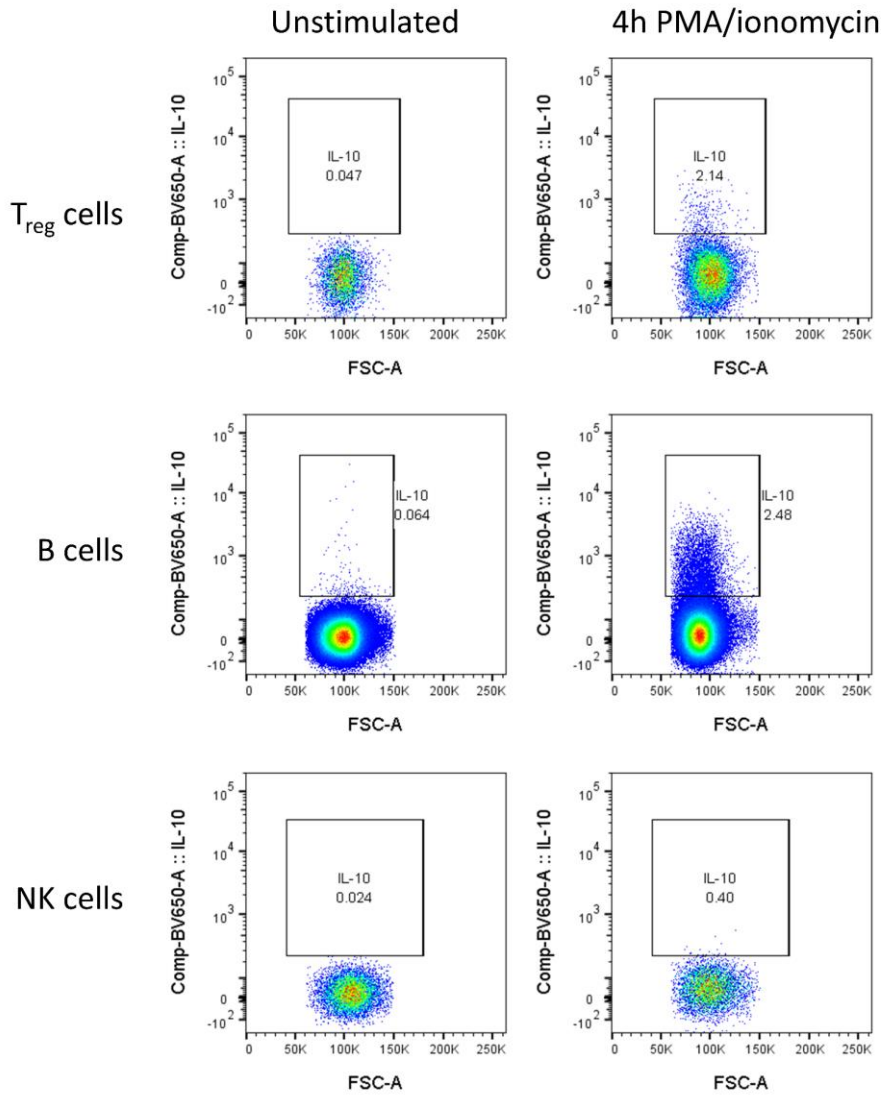
In this study, we provided evidence for a defective anti-inflammatory IL-10 response in sJIA, both in the mouse model and in patients. The IL-10 neutralisation experiments in the mouse model endorse the hypothesis that these IL-10 defects may underlie disease development, rather than being a consequence of the disease. sJIA is considered an autoinflammatory disease, and therefore most attention has been given to the role of innate immune cells in sJIA pathogenesis. To our knowledge, we are the first to elaborate on the importance of regulatory B cells in sJIA patients, and showed an impaired IL-10 production by these cells. Our data allow to speculate that this defect in IL-10 production plays a role in disease development.

4. Supplementary material

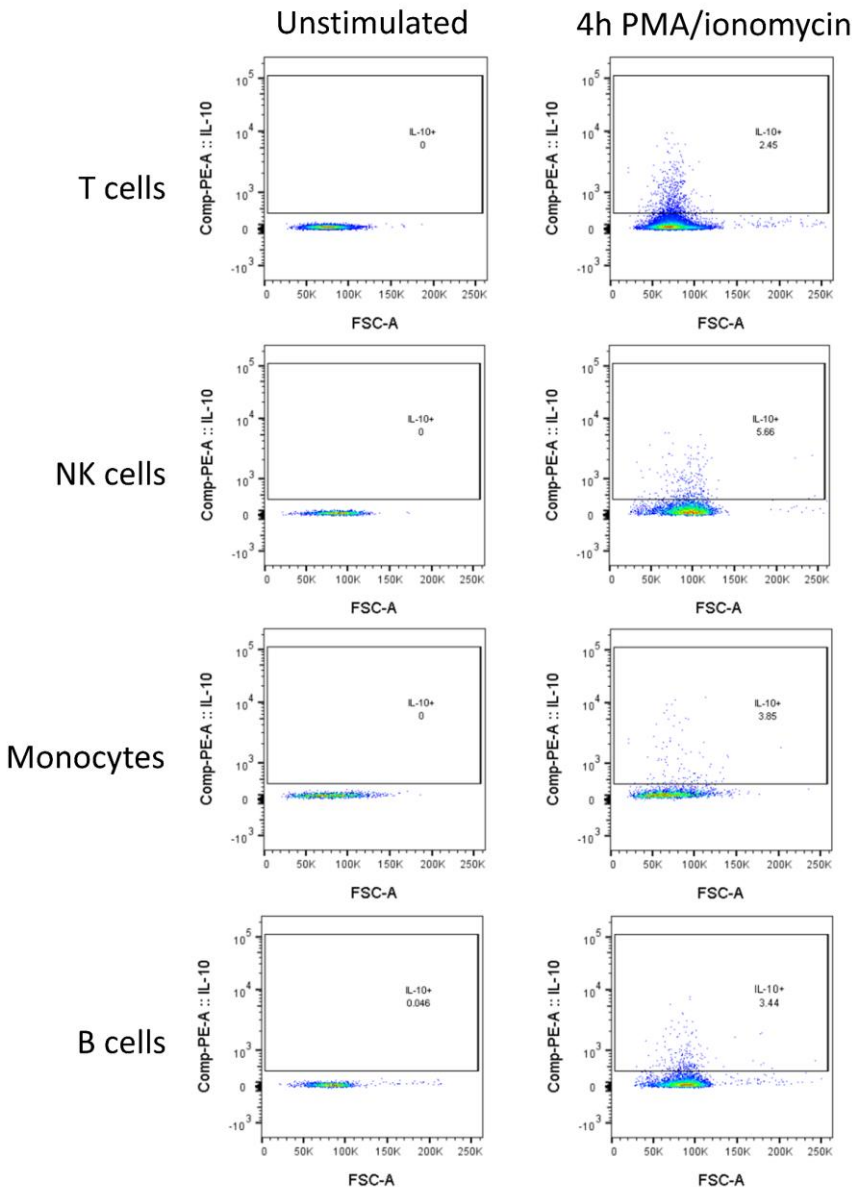


Supplementary Figure 1. *(A) IL-10 protein production measured in the spleen by*

*intracellular flow cytometry in CD4$^+$ Teff cells (CD3$^+$ CD4$^+$ Foxp3$^-$), CD8$^+$ T cells (CD3$^+$ CD8$^+$), γδ T cells (CD3$^+$ Foxp3$^-$ γδTCR$^+$) and CD11b$^+$ cells from CFA-challenged WT mice (open circles) and IFN-γ KO mice (black circles) at day 21 post immunization, and NI littermates (squares). Data show the percentage IL-10$^+$ cells in the specific cell populations. (B) Median fluorescence intensity (MFI) of IL-10 within IL-10$^+$ cells was determined in CD4$^+$ Teff cells (CD3$^+$ CD4$^+$ Foxp3$^-$), CD4$^+$ Treg cells (CD3$^+$ CD4$^+$ Foxp3$^+$), γδ T cells (CD3$^+$ Foxp3$^-$ γδTCR$^+$), CD11b$^+$ cells, B cells (CD3$^-$ CD19$^+$) and NK cells (CD3$^-$ CD122$^+$ CD49b$^+$) in naïve and CFA-injected WT and IFN-γ KO mice. Total splenocytes (C) and enriched splenic CD4$^+$ T cells (D) were stimulated with anti-CD3/CD28-antibodies (aCD3/CD28) in the absence or presence of IFN-γ (150 ng/ml or 300 ng/ml) for 72h, followed by analysis of IL-10 production in the supernatant by ELISA. Dots represent individual mice, with median. Bars show median with range, n = 5. Results are representative for 2 (A, B) or 1 (C, D) independent experiments. * $p < 0.05$; ** $p < 0.01$; Kruskal-Wallis followed by Mann-Whitney U test.*



Supplementary Figure 2. *Representative flow cytometry dot plots for intracellular detection of IL-10 in murine T_{reg} cells ($CD3^+CD4^+Foxp3^+$), B cells ($CD3^-CD19^+$) and NK cells ($CD3^-CD122^+CD49b/DX5^+$). Cells were stimulated for 4h in the presence of PMA, ionomycin and brefeldin A, followed by intracellular staining. Unstimulated cells were used as a gating control*



Supplementary Figure 3. *Representative flow cytometry dot plots for intracellular detection of IL-10 in human T cells (CD3⁺), NK cells (CD3⁻ CD56⁺), monocytes (CD14⁺) and B cells (CD19⁺). Cells were stimulated for 4h in the presence of PMA, ionomycin and brefeldin A, followed by intracellular staining. Unstimulated cells were used as a gating control.*

Supplementary Table 1: Patient characteristics

	Active sJIA			Inactive sJIA	
	plasma	**PBMCs**	**sort**	**plasma**	**PBMCs**
Male/Female No.	5,13	3,2	1,2	4,7	3,2
Age, median (range) in years	4,5 (1-16)	3 (1-12)	5 (3-12)	6 (2-16)	3 (2-13)
Clinical features					
Active arthritis	18/18	5/5	3/3	1/11	0/5
Fever	18/18	5/5	3/3	0/11	0/5
Rash	14/18	5/5	3/3	0/11	0/5
Lymphadenopathy	2/18	1/5	0/3	1/11	0/5
Hepato- or splenomegaly	6/18	1/5	0/3	0/11	0/5
Laboratory features					
WBC count (x10^9/l)	20,72 (4,77-30,3)	22,74 (20,69-30,23)	22,74 (20,75-30,23)	7,82 (3,67-22,85)	7,82 (5,25-22,85)
Neutrophil count (x10^9/l)	15,4 (2,9-27)	18,1 (14,4-26,9)	18,1 (16,4-26,9)	2,7 (1,3-15)	2,7 (1,3-12,5)
RBC count (x10^{12}/l)	3,82 (2,86-4,92)	3,89 (3,15-4,11)	4,07 (3,89-4,11)	4,56 (4,12-4,91)	4,37 (4,12-4,64)
Hemoglobin (g/dl)	10,2 (7,3-12,9)	10,6 (7,9-11)	10,9 (10,6-11)	12,4 (9,9-13,1)	12,5 (9,9-12,9)
Platelet count (x10^9/l)	547,5 (210-894)	578 (380-757)	438 (380-578)	342 (172-759)	369 (280-759)
C-reactive protein (mg/l)	110 (25,1-341,6)	163 (103,4-208,8)	116,7 (103,4-169,7)	0,3 (0,3-12,3)	0,3 (0,3-2,9)
Aspartate transaminase (U/ml)	31 (14-51)	25 (14-31)	22,5 (14-31)	29 (19-43)	28 (19-43)
Alanine transaminase (U/ml)	15,5 (3-38)	10 (7-15)	8 (7-13)	16 (9-27)	21 (13-27)
Lactate dehydrogenase (U/ml)	370 (196-607)	237 (207-373)	290 (207-373)	267 (193-435)	265 (193-317)
Ferritin (µg/l)	865 (49-4469)	975 (476-3959)	513 (476-2425)	ND	ND
Treatment					
NSAID	8/18	3/5	2/3	2/11	0/5
Oral corticosteroids	2/18	0/5	0/3	2/11	2/5
Methotrexate	2/18	0/5	0/3	5/11	2/5
Anti-IL-1	3/18	0/5	0/3	1/11	0/5
Anti-IL-6	0/18	0/5	0/3	6/11	4/5

For age and laboratory features, median with minimal an maximal values are given. NSAID = non-steroidal anti-inflammatory drugs

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CHAPTER 5

IFN-γ stimulates CpG-induced IL-10 production in B cells via p38 and JNK signalling pathways

RESEARCH ARTICLE

Manuscript in press:

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Abstract

The production of IL-10, a potent immunosuppressive cytokine, must be strictly regulated to ensure a balanced immune response. IFN-γ, a key cytokine in multiple immune processes and pathologies, is known as an inhibitor of IL-10 production by monocytes and macrophages, but also has some regulatory functions. In the present study, we explored the role of IFN-γ on Toll-like receptor (TLR)-induced IL-10 production in murine peritoneal and spleen cells and in human peripheral blood mononuclear cells. IFN-γ inhibited IL-10 production induced by TLR2, TLR3, TLR4 and TLR7/8 agonists, but stimulated IL-10 production when cells were triggered with CpG oligodeoxynucleotides, a specific TLR9 agonist. The stimulatory effect of IFN-γ on TLR9-induced IL-10 was restricted to B cells. In line with the increased IL-10, B cells stimulated with CpG and IFN-γ profoundly inhibited CD4 T cell proliferation. Further research into the mechanisms involved, revealed that the mitogen-activated protein kinases p38 and JNK are essential players in this stimulatory effect, and that the phosphatase MKP1 – an inhibitor of p38 and JNK activity – is downregulated after combined stimulation with IFN-γ and CpG. Our data may represent a novel immunoregulatory role of IFN-γ in B cells after triggering of TLR9, by stimulating IL-10 production.

1. Introduction

A robust immune response is crucial in protecting the host against harmful threats. On the other hand, appropriate control of the immune reaction is equally important to avoid excessive inflammation and organ damage. To fulfil the latter task, our immune system is equipped with regulatory circuits provided by regulatory cells and anti-inflammatory cytokines. Among these cytokines, interleukin (IL)-10 is undoubtedly one of the best-known immune messengers with numerous anti-inflammatory capacities (recently reviewed in[46,47]). For example, IL-10 downmodulates antigen presentation and T cell activation and inhibits the secretion of many inflammatory cytokines and chemokines[48,49,109,185]. Defective IL-10 production results in an amplified immune response, provoking excessive inflammation and immunopathology[48,111]. On the other hand, an excess of IL-10 is critically involved in persistence of bacteria and viruses[186]. Strict regulation of IL-10 production is thus essential to ensure a balanced immune response.

IL-10 can be produced by a variety of cells in response to different triggers. Although initially described as a cytokine produced by T helper (Th) 2 cells, it became clear that also other T cell subtypes, monocytes, macrophages, eosinophils, neutrophils, dendritic cells (DC), natural killer (NK) cells and B cells can produce IL-10[47,49,121,187]. B cells are generally thought to enhance inflammatory immune responses, but more recently, it became clear that IL-10-producing B cells (often designated as B10 cells) also play a role in immunoregulation[188].

In B cells and most cells within the innate immune system, IL-10 can be induced following stimulation by pathogen-derived products via pattern recognition receptors (e.g. Toll-like receptors (TLR))[47,121,130,189]. TLRs are transmembrane proteins that consist of an extracellular domain with leucine-rich repeat motifs, and an intracellular Toll/IL-1R (TIR) domain. In humans, 10 TLR family members have been identified. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are found on the cell surface, while TLR3, TLR7, TLR8 and TLR9 are present intracellularly. Each TLR recognizes specific pathogen-associated molecular patterns (PAMP), such as conserved molecules of the microbial cell wall, flagella or nucleic acids[190]. In addition to PAMP, TLRs can also be activated by endogenous stimuli, i.e. danger-associated molecular patterns, stimulating the immune system

in response to tissue damage[190–192]. TLRs are expressed on many cell types, e.g. monocytes, macrophages, conventional and plasmacytoid DCs, neutrophils, NK cells and B cells, although not all cell types express all TLRs[193]. After binding of the TLR ligand to its receptor, TIR-domain-containing adaptor molecules, such as myeloid differentiation primary-response protein 88 and TIR-domain-containing adaptor protein inducing IFN-β, initiate mitogen-activated protein kinase (MAPK) or NF-κB signalling cascades resulting in cytokine production[116].

Interferon-γ (IFN-γ) is often viewed as a pro-inflammatory cytokine as it stimulates the immune response against pathogens and malignant cells by a number of cellular and molecular pathways[62]. In line with its pro-inflammatory effects, IFN-γ reportedly is an important negative regulator of TLR-induced IL-10 production. The inhibition of TLR-induced IL-10 by IFN-γ has been described *in vitro* in monocytes, macrophages and myeloid or bone marrow-derived DCs, after stimulation of TLR2, TLR4 or TLR9[47,103–105,107]. In macrophages, IFN-γ inhibits TLR-induced IL-10 production by blocking the action of the MAPKs extracellular signal-regulated kinase 1/2 (ERK1/2) and p38[102]. Next to directly inhibiting these MAPKs, IFN-γ also antagonises the phosphoinositide 3-kinase (PI3K) – protein kinase B pathway, resulting in suppressed binding of activator protein 1 to the *Il10* promotor and decreased IL-10 production[47,102].

In addition to its pro-inflammatory effects, IFN-γ also possesses profound anti-inflammatory properties, such as inhibition of the development of Th17 cells and stimulation of T_{reg} cell function[63,194]. Furthermore, IFN-γ can, under certain *in vivo* conditions, also stimulate IL-10 production in Th1 cells and neutrophils[178,179]. These data challenge the generally accepted paradigm of IFN-γ and IL-10 as each other's antagonists, a viewpoint mainly resulting from studies where IFN-γ was found to inhibit TLR-triggered IL-10 production in monocytes or macrophages[102,103,105–107]. In the current work, we have addressed this challenge through exploring the role of IFN-γ on the production of IL-10, in a heterogeneous population of cells from mice and human, and after stimulation with different TLR triggers. We unexpectedly found a stimulatory role of IFN-γ on IL-10 production after triggering of TLR9 and further unravelled the cellular and molecular aspects involved in this observation.

2. Results

2.1 IFN-γ inhibits IL-10 production induced by most TLR agonists but stimulates TLR9-induced IL-10

The production of TLR-induced IL-10 and regulation by IFN-γ has predominantly been studied in monocultures of monocytes and macrophages, or their derived cell lines, and upon stimulation with TLR2-, TLR3- or TLR4-agonists. These studies revealed a profound inhibition of TLR-triggered IL-10 by IFN-γ[47,103,105,107]. However, the regulation of IL-10 production by IFN-γ in other cell types and upon stimulation with different TLR triggers is less known.

In a first set of *in vitro* experiments, peritoneal cells were stimulated with several TLR agonists, in the presence or absence of IFN-γ, followed by measurement of IL-10 production in the supernatant. The following TLR triggers were used: peptidoglycan (PGN; TLR2 agonist), polyinosinic-polycytidylic acid (PIC; TLR3 agonist), lipopolysaccharides (LPS; TLR4 agonist), resiquimod (R848; agonist for both TLR7 and TLR8) and unmethylated CpG oligodeoxynucleotides (CpG ODN or CpG) (TLR9 agonist; ODN1826). All TLR agonists induced IL-10 in peritoneal cells (Figure 1A). In line with the existing literature, IFN-γ inhibited TLR2-, TLR3- and TLR4-induced IL-10 production. IFN-γ also suppressed IL-10 production after TLR7 and TLR8 triggering by R848. Surprisingly, IFN-γ did not inhibit IL-10 production after triggering TLR9 with CpG. On the contrary, addition of IFN-γ led to a significant increase in CpG-induced IL-10 production. Of note, IFN-γ itself did not induce IL-10 production. The stimulatory and inhibitory effects of IFN-γ on respectively CpG- and LPS-induced IL-10 were consistently found in different mouse strains, i.e. BALB/c, DBA/1 and C57BL/6 mice (Figure 1B), and were also seen in splenocytes (Figure 1C), indicating that the results are not strain- or tissue-dependent. To verify whether the stimulatory effect of IFN-γ on CpG-induced IL-10 was restricted to *in vitro* conditions, BALB/c wild type (WT) and IFN-γ deficient (IFN-γ KO) mice were injected intraperitoneally with CpG and serum IL-10 levels were analysed 24 hours later. A single CpG-injection induced detectable serum levels of IL-10 and these levels were significantly lower in IFN-γ KO mice as compared to WT mice (Figure 1D), confirming the stimulatory effect of endogenous IFN-γ on CpG-

induced IL-10 production *in vivo*. Finally, the opposite effects of IFN-γ on LPS- versus CpG-induced IL-10 were also seen upon stimulation of human peripheral blood mononuclear cells (PBMCs) from healthy donors (Figure 1E), highlighting the translational relevance of our findings.

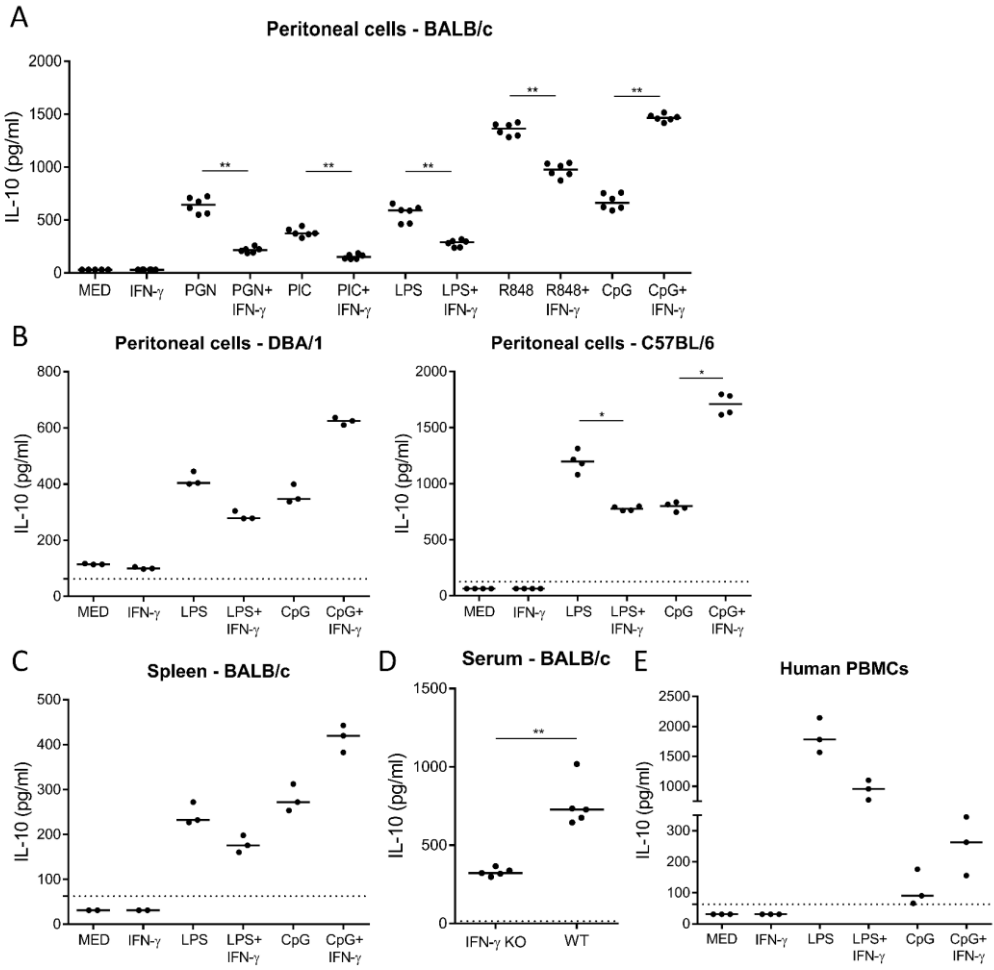


Figure 1: Induction of IL-10 by TLR agonists and differential effect of IFN-γ. *(A) IL-10 production at 48h after in vitro stimulation of peritoneal cells (from BALB/c mice) with PGN (TLR2), PIC (TLR3), LPS (TLR4), R848 (TLR7/8) and CpG (TLR9) in presence or absence of IFN-γ. IL-10 production by peritoneal cells from DBA/1 and C57BL/6 mice (B) and spleen cells from BALB/c mice (C), after 48h stimulation with LPS and CpG ± IFN-γ. D) Serum IL-10 levels of IFN-γ KO and WT BALB/c mice, 24h post injection with 200μg CpG.* ***E)*** *IL-10 production after stimulation of PBMCs*

*of healthy subjects with LPS and CpG ± IFN-γ. Dots represent data points with median from a pool of 5-10 mice (A-C), individual mice (D) or individual healthy subjects (E). Results are representative of 2 independent experiments (A, B), 10 independent experiments (A: LPS and CpG ± IFN-γ stimulation, C) or 3 independent experiments (D, E). IL-10 concentration was measured in the supernatant (A-C, E) or in the serum (D) by ELISA. * $p < 0.05$; ** $p < 0.01$; Kruskal-Wallis followed by Mann-Whitney U test. MED = medium.*

Taken together, we confirmed studies reporting on the inhibitory role of IFN-γ on TLR-induced IL-10, but also observed an opposite effect of IFN-γ when CpG was used as TLR agonist. The increase of this TLR9-induced IL-10 by IFN-γ is a solid and physiologically relevant finding, as it was found in isolated cells from different organs and mouse strains, as well as *in vivo* and in human PBMCs.

2.2 B cells are responsible for the increase in CpG-induced IL-10 by IFN-γ

Primary cell cultures of peritoneal cells, as well as splenocytes and human PBMCs, consist of different types of immune cells. Therefore, the opposite effects of IFN-γ on TLR2-, TLR3- TLR4- and TLR7/8-triggered IL-10 production on the one hand, and TLR9-triggered IL-10 on the other hand, may be mediated by differential target cells of the TLR agonists. To investigate this hypothesis, experiments were performed on isolated subpopulations from peritoneal cells, spleen cells and human PBMCs. We selected LPS and CpG as TLR agonists as representatives of the respective inhibitory and stimulatory effects of IFN-γ on IL-10 production (Figure 2).

Peritoneal cells were sorted in their main subpopulations, being B1 cells ($CD19^+$ $CD23^-$), B2 cells ($CD19^+$ $CD23^+$), macrophages ($F4/80^+$) and the rest fraction ($CD19^-$ $F4/80^-$). Upon TLR stimulation, IL-10 was detected in stimulated B1 cells and macrophages (Figure 2A), but not in B2 cells and the rest fraction (data not shown). In peritoneal B1 cells, LPS was found to induce low levels of IL-10, which were not altered by addition of IFN-γ. Conversely, CpG induced relatively high levels of IL-10 that were further increased in presence of IFN-γ (Figure 2A, center panel). Peritoneal macrophages were found to spontaneously produce IL-10, which was

increased upon stimulation with LPS but not by CpG. In these macrophages, IFN-γ inhibited IL-10 production in all conditions.

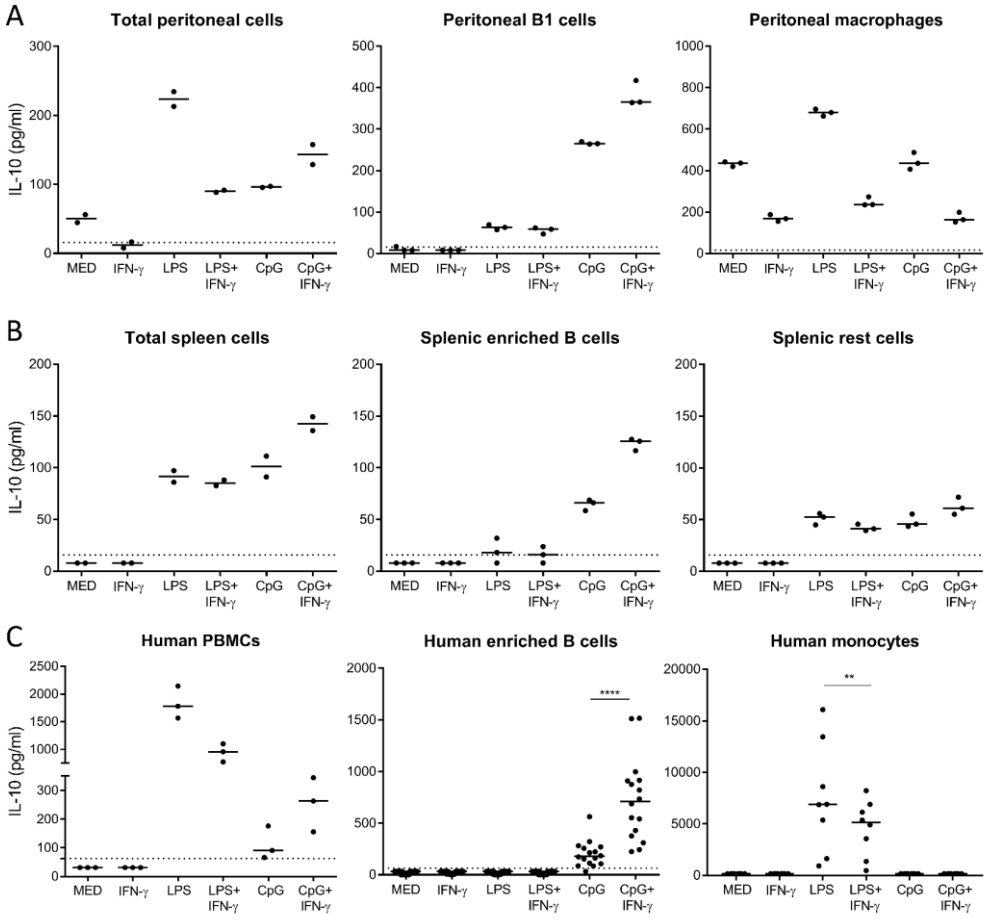


Figure 2: Regulation of LPS- and CpG-induced IL-10 by IFN-γ in isolated subpopulations of mouse and human cells. *(A) Peritoneal cells were left unsorted (left panel) or sorted in B1 cells ($CD19^+CD23^-$; 29.9% of live cells; purity 91.5%), macrophages ($F4/80^+$; 39.5% of live cells; purity 94.1%), B2 cells ($CD19^+$ $CD23^+$; 13.6% of live cells; purity 95.9%) and the rest fraction ($CD19^-$ $F4/80^-$; 9.62% of live cells; purity 98.3%). No IL-10 was detected in B2 cells and macrophages, therefore only B1 cells and macrophages are shown. (B) Spleen cells were left unsorted (left panel) or enriched for $CD19^+$ cells (purity 86,3%; middle panel), the rest fraction is shown in the right panel (this fraction still contained 19,6% $CD19^+$ cells). (C) Human PBMCs from healthy subjects were left unsorted*

*(left panel) or enriched for B cells (purity 92.4% - 97%) and monocytes (purity 97.1% - 98.3%). Cells were stimulated for 48h with LPS and CpG ± IFN-γ, IL-10 was measured in the supernatant by ELISA. (A, B) Dots represent duplicate or triplicate measurements with median of a pool of 16 mice and are representative of 2 (A) or 4 (B) independent experiments. (C) Dots represent individual donors with median from 2-5 independent experiments. ** p < 0.01; **** p < 0.0001; Friedman followed by Wilcoxon matched-pairs signed rank test. MED = medium. Purity determined by flow cytometry as % of live single cells.*

The stimulatory effect of IFN-γ on CpG-induced IL-10 by peritoneal B1 cells was confirmed in B cells enriched from splenocytes (Figure 2B). Similar to the pattern observed in peritoneal cells, LPS barely induced IL-10 in splenic B cells. In the remaining cell fraction, LPS and CpG induced moderate levels of IL-10 without pronounced effects of IFN-γ. When enriched B cells from human PBMCs were stimulated, IL-10 was only detected after CpG challenge, and IL-10 levels were found to be increased by IFN-γ (Figure 2C). On the other hand, in enriched human monocytes, only LPS induced IL-10 production, which was inhibited by IFN-γ.

Taken together, monocultures of the main IL-10-producing cell types demonstrated that the stimulatory effect of IFN-γ on the production of IL-10 was restricted to TLR9-induced IL-10 in B cells. While CpG induced IL-10 mainly in these B cells, the reversed effects of IFN-γ on LPS-induced IL-10 were only present in monocytes and macrophages.

2.3 Characterisation of B cells stimulated with CpG and CpG+IFN-γ

2.3.1 IgM production by CpG-stimulated B cells is not increased by IFN-γ

To evaluate whether IFN-γ has a general stimulatory effect on CpG-stimulated B cells, the production of another B cell cytokine (i.e. IL-6) and immunoglobulins (i.e. IgM and IgG) was analysed in the supernatant of enriched B cells from mouse peritoneum and spleen cells and from human PBMCs. Herefore, cells were stimulated with CpG with or without IFN-γ. LPS was included as a control (Supplementary Figure 1).

The production of IL-6 was increased after stimulation of peritoneal B cells with LPS, while CpG only modestly increased IL-6 levels in peritoneal and splenic B cells. Addition of IFN-γ slightly increased IL-6 levels in all conditions. In human B cells, only CpG increased IL-6 production, which was significantly upregulated by IFN-γ. Analysis of immunoglobulins showed that murine B cells secrete IgM after stimulation with LPS and CpG, while in human B cells, only CpG induced IgM production. IFN-γ did not further increase IgM production in any of the conditions. Next to IgM, IgG levels were analysed, but did not reveal detectable results (data not shown). Of note, human B cells did not respond to LPS with regard to the production of IL-10, IL-6, IgM or IgG.

2.3.2 IFN-γR and IL-10 expression in specific B cell subtypes

B cells can be subdivided in different B cell subtypes, such as B1 and B2 B cells in the peritoneum, follicular (FO), marginal zone (MZ) and transitional B cells in the spleen and plasmacells, transitional, naïve and memory B cells in human PBMCs[184]. To verify whether all these subsets are capable of responding to IFN-γ stimulation, we analysed their IFN-γ receptor (IFN-γR) expression by flow cytometry. As can be seen in Supplementary Figure 2A, all subsets of murine B cells partially expressed the IFN-γR. The highest percentage of IFN-γR$^+$ cells was found within the B2 cell population in the peritoneum, and within marginal zone and transitional B cells in the spleen. Stimulation with IFN-γ, CpG or CpG+IFN-γ had no effect on IFN-γR expression. Strikingly, in human B cells, IFN-γR was barely detectable in unstimulated cells but was significantly upregulated upon stimulation with CpG. Addition of IFN-γ did not further increase IFN-γR expression. Of note, the median fluorescence intensity of IFN-γR in the specific B cell subtypes showed a similar picture (data not shown).

Next to expression of the IFN-γR, we evaluated whether all B cell subtypes were capable to produce IL-10 and therefore measured IL-10 production with intracellular flow cytometry in stimulated human B cells (Supplementary Figure 2B). We confirmed that CpG induced the production of IL-10, which was further increased by addition of IFN-γ, and observed IL-10 production in naïve, transitional and memory B cells. In plasma B cells, almost no IL-10 was detected.

Together, we showed that all mouse B cell subtypes partially express the IFN-γR and thus can respond to IFN-γ. However, human B cells showed a very low basal IFN-γR expression, but strongly upregulated the IFN-γR after stimulation with CpG. In addition, we observed that in CpG-stimulated human B cells, naïve, memory and transitional B cells do produce IL-10, but plasma B cells do not.

2.3.3 B cells stimulated with CpG+IFN-γ have an increased suppressive function

We showed that IFN-γ increased CpG-induced IL-10, as well as IL-6, in human B cells, but the biological significance of this observation remained unclear. We investigated whether the stimulated B cells have the ability to inhibit CD4 T cell proliferation, a well-described procedure to determine regulatory activities of B cells[171,195]. To this end, B cells were stimulated with IFN-γ, CpG or CpG+IFN-γ for 48h and subsequently co-cultured with CFSE-labelled anti-CD3/CD28-stimulated $CD4^+$ T cells. After 72h of co-culture, the percentage of $CD4^+$ T cell proliferation was measured (Figure 3A). Next to co-culture of stimulated B cells and CFSE-labelled T cells, we also included a condition in which CFSE-labelled T cells were incubated with the supernatant of stimulated B cells either alone (Figure 3B) or together with stimulated B cells (Figure 3C). We observed that CpG-stimulated B cells or their supernatant profoundly inhibited $CD4^+$ T cell proliferation, which was even more pronounced after stimulation with CpG+IFN-γ. Of note, IFN-γ and/or CpG itself did not directly inhibit the proliferation of CD4 T cells (data not shown).

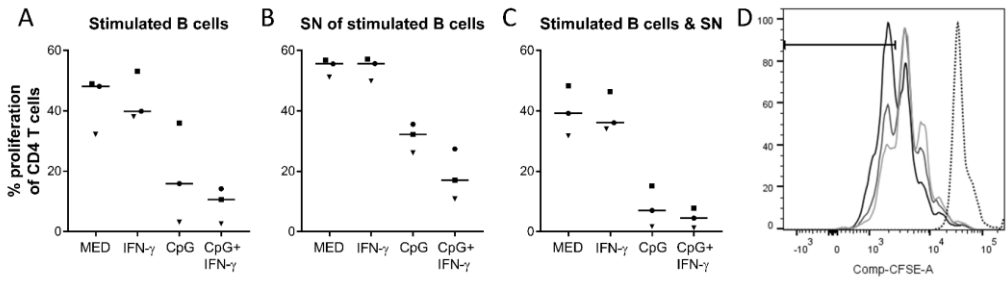


Figure 3: CpG-stimulated B cells inhibit $CD4^+$ T cell proliferation. *Enriched human B cells were stimulated with IFN-γ, CpG or CpG+IFN-γ for 48h. Next, CFSE-*

labelled CD4⁺ T cells of the same donors were stimulated with anti-CD3/CD28-antibodies and co-cultured with the stimulated B cells (A), their supernatant (SN) (B) or both stimulated B cells and their supernatant (C) for 72h. CD4⁺ T cell proliferation was measured by flow cytometry, by analysis of the CFSE signal within single live CD4⁺ T cells. Unstimulated and anti-CD3/CD28-stimulated CFSE-labelled CD4⁺ T cells (without B cells) were included as a control. Dots reprent individual donors with median, every donor has a different symbol (circle, square or trangle). (D) Representative histogram showing the proliferation of CD4⁺ T cells after 72h of co-culture with non-stimulated B cells (medium, black line), CpG-stimulated B cells (dark grey line) and B cells stimulated with CpG+IFN-γ (light grey line). Dotted line shows the unstimulated CFSE-labelled CD4⁺ T cells as a control. The gate shows the 4th proliferation cycle, the percentage of CD4⁺ T cells of the 4th generation is depicted in the graphs. Graphs are representative of 2 independent experiments. MED = medium.

2.4 The mechanism behind the stimulatory effect of IFN-γ on CpG-induced IL-10 involves p38 and JNK

Our experiments showed an inhibitory effect of IFN-γ on IL-10 production in response to TLR2, TLR3, TLR4 and TLR7/8 stimulation, and exposed an opposite scenario in response to TLR9-triggering of B cells. While the mechanism behind the inhibitory function of IFN-γ on TLR-induced IL-10 production has been well described[47], it is not known how IFN-γ exerts its stimulatory effect on TLR9-induced IL-10.

2.4.1 The increase in CpG-induced IL-10 by IFN-γ is also present on mRNA level

Firstly, we questioned whether the stimulatory effect of IFN-γ on CpG-induced IL-10 also occurred on a transcriptional level. Therefore, IL-10 mRNA levels were analysed after *in vitro* stimulation of peritoneal, spleen and human cells (Figure 4). In all cell populations, we observed a significant increase in IL-10 mRNA expression after stimulation with CpG+IFN-γ when compared to CpG alone, indicating that the stimulatory effect of IFN-γ on CpG-induced IL-10 was equally present on the transcriptional level. In addition, we confirmed the transcriptional

regulation of IL-10 by IFN-γ by analysis of nascent IL-10 mRNA expression in human B cells (data not shown).

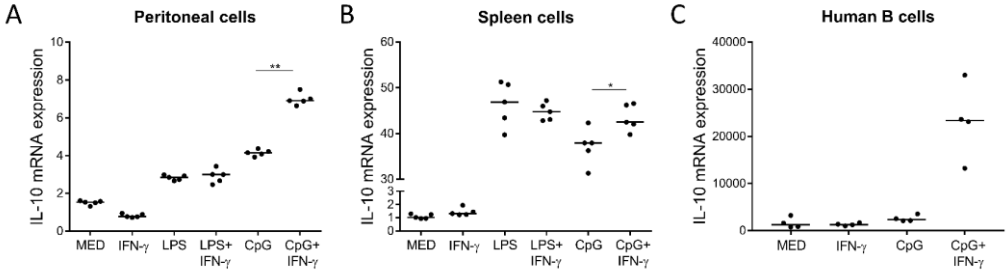


Figure 4: IFN-γ increases CpG-induced IL-10 on a transcriptional level. *IL-10 mRNA expression after stimulation of peritoneal cells (A), spleen cells (B) and enriched human B cells (C) with LPS and CpG ± IFN-γ for 24h. IL-10 mRNA levels were normalized for GAPDH expression by the $2^{-\Delta\Delta Ct}$ method. Dots represent replicates of a pool of 10 mice (A, B) or individual donors (C), with median. Graphs are representative of 4 (A) or 2 (B, C) independent experiments. * p < 0.05; ** p < 0.01; Kruskal-Wallis followed by Mann-Whitney U test. MED = medium.*

2.4.2 Increased CpG-induced IL-10 by IFN-γ is not caused by increased TLR9-expression, B cell viability, increased IL-6 production or IL-10 mRNA stability

Since IFN-γ has been described to increase the expression of TLR9 in mouse myeloid DCs and macrophages[196–198], to stimulate the proliferation of B cells[199] and to potentially influence mRNA stability[200,201], we verified whether the stimulatory effect of IFN-γ on CpG-induced IL-10 could be explained by any of these effects. Therefore, we measured TLR9 expression by intracellular flow cytometry (Supplementary Figure 3A). B cell proliferation and viability were measured by direct cell count using manual microscopy counts with trypan blue and via Incucyte Live Cell Imaging with Annexin V, a marker to analyse apoptosis (Supplementary Figure 3B). IL-10 mRNA stability was checked using Actinomycin D, a blocker of *de novo* mRNA synthesis, and subsequent measurement of mRNA levels at different time points (Supplementary Figure 3C). In addition, since we also observed an increased IL-6 production after stimulation with CpG+IFN-γ, we analysed the production of IL-10 after neutralisation and after addition of IL-6 (Supplementary Figure 3D).

Altogether, none of these mechanisms could adequately explain the increase in IL-10 production by IFN-γ in B cells.

2.4.3 Type I IFN production cannot explain the increase of CpG-induced IL-10 by IFN-γ

In another approach, we investigated whether secondary cytokines were involved in the observed increase in IL-10 after stimulation with CpG and IFN-γ, with a multiplex cytokine array (measuring IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-18, GM-CSF, TNF-α, IL-21, IL-22, IL-23 and IL-27) on the supernatant of stimulated mouse peritoneal cells. Analysis of these cytokines drove our interest towards IFN-α and IFN-β. Both cytokines were elevated after CpG+IFN-γ when compared to CpG alone, and in fact showed a similar pattern as observed for IL-10 production (Supplementary Figure 4A). It is known that type I interferons can induce IL-10 production[202–204], although IFN-α is not able to increase CpG-induced IL-10 in B cells[131,137]. We therefore first focussed on the role of IFN-β on IL-10 production using neutralising antibodies. IFN-β was indeed a potent inducer of IL-10 production, as its neutralisation decreased IL-10 production in all conditions. However, when the percentage increase in IL-10 production after stimulation with CpG+IFN-γ was compared to CpG alone, we observed a similar increase in IL-10 with and without IFN-β neutralisation (Supplementary Figure 4B). We also investigated the influence of both IFN-α and IFN-β by using IFNAR1 KO mice, which lack the type I IFN receptor, and therefore do not respond to either IFN-α or IFN-β. Enriched B cells from the peritoneum and the spleen of WT and IFNAR1 KO mice were stimulated with LPS or CpG with and without IFN-γ, and IL-10 production in the supernatant was analysed (Supplementary Figure 4C). No difference in the induction of IL-10 after stimulation with CpG+IFN-γ was observed in IFNAR1 KO mice compared to WT mice, indicating that type I IFNs are not involved in the specific increase of CpG-induced IL-10 by IFN-γ.

2.4.4 B cell activating factor is not involved in CpG-induced IL-10 production in B cells

B cell activating factor of the tumour necrosis factor family (BAFF) is a critical B cell survival and maturation factor that is predominantly produced by myeloid cells, but also B cells can produce BAFF. BAFF can bind to three different receptors: BAFF receptor (BAFF-R), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and B cell maturation antigen (BCMA). BAFF binds strongly with BAFF-R and TACI and only weakly with BCMA[205].

In human regulatory B cells, BAFF has been described to induce IL-10 production via its receptor TACI[135]. In human intestinal epithelial cells, BAFF expression can be induced by IFN-γ via Janus kinase/Signal Transducer and Activator of Transcription signalling pathways[206]. We hypothesised that in our experiments, the BAFF/TACI pathway may explain the increase of CpG-induced IL-10 by IFN-γ. We therefore analysed BAFF and TACI mRNA expression levels in human B cells after 6h, 16h, 24h and 48h of stimulation with CpG and/or IFN-γ. BAFF mRNA levels were strongly upregulated after stimulation with IFN-γ alone, but only mildly after 48h of CpG+IFN-γ stimulation (Figure 5A). Interestingly, TACI mRNA expression was induced after 24h and 48h stimulation with CpG and CpG+IFN-γ. However, at the protein level, we were unable to detect soluble BAFF protein by ELISA in the supernatant of stimulated B cells at any time point and condition (data not shown). Flow cytometric analysis of membrane-bound BAFF and BAFF-R revealed no differences between the conditions, while the expression of TACI was increased after CpG stimulation, but unaltered between CpG- and CpG+IFN-γ-stimulated cells (data not shown). In addition, IL-10 production was not decreased after neutralisation of BAFF with anti-BAFF-antibodies. In fact, a similar increase in IL-10 after stimulation with CpG+IFN-γ was observed in all experimental conditions (Figure 5B). Likewise, no additional IL-10 production was seen upon stimulation of B cells with recombinant BAFF, in any condition (Figure 5B). Lastly, neutralisation of TACI, using antibodies that block receptor-ligand interaction, again showed no effect on IL-10 production (Figure 5C).

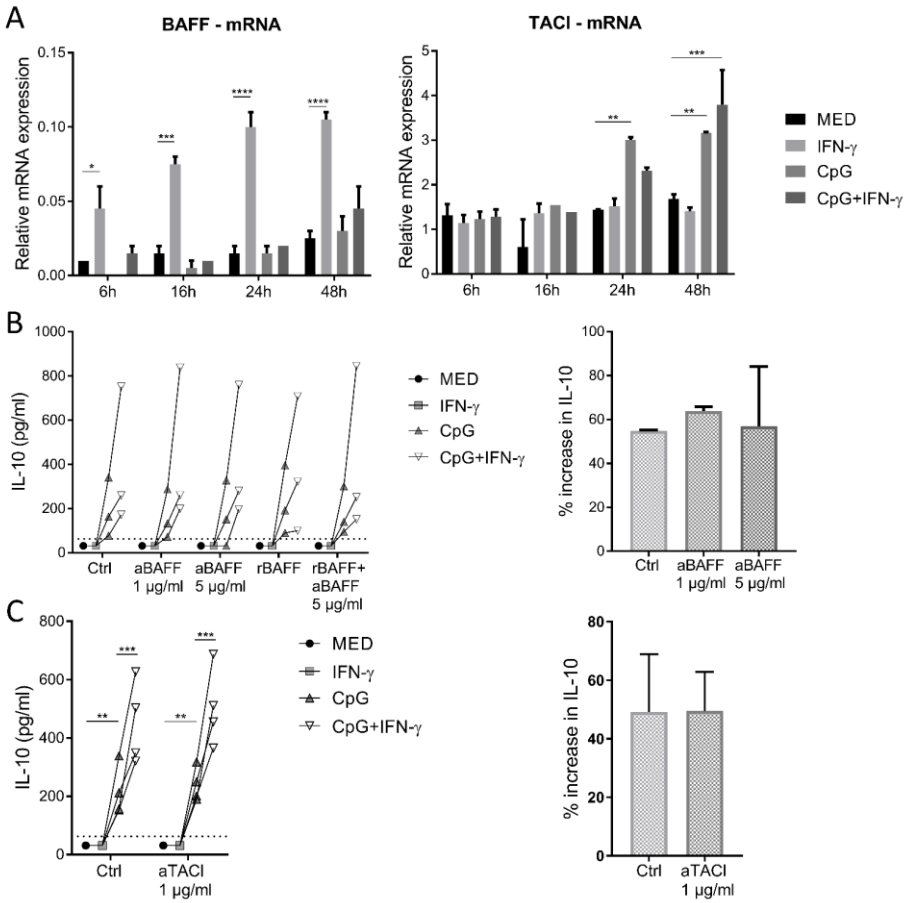


Figure 5: Exploring the BAFF pathway in CpG-induced IL-10 expression. *(A) Relative mRNA expression of BAFF and its receptor TACI, normalised for expression of the housekeeping gene POLR2A, after stimulation of enriched human B cells with IFN-γ, CpG or CpG+IFN-γ for 6h, 16h, 24h and 48h. (B) Left panel: IL-10 production after neutralisation of BAFF with anti-BAFF-antibodies (aBAFF; 1 µg/ml & 5 µg/ml) and/or addition of recombinant BAFF (rBAFF; 500 ng/ml) in enriched human B cells stimulated with IFN-γ, CpG or CpG+IFN-γ for 48h. (C) Left panel: IL-10 production after neutralisation of TACI with anti-TACI-antibodies (1 µg/ml), blocking the receptor-ligand interaction, in enriched human B cells stimulated with IFN-γ, CpG or CpG+IFN-γ for 48h. (B, C) Right panel: data from left panel depicted as the percentage increase in IL-10 production after stimulation with CpG+IFN-γ compared to CpG alone. (B, C) IL-10 was measured in the supernatant by ELISA. Bars show median with range of 2 (A) or 3 (B, C) different donors, symbols represent individual donors connected by lines (B, C). Graphs are representative of 2 independent*

*experiments. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001 Two-Way ANOVA followed by Tukey's multiple comparisons test (A) or Friedman followed by Wilcoxon matched-pairs signed rank test (C). MED = medium, Ctrl = isotype-treated.*

2.4.5 IFN-γ increases CpG-induced IL-10 production in B cells via p38 and JNK signalling pathways

Another explanation for the stimulatory effect of IFN-γ on CpG-induced IL-10 was sought at the level of signal transduction. In monocytes and macrophages, the MAPKs c-Jun N-terminal kinases (JNK), p38 and ERK1/2 are involved in TLR-induced IL-10 production and are tightly controlled by IFN-γ[121]. In B cells, it has been demonstrated that p38, JNK and ERK1/2 are important for IL-10 production[129–132]. We confirmed the importance of these MAPKs in IL-10 production by B cells. Indeed, blocking of ERK1/2 – via blocking of MEK1/2, upstream of ERK1/2 – p38 and JNK decreased CpG-induced IL-10 production by B cells (Figure 6A).

We hypothesized that the increased IL-10 production in B cells after stimulation with CpG in combination with IFN-γ may be due to an increased activation of MAPKs JNK, p38 and ERK1/2. To asses this hypothesis, we verified IL-10 production by human B cells after stimulation with IFN-γ, CpG and CpG+IFN-γ in the presence of inhibitors for these MAPKs (Figure 6B). Inhibition of ERK1/2 led to a similar decrease in IL-10 production in both CpG- and CpG+IFN-γ-stimulated cells. On the other hand, inhibition of p38 and JNK completely abolished the increased IL-10 production caused by IFN-γ. Furthermore, we checked the phosphorylation of these MAPKs at different time points (15 mins, 30 mins, 1h, 6h, 14h, 20h and 38h) after stimulation of B cells with IFN-γ, CpG and CpG+IFN-γ by flow cytometry (data not shown). The strongest MAPK phosphorylation was observed after 38h. Therefore, this time point was chosen as a representative and is depicted in Figure 6C (gating strategy in Supplementary Figure 5). Surprisingly, we could not observe a difference in activation status of p38 between CpG and CpG+IFN-γ stimulated cells at the different time points post-stimulation, and we only observed a small, non-significant, increase in JNK phosphorylation. As we expected, the phosphorylation status of ERK1/2 was unaltered after stimulation with CpG+IFN-γ when compared to CpG. However, all MAPKs were clearly activated after CpG stimulation. Thus, even though

inhibition of p38 and JNK completely abolished the increase in IL-10 after stimulation with CpG+IFN-γ (Figure 6B), this effect cannot be ascribed to an increased phosphorylation status of these MAPKs (Figure 6C). We therefore analysed whether IFN-γ influences the presence of MAPK phosphatases (MKP), which provide an important negative feedback mechanism for MAPK activation, or the activation of protein kinase B (AKT), which increases MAPK-induced IL-10 in macrophages[121,207]. Indeed, IFN-γ can decrease the expression of phosphatases MKP1 (or DUSP1) and MKP2 (or DUSP4), known to inactivate the MAPKs JNK, p38 and ERK1/2, and thus increase their activation[207,208]. Additionally, IFN-γ can modulate AKT activity and in this way influence IL-10 production[121,209]. We analysed the activity of AKT via Western Blot and the expression of MKP1 and MKP2 on the mRNA level at 24h and 48h of stimulation. However, we could not observe an increased AKT phosphorylation after stimulation with CpG+IFN-γ compared to CpG alone, and MKP2 expression could not be detected (data not shown). MKP1 mRNA levels were decreased upon stimulation with IFN-γ. CpG-stimulation further decreased MKP1 mRNA levels, and these were even lower after stimulation with CpG+IFN-γ (Figure 6D).

Taken together, we showed that p38 and JNK are crucial players in the increase of CpG-induced IL-10 by IFN-γ. Although we could not detect an increased phosphorylation of these MAPKs after stimulation with CpG+IFN-γ at the time points analysed, we showed that the expression of MKP1 was decreased, possibly leading to a prolonged MAPK activation.

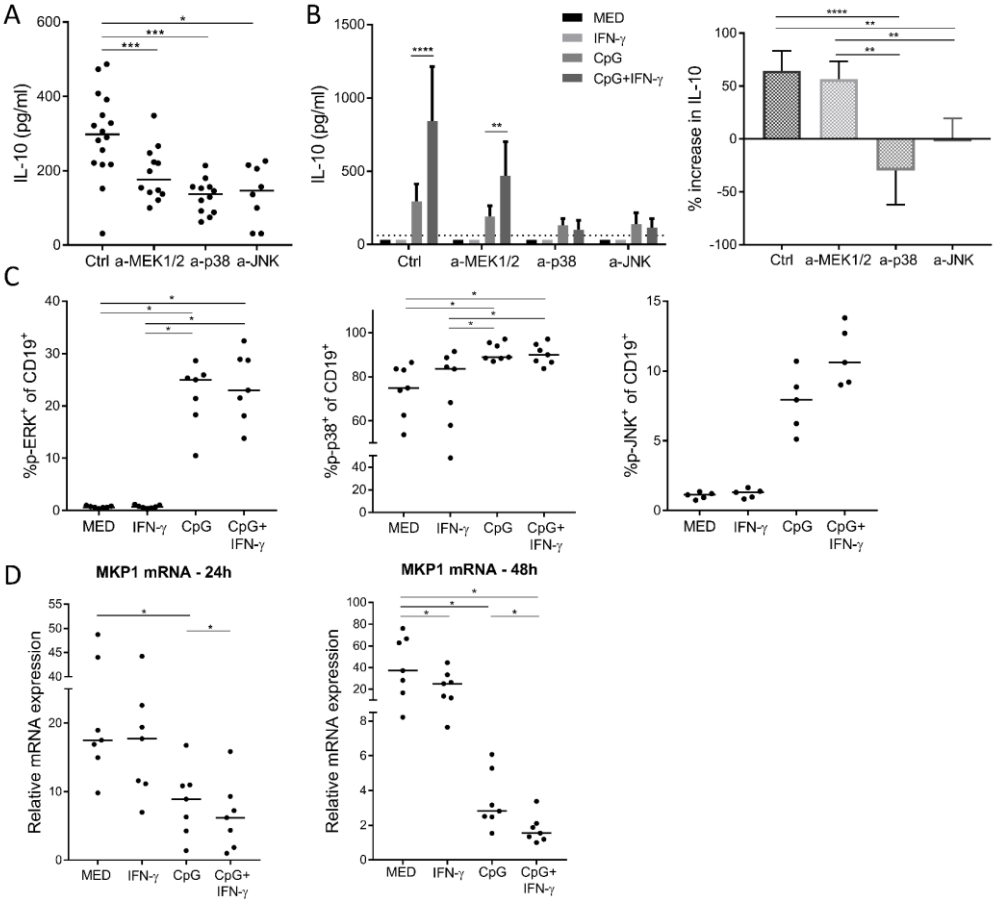


Figure 6: MAPKs ERK1/2, p38 and JNK and phosphatase MKP1 in human B cells. *IL-10 production after inhibition of ERK1/2 (via upstream MEK1/2), p38 or JNK in enriched human B cells stimulated with CpG (A) or IFN-γ, CpG and CpG+IFN-γ (B) for 48h. (B-right panel) Data from B-left panel shown as the percentage increase in IL-10 production after stimulation with CpG+IFN-γ compared to CpG alone. IL-10 was measured in the supernatant by ELISA. (C) Percentage phosphorylation of the MAPKs ERK1/2, p38 and JNK in B cells, determined as percentage p-ERK1/2^{+}, p-p38^{+} or p-JNK^{+} cells from single CD19^{+} cells, after stimulation with IFN-γ, CpG and CpG+IFN-γ for 38h, gating strategy is shown in Supplementary Figure 5. (D) Relative mRNA expression level of MKP1, normalized for GAPDH expression by the $2^{-\Delta\Delta Ct}$ method, after 24h and 48h stimulation with IFN-γ, CpG and CpG+IFN-γ. Dots represent individual donors with median (A-D), bars represent the median with range (B) for 12 (a-MEK1/2 and a-p38) or 8 (a-JNK) different donors, obtained in 4 (a-MEK1/2 and a-p38) or 3 (a-JNK) independent*

*experiments. (C, D) Graphs are representative of 3 (ERK1/2 & p38) or 2 (JNK, MKP1) independent experiments. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$ Friedman followed by Wilcoxon matched-pairs signed rank test or Two-Way ANOVA followed by Tukey's multiple comparisons test (B-left). Ctrl = vehicle (DMSO), MED = medium.*

2.4.6 IL-10 induced by stimulation of B cells with TLR7/8 ligands is also increased by IFN-γ

In the previous part, we showed that IFN-γ increased CpG-induced IL-10 via the TLR9-activated MAPK p38 and JNK. Interestingly, TLR7 and TLR8 share their signalling pathway with TLR9[116,210,211]. Correspondingly, we expect that IFN-γ will also increase IL-10 induced by TLR7/8 activation of B cells. To check this hypothesis, enriched peritoneal B cells were stimulated with TLR agonists, including R848, wich is an agonist for TLR7/8, in the presence and absence of IFN-γ. The $CD19^-$ rest fraction was also included (Figure 7). We observed that all TLR agonists could induce small amounts of IL-10 in B cells, with the most pronounced IL-10 production after stimulation with R848 or CpG. In contrast to the data obtained in total peritoneal cells (Figure 1) and in the $CD19^-$ rest fraction (Figure 7), in enriched B cells addition of IFN-γ did not influence IL-10 induced by stimulation of TLR2 (PGN), TLR3 (PIC) and TLR4 (LPS), but did increase IL-10 production after stimulation of TLR7/8 and TLR9. Since TLR7, TLR8 and TLR9 share the same signalling pathway, these data strengthen the conclusion that IFN-γ increases IL-10 in B cells by influencing MAPK signalling.

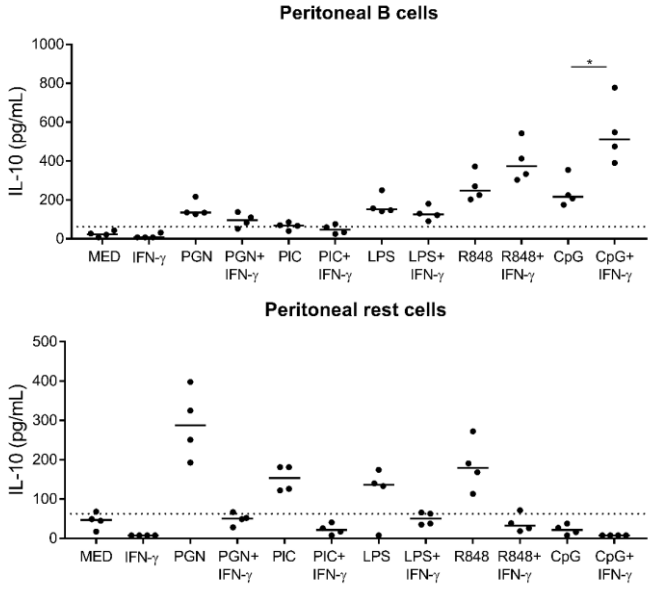


Figure 7. IL-10 production after stimulation of B cells with several TLR agonists. *Enriched peritoneal B cells (top panel) and the rest fraction ($CD19^-$, bottom panel) were stimulated for 48h with PGN (TLR2), PIC (TLR3), LPS (TLR4), R848 (TLR7/8) and CpG (TLR9) in the presence or absence of IFN-γ. IL-10 production was analysed in the supernatant by ELISA. Dots represent individual mice, with median. Graph is representative for 2 individual experiments. * $p < 0.05$; Kruskal-Wallis followed by Mann-Whitney U test. MED = medium.*

3. Discussion

Our data confirm reports on the inhibition of IL-10 by IFN-γ, when agonists of TLR2, TLR3, TLR4 and TLR7/8 are used as triggers. Furthermore, we exposed a previously unreported and surprisingly opposite role for IFN-γ when CpG, a TLR9 agonist, was used as IL-10-inducer. The stimulatory effect of IFN-γ on CpG-triggered IL-10 was seen in mixed cell populations from different murine organs as well as *in vivo* and in human PBMCs, which points to the possible physiological relevance of our findings.

We found that the stimulatory effect of IFN-γ on CpG-induced IL-10 is restricted to B cells. In contrast to CpG, in our experiments LPS barely

induced IL-10 production in B cells, and IL-10 levels were not affected by IFN-γ. Conversely, a study of Holan *et al.* (2014) reported a high LPS-induced IL-10 production in splenic B cells, which was further increased by IFN-γ[133]. Possible explanations for this discrepancy may lie within the significantly higher dose of LPS used by Holan *et al.*, or differences in B cells subpopulations within the enriched splenic B cells.

Analysis of several B cell subtypes revealed that they all express the IFN-γ receptor, either constitutively (murine B cells) or after CpG-triggering (human B cells) and thus can respond to IFN-γ. Nevertheless, in peritoneum, B1 cells ($CD19^+CD23^-$) were found to be the most important IL-10-producing B cells, which is in accordance with previous studies[188]. In mouse splenocytes and human PBMCs, B1 cells are difficult to identify, and they are not particularly linked with IL-10 production or a regulatory phenotype[212–214]. In human PBMCs, we identified naïve B cells ($CD19^+CD27^-$) and to a lesser extent transitional ($CD19^+CD24^{hi}CD38^{hi}$) and memory B cells ($CD19^+CD27^+$) as IL-10-producing target cells for CpG+IFN-γ stimulation, while plasma B cells ($CD19^+CD24^-CD38^{hi}$) were not responsive. Our results are in line with previous reports, which showed that $CD27^-$ B cells (corresponding to naïve B cells), transitional B cells, $CD24^{hi}CD27^+$ (overlapping with the phenotype of memory B cells) and $CD73^-CD25^+CD71^+$ B cells can all produce IL-10[138,170,183,184,215,216]. Apparently, no clearcut phenotypic markers can be used to characterize IL-10-producing B cells. We believe that any B cell subtype might be able to produce IL-10 depending on the condition, and confirm that IL-10 remains the only valid marker to identify IL-10-producing B cells[217].

To unravel the mechanism behind the stimulatory effect of IFN-γ on CpG-induced IL-10, we studied the potential involvement of the type I IFNs IFN-α and IFN-β, since these have been described to increase TLR-induced IL-10 production in murine macrophages[218]. Interestingly, it has been reported that human B cells can produce IFN-α after stimulation with CpG class C[219]. However, while class A and class C CpG oligonucleotides are indeed potent inducers of type I IFNs, class B CpG (the one we used in our assays) is a weak inducer[220]. We performed experiments with recombinant IFN-β, anti-IFN-β-antibodies and IFNAR1 KO mice to evaluate the role of IFN-α and IFN-β in our assay, and

demonstrated that type I IFNs are not involved in the specific increase of CpG-induced IL-10 by IFN-γ.

BAFF is a B cell survival factor that can induce IL-10 in human regulatory B cells via its receptor TACI[135]. In addition, BAFF is known to increase CpG-induced IL-10 in human B cells[134]. IFN-γ on the other hand, can increase BAFF expression in human intestinal epithelial cells[206]. We found that IFN-γ was equally capable of stimulating BAFF expression in human B cells, but we could only confirm this at mRNA level. However, when B cells were stimulated with IFN-γ in combination with CpG, BAFF mRNA expression was not profoundly increased. TACI expression was increased after CpG-stimulation, as described in literature[221]. From our experiments with recombinant BAFF and neutralising BAFF- and TACI-antibodies, we concluded that the increased IL-10 after CpG+IFN-γ could not be attributed to involvement of BAFF or its receptor. We could not confirm the observations of Saulep-Easton *et al.* (2016) and Yehudai *et al.* (2012) that recombinant BAFF increases CpG-induced IL-10 in human B cells. However, our results are in line with the observations of Hua *et al.* (2016), who demonstrated that BAFF does not promote IL-10 production by CpG-stimulated B cells. In fact, the authors showed that another member of the same family, i.e. a proliferation-inducing ligand (APRIL) did increase IL-10 production by CpG-stimulated B cells[132]. In our study, we did not specifically investigate the role of APRIL. However, since APRIL also binds to TACI for the induction of IL-10[132] and since we did not observe any differences in IL-10 production after neutralisation of TACI, our data suggest that BAFF and APRIL are not involved in the increase of CpG-induced IL-10 by IFN-γ.

The signalling pathways underlying TLR-induced IL-10 production have mainly been studied in macrophages and DCs. In B cells, these pathways are less well described. In line with some previous reports, we showed that the MAPKs ERK1/2, p38 and JNK are implicated in CpG-induced IL-10 production in human B cells[129–132]. It has been described that IFN-γ negatively regulates TLR-induced IL-10 through the ERK1/2, p38 and PI3K signalling pathways in macrophages[102]. On the contrary, we showed that in B cells, IFN-γ positively regulates CpG-induced IL-10 production via the p38 and JNK signalling pathways. However, we could not observe an increased phosphorylation of these MAPKs after stimulation with

CpG+IFN-γ compared to CpG alone at the time points analysed, indicating IFN-γ exerts its positive effects on IL-10 production not by increasing MAPK phosphorylation, but potentially by decreasing the MAPK phosphatases. Valledor *et al.* (2008) reported that in murine macrophages, IFN-γ prolongs M-CSF-induced ERK1/2 activation by inhibiting the expression of the MAPK phosphatases MKP1, -2 and -4. The phosphatase MKP1 preferentially inhibits p38 and JNK activation, and has only small effects on ERK1/2. MKP2 and MKP4 on the other hand, have similar effects on p38, JNK and ERK1/2 MAPKs[207]. Indeed, we observed lower MKP1 mRNA levels after stimulation with CpG+IFN-γ when compared to CpG alone. This lower MKP1 expression suggests a prolonged activation of the MAPKs p38 and JNK, although this could not be detected at the time points studied, but which confirms that the MAPKs p38 and JNK are involved in the increased CpG-induced IL-10 by IFN-γ.

An additional confirmation for the involvement of p38 and JNK MAPK in the specific increase of CpG-induced IL-10 by IFN-γ comes from our observation that IFN-γ also increased B cell-derived IL-10 production after stimulation of TLR7 and TLR8, two TLRs that use the same signalling pathways as TLR9. Notably, in total peritoneal cell cultures and $CD19^-$ peritoneal fractions, IFN-γ decreased TRL7/8-triggered IL-10 levels. Considering that macrophages are the major cell population within the $CD19^-$ peritoneal cell fraction, this discrepancy is most likely explained by a differential effect of IFN-γ in B cells compared to monocytes/ macrophages.

The findings in the present study challenge the widely accepted view of IFN-γ and IL-10 as antagonistic cytokines. Along with a few other studies reporting on a stimulatory role for IFN-γ on the production of IL-10[178,179], our data may provide an explanation as to why, in some animal models, lower levels of IL-10 were observed when IFN-γ was neutralised or depleted. For example, in a mouse model for macrophage activation syndrome, where repeated CpG injections resulted in a cytokine storm (including IFN-γ and IL-10), IFN-γ KO mice showed decreased serum levels of IL-10[222].

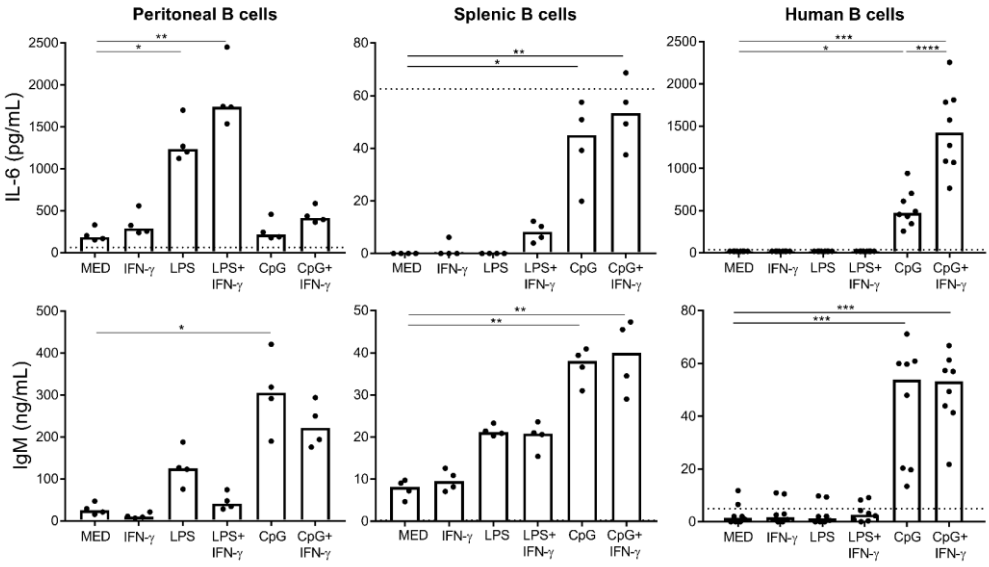
In a viral context, our data may provide an explanation for the persistence of infections. When DNA viruses infect B cells (e.g. Epstein-Barr virus), TLR9 is activated[223], accompanied by production of IFN-γ[224]. Our results

indicate that IFN-γ could increase TLR9-induced IL-10 production in these B cells, potentially contributing to the chronicity of the infection. Likewise, during murine cytomegalovirus infection, which also triggers TLR9, it has been reported that IL-10-producing B cells constrain virus-specific $CD8^+$ T cell responses. The high levels of IFN-γ found in MCMV-infected mice might further increase IL-10 production by B cells, resulting in impaired viral clearance[225].

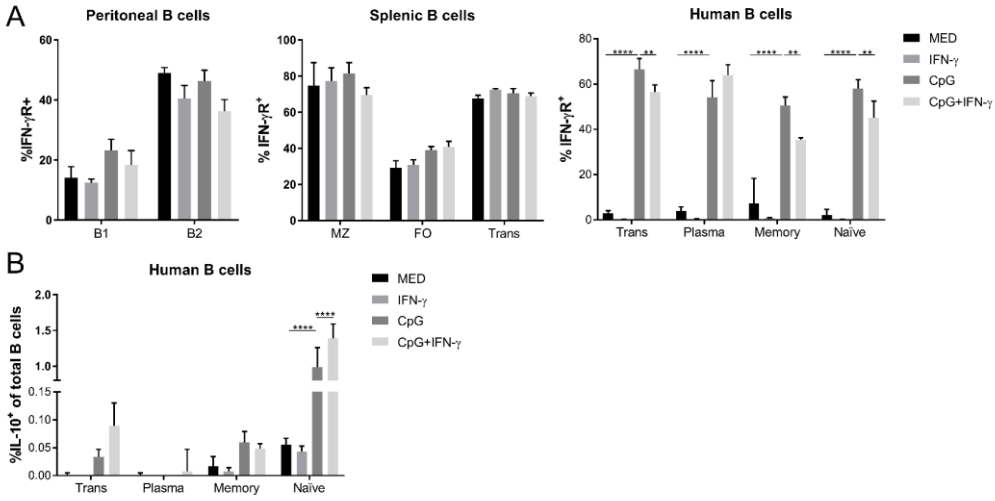
CpG DNA is currently used as adjuvant in designing vaccines targeting infectious diseases and cancer[226,227]. In these conditions, endogenous production of IL-10 hampers an optimal immune response[228–231]. Upon vaccination, IFN-γ production is often used as a readout for Th1 responses, cell-mediated cytotoxicity and NK cell activity[228,232,233]. However, our results show that the presence of IFN-γ may also have an inhibitory effect on the immune response through stimulation of IL-10, a mechanism that may explain the absence of an adequate vaccine response in some patients[231].

Finally, the stimulation of CpG-triggered B cells by IFN-γ towards a more regulatory phenotype, as demonstrated in our study by a more vigorous IL-10 production and inhibition of $CD4^+$ T cell proliferation, may represent a so far unappreciated and novel immunoregulatory role of IFN-γ in conditions related to TLR9 activation. Of special interest are animal models for autoimmune diseases that rely on Complete Freund's Adjuvant (CFA), an oil adjuvant in which autoantigens are emulsified, e.g. collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE), respectively a mouse model for rheumatoid arthritis (RA) and multiple sclerosis (MS). CFA contains heat-killed *Mycobacteria* and therefore a high amount of CpG[181]. In CIA and EAE, both endogenous IL-10 and IFN-γ have a clearcut disease-protective role, and it is intriguing to speculate that at least part of the protective role of IFN-γ resides in the stimulation of CpG-induced IL-10[153,154,234–236]. Furthermore, together with the knowledge that autoimmune symptoms are exacerbated in the absence of TLR9[237–239] and the observation that CpG stimulation can inhibit disease in experimental autoimmune animal models[240,241], our data and others [237,242] identify TLR9-triggering as a potential key event in the regulation of autoimmunity.

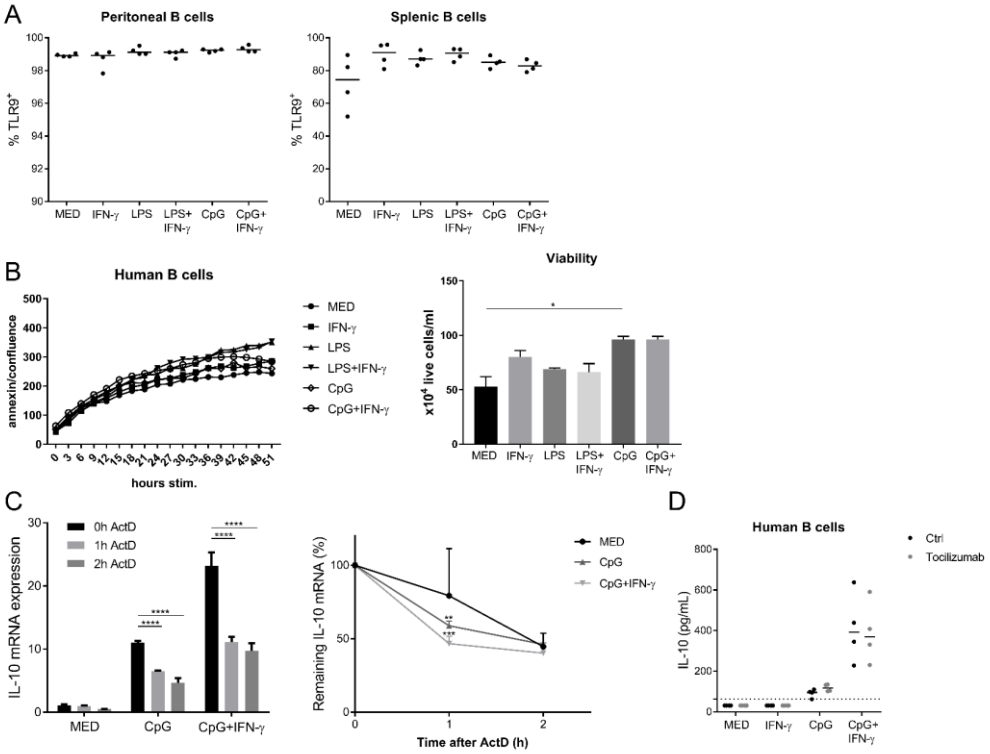
4. Supplementary material



Supplementary Figure 1: Production of IL-6 and IgM by enriched murine and human B cells. *Enriched B cells from murine peritoneal and splenic cells and human PBMCs were stimulated with LPS and CpG with or without IFN-γ. After 48h, IL-6 (upper panels) and IgM (lower panels) levels were measured in the supernatant by ELISA. Dots represent individual mice or donors, bars show the median. Graphs are representative of 2 independent experiments. * p < 0.05; ** p < 0.01; *** p<0.001; ****p<0.0001; Kruskal-Wallis followed by Mann-Whitney U test (peritoneal and splenic B cells) or Friedman followed by Wilcoxon matched-pairs signed rank test (human B cells). MED = medium.*

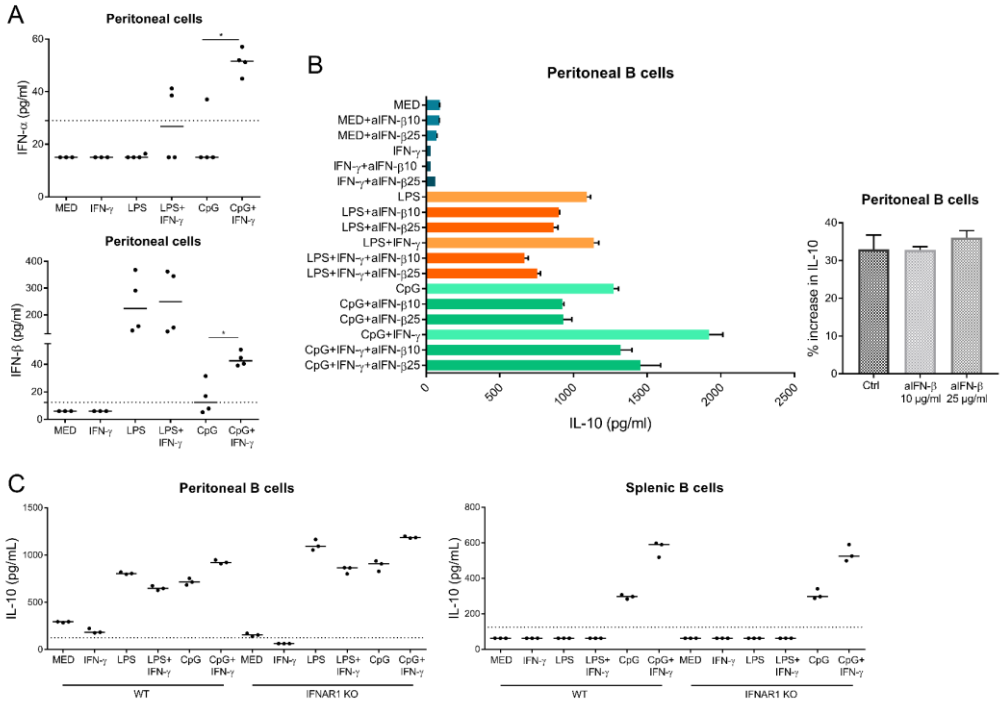


Supplementary Figure 2: Expression of IFN-γR and IL-10 in different B cell subtypes. *(A) Enriched B cells from murine peritoneal and splenic cells and human PBMCs were stimulated with CpG with or without IFN-γ. After 48h, IFN-γR expression was analysed by flow cytometry within peritoneal B1 ($CD19^+CD23^-$) and B2 ($CD19^+CD23^+$) cells, splenic marginal zone (MZ; $CD19^+CD21^{hi}IgM^{hi}IgD^{lo}$), follicular (FO; $CD19^+CD21^{lo}IgM^{lo}IgD^{hi}$) and transitional (Trans; $CD19^+CD21^{lo}IgM^{hi}$) B cells and human transitional ($CD19^+CD24^{hi}CD38^{hi}$), plasma ($CD19^+CD24^-CD38^{hi}$), memory ($CD19^+CD27^+$) and naïve ($CD19^+CD27^-$) B cells. (B) Enriched human B cells were stimulated with CpG with or without IFN-γ for 48h. IL-10 expression in the different B cell subtypes was determined with intracellular flow cytometry. Bars show the median with range of individual mice or donors (n=4). Graphs are representative of 2 independent experiments. ** $p < 0.01$; ****$p<0.0001$; Two-Way ANOVA followed by Tukey's multiple comparison test. MED = medium. Gating strategy is shown in Supplementary Figure 6.*

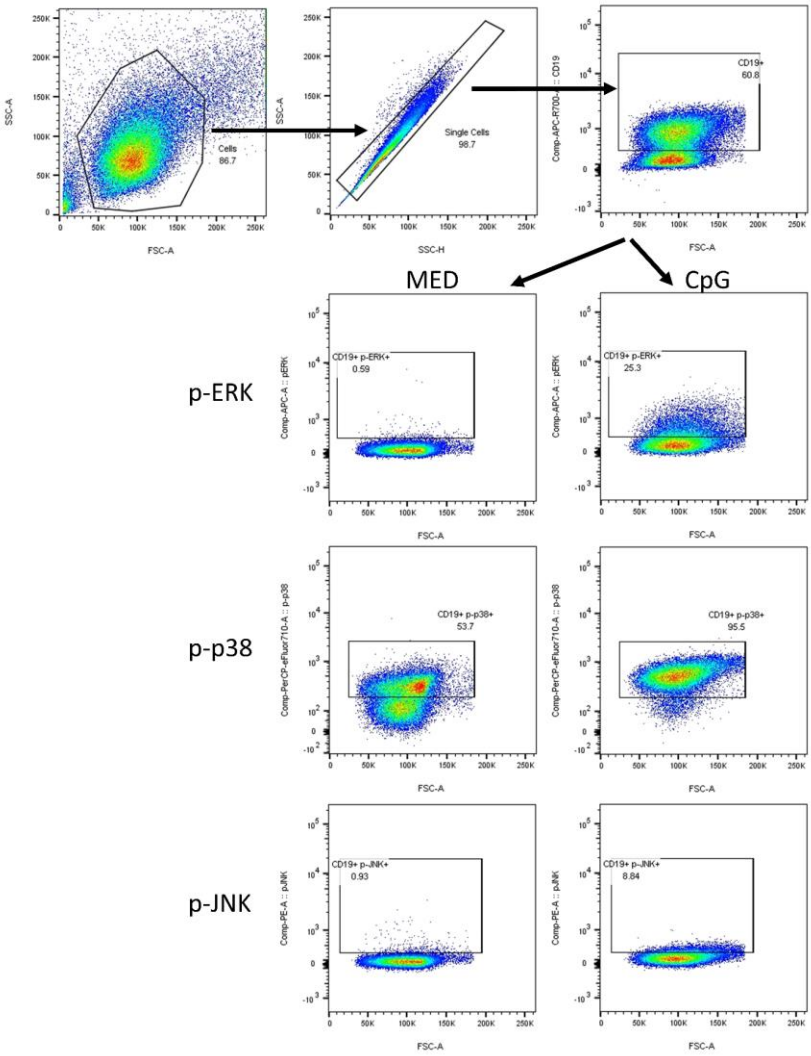


Supplementary Figure 3: Analysis of TLR9 expression, B cell viability, IL-10 mRNA stability and role of IL-6 production. *(A) Peritoneal and splenic cells were stimulated for 48h with LPS or CpG ± IFN-γ, followed by intracellular staining of TLR9 expression by flow cytometry. Percentage of TLR9⁺ cells within single live CD19⁺ B cells is shown. Dots represent individual mice with median, graphs are representative of 2 independent experiments. (B) Left panel: Human B cells were stimulated with LPS or CpG ± IFN-γ, in the presence of the apoptotic marker Annexin V. Every 3 hours, the amount of apoptotic cells was measured via Incucyte Live Cell Imaging and plotted relative to the cell confluence. The median of 4 different donors is shown, graphs is representative of 1 independent experiment. Right panel: human B cells were manually counted with trypan blue after 48h of stimulation, the number of live cells is shown. Bars represent the median with range, n = 2, graph is representative of 3 independent experiments. (C) Left panel: Relative IL-10 mRNA expression levels, normalized for GAPDH expression by the $2^{-\Delta\Delta Ct}$ method, 13h after stimulation with CpG ± IFN-γ and after 0, 1 and 2h of actinomycin D (ActD) treatment. Percentage remaining IL-10 mRNA is shown in the right panel. Bars or symbols show median with range of 3 data points obtained from a pool of 14 mice. (D) Human B cells were stimulated for 48h with CpG ± IFN-γ in the presence or absence*

*of tocilizumab, an anti-IL-6R antibody. IL-10 was measured in the supernatant by ELISA. Dots represent individual donors, graph is representative for 1 independent experiment. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, compared to MED; Kruskal-Wallis followed by Mann-Whitney U test or Two-Way ANOVA followed by Tukey's multiple comparisons test. MED = medium, ctrl = untreated.*

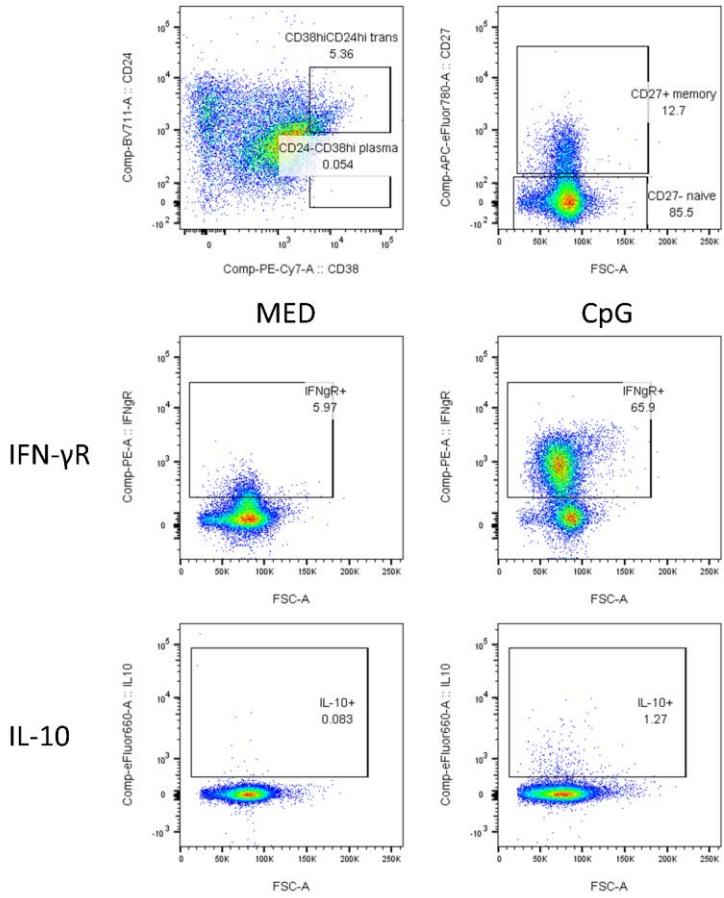


Supplementary Figure 4: Production and neutralisation of type I interferons. *(A) Peritoneal cells were stimulated in vitro with LPS and CpG ± IFN-γ. After 48h, IFN-α and IFN-β levels were measured in the supernatant by procartaplex. Dots represent replicates from a pool of 5 mice. (B) Enriched peritoneal B cells were stimulated for 48h with LPS and CpG ± IFN-γ in the presence or absence of anti-IFN-β-antibodies (aIFN-β; 10 µg/ml and 25 µg/ml). After 48h, IL-10 levels were measured in the supernatant by ELISA (left panel). Right panel: data from the left panel depicted as the percentage increase in IL-10 production after stimulation with CpG + IFN-γ compared to CpG alone. (C) Enriched peritoneal and splenic B cells from WT and IFNAR1KO mice were stimulated with LPS or CpG ± IFN-γ. After 48h, IL-10 was measured in the supernatant by ELISA. Dots represent replicates from a pool of 10 mice, with median. Graphs are representative of 2 independent experiments. * p < 0.05; Kruskal-Wallis followed by Mann-Whitney U test Ctrl = untreated, MED = medium.*



Supplementary Figure 5: Gating strategy to determine the phosphorylation of ERK1/2, p38 and JNK. *Demonstration of the gating strategy for the flow cytometric analysis of enriched human B cells. Cells were stained with monoclonal antibodies specific for CD19 (APC-R700) and pERK1/2 (APC), p-p38 (PerCP-efluor710) and p-JNK (PE) according to surface and Phosflow protocols (BD Biosciences) respectively. Lymphocytes were identified by their scatter properties (FSC-A x SSC-A plot), and then doublets were excluded by gating on SSC-H x SSC-A. CD19⁺ B cells were selected, followed by analysis of the percentage phosphorylated ERK1/2, p38*

and JNK. Plots with the percentage phosphorylated MAPK are shown for medium (MED) and stimulated (CpG) conditions.



Supplementary Figure 6: Gating strategy to determine IFN-γR and IL-10 in different human B cell subsets. *Human B cells were stained with monoclonal antibodies specific for CD19 (APC-R700), CD24 (BV711), CD27 (APC-efluor780) and CD38 (PE-Cy7) to determine the different B cell subsets. After exclusion of doublets and dead cells, CD19⁺ B cells were selected and transitional B cells (CD24^{hi}CD38^{hi}), plasma cells (CD24^{-}CD38^{hi}), memory B cells (CD27⁺) and naïve B cells (CD27^{-}) were gated as shown in the top panels. The percentage of IFN-γR⁺ and IL-10⁺ cells was determined after stimulation. Representative plots for medium (MED) and CpG-stimulated (CpG) conditions are shown.*

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Study conception and design: Imbrechts, Brisse, Vandenhaute, Goris, Wouters, Matthys.

Acquisition of data: Imbrechts, De Samblancx, Fierens, Vandenhaute, Mitera, Smets, Goris.

Analysis and interpretation of (part of) the data: Imbrechts, De Samblancx, Fierens, Brisse, Vandenhaute, Mitera, Smets, Goris, Wouters, Matthys.

CHAPTER 6

DISCUSSION AND CONCLUSION

sJIA is a severe autoinflammatory disease, which is thought to be caused by a relatively innocent trigger in predisposed children. It has been suggested that one of these predisposing factors may come from the inability of anti-inflammatory mediators to adequately resolve the ongoing inflammation[8]. The observation that IL-10 polymorphisms resulting in a lower IL-10 production are associated with sJIA disease development fits this viewpoint[18,19].

In this project, we showed that IL-10 production is defective in sJIA, both in a mouse model and in patients (**chapter 4**). More specifically, we revealed a defective IL-10 production by B cells, NK cells and T_{reg} cells in the mouse model, and a decreased IL-10 production in a subset of B cells in sJIA patients. Furthermore, we showed an imbalance between IL-10 and pro-inflammatory mediators, pointing to an insufficient IL-10 production to terminate the inflammatory process. In line with this, neutralisation of IL-10 signalling in CFA-injected WT mice resulted in the development of sJIA-like symptoms, further confirming the hypothesis that a defective IL-10 production contributes to sJIA disease development.

In the second part of this project (**chapter 5**), we elaborated on our remarkable observation of a defective IL-10 production in the absence of IFN-γ, which is counterintuitive in view of the traditional paradigm of IL-10 and IFN-γ as each other's antagonists. We showed that IFN-γ stimulates CpG-induced IL-10 production via a mechanism involving p38 and JNK, a finding that might represent a new anti-inflammatory property of the traditionally considered pro-inflammatory IFN-γ.

1. Insufficient IL-10 production in autoinflammatory and autoimmune diseases

Similar to our observations in sJIA patients, several autoimmune and autoinflammatory diseases seem to be associated with a defective IL-10 production. The prototypical example is inflammatory bowel disease (IBD), a heterogeneous group of intestinal disorders, of which Crohn's disease and ulcerative colitis are the main subtypes. In IBD patients, polymorphisms in IL-10 or the IL-10R are associated with disease development. Moreover, patients with loss-of-function mutations in the

IL-10/IL-10R pathway develop severe early-onset IBD, and rely on hematopoietic stem cell transplantation for successful treatment[46,243–245]. Interestingly, these patients may also present with arthritis[245]. Corresponding to the IL-10 defects in patients, IL-10 KO mice spontaneously develop IBD[246].

In keeping with our observations in sJIA patients, it has been reported that B cells from patients with Crohn's disease produce less IL-10 after stimulation with CpG compared to healthy controls. In addition, a lower percentage of IL-10-producing $CD24^{hi}CD38^{hi}$ B cells was observed *ex vivo*[247]. Similarly, B cells from patients with MS produce less IL-10 than healthy control cells upon *in vitro* stimulation of CD40, CD40+BCR or TLR9, and patients also present with reduced numbers of IL-10-producing B cells *ex vivo*[138,248,249]. Interestingly, the positive effects of immune-mediated therapy in MS patients might be attributed to an expansion of $CD24^{hi}CD38^{hi}$ B cells[189]. Also in patients with SLE, RA and psoriasis, a defective IL-10 production by B cells and impaired B cell suppressive functions have been reported[139,171,183,250].

Until now, no general explanation for the impaired IL-10 production by B cells has been found yet. In SLE, it has been suggested that IFN-α levels might influence the B cell fate, with low levels of IFN-α resulting in the expansion of IL-10-producing $CD24^{hi}CD38^{hi}$ B cells and plasmablasts, while high IFN-α levels only increase plasmablast differentiation and fail to increase $CD24^{hi}CD38^{hi}$ B cell expansion[251]. However, in contrast to SLE, we could not observe increased IFN-α levels in sJIA patients, suggesting that other mechanisms are involved.

Together, a defective IL-10 production by B cells seems to contribute to the pathogenesis of several autoimmune and autoinflammatory diseases. However, it remains unclear whether this defect underlies disease development, or rather is a consequence of the disease. We showed that neutralisation of the IL-10R in a sJIA mouse model results in sJIA-like symptoms, pointing to a defective IL-10 production underlying disease development. Additionally, in mouse models for colitis, MS and arthritis, depletion of IL-10-producing B cells or induction of a B cell-specific defect in IL-10 exacerbates disease development, and transfer of IL-10-producing B cells ameliorates disease in mouse models for MS, arthritis and SLE[247,252–255]. Furthermore, it has been reported that inflammatory signals can

expand the numbers of IL-10-producing B cells[256], confirming the hypothesis that a decreased IL-10 production by B cells underlies disease development, rather than being secondary to the inflammatory environment.

2. IL-10 supplementation or stimulation of IL-10 production as a potential therapy

Since a defective IL-10 production by B cells seems to underlie disease development in sJIA and other autoinflammatory or autoimmune diseases, enhancement of IL-10 production, either via IL-10 supplementation, stimulation of IL-10 production or by adoptive cell transfer, might be a potential treatment strategy for patients not responding to current therapies.

Supplementation of IL-10, for example via viral gene delivery, was shown to be successful in animal models for rheumatoid arthritis, type 1 diabetes and inflammatory bowel disease. However, administration of recombinant IL-10 showed limited success in several clinical trials[185,257]. In rheumatoid arthritis, the effect of serial administration of recombinant IL-10 was first evaluated in two studies conducted in 1995 and 1997, with conflicting results. One study showed some efficacy without serious complications after subcutaneous injection of IL-10 for 28 days, while the other observed side effects after serial intravenous administration of higher doses[257]. More recently, the safety and efficacy of Dekavil (F8-IL10), a fusion protein of IL-10, was shown in a Phase I clinical trial in patients with active rheumatoid arthritis receiving methotrexate. Dekavil is an "immunocytokine" that is composed of an antibody fragment F8, specific to the extra domain A of fibronectin (produced in response to tissue injury), which is fused to IL-10, thereby localizing IL-10 activity at the site of inflammation. Phase II clinical trials are currently ongoing[257–259]. In psoriasis, daily injections directly under the skin lesions of recombinant IL-10, combined with IL-4 and IL-11, resulted in a positive response[257]. Furthermore, subcutaneous injection of IL-10 decreased the incidence of relapse and prolonged the disease-free interval in a phase II clinical trial[260]. In patients with Crohn's disease, it was suggested that IL-10

administration could have significant therapeutic benefits, but unfortunately only mild and non-significant clinical improvement was seen[261]. Likewise, treatment of patients with multiple sclerosis did not result in beneficial effects[257].

Thus, although IL-10 is a powerful anti-inflammatory cytokine, until now, administration of recombinant IL-10 fails to significantly ameliorate disease symptoms. Potential explanations for this failure are the short half-life of IL-10 (2.7-4.5h), the complex regulation and tight control of IL-10 production *in vivo*, and ineffective delivery to the site of inflammation. Therefore, alternative strategies to replenish or stimulate endogenous sources of IL-10 might be more successful[257].

In this light, stimulation of B cells to increase their production of IL-10 might be an interesting alternative. TLR-, BCR- and CD40-signalling pathways have been reported to induce IL-10 production by B cells and are therefore potential targets for new treatment strategies[189]. For example, in a mouse model for multiple sclerosis, stimulation of TLR9 with CpG ameliorated disease, and this positive effect was partly attributed to an increased production of IL-10 by B cells[240]. Correspondingly, TLR9 KO mice develop more severe EAE symptoms, but the role of B cells was not analysed here[262]. It would be interesting to investigate whether TLR9 stimulation will also ameliorate disease in the sJIA mouse model, via provision of IL-10.

Adoptive transfer of IL-10-producing B cells might be another treatment strategy, which has proven to be effective in several animal models for autoimmune diseases, such as arthritis and EAE[252–255]. However, several questions remain to be elucidated before this strategy can potentially be translated to the clinic. For example, isolation of IL-10-producing B cells is hampered by the lack of specific phenotypic markers. Indeed, although transitional B cells ($CD24^{hi}CD38^{hi}$) are generally considered as the regulatory B cell subset contributing most to IL-10 production, almost all B cell subsets can produce IL-10, depending on the context and specific triggers used. In addition, the optimal stimuli to enrich IL-10-producing B cells *in vitro* are not determined yet. Furthermore, it is still unclear whether or not the isolated B cells will keep their regulatory phenotype once they are transferred to an inflammatory environment[189].

Together, cell-specific stimulation of IL-10 production or adoptive transfer of IL-10-producing B cells might provide future valuable alternatives for the direct supplementation of IL-10, which has proven to be not very effective, in diseases were a defective IL-10 production by B cells is thought to underlie disease development (such as sJIA). However, further research has to be performed to determine the feasibility and efficacy of these strategies.

3. Neutralisation of IL-10 signalling as an alternative mouse model for sJIA in IFN-γ competent mice?

Three animal models of sJIA have been described until now, of which the CFA-induced model in IFN-γ KO mice results in a picture closest to patient's situation, as evident from the many pathological and biological similarities. However, although sJIA patients display a defect in IL-18-induced IFN-γ production by NK cells[41], no genetic mutations in IFN-γ or its signalling pathways have been reported.

With respect to the pathogenesis of sJIA, it is hypothesized that a relatively innocent trigger induces disease development in predisposed children. Since polymorphisms resulting in a lower IL-10 production have been described, a decreased IL-10 production may be one of these predisposing factors. Neutralisation of IL-10 signalling in CFA-injected WT mice may therefore be a more clinically relevant mouse model to study sJIA pathogenesis.

Several sJIA-like symptoms were observed in CFA-injected mice treated with αIL10R. All mice presented with weight loss, splenomegaly and lymphadenopathy. Most of the mice also developed a skin rash. Haematologically, neutrophilia, thrombocytosis, lymphopenia and increased immature WBC were observed. Anaemia and decreased haemoglobin were present in part of the mice, together with an increased number of immature RBC. NK cell numbers were decreased to a similar extent as compared to CFA-injected IFN-γ KO mice, indicating that IL-10 and IFN-γ are equally important for maintaining NK cell numbers in CFA-injected mice. NK cell function was not analysed, but it has been reported that IL-10 has a stimulatory effect on NK cell cytotoxicity[102,108], potentially

suggesting a decreased NK cell function in the absence of IL-10. Evaluation of cytokine levels revealed only a limited increase in IL-6 and IL-17 levels in αIL10R-treated mice. Although anti-IL-17 treatment resolves disease in CFA-injected IFN-γ KO mice and anti-IL-6 treatment is successful in sJIA patients[101], the low IL-6 and IL-17 levels in αIL10R-treated mice indicate that sJIA disease development is not solely dependent on these cytokines. Importantly, the use of anti-IL-10R-antibodies limited the time to follow-up sJIA disease development, since the effect of αIL-10R treatment was abolished after 2-3 weeks of injections. As arthritis typically develops later in the disease course (i.e. after day 25), this feature was not observed in the αIL10R-treated mice. However, an indication of emerging joint inflammation was found by analysing osteoclast precursor cells in the spleen, with an increased percentage of these precursors in some of the αIL10R-treated mice. Likewise, stimulation of spleen cells with RANKL and M-CSF resulted in a trend towards the formation of more and larger TRAP-positive osteoclast cells in αIL10R-treated mice compared to WT CFA mice, suggesting that these mice might be more prone to develop arthritis. A potential role for IL-10 in the development of arthritis was also demonstrated in patients with loss-of-function mutations in IL-10 or its receptor, who present with IBD but may show arthritis as well[245]. Notably, induction of the sJIA mouse model in IL-10 KO mice might be a valuable alternative for the αIL10R treatment that will allow to study the development of arthritis during a longer period of time.

Together, neutralisation of IL-10 signalling in CFA-injected WT mice results in the development of many sJIA-like symptoms and represents an alternative, clinically relevant model for sJIA in IFN-γ competent mice. More importantly, these data also underline the vital role of IL-10 in the pathogenesis of sJIA. A visual overview of how IL-10 deficiency may contribute to sJIA development is shown in Figure 1.

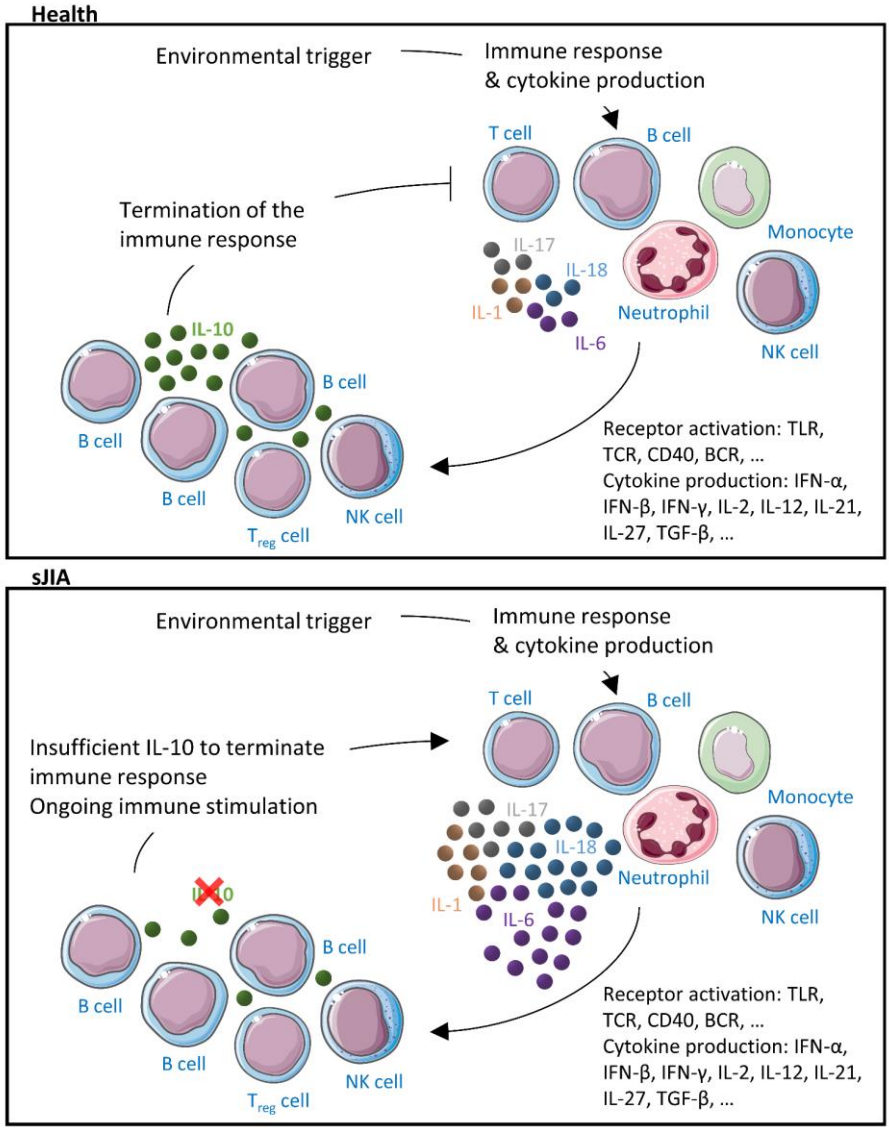

Figure 1: Overview of the contribution of an IL-10 deficiency to sJIA disease development. *In a normal condition (top panel), the immune response elicited by an environmental trigger is dampened via the production of IL-10, produced by B cells and to a lesser extent by T_{reg} cells and NK cells. In sJIA, the insufficient IL-10 production by B cells fails to terminate the immune response and subsequently allows for an ongoing immune stimulation and disease development. TLR = Toll like receptor, TCR = T cell receptor, BCR = B cell receptor.*

4. Regulation of IL-10 by IFN-γ: a new anti-inflammatory effect of IFN-γ through increasing IL-10 production?

IFN-γ is generally considered a pro-inflammatory cytokine because of its well-known immune-stimulating functions, such as the activation of macrophages, antigen presentation and the induction of Th1 polarisation. However, IFN-γ also has regulatory functions, such as the modulation of T_{reg} cell differentiation, inhibition of development and function of Th17 cells and limitation of inflammation-associated tissue damage[143,144,146].

In addition to its previously described regulatory properties, we showed that IFN-γ can increase TLR7/8- and TLR9-induced IL-10 production in B cells via a mechanism involving p38 and JNK signalling pathways. In addition, IFN-γ further enhanced the suppressive functions of CpG-stimulated B cells, measured by the ability of stimulated B cells to inhibit $CD4^+$ T cell proliferation. This effect is most likely explained by the increased production of IL-10, since addition of the supernatant of stimulated B cells (without the B cells) resulted in a similar inhibition of $CD4^+$ T cell proliferation. Of note, the increased IL-10 production by B cells did not have an autocrine effect on antibody production at the time point studied, since the secretion of IgM was not altered by IFN-γ.

In contrast to our observation of an increased IL-10 production by IFN-γ, several reports previously showed that IFN-γ is a negative regulator of IL-10 production, contributing to the traditional viewpoint of IL-10 and IFN-γ as each other's antagonists. Indeed, it has been described that IFN-γ inhibits IL-10 production in PBMCs, monocytes or macrophages stimulated with TLR agonists (such as Pam3Cys, LPS, *Borrelia Burgdorferi* lipoproteins, *Mycobacterium leprae*)[103,105–107,263,264]. IFN-γ inhibits TLR-induced IL-10 production in monocytes and macrophages both directly, by interfering with MAPK activation, and indirectly, by enhancing the production of GSK3[47,103,121]. In human monocytes stimulated with *Borrelia burgdorferi*, the downregulation of IL-10 by IFN-γ was mediated by IL-12[263]. In DCs, IFN-γ inhibits LPS- and CpG-induced IL-10 production

in a STAT1-dependent way[104]. Furthermore, next to attenuating TLR-induced IL-10 production, IFN-γ also interferes with IL-10 signalling itself. In LPS-activated macrophages, IFN-γ priming before addition of IL-10 results in a switched balance of STAT activation by IL-10. While addition of IL-10 normally results in STAT3 activation and concomitant expression of IL-10 responsive genes, IFN-γ priming skewed IL-10-induced STAT activation towards the activation of STAT1 instead of STAT3, allowing the activation of inflammatory gene expression[107].

Conversely, and in line with our observations, some more recent studies also reported on the positive effect of IFN-γ on IL-10 production. For example, in a sepsis mouse model, IFN-γ production by NKT cells increased IL-10 production by neutrophils[178]. Similarly, in a model for spinal cord injury, IFN-γ-dependent secretion of IL-10 by Th1 cells was required for recovery[179]. In murine B cells, LPS-induced IL-10 production has been shown to be increased by IFN-γ, which is in contrast to the negative effect of IFN-γ on LPS-induced IL-10 in myeloid cells, suggesting that the effect of IFN-γ on TLR-induced IL-10 is cell type-dependent[133]. Interestingly, our own results also indicate that the effect of IFN-γ on TLR-induced IL-10 production not only depends on the TLR trigger used, but also on the IL-10-producing cell type. More specifically, IFN-γ decreased IL-10 production by monocytes, macrophages or non-B cells, and increased (or did not alter) IL-10 production by B cells. Hypothetically, this cell type-dependent regulation of IL-10 by IFN-γ might play a role in the regulation of the immune response, where the downregulation of IL-10 in cells of the innate immune system (monocytes, macrophages and DC) enhances antigen presentation and pathogen clearance, subsequently followed by an increased IL-10 production by B cells to limit excessive immune responses and collateral damage after the pathogen has been cleared.

Although the mechanism behind the negative regulation of IL-10 by IFN-γ has been comprehensively studied, little is known about the precise mechanism by which IFN-γ may upregulate IL-10 production, except for the observation that this increase was also visible on the mRNA level[133,178,179]. In our study, we confirmed that IFN-γ increases IL-10 transcription and revealed part of this mechanism by showing that, in CpG-stimulated B cells, p38 and JNK signalling pathways are essential for

the increase in CpG-induced IL-10 by IFN-γ. In addition, we proposed that the downregulation of the MAPK phosphatase MKP1 by IFN-γ contributes to the increased IL-10 production (Figure 2).

An overview of the differential regulation of IL-10 by IFN-γ, as shown in literature and by our own data, is depicted in Table 1.

Table 1: overview of the regulation of IL-10 by IFN-γ in different cell types

IL-10-producing cell-type	**Stimulus/ model/disease**	**Effect IFN-γ**	**Mechanism**	**Ref**
Murine macrophages (RAW264.7)	LPS (TLR4)	↓	AKT ↓ GSK3↑	[265]
Human macrophages	LPS (TLR4)	↓		[103]
Human macrophages	Zymosan (TLR2)	↓		[103]
Human macrophages	Pam_3Cys (TLR2)	↓	ERK, JNK, p38, AKT ↓ GSK3↑	[103]
Human macrophages	Immune complexes	↓		[103]
Human adherent PBMCs	*Mycobacterium leprae*	↓		[263]
Human PBMCs	Staphylococcus aureus Cowan	↓		[105]
Human PBMCs	LPS	↓		[105]
Human monocytes	Staphylococcus aureus Cowan	↓		[105]
Human monocytes	LPS	↓		[105,106,264]
Human monocytes (THP1)	*Borrelia burgdorferi* lipoproteins	↓		[106]
Human APC	CD40	↓	Via JAK1	[266]
Murine DC	Antigen restimulation with	↓		[267]

	papillovirus-like particles			
Murine DC	LPS	↓	STAT1	104
Murine DC	CpG	↓	STAT1	104
Murine neutrophils	Sepsis mouse model	↑		178
Murine CD4 T cells	Antigen restimulation with papillovirus-like particles	↑		267
Murine Th1 cells	Spinal cord injury mouse model	↑		179
Murine T cells	Concanavalin A	=		133
Murine B cells	LPS (TLR4)	↑/=		133,180
Murine B cells	Peptidoglycan (TLR2)	=		180
Murine B cells	polyinosinic-polycytidylic acid (TLR3)	=		180
Murine B cells	Resiquimod (R848; TLR7/8)	↑		180
Murine B cells	CpG (TLR9)	↑		180
Human B cells	CpG (TLR9)	↑	P38, JNK MKP1↓	180

↑ : upregulation of IL-10 by IFN-γ; ↓ : downregulation of IL-10 by IFN-γ; = : no effect of IFN-γ on IL-10 production.

The biological relevance of the increased CpG-induced IL-10 production by IFN-γ can be found in several processes. For example, DNA viruses infecting B cells, such as Epstein-Barr virus, will activate TLR9 and at the same time induce the production of IFN-γ[223,224]. Our data showed that combination of these stimuli results in an increased production of IL-10, a mechanism that may be beneficial for the virus and potentially contributes to the chronicity of the infection. On the other hand, also endogenous DNA molecules released from damaged tissue or dead cells are able to activate TLR9[192]. Together with IFN-γ produced during the inflammatory process, this will result in an increased IL-10 production, which may be a

mechanism to temper the ongoing inflammation and to limit additional organ damage. Furthermore, in several mouse models that are induced with CFA, both IL-10 and IFN-γ play a disease-protective role. This is the case in the sJIA mouse model (as described in chapter 4), EAE, CIA, experimental autoimmune uveitis and autoimmune myocarditis[63,153,154,234,268–271]. In the sJIA mouse model, we showed that IFN-γ KO mice present with a defective IL-10 production by B cells. Since CFA contains heat-killed *Mycobacteria* and therefore also a lot of CpG motifs[181], this observation might be explained by the stimulatory effect of IFN-γ on CpG-induced IL-10 production in B cells. Although the effect of IFN-γ on IL-10 production by B cells has not been studied in other CFA-induced mouse models, it is intriguing to speculate that the disease-protective role of IFN-γ in CFA-induced mouse models is partially explained by an increased IL-10 production by B cells, next to other contributing mechanisms, such as decreased IL-17 levels.

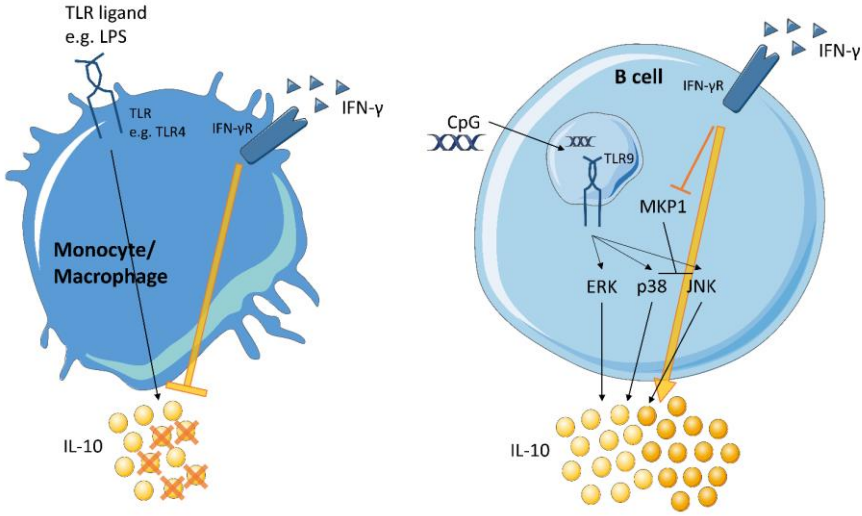

Figure 2: Proposed mechanism behind the increase of CpG-induced IL-10 by IFN-γ in B cells. *While IFN-γ inhibits TLR-induced IL-10 production in monocytes and macrophages (left), it stimulates CpG-induced IL-10 production in B cells (right). Stimulation of B cells with CpG induces the production of IL-10 via the MAPK ERK, p38 and JNK. Simultaneous addition of IFN-γ increases this IL-10 production by*

inhibiting the MAPK phosphatase MKP1, an inhibitor of p38 and JNK, resulting in enhanced IL-10 production. Big orange arrows: effect of IFN-γ on IL-10 production.

5. Future perspectives

In the future, it might be important to evaluate whether the defective IL-10 production by B cells in sJIA can be reversed. One potential way to achieve this, is to increase B cell-specific IL-10 production via stimulation with low doses of CpG, a treatment strategy that was proven effective in the EAE mouse model[240]. To this end, both preventive and curative treatment of sJIA mice with CpG should be tested. Since CpG+IFN-γ further increases IL-10 production by B cells, and since IFN-γ plays a protective role in the sJIA mouse model, simultaneous administration of CpG and IFN-γ can also be evaluated. However, injection of CpG in the sJIA mouse model should be studied with caution, as repeated injection of high doses of CpG (10 times higher than the dose used as treatment in the EAE model) results in the development of macrophage activation syndrome. Therefore, other B cell-specific stimuli can also be evaluated.

sJIA is considered an autoinflammatory disease and therefore, the role of B cells in sJIA pathogenesis is barely studied. In fact, to our knowledge, our report is the first to investigate the importance of B cells in sJIA, via analysis of their IL-10 production. To get a broader understanding of the role of B cells in sJIA pathogenesis, it would be interesting to induce the sJIA mouse model in mice lacking B cells (either in B cell KO mice or by using B cell-depleting antibodies) or in mice with a B cell-specific defect in IL-10 production. Furthermore, an in-depth analysis of B cells from sJIA patients may provide some clues about their role in disease development, aside from their defective IL-10 production.

SUMMARY

Systemic juvenile idiopathic arthritis (sJIA) is a rare but severe childhood immune disorder and is a subtype of JIA, a heterogeneous group of arthritic diseases. In contrast to other JIA subtypes, sJIA is characterised by systemic features, such as fever, rash and enlargement of lymph nodes, spleen or liver, which are often more prominent than joint inflammation. The aetiology of sJIA remains largely unknown. One hypothesis is that sJIA results from the inappropriate control of the immune response to an initially harmless trigger in predisposed children. In line with this, polymorphisms in the anti-inflammatory cytokine IL-10, resulting in a lower IL-10 production, are associated with sJIA disease development.

In the first part of this thesis, we investigated whether a defective IL-10 production underlies the pathogenesis of sJIA. To this end, we took advantage of a recently developed mouse model for sJIA and used plasma and blood cells from sJIA patient. In the mouse model, injection of IFN-γ deficient (KO) mice with Complete Freund's Adjuvant (CFA) results in the development of sJIA-like symptoms, while CFA-injected wild type (WT) mice only develop a subtle inflammatory reaction and serve as a control. In the diseased CFA-injected IFN-γ KO mice, we observed a cell-specific defect in IL-10 production in B cells, regulatory T cells and NK cells, with B cells being the major source of IL-10. Furthermore, neutralisation of IL-10 signalling in CFA-injected WT mice resulted in an immune-inflammatory disorder that is clinically and haematologically reminiscent of sJIA, indicating that a defective IL-10 production contributes to the development of sJIA symptoms. In line with the observations in the mouse model, we observed low levels of IL-10 in the plasma of sJIA patients and demonstrated a decreased IL-10 production by B cells, both *ex vivo* and after *in vitro* stimulation. Together, our data show that a decreased IL-10 production may underlie sJIA development.

The defective IL-10 production in sJIA mice which genetically lack IFN-γ came as a surprise considering the traditional paradigm of IL-10 and IFN-γ as each other's antagonists. Since the sJIA mouse model is induced

with CFA, an adjuvant that contains several Toll-like receptor (TLR) ligands, the influence of IFN-γ on TLR-induced IL-10 production was explored in the second part of this thesis. The study was performed by using different cell types obtained from naïve mice and from human healthy donors. In agreement with the traditional viewpoint of IL-10 and IFN-γ as antagonists, we confirmed that IFN-γ inhibits TLR-induced IL-10 production by monocytes and macrophages. In contrast, we observed an opposite scenario when B cells were stimulated with CpG, a TLR9 ligand. Thus, IFN-γ stimulated TLR9-induced IL-10 production in B cells, and this observation is in line with the defective IL-10 production in the CFA-injected IFN-γ KO mice. Further research into the mechanisms involved revealed that the increase of TLR9-induced IL-10 by IFN-γ was restricted to B cells. We excluded involvement of B cell proliferation, increased TLR9 expression, type I IFN production, IL-6 production, IL-10 mRNA stabilisation and B cell activating factor but demonstrated that the mitogen-activated protein kinases (MAPK) p38 and JNK are essential players in the stimulatory effect of IFN-γ on CpG-induced IL-10. Furthermore, we showed that the MAPK phosphatase MKP1, which is an inhibitor of p38 and JNK, is downregulated by IFN-γ, potentially contributing to the increased IL-10 production. Together, these data may represent a novel anti-inflammatory property of the traditionally considered pro-inflammatory IFN-γ, by stimulating TLR-induced IL-10 production in B cells.

SAMENVATTING

Systemische juveniele idiopathische artritis (sJIA) is een zeldzame maar ernstige pediatrische inflammatoire aandoening en is een subtype van JIA, een heterogene groep van artritische aandoeningen. In tegenstelling tot de andere JIA-subtypes wordt sJIA voornamelijk gekarakteriseerd door systemische ziektesymptomen zoals koorts, uitslag en een vergroting van de lymfeknopen, milt of lever, die vaak prominenter aanwezig zijn dan de artritis. De oorzaak van de ziekte is echter nog onbekend. Eén van de hypotheses is dat sJIA wordt veroorzaakt door een slechte downregulatie van de immuunrespons tegen een onschadelijke trigger. De observatie dat polymorfismen in het anti-inflammatoire cytokine IL-10, die resulteren in een lagere IL-10 productie, geassocieerd zijn met de ontwikkeling van sJIA komt overeen met deze hypothese.

In het eerste deel van deze thesis werd onderzocht of een defect in de productie van IL-10 aan de basis kan liggen van de ontwikkeling van sJIA. Om deze vraag te beantwoorden, hebben we gebruik gemaakt van een recent ontwikkeld muismodel voor sJIA en van sJIA patiënten stalen. Het sJIA muismodel wordt geïnduceerd door injectie van Compleet Freund's Adjuvans (CFA) in IFN-γ-deficiënte (KO) muizen. CFA-injectie in wildtype (WT) muizen resulteert daarentegen enkel in een beperkte inflammatoire reactie en werd gebruikt als controle. In de zieke IFN-γ KO muizen observeerden we een verlaagde IL-10 productie door B cellen, regulatorische T cellen en NK cellen, met B cellen als de belangrijkste bron van IL-10. Neutralisatie van IL-10 signalisatie in CFA-geïnjecteerde WT muizen resulteerde in de ontwikkeling van ziektesymptomen die klinisch en hematologisch sterk lijken op sJIA. Dit wijst erop dat een defecte IL-10 productie bijdraagt aan de ontwikkeling van sJIA symptomen. In overeenkomst met de resultaten in het sJIA muismodel werd een opvallend lage IL-10 productie geobserveerd in het plasma van sJIA patiënten. Daarnaast werd aangetoond dat B cellen van sJIA patiënten een verminderde productie van IL-10 vertonen, zowel *ex vivo* als na *in vitro*

stimulatie. Samenvattend kan hieruit besloten worden dat een defecte IL-10 productie bijdraagt aan de ontwikkeling van sJIA.

De observatie van een defecte IL-10 productie in de afwezigheid van IFN-γ in het sJIA muismodel (i.e. CFA-geïnjecteerde IFN-γ KO muizen) was verrassend aangezien IL-10 en IFN-γ traditioneel beschouwd worden als elkaars antagonisten. Omdat CFA uit verschillende Toll-*like* receptor (TLR)-liganden bestaat, werd een verklaring voor deze opvallende observatie gezocht door het effect van IFN-γ op TLR-geïnduceerde IL-10 productie te bestuderen *in vitro* met behulp van verschillende celtypes van gezonde muizen en humane donoren. In overeenkomst met het traditionele paradigma, konden we bevestigen dat IFN-γ de productie van TLR-geïnduceerde IL-10 productie door monocyten en macrofagen inhibeert. We observeerden echter een omgekeerd scenario wanneer B cellen werden gestimuleerd met CpG, een TLR9 ligand. Dus, IFN-γ verhoogde de productie van TLR9-geïnduceerde IL-10 productie in B cellen, en deze observatie komt overeen met de gedaalde IL-10 in de afwezigheid van IFN-γ zoals vastgesteld in het sJIA muismodel. Verder onderzoek naar het mechanisme dat verantwoordelijk is voor deze verhoogde IL-10 productie door IFN-γ toonde aan dat het effect specifiek was voor B cellen en niet verklaard kon worden door een verhoogde B cel proliferatie, verhoogde TLR9 expressie, IL-10 mRNA stabilisatie of door inductie van type I IFN, IL-6 of B cel-activerende factor. De mitogeen-geactiveerde proteïne kinases (MAPK) p38 en JNK bleken essentieel te zijn voor de stijging in TLR9-geïnduceerde IL-10 productie door IFN-γ. Mogelijks draagt ook de downregulatie van het MAPK fosfatase MKP1, een inhibitor van p38 en JNK, door IFN-γ bij tot de geobserveerde stijging in IL-10 productie. De stimulatie van TLR9-geïnduceerde IL-10 productie door IFN-γ kan gezien worden als een nieuwe anti-inflammatoire functie van het traditioneel beschouwde pro-inflammatoire IFN-γ.

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