Follicular T helper cells and IL-21 in Rheumatic Diseases

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THE FIVE ORIGINAL PAPERS ARE

1. Increased Interleukin 21 (IL-21) and IL-23 Are Associated with Increased Disease Activity and with Radiographic Status in Patients with Early Rheumatoid Arthri- tis. Tue Kruse Rasmussen, Thomas Andersen, Malene Hvid, Merete Lund Hetland, Kim Hørslev- Petersen, Kristian Stengaard-Pedersen, Christian Kanstrup Holm, and Bent Deleuran. Journal of Rheumatology 2010 October 37(10):2014-2020.

2. Increased plasma levels of IL-21 and IL-23 in spondyloarthritis are not asso- ciated with clinical and MRI findings. Thomas Andersen*, Tue Kruse Rasmussen*, Malene Hvid, Christian Kanstrup Holm, Karen Jong-Nyo Berenth Madsen, Anne Grethe Jurik, Marianne Hokland, Kristian Stengaard-Pedersen, Berit Schiøttz-Christensen, Bent Deleuran. Rheumatology International 2010 32(2):387-393. *Shared first authorship.

3. Overexpression of MicroRNA-155 increases IL-21 mediated STAT3 Signaling and IL-21 Production in Systemic Lupus Erythematosus. Tue Kruse Rasmussen, Thomas Andersen, Rasmus Otkjær Bak, Gloria Yiu, Kristian Stengaard- Pedersen, Jacob Giehm Mikkelsen, Paul Joseph Utz, Christian Kanstrup Holm, Bent Winding Deleuran. JArthritis Res Ther. 2015; 17(1) 154.

4. MicroRNA-21 can regulate apoptosis of CD4+ T cells in Systemic Lupus Erythematosus. Tue Kruse Rasmussen, Rasmus Otkjær Bak, Thomas Andersen, Anders Dige, Christian Holm, Jacob Giehm Mikkelsen, Bent Deleuran. Rheumatology 2015 5:155.

5. Development of Th17-associated interstitial kidney inflammation in lupus-prone mice lacking the gene encoding Signal Transduction and Activator of Transcription-1 (STAT-1). Gloria Yiu, Tue Kruse Rasmussen, Bahareh Ajami, David J. Haddon, Alvina D. Chu, Stephanie Tangsombatvisit, Winston A. Haynes, Vivian Diep, Larry Steinman, James Faix, Paul J. Utz. Arthritis Rheumatol. 2016 May; 68(5): 1233-1244.

INTRODUCTION

The treatment of rheumatic disease has undergone a revolution during the last two decades. With the introduction of the first TNFα-blockers, a whole new generation of drugs was introduced. These have since been expanded significantly to cover diverse targets throughout the immune system. The first 'biologics' provided new treatment option for patients, who had exhausted the traditional options, and brought hope of both improvement of daily-living and potentially a cure. Much has happened since their introduction, and while these new biologics provide crucial and even life-saving treatment options, their initial promises of a life without disease symptoms for all patients have been dimmed, while they burdening the health care system with heavy cost. Belimumab, a drug targeting B cells, was recently approved and introduced to the market, but still fail to show truly convincing treatment efficacy.

TNFα-blockers were the first line of drugs designed to modulate the immune system. Along with their introduction our understanding of the immune system grew substantially, and we now face the second wave of biologics. New classes of drugs have been introduced such as 'small molecules' and siRNA therapy. With hopes of better efficacy and higher specificity, these new drugs aim to target key processes driving and maintaining autoimmune disease. To realize the potential of these new drug therapies intricate knowledge of the processes initiating and maintaining the diseases is key.

Follicular T helper (Tfh) cells and interleukin (IL-)21, with their key functions in controlling B cells and (auto-)antibody production, present appealing targets for tailoring new treatment options for autoimmune diseases characterized by autoantibodies. This present PhD thesis aims to further investigate the role of Tfh cells and IL-21 in rheumatic disease, and elucidate new molecular mechanisms by which they function, to further and improve new treatment options for patients.

Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is a multifactorial autoimmune disease. SLE is caused by aberrant immune responses mainly affecting the skin, but spares no organ system. Accordingly, SLE is diagnosed based on 11 diverse criteria of which at least four must be fulfilled [1, 2]. SLE is a prototypic autoimmune disease characterized by the presence of autoantibodies directed at DNA and RNA associated antigens, and it has multiple contributing factors such as genes, environment, and innate immunology [3]. Many advances in the care and treatment of SLE patients have

been made in the last decade. In the 1950s SLE patients had a 50% 5-year survival rate [4], contrasted by current 5-year survival rates of >90% [5].

The worldwide prevalence of SLE is approximately 20-130 per 100.000 making it a quite rare disease. It usually debuts in younger women around 20-30 years of age. How- ever, differences in geographic and ethnic distribution cause large variations in prevalence rates. In Denmark the prevalence is estimated at 22 per 100.000 people and in the rest of Northern Europe including the UK the prevalence rates are approximately 20-50 [5, 6]. The United States has some of the highest prevalence rates worldwide with 110-150 per 100.000 due to the markedly higher prevalence rates in african-americans [5]. Different ethnic groups besides African-americans also have increased risk of SLE including people with Asian background having two to three times higher rates than caucasians [5]. The strongest risk factor for SLE is, however, female gender with women making up more than 90% of patients. This gender bias in SLE is still poorly understood. It is hypothesized that hormonal changes in estrogen may play a role. During early pregnancy levels of estradiol and progesterone rise along with the risk of disease flare [7]. Further, hormone replacement therapy (HRT) suspected of increasing the risk of flare [8].

While the ethnic variations in SLE presumably reflect different compounded genetic risk factors within these groups other more distinct genetic factors also contribute to SLE pathogenesis. Although rare, a few single gene deficiencies have been shown to increase risk of SLE such as the genes encoding TREX1, C1q, and C4 [9]. TREX1 is a DNA exonuclease functioning to degrade anomalous DNA structures thus preventing them from activating the immune system. Loss of function of TREX1 confers a inflammatory immune response with severe neurological brain disease (also known as Aicardi-Gouti`eres syndrome)[10]. Both genetic deficiencies in C1q and autoantibodies directed at C1q are high risk factors for SLE. This is presumed to be caused by the loss of opsonization of immune complexes (ICs) conferred by C1q binding to the Fc regions of the antibodies leading to aberrant processing of the ICs [11, 12] These genes can, however, only account for a small number of cases and it is presumed that a mix of variations and mutations in a large number of genes is present in SLE patients [3]. Single-nucleotide polymorphisms (SNPs) are also implicated in SLE, and recent genome-wide association studies (GWAS) have increased the number of SNPs, linked to SLE, dramatically over the last few years. Still, it is estimated that SNPs only account for up to 15% of heritable risk of SLE [3, 9].

Multiple immune subsets are involved in SLE pathogenesis

Dysregulated Th cell and B cell interactions are believed to be responsible for the production of autoantibodies. Self-reactive plasma cells produce autoantibodies often directed at DNA- and RNA-associated epitopes (also referred to as antinuclear antibodies or ANAs) (Figure 1)[13]. The reason that these autoantibodies are directed at nuclear epitopes is uncertain, but it could be speculated that this is due to the rare nature and nuclear localization of these molecules. These DNA- and RNA-associated proteins are less likely to be exposed to the immune system during maturation, as they are located in the nucleus, and do not undergo the same mechanisms for self-presentation as cytosolic or secreted proteins. Thus, these nuclear components could be more likely to be recognized as non-self upon presentation [14]. Further, the increased tendency of leukocytes to undergo apoptosis is characteristic of SLE, and could thus explain the increased exposure of these proteins to the immune system [15, 16, 17]. Once in the blood and tissues, autoantibodies form immune complexes (ICs) by cross- linking with their antigens. The ICs deposit in tissues such as the skin, kidney and the central nervous system (CNS) causing inflammatory reactions by activating the complement cascade [18]. The activation of the complement cascade causes activation of other parts of the immune system and in both SLE and mouse models of SLE Th17 cells have been found to be increased in inflamed kidneys during glomerolunephritis (GN) [19, 20].

Previous therapeutic strategies have focused on broad dampening of immune responses by applying cytotoxic drugs and corticosteroids. This has improved disease outcome significantly, but leaves many patients with severe side effects sometimes outweighing the benefits. Novel treatment strategies attempt to regulate key functions in the pathological process thus if effective reducing side effects and improving treatment efficacy [3, 21]. Belimumab (marketed as Benlysta) is one such new drug and it is the first newly developed drug approved for SLE in over 50 years [22, 23]. Belimumab is a fully humanized antibody against B-cell activating factor (BAFF), also known as B-lymphocyte stimulator (BLyS). BAFF is over expressed in B cells and function to in- duce maturation of naive B cells. Inhibiting BAFF thus represses development of new self-reactive B cells clones and thereby autoantibody production. While efficacy of belimumab underlines the importance of B cells in SLE pathology, the improvements in disease activity observed in the clinical trials were limited. In the high-dose belimumab group 58% met the primary endpoint (reduction in SLEDAI by 4 points over 52 weeks) compared to 51% in the low-dose group and 44% in the placebo group [22]. For context one-years treatment with belimumab costs approximately US$35.000 compared to US$100-1000 per year for standard drugs such as corticosteroid, methotrexate, and mycophenolate mofetil. Further, the patients who benefited the most where those with high dsDNA and/or low complement (C3, C4), thus highlighting the need to stratify better patients before treatment is initiated. Combined, the moderate efficacy and very high cost has caused questions as to belimumabs viability in the treatment of SLE [24, 25].

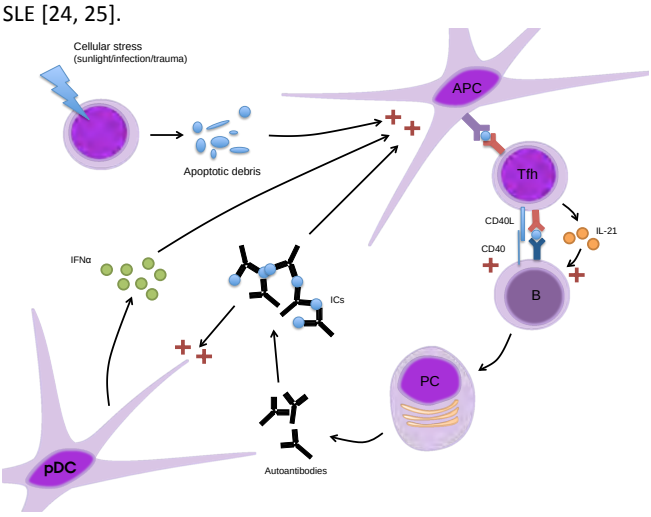

Figure 1. The Immunological Background of Systemic Lupus Erythematosus. Upon cellular stress from sunlight, trauma and possibly viral infection, apoptosis of affected cells is increased. The debris formed contains nuclear and DNA/RNA-associated proteins such as histone, ribo-nucleoproteins (RNPs) and double- or single-stranded (ds/ss)DNA. Combined with incompletely clearing of this debris by macrophages in the surrounding tissue there is a build up of debris in the tissue. Antigen-presenting cells (APCs) such as dendritic cells take up this debris and present it to circulating T

cells. The activated T cells in turn prime B cells to produce antibodies directed against these same epitopes (now autoantigens), in part driven by IL-21. B cells mature into plasma cells (PCs) and produce immunoglobin (Ig)-switched autoantibodies which are capable of forming im- mune complexes (ICs) by cross-linking to cellular debris. ICs on their own stimulate DCs to become more activated and induce interferon (IFN)-α production from plasmacytoid (p)DCs. © 2014 Tue Kruse Rasmussen.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease responsible for debilitating joint destruction and premature death of afflicted patients. With no cure, life-long medical costs, and estimated to affect 1% of the general population in the western world, it is a major health cost both in terms of quality of life but also financially [26, 27]. New insights into RA pathology are developing and improving rapidly and involves mul- tiple aspects of immunity. Abnormalities have been described in a range of immune functions and both innate mechanism and highly evolved adaptive functions seems to operate side-by-side to create and propagate the inflammatory reactions attacking the joints [28, 29, 30].

In terms of pathophysiology, RA can be divided into four stages of disease progression. In the induction phase of the disease, the innate immune system is activated, by factors yet unknown, to mount an immune response. This in turn primes the adaptive immune system to become more susceptible. In the inflammation phase self-antigen, or antigens mimicking these, are presented to the adaptive immune system. If self-tolerance is lost at this point an inflammatory reaction develops in the synovium with initial swelling and soreness as the first disease manifestations. Although no known pathogens are present, several high impact risk factors have been found of which smoking is critical in this initial phase [31]. In the self perpetuation phase the breakdown of cartilage continuously presents new autoantigens to the immune system, which in turn induces further inflammation and destruction of cartilage and bone. Finally, in the destruction phase irreversible destruction of the joints by pannus formation and osteoclast driven bone erosion ensues [29, 31].

Although this linear representation of disease development is helpful in understanding RA the actual disease can progress through these phases in a non-linear fashion. Thus patients can debut at different stages and fluctuate between them as disease progresses. This is highlighted by the fact that CTLA4 (abatacept) agonists are surprisingly effective even in chronic RA. CTLA4 is a co-repressor active during priming and activation of naive T cells in response to immune stimulation. The fact that stimulation of CTLA4 is effective in treatment reflects that there is ongoing immune activation even in late phase RA [32, 33].

Both innate and adaptive immune mechanisms appear to coincide in RA. However, several mouse models of RA (CIA, K/BxN, and AIA) have demonstrated the unique role of CD4+ T cells in RA as all these models require CD4+ T cells to develop fulminant disease [34, 35, 36]. This is supported by the efficacy of CTLA4 therapy showing an ongoing T cell activation in RA. CD4+ T helper (Th) cells are a highly differentiated, but varied group of immune cells capable of directing a range of immune functions. Two dual functions of Th cells are emerging in RA. Interleukin-17A (IL-17A) producing Th (Th17) cells are highly pro-inflammatory cells through stimulation of innate immune cells to secrete cytokines such as TNFα and matrix-metalloproteases (MMPs) [37, 38]. However, clinical trials have failed to produce convincing results for anti-IL-17A therapy's efficacy in a large cohort of randomized RA patients [39, 40], but had better results in a smaller, more selected, cohort treatment refractory RA patients [41]. Follicular T helper (Tfh) cells are another, recently characterized, subset of CD4+ T cells responsible for directing B cell responses in lymphoid tissue. Tfhs and their main signaling molecule, IL-21, have been shown to be central in the processes leading to loss-of-tolerance and the production of autoantibodies (discussed in detail below) [42].

Follicular T Helper Cells

The term 'follicular T helper cells' was originally coined by Breitfeld et al. in 2000 [43]. They were first described in a series of papers, early in the 21st century, demonstrating Tfh cells capacity to induce high levels of IgG production from B cells, their increased expression of CXCR5 and the high degree of follicular homing conferred by this chemokine receptor [44, 45, 46, 47]. Numerous studies have since added to this knowledge providing a more detailed phenotype of the Tfh cells and they are now more or less stringently defined as T helper cells that express high levels CXCR5, ICOS and PD-1 and secrete IL-21 [42, 48, 49].

Yu et al. were the first to report that the B-cell lymphoma 6 (Bcl-6) protein control lineage commitment of Th cells to Tfh cells [50]. Bcl-6 is encoded by the Bcl6 gene - a gene first described by its proto-oncogenic role in B cell lymphomas [51]. With remark- able foresight Dent et al. had 10 years previously setup the framework in which Bcl-6 function by providing the first mechanistic insights in to Bcl-6's role as a major regulator of B cell fate and GC formation [52]. Functionally, Bcl-6 is a transcriptional repressor, and works by binding to - and inhibiting the function of Tbx21 and RORC2 (human equivalents of Tbet and RORγt), thus inhibiting Th1 and Th17 lineage commitment, respectively [50]. Murine T cells lacking the Bcl-6 gene are unable to produce Tfh cells and have disrupted germinal center formation and responses while T cells overexpressing Bcl-6 develop increased Tfh cell numbers [53]. Recently, Liu et al. were able to expand on these findings by showing that achaete-scute homologue 2 (Ascl-2) is responsible for the early commitment of naive CD4+ T cells to Tfh cells [54]. While the Ascl-2 protein like Bcl-6 also is a repressor of competing lineage transcription factors it is also a transcription factor and selectively upregulates CXCR5 and downregulates CCR7 expression. This primes the Th cells for follicular homing and early Tfh lineage commitment. Once the early Tfh cell is in the follicle, stimulation through CXCR5 by CXCL13, amongst others, induce transcription and up-regulation of Bcl-6 thus finally committing the cell to the Tfh lineage and priming its capacity to provide costimulatory signals to B cells [42, 50, 54].

Generation of Autoantibodies

Antibodies are produced by B cells and plasma cells in response to immunological chal- lenges to the immune system. Activated B cells will migrate to the follicles where they scan for non-self antigens bound to follicular dendritic cells (FDCs). Once in the follicle the B cells will pair (guided by FDCs) with their respective antigen-specific Tfh cell. Depending on binding-affinity of the MHC-II molecule on the B cell (presenting the antigen) and the T cell receptor (TCR) the B cell will receive costimulatory signals to either proliferate and mature into a memory B cells/plasma cell or to become anergic and undergo apoptosis [55]. As the B cell is in an apoptotic state by default it will need survival signals to avoid apoptosis. These costimulatory signals consists of both cytokine-mediated signals, such as IL-21, and receptor-ligand interactions, such as CD40, PD-1, OX40, and ICOS. If the B cell is allowed to pass this threshold it will undergo Ig class-switch to produce IgG or IgA molecules and somatic hypermutation (SHM)

thus increasing the affinity of the antibody driven by the signals given to it from the Tfh cell [55, 56].
The importance of these costimulatory signals is highlighted by the effects of im balances in Tfh numbers. Imbalances in the number of Tfh cells, and consequently their capacity to provide B cell help, is crucial for optimal non-autoreactive antibody production [42]. If there is increased numbers of Tfh cells, B cells will receive excessive survival signal and produce self-reactive antibodies as demonstrated in mice [57, 58] and several autoimmune diseases including SLE and RA [59, 60, 61, 62, 63]. Conversely, low numbers of Tfh cells causes disrupted germinal center (GC) formation and immunodeficiency [42]. In mice, inhibiting Tfh cell differentiation disrupts GCs formation and leaves B cells unable to progress from early centrocyte phase (early developmental stage in B cell maturation in the follicle) and incapable of producing high affinity antibodies [50, 64, 53]. Similarly in humans, genetic mutations in genes conferring costimulatory signals disrupts Tfh cells and B cells interactions. Thus patients
with CD40L deficiency develop hyper-IgM syndrome reflecting that they are not able to class-switch or SHM their antibodies leaving them developmentally 'stuck' as IgM antibodies [65] and similar defects are seen in ICOS and STAT3 deficiencies [66, 67].

While Tfh cells undoubtedly hold key non-redundant functions in controlling B cell maturation and antibody production the actual mechanism for loss-of-tolerance is still unclear. Either self-antigens are directly presented to self-reactive B cells and Tfh cells or loss-of-tolerance happens during maturation of the B cell [48]. The latter is thought to happen as part of the somatic hypermutation where no control mechanism for deletion of accidental self-reactivity against extra-follicular antigens exist [68].

IL-21 Functions and Signaling
Interleukin 21 was first discovered in 2000 by Parrish-Novak et al., as a regulator of NK cell function [69]. IL-21 is secreted by activated T cells and especially Tfh cells and is a member of the IL-2 family of cytokines [69,70,71]. Since its discovery IL-21 has been found to regulate a plethora of both adaptive and innate immune functions [72].

Interleukin 21 signals using a unique IL-21 receptor subunit combined with the common gamma-chain. Receptor ligation leads to phosphorylation of signal transducer and activator of transcription (STAT) molecules 1, 3, and 5 of which STAT3 is the dominant [73,74]. Modulation of STAT signaling both pathological and therapeutic is believed to be integral in the processes driving and controlling autoimmune disease [75]. Phosphorylation of STAT3 happens at two distinct locations. Phosphorylation of Tyrosine 705 (Y705) is mainly inducible by cytokine stimulation while phosphorylation of Serine 727 (S727) is more affected by the basal activation state of the cell [76,77]. In the context of RA, Ju et al. were able to show that STAT3 expression in synovial tissue was correlated to synovitis score and further that inhibition of STAT3 reduced levels of pro-inflammatory Th17 cells [78]. In a murine model of SLE, Hale et al. demonstrated that STAT responsiveness mirrored disease activity and progression [79]. Apparent in the latter study was also that STAT responsiveness dropped as disease progressed and became more and more chronic. This decrease in STAT responsiveness was followed by an increase in the STAT regulating protein *suppressor of cytokine signaling 1* (SOCS1). STAT signaling is in general a very tightly controlled process [80]. Three major classes of inhibitory molecules exist; SOCS (suppressors of cytokine signaling) proteins, PIAS (protein inhibitors of activated stats) and PTPs (protein tyrosine phosphatases). PIAS proteins and PTPs function by inhibiting DNA binding of STAT dimers and actively dephosphorylating STATs, respectively [81]. SOCS proteins function by both actively dephosphorylating STATs but also by inhibiting the interaction between JAKs and STATs. In contrast to PIAS proteins and PTPs, SOCS proteins offer a more direct regulation of cytokine signaling, as they are often induced by the cytokine that they in turn inhibit, providing instantaneous negative feedback [81,82]. A detailed understanding of the functions and regulation of STAT signaling pathways is of primary interest when considering the functions controlled by STAT signals. STAT molecule mediated cell survival and apoptosis signals as well as controlling key molecules in the context of immunity such as transcription factors and cytokines.

Through STAT molecules IL-21 has several effects on T and B cells. When stimulated with IL-21 both T and B cells upregulate Bcl-6 [53,83]. As described above Bcl-6 in turn acts as a transcriptional repressor to inhibit competing lineage determining transcription factors and increase CXCR5 and PD-1 expression on T cells. The induction of Bcl-6 by IL-21 is effectively blocked in vitro by the addition of TGFbeta (a combination know to favor Th17 development). And while IL-21 regulates Bcl-6 expression to promote Tfh cells, increased Bcl-6 expression does not affect IL-21 expression itself [53]. Conversely, lack of IL-21 signals to the B cells leads to impaired affinity maturation, reduced Ig class-switch and impaired generation of late-memory B cells. While GC formation is possible in the absence of IL-21 the structure is disrupted and long-tern maintenance is reduced [83]. Upon IL-21 stimulation of T cells, STAT3 is phosphorylated and directly binds to response elements activating transcription of the il21 gene [84,85]. This autocrine loop has been shown to promote Th17-mediated inflammatory responses *in vitro*, but its significance *in vivo* is still uncertain [42,85]. The ability of IL-21 to induced Tfh cells combined with its ability to positively regulate its own production in an autocrine manner presents an potential downward slope which could promote and maintain autoimmune responses. Unchecked this loop would continuously produce larger numbers of Tfh cells known to promote autoimmunity [42].

MicroRNA Biology and Function in Autoimmune Disease
MicroRNAs are a novel class of gene expression regulators found in eukaryote cells. They are short single-stranded RNA molecules consisting of 20-23 nucleotides with sequences highly complementary to their target gene mRNAs. By binding to their targets they form transiently double-stranded RNA molecules, which are degraded by the RNA-induced silencing complex (RISC) [86,86]. This process is referred to as RNA interference (RNAi) [88].

Although often subtle in their effects, evidence of miRNA mediated regulation of immunological processes have grown over the last few years [89,90,91,92]. A recent study demonstrate that in mice miR-146a acts as a repressor of autoimmunity and loss of miR-146a conferred an inflammation and oncogenic transformation [93]. Conversely, overexpression of the miR-17-92 cluster induces lymphoma and systemic autoimmunity by inhibiting key tumor suppressors [94]. In humans decreased miR-142 in T and B cells caused them to become activated and hyper-stimulated due to alteration in DNA methylation [95]. Using high-power microRNA array analyses, Zhu et al. were able to demonstrate both up- and down-regulation of a range of miRNAs in SLE and the lpr-model [96].

In the context of SLE, deletion of miR-155 in the Fas/lpr murine model of SLE (Fas deficient) significantly reduced autoantibody production and ameliorated disease severity. This effect was attributed to decreased B cell receptor (BCR) signaling, caused by lack of repression of SH2 domain-containing inositol 5'-phosphatase 1 (SHIP-1) by miR-155. SHIP-1 is an inhibitor of the B cell receptor (BCR) signaling [97]. Thus, deleting miR-155, increased SHIP-1 and its inhibition of BCR signaling, and less autoantibodies were consequently produced. Another study showed that knockout of miR-155, in a murine model of experimental autoimmune encephalomyelitis (EAE), rendered the mice highly resistant to T cell-dependent tissue inflammation [98].

MicroRNA-21 is a important regulator of apoptosis and has recently been implicated in SLE [92,99,100]. MicroRNA-21 functions as an oncomir in cancer, where high levels of miR-21 suppress the tumor suppressor programmed cell death 4 (PDCD4), thus inhibiting apoptosis [101,102]. As SLE is a disease characterized by increased apoptosis of lymphocytes [15,17,103,104] it has been speculated that miR-21 could contribute to this effect [92].

Combined, these findings highlight that miRNAs are important regulators of immune function. When targeting key signaling molecule or regulators of signaling, they enable widespread effects on immune function and balance. The cause of the variations in miRNA levels between healthy controls and patients suffering from autoimmune diseases are often uncertain and rarely addressed. However, Bcl-6 is an important repressor of miRNA with broad effects on multiple miRNAs [50]. As Bcl-6 expression in T cells is increased in SLE, this offers a possible explanation for many of the observed miRNA effects seen in SLE. Further, epigenetic changes such as (de-)methylations and histone modifications have proven efficient regulators of miRNAs expression [105], and SLE is a disease characterized by a high degree of epigenetic changes [106].

Interferon-alpha in SLE

Soluble factors conferring resistance to viral infections was first discovered almost 50 years ago [107]. These molecules were later dubbed 'interferons' and divided into three distinct groups - type I/II/III interferons [108]. It is still regarded as a key function in interferon biology to combat viral infections, but during the last two decades a series of diverse functions have been added to the long list of interferon regulated effects [108]. The type I interferon, interferon-alpha (IFNα), can in many ways be seen as an immune adjuvant. It has pleiotropic effects on the immune system, serving as an activator and inducer of immune responses [109].

In the context of SLE apoptotic material combined with immune complexes (ICs) have been shown to be potent inducers of IFNα production from plasmacytoid dendritic cells (pDCs) [110]. ICs are internalized by Fcγ-RIIA on the surface of pDC and once internalized activate Toll-like receptor (TLR-) 7 and 9 to induce IFNα production [110]. Plasmacytoid DCs are assumed to be the main source of IFNα in SLE, by their capacity to quickly and efficiently produce large amount of IFNα, and serum levels of IFNα have been shown to be elevated in SLE and regulate DC function [113]. However, both the numbers of circulating pDCs, and their capacity to produce IFNα, is decreased in SLE [114,115]. While part of the explanation for this probably is that circulating pDCs have migrated to inflamed tissues, and are actively producing large amounts of IFNα locally, it also indicates that IFNα effects in SLE could be due to other factors than purely expression levels.

A landmark paper, concerning IFN biology in SLE, was published by Baechler et al. in 2003 showing that not so much the quantity of IFNα, but more so the quality of the response to IFNα, is important. By identifying 161 IFNα inducible genes, the authors showed, that the degree of gene expression of these genes could be used to stratify SLE patients in terms of disease activity, key clinical outcomes, and disease severity. This IFN gene signature can be compressed into a distinct 'IFN signature', or profile, of expression levels of IFNα inducible genes [116]. This IFN signature has since been recapitulated and reconfirmed by several other groups in both SLE and other autoimmune diseases [117,118,119,129,121,122,123]. The cause for why SLE patients display the IFN signature is still uncertain but presumably involves epigenetic changes to interferon-regulated genes. It does, however, still represent one of the only validated and convincing stratification of SLE patients to date.

RESULTS

IL-21 Plasma Levels in Rheumatic Disease

To assess whether patients with rheumatic disease have changes in plasma levels of IL-21, and the related cytokine IL-23, we quantified the plasma levels of these cytokines in RA, SpA, and SLE patients by ELISA.

At the time of diagnosis early-stage RA patients, with disease duration of less than six months, had significantly increased plasma levels as compared to both late-stage RA patients (disease duration >8 years) and HCs (Figure 2). The levels increased from baseline to 3 months after diagnosis, but declined at 12 months after diagnosis. Late-stage RA patients did not have increased IL-21 plasma levels as compared to HCs. Plasma levels of IL-23, a related cytokine functioning as an inducer and growth factor for Th17 cells, showed a similar pattern with initial increase but low levels in late-stage disease. Plasma levels of both IL-21 and IL-23, at the time of diagnosis, correlated significantly with the disease activity score DAS28 (both $p<0.05$) and erythrocyte sedimentation rate (ESR) at 12 months (both $p<0.01$).

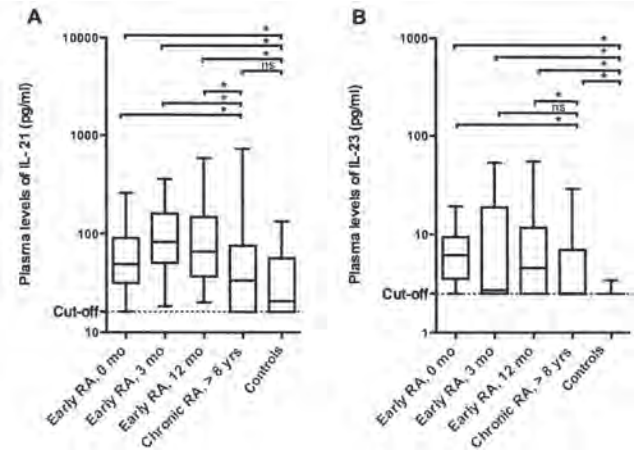

Figure 2. IL-21 Rheumatoid Arthritis Plasma Levels]{IL-21 and IL-23 Rheumatoid Arthritis Plasma Levels. Plasma levels of IL-21 and IL-23 were measured in the CIMESTRA cohort of early-stage RA patients, late-stage RA patients, and HCs. *p<0.05 (Mann-Whitney or non-parametric paired T test where appropriate).

In SpA patients plasma levels of IL-21 were also increased compared to HCs (Figure 3A), although the levels were lower as compared to early-stage RA patients. In SpA, however, neither IL-21 nor IL-23 plasma levels showed any correlation to disease activity or chronicity. This was assess by both physician/patient based parameters such as BASDAI, BASMI, BASFI, and MRI of the spine and sacroiliac joints (SIJ).

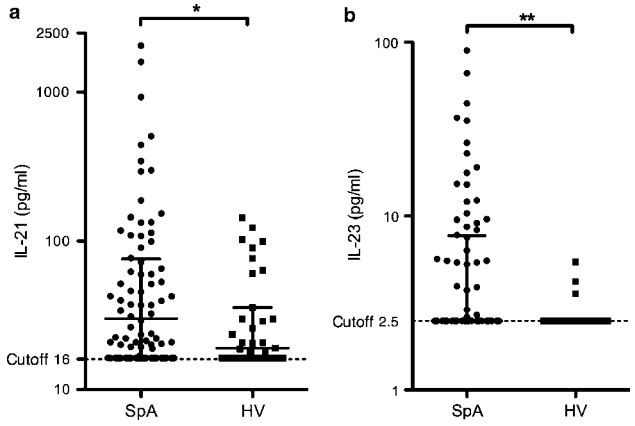

Figure 3. IL-21 Spondyloarthopathy Plasma Levels]{IL-21 and IL-23 Spondyloarthopathy Plasma Levels. Plasma levels of IL-21 in one cohort of SpA patients and one cohort of SLE patients as well as age and gender matched healthy volunteers (HV). *p<0.05, ****p<0.0001 (Mann-Whitney).

SLE patients also had increased plasma levels of IL-21 compared to HCs. Plasma levels in SLE patients were comparable to those found in early-stage RA patients. However, similar as to what was observed in SpA patient, IL-21 plasma levels did not show any correlation to disease activity, as measured by SLEDAI or chronicity as measured by SLICC (data not shown).

Production of IL-21 by T Helper Cells

Cytokine plasma levels reflect both systemic and local production of the investigated cytokines, irrespective of their cellular origin. To assess which subsets were the main producers of IL-21 we performed intracellular cytokine staining (ICS) of immune subsets in RA, SpA, and SLE. Representative flow cytometry plots are shown for RA patients (Figure 4).

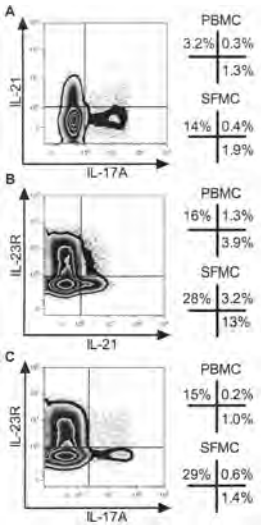

Figure 4. Rheumatoid Arthritis Flow Analysis. Intracellular cytokine staining of IL-21 and related cytokine IL-17A in CD3+CD4+CD45RO+ T cells combined with IL-23R staining. Median percentage positive cells for each quadrant is shown on right hand side. Data is shown for both PBMCs and SFMCs (n=9).

IL-21 production was significantly increased in SFMCs compared to PBMCs from RA patients (Figure 5). However, IL-17A (another pro inflammatory cytokine implicated in RA) producing T cells were not increased in SFMCs compared to PBMCs from RA patients. Similarly, SpA patients had increased IL-21 production in SFMCs compared to PBMCs but not IL-17A (Figure 6). Furthermore, percentages of IL-21 producing T cells in RA and SpA patients was increased compared to HCs (data not shown). Interestingly, IL-21 producing T cells from both RA patients and SpA patients were not double positive for IL-21 and IL-17A but single positive. This adds to the notion that IL-21 is not a Th17 related cytokine but more so belongs to its own separate subset of T cells.

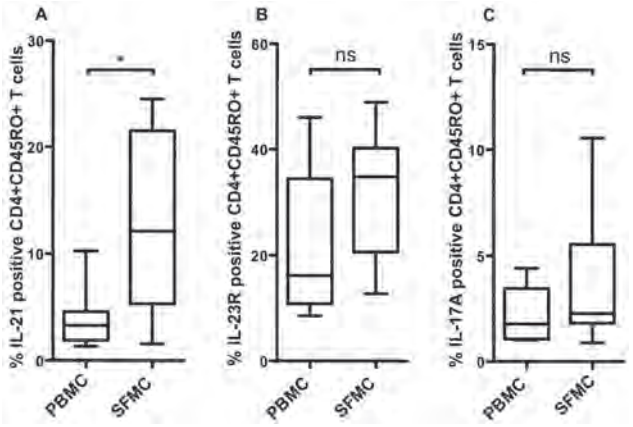

Figure 5. Rheumatoid Arthritis Cytokine production. Production of IL-21 and IL-17A and expression of IL-23R by CD4+CD45RO+ T cells from RA patients (all n=9). Production of IL-21 is significantly increased in SFMCs compared to PBMCs. *p<0.05 (Paired T test).

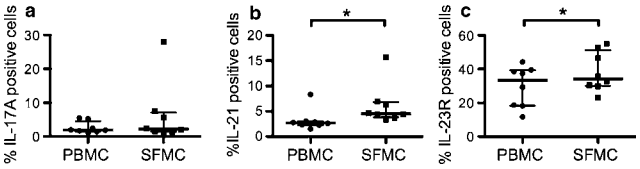

Figure 6. Spondyloarthopathy Cytokine production]{Spondyloarthopathy Cytokine production. Production of IL-21 and IL-17A and expression of IL-23R by CD4+CD45RO+ T cells from SpA patients (n=8). Production of IL-21 was significantly increased in SFMCs compared to PBMCs. *p<0.05 (Mann-Whitney)

SLE patients had significantly increased IL-21 producing CD4+ naive and memory T cells compared to HCs (Figure 7). Another IL-21 producing immune subset, NKT cells, did not show increased IL-21 production.

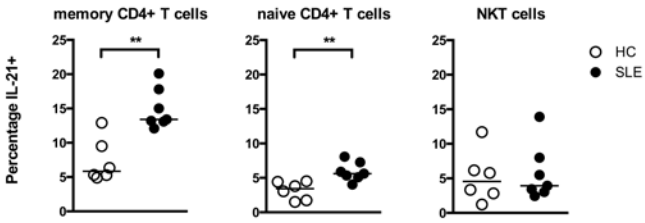

Figure 7. Systemic Lupus Erythematosus Cytokine Production. Production of IL-21 by CD4+CD45RO+ (memory) T cells, CD4+CD45RO- (naive) T cells, and NKT Cells from HCs and SLE patients (n=6-8). Production of IL-21 is increased in both naive and memory T cells, but more so in the latter. **p<0.05

MicroRNAs in Systemic Lupus Erythematosus

We hypothesized, that STAT3 responsiveness to IL-21 is dysregulated in SLE. We further hypothesized, that miR-155 can regulate IL-21/STAT3 signaling, and thus IL-21 production, by targeting SOCS1 in CD4+ T cells from SLE patients. Finally, we hypothesized that miR-21 can regulate apoptosis in CD4+ T cells by targeting PDCD4.

MicroRNA-155 in SLE

STAT3 is the main signaling molecule for IL-21 and has two separate phosphorylation sites. Tyrosine (Y)705 is inducible by cytokine stimulation, and Serine (S)727 controls the basal activity of STAT3 [76]. To assess if the signaling capacity of IL-21 was altered in SLE, we stimulated PBMC from SLE patients and controls and measured STAT3 phosphorylation in CD4+ T cells, B cells, and NK cells (Figure 8). STAT3 phosphorylation was significantly depressed in CD4+ T cells and B cells at the Y705 site, but not at S727. NK cells also had decreased STAT3 phosphorylation, however this was not significant. Interestingly, CD4+ T cells had marginally increased STAT3 phosphorylation at S727. Generally, the induction of phosphorylation was lower at S727 compared to Y705 reflecting their different functions.

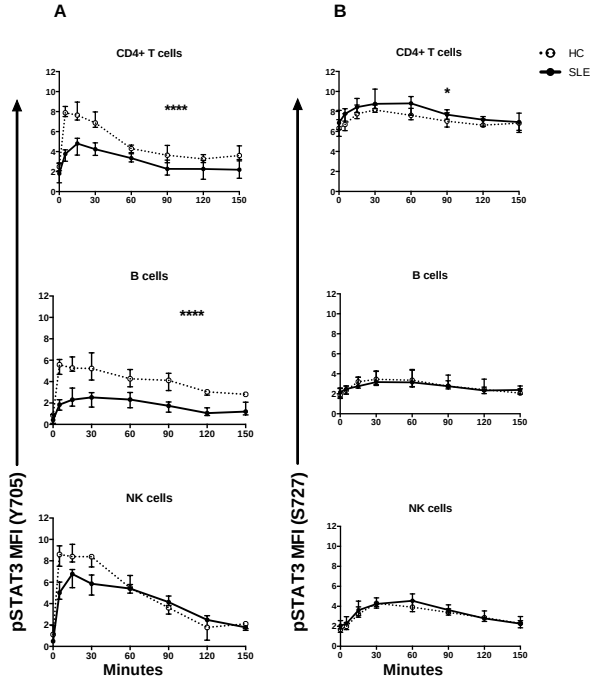

Figure 8. STAT3 Phosphorylations by IL-21 in Systemic Lupus Erythematosus. CD4+ T cells, B cells, and NK cells were stimulated with IL-21 and STAT3 phosphorylation was measured as Tyrosine 705 (A) and Serine 727 (B). Tyrosine 705 is primarily inducible by cytokine stimulation, while Serine 727 serves as a regulator of basal STAT3 activity. CD4+ T cells and B cells had significantly depressed STAT3 phosphorylation levels upon IL-21 stimulation at Tyrosine 705. *p<0.05, ****p<0.0001 (Repeated Measures Two-way ANOVA)

IL-21 Regulates miR-155 Expression

The levels of miR-155 were determined in unstimulated purified CD4+ T cells and showed that CD4+ T cells from SLE patients have significantly lower levels of miR-155 compared to HCs (Figure 9A). To determine if IL-21/STAT3 are capable of regulating miR-155 expression, we stimulated CD4+ T cells with IL-21 and measured miR-155 expression levels. Levels of miR-155 were significantly decreased in CD4+ T cells from SLE patients compared to HCs. Further, induction of miR-155 by IL-21 was reduced in SLE patients compared to HCs and dependent on STAT3 (Figure 9B-C). Expression levels of SOCS1 were increased in SLE patients, in line with the decreased levels of miR-155 (Figure 9D). Finally, levels of miR-155 and SOCS1 correlated negatively when examining SLE patients and HCs combined. While not conclusive these results imply that IL-21 regulates miR-155, which in turn regulates SOCS1 in SLE (Figure 9E).

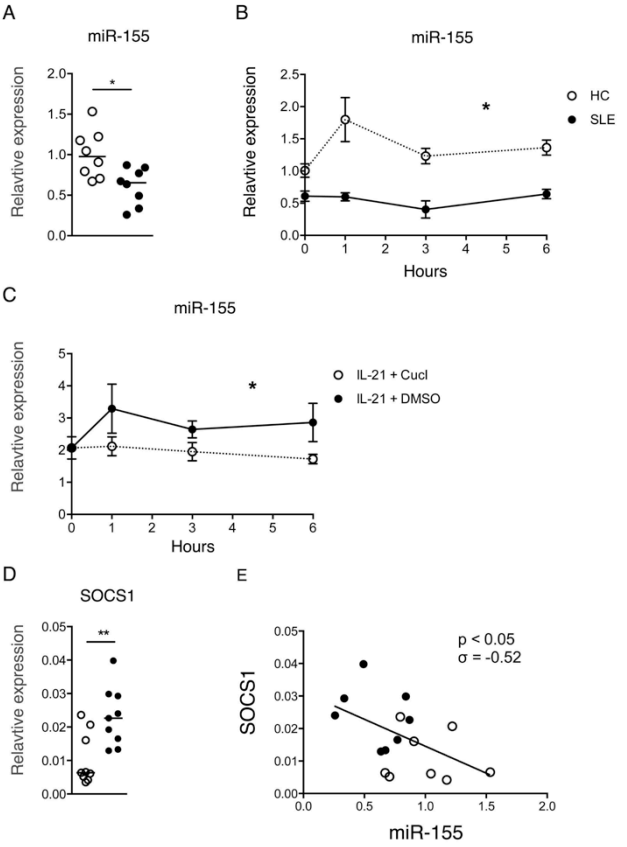

Figure 9. MicroRNA-155 in Systemic Lupus Erythematosus. (A) Expression levels of miR-155 in CD4+ T cells from SLE patients and HCs. SLE patients have decreased levels of miR-155 in CD4+ T cells. (B) Expression levels of miR-155 upon IL-21 stimulation in CD4+ T cells from SLE patients and HCs. Induction of mir-155 by IL-21 is significantly reduced in CD4+ T cells from SLE patients. (C) Blockage of STAT3 inhibits miR-155 induction. (D) Expression levels of SOCS1 (one of miR-155's target genes) are increased in CD4+ T cells from SLE patients compared to HCs. (E) Expression levels of SOCS1 and miR-155 correlate negatively. *p<0.05, **p<0.01 (Mann-Whitney, RM two-way ANOVA, and Spearman' correlation where relevant)

MicroRNA-155 Regulates STAT3 Phosphorylation

The functional effects of miR-155 was investigated by transducing PBMCs with a miR-155 encoding gene, combined with GFP as a marker for successful transduction (Figure 10A). The cells were transduced with a lentiviral vector, thus achieving a permanent and stable expression of the microRNA, as oppose to non-

retroviral transfection, which is only transient and does not produce stable expression of the desired gene. Upon transduction both CD4+ and CD8+ T cells increased their STAT3 responsiveness to IL-21 (Figure 10B). This difference was, however, only significant after 15 minutes of stimulation. This could reflect the fact that STAT signaling is a very tightly controlled process. Thus, in miR-155 overexpressing cells, there is presumably increased inhibition by various signaling inhibitors to repress the aberrant signaling. However, when supraphysiological levels of IL-21 are added, this repression is unable to counter the induction of phosphorylation by IL-21 and the levels of STAT3 phosphorylation are differentially increased in miR-155 overexpressing cells as seen at 15 minutes of stimulation.

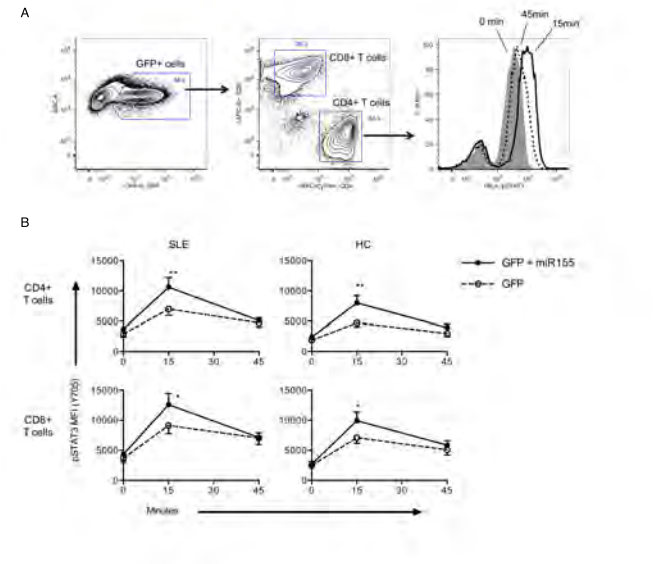

Figure 10. Effects of miR-155 Transduction on IL-21 Production. (A) Representative plots showing the gating strategy used in analysis of IL-21 production in CD4+ and CD8+ T cells. After transduction almost all CD4+ T cells were CD45RO+ (memory). This is due to the pre stimulation with CD3+CD28 and IL-2, which differentiated the cells into memory cells. Graphs show ratio (miR-155/GFP) (B) and percentage (C) of IL-21+ CD4+ and CD8+ T cells overexpressing miR-155. IL-21 production was significantly increased in CD4+ T cells overexpressing miR-155. *p<0.05, **p<0.01 (Mann-Whitney)

Functions of MiR-155 in SLE

In summary, we find that STAT3 responsiveness to IL-21 is reduced in CD4+ T cells and B cells from SLE patients. Further, induction of miR-155 is also reduced, leading to lack of inhibition of SOCS1. Increased levels of SOCS1, in turn, lead to further inhibition of STAT3. Finally, the reduced STAT3 responsiveness differentially inhibits autocrine-induction of IL-21 in CD4+ T cells from SLE patients (Figure 11).

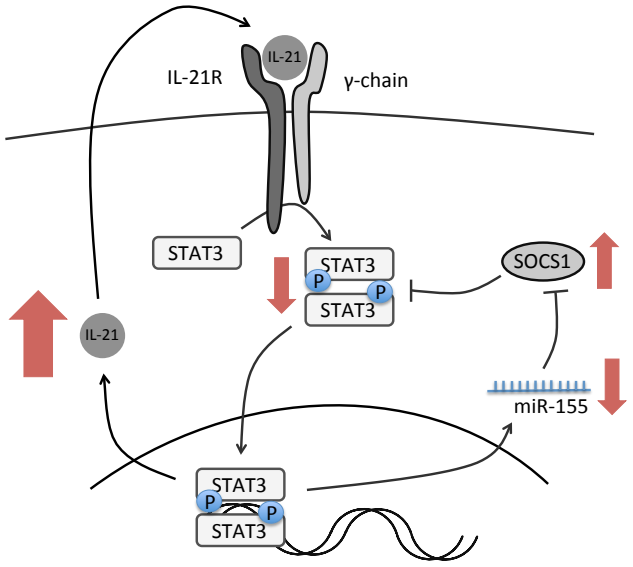

Figure 11. Schematical Representation of miR-155 Effects in SLE. Decreased STAT3 phosphorylation leads to suppressed induction of miR-155 by IL-21, and lower levels of IL-21 production. Loss of inhibition of SOCS1 by miR-155 leads to increased SOCS1 levels which in turn inhibit STAT3 phosphorylation. Red arrows indicate where SLE patient differ significantly from HCs.

Interferon-α and Autoantibodies in Systemic Lupus Erythematosus

The type I interferon, IFNα, is implicated as a key pathological factor in SLE. In an effort to study the individual contributions of the signaling proteins IFNAR2, IRF9, and STAT1 in the type I interferon signaling pathway we created knock-out mice for these proteins on a Balb/c background and backcrossed these into a lupus prone mouse strain (Fas/lpr). This allowed us to dissect the mechanistic properties of each individual protein and its possible pathological contribution.

Disruption of the Interferon Signaling Pathway Improves Proteinuria

To assess the impact of IFN signaling on kidney disease we measured proteinuria in all three knock-outs and controls. Both IRF9-/-, STAT1-/-, and IFNAR2-/- had significantly less proteinuria as compared to Fas/lpr control mice (Figure 12).

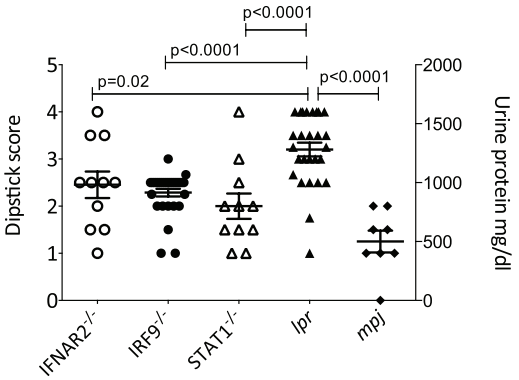

Figure 12. Proteinuria in IFN Signaling Knock-out Mice. Proteinuria in IFN signaling knock-out mice as measured by dipstick method. All knock-outs display improved (decreased) proteinuria compared to lpr control mice.

Stat1-/- Mice Show Interstitial Kidney Inflammation and Interstitial Leukocyte Infiltration

Having shown that the knock-out all mice had improved proteinuria, we wanted to further investigate, this by doing kidney histology. To our surprise, Stat1-/- showed marked and severe interstitial kidney inflammation, as assessed by the NIH Activity and Chronicity Index, scored by an independent, blinded observer (Figure 13A-D). Investigating this further by staining kidney sections for CD45 (as a marker for leukocytes), we found that Stat1-/-mice had marked infiltration of the interstitium by CD45+ leukocytes, but relatively well preserved glomerular structure (Figure 13E).

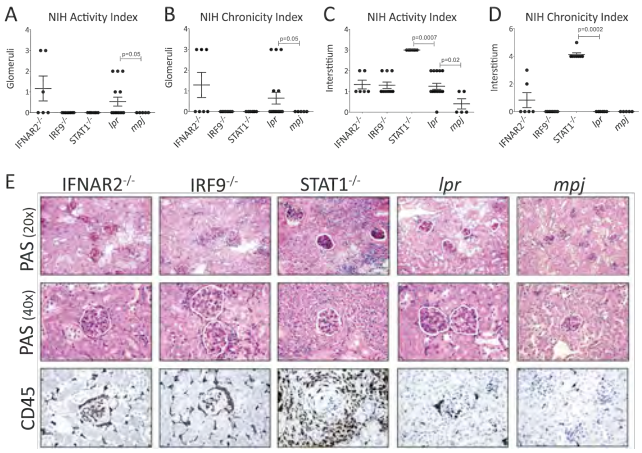

Figure 13. Kidney Scores and Histology in IFN Signaling Knock-out Mice. (A-D) NIH Activity and Chronicity scores for glomeruli and interstitial, scored by an independent-blinded observer. Stat1-/- mice display significantly increased interstitial kidney disease as measured by the NIH Activity and Chronicity scores compared to lpr control mice. (E) Histological slides of kidney sections from knock-out mice stained with periodic acid-Schiff (PAS) stain for structural overview and CD45 as a marker for leukocyte infiltration.

Th17 Cells are Increased in Stat1-/- Mice

Having shown that Stat1-/- mice have significantly worsened interstitial kidney disease and leukocyte infiltration compared to controls and Ifnar2-/- and Irf9-/-, we wanted to investigate the origin of this inflammatory response. Th17 cells have previously been implicated in kidney inflammation. By staining kidney sections for Th17 cells and their responder cells (macrophages), we were able to detect and visualize the presence of Th17 cells in the inflamed kidneys (Figure 14). We found that Stat1-/- mice had increased Th17 cells and macrophages infiltrating the kidney interstitium. By doing intracellular cytokine staining for IL-17A and IFNγ, we were able to show that Stat-/- mice have increased Th17 cells, and reduced IFNγ producing CD4+ T cells, in their lymph nodes and spleen.

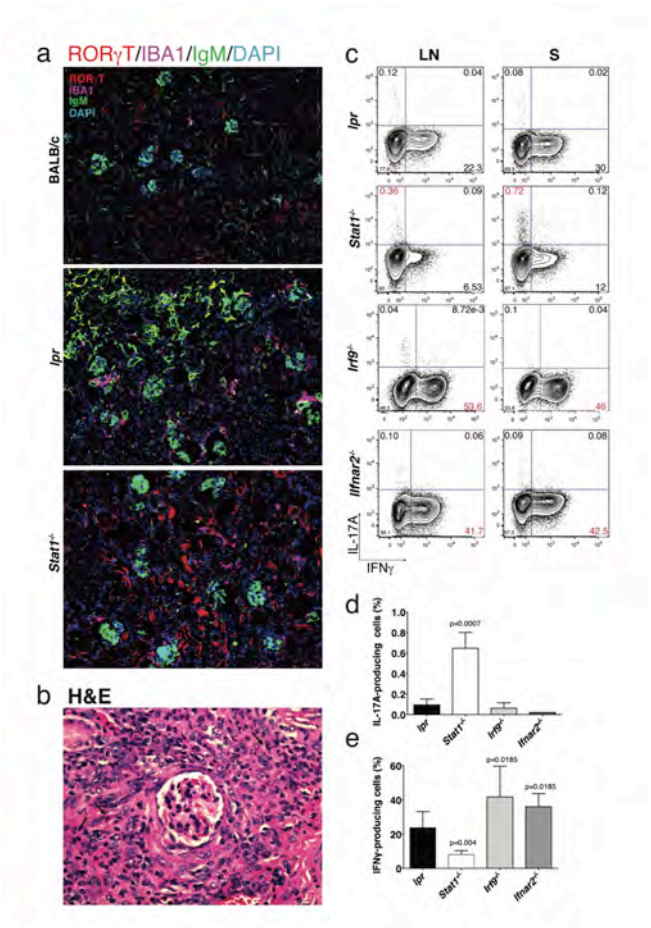

Figure 14. Th17 Cells are Increased in Stat1-/- Mice. (A) Immunofluorescence staining for Th17 (RORγt) and macrophage markers (IBA1) in murine kidney sections. (B) Hematoxylin and eosin (H\&E) stain for eosinophils in a kidney section from a Stat1-/- mouse. (C) Intracellular cytokine stainings for IL-17A and IFNγ in CD4+ T cells from lymph nodes and spleen. (D-E) Summary graph of ICS data. T test were done using Mann-Whitney's U test of indicated KO mouse compared to lpr controls.

Decreased Autoantibodies in Stat1-/- Mice

Disruption of IFNα signaling by knocking-out IFNAR2, IRF9, and STAT1 overall improved disease severity in all three knockouts. To investigate if this disruption impacted autoantibody production, we performed highly multiplexed autoantibody microarrays for selected SLE-associated autoantigens on serum from the mice (Figure 15A-B). Stat1-/- mice had significantly reduced production of autoantibodies, as compared to the lpr mice, for both IgM and IgG subtypes. The significance of this finding is highlighted by the fact that Irf9-/- mice had increased autoantibody production, while Ifnar-/- mice did not display any changes in autoantibody profiles compared to lpr mice.

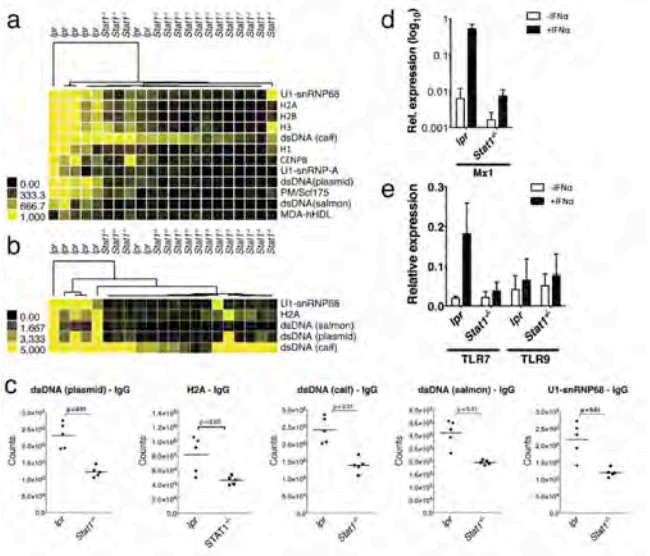

Figure 15. Decreased Autoantibodies in Stat1-/- Mice. Serum levels of IgM (A) and IgG (B) autoantibodies in Stat1-/- and lpr mice as measured by protein microarrays. SAM analyses were used to identify significant differences in autoantibody levels between the groups, and data is presented in heat map format with hierarchal clustering trees. (C) ELISA confirmation of IgG autoantibodies (D-E) IFNα mediated induction of Mx1, TLR7, and TLR9 in B cells from Stat1-/- and lpr mice. T test were done using Mann-Whitney's U test.

Intracellular signaling Responses

Each components of the IFNα pathway, that was knocked-out, has different functions in the signaling pathway. IFNAR2 is a receptor component necessary for docking IFNα, with the IFN receptor complex, and STAT1 is the primary STAT phosphorylated by IFNα, while IRF9 attaches to the STAT dimer facilitating nuclear localization. To investigate the impact of knocking these genes out on signaling responses we performed highly multiplexed STAT phosphorylation assays across immune subsets with relevant stimulations. As expected, we found that Stat1-/- had no detectable STAT1 phosphorylation. While this is trivial the experiment revealed that loss of STAT1 led to significant overflow to other signaling molecules including STAT3/4/5 upon IFNα stimulation (Figure 16).

As expected, loss of IRF9 seemed not to affect STAT phosphorylation significantly. As per IRF9's role as a chaperone protein, facilitating nuclear transition, it would not affect actual phosphorylation upstream of its interactions with the STATs, but could easy affect downstream gene transcription. Interestingly, loss of IFNAR2 led to reduced IFNα signaling across all immune subsets and STATs, but only affected IFNγ signaling in B cells, and not CD4+/CD8+ T cells, indicating a possible distinct role for IFNγ in this subset.

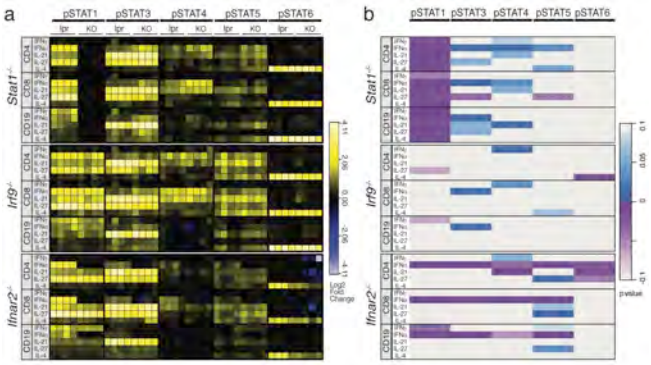

Figure 16. STAT Networks in Interferon Signaling Knockouts. (A) Heatmaps of STAT1/3/4/5/6 phosphorylation in CD4+ T cells, CD8+ T cells, and B cells stimulated with IFNγ, IFNα, IL-21, IL-27, and IL-4. (B) Heatmap of p-values comparing KOs to lpr for each respective stimulation and STAT molecule. Blue indicates higher phosphorylation levels and purple indicates lower phosphorylation in KO mice compared to lpr mice.

DISCUSSION

IL-21 and Follicular T helper cells in Rheumatic Disease

In the present study, we found that patients with RA, SpA, and SLE have increased plasma levels of IL-21 compared to HCs. For RA patients this increase is most pronounced in the early stages of disease, where patients have several fold higher plasma levels, compared to HCs. Later in disease, RA patient have only slightly elevated plasma levels compared to HCs.

This apparent decline in IL-21 plasma levels, with progression of disease, could reflect either natural attenuation or treatment-induced attenuation of IL-21 production. As the disease becomes chronic and more established, it could be speculated, that the initiating process of T and B cells cross-talk, driven in part by IL-21, becomes less important in maintaining the aberrant autoimmune response. Once formed and matured, the autoreactive B cells do not require IL-21 to produce their autoantibodies. Local inflammatory responses in the joints could function independent of IL-21 or rely on local production of IL-21. This notion is supported by the increased IL-21 and IL-21+ Th cells seen in synovial fluid from RA patients. Alternatively, the decline in IL-21 could primarily be mediated by the aggressive pharmacological treatment the patients receive. Lending support to this is the observation that plasma levels start to fall of after 3 months of treatment, which includes intra-articular steroid injections. This also coincides with the time when the medications take most effect, and patients experience the greatest improvement in disease symptoms. The basal plasma levels of IL-21 also correlated positively with disease activity at 12 months after diagnosis, but not at 0 and 3 months. The fact that the IL-21 plasma levels only correlate at 12 months could indicate a 'mass effect' by the treatment regime. Given that aggressive treatment separately reduces both disease activity, as measured by DAS28, and IL-21 plasma levels, it would force the parameters to correlate, irrespective of any causal link between the two.

IL-21 producing CD4+ T cells were also increased both systemically in RA, SpA, and SLE and locally in the inflamed joints of RA and SpA patients. In all three rheumatic disease were CD45RO+ T cells the main producers of IL-21. Systemic levels of IL-21+ cells were at comparable levels in RA and SpA patients while SLE patients had almost 3 fold higher circulating CD4+CD45RO+IL-21+ T cells. IL-21 has, based on murine studies, been suggested to, phenotypically, be a Th17-associated cytokine, but in our studies of both RA

and SpA were the IL-21+ Th cell mainly single positive for IL-21 and not IL-21/IL-17A double positive. This distinction is important as the pathological effects mediated by Th17 and Tfh cells are fundamentally different.

The effects and functions of the increased plasma levels of IL-21 are still uncertain. In line with our results, Gottenberg et al. found that early-stage RA patients have increased plasma levels of IL-21 [125]. They also found that IL-21 plasma levels correlated with markers of B cell activation such as IgM-RF and anti-CCP antibodies (ACPA) - IL-21 tended towards correlating with IgM-RF and anti-CCP antibodies in our data set. It is plausible, that increased systemic levels of IL-21 could help drive aberrant B cell responses in secondary lymphoid tissues in the synovium. It can thus be speculated, that IL-21 could in-part drive the local inflammatory response by promoting B cell activation and production of IgM-RF and ACPAs. However, while both IgM-RF and ACPAs are important markers for RA their pathological roles are uncertain. IgM-RF serves as an important prognostic marker in RA, but does not show any correlation with disease activity and is also found in healthy controls, especially with increasing age [126]. Anti-CCP antibodies offer a high sensitivity to RA, but has similarly not been shown to directly play a pathological role or correlate with disease activity [127,128,129]. Cigarette smoke has been shown to act differentially in ACPA positive and negative RA patients. People who are ACPA positive have significantly increased risk of developing RA if they smoke cigarettes as oppose to people who are ACPA negative, where the risk is similar to the general population [31]. The reason for this differential effect is presumably gene-environment interplay where smoking and other airway irritants leads to citrulination of specific peptides and generation of autoantibodies (i.e. ACPA) in susceptible individuals [130]. Based on these findings it has been speculated if ACPA could define separate subsets of RA patients suffering from distinct diseases with different pathology and needing different treatment regimes but with a shared symptomatology. Interestingly, ACPA has been shown to be a strong predictor of treatment outcome for rituximab (anti-CD20/B cell depletion) [131]. Based on this it could thus be speculated that ACPA+ RA patients would prove more susceptible to treatment directed against IL-21, as they are more characterized by B cell activation.

Another possible mechanism by which IL-21 promotes inflammatory processes in autoimmune disease is by inducing and promoting differentiation of Th17 cells. Based on murine studies Th17 cells have been implicated as key inducers of inflammation in tissues [37,38]. Central in the Th17 mediated inflammatory reaction in RA is induction of matrix-metalloproteases (MMPs) [132,133]. IL-21 in cooperation with TGFβ has been shown to be a key inducer of Th17 cells and help drive their effector functions [134,135]

MicroRNAs in Systemic Lupus Erythematosus

The role of microRNAs in regulation of important immune functions has become evident over the last few years. We here show, that miR-155 is regulated by IL-21 in a STAT3-dependent manner. Furthermore, we uncover a STAT3 signaling dysfunction and describe a novel role for miR-155 in regulating STAT3 signaling and IL-21 production in SLE.

Little is know about temporal regulation of cytokine signaling networks in autoimmune diseases. In our study, T and B cells from chronic SLE patients display decreased STAT3 phosphorylation in response to IL-21 stimulation. This depression was maintained over time and marked, as only half of normal phosphorylation levels were achieved at maximum, compared to HCs. Seeing as our patients were well-treated pharmacologically, and had low disease activity, it is reasonable to assume that the observed levels were affected by both disease mechanisms, but also by treatment responses. Using a murine model of SLE Hale et al. were able to address parts of this question. By sequentially measuring STAT responsiveness over time, as the mice aged, the authors were able to show that as disease progressed, STAT responsiveness declined [79]. Further, coinciding with the decrease in STAT responsiveness levels of the signaling suppressor SOCS1 rose significantly. Thus, based on these findings, it would seem that increased STAT responsiveness characterizes early disease, while later and more chronic stages are characterized by attenuation and suppression of the increased STAT signaling. It seems plausible that early in disease aberrant and uncontrolled signals are delivered through the STAT pathway, by IL-21, conveying inappropriate survival and differentiation signals to B cells promoting autoreactivity. While later in disease, these aberrant responses are attenuated by the immune systems in an attempt to reduced and inhibit further autoimmunity. While this is speculative, it has been shown that high levels of IL-21 producing Tfh cells can in themselves promote autoimmunity (reviewed in [42]).

The role of miR-155 in STAT signaling has not been well described. Our results demonstrate a novel role for miR-155 in regulating STAT3 signaling and could present a possible explanation for the attenuation of STAT signaling seen in SLE. We show that SLE patients have lower levels of miR-155 compared to HCs and correspondingly high levels of SOCS1. While not conclusive, in terms of cause and effect, this link indicates that miR-155 can regulate STAT signaling through SOCS1. To address if miR-155 can indeed regulate STAT3 signaling, we overexpressed miR-155 in PBMC from SLE patients and HCs. This allowed us to address the mechanistic effects of miR-155, in the relevant *in vitro* cell system. We used a lentiviral system to introduce the miR-155 gene into the cells. This approach ensures a stable and permanent genetic modification thus better mimicking physiological condition as oppose to non-viral transfection. While overexpression of miR-155 in CD4+ and CD8+ T cells increased STAT3 phosphorylation, it only did so after 15 minutes of stimulation. This was contrary to our initial expectations as we would have expected that baseline levels were also increased. However, in hindsight this would probably have been an unlikely outcome. Increased baseline phosphorylation of STAT3 would presumably over-activate the T cells, and cause them to either proliferate uncontrollably or undergo apoptosis, as these are the key functions ultimately mediated by STAT3. This state would be highly unstable and would quickly be countered by regulatory self-preserving mechanisms within the cell. Thus the transfected cells quickly adapt to their new genetic composition by countering the increased STAT activation, and bringing it down to normal levels. However, once stimulated the imposed counter-regulatory mechanisms in place in miR-155 overexpressing cells are incapable of inhibiting the full response, compared to GFP controls, and higher levels of STAT3 phosphorylation are achieved in the miR-155 overexpressing cells. To assess the impact of miR-155 overexpression on IL-21 production, the transduced cells were stimulated and IL-21 production was measured. We found that overexpression of miR-155 differentially increased IL-21 production in SLE patients compared to HCs. This indicates that miR-155 is indeed repressed in SLE, and that the supposedly attenuated IL-21 production can be 'rescued'

by overexpressing miR-155. This suggests that repression of miR-155 is part of a counter-regulatory mechanism to reduce pathologically increased IL-21 production. Our findings thus offer possible insights into the progression of SLE and we speculate that early stages of SLE are characterized by high levels of IL-21 and IL-21 production as well as increased STAT responsiveness. As disease progresses, and becomes more established, IL-21 production and STAT responsiveness are attenuated in part by miR-155 and SOCS1, as well as other counter-regulatory mechanisms.

Interferon-alpha and Autoantibodies in Systemic Lupus Erythematosus

Interferon signaling and responsiveness has in the last decade become of primary interest in SLE. It is presently the only available and convincing way to stratify SLE patient in terms of disease activity and prognosis. Using a murine model for SLE and knocking-out key signaling molecule in the interferon signaling pathway, we were able to individually assess the impact of each of the proteins on disease outcome and its immunological impact. We demonstrate that loss of STAT1 ameliorates disease severity and reduces autoantibody production. However, simultaneously to these improvements, a marked worsening in interstitial kidney disease was observed. Our results suggest, that this inflammatory process was mediated by Th17 cells and macrophages infiltrating the interstitium. This finding could highlight a potential caveat to the novel JAK-STAT inhibitors and their possible role in treatment of SLE. Tubulo-interstitial kidney disease is a rare disease usually caused by allergic reactions and adverse effects to pharmacological treatment [136,137]. It has an unpredictive disease course and rarely responds to treatment leaving sequential withdrawal of possible offending drugs and supportive therapy as the main options. Inducing such a disease in sick patients, already at risk of severe kidney disease, would be highly unfortunate.
Interestingly, while we observed the greatest amelioration in disease severity and autoantibody production in STAT1-/-, uncoupling IFNα signaling in IFNAR2-/- had little effect. Our signaling experiments clearly show that IFNAR2-/- have no detectable IFNα signaling through either STAT1/3/4/5, while STAT1-/- have loss of STAT1 phosphorylation to all stimuli. This hints that perhaps it is not so much IFNα in it-self, that is pathological, but more so its continuous induction of STAT1. While it is reasonable to assume that the greatest induction of STAT1 in SLE patients is indeed IFNα driven, this could be missing the point; the point instead being, that it is in fact aberrant STAT1 phosphorylation, that is pathological, regardless of the source of the phosphorylation. This assumption fits nicely with the concept of the IFN signature, and its stratification of SLE patients by disease activity and autoantibody production. Hence, patients with high IFN signature, presumably by some genetic predisposition, experience worse effects of STAT1 induction and consequently develop a severe disease course and more autoantibodies compared to IFN-low patients.

Using a composite IFN signature panel, we were able to stratify and group SLE patients into IFN high and IFN low groups. We show, that IFN high SLE patients have markedly higher levels of autoantibodies, compared to IFN low, and that these were mainly directed against small nuclear RNA-associated proteins. Although still incomplete, our studies of the signaling responses indicate that SLE patients have suppressed STAT1/3/5 signaling globally. Further, IFN high patients have increased STAT1/3 reactivity to both IFNα, IFNγ, and IL-21 in key subsets of T and B cells. This could be interpreted so that STAT signaling is suppressed by cellular mechanisms, in an attempt to dampen the effects of the signals delivered. However, upon stimulation of IFN high patients this suppression is overcome, and a relatively higher degree of phosphorylation is produced compared to HCs and IFN low patients, reflecting their high IFN profile (i.e. high levels of IFN inducible genes). Increased signaling responses to Tfh and B cells would deliver inappropriate survival and differentiation signals promoting production of autoreactive antibodies. This supports the notion that IFN signature and STAT signaling are important pathological factors controlling disease activity and autoantibody production.

CONCLUSION

The studies comprising this thesis address multiple aspects of IL-21 and Follicular T cell immune functions. We show, that IL-21 plasma levels and T cells producing IL-21 are increased in several rheumatic diseases, and that they are linked to disease activity and progression. Addressing the regulation and signaling of IL-21, we show that miR-155 is capable of modifying IL-21-mediated signaling responses and production. We further show, that STAT signaling appears to be regulated by SOCS1 in SLE. Finally, we add new knowledge to the impact of interferon signaling on SLE pathology. Using a murine model of SLE, we demonstrate a key role for STAT1 in ameliorating SLE pathology, but also highlight a possible adverse effect of pharmacological inhibition of STAT1, by the concomitant development of interstitial kidney disease. In a cohort of SLE, we further show, that aberrant STAT1/3 signaling and autoantibody production differentiates IFN high and low SLE patients.

IL-21, with its non-redundant effects on B cells and antibody production, is of obvious interest in the context of autoimmune diseases. However, the interplay between IL-21 and IFNα, and its impact on autoantibody production has not previously been studied in detail. IL-21 is induced by IFNα, and can be taken as part of the IFN signature. It could thus be speculated that patients with a high IFN signature have increased STAT responsiveness and produce excess amounts of IL-21. Such a state would render them particularly prone to autoimmunity. Based on our work and others it would, however, seem that this state is mainly characteristic of early disease stages, as later stages appear to be characterized by counter-regulatory mechanisms, as indicated by our studies of miR-155 and STAT3 in SLE. Combined, these findings indicate that early treatment directed against IFNα, IL-21, or STATs would serve the patients best. Treatment initiated later could possibly induce unwanted side-effects, such as interstitial nephritis.

In conclusion, the present thesis adds important insights into IL-21's and IFNα regulation of Follicular T and B cells responses and production of autoreactive antibodies. It further highlights the importance of STAT signaling responses in relation to the development and maintenance of autoimmune diseases such as RA and SLE. This knowledge will aid and further the development of novel treatment strategies to ultimately better patient outcome.

SUMMARY

Rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are lifelong diseases with increased mortality and chronic pains. They are both characterized by immunological imbalances causing the immune system attack and destroy the bodies own tissues (called autoimmune disease). The best treatment, we are currently able to offer these patients, cause significant side-effects and can not prevent significant loss of quality of life.

At the heart of the disease mechanisms in RA and SLE are subsets of immune cells called T and B cells. These cell types produce proteins (called antibodies), which under normal circumstances protect the body against disease. In RA and SLE these cells produce antibodies that are directed at the bodies own tissues (called autoantibodies), causing inflammation and tissue damage. The cause of this loss of tolerance is still unknown. Interleukin 21 (IL-21) and is thought to exert key functions in controlling and directing the T and B cell responses leading to formation of antibodies and autoantibodies alike. IL-21 is a signaling molecule secreted by a subpopulation of T cells called follicular T helper (Tfh) cells. IFNα is another signaling molecule of key importance in autoimmune disease. Stratification of SLE patients by their responsiveness to IFNα has proven a crucial tool in stratifying patients in terms of disease development and treatment response.

The aim of this PhD study is to investigate the role of IL-21 and IFNα, and their effects on Tfh cells and B cells and the formation of autoantibodies in RA and SLE.

The first part of this PhD addresses whether plasma levels of IL-21 influence disease activity in rheumatic disease. We further investigate the distribution of IL-21-producing Tfh cells in these patients. We find that IL-21 plasma levels correlate to disease activity and radiological progression in RA, and that the IL-21-producing Tfh cell are increased in the blood and synovial fluid of these patients. These findings support the idea that IL-21 and Tfh cells are linked to the development and perpetuation of these diseases. In the second part of this PhD we investigate how small RNA molecules, called microRNAs, can regulate immunological processes. We find that microRNA-155 can regulate IL-21's capacity to signal, while microRNA-21 is important for survival of T cells. The third, and last part of this PhD, concerns IFNα signaling and its impact on the development of SLE and the formation of autoantibodies. We find that IFNα signaling is altered in a murine model of SLE, and that inhibition of this signaling pathway leads to severe kidney disease. The latter is of key importance as inhibition of IFNα is currently in early trial as a new treatment form for SLE patients. In SLE patients, we find that IFNα responsiveness, as measured by a so-called 'IFN signature', is crucial in terms of development of the disease as well as serious complications such as kidney disease and involvement of the central nervous system (CNS). Interferon alpha does this by affecting intracellular signaling responses and the formation of autoantibodies.

The data presented in this thesis supports that IL-21 and Tfh cells have a key role in the disease processes characterizing RA and SLE. We further describe a novel mechanism for microRNA-155 and microRNA-21 in regulating immunological processes in these diseases. Finally we show, that IFNα has important functions in the formation of autoantibodies in SLE. In conclusion, this thesis adds new and important knowledge on the interplay between Tfh cells and B cells and their formation of autoantibodies in rheumatic disease. This knowledge will guide and further the development of new treatment strategies to better patient outcome.

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