Dissertation

Transcriptional profiling of peripheral blood monocytes from SLE, RA and AS patients: how monocytes modulate the impact of cytokines

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1. Introduction

1.1. Alteration of the immune system in autoimmunity

The immune system is an integral part of the human body whose principal role is to provide defence against infections and diseases. Different organs, tissues, cells and their secreted components compose the immune system and they all act together in an endeavour to recognise and eliminate unknown molecules. Since the human body is an ideal environment for many microbes, an interaction between the immune system and microbes is inevitable and essential for the host survival. The interaction ranges from the fight against "foreign" microbes (pathogens) to the symbiotic life with "self" microbes (microbiota)[1]. Some pathogens such as the Ebola virus, Mycobacterium tuberculosis, Plasmodium parasites are responsible for the development of life-threatening diseases such as hemorrhagic fever, tuberculosis and malaria. Principally, in these and many other infectious diseases, the immune system might lose the battle against the microbes. On the other hand, the symbiotic life with microbiota shows that the immune system accommodates microorganisms that live inside and on the human body, mainly in the gut, on the skin and mucosal surfaces. In this symbiotic life the exchange of energy and metabolites between these two entities is an essential for host survival and vital for the maintenance of homeostasis in the body[2].

The immune system is familiar with the antigens expressed on the body's own cells (autologous, self-antigens) and they coexist peacefully in a state known as self-tolerance. If this state breaks down, the activity of the immune system might be directed towards the body's own cells and tissues, which can ultimately results in the development of autoimmune diseases. These diseases are characterized by the production of various inflammatory mediators and the presence of auto-reactive cells. The main producers of mediators that initiate and maintain inflammation are the cells of the innate immunity, like monocytes/macrophages and neutrophils. The detection of auto-reactive T-cells and B-cells, and the production of auto-antibodies suggests that the improper activation of the adaptive arm of immunity is also present[3].

Autoimmune diseases occur in approximately 3-5% of the human population and they are phenotypically heterogeneous[4]. From a clinical perspective, they are classified into two groups: "organ-specific" and "systemic" autoimmune diseases. In the first group of diseases, an auto-antigen is expressed in a particular organ and marks this organ as a target for activation of the immune system. For instance, β-cells of the pancreas, brain and spinal cord, thyroid peroxidase and/or thyroglobulin, and TSH-receptor are considered as organ-specific auto-antigens. They are targeted by the immune system in the following diseases: type I

diabetes, multiple sclerosis, Hashimoto's thyroiditis and Graves' disease. The second group of autoimmune diseases are characterized by systemic manifestations. Namely, the antigens, like DNA, RNA, histones and citrullinated peptides are widely expressed in the body and therefore many organs are targeted by the activated immune system[5]. This group of autoimmune diseases includes: systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), ankylosing spondylitis (AS), polymyositis and dermatomyositis. The presence of antigens that are widely expressed in the body and the systemic nature of the resulting disorders may be the cause of many common signs and symptoms that accompany various autoimmune diseases.

The aetiology of autoimmunity is unknown. However, autoimmunity is always considered as cross-talk between host genes and environmental factors[3]. It is known that one or more autoimmune diseases frequently affect members of the same family. Speculations that there are susceptible genes that contribute to the pathogenesis of autoimmune diseases were confirmed by genome-wide association studies (GWAS). These studies showed that gene polymorphisms in autoimmune diseases are not disease-specific[6]. For example, the polymorphisms of genes, such as MHC class II, PTPN22 and STAT4, have been associated with the pathogenesis of SLE and RA[7-8]. GWAS also showed that most of the risk alleles are found in regions of the genome associated with immune functions. The implicated genes are related to the activation of both the innate and adaptive immunity such as CTLA4, IRF5, MHC class II, PTPN22, STAT4 and TNFSF4[9-10].

Most autoimmune diseases predominantly affect females and there are several lines of evidence that the female sex hormones are responsible for sexual dimorphism in autoimmunity[11]. It is known that estrogen and prolactin possess immunomodulatory roles that allow survival of autoreactive B-cells in lupus patients and thus, they may facilitate the breakdown of tolerance to self-antigens[12]. Furthermore, there are speculations that sex hormones are also important in the pathogenesis of RA, since estrogen stimulates proliferation of synovial cells and remission of disease accompanies RA patients during pregnancy[12-13]. It is interesting that pregnancy, characterized by a physiological immunosuppression, is beneficial for RA patients but can induce flares in SLE patients[14]. Considering that a small percentage of females develop these types of autoimmunity, the sex hormones are not an exclusive factor in the pathogenesis of SLE and RA.

Other factors involved in the pathogenesis of autoimmune diseases are related to the environment. Certain types of infections are considered as environmental triggers of autoimmunity. Thus, viral infections induced by Cytomegalovirus, Epstein - Barr virus and Parvovirus B19 are considered to be triggers of SLE pathogenesis[15-16]. There are also studies that relate the pathogenesis of RA to infections, since bacterial and viral DNA were found in the joints of RA patients[17-18]. The exact mechanism of how an infection can initiate the breakdown of immune tolerance and how it induces the development of autoimmune disease is unknown. Nevertheless, one postulated mechanisms is a molecular mimicry or cross-reactivity. It is assumed that a microbial antigen bears a peptide epitope that has a very high sequence homology with a self-antigen. Thus, the immune system once activated by pathogens, due to its cross-reactivity, might be misdirected toward the body's own molecules.

It is also interesting that some types of infections might have a protective role for the development of autoimmunity. In developed, and more recently in developing countries, the incidence of infections has decreased, while the incidence of autoimmune and allergic diseases has increased[19]. Thus, according to the "hygiene hypothesis", a deficiency in microbial flora is related to an increased incidence of immune mediated diseases[20]. Basically, the reduction of microbial flora from the environment is associated with measures that limit the spread of infections, for example, obligatory vaccination, wide use of antibiotics, decontamination of water, sterilization and pasteurization of milk and other food products. Thus, the activation of the immune system by microbial flora that was common and inevitable in our ancestors is weakened or completely diminished. A possible consequence is that the immune system once used to be activated by microbial flora in a condition without them is still activated but unfortunately by self molecules. Thus, the misdirection of the immune system ultimately results in the destruction of the host's body.

As previously mentioned, autoimmune diseases show gender dimorphism and since they predominantly affect females, it is also assumed that environmental factors, like hair dye, lipstick and silicon implants are playing an important role in their pathogenesis[21-25]. These assumptions might sound reasonable, but so far only small groups of patients have been included in these types of studies. Therefore, research provided by larger cohort of patients is required in order to provide more convincing results related directly to the described female-like behaviour. However, one of the convincing results for gene-environmental interaction is smoking[26]. A study performed by Padyukov *et al.* (2004) showed that smoking in genetically

predisposed individuals was associated with auto-antibody production many years before the onset of RA[26].

In this study we have been focused on three systemic autoimmune diseases, SLE, RA and AS. They are characterised by many overlapping syndromes, large heterogeneity among patients with the same disease, chronic inflammation maintained by cytokines, and similar therapeutic interventions[27]. Some basic information and currently proposed models of SLE, RA and AS pathogenesis are described below.

1.1.1. An insight into the pathogenesis of Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by a broad spectrum of clinical manifestations. As a systemic disease it affects multiple organs and includes syndromes like fatigue, fever, weight loss, skin rash, profound anaemia, lymphopenia, arthritis, seizures, pericarditis, pleuritis, psychosis and very severe renal failure (glomerulonephritis)[28]. SLE predominantly affects women, where female to male ratio is 9:1. It typically appears in women of child-bearing age. The incidence of SLE in Europe ranges from 3.3–4.8 cases per 100 000 persons per year[29-31]. The estimated prevalence of SLE is 31 per 100 000 women of European ancestry, and the prevalence is three to four times higher among African-American women compared to women of Caucasian origin[32].

Although great progress has been made in understanding the contribution of genetic and environmental factors in SLE pathogenesis, the exact mechanisms that underline disease pathogenesis is still unknown[15-16,32-34]. Nevertheless, it is known that SLE is characterized by chronic inflammation, and that nearly all lupus patients exhibit increased level of anti-nuclear antibodies (ANA) in the peripheral blood, such as anti-RNA, anti-DNA, anti-histone, anti-nucleosome antibodies[35]. Additional features of SLE are the presence of auto-reactive T- and B-cells, an alteration of the type I IFN system and an inefficient clearance of apoptotic cells debris by monocytes/macrophages. Thus, both innate and adaptive immunity play an important role. A relatively simplified scheme of SLE aetiopathogenesis is presented in Figure 1.1. The scheme shows that viral infection and UV light are able to provoke cell apoptosis and/or necrosis, which are accompanied by defective clearance of cellular debris. These alterations represent the main source of auto-antigens, including RNA, DNA, histones, and nucleosomes. Furthermore, viral infections induce production of various cytokines including type I IFN. This cytokine is mainly produced by plasmocytoid dendritic cells (pDCs) and has a great potential to interfere with viral replication. In genetically predisposed

individuals the presence of nuclear auto-antigens can cause the breakdown of immune tolerance and can lead to the production of ANA[36]. Auto-antigens and auto-antibodies form immune complexes that can further amplify production of type I IFN. One of the roles that type I IFN exhibits is maturation of monocytes into DCs, whose primary function is to process and present the auto-antigens to CD4 and CD8 T-cells. In addition, type I IFN and immune complexes are also able to activate B-cells. Their activation is associated with increased productions of auto-antibodies that presents a permanent source for the formation of immune complexes and therefore facilitate maintenance of the self-destructive nature of SLE.

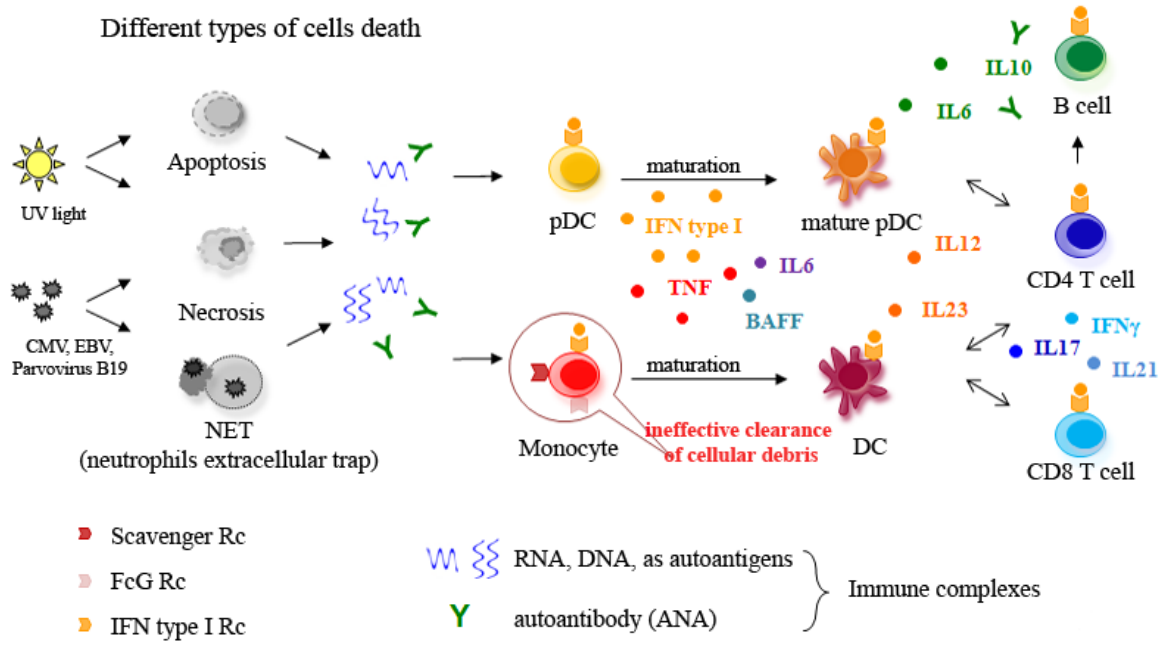

Figure 1.1 A proposed model of SLE pathogenesis. Scheme is based on the models suggested by Baechler *et al.* (2004) and Roennblom *et al.* (2008).

Besides type I IFN, a large number of other cytokines have been reported as important players in lupus pathogenesis, including BAFF, APRIL, TNFα, IFNγ and IL6. These cytokines are produced by a variety of different cell types such as pDCs, myeloid DCs (mDCs), monocytes, macrophages, T-cells and B-cells. Currently, there are several clinical trials that consider the neutralisation of various cytokines as potential therapeutic intervention in SLE[37].

Taken as a whole, once the immune system is activated, it is characterized by a self-sustained and auto-amplified nature that leads to chronification of the inflammatory response. Recently it has been shown that a novel type of cell death program identified in neutrophils, termed as neutrophils extracellular trap (NET), is also involved in the pathogenesis of SLE[38]. The NET formation captures and kills microorganisms by forming traps. They are mainly composed of

chromatin that is expelled from the nucleus and principally this structure represents a source of vast amounts of auto-antigens. In genetically predisposed individuals these conditions might lead to a break of immune tolerance and the pathogenesis of SLE[39].

1.1.2. An insight into the pathogenesis of Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease that mainly targets the synovial membrane, cartilage and bone. RA preferentially affects the small diarthrodial joints of the hands and feet[40]. The inflammation that affects a lining layer of the joints, known as synovium, leads to the development of a fibrovascular tissue known as pannus tissue, which invades and destroys articular structures[40]. The synovium in healthy individuals is an acellular structure, but in RA patients it is infiltrated with T-cells, B-cells, macrophages and fibroblasts. All these cells are able to produce many inflammatory mediators including cytokines and degrading enzymes. Therefore, the synovium of RA patients is characterized by the presence of TNFα, IL1β, IL6, IL15, BAFF, APRIL, matrix metalloproteinases and serine proteases[40-43].

The prevalence of RA is 0.5-1% of the adult population worldwide, with a few exceptions where it is higher in populations of Pima and Chippewa Indians (5.3% and 6.8%, respectively) and very low in the Asian population (0.2-0.3%)[44]. RA is 3 to 4 times more frequent in females than in males and contrary to SLE, which predominantly affects females in child-bearing ages, RA is observed predominantly in middle-aged and older patients[45]. Its incidence increases with age and a peak of RA onset is in the fifth decade of life. It is observed that with increasing age the female/male ratio decreases to 2:1[45].

The hypothesis that self-reactivity plays a role in RA was evaluated with the identification of auto-antibodies in affected patients. Approximately two-thirds of RA patients are seropositive for rheumatoid factor (RF, an anti-immunoglobulin antibody against the Fc portion of IgG) and/or anti-cyclic citrullinated peptide antibodies (ACPA or anti-CCP)[46]. However, the presence of auto-antibodies is not sufficient for the onset of disease, considering the fact that they are also detected in many healthy individuals, as well as in patients with other inflammatory arthropathies[40]. The aetiopathogenesis of RA is not completely understood. A proposed model points towards an initial alteration of the innate immune system (Figure 1.2).

Unlike to SLE, the auto-antigens in RA patients have not been identified so far. It has been suggested that Toll-like receptors (TLRs) agonists are strong stimulators of various cell types,

and that they play an important role in the pathogenesis of RA[43]. Beside bacterial and viral components, which are prototypical triggers of TLRs, many endogenous ligands are also potent activators of TLRs. For example, fibrinogen, heat-shock proteins, hyaluronan fragments and oxidised low-density lipoprotein are all endogenous TLR ligands and are typically present in larger amounts in tissues that are infected or injured[43]. Ligands for TLR initiate the activation of macrophages, fibroblasts, DCs and mast cells. Cell activation induces production of cytokines that further facilitates an activation of other cells, inducing T-cells and B-cells. Once the immune system is activated and inflammation is not resolved, the whole process is auto-amplified, where the permanent production of cytokines attracts more cells from peripheral blood, and where migration of cells into already inflamed joints further promotes disease progression.

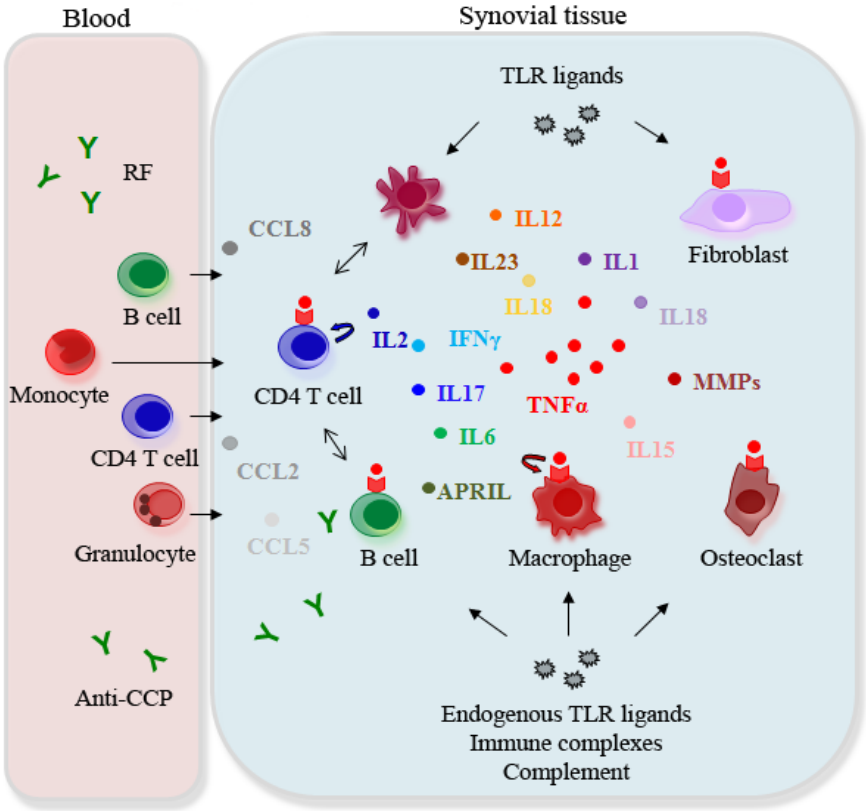

Figure 1.2 A proposed model of RA pathogenesis. Scheme is based on the models suggested by Feldmann *et al.* (2004) and McInnes *et al.* (2007).

1.1.3. An insight into the pathogenesis of Ankylosing spondylitis

Ankylosing spondylitis (AS), also known as Morbus Bechterew, is a chronic inflammatory disease that affects the spine and the sacroiliac joints[47]. It may also affect the peripheral joints and organs such as the uveal tract, skin and bowel[48]. Beside inflammation, two other

processes accompany AS pathogenesis: bone erosion and new bone formation (Figure 1.3)[49]. Bone erosion often occurs simultaneously with inflammation and at the same location. These two phases represent the destructive phases of AS accompanied by the loss of cartilage and bone[50]. New bone formation presents the process that takes place relatively late in the pathogenesis of AS, and it resembles an attempt of the body to repair damaged bones. New bone formation is characterized by generation of syndesmophytes, which represents the bony spurs that bridge and fuse vertebras of the spine, causing pain, stiffness and spinal immobility (bamboo spine)[49]. Contrary to SLE and RA, which are the prototypical female diseases, AS preferentially affects males, with a male to female ratio of 2:1[51]. AS affects young people, where about 80% of patients develop the first symptoms of disease under the age 30 years[48]. The incidence of AS is between 0.5 and 14 per 100 000 people, and the prevalence is between 0.1% and 1.4% depending on the ethnic background[48]. The most of the epidemiological studies performed so far, included patients of Caucasian origin. Thus, the prevalence and incidence of AS in the worldwide population are still unknown.

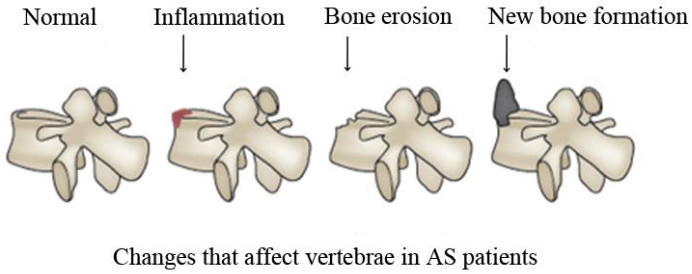

Figure 1.3 Three different processes characterize the pathogenesis of AS: inflammation, bone erosion and new bone (syndesmophyte) formation. The scheme is taken from Tam *et al.* (2010).

The aetiology of AS is unknown, although both genetic and environmental factors are related to its pathogenesis. The first and most frequently documented genetic factor associated with AS is an expression of the tissue antigen HLA-B27. The HLA-B27 gene consists of multiple alleles that encode at least 45 different subtypes. The HLA-B27 presence was detected in approximately 90-95% of AS patients. Nevertheless, its expression is not restricted just to AS patients since it is also identified in patients with psoriatic arthritis, reactive arthritis, inflammatory bowel disease and even in 4-8% of the healthy population[52]. Recently it has been reported that polymorphisms of genes, such as IL23R, IL1R2, ERAP1 and ANTXR2 are also associated with the pathogenesis of AS[53]. Beside genetic factors, environmental factors, like bacterial infections, are also considered to be the important players in the pathogenesis of

this disease. The infections with *Chlamydia trachomatis, Shigella, Salmonella, Yersinia,* and *Campylobacter* spp. are associated with the development of reactive arthritis[54]. About 10-20% of HLA-B27 positive patients with reactive arthritis develop AS after 10-20 years[48]. A possible role of infection in AS pathogenesis is further supported by the fact that more than 50% of HLA-B27 positive patients with Crohn's disease develop also AS[55]. Since, Crohn's disease is characterized by colitis and by leakage of the gut mucosa, it is proposed that interactions between the gut microflora and the immune system in genetically predisposed individuals may be responsible for the development of AS.

The production of multispecific autoantibodies is detectable in patients with AS[56]. They are directed towards the multiple antigenic targets that compose the extracellular matrix of connective and skeletal tissues, including glypican 3, glypical 4, osteoglycin, connective tissue growth factor[56]. The production of autoantibodies in AS has been identified recently, but unlike to those in SLE and RA, it has not been accepted as the main feature of AS pathogenesis.

The histological analysis of joints from AS patients identified the existence of infiltrates composed of T-cells, B-cells, bone marrow derived macrophages and osteoclasts[57-58]. A synovitis (inflammation of synovial membrane) in AS is less common than in RA patients[49]. However, if it is present the pathological features of synovitis are similar to those in RA. The hypothesis that TNFα might mediate pathogenesis of AS has been supported by clinical trials when patients treated by anti-TNFα drugs showed withdrawal of the symptoms and significant reduction in the amounts of acute-phase reactant proteins in the blood[49].

1.2. Chronic inflammation drives the pathogenesis of SLE, RA and AS

In general, the immune response is accompanied by an inflammation as an immediate response to pathogens, noxious stimuli and tissue injuries[59]. Although inflammation is experienced as an unpleasant reaction with redness, swelling, heat, fever and pain, it is part of the host's defence mechanisms that provides survival during infection or injury[60]. Once the inducer of the immune response is eliminated, the activation of the immune system, together with inflammation is terminated and tissue injuries are repaired. Although inflammation is beneficial, it is also associated with a transient decline in tissue functions, which in genetically predisposed individuals might lead to a permanent activation of the immune system and pathogenesis of diseases[59]. All three rheumatic diseases that are focus of this study, SLE, RA and AS, are characterised by chronic inflammation[37,50,61]. The hallmarks of

inflammation in these autoimmune diseases are an excessive and abundant production of pro-inflammatory mediators and the presence of pathogenic memory[60,62-63]. The cells of the innate and adaptive immunity are the principle source of cytokines, while the pathogenic memory is imprinted in cells of the adaptive immunity. Therefore, many components of the immune system are involved in perpetuating chronic inflammation and autoimmunity.

1.2.1. An abundant production of cytokines contributes to the maintenance of chronic inflammation in SLE, RA and AS

Both acute and chronic inflammation are portrayed with production of many cytokines including TNFα, IL1, IL6, IL8, CCL2, CXCL10, type I IFN and IFNγ. Principally, cytokines exhibit beneficial roles for the host under physiological conditions and they regulate a wide variety of biologic activities including cell development, cell recruitment, cell proliferation, cell death, immune-regulation and immune-effector functions[62]. They also participate in the host defence where their role is indispensible for the proper activation of the immune system. However, if they are secreted extensively they initiate and maintain chronification of inflammation[41]. Since cytokines possess roles that might be beneficial and detrimental for the body, they are appreciated both as therapeutics and therapeutic targets[64]. For example, cytokine based therapies, which includes application of IFNα or IFNβ, have been approved for treatment of some malignancies, chronic viral hepatitis and multiple sclerosis[64-65]. A therapy based on suppressing cytokine effects like, anti-TNFα therapy, has been approved for the treatment of RA, AS and Crohn's disease[50,66-67]. Since the main focus of this study was to reveal the effects of TNFα, type I IFN and IFNγ in the pathogenesis of autoimmune diseases, the physiological and pathophysiological roles of these cytokines are described in more detail.

1.2.2. Physiological and pathophysiological roles of TNFα and insight into TNFα signalling pathways

TNFα is a cytokine secreted by various cells types, but its primary source in the body are cells of the immune system, such as monocytes/macrophages, NK cells, T cells[68-69]. TNFα is produced following cell activation with different stimuli including bacteria, viruses, cytokines (IL1, IL17 and IFNγ), immune complexes, complement factors, irradiation, ischemia and trauma[68,70]. The role of TNFα is essential for defense against intracellular pathogens, such as *Mycobacterium* and *Listeria*, which are the cause of tuberculosis and listeriosis in humans[71-72]. In these infections, the secretion of TNFα together with IFNγ is indispensible for a proper

activation of macrophages and the development of a cell-mediated immune response. The response to TNFα is induced by its binding to the appropriate receptors that are expressed on the cell surface: TNFR1 (also known as p55 TNFR, CD120a, TNFRSF1A) or TNFR2 (p75 TNFR, CD120b, TNFRSF1B). TNFR1 is constitutively expressed on all human cell types, apart from erythrocytes, while TNFR2 is an inducible receptor, predominantly expressed on endothelial and hematopoietic cells[70]. The biological response to this cytokine affects the function of many different cell types, including cell proliferation, differentiation, necrosis, apoptosis, fever, secretion of other cytokines and acute-phase response proteins[68].

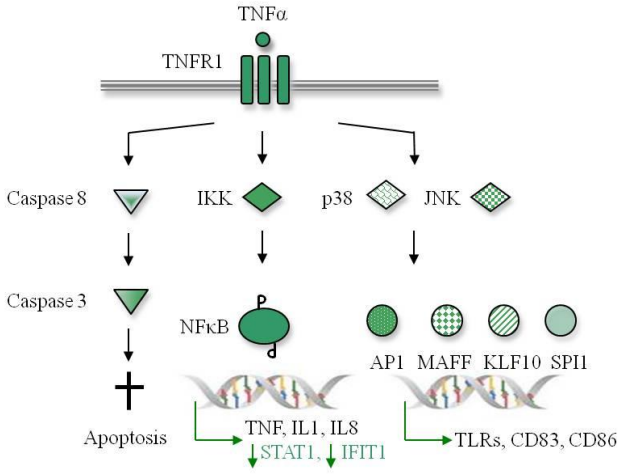

Figure 1.4 An overview of TNFα signaling pathways.

Different signalling pathways mediate the effects induced by TNFR1. The death domain of this receptor is responsible for activation of a pro-apoptotic pathway and initiation of programmed cell death. TNFR1 also contains TRAF2 and RIP domains whose activations mediate NFκB and JNK-AP1 signalling pathways[73]. The activation of transcription factors NFκB and AP1 is essential for the expression of large numbers of pro-inflammatory genes including cytokines and immunoregulatory molecules (Figure 1.4). Thus, activation of TNFR1 mediates a wide variety of biological functions in the cells and it ranges from dying of cells to promoting their survival and sustaining inflammation[74].

TNFα production can be considered as double-edged sword, when secreted properly it is part of the host's defence mechanism, but when secreted extensively it is related to the pathogenesis of different diseases. TNFα can be secreted in large amounts at once, and under this condition it is involved in the development of life-threatening septic shock[75]. But it can be also produced chronically at a low-level, when its role is implicated in tissue injuries and pathogenesis of chronic rheumatic diseases such as RA, SLE, AS and Crohn's disease[37,41,76-

[77]. Thus, it is reasonable to assume that TNFα might exhibit therapeutic effects, but also neutralisation of this cytokine might be relevant for therapeutic intervention. Since, this cytokine was initially identified as a molecule that causes necrosis of tumor cells, it was assumed that TNFα might be beneficial for the treatment of cancers. However, the effects of this therapeutic intervention were disappointing since, it was accompanied with major side effects like, shock, cachexia and toxicity[78]. Another therapeutic approach focused on neutralization of this cytokine by appropriate monoclonal antibodies. Considering the fact that bacterial sepsis is accompanied with increased levels of TNFα in blood, it was assumed that anti-TNFα therapy would be indispensable for treatment of septic patients[78]. Nevertheless, this therapeutic intervention was also ineffective. A new trial of anti-TNFα therapy was initiated in the treatment of rheumatic patients. Namely, the kinetics of TNFα release is quite different in sepsis and in chronic rheumatic diseases. Neutralization of massively released TNFα, as one of main characteristics of sepsis, was disclosed as too late for therapeutic intervention. Actually, the protective effect of anti-TNFα therapy might be apparent only if it is administrated before infection[79]. Nevertheless, neutralisation of small amounts of chronically released TNFα, as is typically observed in chronic rheumatic diseases, has been identified as successful therapeutic intervention[70]. Thus, the treatment of chronic rheumatic patients with TNFα antagonists was approved. Currently there are five TNFα antagonists in clinical practice: Infliximab (chimeric monoclonal antibody), Etanercept (soluble receptor), Adalimumab (humanized monoclonal antibody), Certolizumab (PEGylated Fab' fragment of anti-TNFα antibody) and Golimumab (humanized monoclonal antibody).

1.2.3. Physiological and pathophysiological roles of type I and type II IFNs and insights into their signalling pathways

Interferon (IFN) was one of the first cytokines to be discovered and cloned[80-81]. Its initial discovery in the 1970s was related to IFNβ and nowadays it is known that many functionally related proteins compose the IFN family. The main inducers of IFNs secretion are microbial components that bind and activate TLRs. For example, a viral dsRNA activates TLR3, a viral ssRNA activates TLR7 and TLR8, while bacterial LPS and oligodeoxyribonucleotides (CpG) trigger activation of TLR4 and TLR9, respectively[82]. Beside microorganisms, the production of IFNs might be induced by endogenous ligands such as the immune complexes. They are composed of two components: auto-antigens and auto-antibodies. The first component originates from cellular debris of apoptotic and necrotic cells and the second one is produced by auto-reactive B-cells[83-84].

The IFN family is classified into three major types: type I, type II and type III. Type I IFNs are composed of IFNα (13 subtypes), IFNβ, IFNω, IFNκ and IFNε. Type II IFN constitutes one molecule IFNγ, and type III IFNs comprises three subtypes of IFNλ. The classification of IFNs is based on structural homologies of these molecules and on the kind of receptors that are utilize by different types of IFNs.

Type I IFN molecules are genetically and structurally very similar. The genes that encode their production are located on the short arm of chromosome 9, and these molecules share amino-acid sequence homologies in the range of 30-85%[85-86]. Type I IFNs can be produced by almost all nucleated cells as a response to viral infection, but their main producers are pDCs. Type I IFNs act through the activation of the cell surface receptors: IFNAR1 and IFNAR2 that assembled into a heterodimeric complex. Although type I IFNs bind to the same receptors, many studies suggest that different subtypes of type I IFNs regulate slightly different biological responses[87]. One of the main differences was related to the magnitude of changes in gene expression because all subtypes are able to induce expression of so called interferon-stimulated genes (ISGs).

Type II IFN, with IFNγ as its only member, exhibits distant amino-acid sequence homology with type I IFNs. It is produced by NK-cells and activated T-cells. It binds to different receptors compared to type I IFN, namely to IFNGR1 and IFNGR2. IFNGR1 is constitutively expressed on all cells and expression of IFNGR2 is tightly controlled and less widely distributed[86].

Type III IFN composes three subtypes of IFNλ; IFNλ1, IFNλ2 and IFNλ3. They might be produced by all nucleated cells but frequently they are co-produced with IFNβ[86,88]. Type III IFNs bind to IFNLR1 (IL28RA) that assembles with IL10R2 and forms a heterodimer receptor complex. The responses to type III IFNs are very similar to those triggered by type I IFNs. Nevertheless, the type III IFN response is more restricted, since the type III IFNR complex is expressed only on epithelial cells and pDCs[86].

IFNs regulate a wide variety of biological functions, like resistance to viral infections, enhancement of activity of innate and acquired immune responses, survival and death of normal and tumor cells[86]. The cell responses to type I and type II IFNs are characterized by the expression of very similar genes although these two types of IFNs act by binding to different types of receptors. Type I IFNs utilise the IFNAR1-IFNAR2c receptor complex and IFNγ binds to IFNGR1-IFNGR2. However, both types of IFNs are able to activate the JAK-

STAT signalling cascade and regulate the expression of ISGs. While type I IFNs preferentially activate the trimeric IFN-stimulated gene factor 3 (ISGF3, a transcriptional complex composed of phosphorylated STAT1, phosphorylated STAT2 and unphosphorylated IRF9), IFNγ favours STAT1 homodimers (both subunits are phosphorylated). Furthermore, both types of IFNs are able to activate gene expression by alternative pathways, and for example, the activation of kinases, like phosphatidylinositol 3-kinase (PI3K), extracellular response kinases (ERKs) and p38 leads to an activation of transcription factors NFκB, AP1 and PU.1[85-86,89]. Thus, the effects of IFNs are manifested in changes of gene expression that is regulated by STAT1, but also by many other transcription factors, including AP1, IRF1, IRF4, IRF8, PU.1 and NFκB (Figure 1.5).

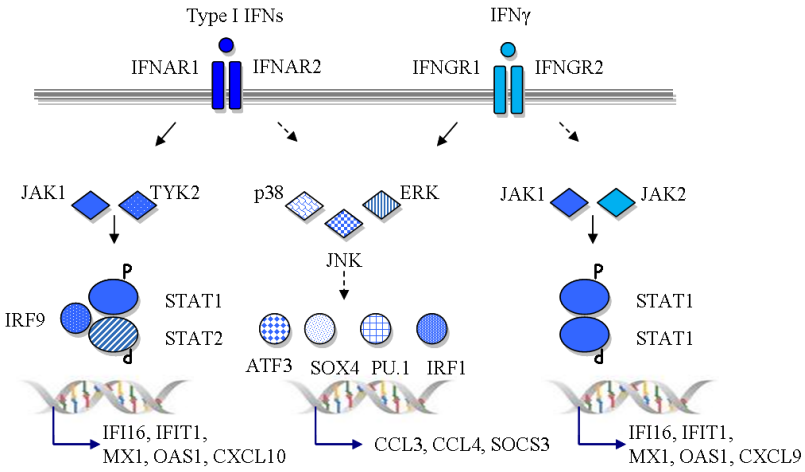

Figure 1.5 An overview of type I and type II IFNs signaling pathways.

Production of IFNs augments the activity of many different cell types, including those that belong to the innate and adaptive immunity. The prototypical response to IFNs is an induction of ISGs whose expression results in synthesis of proteins that interfere with viral replication. ISGs also encode proteins that constitute the IFNs signalling pathways and therefore, enable a sustaining of responses induced by IFN in an autocrine manner. These cytokines play a vital role in mediating cellular immune responses since they increase the expression of MHC class I and II molecules on the cell surface. Therefore, antigen processing and presentation is facilitated together with activation of CD4 and CD8 T-cells. These steps ultimately lead to clearance of the virus and viral-infected cells[90]. The up-regulation of MHC class II molecules is selectively induced by type II IFN, where the expression of these receptors is a prerequisite for the activation of CD4 T-cells[91]. The important role of IFNγ considers its ability to drive differentiation of the naïve T-cells into the Th1 phenotype. Furthermore, IFNs increase the

production of chemokines, such as CXCL9, CXCL10 and CXCL11, which leads to the recruitment of leukocytes at the site of inflammation and facilitate host defence mechanisms. Upon infection, IFNs are secreted for a few hours, and the subsequent secretion of other cytokines, including TNFα, shut down the production of IFNs. Therefore, with elimination of pathogen and under physiological condition, the inflammatory response driven by IFNs might be terminated[92].

Beside the physiological role of IFNs, this group of cytokines exhibit an important role in the pathogenesis of systemic autoimmune diseases[92]. They are involved in the development and maintenance of chronic inflammation in patients with SLE, RA, dermatomyositis, Sjoegren's syndrome, psoriasis, diabetes and active tuberculosis[93-100].

Thus, IFNs act as double-edged swords, similarly to TNFα, and these cytokines are considered to be both therapeutics and therapeutic targets. Their therapeutic effects are based on their properties to inhibit viral replication and tumor growth[64]. The neutralisation of different types of IFNs is currently investigated by applying monoclonal anti-IFNα and anti-IFNγ antibodies in treatment of chronic rheumatic diseases[37].

1.2.4. Cross-talk of cytokines in the pathogenesis of autoimmune diseases

In the previous paragraphs the effects of cytokines have been described in a rather simplified context because under inflammatory conditions one cytokine does not act alone but in combination with many other inflammatory mediators. How the response to various cytokines is coordinated by a particular cell type has remained poorly understood so far. Nevertheless, it is known that usually the same cytokines constitute the inflammatory milieu in different diseases. For example, elevated levels of cytokines, such as type I IFN, IFNγ, BAFF, IL6 and TNFα, were reported in SLE, RA and AS[37,101-103]. It was reported that many cytokines are involved in the pathogenesis of RA but the role of TNFα was considered as dominant. Treatment with anti-TNFα drugs was characterized by neutralisation of TNFα and reduced production of other cytokines, including IL1 and IL6[104]. Therefore, it has been hypothesised that there is a cytokine hierarchy within the cytokine networks[105].

One important aspect of this study is a better understanding of the responses induced by TNFα, type I and type II IFNs in chronic rheumatic diseases. There are several lines of evidence that the effects of these cytokines are interconnected within the complex cytokine

networks[105]. For an appropriate interpretation of data presented in this study, it is helpful to have an insight into so far known data considering the cross-talk of TNFα and IFNs.

It has been shown that TNFα influences the effect of IFNs and vice versa. For example, TNFα inhibits the generation of pDCs from their progenitors, which are the major producer of type I IFN in the body[97]. TNFα also inhibits the release of type I IFN by immature pDCs following exposure to the influenza virus[97]. The most striking interplay of TNFα and IFNs is demonstrated in rheumatic patients. RA patients treated with anti-TNFα revealed an increased expression of genes related to IFNs stimulation[106-107]. Similar results were reported for SoJIA patients that were also treated with anti-TNFα drugs[97]. Based on these studies it was obvious that TNFα exhibits the suppressive effect on the production of IFNs and on the gene expression induced by these cytokines. Nevertheless, there is also evidence that IFNs decrease the production of TNFα either directly or indirectly by attenuating the activation of T-cells[108-109]. It has been shown that NFκB, a prototypical transcription factor activated by TNFα, acts as a negative regulator for expression of IFN stimulated genes (ISG), including STAT1[110-111]. Since, ISG might be regulated both by STAT1 and NFκB but in opposite directions, a question that appears consider if these genes are a valuable source for estimating the balance between TNFα and IFNs.

1.3. Physiological role of monocytes and their involvement in the pathogenesis of chronic rheumatic diseases

As previously mentioned cytokines and pathogenic memory imprinted in the cells of the adaptive immunity are essential for the maintenance of chronic inflammation, which further perpetuates the autoimmune processes[63,112]. Nevertheless, how cells of the innate immunity, including monocytes, respond to these alterations and how they contribute to pathogenesis of diseases are mainly unknown. This study has been focused on the role of monocytes in the pathogenesis of SLE, RA and AS. The facts considering monocytes physiological and pathophysiological roles are described in the following chapters.

1.3.1. Development and functions of monocytes

Monocytes are circulating cells that constitute ~5-10% of peripheral blood leukocytes in humans[113]. Like all the other leukocytes, they originate from hematopoietic stem cells in the bone marrow, which proliferate and differentiate through several commitment steps (Figure

1.6). Hematopoietic stem cells differentiate into common myeloid and lymphoid progenitors, where the common myeloid progenitors (CMP) further differentiate into granulocyte/macrophage progenitors (GMPs) and macrophage/DC progenitors (MDCs). GMPs give rise to blood monocytes and granulocytes, whereas MDCs differentiate to tissue-specific macrophages and most DCs[114].

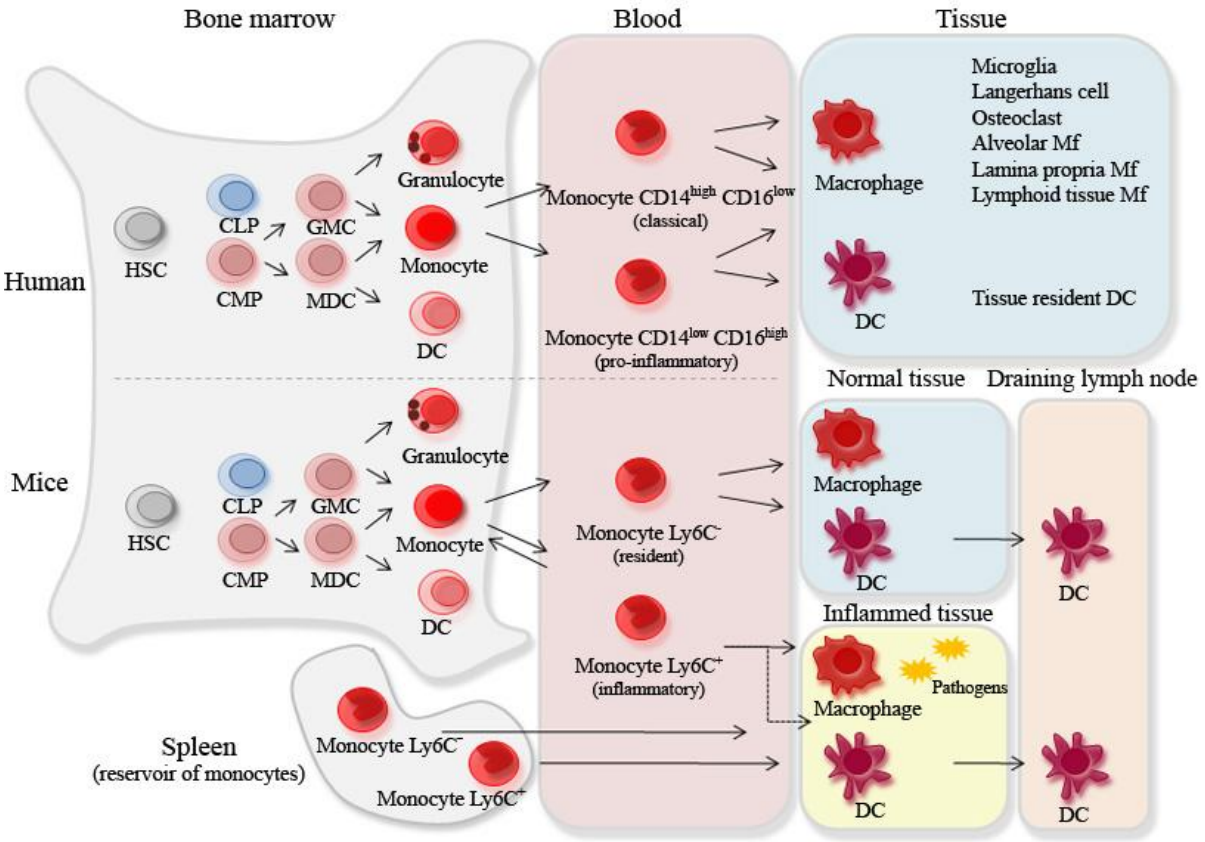

Figure 1.6 Development of monocytes and their differentiation into macrophages and DCs. Scheme is based on models proposed by Gordon *et al.* (2005), Swirski *et al.* (2009) and Geissmann *et al.* (2010).

Monocytes migrate from the bone marrow in peripheral blood as non-proliferating cells. Their half-life in peripheral blood is 1-3 days, and it is assumed that monocytes at least partially mature in circulation[115-116]. During their lifetime in circulation, they perform surveillance of the organism and comprise the first line of defence to invading pathogens and tissue injuries. They can reach any part of the body, and they are the principle source for repopulation of tissue macrophages and DCs[114,117-120]. Depending on the tissue environment, monocytes differentiate into macrophages that acquire tissue specificity, like splenic macrophages, alveolar macrophages in the lung, Kupffer cells in the liver, microglia cells in the brain, or osteoclasts in the bone. Monocytes might also differentiate into mDC under inflammatory

conditions[114]. Thus, the plasticity of monocytes to differentiate into various cell types exhibits their potential to take part in a broad range of cellular processes[114]. Since, the main function of macrophages is the phagocytosis and the central function of mDC is to process and present antigens to T-cells, monocytes are considered as the cell type that is on the cross-road between the innate and adaptive immunity.

One phenotypical characteristic of monocytes is an expression of CD14 molecule on their surface. CD14 is considered as monocyte-specific linage marker and its principal role is to cooperate with TLR4 and MD-2 in the binding of LPS, which is the component of the outer membrane of Gram-negative bacteria. Monocytes also express the receptors that mediate phagocytosis such as CD16 (FcγRIII), CD32 (FcγRII) and CD64 (FcγRI)[119]. Furthemore, this cell type expresses molecules indispensable for the antigen presentation and activation of T-cells, including MHC class II molecules, CD80 and CD86. Based on the expression of surface markers CD14 and CD16, the whole population of peripheral blood monocytes in humans is divided into two subsets: the "classical or resident" monocytes, which highly express CD14 molecules and weakly CD16, ($CD14^{hi}CD16^{low}$), and the "inflammatory" monocytes that express weakly CD14 but strongly CD16 ($CD14^{low}CD16^{hi}$)[116]. Studies in mice have shown that the counterparts of the human monocytes subsets are also detectable in mice, where the resident monocytes are determined as $Gr1^{low}$ ($Ly6C^{-}$) and the inflammatory monocytes as $Gr1^{hi}$ ($Ly6C^{+}$)[119]. In mice, these two subsets possess different functions and differentiation fates. The main role of resident monocytes is to renew tissue macrophages and DCs, but also to patrol blood vessels, to scavenge oxidized lipids, dead cells and potential pathogens[118]. The crucial role of inflammatory monocytes is to produce the pro-inflammatory mediators, migrate to the site of inflammation and subsequently differentiate into DCs[114,121-122]. It has been shown in the mouse model that in the absence of inflammation, when the body does not exploit the potential of inflammatory monocytes, this subset is able to convert into a resident and acquire a more beneficial role for the host[121]. More precisely, the inflammatory monocytes were able to return (home back) to the bone marrow. Their reappearance in peripheral blood was accompanied with changes of their phenotype[117]. The changes were characterised by the loss of the Gr1 molecule and the conversion of $Gr1^{hi}$ cells into $Gr1^{low}$ cells. Therefore, the inflammatory monocytes were able to convert into the resident subsets (Figure 1.6). However, it is still unknown if resident monocytes are also generated in the bone marrow independently of phenotypical changes[123]. Although these studies were performed in mice, it is assumed that phenotypical changes might also take place in humans[118]. For

example, it has been shown that 2 hours after autologous injection of radioactively labelled monocytes in humans, about 12% of injected cells were detectable in the bone marrow[124]. Although that study did not focus on following the changes in monocyte phenotypes, which is still limiting in humans, the results indicated that the peripheral blood monocytes were able to home back to the bone marrow.

As mentioned before, the well accepted fate of monocytes postulates that this cell type circulates in the peripheral blood and upon entering tissues irreversibly differentiates into macrophages or DCs. Nevertheless, it has been shown in the mouse model that monocytes might reside in the spleen as undifferentiated cells, and that spleen monocytes even outnumber peripheral blood monocytes[125]. Spleen monocytes can be rapidly mobilized in response to injury and their role is vital for host survival during life-threatening conditions[125]. However, whether or not a similar process takes place in humans is still unknown.

1.3.2. Role of monocytes in the pathogenesis of SLE, RA and AS

It is known that macrophages infiltrate inflamed tissues, including synovium in RA, kidneys in SLE and sacroiliac joints in AS. In addition, macrophages produce a wide array of inflammatory mediators that sustain chronic inflammation[41,71,113,126-128]. Therefore, it is speculated that cytokines alter the function of monocytes in these diseases. As already mentioned, type I IFN was able to induce the maturation of monocytes into DCs[129]. This alteration is associated with an inappropriate clearance of apoptotic and necrotic cells, the rising amounts of nuclear antigens, production of anti-nuclear autoantibodies and development of SLE[129-131].

Monocytes/macrophages (Mo/Mf) also play an important role in the pathogenesis of RA. It is known that the synovial membrane of RA patients is infiltrated with activated macrophages that produce large amounts of inflammatory mediators including TNFα, MMP1, MMP3, MMP9 and MMP12[41,43,66,132-133]. The role of Mo/Mf in the pathogenesis of RA became more apparent concerning that treatment of RA patients with various types of drugs directly or indirectly affected the number and function of monocytes. For example, treatment with gold salts is accompanied by reduced infiltration of Mo/Mf within synovium. Treatment with methotrexate promotes the secretion of anti-inflammatory cytokines and suppresses secretion of pro-inflammatory cytokines by Mo/Mf. Anti-TNFα drugs target TNFα, which is mainly produced by Mo/Mf and in addition they are able to reduce the number of monocytes in peripheral blood and synovium[133-137].

The role of monocytes in the pathogenesis of AS has remained largely unknown. Nevertheless, it is well accepted that inflammation of sacroiliac joints of AS patients accompanies an infiltration of macrophages and T cells[128,138]. Furthermore, an increased level of serum TNFα was identified in AS patients, but how the function of cells is altered in this condition has not been provided to date[103]. Interestingly, both AS and RA are accompanied by inflammation of joints, activation of monocytes and with similar response to anti-TNFα therapy[76]. Nevertheless, monocytes proteomes from RA and AS patients exhibited differences[76]. For example, the changes in expression of proteins that belong to the ubiquitin proteasome pathway were restricted only to AS monocytes.

1.4. Gene expression profiling in chronic rheumatic diseases

Gene expression profiling is a technique for measuring the activity of all genes simultaneously in particular biological sample. The sample material may be different, including various cell types, blood or tissues. In each cell in the body, at any given time, hundreds or thousands genes are active. Their activity is revealed by transcription (expression) from genomic DNA, and thus, measuring the abundance of mRNA in the cells provides a comprehensive insight into cell functions under physiological and pathophysiological conditions. Since, gene expression profiling measures transcriptional activity of genes and relates it with the cell functions, the synonyms that are often used for this method are transcriptomics and functional genomics.

1.4.1. The main characteristics of global gene expression profiling

Gene expression profiling appeared with the development of microarray technology that principally combined ideas of Southern- and Northern-blotting methods with increased amount of information generated by DNA sequencing at the end of the 20th century. The idea that mRNA might be measured based on a base-pairing of complementary nucleic acid sequences is not new. Nevertheless, the possibility to measure mRNAs from all active genes in the cells became realistic when the sequence of human genome was completed at the beginning of the 21st century.

Currently, there are several manufacturers that provide different technologies for measuring the levels of gene expression including Affymetrix, Illumina and Agilent. In this study the Affymetrix whole transcriptome arrays have been utilized. Affymetrix arrays are characterized by millions of synthetic oligonucleotides (probes or oligos) which are

synthesized *in situ* on a solid surface, measuring 1.28 x 1.28 cm in size, by photolithographic technology[139]. The oligonucleotides consist of 25 nucleotides in length and recognize the complementary sequences of almost every transcript near its 3' end. Since, the 25-mer oligos are relatively short sequences; the Affymetrix arrays are designed in such a way that 11-20 independent probes detect each transcript. This approach provides a high sensitivity for transcript detection without loss of specificity.

An overview of the procedure applied for mRNA measurement is presented in Figure 1.7.

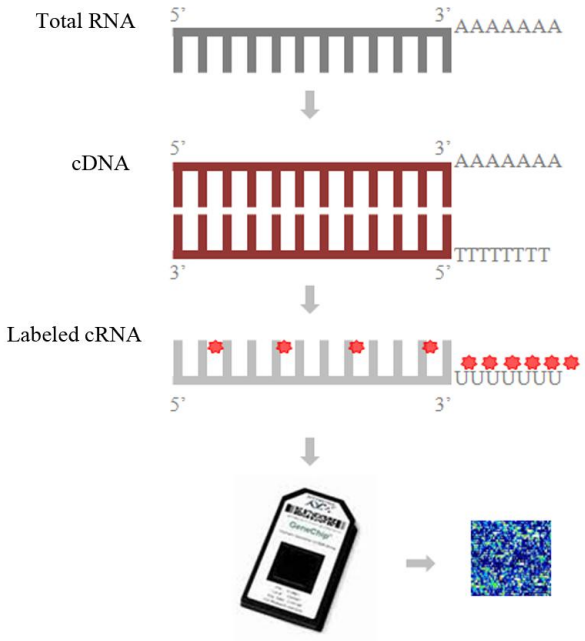

Figure 1.7 Overview of procedures used for transcripts measurement. For measuring mRNAs, total RNA was extracted and converted into cDNA by reverse transcriptions. cDNA is further processed and amplified by *in vitro* transcription into antisense RNA (cRNA) that incorporate biotinylated nucleotides (marked as red stars). cRNA is fragmented and hybridised on the microarrays. Binding of labelled cRNA is visualised by streptavidin-phycoerythrin and the readout is performed with a specific high-density scanner.

One additional characteristic of the Affymetrix arrays is the method applied for detection of transcripts. The signal value of each transcript is determined based on differences of signals between perfect match (PM) and mismatched probes (MM). Principally these signals reflect the amount of mRNA in the sample, where high signals are related to PM and low signal with MM probes (Figure 1.8). The PM probe is 25-mer oligos designed to be complementary to a reference sequence and thus, "fully" hybridized with the transcript from the sample. In contrast, the MM probe is also 25-mer in length and it is also complementary to their reference sequence with the exception that the central nucleotide at the 13th position

mismatches to the reference sequence. Since, MM probes cannot be "fully" hybridized, they cannot provide the high signal intensities. They serve as a specificity control and their signal values are compared to the signal values of corresponding PM probes. A collection of 11-20 PM and MM probes determines one probe-set.

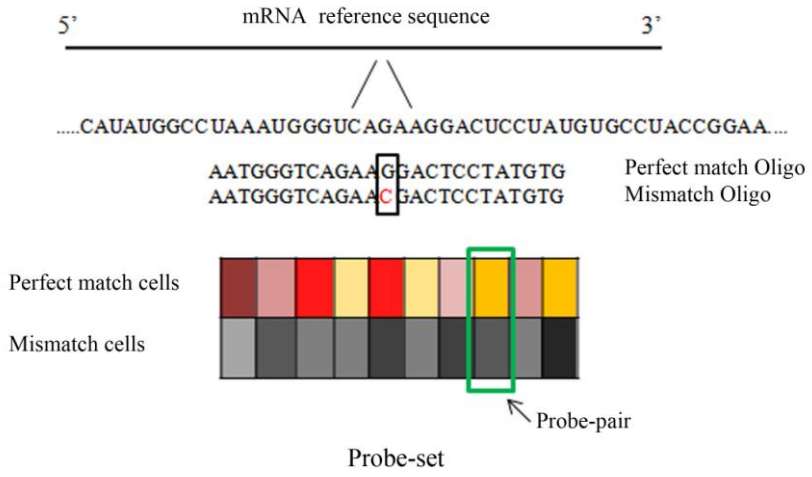

Figure 1.8 Design of Probe-set. Each square represents fluorescence intensity, where bright cells are related to the perfect match oligos (display high signal intensities) and dark cells to the mismatch oligos (display low or no signal intensities).

The Affymetrix GeneChips used in this study are Human Genome (HG) arrays: HG-U133A and HG-U133 Plus 2.0. The older generation of arrays, the HG-U133A, does not cover the whole human genome. According to the Affymetrix array reports, the HG-U133A chip includes 22 283 probe-sets and covers around 14 500 genes, and the HG-U133 Plus 2.0 array contains 54 675 probe-sets and covers around 38 500 human genes. Since, it is known that the human genome contains only 20-25 000 protein-coding genes, the number of 38 500 genes determined by the Affymetrix arrays is related not just to the known genes, but also to those ascertained as potential genes. These potential genes are revealed by mathematical predictions that particular DNA sequences might be protein coding genes. They are usually named as the chromosome open reading frames, the hypothetical proteins FLJ, DKFZ, LOC, MGC, family with sequence similarity (FAM) and KIAA genes. Since the HG-133U Plus 2.0 arrays are extended with the number of probe-sets compared to HG-133A arrays, it is possible to compare the results generated by these two types of arrays. Nevertheless, this approach is associated with the loss of information for those genes that are included in the later generation of arrays.

Noteworthy to mention is that one gene might be determined with more than one probe-set, either because the sequence of the transcript requires more probe-sets to be specifically covered, or because this type of Affymetrix arrays includes different splice variants from a single gene.

Gene expression profiling produces a huge amount of data. To extract the correct and high-quality data, it is essential to utilize the appropriate bioinformatics expertise and tools. In this study, the primary data analysis was done by applying the MAS5.0/GCOS algorithm, which is developed by Affymetrix for analysis of multiple PM and MM oligos per probe-set. The following step focused on selection of differentially expressed probe-sets, and it was performed by a specifically developed BioRetis database[140]. This online database provides a panel of validated parameters that ensure selection of differentially expressed probe-sets with the minimal false discovery rates.

1.4.2. Application of gene expression profiling in clinical practice

A transcription is a highly dynamic cell trait and various factors are able to rapidly induce its changes. Thus, to know when, where and to what extent which genes in the cells or tissues are expressed is an approach that provides a detailed insight into cell functions under various conditions, including those that accompany diseases. The initial breakthrough of transcriptomics appeared in the field of cancer, when transcriptome of leukaemia patients revealed differences between acute myeloid and acute lymphoblastic leukaemia[141]. The relevance of this study was enormous, firstly because it was difficult to discriminate these two diseases, and secondly because they have different clinical courses and show different responses to therapy. The same strategies have been introduced in the field of rheumatology, aiming to understand the underlying pathomechanism of various diseases, to improve diagnosis, subclassify patients with the same disease, facilitate identification of new therapeutic targets, and eventually predict the response to therapy.

Many rheumatic diseases, like RA, SLE, AS, psoriatic arthritis, Systemic sclerosis (SSc) and Sjoegren's syndrome (SS) are characterised by a shared phenotype. For example, the joint inflammation is not exclusively characteristic of arthritis, it also accompanies patients with SLE, AS, psoriasis and SS[49,142]. Furthermore, there is a big heterogeneity between patients with the same disease, for instance lupus patients might exhibit skin manifestation, or haematological disorders, or glomerulonephritis, or a combination of all these clinical manifestations[143]. Moreover, the availability of increasing number of biologicals strengthens

the need for identification of diagnostic biomarkers, which should determine the most beneficial treatment for each individual patient.

Although, modern biologicals revolutionised the field of rheumatology, their application are still based on a "quesswork" approach. That is to say that, the needs of patients are not the same, and while one biological leads to a significant improvement in one group of patients, it may not demonstrate any effect in the other groups. Thus, the necessity to distinguish patients that will respond to anti-cytokine therapy (anti-TNFα, or anti-IL1, or anti-IL6, or anti-IL17), or to Rituximab (induces B-cell depletion), or to Abatacept (inhibits T-cell activation), or that will not respond to any of the available biologicals, will be an important step in personalising treatment of patients with different rheumatic diseases[144].

1.4.3. Transcriptomes from chronic rheumatic diseases and the dilemma associated with detection of IFN signature

Transcriptomes from many rheumatic diseases have been generated so far and it is well accepted that the systemic nature of these disorders is accompanied with changes in gene expression within PBMCs. So far, gene expression profiles have been reported from patients with SLE, RA, psoriatic arthritis, dermatomyositis, Systemic sclerosis (SSc), Sjoegren's syndrome and SoJIA patients[93,95-97,145-147]. Furthermore, gene expression profiling has been applied in studding responses that were induced by a particular treatment, including anti-TNFα therapy and Rituximab treatments[97,106,148]. The gene expression profiles were able to identify differences in diseases compared to healthy donors, but principally they were revealed as very robust profiles. Although, there are attempts to relate differential expression of genes within PBMCs to the numbers of B-cells, T-cells, monocytes, or to their activation, principally it remains unknown what is the contribution of a particular cell type within PBMCs[145]. The basic idea to extract the relevant information from the profiles and bring them into clinical practice has not been achieved.

A substantial improvement in deciphering alteration from chronic rheumatic diseases appeared when a type I IFN signature was identified in lupus patients[93]. Later, the IFN regulated genes were also detected in patients with other rheumatic diseases such as RA, dermatomyositis, psoriasis, or in RA patients after anti-TNFα treatment[93-98,106]. Therefore, it seems that many chronic rheumatic diseases contain the type I IFN signature or that in some diseases it appears after treatment. However, it is frequently overlooked that the genes referred as the type I IFN signature in these diseases are inherently different. For instance, in

SLE patients, this signature is related to 22 genes, in dermatomyositis to 93 genes, in RA patients after therapy to 13 or 6 genes, depending on the study[93-94,96,148]. IFN signatures were disclosed as different not just in terms of number of genes but also in terms of the magnitude of their changes. For example, IFN-regulated genes were identified in SLE patients as strongly up-regulated and in RA patient as genes with slightly elevated expression. Therefore, a discrepancy is apparent when considering the similarities and differences between type I IFN signatures in SLE and RA. The type I IFN signature was found in a subgroup of RA patients, but also in a group of patients treated with anti-TNFα drugs and Rituximab[96,106,148]. In addition, the presence of type I IFN signature in RA, either before or after treatment, does not involve the same genes. Principally, in the aforementioned studies, the type I IFN signature was not analysed in a comprehensive way, considering the fact that only highly up-regulated genes induced by IFN *in vitro* were utilised for analysis. Therefore, it is important to know that the type I IFN signature, developed by Baechler *et al.*, included in total 315 genes that were both up- and down-regulated[93]. So far, only a small number of IFN up-regulated genes were utilized for the analyses[93,95].

Although type I IFN signatures in rheumatic diseases has not been consistently interpreted, its identification was an important step in deciphering alteration from diseases. It demonstrated that the cytokine influence might be detected within disease transcriptomes. A significant step further was provided by identifying that type I IFN signature correlated with disease activity in patients with SLE and dermatomyositis[95]. Furthermore, it was notable that the type I IFN signature appeared in patients treated with anti-TNFα drugs and it was postulated that there is a possible cross-talk between TNFα and type I IFN[97,106]. Nevertheless, how the effects of these cytokines are interconnected in different rheumatic diseases has remained unknown to date.

1.4.4. Biological specimens utilized for transcriptome analysis

An important consideration before starting microarray experiments is, which sample material should be utilized. Some studies have focused on tissues, like glomeruli and synovium, given that the pathophysiological processes are the most prominent at the place of inflammation[149-150]. However, one major limitation for tissue analysis is restricted access to this type of sample material. Another strategy focused on cells from peripheral blood as the sample material. Blood is easily accessible and often used for diagnosis, and principally it is as a pipe line of the immune system. Therefore, the alterations that affect the immune system are

imprinted in the peripheral blood leukocytes[151]. In most previous studies, the peripheral blood mononuclear cells (PBMCs) were used for microarray experiments. After removal of erythrocytes and granulocytes, a cellular mixture composed of T-cells, B-cells, monocytes, and NK-cells is used for analysis. For example, PBMCs have been utilized for transcriptome studies in SLE, RA, psoriatic arthritis, dermatomyositis and SoJIA patients[93,95-97,145,152]. The transcriptomes of these disorders showed differences between patients and healthy donors. Nevertheless, the main difficulties appeared with the interpretation of these data since the quantitative and qualitative differences affect various leukocyte populations under inflammatory conditions. For example, it is known that monocytosis accompanies RA patients and that both monocytosis and CD4 T-cell lymphopenia are present in SLE patients[94,145,153]. Furthermore, PBMCs from patients might be "contaminated" with immature granulocytes, which are not present in healthy donors[154]. Therefore, it is not possible to discriminate alteration in gene activities caused by a real up- or down-regulation from alteration induced by an increase or decrease in absolute cell numbers of a particular leukocyte population. There are some attempts described that have tried to overcome these difficulties, like a virtual dissection of PBMCs profiles by sets of genes that characterise populations of T-cells, B-cells, or monocytes[145,155-156]. Nevertheless, the main potential of transcriptome analysis to completely uncover alteration within cells remained largely unexploited[157-158]. Thus, a focus on cell type specific transcriptomes is a more laborious and more expensive approach, since many different cell types should be analysed separately. However, it is a more detailed and accurate approach that provides a very comprehensive insight into cell-specific alterations in various diseases, which finally would provide a better understanding of disease pathogenesis.

2. Aim of the study

2. Aim of the study

The aim of this study was to characterize the role of pro-inflammatory cytokines in the pathogenesis of chronic rheumatic diseases. For this purpose the gene-expression profiling was utilised, as a strategy that provides an overall insight into alterations within the cells at the transcriptional level.

Three rheumatic diseases have been studied in detail: systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and ankylosing spondylitis (AS). The common characteristics of these disorders are autoimmunity, chronic inflammation and abundant production of various cytokines. The analyses of SLE, RA and AS simultaneously by gene-expression profiling provide a detail insight into similarities and differences that characterize these rheumatic diseases.

Peripheral blood monocytes have been utilised for gene-expression profiling for the following reasons: they have a short half-life in circulation, they are sensitive in detecting and rapid in responding to the various alterations in the body. Therefore, development of the gene expression profiles from SLE, RA and AS monocytes will address the initial question of this study:

1. Is the systemic nature of different rheumatic diseases reflected in a disease-dependent manner at the level of peripheral blood monocytes?

To estimate the impacts of cytokines in altering the profiles of diseases, the cytokine-specific gene-expression profiles were generated by stimulating monocytes *in vitro* with TNFα, IFNα2a and IFNγ. The gene expression profiles from SLE, RA and AS were compared with the *in vitro* generated cytokine-specific signatures. This experimental strategy is applied to address the following questions:

2. Do monocytes display the cytokine-specific gene-expression profiles after *in vitro* stimulation?

3. Do comparisons between disease-specific and cytokine-specific signatures show a dominance of a particular cytokine in the pathogenesis of disease?

4. Is it possible to identify the interplay of cytokines within a complex cytokine network that characterizes the inflammatory milieu in SLE, RA and AS?

The answers to these questions would be indispensable in estimating an individual cytokine profile in various rheumatic diseases. Furthermore, they should lead to an improvement of diagnosis, identification of new therapeutic targets and ultimately they should facilitate tailoring treatment to the needs of each individual rheumatic patient.

3. Materials and Methods

3. Materials and methods

The materials and methods section is divided in seven parts. The first part provides an overview of devices and materials used in this study. The second part offers an insight into the clinical characteristics of patients. The third part focuses on development of cytokine-specific signatures in monocytes after their *in vitro* stimulation. The fourth part considers sample preparation for microarray analysis as well as processing of GeneChip arrays. The fifth part concerns features of software tools used for gene expression analysis, together with explanation how to "read" the figures created. The sixth part considers promoter analysis and the seventh part visualisation of transcriptome data in forms of molecular networks and signalling pathways.

3.1. An overview of utilized devices, software tools and consumables

The following tables show devices, software tools, consumables, buffers, solutions and antibodies that were utilized in this study.

Table 3.1 Devices used in this study

Type of Device	***Manufacturer***
AutoMACS™ Separator	Miltenyi Biotech, Bergisch Gladbach (DE)
Fluorescent Activated Cell Sorter (FACS) *for analysis:* FACSCalibur; FACS LSRII *for cell isolation:* FACSAria™; FACSDiva™	Becton Dickinson, Heidelberg (DE)
Incubator	Binder, Tuttlingen (DE)
Laminar flow (Lamina HERA safe)	Heraeus, Hanau (DE)
Light microscope	Helmut Hund GmbH, Wetzlar (DE)
Analytical Balance	Sartorius, Goettingen (DE)
Centrifuges (5415R, 5810R)	Eppendorf, Hamburg (DE)
Casy® Cell Counter Model TT	Schaerfe System GmbH, Reutlingen (DE)
Eppendorf Pipette Research® Set (volumes 10 µl; 200 µl; 1000 µl)	Eppendorf, Hamburg (DE)
Electronic pipettor	Drummon, Broomall (USA)
Agilent 2100 Bioanalyser	Agilent, Waldbronna (DE)
NanoDrop ND-1000 spectrophotometer	NanoDrop Technology, Wilmington (USA)
GeneChip® Scanner 3000	Affymetrix, Santa Clara, CA (USA)
GeneChip® Fluidic station 400	Affymetrix, Santa Clara, CA (USA)
GeneChip® Hybridization Oven 640	Affymetrix, Santa Clara, CA (USA)

Table 3.2 Software tools applied in this study

Application	*Name*	*Manufacturer*
Acquisition and analysis of FACS data	Cell Quest FACSDiva FlowJo 7.2	Becton Dickinson Becton Dickinson TreeStar Inc, Ashland (USA)
Microarray analysis	GCOS/ MAS5.0 BioRetis data base	Affymetrix, Santa Clara, CA (USA) BioRetis GmbH, Berlin (DE)
Hierarchical clustering (HC)	Genesis MultiExperiment Viewer 4.4 (MeV)	Institute for Genomics and Bioinformatics, Graz (A) TIGR Institute, Maryland (USA)
Principal component analysis (PCA)	Genesis	Institute for Genomics and Bioinformatics, Graz (A)
Terrain map analysis	MultiExperiment Viewer 4.4 (MeV)	TIGR Institute, Maryland (USA)
Promoter analysis:	whole genome rVISTA	Genomic Division, Berkeley, (USA)
Statistics and Diagrams	Office Excel 2007 GraphPad Prism4	Microsoft GraphPad Software, La Jolla (USA)
Data base for microarray analysis	Office Access 2007	Microsoft
Tables	Office Excel 2007	Microsoft
Text	Office Word 2007	Microsoft
Pictures	Adobe Photoshop CS2	Adobe System, San Jose (USA)

Table 3.3 Consumables and Reagents

Consumables and Reagents	*Manufacturer*
BD Vacutainer tubes™ Glass, Sodium Heparin, 10 ml	Becton Dickinson, Heidelberg (DE)
Falcon tubes 15ml, 50 ml	Becton Dickinson, Heidelberg (DE)
FACS tubes 5 ml, 12 mm × 75 mm, steril	Becton Dickinson, Heidelberg (DE)
Eppendorf tubes 1,5 ml	Eppendorf, Hamburg (DE)
Tips for pipettes: 10 µl; 100 µl; 1000 µl	VWR, Darmstadt (DE)
RNase free tips: 10 µl; 100 µl; 1000 µl	NerbePlus, Winsen/Luhe (DE)
Affymetrix GeneChip® Human Genome U133A 2.0 Array	Affymetrix, Santa Clara, CA, USA
Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array	Affymetrix, Santa Clara, CA, USA
GeneChip® Hybridization, Wash and Stain Kit	Affymetrix, Santa Clara, CA, USA
GeneChip® Sense Target Labelling and Control Reagents	Affymetrix, Santa Clara, CA, USA
RNeasy Mini Kit	Qiagen GmbH, Hilden (DE)
RNase-Free DNase Set	Qiagen GmbH, Hilden (DE)
QIAshredder	Qiagen GmbH, Hilden (DE)
Recombinant human TNFα	PeproTech GmbH, Hamburg (DE)
Recombinant human IFNγ	PeproTech GmbH, Hamburg (DE)
Recombinant human IFNα2a	ImmunoTools, Friesoythe (DE)

Brefeldin A	Sigma-Aldrich (DE)
CD15 MicroBeads, human	Miltenyi Biotech, Bergisch Gladbach (DE)
CD14 MicroBeads, human	Miltenyi Biotech, Bergisch Gladbach (DE)
Propidium Iodide (PI)	Sigma-Aldrich (DE)

Table 3.4. Solutions and buffers

Solution	*Amount*	*Component*	*Manufacturer*
PBS phosphate buffered saline	2,7 mM	KCl	Merck
	1,5 mM	KH_2PO_4	Merck
	8,0 mM	Na_2HPO_4 x $2H_2O$	Merck
	137 mM	NaCl	Merck
PBS/BSA	0,5% (w/v)	Bovine serum albumine in PBS	PAA Laboratories, Pasching (A)
PBS/BSA/EDTA	5 mM	Ethylenediaminetetraacetic acid in PBS/BSA	Sigma-Aldrich (DE)
Fixation solution	2 % (w/v)	Paraformaldehyde in PBS	
Permeabilisation sol. (intracellular staining)	0,5% (w/v)	Saponin in PBS	Sigma-Aldrich (DE)
Permeabilisation sol. (nuclear staining)	70 % (w/v)	Methanol in PBS	Carl Roth GmbH
Buffer EL		Erythrocyte Lysis Buffer	Qiagen, Hilden (DE)
Buffer RLT		RNeasy Lysis Buffer	Qiagen, Hilden (DE)

Table 3.5. Antibodies used in this study

Specificity	*Clone*	*Conjugate*	*Manufacturer*
Antibodies used for surface staining			
CD14	M5E2	FITC	BD Bioscience
CD14	M5E2	PE-Cy7	BD Bioscience
CD69	FN50	PE	BD Bioscience
CD83	HB15e	APC	BD Bioscience
CD64	10.1	FITC	BD Bioscience
CD32	FLI8.26	APC	BD Bioscience
CD244	2-69	FITC	BD Bioscience
CD120b	hTNFR-M1	APC	BD Bioscience
CD128b	6C6	PE-Cy5	BD Bioscience
Antibodies used for intracellular staining			
CXCL9 (MIG)	B8-11	PE	BD Bioscience
CXCL10 (IP10)	6D4/D6/G2	PE	BD Bioscience
IL1β	AS10	FITC	BD Bioscience
IL6	AS12	FITC	BD Bioscience
IL8	AS14	PE	BD Bioscience
Antibodies used for nuclear staining			
pNFκB p65 (pS529)	K10-895.12.50	Alexa Fluor® 647	BD Bioscience
pSTAT1 (pY701)	4a	Alexa Fluor® 647	BD Bioscience

3.2. Clinical characteristics of patients recruited for this study

Patients recruited for this study were diagnosed with SLE, RA, or AS. Each of these diseases has its own inclusion criteria for diagnosis, as described in more detail below. The selected patients were not treated with biologicals and/or corticosteroids, with the exception of one RA patient who received treatment with prednisolone (corticosteroid). The control group included age-matched healthy donors. The Ethics Committee of the Medical Faculty of Charité University Hospital-Berlin approved the study. Written informed consent was obtained from all patients and healthy donors.

3.2.1. Clinical characteristics of selected SLE patients

Nine SLE patients were involved in this study, 7 females and 2 males. Seven patients were of Caucasian and 2 of Asian origin. Patients were selected after meeting at least 4 out of 11 ACR criteria[159]. All patients showed active diseases with a mean disease activity score (SLEDAI) of 17 (SD=6). Patients had a mean CRP of 57 mg/l (SD=70 mg/l), and mean ESR of 40 mm/h (SD=37 mm/h). Patients' sera were positive for anti-dsDNA autoantibodies. All SLE patients were treated with non-steroidal anti-inflammatory drugs (NSAIDs) and/or disease-modifying anti-rheumatic drugs (DMARDs). In 4 patients an active renal involvement was diagnosed and they received bolus therapy with cyclophosphamide (800 mg).

3.2.2. Clinical characteristics of selected RA patients

Eight RA patients were selected according to the ACR criteria. Disease activity in these patients was defined as a mean DAS28 of 6.1 (SD=1.1). The mean of CRP value was 50.3 mg/l (SD=53.5 mg/l) and ESR was presented with a mean of 38.3 mm/h (SD=26.6 mm/h). Out of 8 patients, 6 were females and 2 males. The mean age of patients was 51 (range from 20-65). Four patients were positive for rheumatoid factor, with a mean of 210 U/ml (SD=198 U/ml), and 2 patients were HLA-DR positive. Seven out of 8 patients were treated with NSAIDs and one patient was treated only with methotrexate (MTX). Three out of 7 patients treated with NSAIDs received an additional therapy, where one patient received MTX, one MTX and leflunomide, and one prednisolone (15 mg/d) in combination with sulfasalazin.

3.2.3. Clinical characteristics of selected AS patients

In total, 14 AS patients were recruited in this study. Disease activity was defined by the Bath Ankylosing Spondylitis Disease Activity Score (BASDAI) of ≥6.3 (SD=1.8). Patients had a

mean CRP of 32.9 mg/l (SD=44.1 mg/l) and a mean ESR of 56.3 mm/h (SD=34.6 mm/h). The mean age of patients was 36 years (range 27-57), where 11 were males and 3 females. All patients were HLA-B27 positive. They were treated with NSIADs but not with DMARDs.

3.2.4. Characteristics of healthy donors involved in this study

There are two groups of healthy donors, one group was included in generation of disease-specific profiles, and the second one was involved in the generation of cytokine-specific signatures.

In total, 12 healthy donors (ND) were utilized for development of SLE, RA and AS profiles. They were without any medications or indications of inflammation (ESR<30 mm/h and CRP<5 mg/l). Their age was in the range of 20-60 years, where 6 donors were males and 4 females. Two male donors were used twice in the analyses.

The second group of healthy donors, utilized for generation of cytokine-specific signatures, comprised 7 healthy individuals. They were not taking any medication. Six were of Caucasian and one was of Asian origin. All were non-smokers, four donors were females (24-29 years old) and three were males (24-33 years old). Three donors were used more than once for stimulation and generation of cytokine-specific signatures.

3.3. Blood collection and monocyte isolation from SLE, RA and AS patients

Fifty milliliters of blood from patients or healthy donors was collected in Vacutainer Heparin tubes (Becton Dickinson, Heidelberg, Germany). Blood samples were lysed by EL buffer (Qiagen, Hilden, Germany) at 4 ºC according to the instructions of the manufacturer. Subsequent depletion of granulocytes was performed by using anti-CD15-conjugated microbeads (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) and the automated separation system auto-MACS (Miltenyi Biotec). CD15 depleted fraction was stained with anti-CD14-FITC antibody (Becton Dickinson, BD), which represents monocyte linage marker. In addition propidium iodide (PI) was applied for exclusion of dead cells (Figure 3.1). Using FACSAria cell sorter (BD) monocytes were isolated with purities and viabilities of >99%. Purified cells were immediately lysed in RLT buffer (Qiagen, Hildesheim, Germany) and stored at –80 °C until isolation of total RNA.

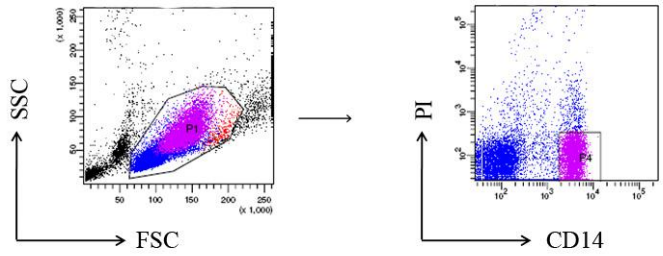

Figure 3.1 Isolation of highly purified monocytes. Monocytes were isolated from PBMCs based on their size and granularity by forward and side scatters (FSC and SSC), and based on the expression of CD14 molecule on their surface.

3.4. *In vitro* stimulation of monocytes in whole blood by TNFα, IFNα2a and IFNγ

Whole blood from healthy donors was used for monocytes stimulation *in vitro* with TNFα, IFNα2a and IFNγ. In total 250 ml of peripheral blood from healthy donors was collected in Vacutainer Heparin tubes (Becton Dickinson, Heidelberg, Germany). Three donors were used more then once for blood donation. Samples that represented unstimulated controls were immediately processed. Other samples were incubated for 1.5 h at 37 ºC, either without stimulus or were stimulated with 100 ng/ml TNFα, or 100 ng/ml IFNα2a, or 100 ng/ml IFNγ. After stimulation, blood samples were processed the same way as the samples from patients.

The activation of monocytes was confirmed by following the expression of surface markers, production of cytokines and phosphorylation of transcription factors.

3.4.1. Markers used for following the activation of monocytes *in vitro* by TNFα

Expression of surface marker CD83, production of cytokines IL8 and IL1β, and phosphorylation of transcription factor NFκB (pNFκB) were used for monitoring the activation of monocytes by TNFα. With the exception of NFκB phosphorylation, which was monitored 30 min after stimulation, the other markers were utilized for following the cell activation 1.5 h after stimulation (Figures 3.2.A and 3.2.B). Isolation of *in vitro* stimulated monocytes was performed in the same ways as described before (paragraph 3.3). Expression of surface markers and production of cytokines were measured by flow cytometry on FACSCalibur.

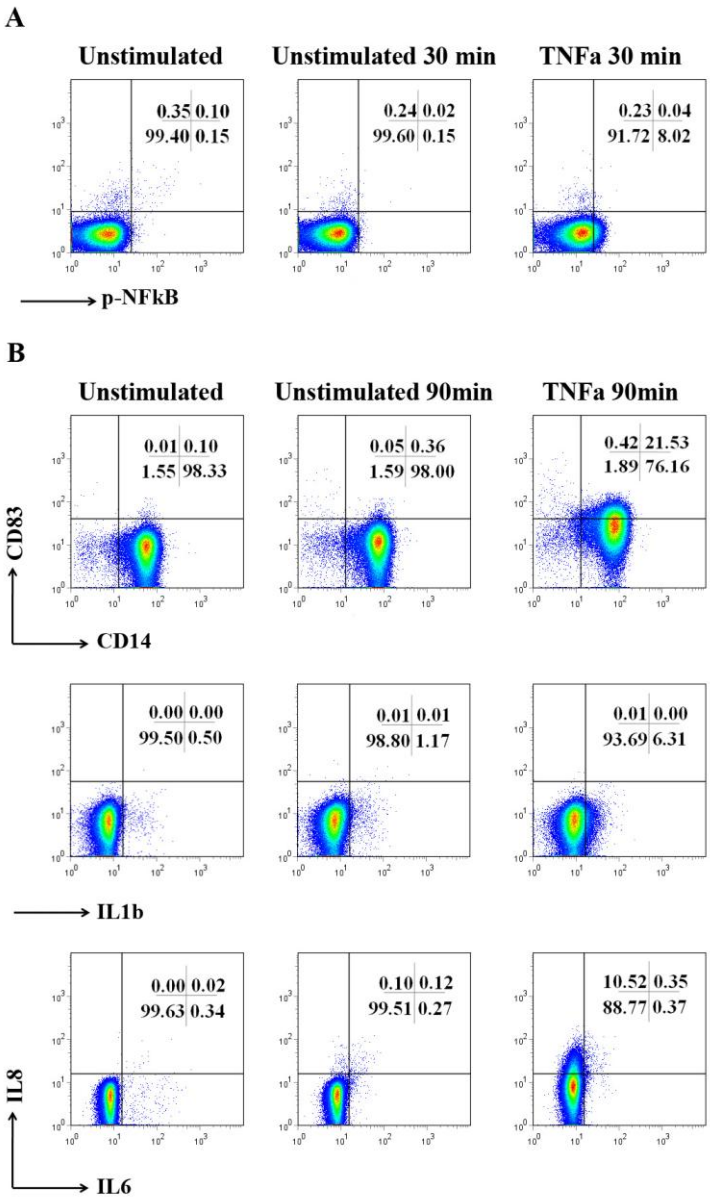

Figure 3.2 Following monocyte activation *in vitro* by TNFα. Figure 3.2.A Phosphorylation of the transcription factor NFκB was measured 30 min after stimulation. **Figure 3.2.B** The expression of the surface marker CD83 and the production of cytokines IL1β, IL6 and IL8 were measured 1.5 h after stimulation.

3.4.2. Markers used for following the activation of monocytes *in vitro* by IFNα2a and IFNγ

CD69 as a surface marker, CXCL10 and CXCL9 as cytokines, and phosphorylation of transcription factor STAT1 (pSTAT1) were used for monitoring the activation of monocytes after IFNα2a and IFNγ stimulation. Phosphorylation of STAT1 was utilized for following the early responses to IFNα2a and IFNγ, 30 min after cell stimulation (Figure 3.3.A). Increased

expression of CD69, CXCL9 and CXCL10 confirmed activation of monocytes 1.5 h after stimulation (Figure 3.3.B). Isolation of the cells and measurement of their activation was performed in the same way as described for TNFα activation.

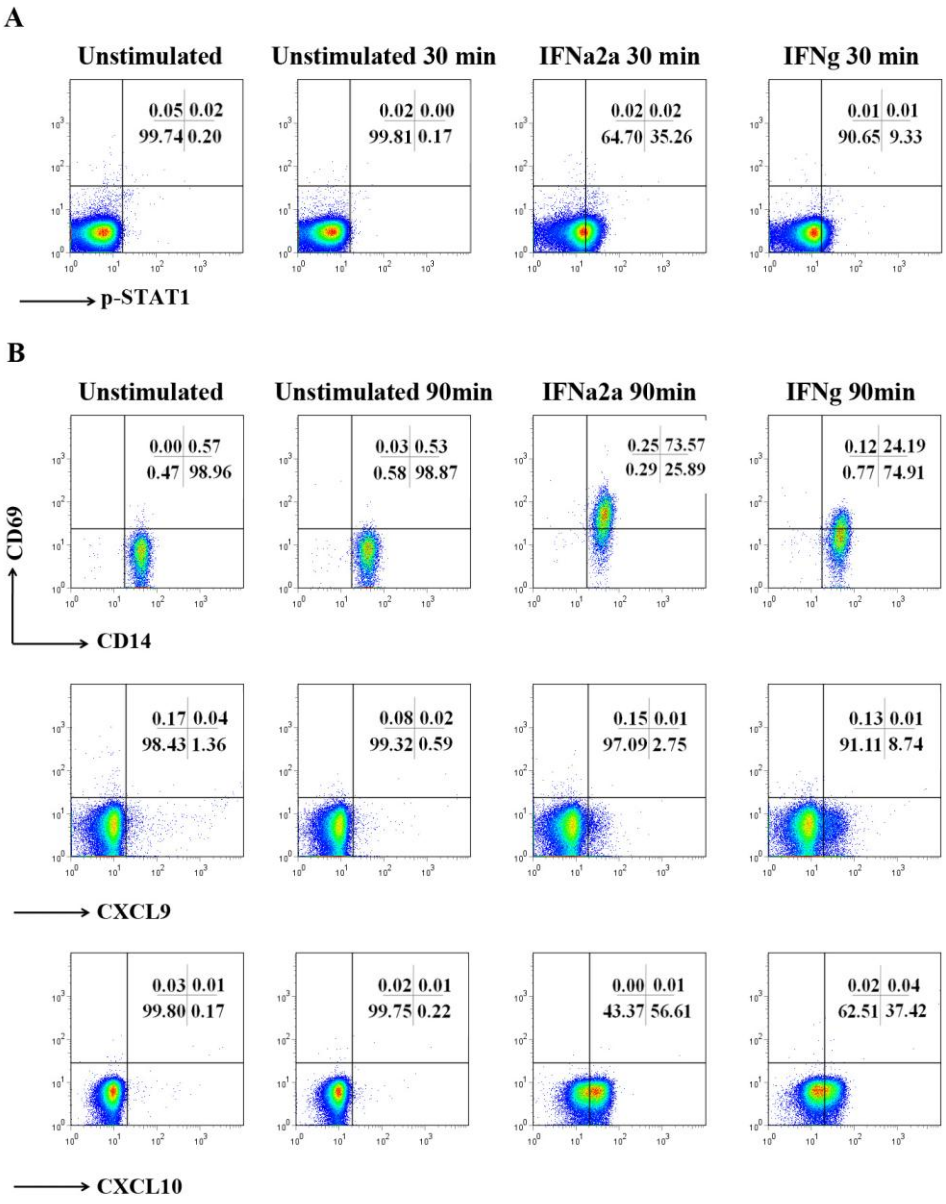

Figure 3.3 Following monocyte activation *in vitro* by IFNα2a and IFNγ. Figure 3.3.A Phosphorylation of the transcription factor STAT1 was measured 30 min after stimulation. **Figure 3.3.B** The expression of the surface marker CD69 and the production of cytokines CXCL9 and CXCL10 were measured 1.5 h after stimulation.

3.4.3. Procedure for staining of surface and intracellular markers

Surface staining was performed with anti-CD14-FITC, anti-CD69-PE and anti-CD83-APC antibodies. After monocytes isolation, cells were stained for 15 min with a combination of

anti-CD14 and anti-CD83 antibodies for following stimulation with TNFα, and with a combination of anti-CD14 and anti-CD69 antibodies in order to confirm activation by IFNα2a and IFNγ.

For intracellular staining, monocytes were stimulated in the presence of 5 μg/ml Brefeldin A. Cells were fixed with 2% paraformaldehyde, permeabilized with 0.5% saponine and labelled with one of these antibodies: anti-IL1β-FITC, anti-IL6-FITC, anti-IL8-PE, anti-CXCL9-FITC or anti-CXCL10-PE.

For nuclear staining, phosphorylation of NFκB and STAT1 was detected 30 min after stimulation. Fixed cells were permeabilized with 70% methanol and stained with human anti-pNFκB-p65-Alexa-Fluor-647 or anti-pSTAT1-Alexa-Fluor-647 antibodies.

For all types of staining, surface, intracellular and nuclear, two types of controls were included. The first type of controls included unstimulated monocytes that were immediately processed after blood was drawn and the second type of controls included unstimulated monocytes that were incubated at 37 °C either for 30 min or 1.5 h. All measurements were performed on FACSCalibur.

All antibodies were titrated and used in dilution of 1:10 of initial manufacturer concentration. The exception was anti-TNFα antibody used in dilution of 1:100.

3.4.4. Validation of candidate genes at the protein level by flow cytometry

Blood samples from 6 SLE patients and 14 ND were used for monitoring the expression of up to 50 surface markers at the protein level. These markers are related to all types of leukocytes in the blood and therefore, there are two groups of markers, one that is linage-specific and the other one that is related to activation of leukocytes. The following markers were used as linage-specific: CD3, CD4, CD8, CD14 and CD19. Out of 50 markers, 13 were considered as relevant for monitoring monocytes activation and they were analyzed in more details. The analysis of markers related to an activation of T-cell, B-cells, NK-cells, granulocytes, DCs was not included in this study.

The analysis of monocytes markers included expression of: CD32, CD64, CD244, CD163, CD128b, CD119, CD35, CD120b, CD107, CD88, CD46, CD55 and CD21. Five of them were found to be differentially expressed in monocytes from SLEs patients when compared to healthy donors. The following 5 surface markers were presented in this study: CD32, CD64, CD120b, CD128b and CD244.

A slightly different approach was applied for the staining of surface markers in the framework of an immune monitoring study. Here, the cells were fixed with 1% paraformaldehyde for 5

min prior to staining. In this part of the study monocytes were labeled with anti-CD14-PECy7. The staining and the data acquisition were described elsewhere[160]. Briefly, the expression of the surface markers mentioned above was measured on LSR II analyzer (BD). As a quality control, the measurements of Rainbow Calibration Particles were included before and after acquisition of data from each experiment.

3.5. Sample preparation for microarray analysis and processing GeneChip arrays

After sorting, purified monocytes were lysed in RLT buffer and stored at –80 °C. Monocytes lysate were utilized for isolation of RNA as processing GeneChips arrays as described below.

3.5.1. RNA isolation, cRNA processing, Affymetrix gene chip hybridization and quality checks

Total RNA was extracted by the RNeasy Mini kit (Qiagen). A potential contamination with genomic DNA was excluded by an on-column digestion step with RNase-Free DNase Set (Qiagen). The total amount of isolated RNA was measured by NanoDrop ND-1000 spectrophotometer, and the integrity of RNA was assessed by using Agilent 2100 Bioanalyser. A total RNA (3-5 μg) was utilized for the synthesis of complementary ds-DNA (cDNA), as recommended in the technical manual GeneChip Expression Analysis (Affymetrix, Santa Clara, CA). The *in vitro* transcription necessary for the synthesis of biotinylated complementary RNA (cRNA) was performed using the Enzo RNA Transcript Labelling kit (Affymetrix). The quality of cRNA was checked by hybridizing aliquots of the cRNA on a GeneChip Test3 array (Affymetrix). In total, 15 μg of fragmented cRNA was hybridized to the Affymetrix chips. Hybridization was performed overnight at 60 °C in a Hybridization Oven 640 (Affymetrix). Chips were washed and stained in the Fluidics Station 400 (Affymetrix) according to the procedure 2 described in the technical manual. Finally, the arrays were scanned with an Affymetrix GeneChip Scanner 3000.

For the generation of SLE, RA and AS expression profiles Affymetrix HG-U133A gene arrays were used that contain 22 283 probe-sets. *In vitro* generated TNFα, IFNα2a and IFNγ signatures were developed by using Affymetrix HG-U133 Plus 2.0 arrays with 54 675 probe-sets.

3.5.2. Chip data analysis

For chip data analysis we used all relevant data of the Affymetrix GCOS/MAS5.0 software, imported these into the BioRetis database, performed group comparisons and filtered the

differentially expressed probe-sets as described elsewhere[140]. In detail, each chip was globally normalized and scaled to an average intensity of 150 (target value TGT=150) to adjust for global differences in hybridization. All data of the GCOS software (signals, detection calls, signal log ratios and change calls) were included in group comparisons and statistical analysis that was utilized for filtering differentially expressed probe-sets.

3.5.3. Selection of differentially expressed genes

Gene expression profiles from patients were more heterogeneous compared to those obtained by *in vitro* experiments. Therefore, the less stringent criteria were applied for selection of differentially expressed probe-sets in diseases compared to those from *in vitro* experiments. This is described in detail in the following two chapters.

3.5.3.1. Selection of differentially expressed genes in monocytes of SLE, RA and AS patients

To obtain maximal statistical power, we performed pair-wise comparisons[140]. More precisely, each of 9 SLE, each of 8 RA and each of 14 AS patients was compared to each of 12 healthy donors, resulting in 108, 96 and 168 comparisons, respectively. The differentially expressed probe-sets (Affymetrix-IDs) in these comparisons were obtained by applying the modified default parameters from BioRetis data base[140]. Aiming to estimate heterogeneity between patients, the parameter that describes % of changes in pair-wise comparison was decreased from a default value of >50% to >30%. More precisely, this parameter showed that a particular gene must be up-regulated or down-regulated in more than 30% of calculated comparisons.

3.5.3.2. Selection of differentially expressed genes from *in vitro* stimulated monocytes with TNFα, IFNα2a and IFNγ

For *in vitro* stimulated samples, each of 3 TNFα samples, each of 7 IFNα2a and each of 7 IFNγ samples was compared to each of 11 unstimulated samples incubated for 1.5 h, which resulted in 33, 77 and 77 comparisons for each of the cytokines mentioned above, respectively. Eight samples of unstimulated controls were immediately processed (unstimulated 0 h), and they were not included in comparisons. Nevertheless, they were included in all heat-maps from *in vitro* profiles in order to provide an insight into genes that were unspecifically affected by the *in vitro* procedure[161].

As previously mentioned, the more stringent criteria were used for selection of differentially expressed probe-sets from *in vitro* stimulated monocytes when compared to those from patients. Since *in vitro* stimulation portrayed a more homogenous response, the default parameter that marks % of changes in pair-wise comparisons was increased from >50% to >60%.

3.5.3.3. Selection of cytokine-specific, cytokine-predominant and cytokine-shared probe-sets

The analysis of TNFα, IFNα2a and IFNγ gene expression profiles identified probe-sets with expression specific for either one cytokine or shared between two or three cytokines. The probe-sets that were differentially expressed by two or all three cytokines were considered to be cytokine-shared. The probe-sets that were altered by just one of these cytokines were determined to be cytokine-specific. Although it was possible to identify specific probe-sets for IFNα2a and IFNγ, as shown in Figure 4.16, principally these two types of IFNs induced similar gene-expression profiles.

Among the cytokine-shared probe-sets, it was obvious that one of the cytokines can alter the expression of particular probe-sets more strongly in comparison to the others. Therefore, the additional comparisons were included for selection of probe-sets that were predominantly regulated by TNFα or IFNs. More precisely, the cytokine-shared probe-sets were included in the pair-wise comparisons that directly compared 1) TNFα to IFNα2a, 2) TNFα to IFNγ and 3) IFNα2 to IFNγ. For these additional comparisons the following cut-offs for fold changes (FC) were applied a cut-off FC>1.5 for up-regulated probe-sets, and a cut-off FC<-1.5 for down-regulated probe-sets. Supplementary Table 3 provides all probe-sets regulated by cytokines and includes additional columns that denote the cytokine-specific, cytokine-predominant and cytokine-shared probe-sets.

3.5.3.4. Selection of probe-sets regulated by cytokines in opposite directions

Besides cytokine-specific, cytokine-predominant and cytokine-shared probe-sets, the results from this study revealed also those probe-sets that were differentially expressed in response to two or all three cytokines but whose expression were changed in the opposite directions. For example, out of 570 probe-sets that were changed both by TNFα and IFNα2a, 263 were up-regulated by TNFα but down-regulated by IFNα2a. A total number of 307 probe-sets were down-regulated by TNFα and up-regulated by IFNα2a. The probe-sets that changed in

opposite directions were included in additional columns and coloured in grey, as presented in Supplementary table 3.

3.6. Software tools used for analyses of gene expression data

For hierarchical cluster (HC) analyses, the program Genesis was used[162]. After log2 transformation and normalisation of expression values, genes were clustered using an average linkage clustering as an agglomeration rule, and Euclidian distance or Pearson correlation as a measure for distance. More precisely, Euclidian distance was used for hierarchical clustering of SLE, RA gene-expression profiles and *in vitro* generated signatures, and Pearson correlation was applied for clustering of AS profile.

For generation of terrain maps the program MultiExperiment Viewer 4.4 was applied[163]. The number of neighbours was 40 and the measure of distance was Pearson squared correlation coefficient. Transformation of signals was performed prior to the analyses and it included log2 transformation and normalisation of signal values.

Principal component analysis (PCA) was performed by using the program Genesis, where log2 transformed and normalised signal values were applied prior to the analyses.

3.6.1. A detail insight into HC, PCA and terrain map analysis

HC, PCA and terrain map analyses are different approaches for visualisation of transcriptome data. They utilize different algorithms, and a detail insight into these types of analyses is described in more detail below.

3.6.2. Hierarchical clustering and creation of heat-map

The most conventional way to present gene expression profiles is to apply HC and to visualize data in the form of heat-maps (HMs). Typically, thousands of genes can be identified as differentially expressed, and the aim of clustering is to identify the groups of genes that behave similarly and exhibit similar expression patterns. Therefore, the inherent differences within data might be displayed as different clusters.

An input data for HC are the signal intensity values. The signal values are related to the amount of fluorescently labelled cRNAs and represent the expression levels of all genes that are determined to be differentially expressed. Since, the expression levels of all genes are not equally distributed, and show a 10 000 fold dynamic range, it is recommended to utilise one of the tools for data transformation. The most common way is to apply a log2 (*logarithmus dualis*) transformation. Furthermore, to compensate technical differences caused by

processing arrays it is recommended to apply normalisation of data. In this study log2 transformation and normalisation were performed always prior to HC.

Results of HC analyses are displayed as tree-like dendrograms, one placed on the top and another one on the left side of the HM (Figure 3.4). They illustrate the arrangements of clusters generated by clustering algorithm, where each column represents a different experiment and each row a different gene. The length of the branches is related to the similarity between experiments or genes. If the length is shorter, the experiments or genes are nested closer to each other and show greater similarity.

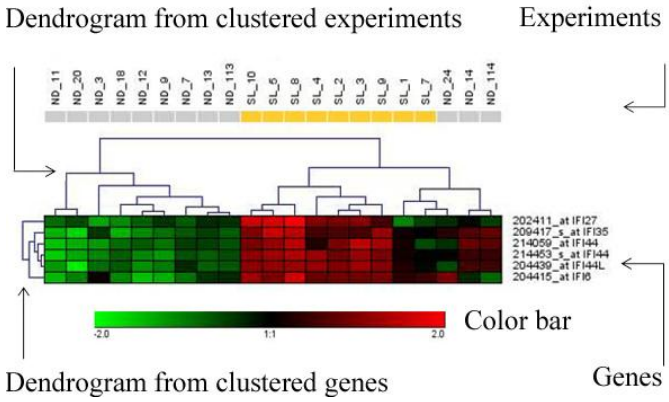

Figure 3.4 Components of heat-map generated by hierarchical clustering.

In this study, two-dimensional clustering was utilized by virtue of the fact that both experiments and genes were clustered. Nevertheless, all HMs presented in this study displayed only dendrograms related to clustering of experiments. Actually, HMs displayed results from dozens of experiments and thousands of genes. Since the aim of this study was to recognise profiles from different experiments, dendrograms related to clustering of the genes were generated by the analyses, but excluded from the visualisation of data. The size of the dendrogram related to clustering of genes is huge and often several times larger that HMs, and for practical reasons they were not included in HMs.

To quantify similarity of profiles, clustering algorithms utilize different measures of distance like the Euclidian distance and Pearson correlation coefficient. Euclidian distance measures the shortest "ordinary" distance between two points. It represents a distance that one can measure with the ruler and it is given by Pythagorean formula. Pearson correlation coefficient measures association in expression between genes. If two genes have different levels of expression (different amounts of mRNA and therefore different signal intensities) but display a "parallel" expression patterns, they are considered to be correlated. As mentioned above, the

Euclidian distance was used for analyses of SLE and RA profiles, as well as for analyses of *in vitro* generated cytokine-specific signatures. The exception was the analysis of AS profile, where three different batches of Affymetrix arrays were applied and where normalisation of data was not sufficient to equalise the systemic technical differences. Therefore, the Pearson correlation coefficient was considered a more suitable measure of distance.

The following step in generation of HMs considers how to link and merge clusters. Average linkage clustering is the most commonly used approach, and it assumes that the distance between two clusters is equal to the average distance from any member of one cluster to any member of another cluster (Figure 3.5).

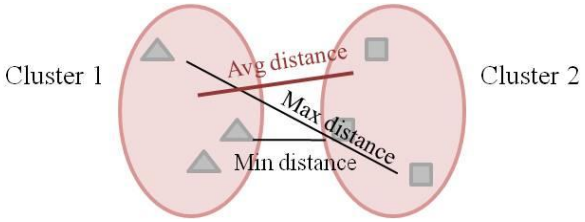

Figure 3.5 Linkages between clusters: minimal distance, maximal distance and average distance.

All HMs included in this study display the relative changes in gene expression, where genes whose expression increased or decreased were coloured in red and green, respectively. Therefore, a colour bar that accompanies HM presents an arbitrary scale, where saturated red represents the maximal positive value and saturated green the maximal negative value. In this study all colour bars are in the range from -2 to +2 values. For practical reasons they were not included below HMs presented in result part, but a prototypical colour bar was demonstrated in Figure 3.4.

3.6.3. Terrain map analysis

Besides the conventional and most common way to present transcriptome data in two-dimensional space by HM, the transcriptome data can be disclosed by terrain map analysis as well. As in the case of HC, this method detects the major clusters within data, but in addition it relates the number of genes that compose the clusters to the corresponding heights. Therefore, transcriptome data are displayed in the form of a landscape-like diagram, in which clusters are represented in the form of hills, and the height of the hills correlate with the absolute number of genes. Genes are shown as dots above the hills (Figure 3.6).

For generating terrain maps, Pearson squared correlation coefficient was considered as the most suitable measure of distance. Since the Pearson correlation coefficient favours clustering

of genes based on their correlation in expression, its squared form tends to cluster strongly correlated and strongly anti-correlated genes. This type of analysis is valuable for identifying the group of genes that show reciprocal expression. Many transcription factors act both as activator and repressor of gene expression, namely they are able at the same time to increase expression of one set of genes and to silence the expression of the other group of genes[164-165]. Since, terrain maps display the transcriptome profiles as groups of co-expressed genes, it is expected that the functionally related genes are grouped together and that they are "guilt by association".

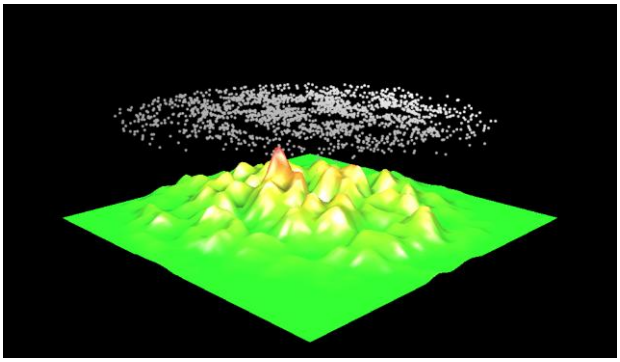

Figure 3.6. Landscape generated by terrain map analysis. Dots represent genes that constitute gene-expression profile.

3.6.4. Principal component analysis (PCA)

PCA is a statistical approach that identifies the key variables (principal components, PCs) within data sets, which are able to detect differences between observations (in our analysis experiments). These components reveal multiple dimensions where the variability of data is most pronounced. Therefore, it is possible to reduce and simplify data presentation from multi-dimensional to three-dimensional space, where the axes x, y and z represent the key variables PC1, PC2 and PC3, respectively.

By applying PCA in analysis of transcriptome data, the most distant and most related experiments are shown in 3D space. Actually, those experiments that are similar are going to group together and they are displayed as a compact cloud in 3D space. Generally, in this study, one experiment represents a gene-expression profile from one person, either patient or healthy donor. To demonstrate the principle of PCA for transcriptome analysis, the same signals applied for HC (6 probe-sets from 21 experiments: 9 from SLE patients and 12 from ND) were also used for PCA (Figure 3.7). The gene-expression profiles from SLE patients, represented by the yellow squares, are separated from the gene-expression profile of healthy donors, represented by the gray squares.

The potential of PCA is more obvious when profiles characterised by thousands of genes and from different diseases are analysed simultaneously.

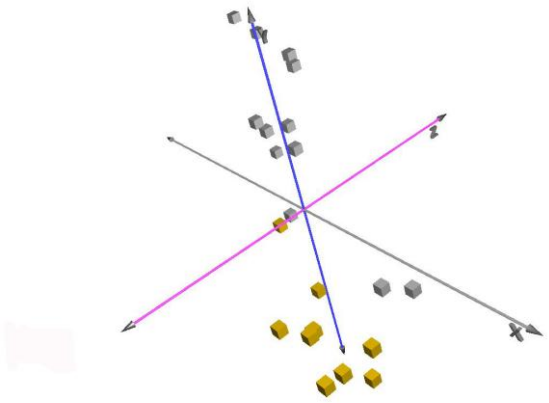

Figure 3.7 A typical 3D graph generated by PCA. Yellow squares represent SLE patients and grey squares healthy donors.

3.6.5. Promoter analysis

Whole genome rVISTA analysis was performed for the identification of transcription factor binding sites (TFBSs), which were conserved between human and mouse. The analysis included those TFBSs that were overrepresented in regions 1 Kb upstream of transcriptional start sites (TSS). This software offers the possibility to analyse the promoter regions of a wide range of genes at the same time. It is assumed that one TF regulates the expression of many genes simultaneously and therefore, TFBS for this TF might be detected in the promoter regions of co-regulated (co-expressed) genes. This type of analysis is valuable for identifying the TFs that are the major driving force in regulating a particular gene expression profile[166]. This approach was applied for the analyses of promoter regions of the profiles from SLE, RA and AS patients.

3.6.6. Ingenuity pathway analysis (IPA)

IPA software provides insight into the possible biological background that portrayed large-scale data, including those generated by gene expression profiling (www.ingenuity.com). IPA knowledge base integrates data from a wide variety of experiments, mainly those related to interactions between genes and/or proteins. Therefore, as the collector of literature-based data, IPA software is suitable for analysis of data where the background of identified changes is unknown. The most common way to disclose results generated by IPA is to present them in the form of known molecular networks or canonical signalling pathways. The input data are

mapped to global molecular networks and canonical pathways integrated by IPA. Therefore, based on connectivity between molecules that are already known from the literature, IPA software aims to identify such functionally related interactions within the data used for the analyses.

The input data in this study were differentially expressed genes with their appropriate fold-changes. Each network can be arbitrarily set to a particular number of molecules and in this study all presented networks were determined with 35 molecules, as shown in Figure 3.8.

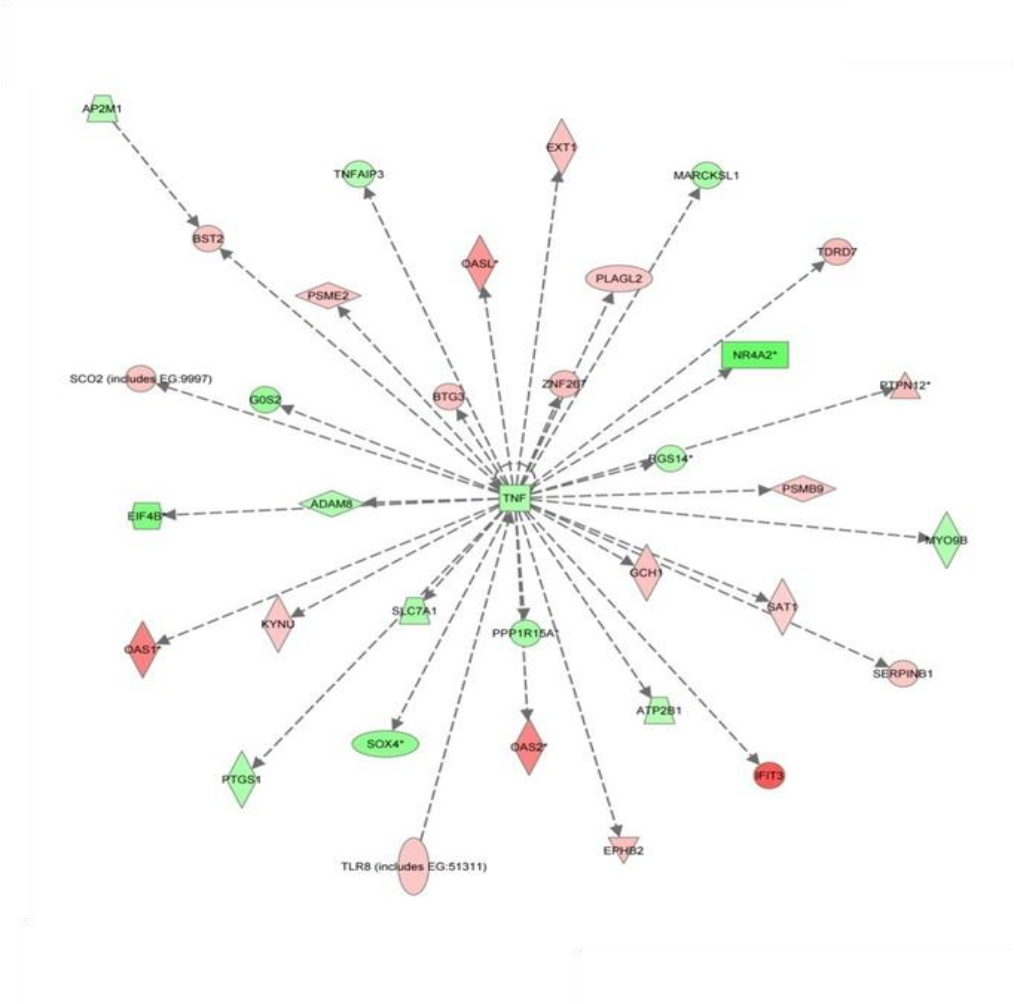

Figure 3.8 Molecular network that contains 35 molecules generated by IPA.

Contrary to networks where the number of connected molecules might be changed, each canonical pathway is predetermined by the particular number of molecules. Identification of canonical pathways is characterized by p-values that are calculated by the right-tail Fischer's exact test, and included Benjamini-Hochberg correction. In this study the canonical pathways that were characterized with $p < 0.05$ were defined as significant.

IPA utilizes the same colouring system to visualize gene expression data as HM. Thus, up-regulated and down-regulated genes are coloured in red and green, respectively. Since the input data are differentially expressed genes with their corresponding fold-changes, the intensity of the node colour indicates the degree of their up- or down-regulation. Each molecule within a particular network and canonical pathway is represented by a node. Different shapes of nodes are related to different functions ascribed to this molecule (Figure 3.9).

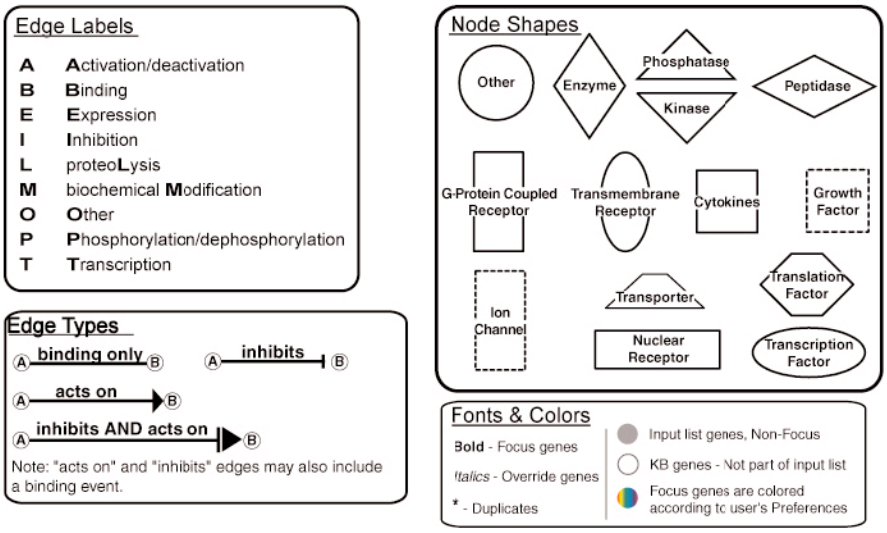

Figure 3.9 The shape of nodes is related to the functions of the molecule.

4. Results

4. Results:

The results chapter comprises four sections. The first section is focused on the development and analysis of gene expression data obtained from purified monocytes of SLE, RA and AS patients. The second section is focused on the development of cytokine-specific signatures in monocytes following their *in vitro* stimulation. The third section considers comparisons of transcriptome profiles from diseases with *in vitro* generated signatures. The forth section presents few markers that were selected from transcriptome data and validated at the protein level in patients with SLE.

4.1. Peripheral blood monocytes from SLE, RA and AS patients displayed disease-specific gene-expression profiles

4.1.1. SLE disease-specific profile in monocytes

Monocytes isolated from peripheral blood of SLE patients were characterized by 1847 probe-sets (about 1220 genes) that were differentially expressed in at least 30% of pair-wise comparisons. Comparisons included 9 SLE patients and 12 normal donors (Figure 4.1 and Supplementary Table 1, WS1). Out of the 1847 probe sets, 920 were up- and 927 were down-regulated. Three SLE patients, referred to as SL-1, SL-2 and SL-7, showed weaker changes in gene expression when compared to other SLE patients. It is worth noting that these patients were characterized by less active disease course.

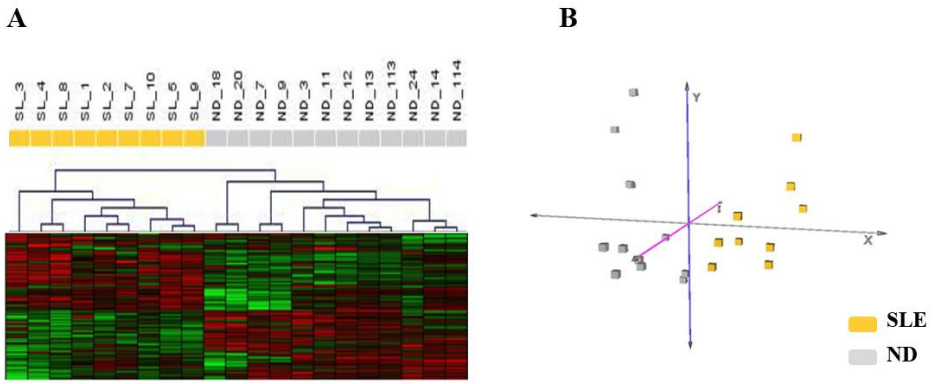

Figure 4.1 Gene expression profile in monocytes from SLE patients. Figure 4.1.A Hierarchical clustering of 1847 probe-sets that were differentially expressed between SLE (coloured in yellow) and ND (coloured in grey). Each row represents one probe-set and each column indicates one subject. **Figure 4.1.B** SLE profile is shown by applying PCA.

The following genes were found to be strongly up-regulated in SLE: IFI27, SIGLEC1, IFI44L, IFIT1 and CCL2. The transcription factors KLF13, JUN and FOSB were identified as down-regulated in SLE monocytes.

When presented in the form of a terrain map, the SLE profile was displayed by one dominant peak, which represents a large group of co-regulated genes (Figure 4.2).

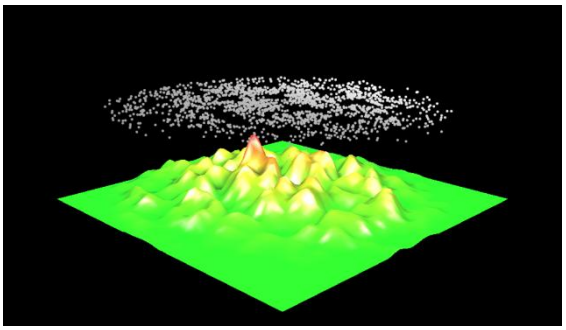

Figure 4.2 Terrain map shows the major clusters within the SLE gene-expression profile in monocytes, and 1847 probe-sets that constitute this profile are represented by grey dots.

4.1.2. RA disease-specific profile in monocytes

Transcriptional profiles of monocytes isolated from RA patients identified 1618 probe-sets (about 1070 genes) as differentially expressed compared to normal donors (Figure 4.3 and Supplementary Table 1, WS2). In at least 30% of pair-wise comparisons between 8 RA patients and 12 normal donors, 1000 probe-sets were up-regulated and 618 probe-sets were down-regulated. An RA specific transcriptional imprint was clearly detectable, although heterogeneity between patients was evident.

The transcriptome from RA monocytes revealed IL8, SOCS3 and the transcription factors MAFF, JUN and FOSB as strongly up-regulated genes. In addition, genes such as HLA-DQ, JARID1D, EIF1AY and FCER1A were found to be strongly down-regulated.

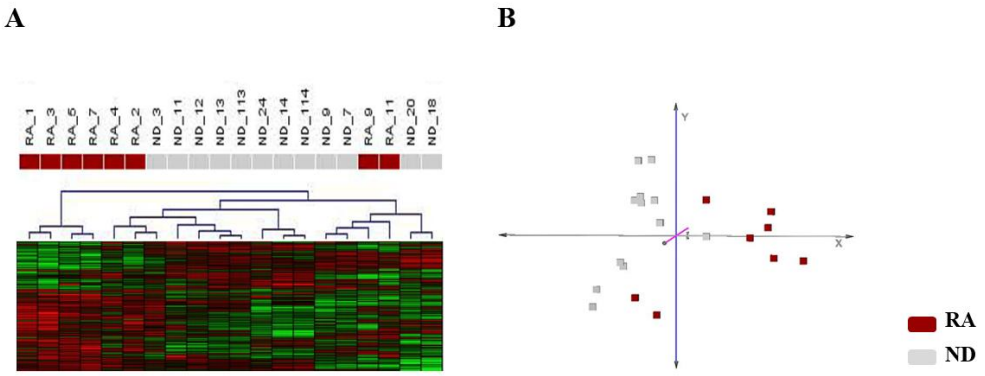

Figure 4.3 Gene expression profile in monocytes from RA patients. Figure 4.3.A Hierarchical clustering of 1618 probe-sets that were differentially expressed between RA (coloured in red) and ND (coloured in grey). Each row represents one probe-set and each column indicates one subject. **Figure 4.3.B** RA profile is shown by applying PCA.

Unlike to the SLE gene expression profile, the terrain map that shown the RA profile revealed a few dominant peaks of co-expressed genes (Figure 4.4).

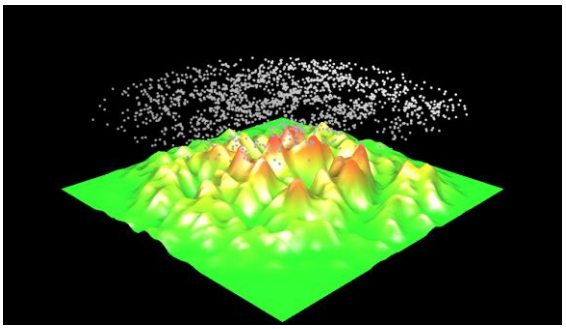

Figure 4.4 Terrain map identifies the major clusters within the RA gene-expression profile in monocytes, and 1618 probe-sets that constitute this profile are represented by grey dots.

4.1.3. AS disease-specific profile in monocytes

The gene expression profile identified in AS patients was generated in pair-wise comparison where monocytes from 14 AS patients were compared to monocytes from 12 normal donors. The AS disease-specific profile comprises 914 differentially expressed probe-sets (about 600 genes), where 602 probe-sets were up- and 312 probe-sets were down-regulated (Figure 4.5 and Supplementary Table 1, WS3). The criteria for selection of differentially expressed genes were the same as those applied for the identification of SLE and RA gene expression profiles. The heterogeneity between AS patients was more obvious as compared to those in SLE and RA. Additionally, the magnitude of changes in gene expression was weaker if compared to the magnitude of changes observed in SLE and RA, and most of the genes in AS exhibited a magnitude of changes in the range of -2 up to +2 fold.

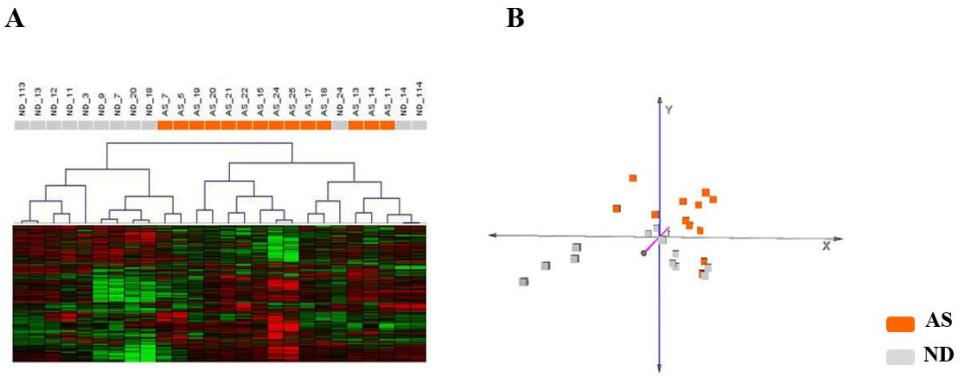

Figure 4.5 Gene expression profile in monocytes from AS patients. Figure 4.5.A Hierarchical clustering of 914 probe-sets that were differentially expressed between AS (coloured in orange) and ND (coloured in grey). Each row represents one probe-set and each column indicates one subject. **Figure 4.5.B** AS profile is displayed by applying PCA.

Genes such as CLU, THBS1, ADAM8, HLA-DRB4, IL1β and IL8 were found to be up-regulated. Genes such as HLA-DQ, HDAC9, CD1C, CD1D, ABCE1, ID2 and TCF7L2 were identified as down-regulated.

As in the case of the RA profile, the AS profile shown by terrain map was also characterized by several dominant peaks (Figure 4.6).

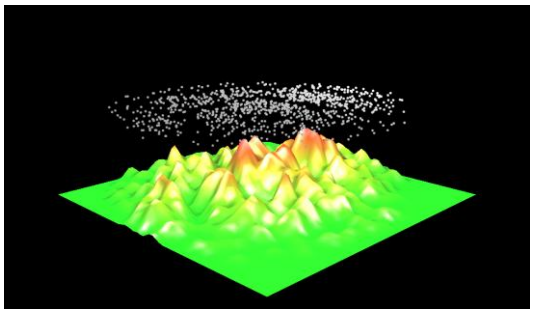

Figure 4.6 Terrain map identifies the major clusters within the AS gene-expression profile in monocytes, and 914 probe-sets that constitute this profile are represented by grey dots.

4.1.4. Transcriptomes of peripheral blood monocytes disclosed SLE, RA and AS as different rheumatic diseases

A principal component analysis (PCA) was performed as a comparative analysis for SLE, RA and AS gene-expression profiles. PCA included all probe-sets that constitute profiles of diseases. When transcriptome profiles from SLE and RA patients were presented together, PCA analysis revealed an obvious difference between them (Figure 4.7.A).

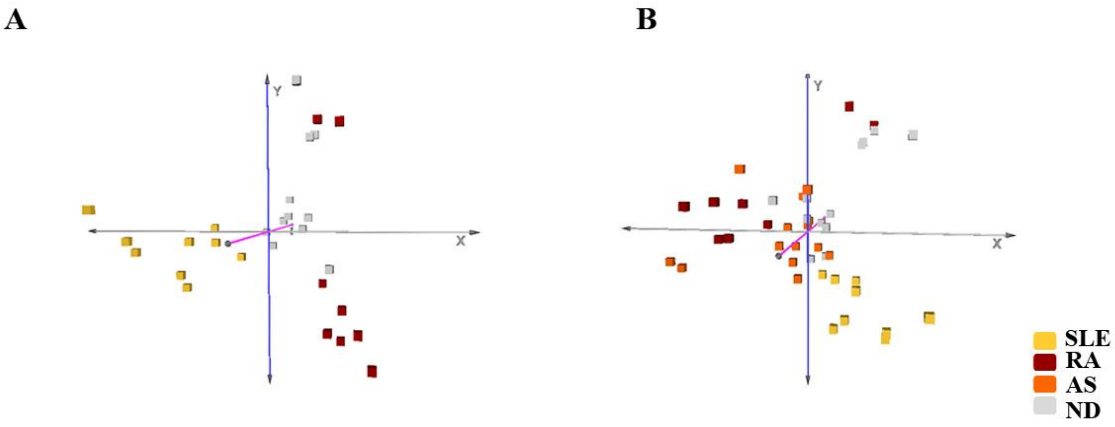

Figure 4.7 Comparative analysis of SLE, RA and AS profiles. Figure 4.7.A PCA represents differences between SLE and RA profiles. Signals from 2707 probe-sets were used for this analysis. This number of probe-sets included 1847 probe-sets of the SLE profile and 1618 probe-sets of the RA profile (758 probe-sets were changed in both diseases, either in the same or in the opposite direction). **Figure 4.7.B** PCA display profiles from all three diseases. Altogether signals from 2890 probe-sets were utilized for analysis. This number represents all probe-sets that were identified in all three diseases. SLE patients are coloured in yellow, RA patients in red, AS patients in orange and healthy donors (ND) in grey.

However, if the AS profile was represented together with SLE and RA, it was found to be closely related to that of healthy donors (Figure 4.7.B). An overlap between SLE, RA and AS profiles is presented in Figure 4.8. The majority of differentially expressed probe-sets were found to be disease specific.

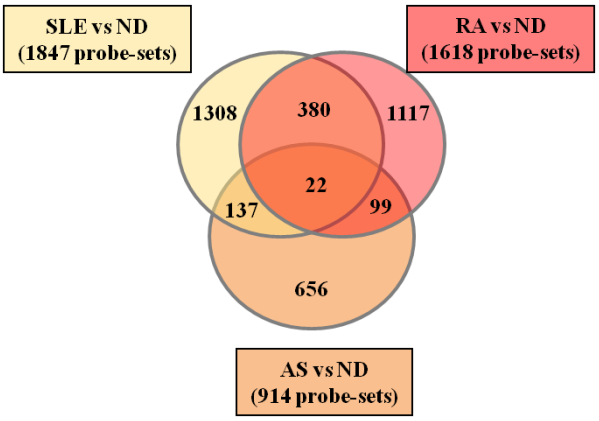

Figure 4.8 A Venn diagram represents 3719 probe-sets that compose SLE, RA and AS gene expression profiles. Values indicate the absolute numbers of the probe-sets whose expression was identified as specific for a particular disease or shared between two or all three diseases. The direction of changes in probe-set expression was considered, and just those probe-sets whose expression altered in same direction comprise the shared profile.

4.1.5. Ingenuity pathway analysis (IPA) revealed cytokines as key payers within the networks and signalling pathways that characterised SLE, RA and AS profiles

4.1.5.1. Molecular networks in SLE, RA and AS identified by IPA

IPA was applied to visualise alterations detected within SLE, RA and AS profiles. IPA assigned SLE, RA and AS profiles to discrete molecular networks. Each molecular network displayed 35 molecules and cytokines, such as TNFα, IL1 and IL8, were identified as the main players in the networks that characterized all three diseases.

TNFα, IL1 and IL8 were differentially expressed in all of the above mentioned rheumatic disorders, but they were either regulated in opposite directions, or they were constituents of distinct molecular networks (Figures 4.9, 4.10, and 4.11). For example, TNFα was down-regulated in SLE and AS, but up-regulated in RA. The cytokine IL8 was down-regulated in SLE but up-regulated in RA and AS. IL1 was the only cytokine found to be up-regulated in all three diseases. However, the molecular networks in SLE, RA and AS that identified IL1 were not the same. Differences between the networks in SLE, RA and AS were emphasised if the direction of changes in gene expression were considered. For example, within TNF networks genes like OAS1, OAS2, IFIT3 and TLR8 were found to be up-regulated in SLE, but down-regulated in RA.

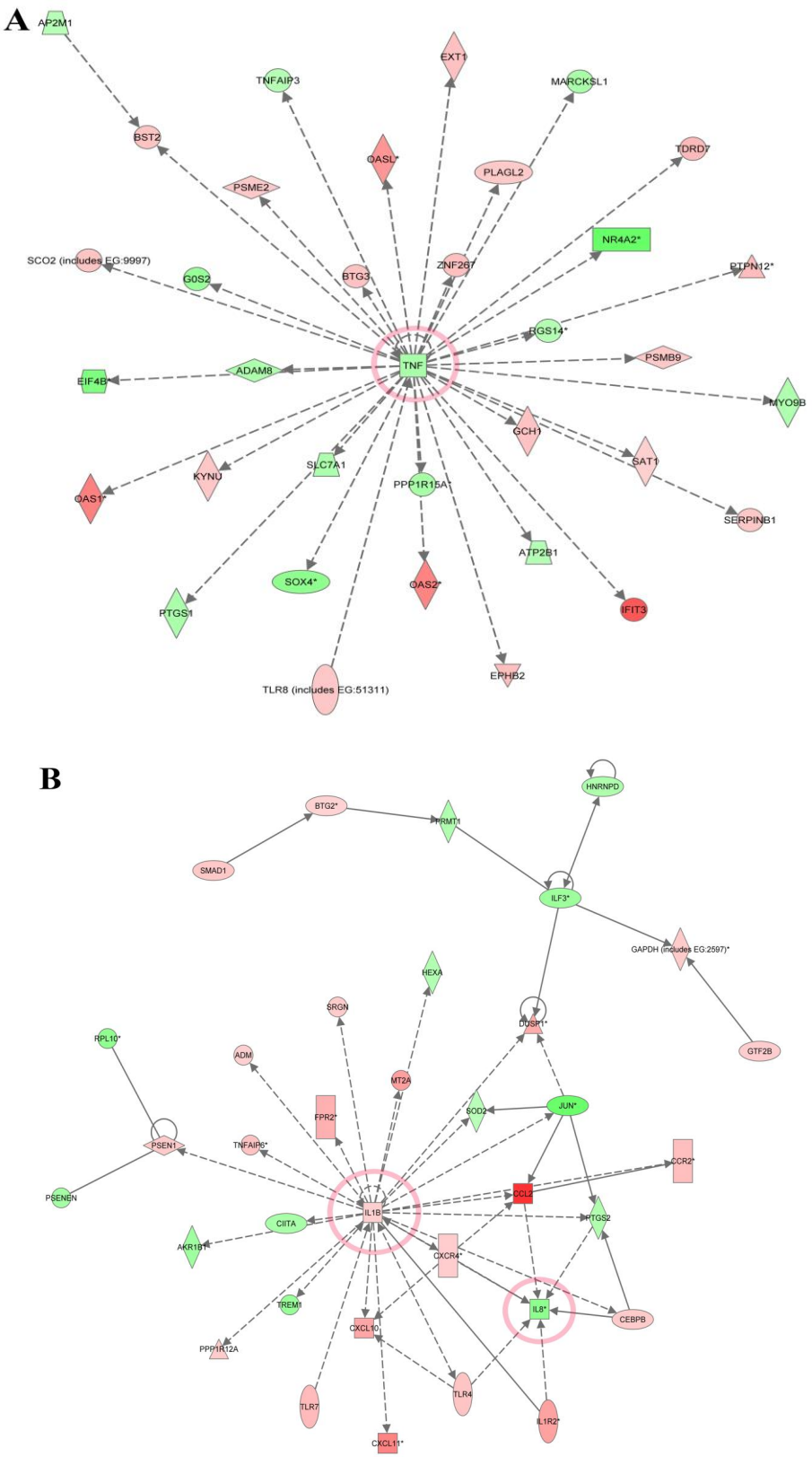

Figure 4.9 Analysis of SLE profile by IPA revealed influence of cytokines in shaping profile of disease. Molecular networks of differentially expressed genes in SLE with TNFα as central player (**Figure 4.9.A**), and IL1 and IL8 as key molecules (**Figure 4.9.B**). Up- and down-regulated genes are shaded in red and green, respectively. The enlarged figures are presented in Appendix (pages 129 and 130).

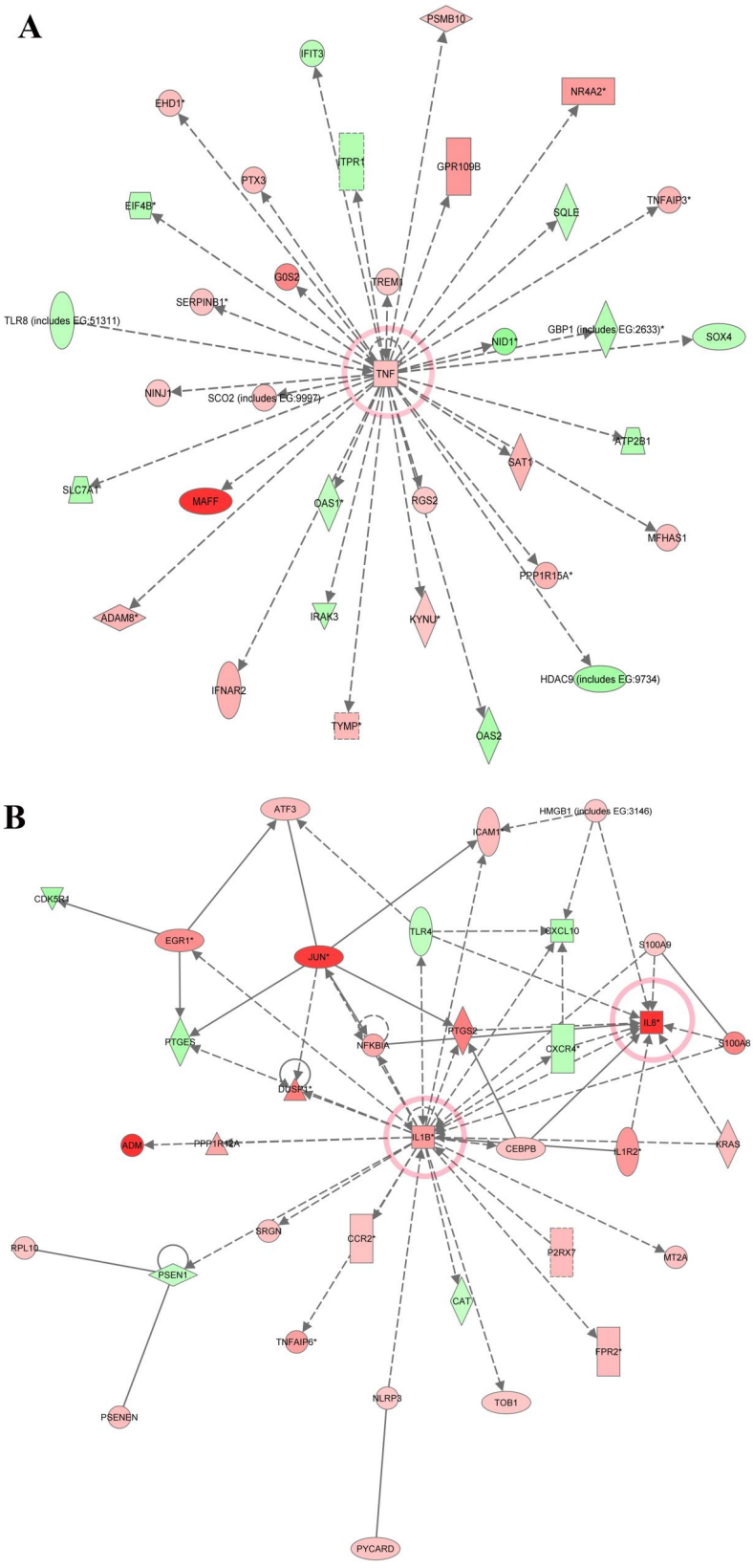

Figure 4.10 Analysis of RA profile by IPA revealed influence of cytokines in shaping profile of disease. Molecular networks of differentially expressed genes in RA with TNFα as central player (**Figure 4.10.A**), and IL1 and IL8 (**Figure 4.10.B**) as key molecules. Up- and down-regulated genes are shaded in red and green, respectively. The enlarged figures are presented in Appendix (pages 131 and 132).

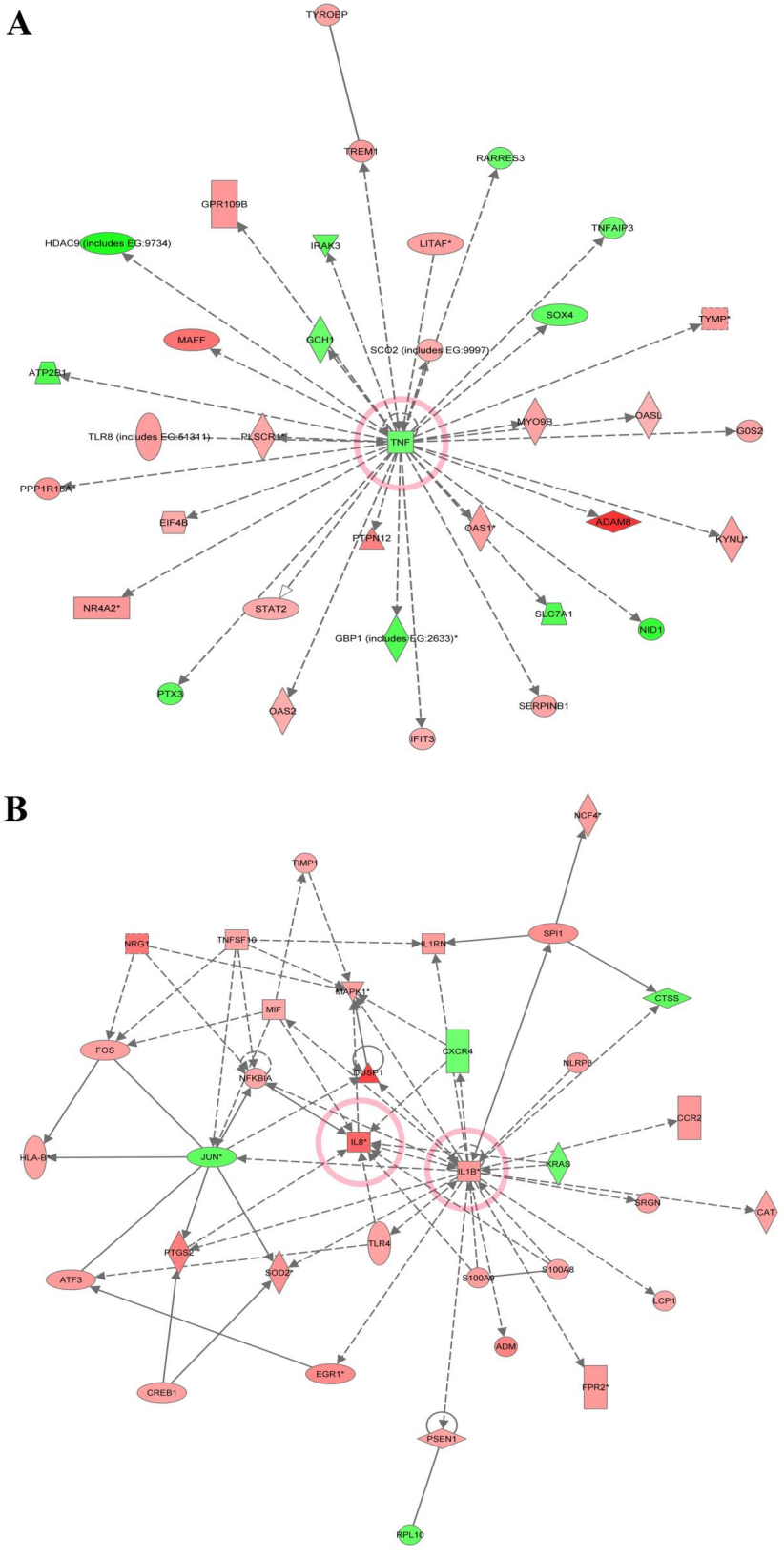

Figure 4.11 Analysis of AS profile by IPA revealed influence of cytokines in shaping profile of disease. Molecular networks of differentially expressed genes in AS with TNFα as central player (**Figure 4.11.A**), and IL1 and IL8 (**Figure 4.11.B**) as key molecules. Up- and down-regulated genes are shaded in red and green, respectively. The enlarged figures are presented in Appendix (pages 133 and 134).

4.1.5.2. Signalling pathways in SLE, RA and AS identified by IPA

All canonical pathways that were identified by IPA as significantly overrepresented in SLE, RA and AS were enlisted in Supplementary table 2, in WS1-3. The top 10 canonical pathways that were related to typical functions of monocytes are included in Table 4.1.

Table 4.1 Selected canonical pathways identified in SLE, RA and AS monocytes

Name of the pathway	SLE profile -log(p-value)*	RA profile -log(p-value)*	AS profile -log(p-value)*
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	6.93	5.35	6.05
Antigen Presentation Pathway	5.40	2.24	3.69
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	4.29	6.24	4.93
Toll-like Receptor Signalling	4.94	2.59	4.64
Complement System	2.18	2.46	2.13
Interferon Signalling	6.16	4.88	4.09
TNFR1 Signalling	4.44	2.68	6.34
TNFR2 Signalling	2.99	2.14	6.26
IL-10 Signalling	3.29	5.71	7.52
IL-6 Signalling	2.68	4.25	5.73

* p-values are calculated by Fischer's exact test and included Benjamini-Hochberg correction

The signalling pathways displayed molecules that were identified in diseases as being differentially expressed. It was obvious that the same signalling pathways can be involved in all three rheumatic diseases, but the molecules that constitute them might be changed in opposite directions. For example, the IFN signalling pathways were obviously activated in SLE. It included an up-regulation of STAT1 and the typical IFN regulated genes, like IFIT1, IFIT3, IFITM1, IFI35, IRF9, MX1 and OAS1. The IFN signalling pathways in RA and AS were also altered. Nevertheless, STAT1 was revealed as being down-regulated, and therefore, an obvious activation of IFN signalling pathways in RA and AS was not detected. An alteration of TNFR1 signalling pathway was detectable in all three diseases. TNFα and NFκB, as key components of this signalling pathway, were down-regulated in SLE but up-regulated in RA (Figures 4.12 and 4.13). Although STAT1 and NFκB were similarly regulated in RA and AS monocytes, the overall activations of IFN and TNFR1 signalling pathways were dissimilar in these two diseases (Figure 4.14).

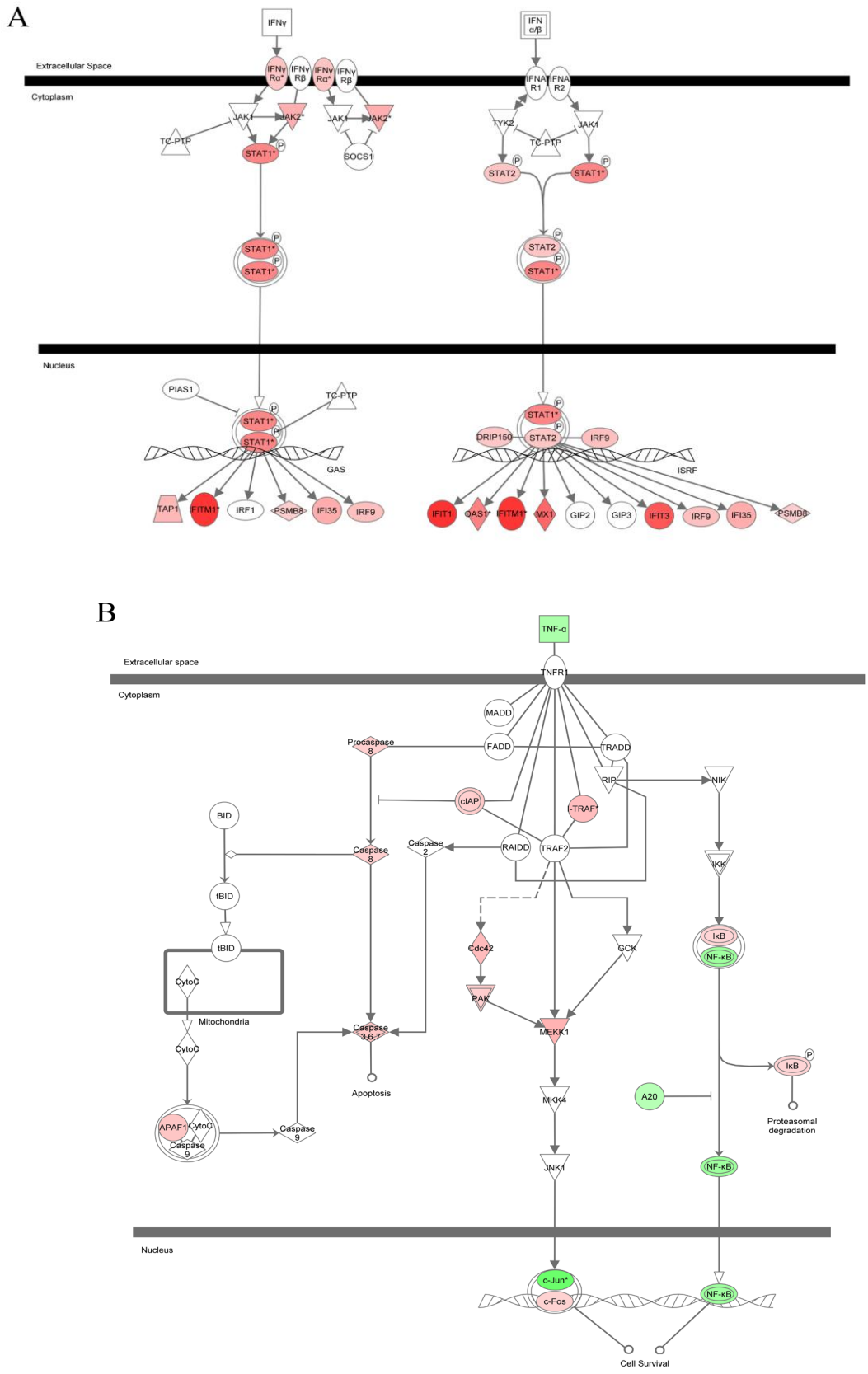

Figure 4.12 Alterations within IFNs and TNFα signalling pathways in SLE monocytes are presented in Figures 4.12.A and 4.12.B, respectively. Up- and down-regulated genes are shaded in red and green, respectively. The enlarged figures are presented in Appendix (pages 135 and 136).

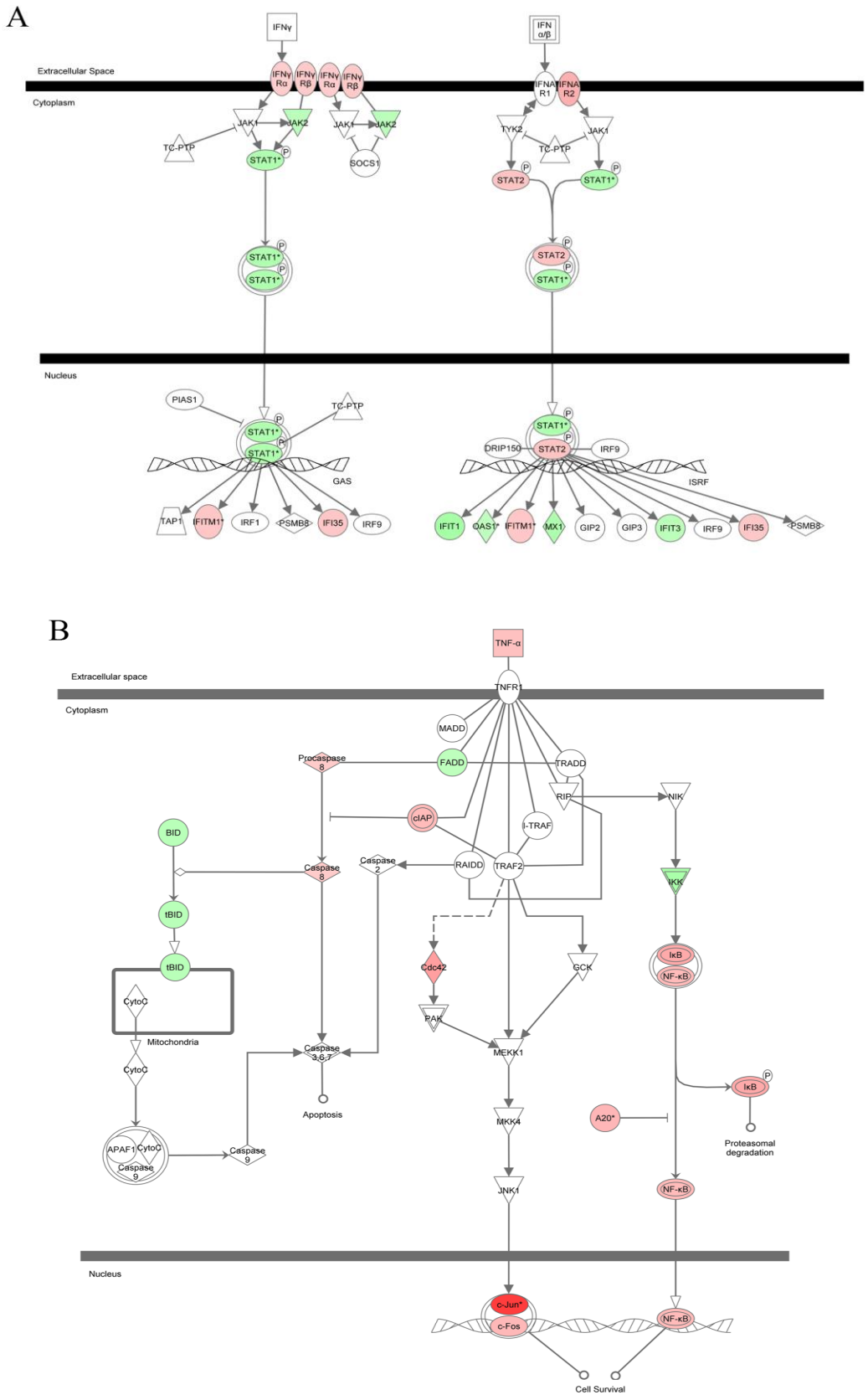

Figure 4.13 Alterations within IFNs and TNFα signalling pathways in RA monocytes are presented in Figures 4.13.A and 4.13.B, respectively. Up- and down-regulated genes are shaded in red and green, respectively. The enlarged figures are presented in Appendix (pages 137 and 138).

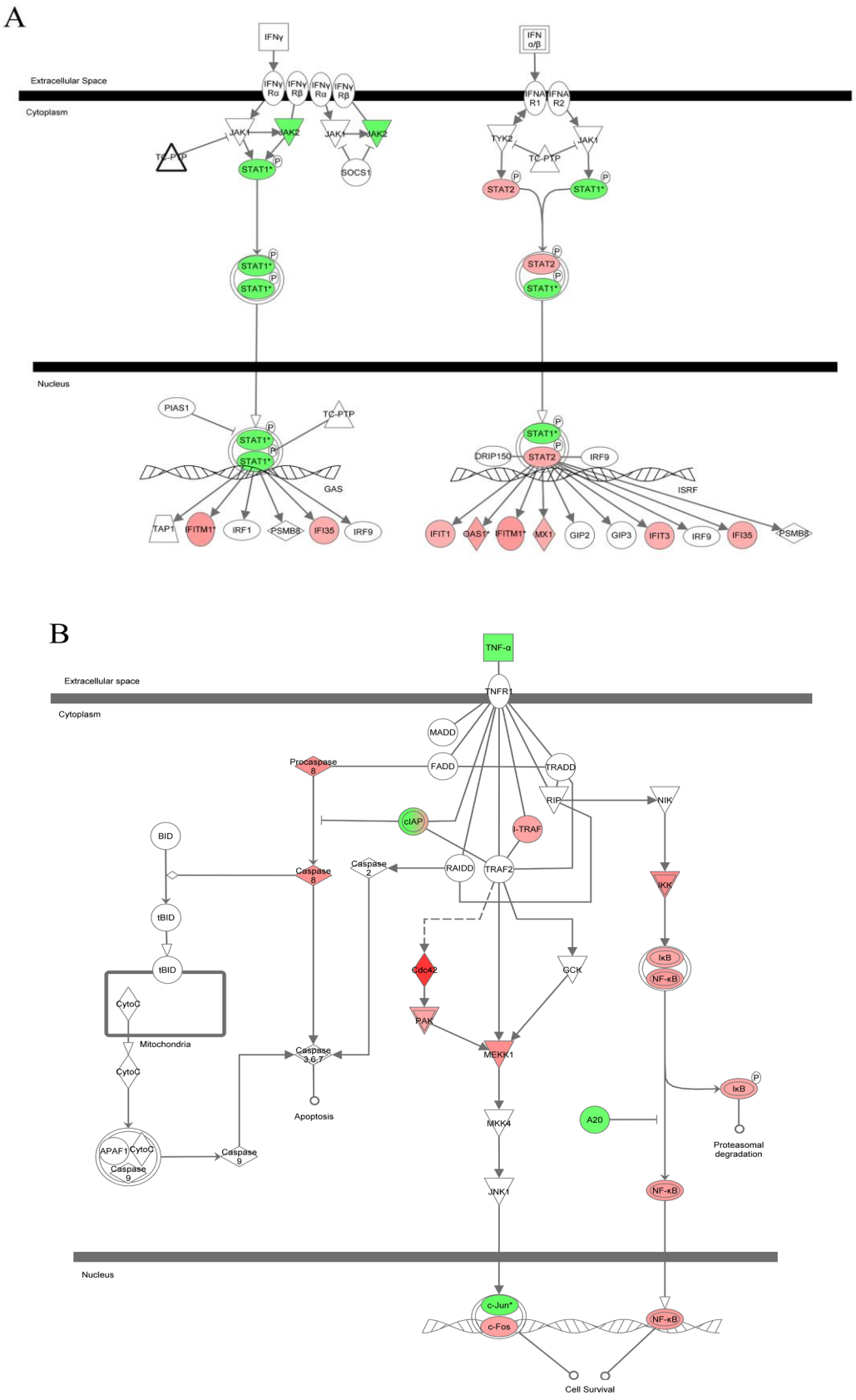

Figure 4.14 Alterations within IFNs and TNFα signalling pathways in AS monocytes are presented in Figures 4.14.A and 4.14.B, respectively. Up- and down-regulated genes are shaded in red and green, respectively. The enlarged figures are presented in Appendix (pages 139 and 140).

4.1.6. Promoter analysis revealed that distinctive transcriptional programs drive gene-expression in SLE, RA and AS monocytes

All genes that constitute the profiles of diseases were included in the promoter analysis by whole-genome rVISTA. More precisely, three groups of genes determined by 1847 probe-sets in SLE, 1618 probe-sets in RA, and 914 probe-sets in AS were analysed. The promoter analysis included identification of transcription factor binding sites (TFBSs) in regions 1 Kb upstream of the transcriptional start sites. Only the conserved regions between human and mice were included in the promoter analyses. The detection of TFBS is accompanied by two parameters, the total number of hits on the genome, which is predetermined by the software, and with the number of hits for submitted regions, which is calculated for the group of genes that is analysed. The TFBSs that were characterized by p-value <0.005 were determined as being overrepresented.

In total, 42 TFBSs in SLE, 59 TFBSs in RA and 47 TFBSs in AS were identified (Table 4.2). A common TFBSs were identified in all three diseases. Nevertheless, disease-specific TFBSs were also evident. For example, TFBSs for EGR1, EGR2 and ISRE were specific for the SLE profile, while TFBSs for AML, AP4, CMYB and NANOG were restricted only to the RA profile. Specific for AS profile were TFBSs for IK3, IRF1 and NFE2.

Based on the results from promoter analysis, we can speculate that the different transcriptional programs, characterized by the interplay of various transcription factors, are responsible for regulating the expression of genes that characterized the profiles of SLE, RA and AS patients.

Table 4.2. TFBSs identified as being overrepresented within the promoter regions of genes that constitute SLE, RA and AS profiles.

Family IDs and Name of TFs	Total number of hits for TF in genome	Number of hits within submitted regions for profiles in			-log10(p-value) for TFBSs in		
		SLE	RA	AS	SLE	RA	AS
3 AHR	1169	94	97	69	3.91	6.72	7.77
4 AHRARNT	3329	249	230	152	6.43	7.42	7.87
9 AML	6163		361			4.14	
14 AP4	17633		920			2.39	
18 ARNT	1593	154	139	78	11.12	10.71	5.46
21 ATF	2726	201	217	140	4.97	12.19	10.48
22 ATF1	3262	233	225	148	4.73	7.22	7.52
23 ATF3	5861		351	228		4.77	5.79
24 ATF4	11203	684	717	414	3.05	13.90	7.28
28 BARBIE	11727		632	383		2.86	2.62

44	CEBP	5645		335	200		4.29	3.05
53	CLOCKBMAL	531	49	52	29	3.61	5.91	3.15
56	MYB	10210		571			3.99	
63	CREBATF	7149	451	439	274	3.19	6.86	6.27
64	CREBP1	1609		128	74		7.55	4.34
65	CREBP1CJUN	1675		112	78		3.49	4.71
68	DEC	4529	287	268	167	2.37	3.52	3.37
78	E2F1DP1	20505	1329	1243	758	10.40	16.03	12.57
79	E2F1DP1RB	1010		82			5.45	
80	E2F1DP2	16766	1027	978	589	4.38	9.40	6.99
81	E2F4DP1	947		75			4.69	
82	E2F4DP2	3239	231	231	137	4.66	8.55	5.43
85	E4F1	540	44	49		2.28	4.73	
89	EGR1	2525	179			3.66		
91	EGR3	72	11			2.78		
92	ELF1	4014	276	252	161	4.30	4.90	4.98
97	ETS1	8230	516	457	311	3.32	3.12	6.48
98	ETS2	4384	284	253	176	2.84	2.79	5.39
110	FREAC2	871	70			3.08		
112	FREAC4	268	27			2.80		
114	FXR	649		49	31		2.87	2.43
115	GABP	2788	204		105	4.84		2.64
126	GLI	19947	1164	1119	629	2.26	7.26	2.54
134	HES1	11971	744	723	423	4.03	9.49	5.49
140	HIF1	5937	554	495	304	33.34	30.89	21.41
149	HNF4ALPHA	14204			465			3.10
156	HSF1	740		60			4.18	
158	ICSBP	631	63	50	37	5.48	3.37	4.46
159	IK1	9108	550	494	308	2.26	2.57	3.03
160	IK3	3298			120			2.44
163	IRF1	7663			269			3.61
166	ISRE	182	24		16	4.26		4.17
181	MAX	410	36	36		2.47	3.39	
191	MINI20	18889	1128	1056	624	3.28	6.65	4.29
197	MYC	12193	819	789	493	9.24	16.23	13.98
202	NANOG	7987		452			3.71	
205	NFE2	3572			129			2.46
206	NFKAPPAB	11792	765	667	454	6.43	5.07	10.05
207	NFKAPPAB50	1196			51			2.53
208	NFKAPPAB65	1037	91	92	63	5.18	7.72	7.61
212	NGFIC	2016	153	123		4.56	2.37	
218	NMYC	8383	530	506	317	3.69	6.89	6.61
219	NRF1	1020	89	68		4.99	2.36	
220	NRF2	12126		652			2.85	
227	OSF2	3280		191			2.42	
239	PEA3	9997	639	565	337	4.79	4.39	3.16
240	PEBP	5538		333	199		4.69	3.32
255	RBPJK	16927		897	543		2.97	2.81
256	RFX1	19071	1134	1075	639	3.08	7.37	5.02
271	SOX9	8655		477			2.98	

273 SP3	15269	946	842		4.73	4.71	
275 SREBP1	3711	244	243	146	2.85	6.00	4.21
277 STAF	12178		669			3.75	
283 STRA13	513	54	51		5.47	6.00	
292 TAXCREB	889		76	43		5.91	3.23
299 TFE	5616		311	192		2.28	2.32
301 TFIII	12074		661	402		3.57	3.26
311 VMYB	853		63			3.25	
312 XBP1	2840	245	224	137	11.74	12.17	8.56
322 ZNF219	6526	434	382		4.92	4.31	

4.2. *In vitro* generated TNFα, IFNα2a and IFNγ reference signatures

Stimulation of monocytes with pro-inflammatory cytokines, such as TNFα, IFNα2a and IFNγ for 1.5 h was used for the development of reference signatures for each of these cytokines. These cytokines profiles were generated by comparing TNFα, or IFNα2a, or IFNγ stimulated monocytes with unstimulated controls incubated for 1.5 h. The comparisons revealed distinctive gene expression profiles where TNFα profile was characterized by 5676 probe-sets, IFNα2a profile by 4566 probe-sets and IFNγ profile by 3897 probe-sets. These numbers of probe-sets are related to ~4000 genes for TNFα profile, ~3250 genes for IFNα2a profile and ~2800 genes IFNγ profiles (Supplementary Table 3, WS1-3). Considering the fact that *in vitro* stimulation of monocytes resulted in more homogenous responses compared to monocytes activation *in vivo*, as shown for SLE, RA and AS profiles, we applied more stringent criteria for the selection of differentially expressed genes. Namely, the number of above mentioned probe-sets was considered as being differentially expressed if they showed changes in at least 60% of pair-wise comparisons (described in section 3.5.5). Combined, TNFα-, IFNα2- and IFNγ- profiles exhibited differential expression of 8941 probe-sets (Figures 4.15 and 4.16, and Supplementary Table 3, WS4).

An analysis of *in vitro* generated cytokine signatures identified probe-sets with expression specific for either one cytokine or shared between two or three cytokines. For example, CCL2, CCL3 and CCL4 were induced by all three cytokines and these chemokines belong to the cytokine-shared profile (Table 4.3). However, stimulation with IFNα2a and IFNγ led to a greater increase in CCL2 as compared to TNFα (FC of 5, 58 and 59 for TNFα, IFNα2a and IFNγ, respectively). Basically, the gene-expression profiles induced by IFNα2a and IFNγ were similar. Thus, the genes altered by IFNα2a and IFNγ were assigned as being specific for IFNs in general. The expression of CCL4 was more strongly increased by TNFα than by IFNα2a or IFNγ (FC of 27, 7 and 4 for TNFα, IFNα2a and IFNγ, respectively). Therefore, if the up-regulation of genes is strongly induced by one cytokine in comparison to the others,

these genes were considered to be predominantly regulated by this particular cytokine. The criteria used for selection of predominantly regulated genes were described in Material and Methods, paragraph 3.5.6. Examples of cytokine-specific genes include CCL20 and CCL23, which were induced only by TNFα. Supplementary Table 3, WS4 includes all 8941 probe-sets and those determined as specific, predominant or shared probe-sets are designated in additional columns.

Table 4.3. Selected genes from *in vitro* generated TNFα, IFNα2a and IFNγ profiles assigned as being cytokine-shared, cytokine-predominant and cytokine-specific. The last column designate genes whose expressions were changed in opposite directions by TNFα and IFNs.

Gene Symbol	TNFα stim FC	IFNα2a stim FC	IFNγ stim FC	Specific for TNFα	Specific for IFNα2a	Specific for IFNγ	Predominant for TNFα	Predominant for IFNα2a	Predominant for IFNγ	Opposite directions
CCL13		15.49	14.74		+					
CCL18	5.83			+						
CCL2	4.86	58.60	59.61					+		
CCL20	247.54			+						
CCL23	226.16			+						
CCL3	10.58	6.15	3.07				+			
CCL4	27.05	7.56	4.06				+			
CCL5	2.71			+						
CXCL1	18.15	-7.72	-2.36							+
CXCL10	6.82	185.31	164.69					+		
CXCL11		554.71	196.12		+					
CXCL3	31.67	-13.40	-7.84							+
CXCL9		34.76	120.51		+				+	
IL15		2.32	1.89		+					
IL18	7.46			+						
IL1A	69.61			+						
IL1B	6.53	3.40					+			
IL23A	4.51			+						
IL27		16.07	7.38		+					
IL8	16.65	-8.35								+

As mentioned before, IFNα2a and IFNγ exhibited similar profiles, and our results identified 2751 probe-sets common for both interferons (IFNs). Within the IFNs profile, the following cytokines were up-regulated: CCL2, CCL3, CCL4, CCL7, CCL8, CXCL9, CXCL10, CXCL11, IL15, IL7 and IL27. Furthermore, we detected an up-regulation of STAT1, STAT2, STAT3, IFI16, ILI27, IFI35 and IFI44. It has been shown before that these genes were regulated by IFNs[93]. Although responses to type I and type II IFNs were essentially the same, it was still possible to identify differences between them. For example, the up-regulation of

IL15R, CD163, CD55, SOCS2 and TNFSF18 was specific for IFNα2a, while the up-regulation of CCR5, CD53, CD97, CIITA, CXCL16, ICOSLG, JAK2, JAK3 and JUN was specific for IFNγ.

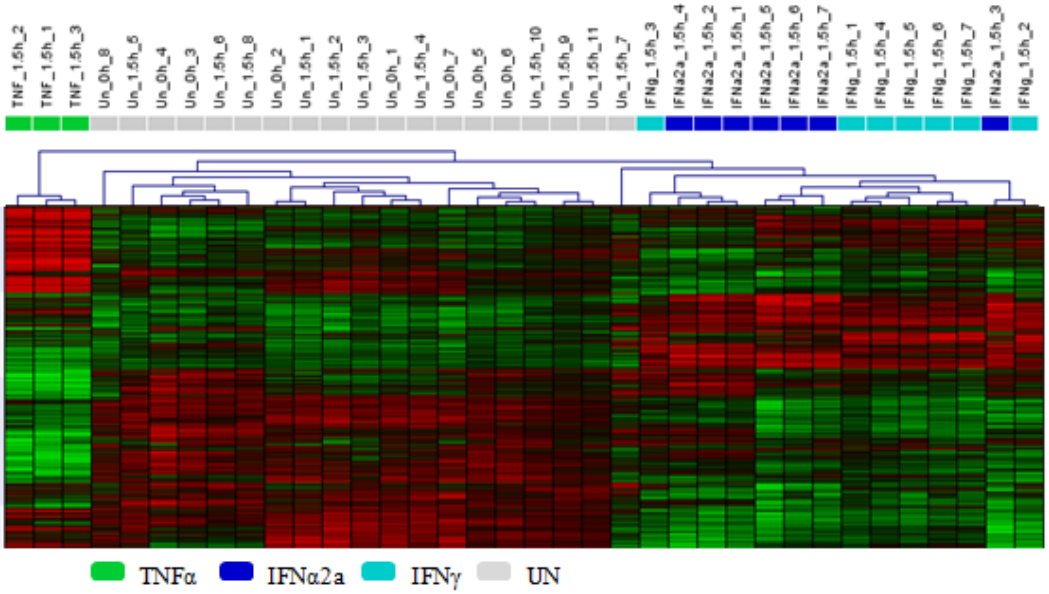

Figure 4.15 Gene expression profiles of monocytes stimulated *in vitro* with TNFα, IFNα2a or IFNγ. In total, 8941 probe-sets were differentially expressed after TNFα, IFNα2a and IFNγ stimulation when compared with unstimulated 1.5 h samples. More precisely, 5676 probe-sets were differentially expressed following TNFα stimulation, 4566 probe-sets following IFNα2a and 3897 probe-sets following IFNγ stimulation. 8941 probe-sets were used for hierarchical clustering of 3 TNFα, 7 IFNα2a, 7 IFNγ and 19 unstimulated samples. Two types of unstimulated samples were utilized: 11 samples, which were incubated for 1.5 h without any stimulus, and 8 samples, which were immediately processed after drawing blood. TNFα, IFNα2a and IFNγ regulated genes were coloured in green, dark blue, and light blue, respectively. Unstimulated samples, both incubated for 1.5 h and immediately processed were coloured in grey.

A Venn diagram presents an overview of probe-sets identified as being cytokine-shared and cytokine-specific (Figure 4.16). The directions of changes in gene-expression like up- or down-regulation were considered in the Venn diagram. That means that only probe-sets that changed in the same direction constitute the cytokine-shared profiles. A small part of TNFα, IFNα2a and IFNγ up-regulated probe-sets was altered by all three cytokines, but a substantial part of up-regulated probe-sets was just induced by TNFα or IFNs. To determine which genes are commonly regulated by all three cytokines, or which ones are rather cytokine-specific, all probe-sets that depict one particular gene have to be considered. Namely, one gene transcript might be presented with more than one probe-set, and the number of cytokine specific probe-sets could give an incorrect estimation of the real number of specific genes.

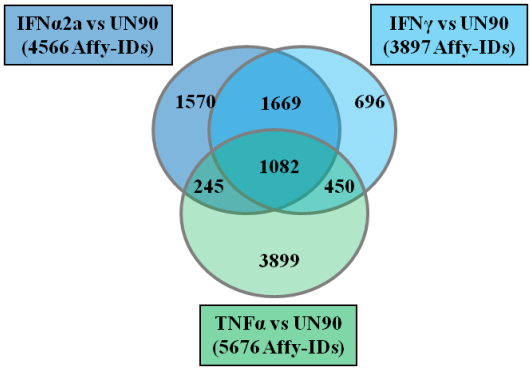

Figure 4.16 A Venn diagram represents 9611 differentially expressed probe-sets that compose TNFα, IFNα2a and IFNγ reference signatures. Values indicate the absolute numbers of probe-sets whose expression was specific for a particular cytokine, shared by two or by all three cytokines. The direction of changes in probe-set expression was considered, and the shared profile constitutes only those probe-sets whose expression was altered in the same direction.

In total, 662 probe-sets were commonly regulated by IFNs and TNFα but in opposite directions. These probe-sets did not belong to the cytokine-shared profile in Figure 4.16. These 662 probe-sets were presented in Figure 4.17, and in addition, they were marked in separate columns in Supplementary Table 3, WS4. Among the genes that were up-regulated by TNFα but down-regulated by IFNs we identified CXCL1, CXCL2, CXCL3, IL8, TNFSF8 and TNFSF10. In contrast, genes such as IFIT1, IFIT3, IFIT5, IFI16, IFI44, IFI44L, OAS1, OAS2 and STAT1 were down-regulated by TNFα and up-regulated by IFNs. These genes were frequently detected in different inflammatory conditions, including SLE and RA, as summarized below.

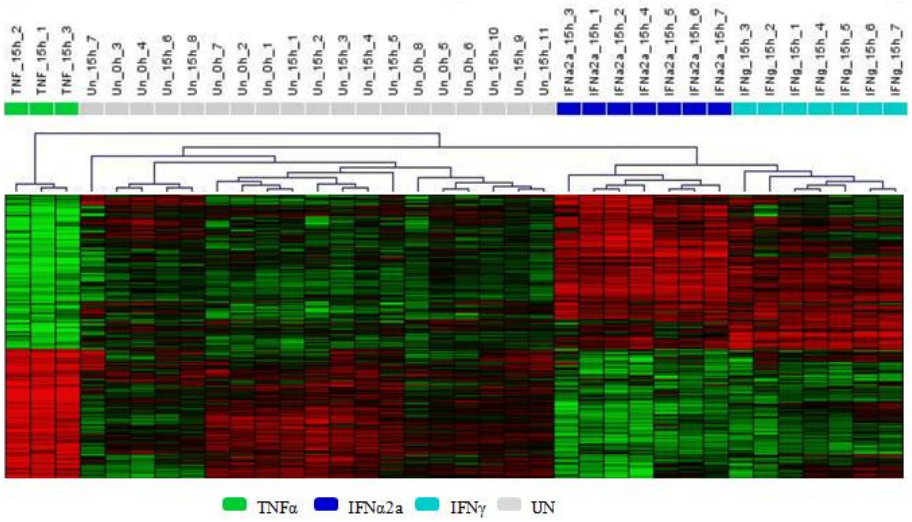

Figure 4.17 Hierarchical clustering of differentially expressed probe-sets that were regulated both by TNFα and IFNs but in opposite directions. 662 probe-sets of *in vitro*-stimulated monocytes were differentially expressed following stimulation with TNFα, or IFNα2a, or IFNγ, but they showed inverse regulation. Namely, these probe-sets were regulated by TNFα in one direction and by IFNs in the opposite one.

4.3. Disease-dependent diversity of IFNα2a-, IFNγ- and TNFα-responses in SLE, RA and AS monocytes

Analyses of transcriptome data from SLE, RA and AS monocytes indicated that cytokines have important roles in inducing changes in diseases. To estimate the role of particular cytokines in a more explicit way, the *in vitro* generated TNFα, IFNα2a and IFNγ reference signatures were utilised for comparisons with the profiles from diseases. The imprints of all three cytokines were apparent in all three diseases. However, it was obvious that they were qualitatively and quantitatively different, and principally they were modulated in a disease-dependent manner.

4.3.1. The monocyte response to IFNα2a was different in SLE, RA and AS

Comparisons between the IFNα2a signature and profiles from SLE, RA and AS shown an overlap of 547, 328 and 230 probe-sets, respectively (Supplementary Table 1 (WS1-3); the last three columns of tables in worksheet 1-3 represent the probe-sets determined as the cytokine imprints). An IFNα2a imprint was clearly identified in 6 out of 9 SLE patients (Figures 4.18.A and 4.18.B). The IFNα2a imprint was not obvious in patients that were characterized by a more inactive disease course (SL-1, SL-2 and SL-7). The IFNα2a imprint in SLE included up-regulation of the following genes: CCL2, CCL8, CD164, CXCL10, CXCL11, FAS, IFI16, IFI27, IFI44, IFI44L, IL15, IL15R, MX1, MX2, OAS1, OAS2, OAS3, OASL, SIGLEC1, SSB, STAT1, STAT2, STAT3 and TNFSF10. The IFNα2a imprint in SLE also included the genes that were down-regulated, such as CENTD2, CYP1B1, GPX4, ID2, IER3, IRS2, JUN, KLF13, KLF2, KLF4, PTARF, TNFAIP2 and TNFRSF10B.

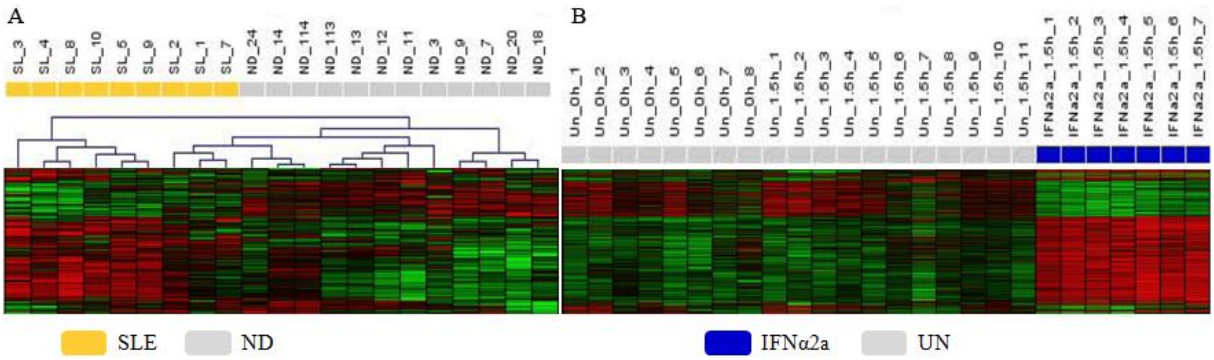

Figure 4.18 Disease-specific IFNα2a imprints in SLE monocytes. Figure 4.18.A 547 differentially expressed probe-sets obtained after IFNα2a stimulation were also identified in SLE monocytes and they were used for a hierarchical clustering of SLE and ND. **Figure 4.18.B** Here, the same 547 probe-sets were extracted from the IFNα2a reference signature and arranged in the same order as identified by HC of SLE and ND in figure 4.18.A.

A distinct IFNα2a imprint was identified in 5 out of 8 patients with RA (Figures 4.19.A and 4.19.B). The IFNα2a imprint in RA included the up-regulation of CD163, CD55, CITED2, IL6ST, FOSL2, MAFF, ATF3 and MT2A, and the down-regulation of CCNG2, CXCR4, ICAM2, FADD, GPX3, NGRN, PURA, TNFSF8 and TP53.

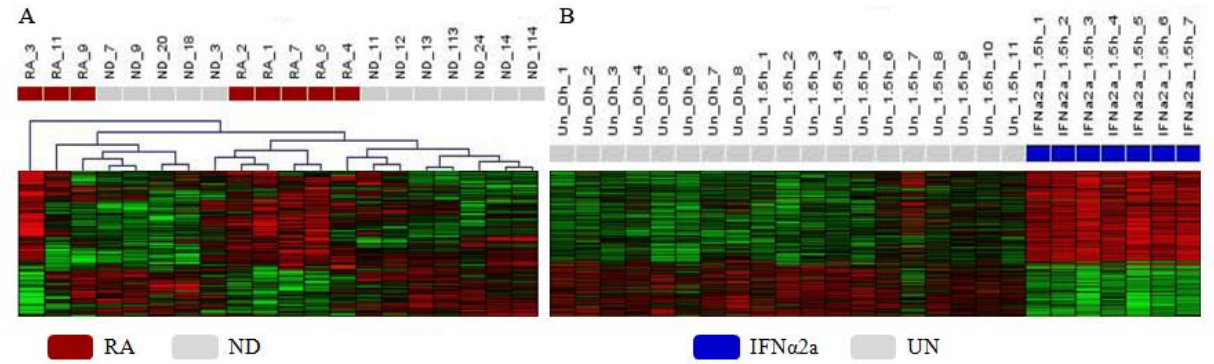

Figure 4.19 Disease-specific IFNα2a imprints in RA monocytes. Figure 4.19.A 328 differentially expressed probe-sets obtained after IFNα2a stimulation were also identified in RA monocytes and they were used for HC of RA and ND**. Figure 4.19.B** Here, the same 328 probe-sets were extracted from the IFNα2a reference signature and arranged in the same order as identified by HC of RA and ND in figure 4.19.A.

An IFNα2a imprint in AS was disclosed by 230 probe-sets. It showed an obvious heterogeneity between AS patients, where the patients AS24 and AS25 exhibited the strong IFNα2a imprint (Figures 4.20.A and 4.20.B). In the majority of other AS patients it was found to be as a rather weak imprint. The following genes composed the IFNα2a imprint in AS: IFIT1, IFIT2, IFIT3 and ATF3, which were up-regulated, and SOX4 and CXCR4, which were down-regulated.

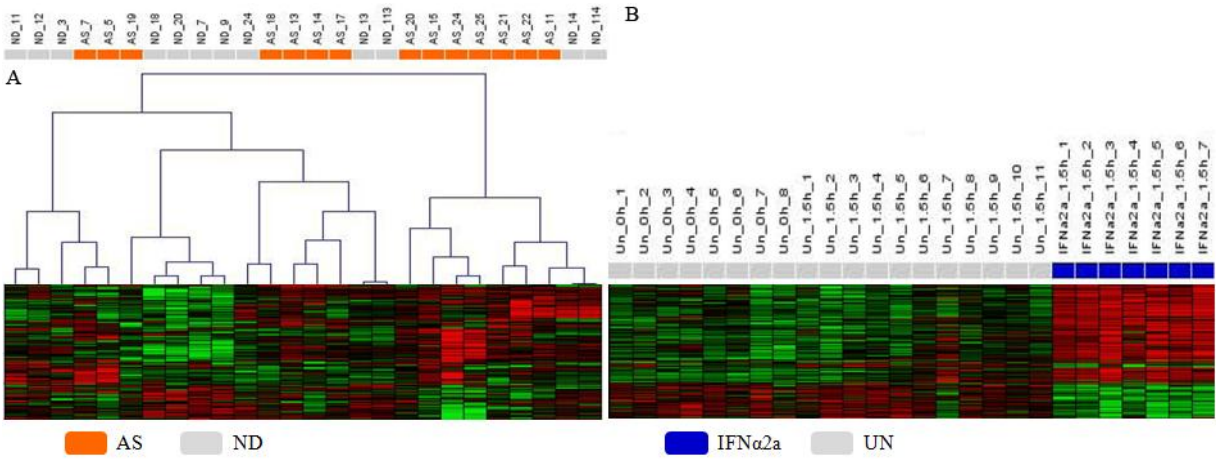

Figure 4.20 Disease-specific IFNα2a imprints in AS monocytes Figure 4.20.A 230 differentially expressed probe-sets obtained after IFNα2a stimulation were also identified in AS monocytes and they were used for HC of AS and ND**. Figure 4.20.B** Here, the same 230 probe-sets were extracted from the IFNα2a reference signature and arranged in the same order as identified by HC of AS and ND in figure 4.20A.

In summary, the IFNα2a imprints that were identified within the SLE, RA and AS profiles were presented together (Figures 4.21.A and 4.21.B). Although an IFNα2a imprint was detected in all three diseases, it was quantitatively and qualitatively different. In SLE monocytes, the IFNα2a imprint was revealed as being more dominant when compared to those in RA and AS. The dominance was demonstrated taking into account the number of the genes and the magnitude of their changes. The IFNα2a imprint identified in RA was distinctive as compared to that in SLE. The IFNα2a imprint in AS was revealed as a very dim and more as an "echo" of this cytokine, but we should be aware that the AS profile was exposed as the weaker one when compared to the profiles from SLE and RA.

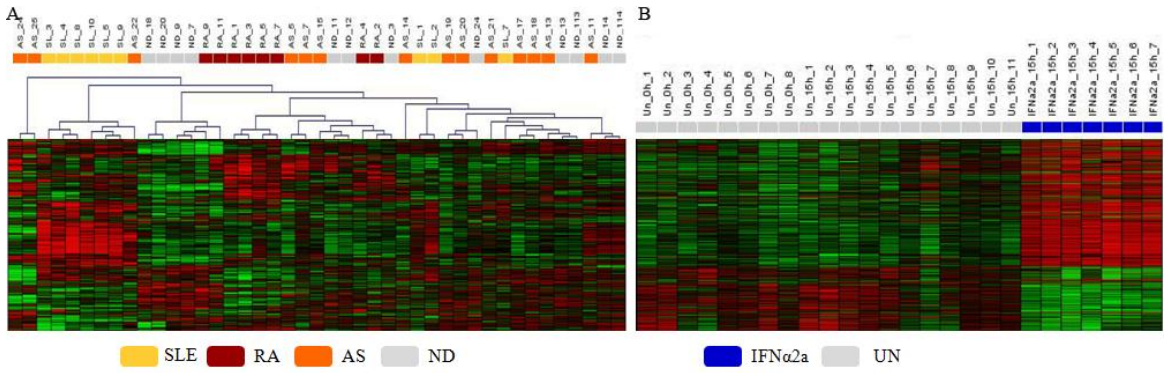

Figure 4.21 Hierarchical clustering of probe-sets identified as the IFNα2a imprints in SLE, RA and AS. **Figure 4.21.A** 777 probe-sets were used for HC of diseases and ND. They represent the IFNα2a imprints in SLE (547 probe-sets), in RA (328 probe-sets), and in AS (230 probe-sets). **Figure 4.21.B** Here, the same 777 probe-sets were extracted from the *in vitro*-generated IFNα2a reference signature and arranged in the same order as identified by HC of SLE, RA, AS and ND in figure 4.21.A.

When the SLE profile was presented in the form of a terrain map and overlaid with the probe-sets that determined the IFNα2a imprint, it was obvious that the highest peak was enriched by IFNα2a regulated genes (Figure 4.22.A). Unlike the SLE profile, the profiles from RA and AS patients displayed the IFNα2a regulated genes as uniformly distributed within the profiles (Figures 4.22.B and 4.22.C).

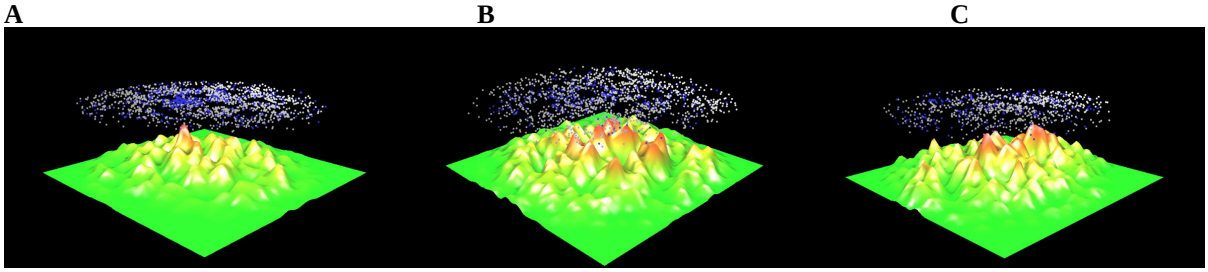

Figure 4.22 Landscapes of SLE, RA and AS profiles with the IFNα2a imprints are presented in **Figures 4.22.A, 4.22.B and 4.22.C**, respectively. Dots over the hills represent probe-sets that constitute the SLE, RA and AS profiles. Probe-sets that were identified as IFNα2a imprints in SLE, RA and AS are displayed as dark blue dots. The enlarged figures are presented in Appendix, page 141.

4.3.2. The monocyte response to IFNγ was different in SLE, RA and AS

Although IFNγ regulated genes in SLE, RA and AS were characterised by a lower absolute number of differentially expressed probe-sets as compared to IFNα2a, the IFNγ imprints in these diseases were very similar to those identified as the IFNα2a imprints. There were a few SLE and RA patients that did not exhibit the IFNγ imprint, and these patients were also negative for the IFNα2a imprint (Figures 4.23 and 4.24).

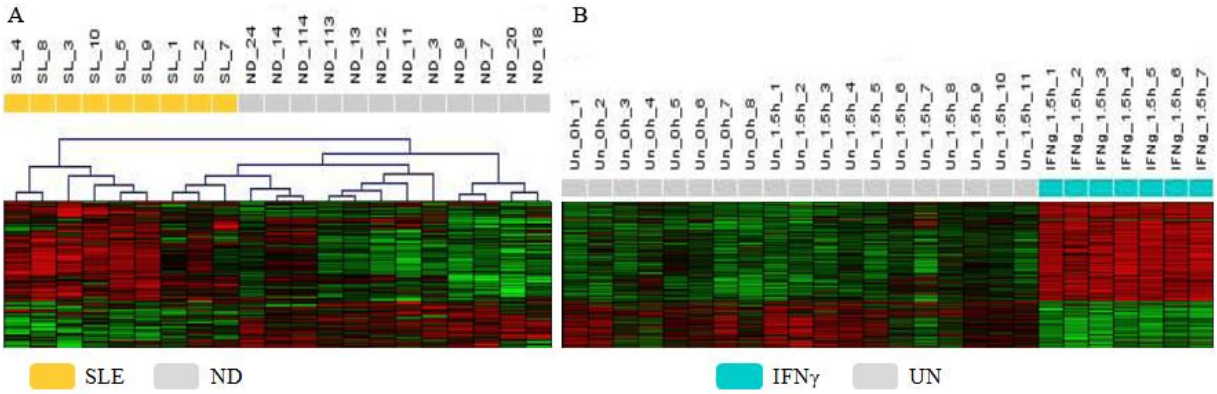

Figure 4.23 Disease-specific IFNγ imprints in SLE monocytes. Figure 4.23.A 421 differentially expressed probe-sets obtained after IFNγ stimulation were also identified in SLE monocytes and they were used for HC of SLE and ND. **Figure 4.23.B** Here, the same 421 probe-sets were extracted from the IFNγ reference signature and arranged in the same order as identified by HC of SLE and ND in figure 4.23.A.

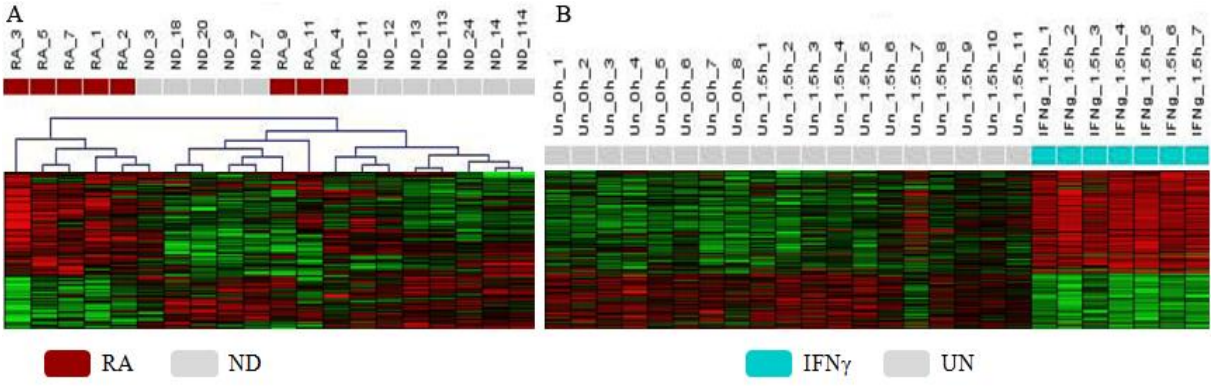

Figure 4.24 Disease-specific IFNγ imprints in RA monocytes. Figure 4.24.A 297 differentially expressed probe-sets obtained after IFNγ stimulation were also identified in RA monocytes and they were used for HC of RA and ND. **Figure 4.24.B** Here, the same 297 probe-sets were extracted from the IFNγ reference signature and arranged in the same order as identified by HC of RA and ND in figure 4.24.A.

In AS, the most prominent IFNγ imprint was identified in AS15, AS20, AS24 and AS25, and these are the same patients that displayed the IFNα2a imprint (Figure 4.25).

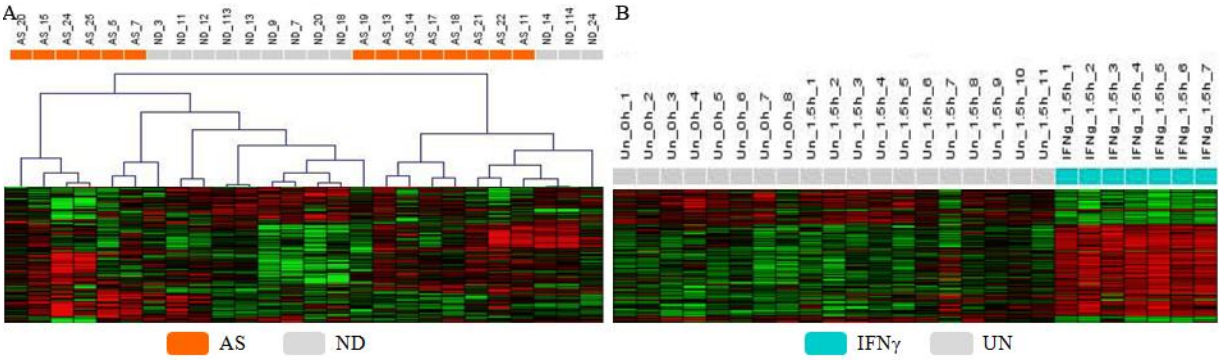

Figure 4.25 Disease-specific IFNγ imprints in AS monocytes. Figure 4.25.A 203 differentially expressed probe-sets obtained after IFNγ stimulation were also identified in AS monocytes and they were used for HC of AS and ND. **Figure 4.25.B** Here, the same 203 probe-sets were extracted from the IFNγ reference signature and arranged in the same order as identified by HC of AS and ND in figure 4.25.A.

When the IFNγ imprints in SLE, RA and AS were combined, it was obvious that the IFNγ imprints were modulated in disease-dependent manner (Figure 4.26). The very uniform IFNγ response, in terms of co-expressed genes, was identified in most of the SLE patients. Similar to the IFNγ pattern in SLE, the IFNγ patterns in RA and AS were also weaker considering both the number of genes that were differentially expressed and magnitude of their changes.

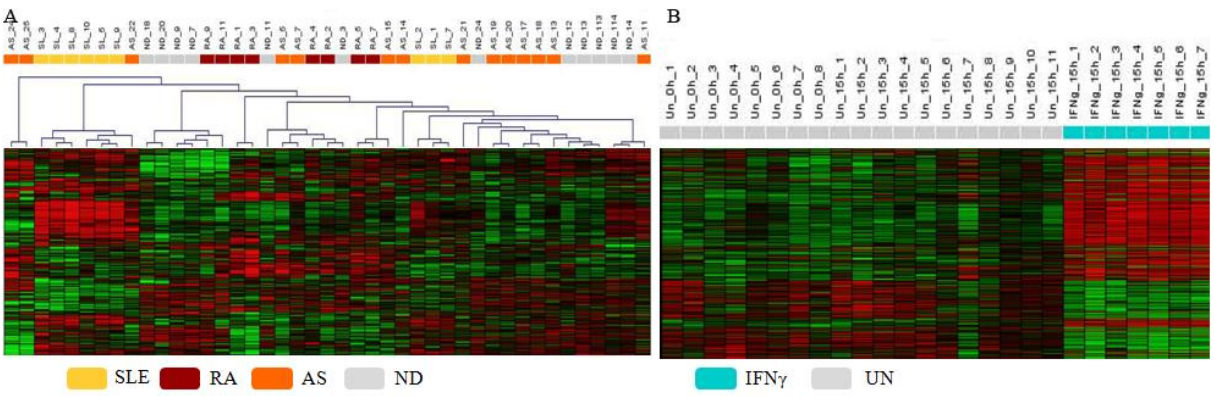

Figure 4.26 Hierarchical clustering of probe-sets identified as the IFNγ imprints in SLE, RA and AS. **Figure 4.26.A** 652 probe-sets were used for HC of diseases and ND. They represent the IFNγ imprints in SLE (421 probe-sets), in RA (297 probe-sets), and in AS (203 probe-sets). **Figure 4.26.B** Here, the same 652 probe-sets were extracted from the *in vitro*-generated IFNα2a reference signature and arranged in the same order as identified by HC of SLE, RA, AS and ND in figure 4.26.A.

Genes regulated by IFNα2a and IFNγ were found to be very similar, and thus, the terrain maps that represent the IFNγ imprints in SLE, RA and AS resembled maps that displayed the IFNα2a imprints in these diseases (Figure 4.27).

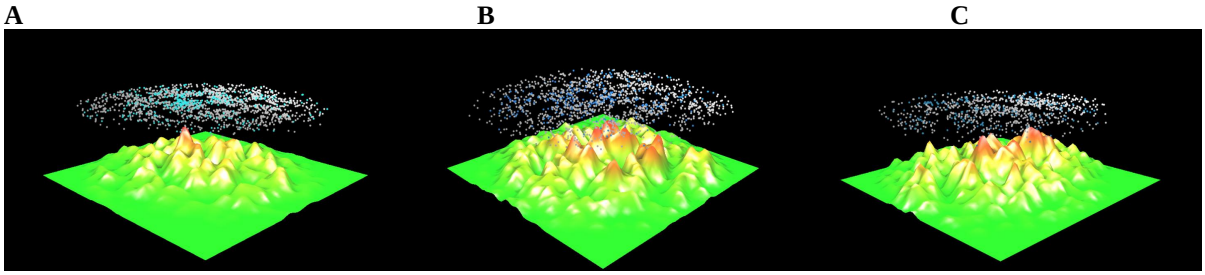

Figure 4.27 Landscapes of SLE, RA and AS profiles with IFNγ imprints are presented in **Figures 4.27.A, 4.27.B and 4.27.C**, respectively. Dots over the hills represent probe-sets that constitute the SLE, RA and AS profiles. Probe-sets that were identified as IFNγ imprints in SLE, RA and AS are displayed as light blue dots. The enlarged figures are presented in Appendix, page 142.

4.3.3. The monocyte response to TNFα was dissimilar in SLE, RA and AS

The *in vitro*-induced TNFα profile from normal donors was compared with profiles from SLE, RA and AS patients. The number of probe-sets identified to overlap was determined to be the TNFα imprint in diseases. More precisely, 303 probe-sets comprised the TNFα imprint in SLE, 395 probe-sets determined the TNFα imprint in RA and 217 probe-sets constituted the TNFα imprint in AS. The following genes were identified as being TNFα regulated in SLE: ALCAM, C3AR1, LYN, MYC, REL, TCF7L2, TLR2 and NFKBIA, which were up-regulated and BAX, CD1D, FLT3, HDAC1, KLF10, LILRA2, NCOA3, NR4A2 and PDGFC, which were down-regulated. Hierarchical clustering with these TNFα regulated genes in SLE allowed a discrimination of all SLE patients from healthy donors (Figure 4.28).

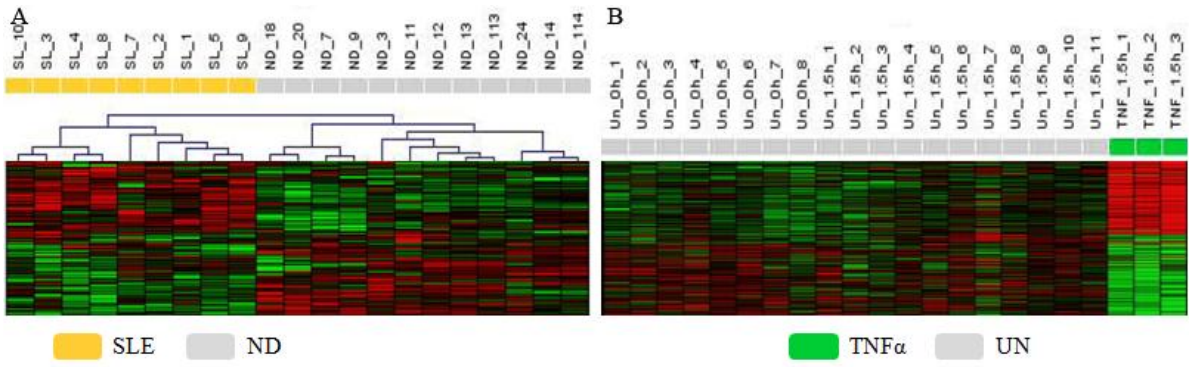

Figure 4.28 Disease-specific TNFα imprints in SLE monocytes. Figure 4.28.A 303 differentially expressed probe-sets obtained after TNFα stimulation were also identified in SLE monocytes and they were used for HC of SLE and ND. **Figure 4.28.B** Here, the same 303 probe-sets were extracted from the TNFα reference signature and arranged in the same order as identified by HC of SLE and ND in figure 4.28.A.

Among the genes regulated by TNFα in RA, the results from this study identified: ATF4, BCL6, CXCL2, EREG, IL8, NFKB2, PLAUR, SPI1, STAB1, TNF and TNFAIP3, which

were up-regulated and CD164, HDAC9, HHEX, IRF2, ITGA4, OAS1, OAS2, STAT1, TLR4 and TLR8, which were down-regulated. There were a few RA patients, RA-4, RA-9 and RA-11, who did not show the TNFα imprint (Figure 4.30).

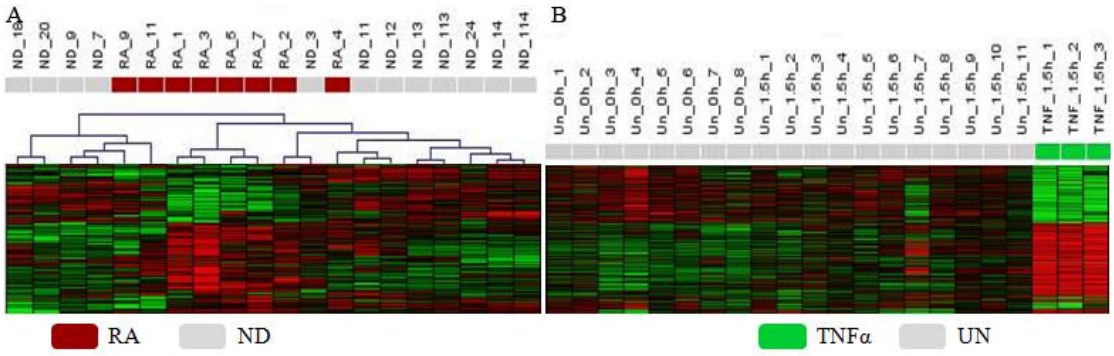

Figure 4.30 Disease-specific TNFα imprints in RA monocytes. Figure 4.30.A 395 differentially expressed probe-sets obtained after TNFα stimulation were also identified in RA monocytes and they were used for HC of RA and ND. **Figure 4.30.B** Here, the same 395 probe-sets were extracted from the TNFα reference signature and arranged in the same order as identified by HC of RA and ND in figure 4.30.A.

A TNFα imprint in AS was characterized by genes such as THBS1, MGAM, SOCS3, IL8, IL1B, BCL3, BCL6, AQP9, LILRB4, SOD2, GPR109B, FPR2, CD44, ATF3, C3RA1, GPR183, EREG, ABCA1, KYNU, HLX, CD83, PLAUR, CFLAR, NFKBIA and CD1D. Similar to SLE and RA, the TNFα imprint in AS revealed heterogeneity among AS patients (Figure 4.31). For instance, the patients AS24, AS25 and AS15 showed the stronger TNFα imprint compared to the other AS patients.

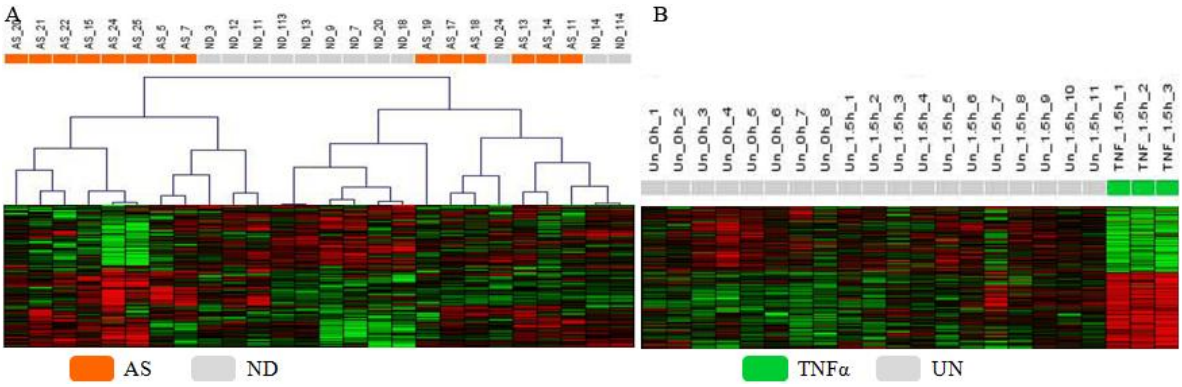

Figure 4.31 Disease-specific TNFα imprints in AS monocytes. Figure 4.31.A 217 differentially expressed probe-sets obtained after TNFα stimulation were also identified in AS monocytes and they were used for HC of AS and ND. **Figure 4.31.B** Here, the same 217 probe-sets were extracted from the TNFα reference signature and arranged in the same order as identified by HC of AS and ND in figure 4.31.A.

The disease-dependent response to TNFα became apparent by analysing all TNFα regulated genes in SLE, RA and AS together by hierarchical clustering (Figure 4.32). The analysis

demonstrated that TNFα imprint in RA prevailed over those determined in SLE and AS. The number of TNFα regulated genes, including those determined as TNFα-specific and TNFα-predominant, was larger in RA than in SLE and AS. The TNFα imprint in AS was very heterogeneous, and several patients, AS24, AS25, AS5 and AS7, displayed the imprint that was similar to those in RA.

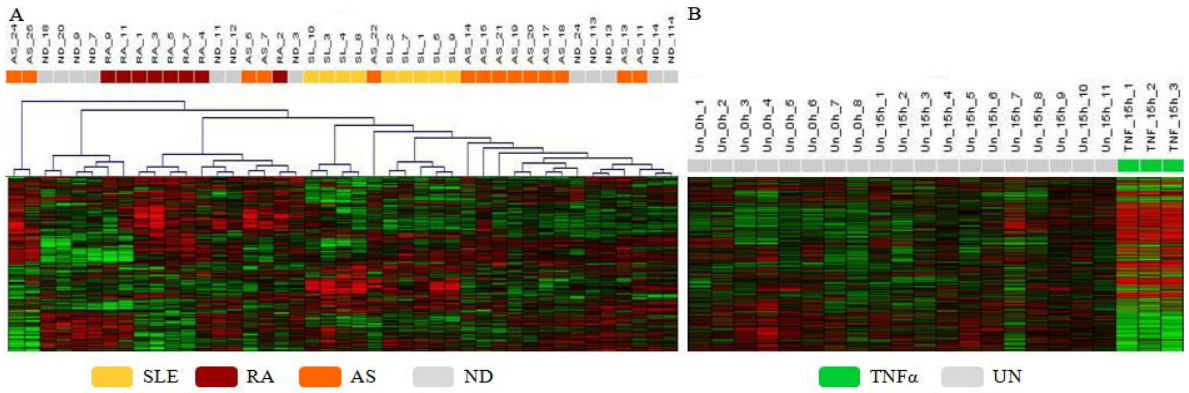

Figure 4.32 Hierarchical clustering of probe-sets identified as the TNFα imprints in SLE, RA and AS. **Figure 4.32A** 672 probe-sets were used for HC of diseases and ND. They represent the TNFα imprint in SLE (303 probe-sets), in RA (395 probe-sets), and in AS (217 probe-sets). **Figure 4.32.B** Here, the same 672 probe-sets were extracted from the *in vitro*-generated TNFα reference signature and arranged in the same order as identified by HC of SLE, RA, AS and ND in figure 4.32.A.

The terrain maps revealed TNFα regulated genes as being uniformly distributed within SLE, RA and AS profiles and with no preferences for dominant clustering (Figure 4.33).

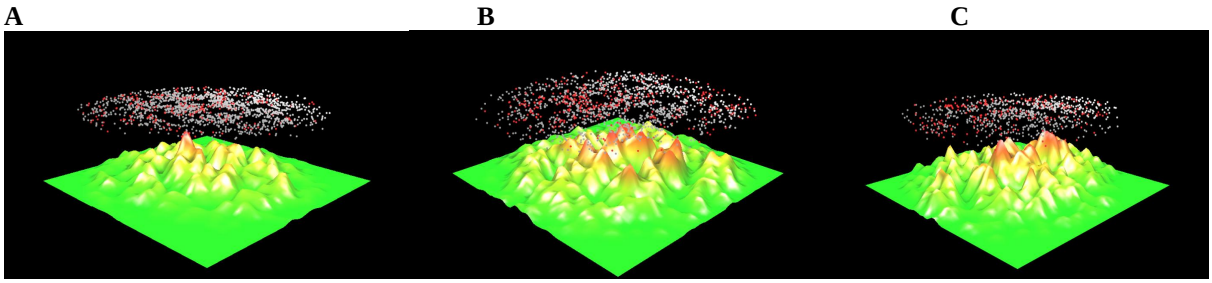

Figure 4.33 Landscapes of SLE, RA and AS profiles with TNFα imprints are presented in **Figures 4.33.A, 4.33.B and 4.33.C**, respectively. Dots over the hills represent probe-sets that constitute the SLE, RA and AS profiles. Probe-sets that were identified as TNFα imprints are displayed as red dots. The enlarged figures are presented in Appendix, page 143.

4.3.4. The TNFα and IFNs imprints in chronic rheumatic diseases were revealed to be disease-dependent

To address the question how specific the cytokine imprints are in SLE, RA and AS, we analysed all genes regulated by TNFα, IFNα2a and IFNγ at the same time. More precisely, all

probe-sets identified as the cytokine imprints in each particular disease were combined. The results showed that a small part of the SLE, RA and AS profiles was changed by all three cytokines. In fact, the major parts of the SLE, RA and AS profiles were changed by cytokines in a specific way (Figures 4.34, 4.35 and 4.36). The absolute numbers of probe-sets identified as cytokine-specific and cytokine-shared in each of three diseases were presented in the Venn diagrams (Figures 4.34.C, 4.35.C and 4.36.C). Taken together, ~30% of SLE profile was regulated by IFNα2a, ~23% by IFNγ and ~16% by TNFα. Therefore, the SLE profile displayed an obvious dominance of IFN-response, where IFNα2a and IFNγ imprints strongly overlapped.

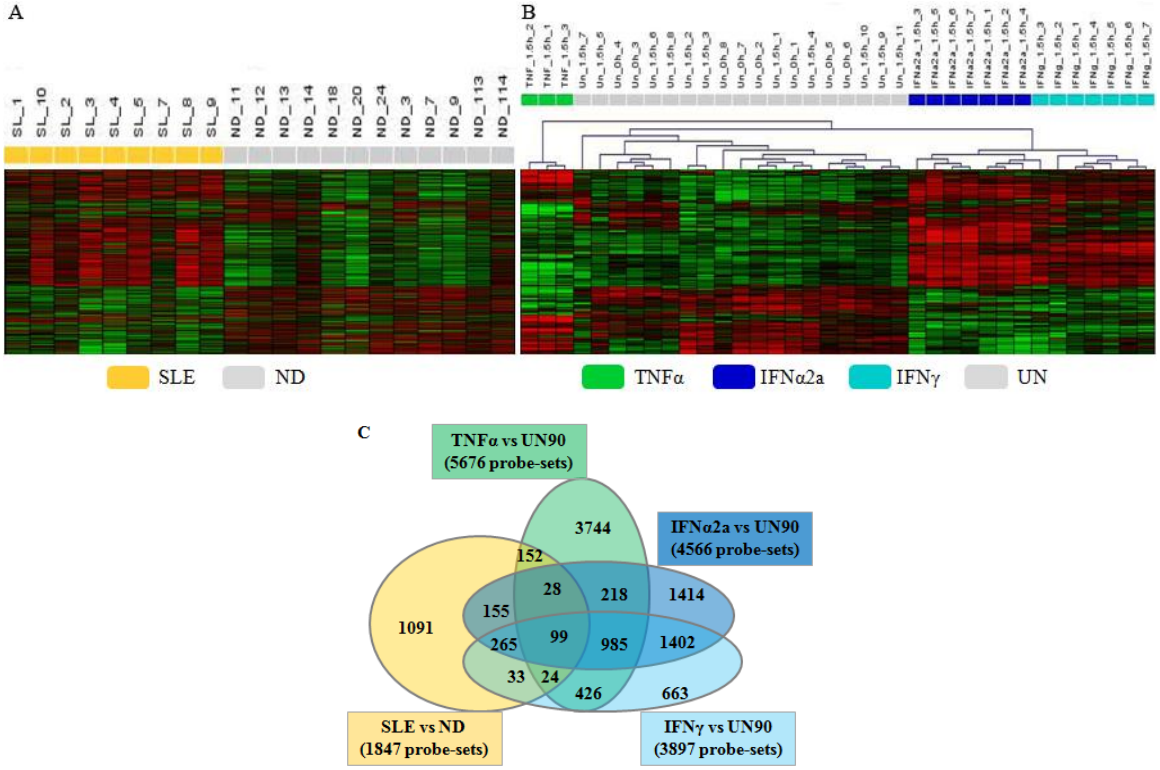

Figure 4.34 IFNα2a-, IFNγ- and TNFα-imprints in SLE partially overlapped. Figures 34.A and 34.B In total, 756 probe-sets obtained after TNFα, IFNα2a or IFNγ stimulation were also identified in SLE monocytes. **Figure 4.34.C** A diagram shows the absolute numbers of probe-sets that comprise the TNFα-, IFNα2a-, and IFNγ-imprints in SLE.

The imprint of cytokines in RA was found in the following ranges: ~20% of profile was influenced by IFNα2a, ~18% by IFNγ and ~24% by TNFα.

Although the AS profile was influenced by TNFα and IFNs, the dominance of a particular cytokine was not evident. For instance, ~25% of the AS profile was altered by IFNα2a, ~22% by IFNγ and ~24% by TNFα. Interestingly, the AS patients with the IFNs imprints showed the TNFα imprint as well.

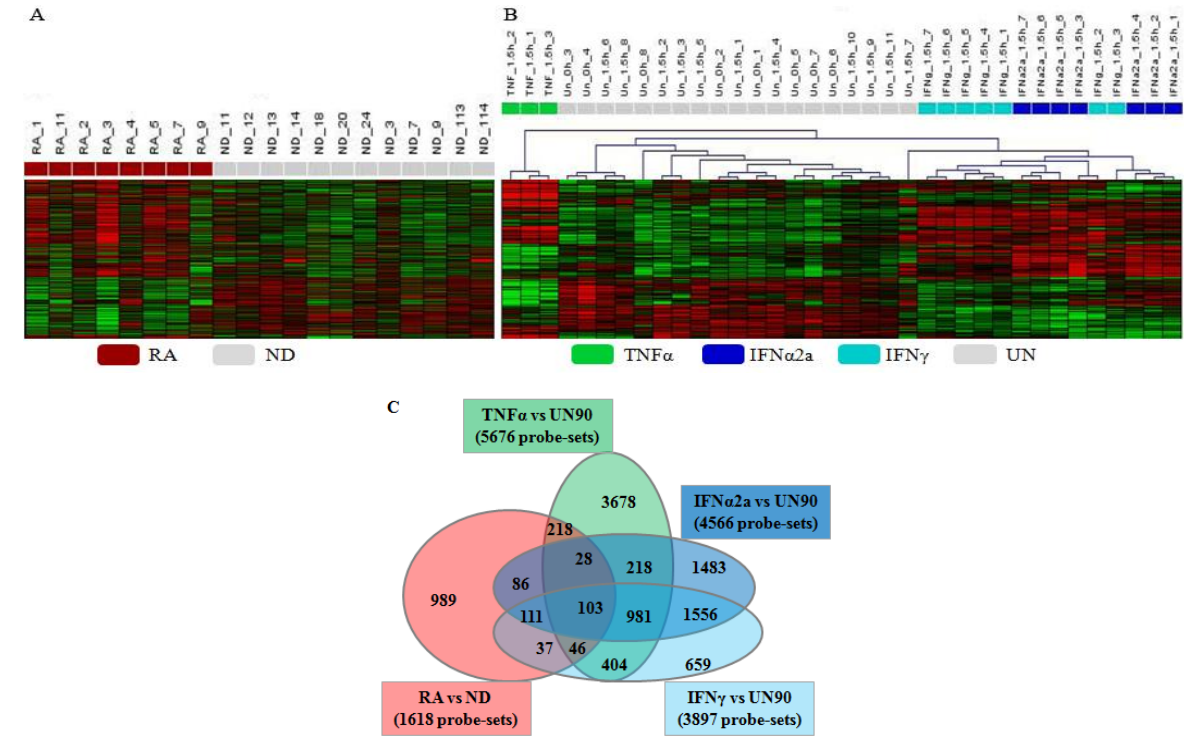

Figure 4.35 IFNα2a, IFNγ and TNFα imprints in RA partially overlapped. Figures 4.35.A and 4.35.B In total, 629 probe-sets obtained after TNFα, IFNα2a, and IFNγ *in vitro*-stimulation were also identified in RA monocytes **Figure 4.35.C** A diagram shows the absolute numbers of probe-sets that compose the TNFα-, IFNα2a-, and IFNγ-imprints in RA.

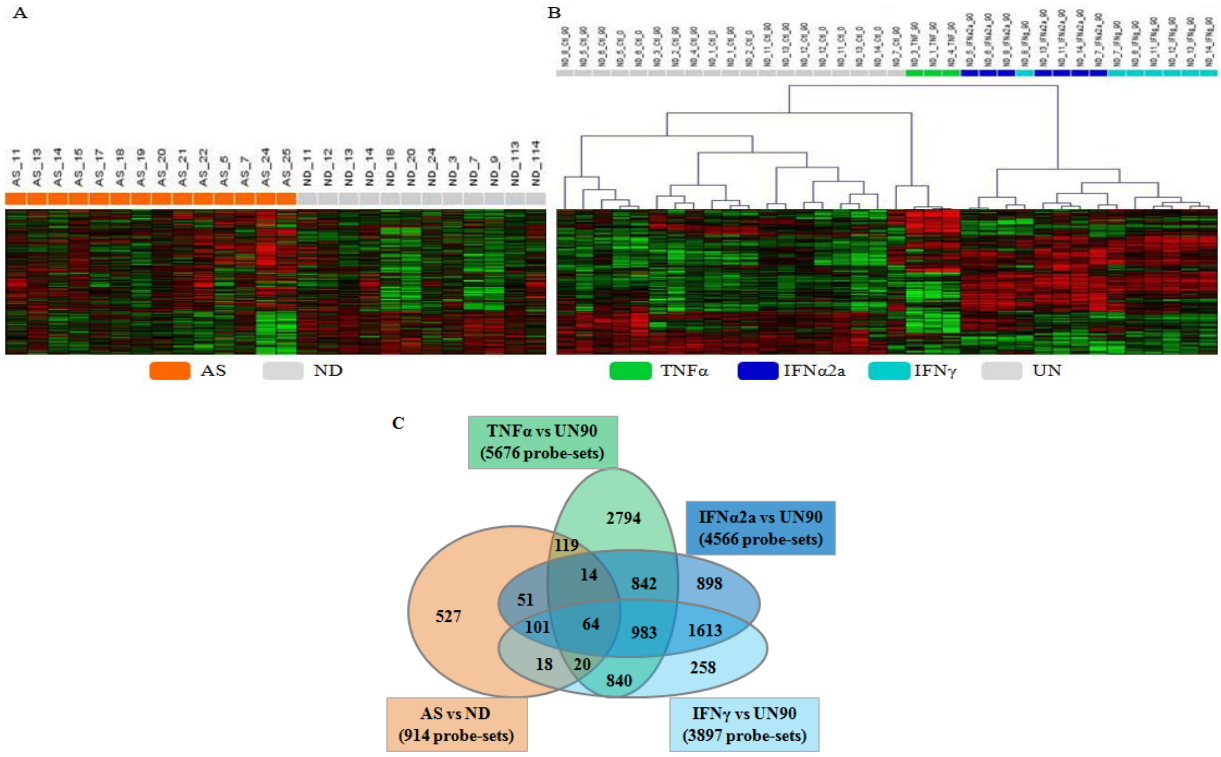

Figure 4.36 IFNα2a, IFNγ and TNFα imprints in AS partially overlapped. Figures 4.36.A and 4.36.B In total, 387 probe-sets obtained after TNFα, IFNα2a, and IFNγ *in vitro*-stimulation were also identified in AS monocytes. **Figure 4.36.C** A diagram shows the absolute numbers of probe-sets that comprise the TNFα-, IFNα2a-, and IFNγ-imprints in AS.

The results showed that the monocyte transcriptomes from SLE, RA and AS patients were imprinted by various pro-inflammatory cytokines. Furthermore, the cytokine imprints were found to be disease-dependent, where the SLE profile was mainly driven by IFNs, while the RA profile was dominated by TNFα. Unlike the profiles in SLE and RA, the profile from AS patients did not show the dominance of a particular cytokine.

4.4. The gene-expression profile from SLE monocytes was utilized in biomarker discovery

A few genes that were differentially expressed in SLE monocytes and/or regulated by cytokines *in vitro* were validated at the protein level by flow cytometry. The main focus was to select the genes that encode the expression of the surface molecules. To validate changes in their expression at the protein level would be relevant for a fast and relatively easy discrimination between monocytes from SLE patients and healthy donors.

The following surface molecules were selected for validation at the protein level: CD32, CD64, CD120B (TNFRSF1B, TNFR2), CD128B (CXCR2, IL8RB) and CD244. All together 6 SLE patients and 14 healthy controls were analysed in this study. The expression of these molecules at transcriptional level is presented in Table 4.4.

The expression of CD64 (Fc fragment of IgG, high affinity receptor) was found to be increased in SLE monocytes as compared to healthy donors. The changes at the transcriptional level were accompanied by alteration at the protein level (Figure 4.37.A).

Table 4.4. Fold changes of differentially expressed genes in monocytes stimulated *in vitro* by TNFα, or IFNα2a, or IFNγ and in monocytes from SLE patients.

Affy IDs	Gene Symbol	TNFvsUN90 fold change	IFNa2avsUN90 fold change	IFNgvsUN90 fold change	SLEvsND fold change
216950_s_at	CD64	-1.67	3.90	10.61	1.85
203508_at	CD244		-2.25	-1.87	
220307_at	CD120b (TNFRSF1B)	-2.74	-1.84	-2.04	
207008_at	CD128b (IL8RB)	-13.67		-2.41	
203561_at	CD32 (FCGR2A)	-1.60			1.23
210889_s_at	CD32 (FCGR2B)		18.20	2.62	

The expression of CD244 (natural killer cell receptor 2B4) was found to be decreased, both at the transcriptional and protein levels (Figure 4.37.B).

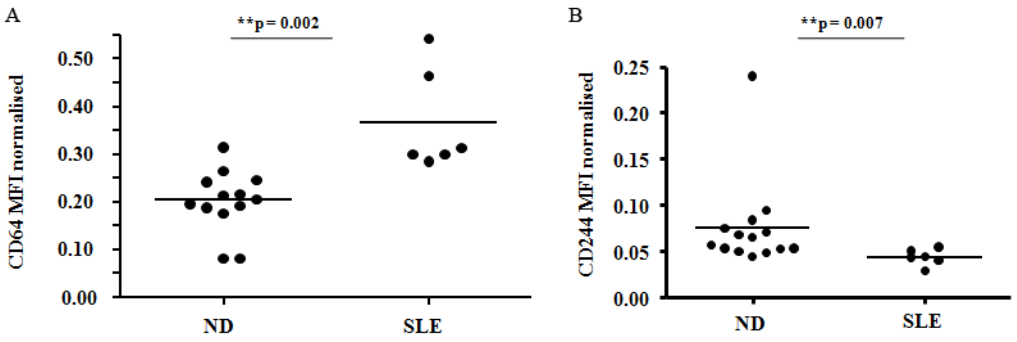

Figure 4.37 Expressions of the CD64 and CD244 at the protein level in monocytes from SLE patients and healthy donors. Normalised MFI (mean fluorescence intensity) values of CD64 and CD244 are represented on the Y-axes in figures 4.37.A and 4.37.B, respectively. Horizontal bars show p-values calculated by Mann-Whitney test.

Two cytokine receptors CD120B (TNF receptor type II) and CD128B (CXCR2, IL8 receptor, beta) did not exhibit changes in SLE monocytes as compared to healthy donors at the transcriptional level. Considering the fact that TNFα and IL8 play important roles in pathogenesis of SLE, and that IFNs and TNFα silenced expression of CD120b and CD128b, respectively, these two receptors were included in analyses at the protein level. The expression of CD120B and CD128B at the protein level was found to be decreased on monocytes from SLE patients when compared to healthy controls (Figure 4.38).

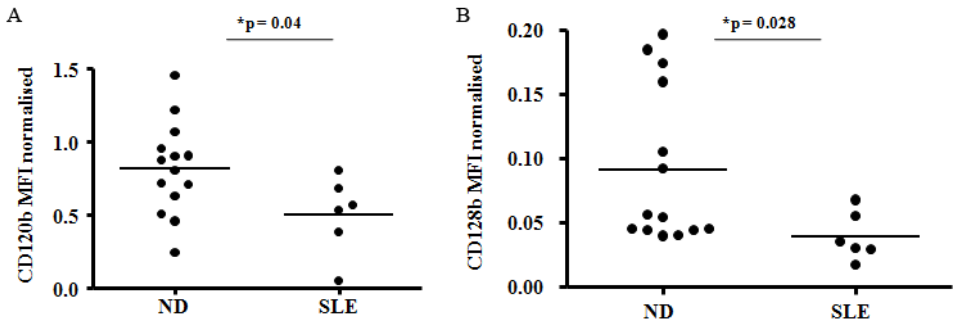

Figure 4.38 Expressions of the CD120b and CD128b at the protein level in monocytes from SLE patients and healthy donors. Normalised MFI (mean fluorescence intensity) values of CD120b and CD128b are presented on the Y-axes in figures 4.38.A and 4.38.B, respectively. Horizontal bars show p-values calculated by Mann-Whitney test.

CD32 (Fc fragment of IgG, low affinity receptor) is the surface marker whose expression was found to be strongly up-regulated by IFNα2a at the transcriptional level (Table 4). Considering the prevailing role of IFNα2a in SLE patients, it was speculated that an increased

expression of CD32 might be detectable on the protein level in SLE monocytes as well. However, monocytes from SLE patients showed decreased expression of CD32 (Figure 4.39). The overall complexity of transcriptome and its translation into proteome was exemplified by the expression of CD32. Namely, it is known that post-transcriptional control plays an important role in determining the response detectable at the protein level.

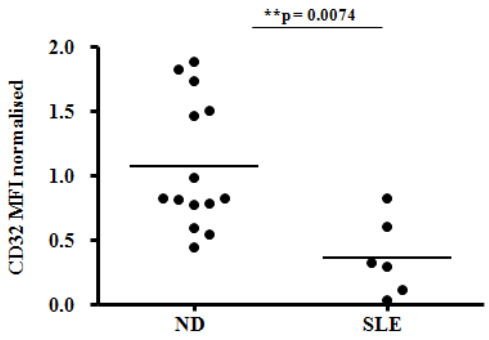

Figure 4.39 Expressions of the CD32 at the protein level in monocytes from SLE patients and healthy donors. Normalised MFI (mean fluorescence intensity) value of CD32 is represented on the Y-axes. Horizontal bars show p-values calculated by Mann-Whitney test.

5. Discussion

5. Discussion

This study demonstrated that the gene-expression profiles from SLE, RA and AS monocytes were found to be disease-specific. Monocytes were identified as an exceptional source for sensing the influence of various cytokines and responding to them in a specific way. The response to cytokines was characterized by particular gene-expression patterns (imprints). To analyse the imprinting of cytokines in various diseases, the cytokine specific gene-expression profiles were generated following monocytes stimulation *in vitro* with TNFα, IFNα2a and IFNγ. A comparison between disease-specific and *in vitro* generated signatures identified TNFα-, IFNα2a- and IFNγ-imprints in all three diseases. The monocyte profile from SLE was predominantly shaped by IFNα2a and IFNγ (IFNs), while the RA profile was mainly patterned by TNFα. Nevertheless, the TNFα imprint was also identified in SLE, and the IFNs imprints were also detected in RA. The AS profile showed the imprints of TNFα and IFNs as well, but a clear dominance of TNFα or IFNs was not detectable. The responses to cytokines in SLE, RA and AS were obviously much more complex than expected, considering the fact that TNFα and IFNs imprints in these rheumatic diseases were quantitatively and qualitatively different. More precisely, the imprints of TNFα and IFNs in SLE, RA and AS were shown to be disease-dependent.

5.1. Systemic nature of SLE, RA and AS was detectable on the level of peripheral blood monocytes

In this study, we have shown that disease-specific gene expression profiles characterized monocytes from SLE, RA and AS patients. In previous studies, PBMCs from SLE, RA and AS patients were utilized for transcriptome analyses, and it was obvious that the changes in gene expression affected different cell types[94,96,167]. However, a detailed insight into alterations that characterize a particular cell type, like monocytes, B-cells, T-cells, has not been provided to date. The changes in cellular composition of PBMCs limited the comparisons between patients and healthy donors and between patients with different diseases. Therefore, the gene-expression profiling of PBMCs was unable to show a real differences between rheumatic diseases.

It is known that many overlapping signs and symptoms characterize patients with SLE, RA and AS. Chronic inflammation, accompanied by the production of cytokines, like TNFα, IL6, type I IFN, IFNγ and BAFF, is evident in all three diseases[5-6,8,37,41,102-103,142]. Besides many similarities between SLE, RA and AS, this study has shown that monocytes were able to disclose these three diseases as different entities. Basically, each of the diseases was

characterized by specific gene-expression profile. For example, a strong up-regulation of genes such as IFI27, SIGLEC1, CCL2, CXCL10 and CXCL11 was specific for the SLE profile. An up-regulation of MAFF, IL8, JUN and CXCL2 was specific for the RA profile and an up-regulation of IL6R, MARCO and IRF5 was detectable only in the profile from AS patients.

The profiles from SLE, RA and AS patients exhibited different magnitudes of changes in gene expression. The total numbers of differentially expressed genes and their fold changes were greater in SLE and RA than in AS. We speculated that either the inflammation was stronger in SLE and RA when compared to AS, or that the alterations that accompanied the pathogenesis of AS were less obvious at the level of peripheral blood monocytes. It is known that chronic inflammation is permanently present in patients with SLE and RA, while in AS patients it is restricted to the early phase of disease development[37,41,49-50]. As previously mentioned, the pathogenesis of AS is characterized by three phases: inflammation, bone destruction and new bone formation. These phases are not necessarily coupled, and it is very likely that each of these phases is characterized by dissimilar transcriptomes[49-50,168]. In this study, the recruitment of AS patients was based on disease activity score determined by BASDAI. In calculation of this score, the parameters related to inflammation, such as CRP and ESR, and different phases of AS pathogenesis were not included. Thus, when all 14 AS patients included in this study were analysed together, it is very likely that the generated AS profile presented "only" the common characteristic for all three phases. The specificity for each particular phase was weakened, and the gene-expression profile of the early inflammatory phase was diluted.

This study demonstrated that SLE, RA and AS profiles were able to detect heterogeneity between patients with the same disease. The majority of SLE, RA and AS patients were characterized by a disease-specific gene-expression profile, although it was possible to identify patients that either displayed a weak disease profile or did not show any changes specific for a particular disease. One of the challenges in the field of rheumatology is to establish the objective criteria that allow a sub-stratification of patients with the same disease[169-170]. Microarray analysis is an expensive and laborious method for routine clinical diagnosis, but it is an exceptional source for identification of potential biomarkers that characterize a particular disease. To understand alterations embodied within the transcriptomes of SLE, RA and AS patients would be essential for identification of the relevant biomarkers. Finally, the extrapolation of identified biomarkers to clinical practice should lead to an improvement of diagnosis.

Ingenuity Pathway Analysis (IPA) was applied for a functional interpretation of SLE, RA and AS profiles. It showed that the profiles from these rheumatic diseases were altered (shaped) by cytokines, such as TNFα, IFNs, IL1 and IL8. This observation was in line with numerous other reports, which demonstrated that cytokines initiate and maintain the chronic inflammation in SLE, RA and AS[41,66,93,103,171-173]. Nevertheless, monocytes' potential to respond to cytokines by particular gene-expression patterns, determined as cytokine imprints, has not been demonstrated so far. To address these findings in a more specific way, the cytokine-specific gene-expression profiles were generated *in vitro* and used to determine the imprint of cytokines in various rheumatic diseases.

5.2. TNFα, IFNα2a and IFNγ modulate the gene-expression profiles in monocytes in a more or less specific way

To our knowledge, the generation and analysis of TNFα-, IFNα2a- and IFNγ-signatures in monocytes was for the first time demonstrated in this study. Monocytes were stimulated *in vitro* by pro-inflammatory cytokines for 1.5 hours. Whole blood from healthy donors was used as a medium for their stimulation. On the one hand, it closely resembles the physiological condition for monocytes activation, and on the other hand the artificial activation of monocytes by various isolation procedures has been avoided. It is known that monocytes are activated by CD14 positive selection and that they easily adhere to plastic surface. These two isolation procedures are related not just to the activation of monocytes but also to their differentiation into macrophages[174]. To have an overview of potential activation of monocytes in whole blood, two types of controls were included[161,175]. The first type represented monocytes that were immediately processed after blood was drawn, and the second type of control denoted monocytes that were incubated for 1.5 hours without any stimulation.

In vitro generated cytokine-signatures were characterized by immense numbers of up- and down-regulated genes. In most of the previous studies, the cytokine signature was characterized only by genes that were up-regulated[93,106,154]. Although almost identical numbers of up- and down-regulated genes were identified, the down-regulated genes were either ignored or excluded from the analyses. To have a complete overview of the cytokine response, we considered that up- and down-regulation of genes are equally important. This approach gave us a possibility to identify the genes that were regulated by various cytokines but in opposite directions. It also facilitated the determination of the interplay between

cytokines, where one cytokine induces the expression of particular genes and at the same time the other cytokine tends to suppress this effect.

Comprehensive analyses of the *in vitro* generated signatures induced by TNFα, IFNα2a and IFNγ suggested that the responses to various cytokines were interconnected. The cytokine responses were characterized by thousands of differentially expressed genes, which were categorized as cytokine-shared, cytokine-predominant and cytokine-specific.

Response to TNFα was different compared to responses induced by IFNα2a and IFNγ. For example, TNFα signature was characterized by a strong up-regulation of CCL20, CCL23, CXCL2, IL1A, IL8, IL18, NFκB1, NFκB2, MYC and TCF7L2 and a strong down-regulation of KLF9, CREB3, CREB5 and FCGR3A. This result is consistent with previous studies, which showed that TNFα induces activation of NFκB and that this transcription factor regulates the expression of previously mentioned cytokines[176-178]. The transcription factor NFκB is one of the best described molecules in the TNFα signalling pathway. Nevertheless, it is also known that many other transcription factors take part in controlling the expression of TNFα regulated genes, for instance AP1, MAFF, KLF10 and SPI1[68,176]. Therefore, the overall response to TNFα is roughly divided into responses that are controlled in a NFκB-dependent and NFκB-independent manner.

Regarding IFNα2a and IFNγ stimulation, the very similar gene-expression profiles were identified, although both cytokines act via binding to different receptors: IFNAR and IFNGR, respectively[87]. However, both types of receptors are able to activate the JAK-STAT signalling pathway and to regulate the expression of interferon-stimulated genes (ISG). The well known type I IFN-regulated genes were also induced by IFNγ. For example, both types of IFNs induced expression of the following genes: IFI35, IFIT1, IFIT4, MX1, MX2, OAS1, OAS2, OAS3, STAT1, TNFSF10 and CXCL10[86]. Considering this fact, we concluded that type I IFN is not the exclusive inducer of these genes. Although, a major part of IFNα2a- and IFNγ-signatures was identical, it was possible to detect differences between them. For example, differential expression of CD47, CD55, CITED2, CSF1R, CSF2RA, CXCL2, EGR1, FCAR, ICAM2, IL6R, IL8, NRF4A2 and TCF4 was specific for the IFNα2a signature, while differential expression of BCL6, C1QB, CD1D, CD300A, CD53, CXCL16, IFNAR1, IFNAR2, IL8RB, TGFB1 and TGFBR1 was specific for the IFNγ response.

A transcription factor STAT1 is activated both by IFNα2a and IFNγ (IFNs). It is considered as a dominant transcription factor for IFNs signalling that controls the expression of many so called typical IFN-regulated genes[86,179]. Nevertheless, many other transcription factors play a role in regulating gene expression after stimulation with IFNs[85,89,180-182]. Therefore, the cell

response to IFNα2a and IFNγ is considered to be regulated in a STAT1-dependent and STAT1-independent manner[85,89].

It is known that the gene-expression profiles induced by TNFα and IFNs are regulated by the interplay of numerous transcription factors. When the main transcription factors for TNFα and IFN signalling pathways were inhibited, NFκB and STAT1, the cell response to TNFα and IFNs was not completely blocked. The activation of the other transcription factors, induced by the alternative signalling pathways still enable the response to these cytokines[68,85,89,176]. The notion that the TNFα response is divided into a NFκB-dependent and NFκB-independent is oversimplified. The same simplification is applied by dissecting the overall IFN response into the part that is controlled in a STAT1-dependent manner and the other part that is controlled independently of STAT1. However, the idea that cytokine response can be dissected is relevant because it emphasises the ability of these inflammatory mediators to act by triggering both the canonical and alternative signalling pathways.

In this study we showed that the up-regulation of NFκB1 and NFκB2 was specific only for the TNFα signature, while the up-regulation of STAT1 was specific for the IFNα2a and IFNγ signatures. Interestingly, TNFα was able to silence expression of STAT1. Therefore, we concluded that TNFα was able to interfere with the typical IFN response, as will be discussed below.

Aside from STAT1, many other genes were shown to be regulated in opposite directions. They were up-regulated by TNFα and down-regulated by IFNs, and vice versa. For example, genes such as IFI16, IFI44, IFI44L, IFIT1, IFIT3, IFIT5, OAS1 and OAS2 were up-regulated by IFNs but down-regulated by TNFα. Therefore, to determine if these genes were up- or down-regulated can be relevant to determine the balance between TNFα and IFNs in conditions that characterized their common production. Interestingly, these genes were frequently detected in various rheumatic diseases, including SLE, RA and AS, and will be discussed below.

In summary, the comprehensive analysis of *in vitro* generated signatures, induced by TNFα, IFNα2a and IFNγ, suggested that the responses to cytokines were different. It was obvious that one part of cytokine signatures can be evoked by all cytokines investigated and it represented a more common inflammatory response. However, the major part of the cytokine signatures was specifically induced by TNFα or IFNs. Remarkably, the part of cytokine signatures was regulated in opposite directions by TNFα and IFNs. This part of cytokine signatures is of great value, because both TNFα and IFNs constitute the inflammatory milieus in various rheumatic diseases. Nevertheless, it has remained unknown how the responses to

these cytokines are interconnected. Thus, to identify the direction of their changes, like up- or down-regulation, can be relevant for determining the dominance of TNFα or IFNs within a particular inflammatory milieu.

5.3. How cytokines modulate the transcriptomes from SLE, RA and AS monocytes

The goal of this study was to estimate the role of pro-inflammatory cytokines in the pathogenesis of SLE, RA and AS. For this reason, *in vitro* generated TNFα-, IFNα2a- and IFNγ-signatures were utilized. Comparisons between *in vitro* generated signatures and SLE, RA and AS profiles identified the cytokine-regulated genes in all three diseases. However, TNFα-, IFNα2a- and IFNγ-regulated genes in SLE, RA and AS were found to be qualitatively and quantitatively different. Considering the overlap of total numbers of disease-associated genes and those identified by *in vitro* stimulation, it was evident that the overall influence of IFNα2a in shaping (altering) the profiles of diseases varied. The IFNα2a imprint was found to be the most dominant in SLE.

5.3.1. Overview of the IFNα2a imprints in SLE, RA and AS

The IFNα2a imprint in SLE was characterized by up-regulation of genes such as CCL2, CCL8, CXCL10, CXCL11, IL15, IFI16, IFI27, IFI35, IFI44, IFI44L, MX1, MX2, OAS1, OAS2, OAS3 and STAT1, and by down-regulation of genes including IL8, IL11RA, CENTD2, ENG, ID2, IER3, JUN, KLF2 and KLF4. It has been already shown that the type I IFN imprint was present in patients with SLE[92-94]. However, the IFN imprint was identified in PBMCs of SLE patients and the cell type that is the principal "carrier" of this imprint has remained unknown so far. We cannot rule out the imprint of type I IFN in other cell types from peripheral blood, but based on the analyses from this study it is obvious that monocytes are its crucial or at least very important carrier.

Previous studies considered 22 up-regulated genes as the type I IFN imprint and all these genes were identified in monocytes from SLE patients as well[93-94]. However, in this study the type I IFN imprint was not just extended by an absolute number of genes, but it was also broadened by inclusion of down-regulated genes. This approach facilitated the ability to analyse cross-talks between cytokines, where different cytokines regulate the expression of the same genes but in opposite directions.

STAT1 is the main transcription factor that characterizes the type I IFN imprints in SLE. It was found to be strongly up-regulated in monocytes from SLE patients and in monocytes stimulated by IFNs. Interestingly, an up-regulation of STAT1 is always accompanied by an

up-regulation of so called typical genes for IFN stimulation, more precisely of genes that are induced by the canonical IFN signalling pathway. The following genes belong to the typical IFN response: IFI16, IFI27, IFI35, IFI44, IFI44L, MX1, MX2, OAS1, OAS2, OAS3 and STAT1[85,92-94,183]. Promoter analysis of all genes that comprised SLE profile identified the transcription factor binding site (TFBS) for ISRE (Interferon Stimulated Response Element) as being over-represented. The ISRE represents the binding site for the heterotrimeric complex consisting of STAT1, STAT2 and IRF9[85]. Therefore, it can be concluded that the IFNα2a imprint in SLE is predominantly regulated by activation of STAT1 (in a STAT1-dependent manner).

The IFNα2a imprint was also identified in RA, but it was characterized by another set of genes including ATF3, CCL3, CCL4, EGR2, FOSL2, JUN, PIM1, SOCS3 and ZFP36. These genes were clearly different from those identified in SLE. Furthermore, their expression was not under the transcriptional control of STAT1 and additionally, STAT1 was shown to be down-regulated in RA patients[181,184]. Promoter analysis of genes that comprise the RA profile did not reveal TFBS for STAT1. Since it is known that transcription factors like AP1, IRF1, IRF4, IRF8 and PU.1 can also control the expression of IFN regulated genes, the imprint of IFNα2a in RA patients was considered to be regulated in a STAT1-independent manner[85,181-182,184].

Regarding the literature data, the type I IFN imprint in RA has been interpreted inconsistently. The cause of misinterpretation was the absence of direct comparisons between the RA profiles and the whole type I IFN signature. In all previous studies only 22 up-regulated genes, which were shown to be type I IFN signature in SLE, were utilized for estimating the type I IFN imprint in RA[93,95-99,106,145]. It is worth mentioning that the type I IFN imprint in RA was not characterized by the up-regulation of all 22 genes and furthermore, an up-regulation of STAT1 was also not identified[96,106,145]. The disease-specific differences of the type I IFN imprints have not been taken into account so far. An additional complexity appeared when the type I IFN imprint was identified in RA patients but only after treatment with anti-TNFα drugs[92,96,106]. More precisely, STAT1 and its co-expressed genes: IFI16, IFI35, IFI44L, IFIT1, MX1, MX2, OAS1 and OAS2 were shown to be up-regulated in RA patients after anti-TNFα treatment. In fact, the type I IFN imprint in RA patients after treatment was very similar to that from SLE patients.

The IFNα2a imprint in AS was characterized by the following genes ATF3, NR4A2, CCL3, FAS, STAT3, TNFSF10, IFI16, IFI35, IFIT1, IFIT2, IFIT3, MX1, OAS1, OAS2 and OAS2. The IFNα2a imprint in AS was found to have relatively weak changes in gene expression. As

previously mentioned, the main characteristics of the gene expression profile in AS were the weak alterations in gene expression and great heterogeneity between patients. Difficulties in uncovering the clear IFNα2a imprint in AS can be demonstrated by the changes in expression of STAT1. The change in STAT1 expression in AS was compared with alterations in its expressions in SLE and RA. For instance, STAT1 (determined by the probe-set 200887_s_at) in SLE was up-regulated. It exhibited increased expression in ~63% and decreased expression in ~3% of pair-wise comparisons. STAT1 in RA was found to be down-regulated, where its expression increased in ~5% and decreased in ~34% of pair-wise comparisons. STAT1 in AS was shown to be down-regulated, and its expression increased in ~17% and decreased in ~43% of pair-wise comparisons. Although STAT1 was identified to be down-regulated in AS, it showed the greater heterogeneity in its expression as compared to that in SLE and RA. The similar variations in expression characterized the other IFN regulated genes in AS, such as IFI16, IFI35, IFIT1, IFIT2, IFIT3, MX1, OAS1, OAS2 and OAS2. They displayed an increased expression in 30-45% of the pair-wise comparisons and a decreased expression in 20-40% of the pair-wise comparisons. Although, all of these genes exhibited the increased expression values, the magnitudes of changes in their expression were rather weak when compared to that in SLE. Promoter analysis of the AS profile identified TFBS for ISRE to be over-represented. Nevertheless, the type I IFN response was very weak if compared to that in SLE and RA.

In summary, the gene-expression profiles from SLE, RA and AS demonstrated that the response to IFNα2a was dissimilar in different diseases. The IFNα2a imprints in SLE and RA were found to be regulated in a STAT1-dependent and STAT1-independent manner, respectively. The IFNα2a imprint in AS was rather weak and very heterogeneous.

5.3.2. The similarities between IFNγ and IFNα2a imprints

As already discussed the *in vitro* generated type I and type II IFNs signatures were shown to be very similar. Considering this fact it was expected that the IFNγ imprints would overlap the related IFNα2a imprints in SLE, RA and AS[87,93]. The IFNγ imprints in SLE and RA were also found to be regulated in a STAT1-dependent and STAT1-independent manner, respectively. Nevertheless, the IFNγ imprints in SLE, RA and AS were characterized by a lower absolute number of genes when compared to the IFNα2a imprints. Interestingly, production of type I IFN and IFNγ was identified both in SLE and RA, but it is often ignored that IFNγ plays a role in their pathogenesis[185-186]. The role of IFNγ is emphasized by the fact

that autoantibodies against both type I and type II IFNs are detectable in patients with SLE[187-189].

The analyses of IFNγ imprints in SLE, RA and AS ascertained the same heterogeneity among patients. The same patients who displayed a strong IFNα2a imprint also showed a strong IFNγ imprint. From these results it can be concluded that the identification of the IFN imprints might be helpful for a sub-classification of patients and for an improvement of diagnosis. There are increased numbers of biologicals that target particular cytokines, like TNFα, type I IFN, IFNγ, IL1 and IL6. One of the aims in the field of rheumatology is to identify which cytokine exhibits the most dominant role in altering profiles of diseases. The analyses of IFNα2a and IFNγ imprints in rheumatic diseases emphasize difficulties in creating a suitable anti-IFN drug. In fact, neutralisation of one member of the IFN family might not result in diminishing the influence of IFNs in general. Namely, the other members of this family might induce the same or very similar response. Thus, to neutralize the various members of the IFN family is a demanding approach for therapeutic intervention. On the other hand, the various members of the IFN family bind two types of receptors, either type I IFNR or type II IFNR. Therefore, a better strategy for silencing the effects of IFNs might be to block receptors for IFN, either receptors for type I or type II IFNs, or if necessary to block both types of receptors at the same time.

5.3.3. Overview of the TNFα imprints in SLE, RA and AS

The gene expression patterns that characterize TNFα response in SLE, RA and AS were specific for each disease. The TNFα imprint was the most dominant in RA patients. The qualitatively and quantitatively different imprints of TNFα were identified in patients with SLE and AS. The TNFα imprints in SLE and RA were weaker in terms of the number of genes that compose the TNFα imprints and in terms of the magnitude of their changes.

To discriminate the TNFα imprints in SLE, RA and AS, we utilized the expression of NFκB, which represents the main transcription factor for canonical TNFα signalling pathway. Since NFκB2 was identified as being up-regulated in RA and down-regulated in SLE, the TNFα imprints in RA and SLE were related to the expression of this transcription factor. Thus, the TNFα imprints in RA and SLE were considered to be regulated in the NFκB2-dependent and NFκB2-independent manner, respectively. TNFα imprint in AS was more similar to that in RA and NFκB2 was also found to be up-regulated. Nevertheless, the TNFα imprint in AS was weaker than that in RA.

As discussed before, the concept of dividing the TNFα imprints into the NFκB2-dependent and NFκB2-independent is relatively simplified. We are aware that many transcription factors regulate the expression of genes after TNFα stimulation[68,176]. Furthermore, there are five transcription factors that constitute the NFκB family: NFκB1, NFκB2, RELA, cREL and RELB[178]. Each family member does not act alone, but takes part in the formation of different homo- or heterodimers. It is also speculated that different dimers regulate the expression of various genes. Therefore, to determine the real pattern of genes that are regulated by particular combinations of NFκB homo- and heterodimers requires more detailed analyses. For example, ChIP-on-chip study, which presents a combination of chromatin immunoprecipitation and microarray technology, can provide a real evidence for genes that are under the transcriptional control of NFκB. However, this type of analyses exceeds the purpose of this study.

Promoter analysis of the SLE, RA and AS profiles disclosed the presence of TFBS for NFκB in all three diseases. This finding was expected for the RA and AS profiles, but its identification in the SLE profile was initially surprising. However, it has been shown recently that NFκB negatively regulates the expression of typical IFN regulated genes[110-111]. Since the SLE profile was characterized by the up-regulation of typical IFN regulated genes, these findings are in agreement with the results from this study.

To determine the heterogeneity between patients based on the TNFα imprint would be essential for an improvement of diagnosis in chronic rheumatic diseases. Anti-TNFα therapy has been approved for the treatment of RA and AS patients and there is some evidence that it might be beneficial for a particular subgroup of SLE patients[47,171,190]. It is important to mention that 30-40% of RA and AS patients either do not respond or respond only moderately to anti-TNFα treatment[190-194]. The anti-TNFα therapy is a pressing clinical and healthcare problem for the reason that it is associated with side-effects and very high costs[195]. Therefore, to predict who will respond to anti-TNFα treatment and to treat just those patients would be beneficial for patients and the health care system.

5.3.4. Monocytes transcriptome towards biomarker discovery

As previously stated, TNFα and IFNs imprints in SLE, RA and AS were found to be disease-specific. Nevertheless, it was remarkable that part of the IFNs imprint in SLE overlapped with part of the TNFα imprint in RA. Interestingly, these parts were composed of genes whose expression changed in the opposite directions by TNFα and IFNs. For example, a substantial number of the IFNs up-regulated genes in SLE were identified to be down-regulated by TNFα

in RA, like IFI44, IFI44L, IFIT1, IFIT5, OAS1, OAS2, OAS3 and STAT1. Taking into account that TNFα exerts a more dominant role in RA than in SLE, and that STAT1 and its regulated genes were decreased in RA by TNFα, the expression of inversely regulated genes is valuable source for estimating the dominance or IFNs and TNFα in various diseases. Interestingly, the above mentioned genes were also reported as being up-regulated after anti-TNFα therapy in RA and SoJIA patients[97,106]. The typical IFN signatures, determined to be STAT1-dependent, appeared in these patients after treatment. Obviously, neutralisation of TNFα in RA and SoJIA patients favours the up-regulation of those genes that were previously silenced by TNFα.

Taken as a whole, the inflammatory milieus that characterize the various rheumatic diseases are different. Even dough the same cytokines might constitute these milieus, their potential in inducing changes is not the same. The results from this study showed that there are different hierarchies of cytokines in various diseases. To determine the role of the most dominant cytokine(s) in a particular disease and for each individual patient would be crucial for selecting the correct anti-cytokine drug(s). There are increasing demands for identification of biomarkers that are related to the dominance of a particular cytokine. Namely, over the last decade the number of anti-cytokine drugs noticeably increased, but a therapy design is still based on a "guesswork" approach. It is unknown if the patient will benefit from a given therapy or not.

This study focused on characterization of SLE, RA and AS monocytes at the transcriptional level. Transcription is the step between information carried by the genes and synthesis of proteins. Thus, the transcriptome analysis is often associated with questions surrounding the final outcome of transcriptional changes, and how they are related to alterations on the protein level. In this study we showed that changes in the gene expression of surface markers CD64, CD244, CD120b and CD128b in monocytes from SLE patients was related to their alteration on the protein level.

A currently ongoing project measures the activation of the transcription factors at the protein level in monocytes from SLE, RA and AS patients. Besides STAT1 and NFκB, many other transcription factors are included in the analyses such as: AP1, TP53, MYC, MAFF, SPI1, NR4A2 and IRF family members. These transcription factors are induced by TNFα or IFNs, and they characterize activation of their canonical and alternative signalling pathways[68,85,89,176]. Therefore, we hypothesised that they would be relevant to determine the dominance of particular cytokines within the disease-specific inflammatory milieu. The following step will be to identify the target genes for these transcription factors. This strategy

should make it possible to extrapolate data from the complex transcriptome analyses to routine clinical practice.

Discovery of biomarkers should enable early diagnosis, stratification of patients with the same disease and implementation of targeted therapy. The ultimate goal is to identify biomarkers that characterise individuals with the highest risk for development of a particular disease. Since the signs of autoimmunity are detectable many years before the onset of diseases, identification of biomarkers that characterize this phase would be of immense importance[170]. The whole management of rheumatic diseases might be shifted from diagnosis and therapeutic stratification to prevention, early detection and early treatment. This approach would certainly benefit patients and the healthcare system.

In this study we showed that monocyte transcriptome is an exceptional source for the identification of disease-associated biomarkers. Monocytes produce a plethora of cytokines and they also respond rapidly to them. Given that cytokines possess both beneficial and deleterious effects, knowledge about high-resolution cytokine signatures, as described in this study, opens new avenues in biomarker discovery.

6. References

6. References:

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7. Appendix

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7.2. Abbreviations

ACPA - antibodies to citrullinated proteins and peptides

ACR - American college of rheumatology determines the criteria for diagnosis of chronic rheumatic diseases, including RA and SLE

ANA - anti-nuclear antibody

AP1 - activating protein-1, a dimeric transcription factor composed of proteins that belong to c-Jun, c-Fos or ATF family

APC - antigen presenting cell

APRIL - member of TNF ligand superfamily. Synonyms are TNFSF13 and CD256

AS - ankylosing spondylitis

BAFF - member of TNF ligand superfamily. Synonyms are TNFSF13B and BLYS.

BASDAI - Bath Ankylosing Spondylitis Disease Activity Score

BCL6 - B-cell CLL/lymphoma 6

C1QB - complement component 1, q subcomponent, B chain

CCLx - chemokines

CD - cluster of differentiation (cluster of designation) is mark for cell surface molecules presented on white blood cells. In total, up to 350 CD molecules have been identified in humans so far

CENTD2 - centaurin, delta 2

CITED2 - Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2

CRP - C-reactive protein

CSF1R - colony stimulating factor 1 receptor

CSF2RA - colony stimulating factor 2 receptor, alpha, low-affinity (granulocyte-macrophage)

CXCLx - chemokines

DAS28 - disease activity score 28

Diarthrodial joints - are freely moveable joints, like those in knee, wrist, and hands

DMARD - disease-modifying anti-rheumatic drugs. This group of drugs includes: methotrexate, leflunomide, azathioprine, cyclosporine, sulfasalazine, choloroquine, but also biologicals like, adalimumab, infliximab, rituximab

EGR1 - early growth response 1, is a transcriptional regulator

ESR - erythrocyte sedimentation rate

FACS - fluorescence activated cell sorting

FCAR - Fc fragment of IgA, receptor for, is receptor expressed on the surface of myeloid lineage cells

FITC - Fluorescein isothiocyanate

GAS - Interferon-gamma activated sequence

GWA - genome wide association study

ICAM2 - intercellular adhesion molecule 2

HLA-DR - human leukocyte antigen DR, is the cell surface receptor complex that belongs to MHC class II molecules, together with HLA-DP, HLA-DQ, HLA-DP and HLA-DM. These types of receptors are expressed on professional antigen presenting cells, like macrophages, DC and B-cells.

HLA-B27 - human leukocyte antigen B27 is a cell surface receptor that belong to MHC class I molecules, together with HLA-A, other members of HLA-B and HLA-C molecules.

HC - hierarchical cluster

HM - Heat-map

IFN - interferon

IFNAR1 - interferon (alpha, beta and omega) receptor 1

IFNAR2 - interferon (alpha, beta and omega) receptor 2

IFNs - type I and type II interferons, in this study IFNα2a and IFNγ

IFN type I: includes 17 subtypes of interferons: 13 subtype of IFNα, IFNβ, IFNω IFNκ and IFNε

IFN type II: synonym for IFNγ

IFN type III: includes 3 subtypes of IFNλ: IFNλ1, IFNλ2 and IFNλ3

ILx - interleukins are group of cytokines that were initially recognised to be produced by leukocytes. This term derives from (*inter*-) as a means of communication, and (-*leukin*) as fact that many of these proteins are produced by leukocytes and act on leukocytes. Later it has been found that interleukins are produced by a wide variety of body cells

IRFx - interferon regulatory factor, family of transcription factors composed of IRF1, IRF2, IRF3, IRF4, IRF5, IRF6, IRF7, IRF8 and IRF9

ISG - interferon stimulated genes, so far it has been identified >300 these genes

ISGF3 - Interferon stimulated gene factor, presents a complex of 3 transcription factors: STAT1, STAT2 and IRF9

ISRE - Interferon stimulated response element, sequence within promoter region of ISG

JAK - Janus kinase, a family of tyrosine kinases

mDC - myeloid DCs

MD-2 (LY96) – myeloid differentiation protein 2

MMP - metalloproteinases MMP1, MMP3, MMP9, MMP12, the proteolitic enzymes that promote joint destruction

MTX - Methotrexate, the most common drug used in treatment of RA patients. It acts as anti-metabolite and anti-folate drug

NFκB - nuclear factor-κB, a transcription factor whose 5 members, p50, p52, RelA (p65), RelB and c-Rel form homo- and heterodimers and regulate expression of many genes

NR4A2 - nuclear receptor subfamily 4, group A, member 2. According to Entrez Gene summary and Swiss-Prot the protein encoded by this gene may act as a transcription factor and as a general coactivator of gene transcription

NSAID - non-steroidal anti-inflammatory drug are group of drugs with analgetic and antipyretic activity. In higher doses they exhibit anti-inflammatory effect. In this group of drugs belong: ibuprofen, naproxen and acetylsalicylic acid.

PBMC - peripheral blood mononuclear cells: monocytes, T cells, B cells, NK cells, DCs

Pannus formation - a sheet of inflammatory granulation tissue, composed of immune cells, blood vessels and fibrous cell, which spreads from the synovial membrane and ultimately invades the joints in RA

pDC - plasmocytoid DC

PRR - pathogen-associated pattern recognition receptors

RA - rheumatoid arthritis

RF - rheumatoid factor, an antibody against the Fc portion of IgG

Sacroiliac joint - are the joint between the sacrum and ilium, which present the larger bones of the pelvic region.

SLE - systemic lupus erythematosus

SLEDAI - systemic lupus erythematosus disease activity score

SoJIA - systemic onset juvenile idiopathic arthritis

STATx - signal transducer and activator of transcription, a family of transcription factors composed of seven members STAT1, STAT2, STAT3, STAT4, STAT5 (STAT5A and STAT5B), and STAT6

TCF4 - transcription factor 4

TGFB1 - transforming growth factor, beta 1

TGFBR1 - transforming growth factor, beta receptor 1

TLR - Toll-like receptor, a type of pattern recognition receptor

TNFα - tumor necrosis factor-alpha

7.3. Abstract

Chronic rheumatic diseases like systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and ankylosing spondylitis (AS) define a group of disorders with unknown aetiology. There are several lines of evidence suggesting that cytokines play an important role in their pathogenesis and in the maintenance of chronic inflammation, including TNFα, type I IFN, IFNγ, IL1, IL6, IL17 and BAFF. To estimate the role of cytokines in chronic rheumatic diseases is intriguing considering that they act together within complex cytokine networks. The role of cytokines has been emphasised by the fact that the increasing numbers of anti-cytokine drugs have been approved for clinical applications. However, a significant proportion of patients showed only a partial response or failed to respond to this type of treatment. Thus, to estimate the response to various cytokines in chronic rheumatic diseases would be essential for a better understanding of disease pathogenesis and for the identification of the most adequate target(s) for therapeutic intervention.

The aim of this study was to determine the role of pro-inflammatory cytokines in the pathogenesis of SLE, RA and AS. Global gene-expression profiling has been considered as a strategy that provides a comprehensive insight into transcriptional alterations that characterize various diseases. Gene-expression profiles generated in monocytes from SLE, RA and AS patients were found to be disease-specific. Functional annotation of disease-specific profiles identified the effects induced by various cytokines, including TNFα, type I IFN, IFNγ, IL1 and IL8. However, the response to these cytokines was disclosed to be different in SLE, RA and AS. To address this question in more detail, the cytokine-specific gene expression profiles were generated by stimulating monocytes *in vitro* with TNFα, IFNα2a and IFNγ (IFNs).

Comparisons between disease-specific and the *in vitro* generated reference signatures showed that the SLE profile was predominantly driven by IFNs, while the RA profile was primarily influenced by TNFα. The IFNs response in SLE was characterized by an activation of the transcription factor STAT1. Interestingly, the activation of STAT1 was found to be silenced by TNFα in patients with RA. However, the IFN imprints were also identified in RA and the TNFα imprint was evident in SLE. It was obvious that the responses to the same cytokines in SLE and RA were identified to be qualitatively and quantitatively different.

Unlike SLE and RA, monocytes from AS showed weak changes in gene expression. The responses induced by IFNs and TNFα in AS were disclosed as rather vain imprints, and the dominance of a particular cytokine was less obvious.

Altogether, this study has demonstrated that monocytes from SLE, RA and AS exhibit disease-specific gene-expression profiles, which can be molecularly dissected when compared to the *in vitro* generated cytokine-specific signatures. The IFNs and TNFα imprints were identified to be disease-dependent and principally they reflected the interplay of cytokines within various inflammatory milieus. The results from this study suggest that estimating the imprints of cytokines in rheumatic diseases would be indispensible for an improvement of diagnosis, proper selection of particular cytokine target(s) for therapeutic intervention and for following up and predicting the response to anti-cytokine drug(s). Ultimately these results should help clinicians to personalize treatment for each rheumatic patient.

7.4. Zusammenfassung

Chronisch-rheumatische Erkrankungen, wie der systemische Lupus Erythematodes (SLE), die rheumatoide Arthritis (RA) und die ankylosierende Spondylitis (AS) sind durch das Auftreten von Autoimmunreaktionen gekennzeichnet, deren Ätiologie bisher weitgehend unbekannt ist. Obwohl in zahlreichen Untersuchungen klar gezeigt werden konnte, dass an der Pathogenese und der Chronifizierung dieser Erkrankungen massgeblich pro-inflammatorische Zytokine beteiligt sind, so wie TNFα, type I IFN, IFNγ, IL1, IL6, IL17 und BAFF, bleibt bisher weitgehend unverstanden, wie diese Mediatoren innerhalb eines komplexen Zytokin-Netzwerks miteinander interagieren. Die pathophysiologische Bedeutung von Zytokinen in SLE, RA und AS wird durch die Tatsache hervorgehoben, dass in den letzten 10 Jahren eine zunehmende Anzahl von Antikörper-basierten anti-Zytokin Medikamenten für klinische Anwendungen zugelassen worden sind.

Ziel dieser Studie ist es, die pathophysiologische Rolle von pro-inflammatorischen Zytokinen im Krankheitsverlauf von SLE, RA und AS zu bestimmen und zu vergleichen. Hierzu wurden zunächst globale Genexpressionsprofile von peripheren Blutmonozyten generiert, die aus dem Blut von Rheumapatienten und gesunden Spendern isoliert worden sind. Mit Hilfe dieses experimentellen Ansatzes ist es möglich, die Genaktivitäten sämtlicher bekannten Gene gleichzeitig quantitativ zu erfassen. Durch den Vergleich der Krankheiten untereinander und dem Vergleich zu den gesunden Spendern konnte erstmals gezeigt werden, dass periphere Monozyten krankheitsspezifische Transkriptionsmuster aufweisen. Nach funktioneller Annotation der krankheits-assoziierten Gene konnte eindeutig die Beteiligung verschiedener Zytokine nachgewiesen werden, allen voraus TNFα, type I IFN, IFNγ, IL1 und IL8. Interessanterweise konnte für diese Zytokine gezeigt werden, dass die durch diese Mediatoren induzierten zellulären Antworten in Abhängigkeit von der jeweiligen Erkrankung unterschiedlich moduliert worden sind. Um diese Beobachtung molekular weiter aufschlüsseln zu können, wurden zusätzlich Zytokin-spezifische Genexpressionsprofile durch *in vitro* Stimulation von Monozyten mit TNFα, IFNα2a und IFNγ (IFN) erstellt.

Vergleiche zwischen den Krankheits-spezifischen und den *in vitro*-generierten Referenzsignaturen zeigten, dass das Transkriptom von Monozyten beim SLE durch IFN-induzierte Gene dominiert ist, während das RA Genexpressionsprofil hauptsächlich durch TNFα beeinflusst ist. Die IFN-Effekte beim SLE wurden ganz offensichtlich primär durch den überexprimierten Transkriptionsfaktor STAT1 vermittelt. Interessanterweise konnte gezeigt werden, dass in Monozyten von RA Patienten die Aktivierung von STAT1 durch die

Überexpression von TNFα inhibiert wurde. Allerdings liessen sich auch IFN-induzierte Signaturen bei RA und TNFα-induzierte Signaturen in SLE Monozyten nachweisen. Diese Ergebnisse lassen die Schlussfolgerung zu, dass die transkriptionellen TNFα und IFN-Antworten auf unterschiedliche Weise krankheitsabhängig bei SLE und RA moduliert werden. Im Gegensatz zu SLE und RA, zeigten Monozyten von AS Patienten generell nur schwache Veränderungen in der Genexpression, und obwohl TNFα und IFN-induzierte Gensignaturen nachweisbar waren, konnte aufgrund der niedrigen Expressionsstärken kein dominierendes Zytokin nachgewiesen werden.

Zusammenfassend konnte diese Studie zeigen, dass Monozyten von SLE, RA und AS Patienten Krankheits-spezifische Genexpressionsprofile aufwiesen, die durch den Vergleich mit *in vitro*-generierten, Zytokin-spezifischen Gensignaturen im Hinblick auf dominierende und interagierende Zytokine wesentlich detaillierter analysiert werden konnten. Die IFN- und TNFα-vermittelten Gensignaturen zeigten krankheitsabhängige Modulationsmuster und spiegelten das Zusammenspiel von Zytokinen innerhalb verschiedener inflammatorischer Milieus wieder. Die Ergebnisse dieser Studie legen den Schluss nahe, dass die Erhebung eines molekularen Zytokinstatus bei Rheumapatienten von grosser Bedeutung für eine verbesserte Differentialdiagnose und eine gezielte Therapieempfehlung sein kann.

Hierdurch würde man dem generell in der modernen Medizin angestrebten Grundsatz der personalisierten Medizin einen entscheidenden Schritt näher gekommen.

7.5. Enlarged Figures

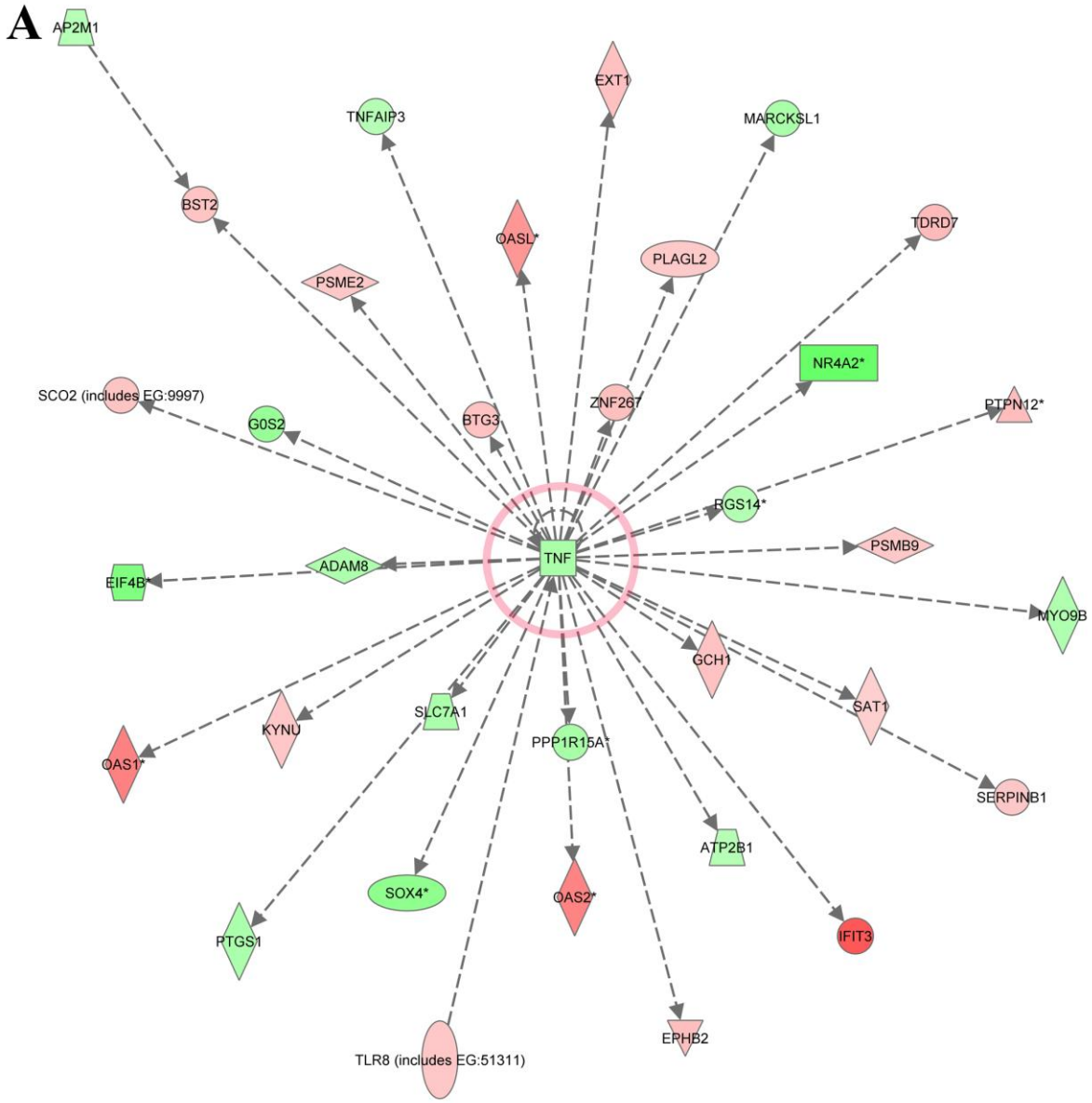


Figure 4.9.A Analysis of the SLE gene-expression profile by IPA revealed the influence of cytokines in shaping profile from disease. This figure shows the molecular network of differentially expressed genes in SLE where TNFα was considered as the central player. Up- and down-regulated genes were shaded in red and green, respectively.

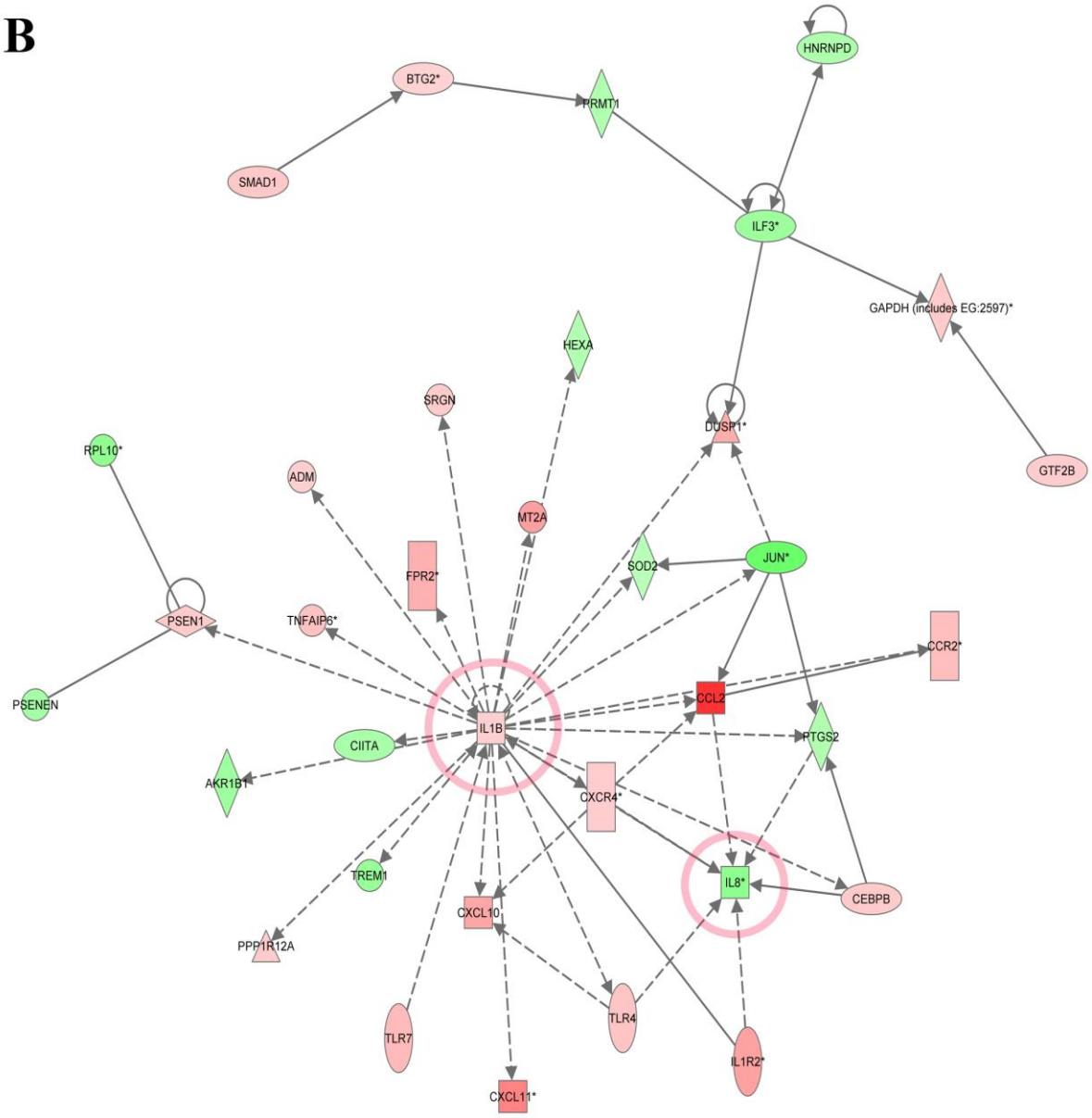


Figure 4.9.B Analysis of the SLE gene-expression profile by IPA revealed the influence of cytokines in shaping profile from disease. This figure shows the molecular network of differentially expressed genes in SLE where IL1 and IL8 were considered as the central players. Up- and down-regulated genes were shaded in red and green, respectively.

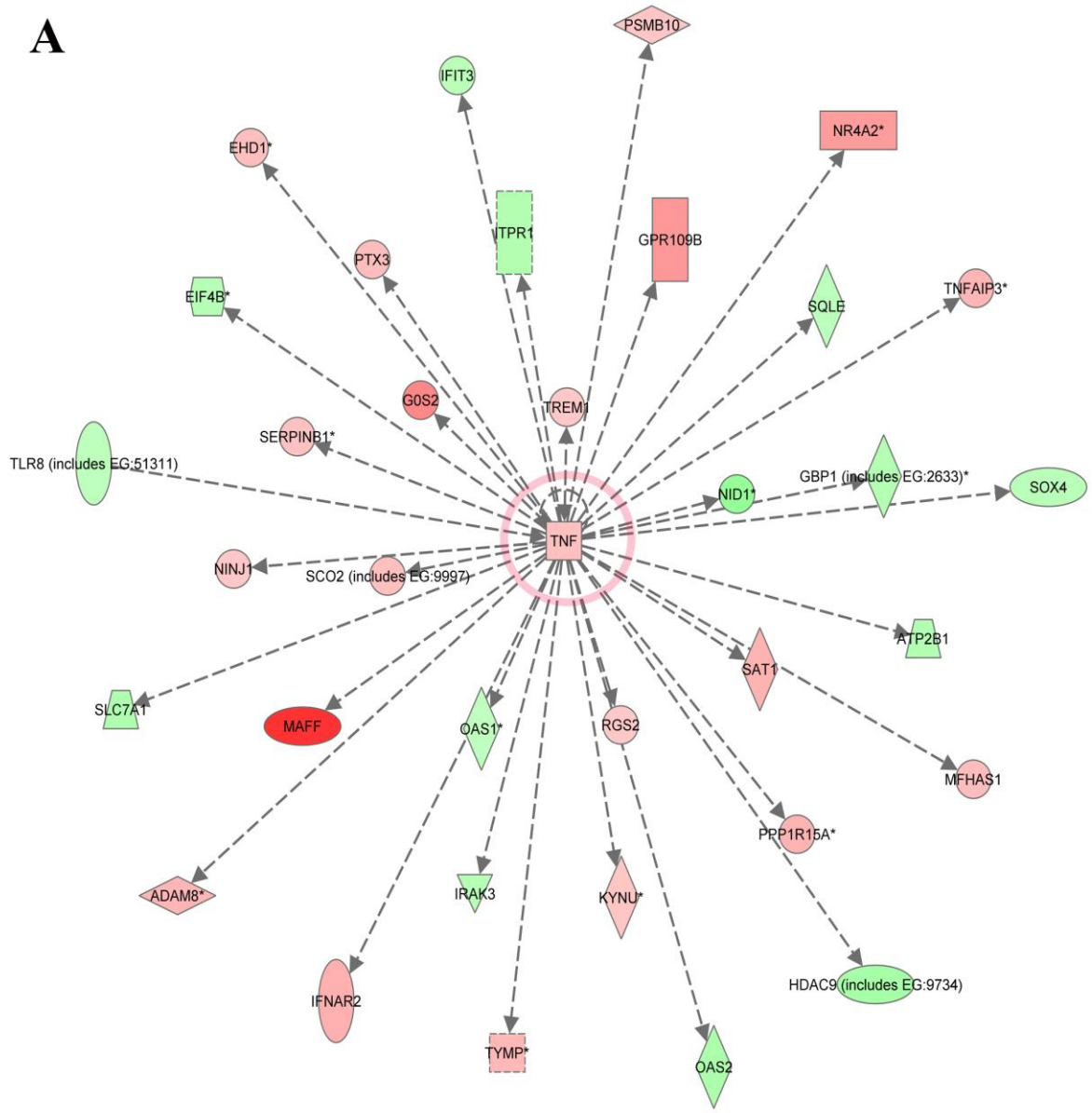


Figure 4.10.A Analysis of RA gene-expression profile by IPA revealed the influence of cytokines in shaping profile from disease. This figure shows the molecular network of differentially expressed genes in RA where TNFα was considered as the central player. Up- and down-regulated genes were shaded in red and green, respectively.

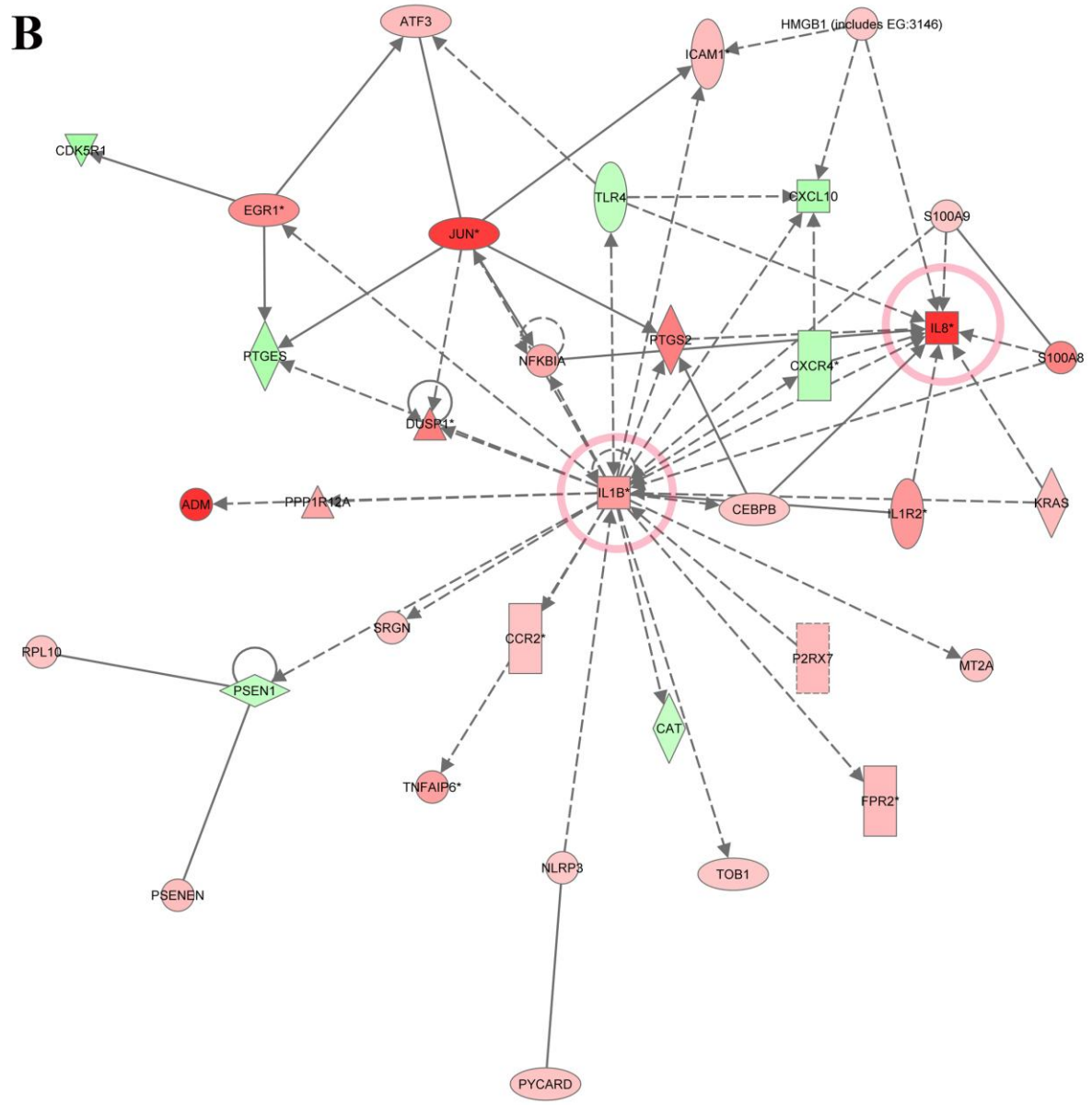


Figure 4.10.B Analysis of RA gene-expression profile by IPA revealed the influence of cytokines in shaping profile from disease. This figure shows the molecular network of differentially expressed genes in RA where IL1 and IL8 were considered as the central players. Up- and down-regulated genes were shaded in red and green, respectively.

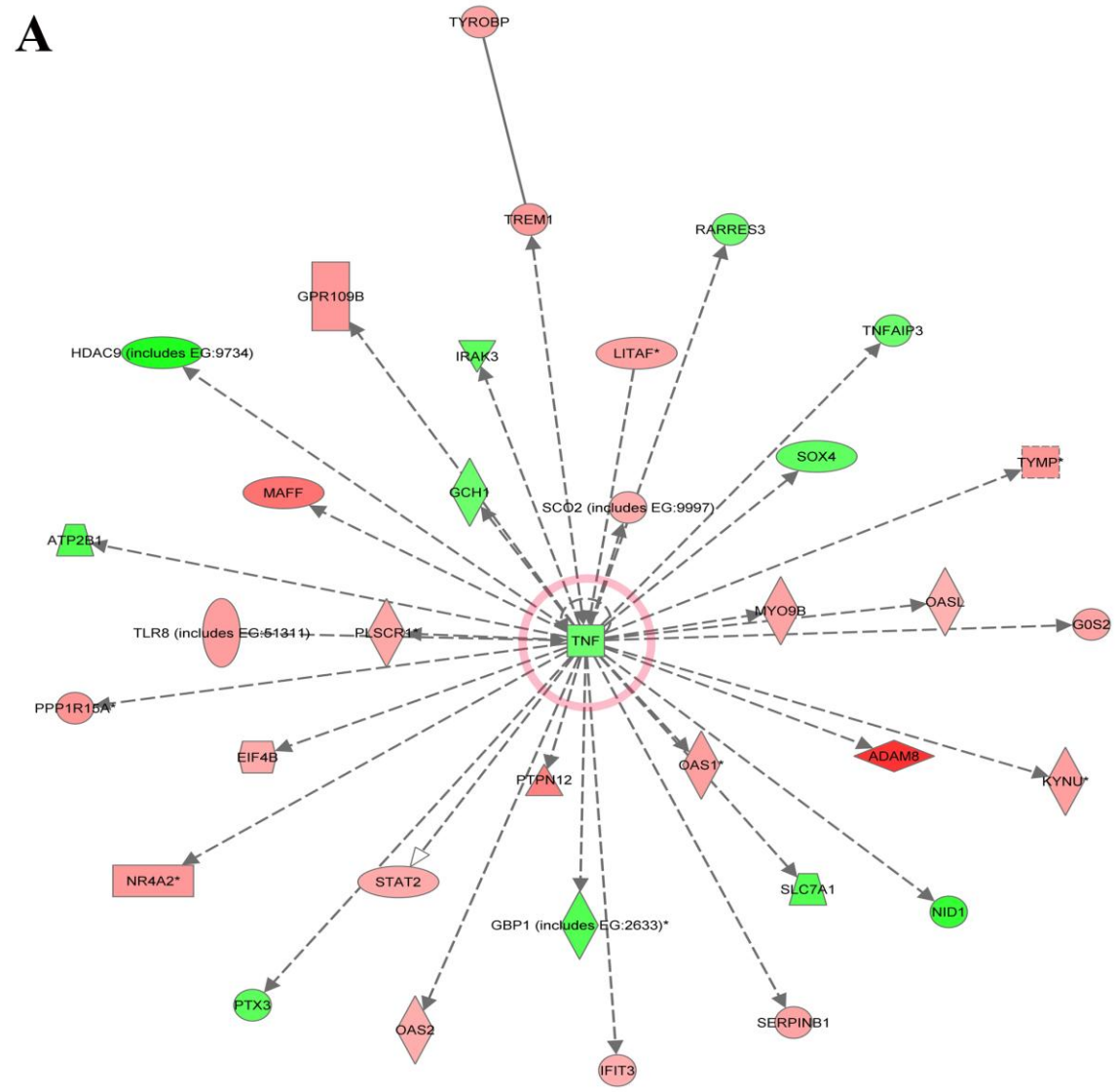


Figure 4.11.A Analysis of AS gene-expression profile by IPA revealed the influence of cytokines in shaping profile from disease. This figure shows the molecular network of differentially expressed genes in AS where TNFα was considered as the central player. Up- and down-regulated genes were shaded in red and green, respectively.

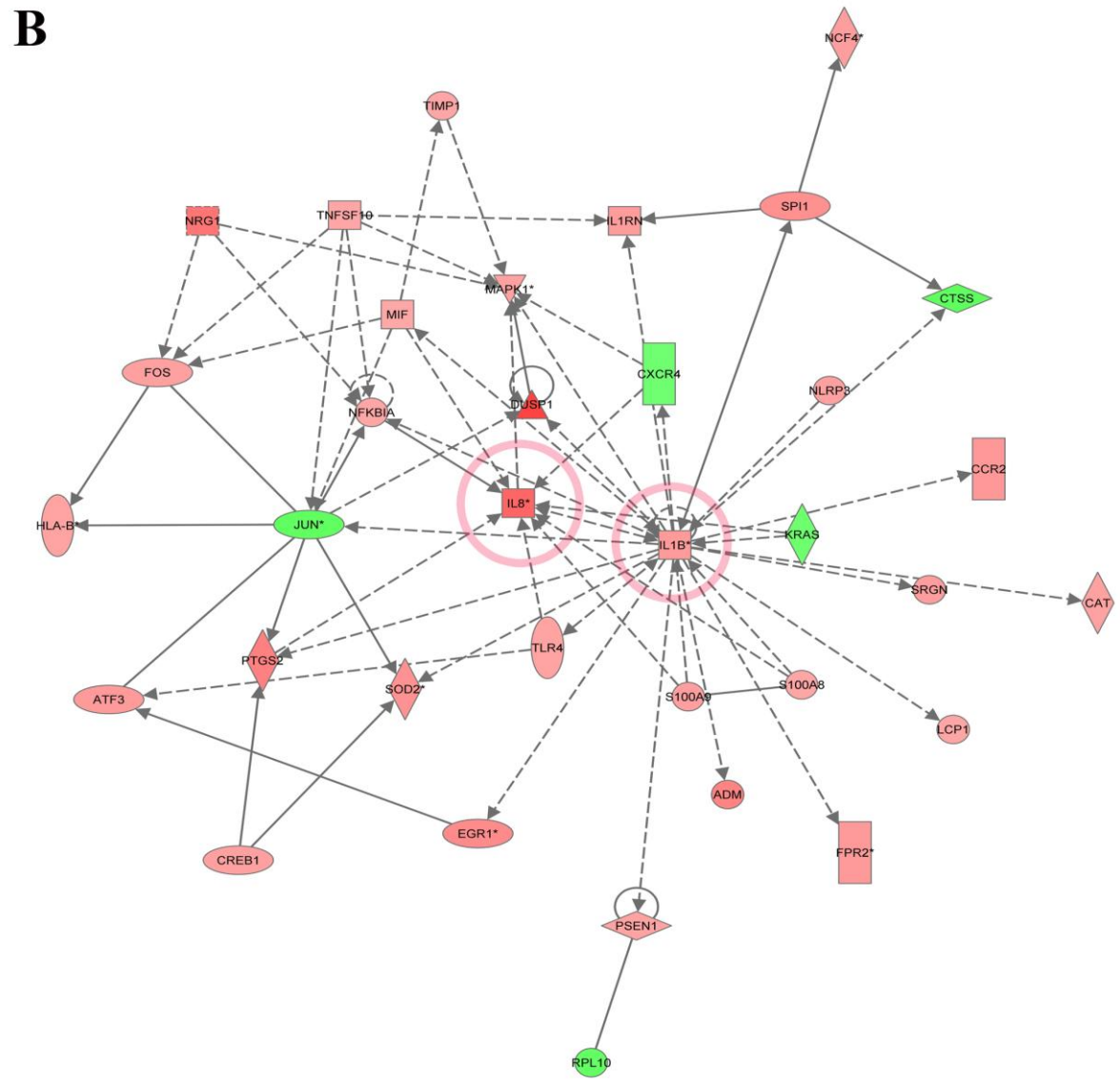


Figure 4.11.B Analysis of AS gene-expression profile by IPA revealed the influence of cytokines in shaping profile from disease. This figure shows the molecular network of differentially expressed genes in AS where IL1 and IL8 were considered as the central players. Up- and down-regulated genes were shaded in red and green, respectively.

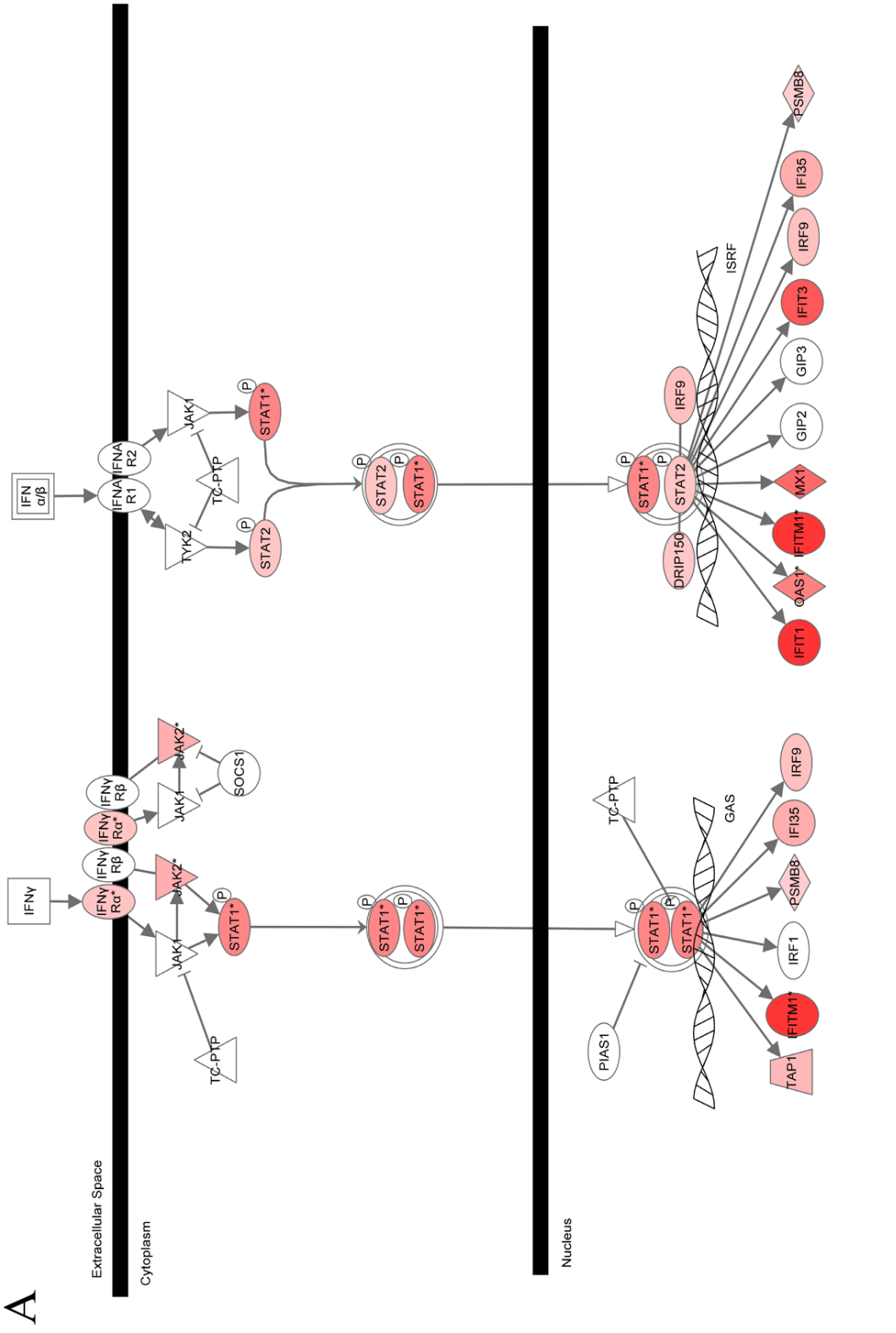


Figure 4.12.A Analysis of SLE gene-expression profile by IPA revealed the alterations within IFN type I and IFN type II signalling pathways. Up- and down-regulated genes were shaded in red and green, respectively.

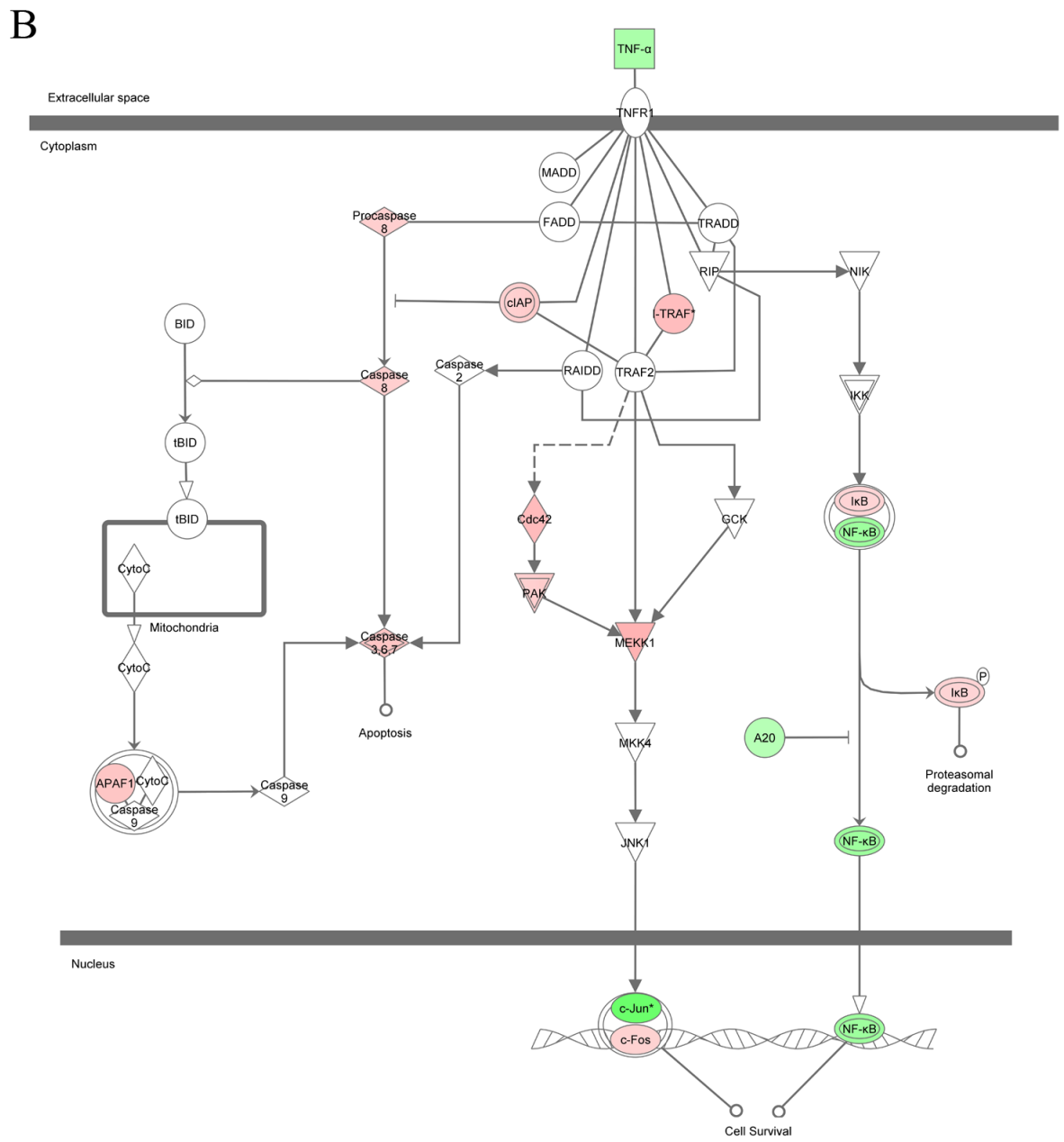


Figure 4.12.B Analysis of SLE gene-expression profile by IPA revealed the alterations within TNFα signalling pathway. Up- and down-regulated genes were shaded in red and green, respectively.

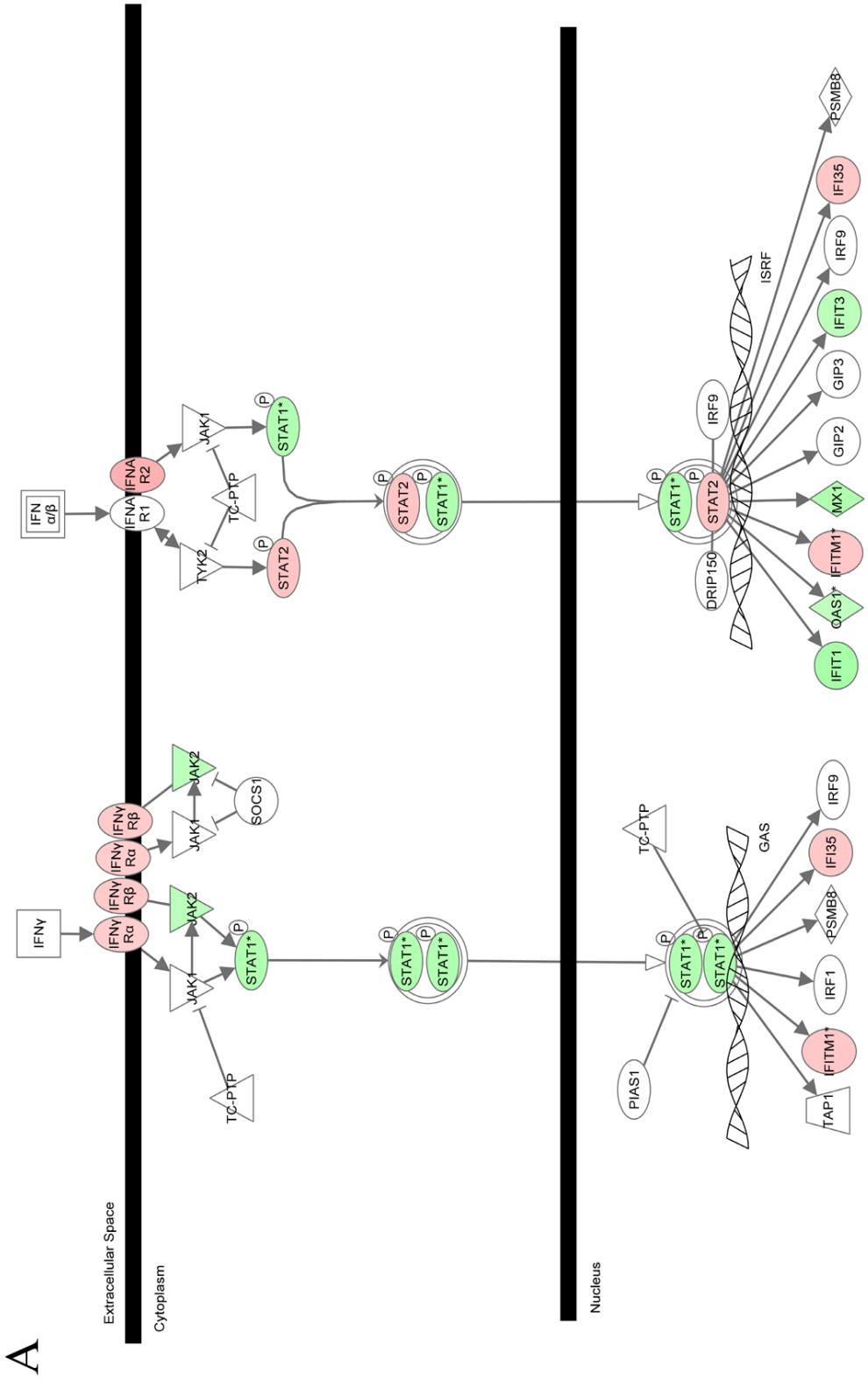


Figure 4.13.A Analysis of RA gene-expression profile by IPA revealed the alterations within IFN type I and IFN type II signalling pathways. Up- and down-regulated genes were shaded in red and green, respectively.

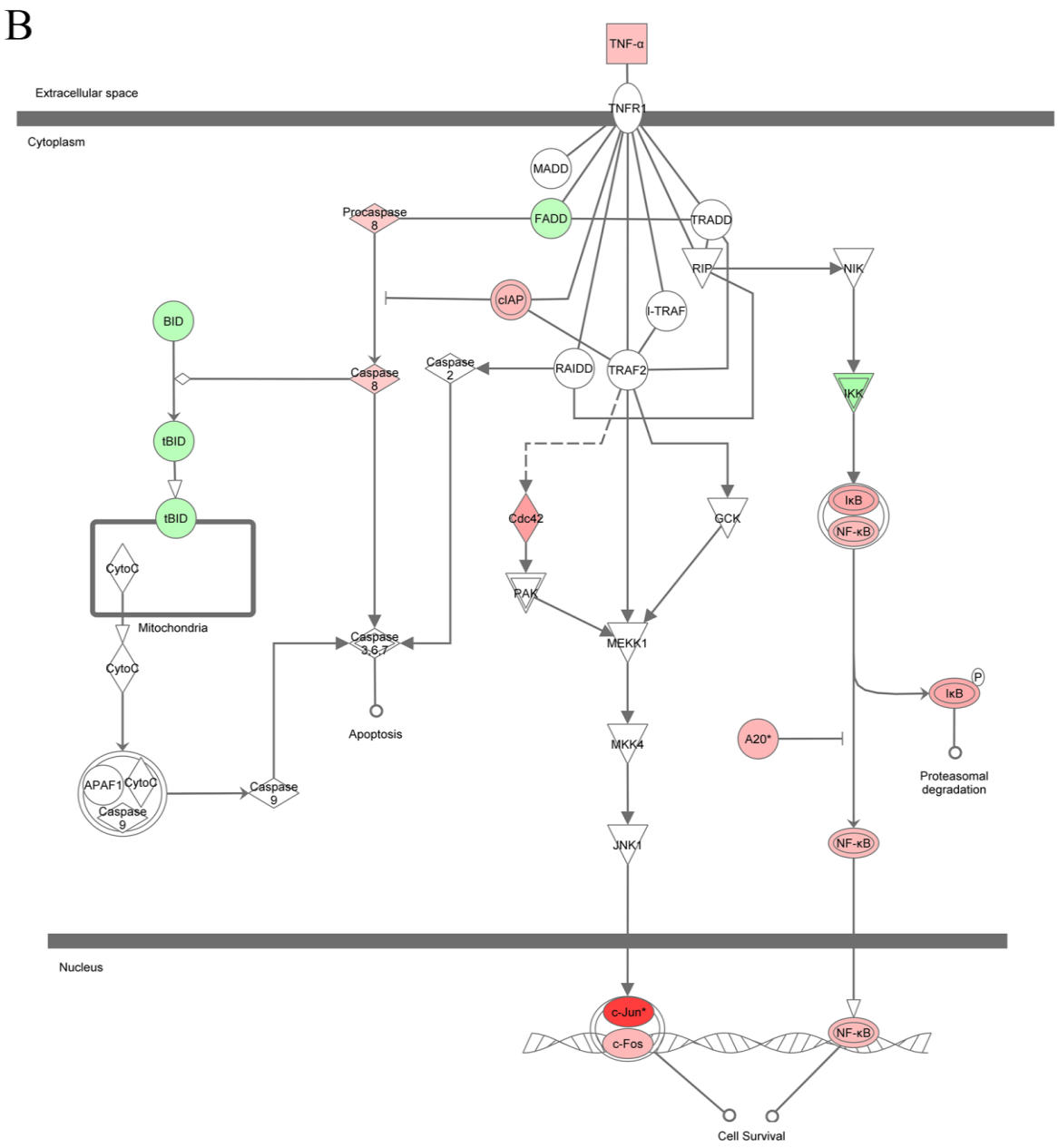


Figure 4.13.B Analysis of RA gene-expression profile by IPA revealed the alterations within TNFα signalling pathway. Up- and down-regulated genes were shaded in red and green, respectively.

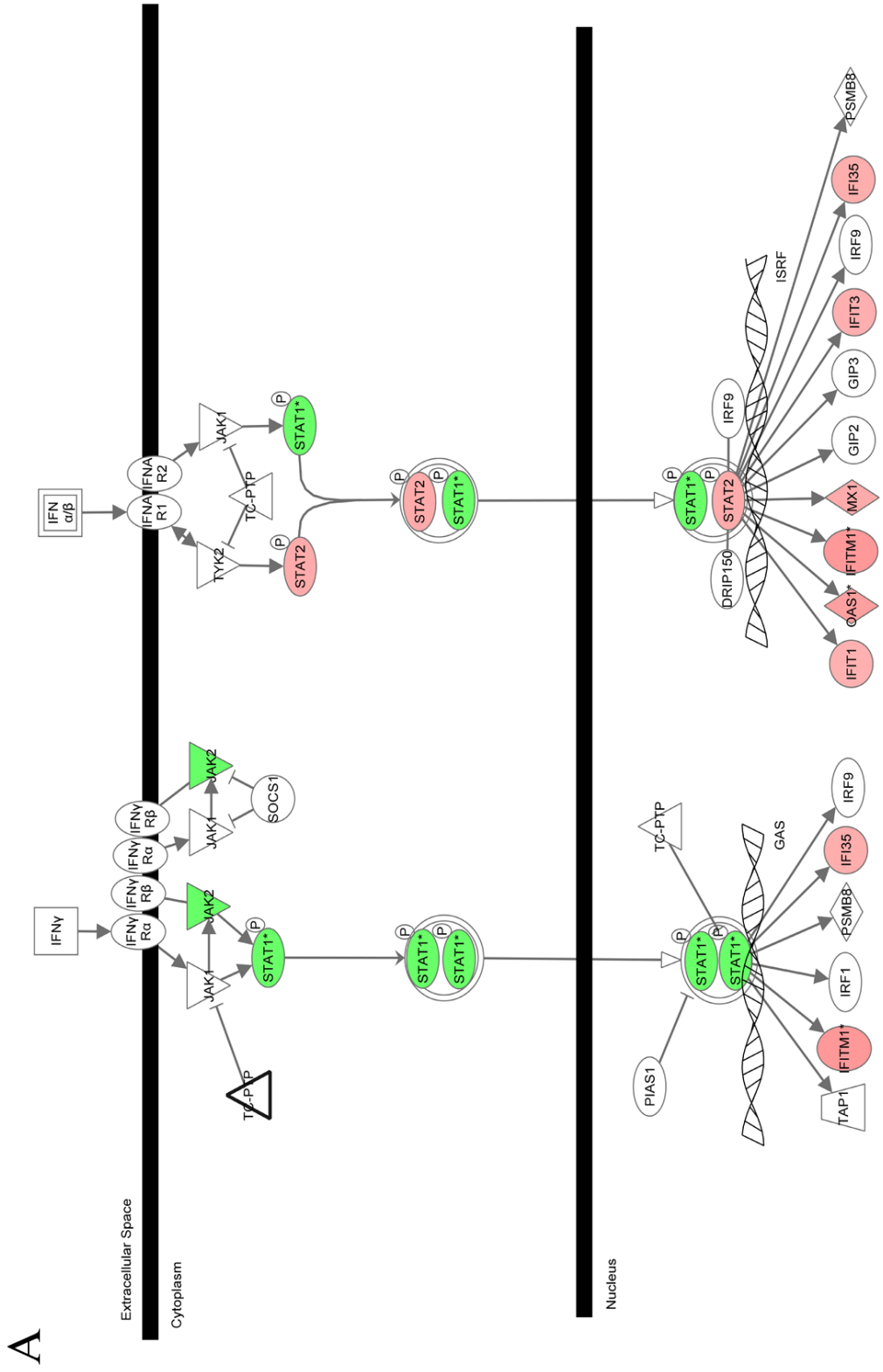


Figure 4.14.A Analysis of AS gene-expression profile by IPA revealed the alterations within IFN type I and IFN type II signalling pathways. Up- and down-regulated genes were shaded in red and green, respectively.

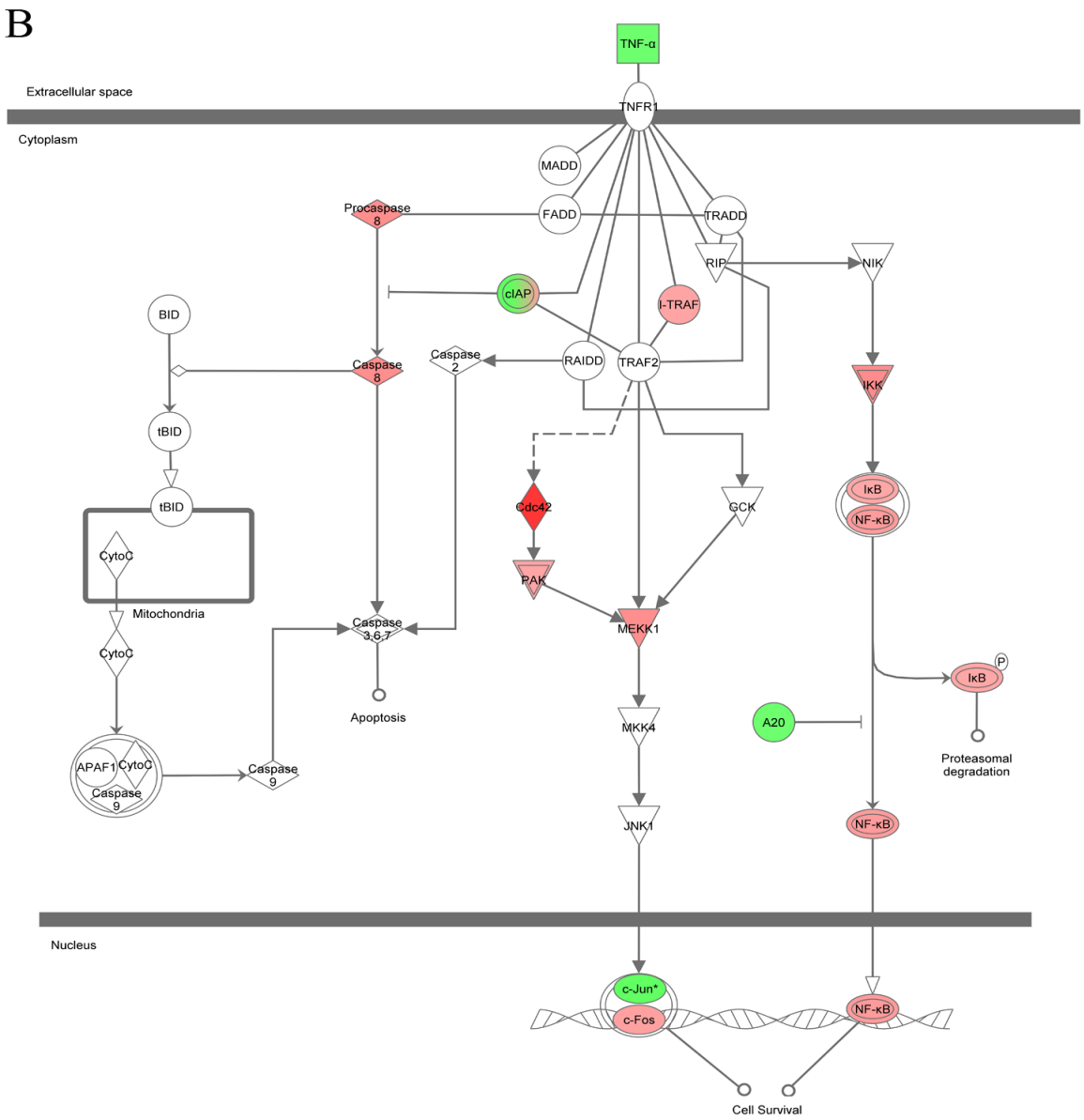
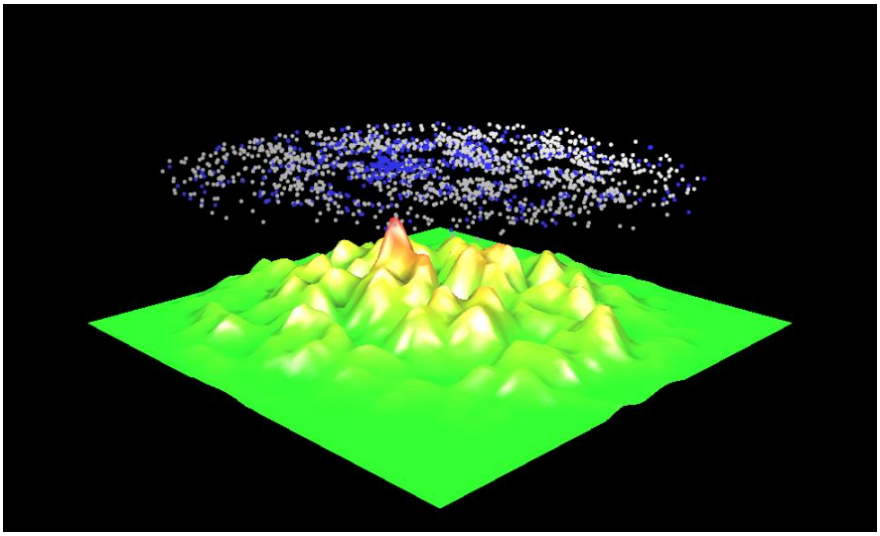
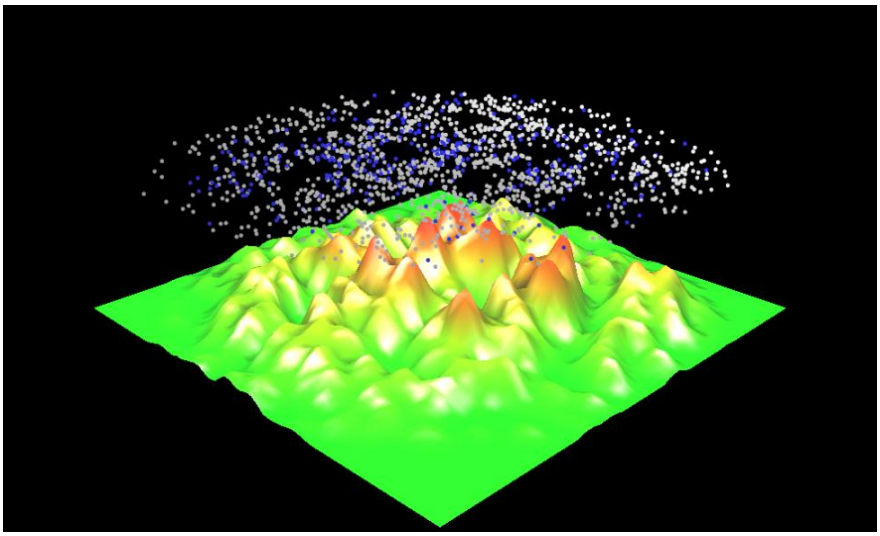


Figure 4.14.B Analysis of AS gene-expression profile by IPA revealed the alterations within TNFα signalling pathway. Up- and down-regulated genes were shaded in red and green, respectively

A



B



C

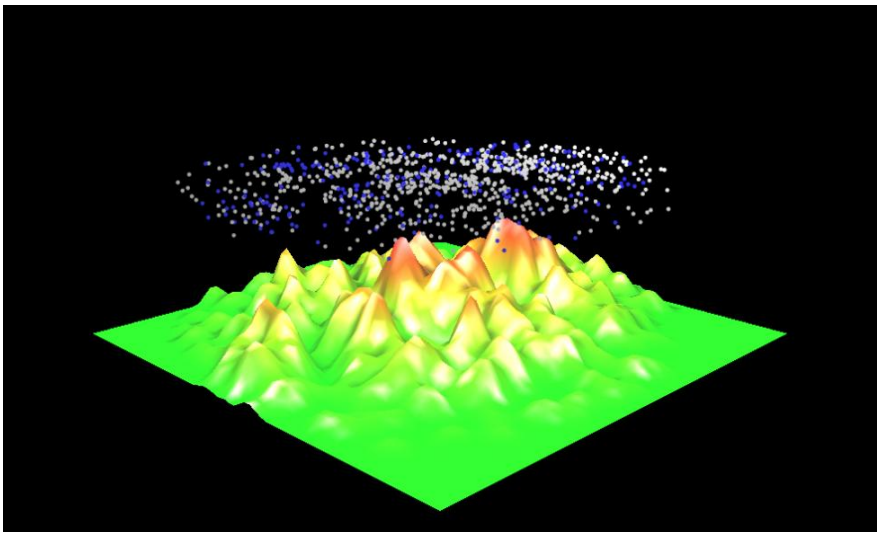
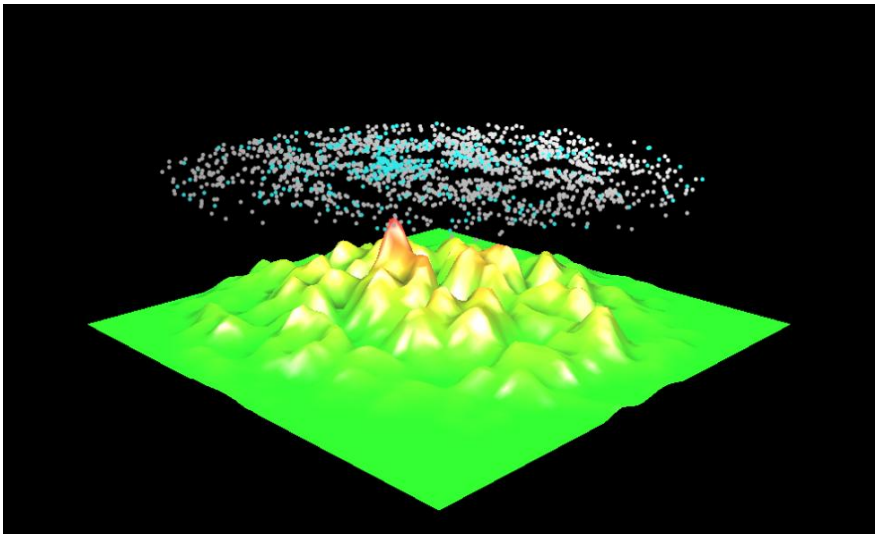
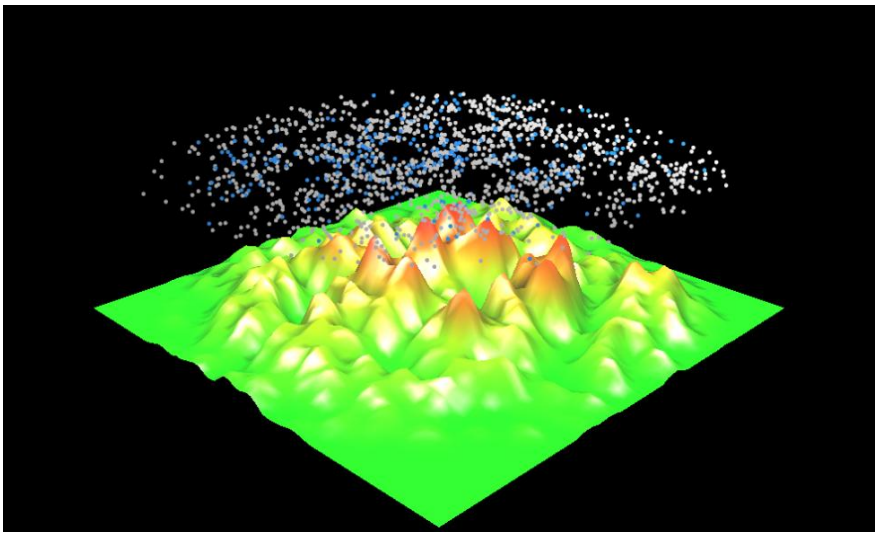


Figure 4.22 Landscapes of SLE, RA and AS profiles with the IFNα2a imprints are presented in **Figures 4.22.A, 4.22.B and 4.22.C**, respectively. Dots over the hills represent probe-sets that constitute the SLE, RA and AS profiles. Probe-sets that were identified as IFNα2a imprints in SLE, RA and AS are displayed as dark blue dots.

A



B



C

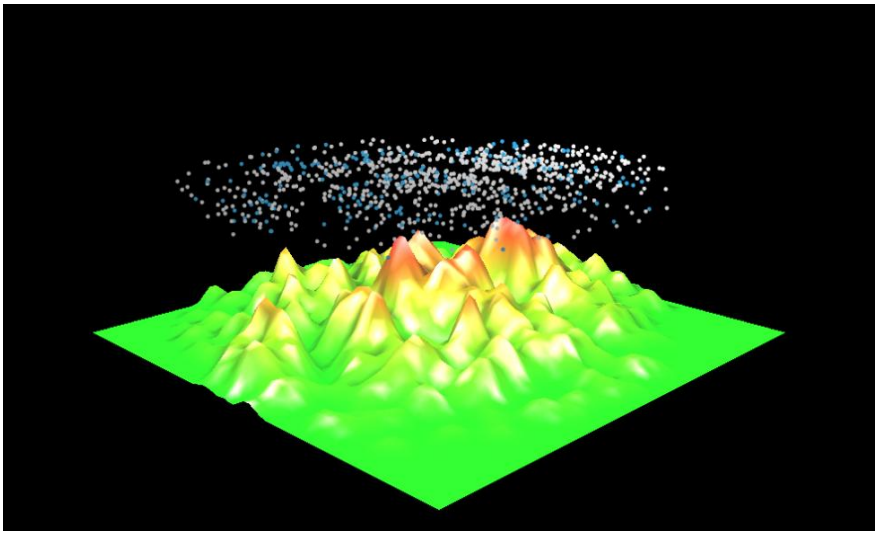
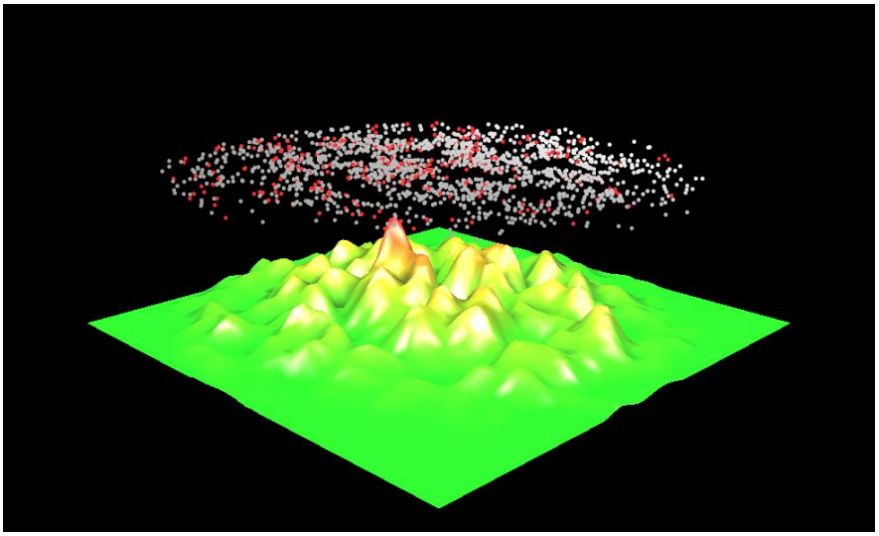
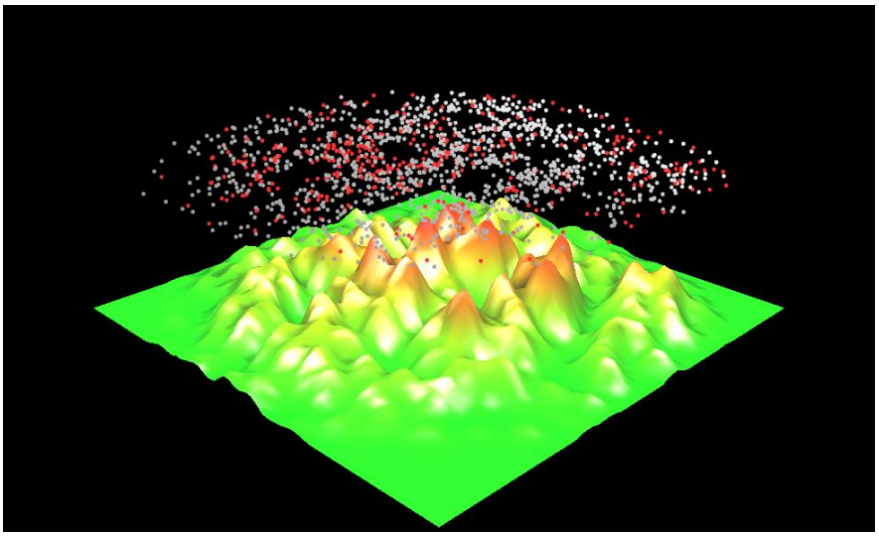


Figure 4.27 Landscapes of SLE, RA and AS profiles with IFNγ imprints are presented in **Figures 4.27.A, 4.27.B and 4.27.C**, respectively. Dots over the hills represent probe-sets that constitute the SLE, RA and AS profiles. Probe-sets that were identified as IFNγ imprints in SLE, RA and AS are displayed as light blue dots.

A



B



C

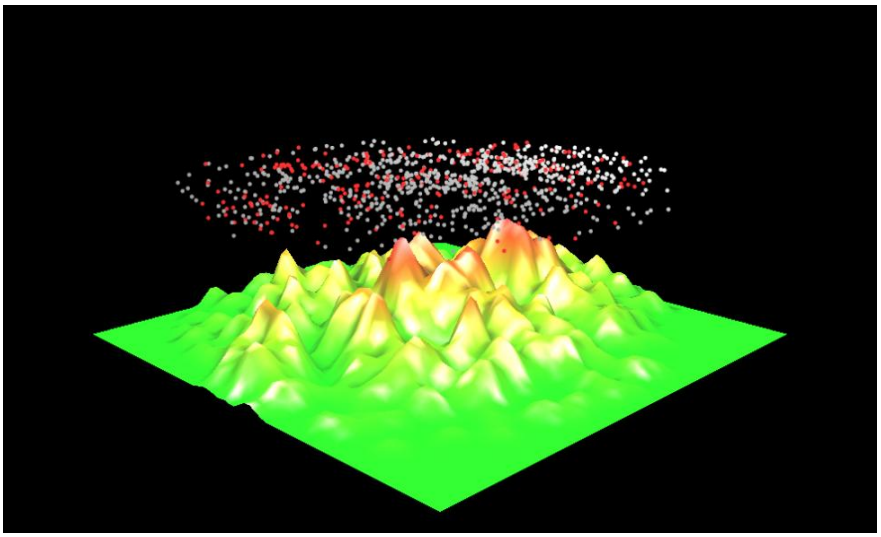


Figure 4.33 Landscapes of SLE, RA and AS profiles with TNFα imprints are presented in **Figures 4.33.A, 4.33.B and 4.33.C**, respectively. Dots over the hills represent probe-sets that constitute the SLE, RA and AS profiles. Probe-sets that were identified as TNFα imprints are displayed as red dots.

7.6. Supplementary Tables

Supplementary Table 1. Differentially expressed probe-sets identified in SLE, RA and AS monocytes. **WS1** contains 1847 probe-sets from SLE profile, **WS2** includes 1618 probe-sets from RA profile, and **WS3** contains 914 probe-sets from AS profile.
The last three columns in **WS1-3** present the fold changes (FD) of probe-sets that compose the TNFα, IFNα2a and IFNγ imprints in SLE, RA and AS.

Supplementary Table 2. Canonical pathways identified by IPA as over-represented in monocytes from SLE, RA and AS patients. WS1, **WS2**, and **WS3** represent identified pathways in SLE, RA and AS, respectively. The p value <0.05 was used as cut-off for selection of the pathways determined as significantly over-represented. The p-value was calculated by the Fischer's exact test, and included Benjamini-Hochberg correction for multiple testing.

Supplementary Table 3. Differentially expressed probe-sets that determined *in vitro*-generated TNFα, IFNα2a and IFNγ signatures. **WS1** includes 5676 differential expressed probe-sets that determined TNFα signature. **WS2** and **WS3** include 4566 and 3897 differentially expressed probe-sets that determined IFNα2a and IFNγ signatures, respectively. **WS4** includes the fold changes (FC) from all probe-sets, which were determined as TNFα, IFNα2a and IFNγ signatures. All together 8941 probe-sets were presented, where TNFα regulated probe-sets were coloured in green, IFNα2a in dark blue and IFNγ in light blue. Probe-sets which were identified as specific for one of these cytokines were included in three additional columns (TNFα specific were coloured in green, IFNα2a specific in dark blue and IFNγ specific in light blue). The probe-sets which were altered by two or all three cytokines, but which were predominantly regulated by one of them, were marked in separate columns. The same colouring code was applied as before. The probe-sets which were altered in opposite directions were assigned in the last three columns of table and they were coloured in grey.

7.7. Affirmation

Hereby I affirm that I wrote the present thesis without any inadmissible help by a third party and without using any other means than indicated. Thoughts that were taken directly or indirectly from other sources are indicated as such. This thesis has not been presented to any other examination board in this or a similar form, neither in Germany nor in any other country. There have been no prior attempts to obtain a PhD at any university.

I have performed this work at Deutsches Rheuma-Forschungszentrum in Berlin under the scientific supervision of Dr. Andreas Grützkau and Prof. Dr. Andreas Radbruch. I have written and submitted this dissertation at Freie Universität in Berlin under scientific supervision of Prof. Dr. Burghardt Wittig.

Berlin, October 2011, Biljana Smiljanovic

date and signature

7.8. Curriculum vitae

Biljana Smiljanovic dipl. pharmacist - med. biochemist

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Experiences	
Since March 2011	Charité University Medicine Berlin Dept. of Rheumatology and Clinical Immunology
July 2005 – March 2011	PhD Study at the German Rheumatism Research Centre (DRFZ), Berlin
October 2004 – July 2005	Aqualab, Belgrade, clinical laboratory, as a medical biochemist
February 2004 – October 2004	Jugomontana, Belgrade, pharmacy and medical equipment, (distributor of Bio-Rad), as a district sale specialist
September 2003 – September 2004	Clinical Centre "Dragisa Misovic", Belgrade, Laboratory of Medical Biochemistry, as a medical biochemist

Education	
July 2005 – March 2011	PhD study at the German Rheumatism Research Centre, Berlin

PhD thesis: Transcriptional profiling of peripheral blood monocytes from SLE, RA and AS patients revealed disease-dependent imprints of TNFα, type I and type II IFNs
Project leaders Dr. rer. nat. Andreas Grüetzkau and Prof. Dr. Andreas Radbruch

1994 – 2003	University of Belgrade, Serbia, Faculty of Pharmacy, MS. Degree – Department of Medical Biochemistry

Diploma thesis: "Biochemical and Clinical Implications of the ErbB/HER tyrosine kinase receptors in Breast Cancer", done by the supervision of Prof. Dr. Nada Majkic-Singh
average mark 9.24 from 10.00

1990 – 1994	The First Belgrade High School, Serbia, natural-mathematical department

Final project: "Haemoglobin", under supervisor dipl. chemist Tatjana Bulatovic

1982 – 1990	Elementary School in Belgrade, Serbia

Training

2000 — The Weitzman Institute of Science, Rehovot, Israel, Department of Biological Regulation, Laboratory of Prof. Dr Yossi Yarden

project: "The Effect of c-Src on EGF-receptor signalling"

1999 — University of Belgrade, Faculty of Biology, Department of Physiology and Biochemistry, Laboratory of Prof. Dr Vukosava Davidovic

The field of research: "Neuroendocrine regulation activities of enzymes antioxidative protection in hypothalamus and brainstem of rats"

Publication

Smiljanovic B., Grün JR., Steinbrich-Zollner M., Stuhlmüller B., Häupl T., Burmester GR., Radbruch A., Grützkau A., Baumgrass R. Defining TNFα- and LPS-induced gene signatures in monocytes to unravel the complexity of peripheral blood transcriptomes in health and disease, *J Mol Med. 2010.*

Smiljanovic B., Grün JR., Biesen R., Schulte-Wrede U., Baumgrass R., Stuhlmüller B., Hiepe F., Burmester GR., Radbruch A., Häupl T., Grützkau A. The multifaceted balance of TNFα and type I / II interferon responses in SLE and RA: how monocytes manage the impact of cytokines, *(under revision).*

General comments to Supplementary Tables 1-3

Supplementary Tables 1 to 3 are available on CD that follows this PhD thesis. In addition, WS1 and WS2 that are included in Supplementary Table 1, and WS1 to WS4 included in Supplementary Table 3 are available on line in a journal article: "The multifaceted balance of TNF-alpha and type I/II interferon responses in SLE and RA: how monocytes manage the impact of cytokines". This article was published by *Smiljanovic et al.* in Journal of Molecular Medicine and it is designated by DOI number: DOI: 10.1007/s00109-012-0907-y.

The minor differences are evident concerning the number of Affymetrix probe-sets that are present in Supplementary tables on CD and those published on line. These differences are commented in the following paragraphs.

Comments to Supplementary Table 1

In more detail, WS1 and WS2 of Supplementary table 1 that are included in this PhD thesis are also available on line in before mentioned article as WS1 and WS2 of Supplementary table 1, respectively. However, the minor differences are present, since control probe-sets that are related to differential expression of ACTB (actin, beta), GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and STAT1 (signal transducer and activator of transcription 1, 91kDa), were not included in the tables that belong to the thesis.

More precisely, the SLE profile presented in WS1 of table that belong to the thesis contains 6 probe-sets less when compared to the table that was published on line in the article. Thus, the total number of probe-sets in WS1 of Supplementary table 1 that belongs to the thesis and to the article is 1847 and 1853, respectively. The following 6 control probe-sets were excluded: AFFX-HUMGAPDH/M33197_5_at, AFFX-HUMGAPDH/M33197_M_at, AFFX-HUMISGF3A/M97935_3_at, AFFX-HUMISGF3A/M97935_5_at, AFFX-HUMISGF3A/M97935_MA_at and AFFX-HUMISGF3A/M97935_MB_at.

Concerning the RA profiles, presented in WS2 of Supplementary Table 1, the number of probe-sets that were excluded is 9. Thus, the RA profiles in the thesis includes 1618 probe-sets and in the article 1627 probe-sets. The following 9 control probe-sets were excluded: AFFX-HSAC07/X00351_5_at, AFFX-HSAC07/X00351_M_at, AFFX-HUMGAPDH/M33197_3_at,

AFFX-HUMGAPDH/M33197_5_at, AFFX-HUMGAPDH/M33197_M_at, AFFX-HUMISGF3A/M97935_3_at, AFFX-HUMISGF3A/M97935_5_at, AFFX-HUMISGF3A/M97935_MA_at and AFFX-HUMISGF3A/M97935_MB_at.

WS3 of Supplementary table 1 is available on CD and can be obtained on request from Dr. rer. Nat. Andreas Gruetzkau (gruetzkau@drfz.de).

Comments to Supplementary Table 2

This Supplementary table is available on the CD that accompanies this PhD thesis. In addition, it can be provided on request by Dr. rer. Nat. Andreas Gruetzkau (gruetzkau@drfz.de).

Comments to Supplementary Table 3

WS1 of Supplementary tables 3 of the thesis is also available on line in the before mentioned article as WS1 of Supplementary table 2. As mentioned before, control probe-sets were excluded and thus, the TNFα profile was presented with 5676 probe-sets in the thesis and with 5683 probe-sets in the article. The following 7 control probe-sets were excluded: AFFX-HSAC07/X00351_5_at, AFFX-HUMISGF3A/M97935_MA_at, AFFX-HUMISGF3A/M97935_MB_at, AFFX-HUMRGE/M10098_3_at, AFFX-HUMRGE/M10098_5_at, AFFX-M27830_5_at and AFFX-M27830_M_at.

WS2 and WS3 of Supplementary tables 3 of the thesis are available on line in the article mentioned before as WS2 and WS3 of Supplementary table 2, respectively. The total number of probe-sets that represent the IFNα2a profile (included in WS2) is 4566 in the thesis and 4571 in the article. The following 5 control probe-sets were excluded: AFFX-HUMISGF3A/M97935_3_at, AFFX-HUMISGF3A/M97935_5_at, AFFX-HUMISGF3A/M97935_MA_at, AFFX-HUMISGF3A/M97935_MB_at and AFFX-HUMRGE/M10098_5_at. The total number of probe-sets that represent the IFNγ profile (presented in WS3) is 3897 in the thesis and 3901 in the article. The following 4 control probe-sets were excluded: AFFX-HUMISGF3A/M97935_3_at, AFFX-HUMISGF3A/M97935_5_at, AFFX-HUMISGF3A/M97935_MA_at and AFFX-HUMISGF3A/M97935_MB_at.

WS4 of Supplementary tables 3 of the thesis is available on line in the before mentioned article as WS1 of Supplementary table 3. WS4 of Supplementary tables 3 of the thesis contains 8941 probe-sets, which is 9 probe-sets less when compared with WS1 of Supplementary table 3 in the

article. The following control probe-sets were excluded: AFFX-HSAC07/X00351_5_at, AFFX-HUMISGF3A/M97935_3_at, AFFX-HUMISGF3A/M97935_5_at, AFFX-HUMISGF3A/M97935_MA_at, AFFX-HUMISGF3A/M97935_MB_at, AFFX-HUMRGE/M10098_3_at, AFFX-HUMRGE/M10098_5_at, AFFX-M27830_5_at and AFFX-M27830_M_at.

Raw data of Affymetrix GeneChips used in this study are available on line in Gene expression omnibus (GEO) DataSet under accession number GSE38351. The exceptions are raw data from chips generated by using monocytes from patients with AS that are not included in GEO DataSet, but they can be provided on request by Dr. rer. Nat. Andreas Gruetzkau (gruetzkau@drfz.de).