Dendritic cells in Sjögren's syndrome — functional analyses

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Contents

Abbreviations

AECC	American-European Consensus group criteria
APC	Antigen presenting cells
BAFF	B cell activating factor
BCA	Bicinchoninic acid
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
cDC	Conventional dendritic cells
DMSO	Dimethyl sulfoxide
DC	Dendritic cells
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FDC	Follicular dendritic cells
FSC	Forward scatter channel
GM-CSF	Granulocyte macrophage-colony stimulating factor
HRP	Horseradish Peroxidase
ICSBP	Interferon consensus sequence-binding protein
IL	Interleukin
IFN	Interferon
Ig	Immunoglobulin
IRF	Interferon regulatory factor
LC	Langerhans cells
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cells
pSS	Primary Sjögren's syndrome
MFI	Median fluorescence intensity
MHC	Major histocomatibility complex
moDC	Monocyte derived dendritic cells
MR	Mannose receptor

NK cells	Natural killer cells
n.s	Non significant
RIPA	Radioimmunoprecipitation assay
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SS	Sjögren's syndrome
SSC	Side scatter channel
TBS	Tris-buffered saline
TLR	Toll like receptor
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
TRIM	Tripartite motif
Treg	Regulatory T cells
WR	Working reagent

Abstract

Sjögren's syndrome (SS) is an autoimmune disease of unknown etiology. It is characterized by chronic inflammation of the exocrine glands which leads to dryness of the mouth and eyes. Dendritic cells (DC) are the most potent antigen presenting cells of the immune system. They take up antigens in the periphery and migrate to secondary lymphoid tissues where specific T lymphocytes recognize the presented antigen and mount an immune response. So far not much work has been done to examine the possible role of DC in SS. Aim of this study was to functionally analyze monocyte-derived DC (moDC) from patients diagnosed with primary SS compared to healthy controls. Peripheral blood mononuclear cells were isolated from freshly heparinized blood samples from pSS patients fulfilling the American European Consesus group criteria (AECC) and gender- and age-matched healthy controls. Monocytes were isolated by plastic adherence, and immature DC were generated by culturing the monocytes with IL-4 and GM-CSF. A fraction of the cells was stimulated with the TLR7/8 ligand CL097 for 48 hours.

In order to investigate functional aspects of moDC, a FITC-dextran assay to explore the endocytic capacity of DC, and a chemotaxis assay to examine migratory capacity of the cells towards CCL19 were performed. Moreover an IL-12p70 ELISA on cell culture supernatants was performed as IL-12 is an inflammatory cytokine which is produced and secreted by mature DC. Finally protein expression of Ro52 and IRF-8 was analyzed on whole cell lysates from moDC by Western blot. Ro52 is a commonly detected autoantigen in patients with SS and IRF-8 is a transcription-factor involved in DC function and differentiation.

The results from the endocytosis assay revealed that immature DC from SS patients have a higher endocytic capacity than the cells from healthy controls. The chemotaxis assay showed that mature moDC from patients have higher migratory capacity than the mature moDC from controls. Furthermore the Western blot analysis indicated that Ro52 expression is significantly lower in mature moDC from patients compared to controls. IRF-8 expression was significantly lower in immature DC from SS patients compared to the control group. The ELISA result proposed a slightly higher production of IL-12p70 in mature moDC from patients than from mature moDC from healthy controls. From the experiments which have been performed in this study we conclude that there indeed is a functional difference between the moDC from SS patient and controls. This difference might possibly be caused by an inappropriate stimulus of the immature moDC in SS patients leading to dysfunctional DC. This study also emphasizes the need to continue examining the possible role of DC in SS.

1. Introduction

1.1 Sjögren's syndrome

Sjögren's syndrome (SS) is a chronic rheumatic autoimmune disease characterized by lymphocyte infiltration in the exocrine glands, primarily the lacrimal and salivary glands. The mechanisms behind the disease are still not understood and there is at this time no cure. Today's treatment is mainly focused on alliviating the symptoms. SS has until recently been an under-diagnosed and under-treated disease mainly because it is a fairly newly discovered disease and has low mortality, but the implications of having this chronic disease is in fact very serious for the patient and goes way beyond just having dry eyes and mouth (1-3).

1.1.1 History

Sjögren's syndrome is a fairly recently discovered disease, although there were some isolated cases reported in the late 1800. It was not described until 1933 when the Swedish ophthalmologist Henrik Sjögren reported on clinical and histological findings in his doctoral dissertation, "Zur Kenntnis der Keratoconjuvitis Sicca"(4). His work was not immediately acknowledged and a mediocre grade on his thesis prevented an academic career. Not until the fifties was his achievement recognized when he published a series of 80 patients of which 62% had arthritis. In 1970 the Swedish government awarded him the title of professor and he was then able to work as a university professor (3).
In 1965, the distinction between primary and secondary SS was suggested and it was not until this time that it became featured as a rheumatic disease. The Ro (SS-A) and La (SS-B) autoantibodies and the labial gland infiltration were first described in the seventies and routine diagnostic tests were available in the 1990s (1, 2).

1.1.2 Classification criteria

The first International Symposium on Sjögren's Syndrome was held in Copenhagen in 1986, where for the first time classification criteria were suggested. Later the Californian and the European classification standard have been established. Today it is the American-European

classification criteria which is used in diagnosis of SS (Table 1) (5). To meet the classification criteria the patient must have:

- Presence of four out of the six items indicative of SS and each patient must have an abnormal biopsy (IV) or the presence of anti-Ro or anti-La autoantibodies (VI).
- Presence of three out of four objective criteria (Items III, IV, V, VI).

Exclusion criteria are: past head and neck injuries, hepatitis C infection, AIDS, pre-existing lymphoma, sarcoidosis, graft versus host disease and use of anticholinergic drugs (5).

Table 1. American-European Consensus Group Classification criteria for Sjögren's Syndrome according to Vitali *et al* (5)

I	Ocular symptoms: 1. Have you had daily, persistent, troublesome dry eyes for more than 3 months? 2. Do you have a recurrent sensation of sand and gravel in the eyes? 3. Do you use a tear substitute more than 3 times a day?
II	Oral symptoms: 1. Have you had a daily feeling of dry mouth for more than 3 months? 2. Have you had recurrent or persistent swollen salivary glands as an adult? 3. Do you frequently drink liquids to aid in swallowing dry foods?
III	Ocular signs-positive result of one of the following tests: 1. Schirmer's I test ($\leq$ 5mm in 5 minutes) 2. Rose Bengal score $\geq$4 according to van Bijsterveld's scoring system)
IV	Histopathology: Abnormal biopsy of minor salivary gland
V	Salivary gland involvement: positive result for one of following diagnostic test: 1. Unstimulated whole salivary flow ($\leq$ 1,5 ml in 15 minutes) 2. Parotid sialography showing the presence of diffuse sialectasias, without evidence of obstruction in the major ducts 3. Salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of tracer
VI	Antibodies to Ro(SSA) or La(SSB) antigens, or both

To classify secondary SS in patients with a potential associated disease fewer criteria need to be met. Only the presence of item I or II plus 2 items from III, IV, V, VI is required to be indicative of secondary SS (5).

1.1.3 Clinical features

SS can occur at any age and in people of both sexes, but it mainly appears in middelaged and elderly women; the ratio between women and men is 9:1 (1, 2). The prevalence of the disease varies in the literature and depending on which classification criteria is used, but recent data suggest a prevalence of approximately 0,3% to 0,6% which is comparable to rheumatoid arthritis (6). SS can occur alone in primary Sjögrens (pSS) or in association with other rheumatic diseases as secondary SS.

SS is characterized by persistent lymphocyte infiltration in the exocrine glands primarily salivary and lacrimal glands leading to dry mouth (xerostomia) as a result of deficient saliva production and dry eyes (keraconjunctivitis sicca) due to lack of tear production.
The patient will generally present subjective symptoms of dry eyes. They can be described as feeling sandy, gritty, itchy or as having a burning sensation. The physical implications of deficient tear production can include destruction of corneal epithelium, conjunctivitis, dilation of the bulbar conjunctival vessels, irregularity of the corneal image and lacrimal gland enlargement. Patients can also have an inability to tolerate smoke and light (photosensitivity) (1, 7, 8). Oral dryness, due to lack of saliva secretion can be quite debilitating in daily life, interfering with the patients' ability to eat, sleep and speak. They can have difficulties in chewing and swallowing without liquid, develop fissures on the tongue, loose taste and smell sensation, have increase in dental caries and develop candidasis (1-3, 8). Chronic persistence can also result in more serious conditions such as chelitis and mouth ulcers (9).
Other common symptoms of pSS are dryness of the skin and vagina, joint pain and a feeling of extreme fatigue (2).

In addition to exocrine dysfunction about 50% of the patients also have systemic manifestations. Other extraglandular manifestations can include; respiratory tract, gastrointestinal tract, genitourinary tract, skin, muscle and joints, neurological and vasculopathies (1, 3, 7). One of the most serious complications of SS is an increased risk of developing non-Hodgkins lymphoma due to chronic B cell stimulation (2).

1.1.4 Pathology

A characteristic feature of SS is a chronic inflammation of the exocrine glands, mainly the salivary and lacrimal glands. Histopathological analysis in salivary gland biopsies show lymphocytic infiltrates consisting of a majority of T cells with fewer B cells, macrophages and mast cells (2). Furthermore it could be shown that 17% of patients have germinal center (GC)-like structures in their salivary glands. These structures consisted of T and B cell aggregates within a follicular DC (FDC) and endothelial cell network (10).

B cells make up for about 20% of the lymhocytes in the lymphocyte infiltrates. There is evidence of hyperactive B cells which have increased amounts of antibodies with autoantibody activity including rheumatoid factors, Ro and La (11). Infiltrating B cells predominantly express the IgG isotype in contrast to IgA which is expressed in normal salivary glands (2). A member of the tumor necrosis factor (TNF) family, B cell activating factor (BAFF), has been found to be upregulated in serum of patients with SS and also in patients with other autoimmune diseases (12-14). BAFF is considered to be involved in the proliferation and survival of B cells and might be involved in the pathogenesis of SS (14).
Infiltrating T cells are mainly activated CD4+ helper T cells which very likely contribute to the hypereactivity of B cells. They produce high levels of IFN-γ and IL-10, while high levels of IL-1β, IL-6 and TNF-α are produced by epithelial cells which might stimulate B cell proliferation and differentiation (2).
It has for a long time been suspected that viruses could be involved in the pathogenesis of SS and several different candidates have been listed, but there was not found a real connection (1, 2, 15, 16). In recent years several studies have revealed an activated type I interferon (IFN) signature in salivary glands of SS patients (15, 17, 18). Plasmacytoid dendritic cells (pDC), which are known as natural IFN producing cells, were found in salivary glands of patients with pSS, but not in controls (15).

SS has a generally low mortality rate, in a study examining long term outcome of ~4500 patients with SS there were 39 deaths recorded of which 1 in 5 was from lymphoma. Since this is mainly a disease of morbidity an important issue is the quality of life for the patients. It has been shown that SS patients and particular those with pSS have very low scores on general quality of life (19).

1.2 The immune system

The body is constantly attacked by harmful microorganisms like bacteria, virus, fungus and protozoa. To protect us from these pathogens our body has aquired the immune system. It is a highly specialized system involving several different cell-types and molecules used for communication between them. Immune cells have to distinguish self from non-self and harmful from benign. The immune system is divided into two different systems, the innate and the adaptive immune systems which both work separately but also communicate and function collectively.

The innate immune response is the body's first line of defence and has a very rapid response time. Within the first 12 hours of an infection it is only the innate immune system which is active. The components of this system are first physical and chemical barriers such as the skin and the low pH of the stomach. If the microorganisms manage to pass these barriers, defence mechanisms in the form of phagocytic cells like macrophages and neutrophils which ingest and destroy foreign material are recruited. Other components of the innate immune response are cytokines, which are secreted by cells in the innate and adaptive immune system, and components of the complement system. They are involved in mediating an immune and inflammatory response.

Many microorganisms manage to bypass all of these defence systems, and in that case the adaptive immune response gets activated. This is a late response and is often called the specific immune response because it targets very specific pathogens and it also has memory. While the innate immune system responds to structures that are shared by many groups of microbes, the adaptive immune system responds to antigens which are very specific for different pathogens.

The major players of the adaptive response are B and T lymphocytes. T lymphocytes interact with cells of the innate immune system and produce effector T cells that have many functions such as secretion of cytokines and activation of other immune cells including B cells.

B lymphocytes are the only cells capable of producing antibodies which can neutralize or initiate phagocytosis of the microbe.

The immune system is very intricate and both the innate and adaptive immune system work together and communicate in several different ways, they are not isolated systems

As mentioned earlier the T lymphocytes are activated by cells of the innate immune system, these are called antigen presenting cells (APC). The most potent APC are the dendritic cells

which are central mediator between the innate and adaptive immune system. Their role is to capture and process antigens and present them to T lymphocytes.

1.3 Dendritic cells

Dendritic cells (DC) got their name from their branchlike, dendritic shape. Their long projections increase surface area and make it possible for them to effectively probe their surrounding tissues for antigens (20). The first DC was visualized 140 years ago by Paul Langerhans, who first characterized Langerhans cells (LC), but further description of the DC did not start until the seventies by Steinman and Cohn (21).Since then DC have been recognized as the most potent antigen presenting cells of the immune system and being central in initiating an immune response. DC capture and process antigens from both pathogens like viruses and bacteria and from apoptotic material. The processed antigens are then presented on major histocompatability complex (MHC) class I and II molecules to naïve T lymphocytes. Recognition of the antigens by T cells leads to either activation of the adaptive immune response or induction of tolerance (22-24).

1.3.1 DC subtypes

Like all blood cells the dendritic cells are of hematopoietic lineage and originate from pluripotent hematopoietic stem cells in the bonemarrow (figure 1.1). Downstream from the multipotent hematopoietic stem cell precursors divide into two main lineages, myeloid and lymphoid (25). DC are mainly generated from the myeloid lineage from a common myeloid progenitor, but can also stem from common lymphoid precursors, both have been isolated from the bone marrow. DC also have several different subsets which have different surface markers and functions (20, 24). Different DC subtypes are present in most of the tissues in the body, in the skin, mucus membranes, blood, lymph and visceral organs.

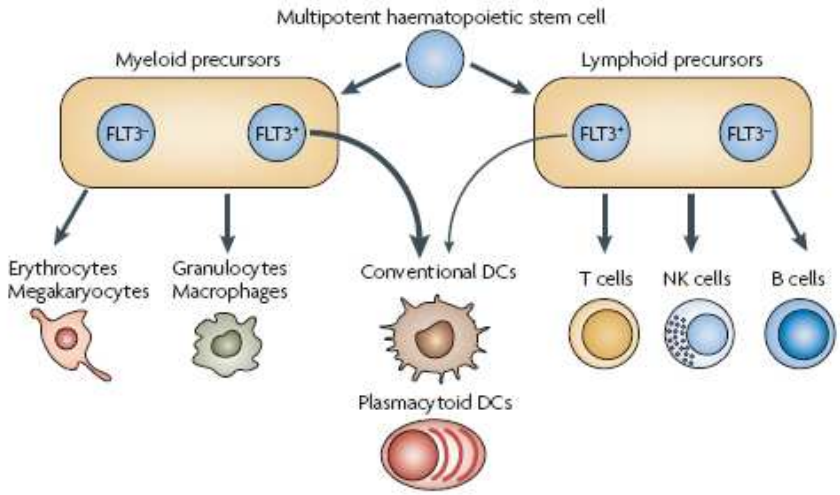

Figure 1.1. Development of DC from a multipotent hematopoietic stem cell.

DC originate from a multipotent hematopoietic stem cell in the bone marrow. Downstream from this stem cell precursors divide into two different lineages, myeloid and lymphoid. DC can originate from both lineages. DC have several different specialised subsets with different functions and surface markers, but the two main subsets are the conventional DC and the pDC. From Shortman .& Naik. *Nat Rev Immunol* 2007 (26).

The two main subsets of DC are plasmacytoid (pDC) and conventional DC (cDC).

Plasmacytoid DC are found as pre DC in steady state. They are circulating, long lived cells which upon stimuli from virus acquire a dendritic form and start producing large amounts of type I interferons (IFN) (26, 27). IFN are cytokines which mediate activation of several other immune cells including T and B lymphocytes and cDC (27). When acquiring a dendritic shape, pDC are also capable of antigen capture and presentation although not as efficient as the cDC (26).

Conventional DC, also known as myeloid DC (mDC), do not develop from pre DC, but have the shape and function of DC in steady state. This subset is known to contain the most potent antigen presenting cells. They can be further divided into subgroups of migratory and lymphoid-tissue-resident DC (26). Migratory DC are the textbook example of DC which catch antigens in the periphery and migrate through lymphatics to secondary lymphoid tissue where they present antigens to T cells. Included in this group are the Langerhans cells (LC) which were the first DC to be described. They have been used as a model to study the function of the DC (26, 28). LC reside in the epidermis and have upon activation been found to loose their connection with neighboring cells and start to migrate to the dermis. From there they have been traced through afferent lymphatic vessels and to T cell areas of cuteneous draining lymphnodes (29).

Another important DC subset are the monocyte-derived DC (moDC). They are inflammatory DC not present in the steady state of the body(26). The ability of human peripheral monocytes to differentiate into DC when cultured with IL-4 and GM-CSF was first published by Sallusto and Lanzavecchia (30). This pathway of DC development has been utilized in vitro by culturing blood monocytes with GM-CSF and IL 4. Today this is an established procedure for generation of DC in the laboratory (30-32). The reason why this is such a conventional way of generating DC instead of just isolating them from the blood is because pDC and cDC are very rare and make up for a very small percentage of the blood cells, and they do not proliferate.

1.3.2 Antigen recognition

The antigen presentation ability of DC is an essential part of their function.
Immature DC recognize pathogens by different surface and intracellular receptors. One important family of receptors are the Toll like receptors (TLR) which is a large family of conserved proteins. These are transmembrane receptors recognizing pathogen-associated molecular patterns (PAMP) on pathogens like viruses, fungi, gram positive, gram negative bacteria and parasites (33, 34). TLR are expressed both on the plasma membrane and intracellularly in a variety of cells of the innate immune system in addition to the DC, including macrophages, mast cells and neutrophils. For instance, TLR3, TLR7, TLR8 and TLR9, which are expressed intracellularly on endosomes, are important for viral recognition. These receptors are activated by viral nucleic acids like RNA and DNA. TLR7 and TLR8 can also be activated by synthetic imidazoquinoline compounds (34). Activation leads to the increased expression of IFNα and inflammatory cytokines (34). TLR activation in immature DC leads to upregulation of several costimulatory molecules like CD80 and CD86 and induction of cytokine production, for example IL-6 and IL-12 (35-37). Secretion of IL-12 by DC in response to TLR activation leads to IFNγ production of T and natural killer (NK) cells. It is also important in promoting differentiation of CD4+ T cells into Th 1 cells, which are essential in immunity towards intracellular pathogens.

1.3.3 Antigen-processing and presentation

After the immature DC recognize and are activated by foreign antigens many intracellular processes start taking place. These include upregulation of co-stimulatory molecules and

production of cytokines which start the maturation process in the DC. Lately it has been proposed that the terms immature and mature DC are quite insufficient (24), but I will stick to these terms throughout this thesis for the sake of simplicity.

Once antigens bind to the immature DC they are taken up in the cell by endocytosis. Immature DC use several different pathways to internalize the antigens such as receptor mediated endocytosis via C-type lectins and Fcγ receptors, phagocytosis and macropinocytosis (22, 38). Once internalized the antigens are taken up into endosomes and phagosomes where they are cleaved into peptides which can be presented to T lymphocytes on major histocomatibility complex (MHC) class II molecules. In immature DC MHC class II molecules are produced in the endoplasmic reticulum and transported in vesicles to endosomes or phagosomes where they bind the processed peptides. The MHC class II/peptide complex is then transported to the surface where it can be recognized by naïve T lymphocytes (39). An intracellular infection, for instance by a virus, leads to presentation of viral peptides on MHC class I molecules. MHC class I exist on all cells of the body and therefore make all cells able to present peptides to CD8+-effector T cells. Usually MHC class I molecules present peptides from degraded proteins in the cytosol or nucleus. Presentation of a foreign or unknown peptide stimulates the CD8+ cell and leads to destruction of the cell (39).

Another important process is the relocation of DC from peripheral tissues to secondary lymphoid organs through the lymphatic vessels (40). DC must undergo several functional and phenotypical changes to be able to migrate to secondary lymphoid tissue. Some of the important functional changes that take place during maturation is downregulation of receptors responding to inflammatory cytokines and upregulating of several trafficking molecules including chemokine (C-C motif) receptor 7 (CCR7) which bind to the chemokine (C-C motif) ligands 19 (CCL19) and CCL21 (40, 41).
CCL19 and CCL21 are chemokines produced by stromal cells in the T lymphocyte area of the lymph node and they are important in homing the DC to this area through chemotaxis (42).

1.3.4 DC are involved in inducing tolerance

DC are considered sentinels of the immune system due not only to their central position in initiating a primary immune response but also because of their involvement in inducing tolerance.

The way T and B cells develop their antigen receptors by genomic recombination leads to the possibility of billions of different combination. Many of these antigen receptors are potentially autoantigenic and recognize self antigens. To avoid autoimmune reactions these T lymphocytes must be deleted. Self–reacting thymocytes (T lymphocyte precursors) are mostly deleted by negative selection in the thymus (39, 43). Both thymic DC and peripheral DC migrated from peripheral tissues have been found to be efficient mediators of negative selection in the thymus (44-46). Some auto-reactive T cells, mainly those with low affinity, manage to escape from the clonal deletion and will have to be controlled by peripheral tolerance to prevent tissue damage (47, 48).

Peripheral tolerance mechanisms include anergy, deletion and suppression to inhibit self-reacting T lymphocytes. Anergy is thought to take place when APC present autoantigen to naïve T cells in the absence of co-stimulatory molecules. This will lead to alterations inhibiting the T cell to react to this antigen if restimulated (39). Activation of an autoreactive T lymphocyte by repeated simulation or in the absence of inflammation can also lead to activation of death receptors and apoptosis (39). Another mechanism to induce tolerance is inhibition of the autoreactive T cell by regulatory T cells (Treg). It is thought that a failure of these mechanisms is the cause of the development of autoimmunity.
Figure 1.2 shows a schematic representation of how immature DC might be involved in the induction of tolerance. Immature DC constantly capture antigens in their environment from apoptotic cells and then migrate to secondary lymphoid tissues. In the absence of inflammation the DC do not mature and they will present the self-antigens to naïve T cells in absence of co-stimulatory molecules. This will then lead to deletion of the autoreactive T cell or induction of Treg (49).

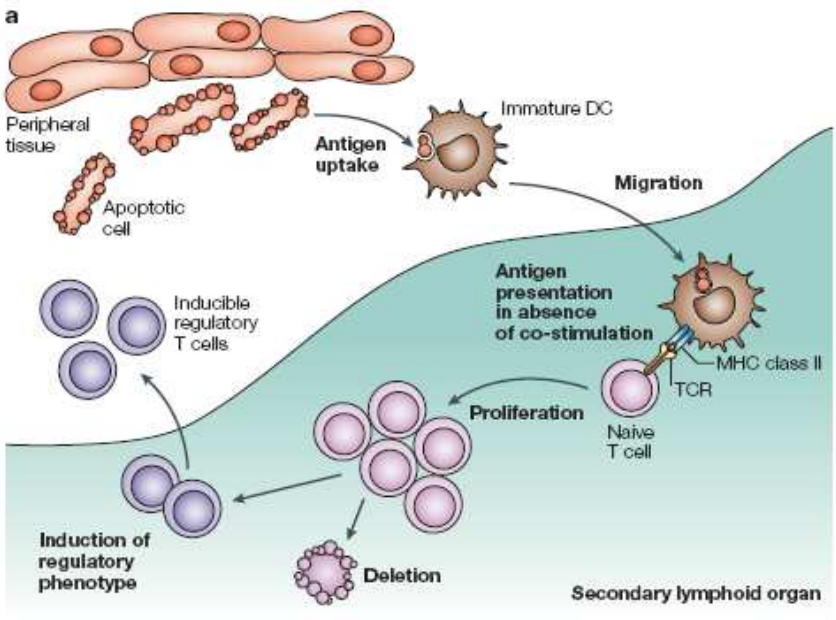

Figure 1.2. Role of immature DC in induction of tolerance. This figure illustrates one hypothesis on the role of immature DC in development of tolerance. The immature DC constantly take up antigens from both the environment and from apoptotic cells from the body's own tissues. They then travel to secondary lymphoid organs where they present the antigens to naïve T cells. In the absence of inflammation the DC stay immature and present the antigens in absence of co-stimulatory molecules. This will lead to either deletion of the T cells or induction of Treg (49). Modified from Banchereau & Palucka. *Nat Rev Immunol*, 2005.

Based on the role DC have both in central and peripheral tolerance there have been speculations on whether or not they protect us from the development of autoimmune diseases. To support this hypothesis it has been reported recently that constitutively DC depleted mice develop spontaneous and severe autoimmune diseases (50).

1.4 Role of Ro52 and IRF in DC

Autoantibodies against the Ro52 protein are found in sera of many patients with SS and they are also used as a diagnostic marker (5). The reason why Ro52 is a target for autoantibodies is not known, and the function of the protein has been unknown for decades. In the recent years it has been found that Ro52 is an E3 ubiqutin ligase with the ability to ubiquitinate itself and other proteins (51). Ro52 is also known as TRIM21 because it contain a tripartite motif (TRIM) (52, 53). Ubiquitin is a small regulatory protein with wide functional diversity. A key task is targeting proteins in the cell for degradation, but it can also regulate other functions

like translation, activation of transcription factors and DNA repair (54). Studies done on a mouse B cell line showed that overexpression of Ro52 can have anti-proliferative and cell-death-mediating properties (51). Its targets for ubiquitination are for the most part illusive, but it has been found to regulate two members of the interferon regulatory factor (IRF) protein family, IRF-3 and IRF-8. It has been suggested that Ro52 functions as a E3 ubiquitin ligase for IRF-8 and that this leads to increased cytokine expression in macrophages (55). Ro52 has also been suggested to regulate levels of IRF-3 by polyubiquitin–mediated degradation (56).

The IRF family of proteins are transcription factors which have been found to be key players in the development maturation and function of DC (57, 58). Figure 3 illustrates the role of different IRF proteins in regulating transcription of IFNα/β and inflammatory cytokines upon activation.
IRF-8, also known as interferon consensus sequence-binding protein (ICSBP), is suggested to have a pivotal role in DC maturation, trafficking, antigen uptake and presentation. It has for instance been shown that IRF-8 knock out mice have defective endocytic capacity and antigen presenting abilities (59). IRF-3 plays a critical role in the induction of type I interferons after a viral infection (60).

Type I IFN are cytokines which mediate early response to pathogens and the most potent stimuli for IFN production is binding of viral nucleic acids (39). IFNα/β produced at an early period in an inflammatory response has the ability to activate other cells. As illustrated in figure 1.3, different IRF proteins participate in these pathways. For instance IRF-8 is both activated by an IFN stimulus and also contributes to an increased IFN production.

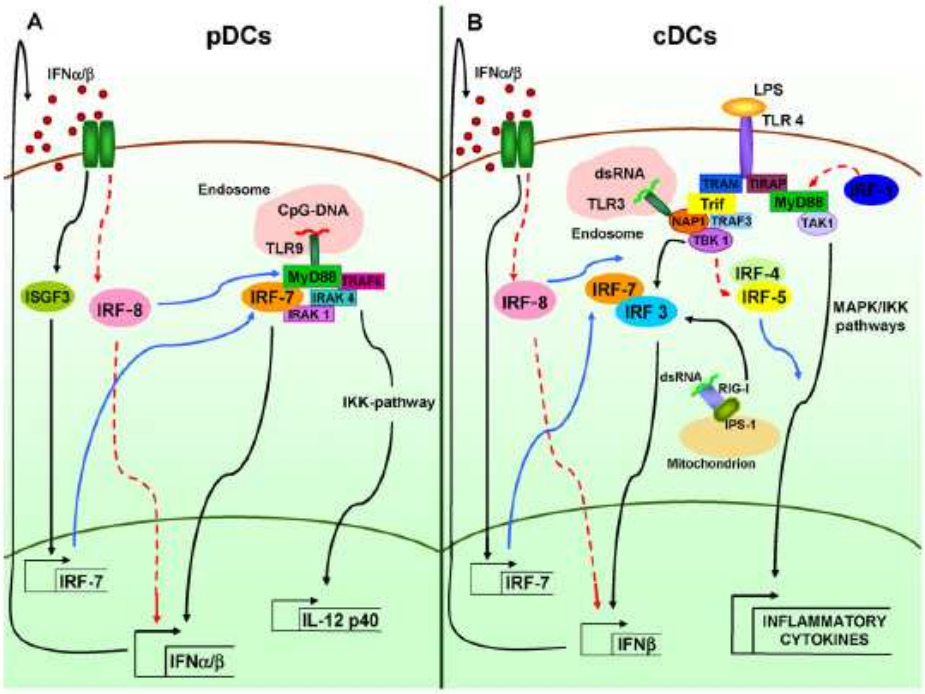

Figure 1.3. Regulation of type I IFN and inflammatory cytokines by IRF proteins. IRF family of proteins are involved in regulation of IFNα/β and inflammatory cytokines in DC through several pathways. For instance is IRF-8 both activated by IFNα/β stimuli and a regulator of transcription of IFNα/β. From Gabriele & Ozato. *Cytokine Growth Factor Rev*. 2007. (57)

1.5 Dendritic cells in SS

Until now not much work has been done on examining the role of DC in SS. Most of the studies performed on SS have focused on B and T cell. Only recently first studies which implicate a role for DC in the pathogenesis of SS have been conducted. The focus mainly has been on the presence of follicular DC (FDC) in salivary gland infiltrates of patients with SS (61). GC-like structures which have been found in minor salivary glands of some patients with SS, consist of T and B cell aggregates within a FDC and endothelial cell network (10).

In SS there has also as mentioned earlier been discovered an activation of type I IFN signature in salivary glands of patient with SS and the presence of type I IFN producing pDC in patients with SS but not in healthy controls (15, 17, 62). Viral infections have been proposed to be the initial cause of the IFN production. The secretion of IFN-α from pDC may lead to apoptosis of cells and autoantibody production towards the apoptotic material (63). This may initiat a vicious cycle where elevated levels of IFN attracts more pDC to the the affected area (64).

A hypothesis that has been proposed on how dysregulation of the DC might lead to autoimmunity is shown in figure 1.4. An immature DC that has taken up an autoantigen is

stimulated by an elevated level of IFNα/β caused by a viral infection. This leads to inappropriate maturation of the DC and presentation of the autoantigen together with co-stimulatory molecules. In a healthy individual this should lead to apoptosis induction in the DC or anergy, where Treg inhibit presentation to autoreactive T cells. In the case of autoimmunity the DC activate autoreactive T cells that then activate autoreactive B cell which start producing autoantibodies towards the autoantigen (64).

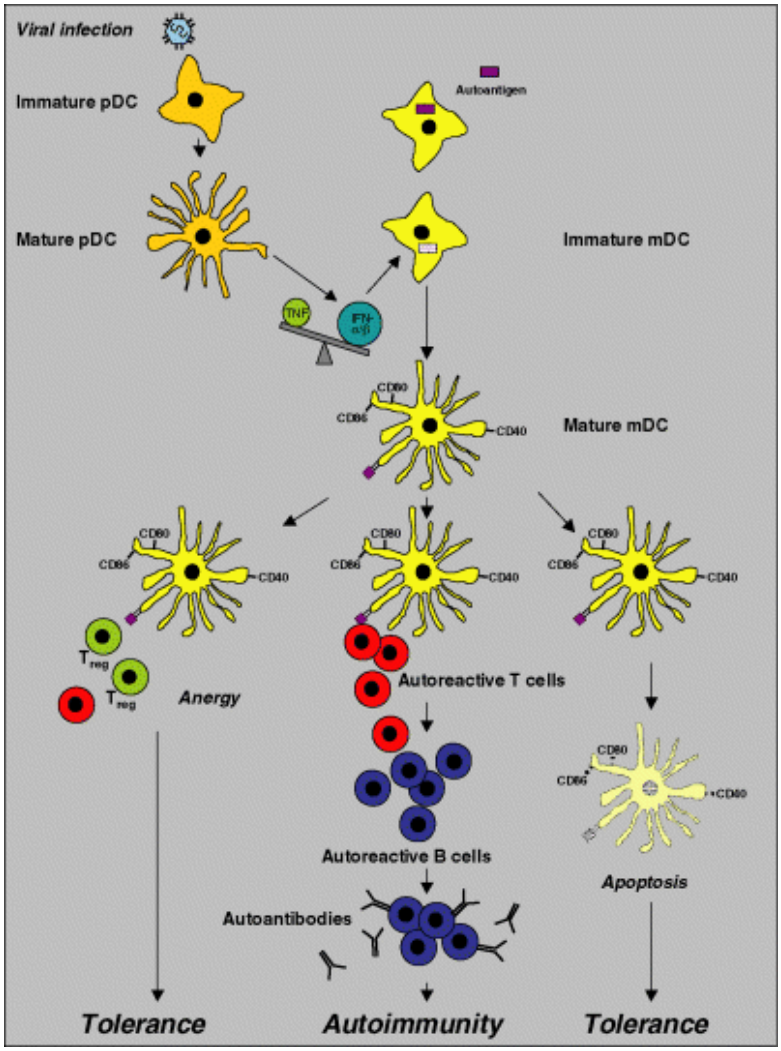

Figure 1.4. The hypothesis on how the dysregulation of the immature DC might lead to autoimmunity.
An initial viral infection stimulates pDC to produce type I IFN. Due to continual stimulation the IFN levels become elevated. This leads to inappropriate stimulation of an immature DC carrying an autoantigen. The immature DC is inappropriately matured and presents both autoantigens and co-stimulatory molecules. In a healthy individual this is controlled by induction of apoptosis or inhibition of autoantigen presentation by Treg. In autoimmunity this leads to activation of autoreactive T cells which again activate autoreactive B cells that start to produce autoantibodies towards the autoantigen presented by the DC. From Vogelsang *et al. Scand J Immunol*, 2006 (64)

1.6 Aim of the study

The aim of this master thesis was to perform functional analyses of moDC from patients with SS and compare them to moDC from healthy controls.
Functional analyses of immature and mature moDC regarding endocytic and migratory capacity were to be performed as both are crucial functions of DC. IL-12p70 production was to be analyzed since this cytokine is one of the most important one for T cell stimulation. Protein expression of Ro52 and IRF-8 was analyzed as Ro52 is a commonly detected autoantigen in patients with SS and IRF-8 is involved in differentiation and function of DC and is one of the targets of ubiquitination by Ro52.

2. Materials

2.1 Study subjects

Heparinized blood from SS patients fulfilling the AECC criteria was collected at the Department of Rheumatology at the Haukeland University Hospital. The control group consisted of gender and age matched healthy persons that were recruited from the same geographical area as the patients.

2.2 Equipment

Name	Company
Cell counter: CASY	Schärfe system GmbH, Germany
Centrifuge: Kubota 8700.	Kubota. Tokyo Japan
Thermo Heraeus multifuge1S-R.	Mandel Scientific company
Mini Spin	Eppedorf, Germany
Heraeus Fresco 17	Thermo Scientific
Flow cytometer: FACS Canto	BD Biosciences, USA
CO_2 Incubator:	Forma scientific, USA
Microplate reader: EMax	Molecular Devices, USA
Microscope: Leica DMIL	Leica, Germany
Mini Trans-blot electrophoretic cell	BioRad, USA
MP3 system	BioRad, USA
10-well, 1.0 mm thickness	
Molecular imager ChemiDoc XRS system	BioRad, USA
Safety cabinet: Nuair biological safety cabinet class II	Nuaire INC, USA

2.3 Plastic ware

Name	Company
50ml tubes	Sarstedt. Germany
15ml tubes	Sarstedt. Germany
6well plates	NUNC. Denmark
Cryotubes	NUNC. Denmark
HTS Transwell- 96 system Insert with 8µm membrane pores	Corning. NY, USA
96 well U shaped plate	NUNC,Denmark

2.4 Kits

Name	Company
BCA™ protein assay kit	Pierce, USA
Immunstar WesternC™ kit	Biorad, USA
ELISA MAX™ SET *Deluxe*. Human IL12p70	BioLegend, USA

2.5 Reagents

Name	Company
25x Proteinase inhibitor, complete EDTA-free	Roche, Germany
BSA	Sigma, USA
CCL19	Immunotools. Germany
CL097	Invivogen, USA
Casyton	Innovatis, Germany
Dextran Fluorescein. 40 000MW	Invitrogen. USA
DMSO	Sigma, UK

Dual color Protein Kaleidoscope Standards	Biorad, USA
EDTA 0,5M	Sigma, USA
GM-CSF	ImmunoTools. Germany
IL-4	ImmunoTools. Germany
Isopropanol	Arcus, Oslo, Norway
Lymphoprep	Axis-Shield Poc AS, Norway
Milk powder	Frema, Germany
Methanol	Merck, Germany
NaF	Merck, Germany
NaOrthovanadat	Merck, Germany
Ponceu S solution	Sigma Aldrich, USA
Penicillin/Streptomyocin	Invitrogen, USA
PMSF	Roche, Germany
RPMI 1640	Lonza, Belgium
Tween20	Merck, Germany
X-Vivo 20 medium	Cambrex (Biowhittaker)

2.6 Antibodies

Name	Company
Primary antibodies	
Actin (I-19) Goat polyclonal	Santa Cruz Biotechnology INC
ICSBP (c 19). Goat polyclonal	Santa Cruz Biotechnology INC
52 kDa Ro/SSA (D12) Mouse monoclonal	Santa Cruz Biotechnology INC

Secondary antibodies

Goat anti-mouse IgG (H+L)/HRP	Bio-Rad. CA, USA
Rabbit anti-goat IgG/HRP	DakoCytomation

2.7 Buffers and media

Name	Reagents
Blocking buffer	TBST 0.1% 5% skim milk powder
1x Blotting buffer pH 8.3	Running buffer without SDS 20% methanol
FACS buffer	0.5 % BSA in PBS
6x Lämmli buffer	375 mM Tris HCl pH 6.8 9% SDS 50% Glycerol 9% β-ME 0.03% Bromphenolblue
PBS	137mM NaCl 2.7mM KCl 8.1mM Na_2HPO_4 1.5mM KH_2PO_4

RIPA	50mM Tris pH 7.4 1% NP40 0.25 % Nadeoxycholat 150mM NaCl 1mM EDTA + 1x proteinase inhibitor (25xstock) 1mM PMSF 1mM Na-orthovanadat 1mM NaF
1x Running buffer	25mM Tris 192 mM Glycine 0.1% SDS Do not adjust pH
10 x TBS	1M Tris pH 8.0 1.4 M NaCl
1 x TBST 0.1%	1 x TBS 0.1% Tween
RP10 medium	RPMI 1640 2 mM glutamine 10 % FBS 50 units/ml Penicillin G sodium 50 µl streptomycin sulfate
Washing buffer (ELISA)	0,05% Tween in PBS

2.8 Software

Name	Company
FlowJo	Tree Star, Inc.
SoftMaxPro	Molecular Devices, USA
Quantity one	BioRad, USA

All reagents not listed here were purchased from Sigma Aldrich, USA or BioRad, USA.

3. Methods

3.1 Generation of monocyte-derived DC (moDC)

To generate DC is a multistep process where the first method is to isolate peripheral blood mononuclear cells (PBMC) which consist of lymphocytes and monocytes. This is done by gradient centrifugation. The second step is to isolate the monocytes in the PBMC from the lymphocytes, which is accomplished by using the ability the monocytes have to adhere to plastic. The third step is to stimulate the monocytes to turn into immature DC, which is done by adding the appropriate cytokines.

3.1.1 Isolation of PBMC from fresh blood.

20-35ml freshly heparinized blood was carefully layered on top of 10ml lymphoprep and centrifuged for 20-30 minutes at 800g, 20ºC. The brake on the centrifuge was turned off to make sure that the layers did not mix.
The PBMC layer, located in-between the plasma and lymphoprep layer, was transferred to a new tube and washed 3x with cold PBS by centrifugation; 4 min, 400g, 4°C.
To determine the number of PBMC in the sample 10µl were removed after the second wash, diluted 1:1000 in casyton, and counted with a CASY® automated cell counter.

3.1.2 Isolation of monocytes by plastic adherence

After the last wash the PBMC were resuspended in X-vivo20 medium and plated out in a concentration of 1 x 10^7 PBMC in 3ml X-vivo medium per well in a 6 well plate.
They were then incubated 1 hour at 37ºC, 5% CO_2.
After incubation the monocytes were washed 2- 3x with PBS (RT) to remove all non adherent cells

3.1.3 Generation of immature moDC

The monocytes were cultured at 37ºC, 5% CO_2 for 5- 6 days in RP10 medium supplemented with IL-4 (20 ng/ml), and GM-CSF (100 ng/ml) which were replenished every 2-3 days.

3.1.4 Maturation of moDC

A fraction of the immature DC were stimulated with imidazoquinoline derivative CL097 (1µg/ml) for 48 hours. This compound activates the TLR 7/8 receptors of the DC which induces activation of the NF-κB pathway in the cells and stimulate maturation.

3.2 Harvesting of moDC

The cells in medium where first transferred from the wells to a new tube and centrifuged for 5 minutes at 400g, 4ºC. PBS with 2mM EDTA was added to the wells to help loosen any remaining cells from the plastic.
After centrifugation the supernatant was collected and stored at -20ºC for later use.
The cell pellet was resuspended in the remaining cells in PBS with EDTA from the wells. Amount of moDC in the suspensions was determined by diluting 10µl 1:1000 in casyton to be counted by a CASY cell counter.
The cells were then centrifuged for 5 minutes at 400g, 4ºC. The supernatant was discarded and the pellet resuspended in PBS or RP10 medium (1 x 10^5 cells pr 100µl). Cells required for different experiments were then distributed to separate tubes.

3.3 Endocytosis

To measure the antigen uptake capacity of the DC dextran conjugated with FITC was used for this assay. Dextran is a water soluble molecule which is taken up via endocytosis by the cells. FITC is a fluorescent dye which can be detected by flow cytometry.
$2x10^5$ moDC in 800µl RP10 medium were divided between two 96 well plates, $5x10^4$ cells in 200µl RP10 medium were plated out in two wells on each plate. They were then pre-incubated approximately 20 minutes, one at 37ºC and the other at 4ºC before FITC-Dextran

(0,25mg/ml) was added to one well of each sample on both plates. They were then incubated for 1 hour at the same temperature.
After incubation the cells were washed 4x with 175µl FACS buffer by centrifugating for 5 minutes at 400g, 4ºC. Thereafter the DC were resuspended in 150µl FACS buffer and immediately analyzed by flow cytometry.

3.4 Chemotaxis

To measure the migratory capacity of the moDC, a chemotaxis assay towards the chemokine CCL19 was performed. This molecule binds to the receptor CCR7, a receptor which is expressed by mature DC, and is found in high concentrations in the lymph nodes and thymus.
235µl RP10 medium + CCL19 (100ng/ml) was added to the bottom chamber of a 96 well transwell plate. To the top of the membrane 5 x 10^4 cells in 80µl RP10 medium was added. The plate was incubated for 16, 5 or 4 hours at 37ºC, 5% CO_2. After incubation the medium with migrated cells from the lower chamber was transferred to appropriate tubes compatible with the CASY cell counter. The wells were washed with 235µl PBS, which was also transferred to the correlating casy tubes and 530µl PBS were added to all the samples (1ml total volume). All the samples were then diluted in 9 ml casyton (1:10 dilution) before the moDC were counted with CASY cell counter.

3.5 Protein Lysate

The cells in PBS or RP10 medium were first centrifuged 5 minutes at 300 g (cells in RP10 medium had to be washed by centrifugation 1x with PBS). The supernatant was discarded, and the pellet resuspended in radioimmunoprecipitation assay (RIPA) buffer (100µl/1 x 10^6 cells). The cells were then incubated on ice for 10 min. After incubation the lysed cells were centrifuged for 5 minutes at 17000g, 4ºC. The supernatant was transferred to a new tube and stored at -80ºC. From the supernatant a 5µl aliquot was taken out and stored at −20ºC to be used in the BCA assay.

3.6 BCA Assay

The bicinchoninic acid (BCA) assay is used for colometric detection and quantification of proteins.
The 5µl aliquots from protein lysates was first diluted 1:5 in PBS.
A standard two-fold serial dilution series were then prepared from BSA, 1,5mg/ml. 10µl of the sample dilution and the dilution series were added in duplicates onto a 96 well plate, and 200µl working reagent was added to all the wells The plate was covered and incubated for 30 minutes at 37ºC before it was analyzed by an ELISA plate reader, absorbance at 595nm.

3.7 SDS-PAGE

SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) is used to separate proteins based on molecular weight. SDS denatures the proteins and covers them with a negative charge allowing them to be separated by mass only.
Polyacrylamide gel has a meshwork of tunnels and fibers which allows the smallest proteins to move faster and thereby move further down the gel. A stacking gel was used which helps focus the proteins and give a clearer band.

3.7.1 Gel casting.

Resolving gel 12 %		**Stacking gel 5 %**	
dH_2O	3,4 ml	dH_2O	2,85ml
30 % Acrylamide	4,0 ml	30 % Acrylamide	0,85 ml
1,5M Tris-HCl pH 8.8	2,5 ml	0,5M Tris-HCl pH 6.8	1,25 ml
10 % SDS	100 µl	10 % SDS	50 µl
10% APS	50 µl	10 % APS	25µl
TEMED	10 µl	TEMED	5µl

For gel casting the BioRad MP3 system, 10 well with 1,00mm thickness was used.
The glass plates were cleaned with 70% ethanol before use and then assembled in the casting frame. The gels were prepared according to the recipies above (APS and TEMED were added immediately before gel casting). The 12% unpolymerized resolving gel was first poured in between the glass plates and layered with isopropanol to give the gel an even surface. The gel was then left to polymerize for 15 – 30 minutes. Thereafter the isopropanol was rinsed off

using distilled water before the stacking gel was layered on top. The comb was then inserted before the stacking gel was left to polymerize for 15 – 30 minutes.
The gels were inserted into the electrode assembly and lowered into the tank. Thereafter the inner camber and about halfway up the outer chamber was filled with 1 x running buffer. The comb was carefully extracted and a syringe used to clean unpolymerized acrylamide from the wells.

3.7.2 Sample preparation

Samples were prepared, consisting of 10µg protein, RIPA buffer and 4µl 6 x Lämmli buffer (volume of 24µl total). The samples were denatured for 5 minutes at 100ºC before being loaded onto the gel. In the first well Dual color protein standard was loaded (6µl). This is a marker with bands of a known size. One sample was used in all gels to make it possible to compare different blots with each other. The electrophoresis was started at 150V and ran until the blue running front left the gel (ca 1 hour).

3.8 Western Blot

Western blot is a method used to identify specific proteins in a sample.
The transfer was performed with BioRad Mini Trans-blot electrophoretic cell system.
A nitrocellulose membrane, filter papers and fiber pads were equilibrated by soaking them in blotting buffer. The nitrocellulose-membrane was placed on top of the polyacrylamide gel in a sandwich between the fiber pads and filter paper. The sandwich was placed in a cassette which was inserted into the electrophoresis module. To maintain an even buffer temperature and ion distribution a frozen ice cooling unit and a magnetic stir bar was added to the tank before it was completely filled with blotting buffer. The transfer was performed for 1 hour at 250 mA.
After transfer the membrane was washed with 1x TBST 0,1% and incubated with Ponceau S solution for about 5 minutes. It was then destained with 1x TBST 0,1% to be able to visualize all proteins on the membrane, and confirm equal loading. The membrane was then incubated 1 hour with blocking solution at RT with agitation to inhibit unspecific binding of the antibodies. Thereafter the membrane was sealed in plastic with primary antibodies and incubated with agitation for one hour RT or overnight at 4ºC.

The membrane was washed 4x 5 minutes in TBST 0.1% with agitation before it was incubated with secondary antibody for 1 hour RT with agitation. After incubation the membrane was washed 4x 5minutes in 1 x TBST 0,1% and 1x 5minutes in 1x TBS with agitation. After the last wash 250µl Luminol/Enhancer solution mixed with 250µl Peroxidase solution from the Immunstar WesternC^{TM} kit and added onto the membrane before it was analyzed using ChemiDoc.

3.9 IL-12p70 ELISA

Enzyme-linked immunosorbent assay (ELISA) is used to detect and quantify antibodies or cytokines in a sample. To examine IL-12 production by the moDC the ELISA^{TM}Set *Deluxe* kit with human IL-12p70 from BioLegend was used and performed according to the protocol.
First diluted capture antibody in coating buffer was added to an ELISA plate and incubated at 4ºC overnight.
The following day the plate was washed 4x with washing buffer before incubation for 1 hour at RT with agitation with assay dilution to block. After incubation the plate was washed 4x with washing buffer. Cell culture supernatant that had been collected during harvesting and a diluted standard was then added. The plate was then incubated for 2 hours RT with agitation. Following incubation the plate was washed 4x again with washing buffer before being incubated for 1 hour at RT with agitation with Detection antibody. The plate was then washed 4x again with wash buffer and incubated for 30 minutes at RT with agitation with Avidin-HRP, which binds to the capture antibody, diluted in assay dilutent. After incubation the plate was washed 5x with washing buffer, soaking 30 seconds-1 minute between washes. After washing the plate was incubated about 20 minutes at RT in the dark with tetra methyl benzidine (TMB) which produces a blue colour proportional to the IL-12p70 concentration in the sample. Stop solution was then added to each well and the plate was read at absorbance 450nm with an ELISA microplate reader and the result was analyzed with SoftMaxPro software.

3.10 Statistics

All analyses were performed with GraphPad Prism 5. Statistics used were D'Agostino and Pearson omnibus normality test to look for normal (Gaussian) distribution. If the populations had normal distribution the difference between them was tested using a two tailed t-test. If the populations did not have normal distribution a Mann-Whitney test was applied to compare the populations. The differences were significant when $p<0,05$.

4. Results

In this study fresh blood was collected from both from a group of patients that had been diagnosed with SS and from a group of healthy controls. Both the patient and the control group consisted of all females exept for one male. The patients ranged from 38 – 79 years of age with a mean age of 60 years and the control group ranged from 38 – 67 years of age with a mean age of 55 years.

PBMC were isolated from the blood within two hours after the blood had been drawn and monocytes were isolated by plastic adherence. Monocyte derived DC (moDC) were generated by culturing them with IL-4 and GM-CSF for 5-6 days. A fraction of the cells was in addition stimulated with the imidazoquinoline compound CL097 TLR7/8 ligand for 48 hours. This generated one population of mature and one of immature moDC from each patient and control. After harvesting and counting, the moDC populations were divided between the different experiments. Endocytosis assay and chemotaxis assay were set up immediately and the rest of the cells were lysed to be used in Western blotting. The cell culture supernatant in which the moDC had been cultured was collected and stored at −20ºC to be used in an ELISA assay.

4.1 DC from SS patients may have higher endocytic capacity than DC from healthy controls

An important function of the DC is endocytosis to internalize antigens that will then be processed and eventually be presented to T cells. To see if there was a functional difference in the endocytic capacity of moDC from SS patients and healthy controls, a dextran assay was performed.

The moDC were incubated for 1 hour with FITC-dextran at 37ºC or at 4ºC respectively, washed four times with FACS buffer and immediately afterwards analyzed by flow cytometry.

Figure 4.1 shows a representative result of the endocytosis assay of immature and mature moDC from one SS patient.

The FSC/SSC plot shows the gating of the moDC based on size (FSC) and granularity (SSC). This was done to exclude other cells like lymphocytes, monocytes, dead cells and cellular debris which are smaller and less granulated. In most experiment about 10 000 cells were

acquired in the gate. The green histograms show fluorescence intensity of the gated moDC, which translates into how much FITC-dextran the cells have taken up. The black histogram show a control group of moDC from each population which was incubated without dextran. The further the histogram shifts to the right, the higher is the fluorescence intensity. The moDC incubated at 4ºC were used as a control. Since cells incubated at 4ºC should be inactive the fluorescence intensity values from these cells are considered to be the result of unspecific binding and not by endocytosis. The fluorescence intensity values from moDC incubated at 37ºC illustrates how much FITC-dextran was taken up by the moDC. Both the immature and mature moDC took up FITC-dextran, but the fluorescence intensity of the immature cells was much higher. This shows that the stimulation of moDC with the TLR7/8 ligand CL097 leads to reduced uptake of FITC-dextran.

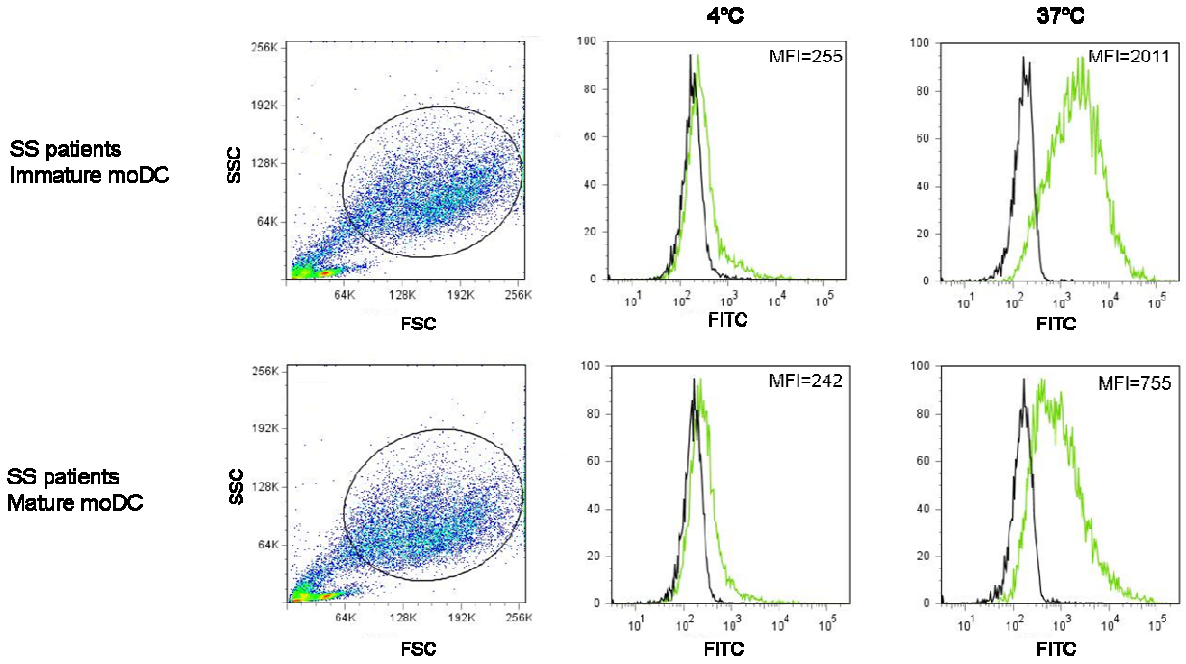

Figure 4.1. Maturation of moDC with TLR7/8 ligand CL097 leads to reduced uptake of dextran.
MoDC were generated by culturing monocytes with IL-4 and GM-CSF for 5-6 days. Some of the immature moDC were stimulated with Toll like receptor 7/8 ligand CL097 for 48 hours. Both mature and immature moDC were incubated for 1 hour with FITC dextran at 37ºC and 4ºC respectively. They were washed four times with FACS buffer and analyzed by flow cytometry. About 10 000 cells were acquired in the gate according to the known size (FSC) and granularity (SSC) of moDC. The representative result of one experiment with cells from an SS patient is shown. The histograms represent the fluorescence intensity of the gated cells. The black histograms represent moDC incubated without dextran and the green histograms represent moDC incubated with dextran. As can be seen by the FITC uptake of the moDC incubated at 37ºC, the immature moDC take up more FITC Dextran than the mature cells (immature moDC have median fluorescence intensity (MFI) = 2011 vs. the mature MFI = 755) showing that stimulation with CL097 leads to reduced endocytosis.

To analyze the endocytic activity of the moDC, the median fluorescence intensity (MFI) from moDC incubated at 4ºC was subtracted from the values of the corresponding population incubated at 37ºC. Statistics used was first a D'Agostino and Pearson omnibus normality test to look for normal (Gaussian) distribution of the different populations. All the populations passed the normality test so the difference between the populations were tested using a two tailed t-test. The following groups were compared:

1. SS patients immature moDC vs. control immature moDC,
2. SS patients mature moDC vs. control mature moDC,
3. SS patients immature moDC vs. SS patients mature moDC
4. Control immature moDC vs. control mature moDC

Figure 4.2 represents the analysis of the median fluorescence intensity (MFI) of all the samples from the different populations. From the SS patients there were 12 immature moDC and 15 mature moDC and from healthy controls there were 10 immature moDC and 9 mature moDC.

No significant difference (n.s.) in FITC-dextran uptake was observed between immature moDC from patients and controls. On the other hand the fluorescent intensity median (M) differs considerably between the two populations: (Patients: M=2685,31 vs. controls M=4134,46) This variation suggests that there is a difference and that the immature moDC from patients have lower endocytic capacity than the immature moDC from controls. Between the mature moDC from patients and controls there is no significant difference. Between immature and mature moDC from SS patients a significant difference ($p=0,0245$), but not between immature and mature moDC from controls. Comparing the median of each population on the other hand suggests that there is an even bigger difference between the immature and mature moDC of controls than patients (Controls: immature M = 4134,46 vs. mature M = 1561,81; Patients: immature M = 2685,31 vs. mature M = 1857,63. $p<0,05$).

After analyzing the graph it was suggested that the samples for immature moDC from controls seemed to be divided into two groups, six samples with very high MFI values and four samples with low MFI values. After analysis of the individual experiments it was proposed that the six highest values might be incorrect due to high lymphocyte contamination and a low percentage of moDC (around 20%). A follow-up test was also performed by a different person in the group with 11 new healthy controls. The result from this test supported the assumption that the samples with the lower values were correct and that immature patients

have a higher endocytic activity than the immature controls (data not shown). Figure 4.2B shows the result after eliminating the six high MFI samples. In this figure it is shown that the immature moDC from patients have higher endocytic capacity than the controls (Patients: M = 2663,27 vs. controls M = 1384,73). The MFI values for mature moDC from patients are also higher in patients than in controls (Patients M= 1857,63 vs. controls M= 479,96). This indicates that both immature and mature moDC from patients have higher endocytic capacity than the controls.

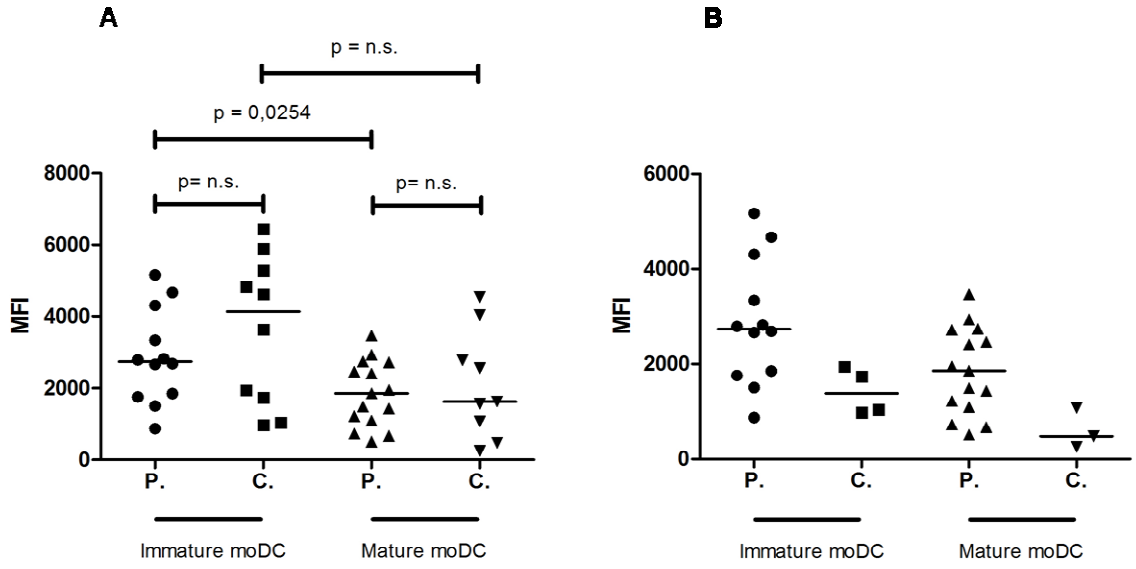

Figure 4.2. MoDC from patients seem to take up less FITC-dextran than controls, but they take up more after correcting the results.

These graphs show the median fluorescence intensity (MFI) of the samples in the dextran assay. The MFI was calculated by subtracting median fluorescence of the moDC incubated at 4ºC from moDC incubated at 37ºC.

A Results from 12 immature and 15 mature moDC from SS patients (P.) and 10 immature and 9 mature moDC from controls (C.). A D'Agostino and Pearson omnibus normality test was used to look for a Gaussian distribution in the different groups and differences between the populations where tested using a two tailed t-test. The statistics indicate that there are non significant (n.s.) differences between immature moDC of patients and controls and between mature moDC. The median (M) values of the two groups suggest that the immature moDC from patients (M=2685,31) take up less FITC-dextran than the immature moDC from controls (M=4134,46).

B corrected results seen in A. The highest values of moDC from controls have been removed. It was suggested that these were most likely not correct due to high lymphocyte contamination and low moDC population (around 20%) in the samples. Due to few samples it was not possible to run statistics on the populations, but one can see that the result of the experiment changes from the result in fig. A. This result shows that immature moDC from patients (M = 2663,27) take up more dextran than the immature moDC from controls (M = 1384,73).

The mature moDC from patients took up less dextran than mature moDC from controls (Patients: M = 1857,63 vs. controls: M = 479,96).

4.2 Mature moDC from SS patients have higher migratory capacity than mature moDC from healthy controls

An important function of the maturing DC is the migration by chemotaxis from peripheral tissues to secondary lymphoid organs to present antigens to naïve T lymphocytes. CCL19 is a known chemokine which has been found to be important in the trafficking of DC by binding to CCR7 receptor, expressed by mature DC. Therefore a chemotaxis assay was set up towards CCL19 to measure the chemotaxis of the immature and mature moDC.

Different incubation times were tested in several experiments, the graph in figure 4.3 shows the results from experiments using 4 hours incubation. There is no difference between immature moDC from SS patients and controls; they both have very low chemotaxis. The mature moDC from SS patients show a slightly higher chemotaxis than the mature moDC from controls (Patients M = 11334,80 cells vs. controls: M= 8632,83 cells).

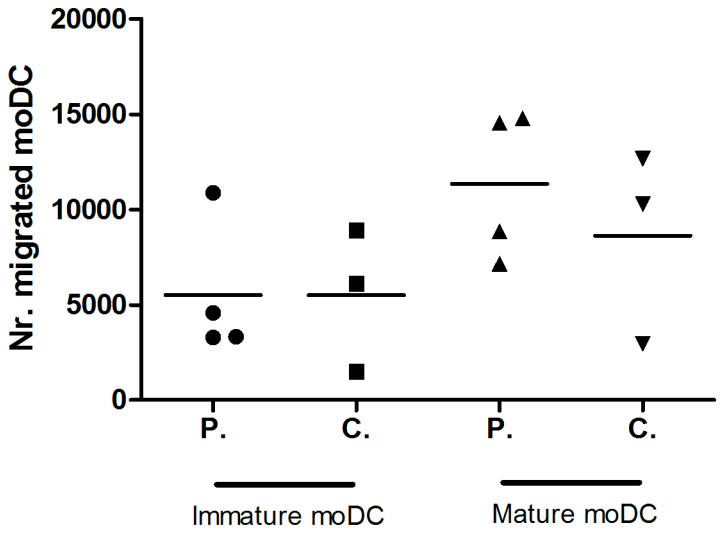

Figure 4.3. Mature moDC from SS patients migrate better than mature moDC from controls. In a transmigration plate $5x10^4$ moDC in medium was placed on top of a membrane with 8µm pores, small enough to only let through actively migrating cells. In the bottom chamber medium with chemoattractant CCL19 was added. This is the results from 4 immature and mature moDC from SS patients (P.) and 3 immature and mature moDC from healthy controls (C.) after 4 hours incubation. The cells that had migrated to the bottom chamber was counted with a CASY cell counter. The result of the experiment indicates that there is no difference in chemotaxis between immature moDC from SS patients and healthy controls. Between the mature moDC there is a difference, the result indicate that mature moDC from SS patients have higher chemotaxis than mature moDC from controls (Patients M = 11334,80 cells vs. controls: M= 8632,83 cells).

A control was set up in some of the experiments to examine the occurrence of spontaneous migration of cells. This was done by adding moDC populations in duplicates to the top chambers of the transmigration plate and in the bottom chambers adding medium supplemented with CCL19 to one well and medium without CCL19 to the other. These control experiments showed that there was no difference in migration towards medium with and without chemo attractant (data not shown).

4.3 Expression of IRF-8 and Ro52

Using protein lysate which had been prepared from the moDC from most of the samples, western blots were set up to analyze the expression of Ro52 and IRF-8.
IRF-8 is a transcription factor which has been found to be involved in regulating differentiation and functional aspects of DC. It has also been found that activation leads to increase in interferon production (57, 65)
The E3 ubiquitin ligase Ro52 is a known SS associated autoantigen. A correlation between IRF-8 and Ro52 has been reported, suggesting that Ro52 ubiquinates IRF-8 and that they possibly act together in a non degradation pathway (55).

For this experiment I used protein lysates from immature moDC from 18 patients and 12 controls and from mature moDC from 19 patients and 13 controls.
To be able to compare different blots, one sample (mature moDC from a control) was applied to all of the gels in the same position when setting up SDS-PAGE. On each gel, protein lysate from all the different populations were added to lower the effect that small differences in the gels and handling of each membrane could have on the result. How the gels were set up is shown in figure 4.4, which shows a representative membrane.
After blotting, the membranes were first incubated with an IRF-8 antibody, then reprobed with a Ro52 antibody and finally they were incubated with an actin antibody which was used as a loading control.
A ChemiDoc molecular imager was used to image the membranes. The blots were then analyzed with Quantity One analysis software, which measured the intensity of each band.
The intensity of the standard band was set to 100% and the other bands on the membrane from different samples were calculated in relation to this standard. This resulted in a value for each band of IRF-8, Ro52 and actin. To exclude differences caused by different amounts of

proteins loaded, a ratio was calculated from the values of each band of IRF-8 and Ro52 with the values of the corresponding actin band. The analysis for all the samples is seen in fig 4.5 (IRF-8) and fig 4.6 (Ro52). The statistics were calculated using a normality test and a two tailed t-test in the same manner as the dextran assay.

The results of the experiments show that the expression of IRF-8 in immature moDC is significantly lower in moDC from SS patients than from the controls (Patients: M= 2,88 vs. controls M = 3,92, $p<0,05$). When comparing the mature moDC there is no significant difference, but they both have lower expression of IRF-8 than the immature moDC.

Between immature and mature moDC from patients there is no significant difference, but it seems like the expression in immature moDCc is higher (Immature moDC M= 2,88 vs. mature moDC M= 1,96). In-between the immature and mature moDC from controls there is a significant difference with the immature moDC being significantly higher than the mature moDC (Immature moDC M= 3,92 vs. mature moDC M= 2,14, $p<0,05$).

There are also some differences in the expression of Ro52 between moDC from patients and controls. Between immature moDC from SS patients and controls there is no significant difference, but the expression seems to be a bit higher in the patients than in the controls (Patients: M= 0,95 vs. controls: M= 0,76). On the other hand there is a significant difference between mature moDC from SS patient and controls. There is a lower expression of Ro52 in mature moDC from patients than from controls (Patients: M= 0,78 vs. controls: M= 1,02; $p<0,05$). Between immature and mature moDC from patients there is a significant difference, with a higher expression in immature than in mature moDC (Immature: M= 0,95 vs. mature: M= 0,78; $p<0,05$).

Between immature and mature controls there is no significant difference, but it seems like the immature moDC have a lower expression than the mature moDC (Immature: M= 0,76 vs. mature: M= 1,02). It seems like stimulation with CL097 result in lower expression of Ro52 in patients vs. a higher expression after stimulation in healthy controls.

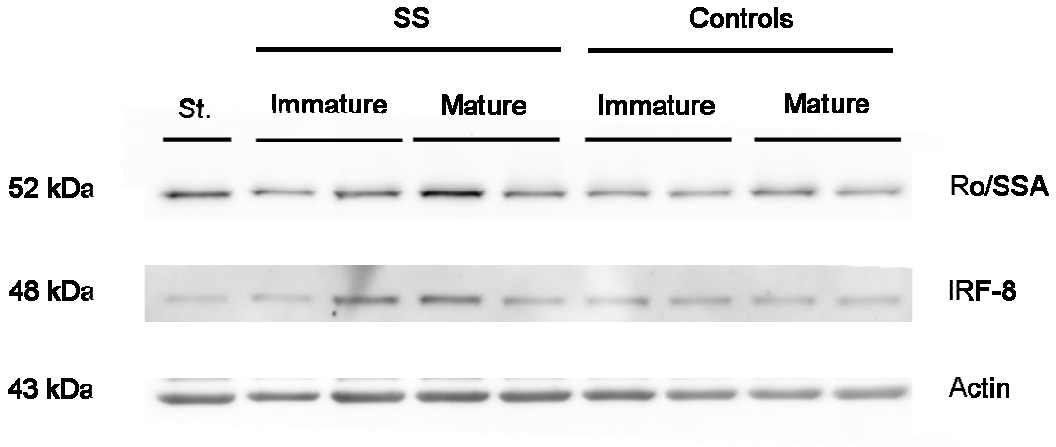

Figure 4.4. Representative result from the Western blot analysis. This figure shows one representative Western blot with Ro52, IRF-8 and actin bands from samples from all the populations. On each gel protein lysate from the different populations; immature and mature SS patients and immature and mature controls was loaded. In the second left lane next to the marker was the standard (St) sample which was applied to all the blots in the same position to be able to compare different membranes. The membranes were imaged using the ChemiDoc system and analyzed with Quantity One analysis software which measured the intensity of each band. The standard was set at 100%, and the intensity of the other bands was determined in relation to this band. Actin was used as a loading control to exclude differences due to amount of loaded protein.

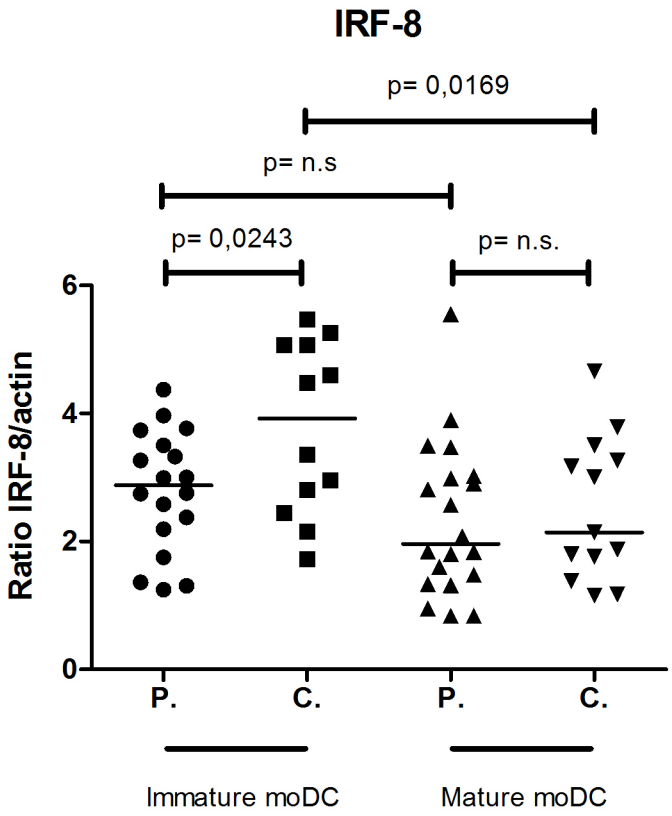

Figure 4.5. Expression of IRF-8 is significantly lower in immature moDC from SS patients than controls. This figure shows the result from Western blots using from 18 immature and 20 mature moDC from patients (P.) and 12 immature and 13 mature moDC from healthy controls (C.). The values for the samples were calculated by making a ratio of the value of the IRF-8 band of each sample and the corresponding actin band. The results show that the expression of IRF-8 is significantly lower in the moDC from the patients compared to the controls (Patients: M= 2,88 vs. controls M = 3,92, $p<0,05$**).** Between mature moDC from SS patients and controls there is no significant difference, but they both have a lower expression than the immature moDC. Between immature and mature moDC from patients there is no significant difference, but it seems like the expression in patients is higher (Immature moDC M= 2,88 vs. mature moDC M= 1,96). Inbetween the immature and mature moDC there is a significant difference with the immature moDC being significantly higher than the mature moDC (Immature moDC M= 3,92 vs. mature moDC 2,14). The results indicate that the immature moDC from SS patients are more similar to the mature moDC than to immature moDC from the healthy controls in the expression of IRF-8.

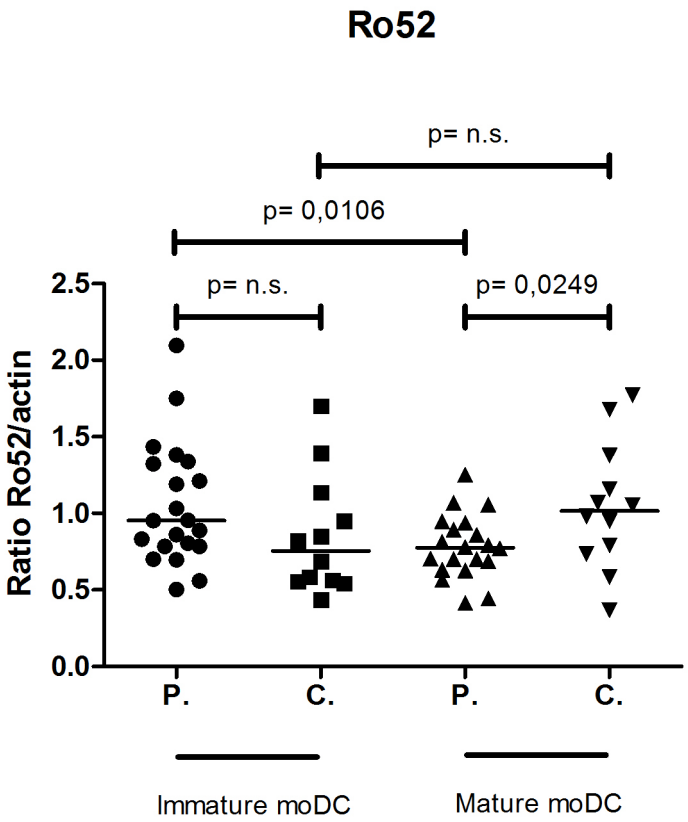

Figure 4.6. Expression of Ro52 is significantly lower in mature moDC from SS patients than from controls. Analysis of all Western blots of Ro52 expression is shown. Samples from18 immature and 20 mature moDC from patients (P.) and 12 immature and 13 mature moDC from healthy controls (C.) was analysed. A ratio was calculated between the value of the Ro52 band and the corresponding actin band. The results show that there was no significant difference between the immature moDC from patients and controls, but they indicate that the expression might be slightly higher in the moDC from SS patients. (Patients: M= 0,95 vs. controls: M= 0,76) Between the mature moDC there is a significant difference, the mature moDC from patients have a lower expression of Ro52 than the control population **(**Patients: M= 0,78 vs. controls: M= 1,02; $p<0,05$**)**. An interesting aspect with this result is that the stimulation of moDC with CL097 seems to lead to opposite responses. In the patients the stimulation leads to downregulation of Ro52 (Immature: M= 0,95 vs. mature: M= 0,78;$p<0,05$) while in the moDC from controls Ro52 seem to be upregulated after stimulation (Immature: M= 0,76 vs. mature: M= 1,02)**.**

4.4 Slight decrease in IL-12p70 production in mature moDC from SS patients.

Stimulated DC are important in regulating T cell differentiation into Th1 and Th2 subsets. IL-12, which is secreted by mature DC is an important mediator of Th1 cell development (39).
An ELISA was performed to measure IL-12p70 production of the different populations of moDC.
This assay was performed with ELISA MAX^{TM} Set Deluxe for quantification of human IL-12p70. The samples were from cell culture supernatant collected from 21 immature and 18 mature moDC cultures from patients and 20 immature and 16 mature moDC cultures from controls.
Only one of the populations, the immature moDC from patients, passed the normality test so a Mann Whitney test was used to compare the groups.

The result of the assay is illustrated in figure 4.7 which shows that there was no difference in the IL-12p70 production between the immature moDC from SS patients and controls, the median of both populations were approximately 1pg/ml. The IL-12p70 production of the mature moDC from both SS patients and controls was very limited, with most of the concentration of IL-12p70 being below 100 pg/ml, but both had a much higher IL-12p70 concentration than the immature moDC. There was also no significant difference between these two populations, but the median of the IL-12p70 secretion of mature moDC from controls was a bit higher than the median of the patient population (M = 66,5 pg/ml vs. M = 40 pg/ml) suggesting that the moDC from patients have a slightly lower IL-12p70 secretion than healthy controls. This result show that stimulation with CL097 increases IL-12p70 production in both moDC from SS patients and healthy controls, but SS patients seem to have a slightly lower production than the controls.

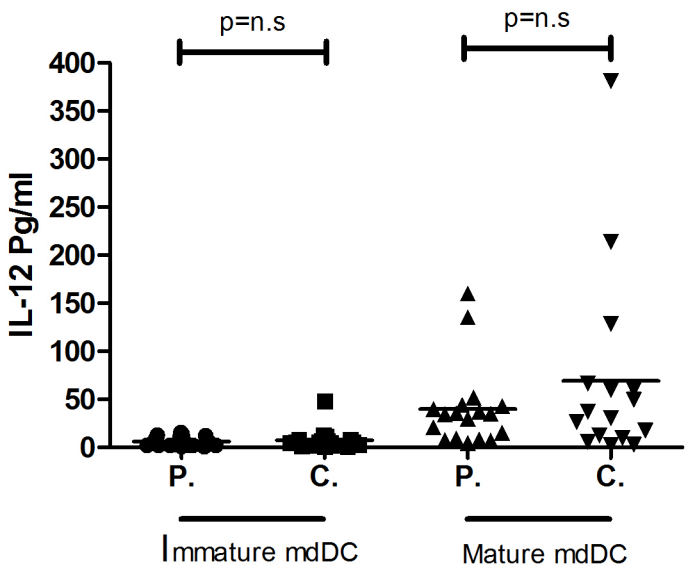

Figure 4.7 No significant difference between IL-12p70 production between SS patients and controls.

An ELISA was set up using cell culture supernatant that had been collected during harvesting. 21 immature and 18 mature moDC samples from SS patients (P.) and 20 immature and 16 mature moDC samples from healthy controls (C.) were used in the assay. Statistics used was Mann-Whitney test to compare the populations. The results show that there was no difference in IL-12p70 production between immature moDC, both moDC from patients and controls had median (M) values of IL-12p70 secretion of about 1pg/ml. The mature moDC from both populations had some secretion of IL-12p70, which showed that stimulation with CL097 increased IL-12p70 production, but is was quite limited. Cell supernatant from most of the samples had IL-12p70 concentrations below 100 pg/ml. There was no significant difference between the SS patients and controls, but the results indicated that the moDC from controls have a slightly higher production than the moDC from patients (M = 66,5pg/ml M= 40 pg/ml respectively).

5. Discussion

SS is an autoimmune disease characterized by a chronic inflammation and lymphocyte infiltration in the exocrine glands. The etiology of the disease is still unknown and at this time there is no cure, all the medication currently on the marked only alleviate symptoms. It is therefore necessary to find out more about the mechanisms behind the disease to be able to improve current treatment and to develop new therapies.
DC are so-called sentinels of the immune system with a pivotal role in initiating an immune response and inducing T cell tolerance to self antigens. During recent years DC have been in the focus of several studies on different autoimmune diseases, mainly because of their role in inducing T cell tolerance, but so far limited efforts have been made in analyzing their possible role in SS.

This study is a part of a more extensive project trying to examine DC in SS patients.
The aim of my project was to look for functional differences between moDC from SS patients and healthy controls. The thesis was built on the hypothesis that immature DC in patients with SS might have had received an improper maturation stimulus and would therefore have a dysfunctional maturation status.
The experiments performed were dextran uptake assays, to look for differences in the endocytic capacity, chemotaxis assays, an ELISA to measure IL-12p70 production and Western blotting to analyze expression of the transcription factor IRF-8 and the E3 ubiquitin ligase Ro52. The results of these experiments showed that there were indeed significant differences between moDC from SS patients and healthy controls. The initial results of the dextran assay suggested that moDC from SS patients had lower endocytic capacity than the controls, but further experiments revealed that the patients DC actually have a higher endocytic capacity than the controls. The chemotaxis assay showed that the mature moDC from patients have a higher chemotaxis than the controls. The Western blot showed a slight increase in the Ro52 expression of immature moDC from patients and a significant decrease in mature moDC from patients, while IRF-8 expression was significantly lower in immature moDC from patients. The results from the ELISA suggested that the IL-12p70 secretion from mature moDC from patients might be lower than from moDC from controls.

The results from the dextran assay where we examined endocytic capacity showed that the stimulation of immature moDC with CL097 resulted in decreased uptake of FITC-dextran.

All the experiments showed this result which made it possible to determine that stimulation with CL097 modulated the endocytic capacity of the moDC reduced dextran uptake. After analyzing all the samples and comparing the different populations the result showed that there was no difference in dextran uptake between mature moDC from patients and controls. Between immature moDC there was a distinct difference, even if not statistically significant. (Patients M = 2685,31 vs. controls M= 4134,46).

However looking closer at all the MFI values of the different samples of the immature moDC from the controls revealed that there is an issue with the result. Although the column for immature moDC passed the normality test, the spread of the fluorescence intensity values seem to be divided into two groups, one group with very high MFI values and one with low MFI values. This gave a confusing result and after discussion with other members of the group we concluded that it was likely that either the samples with very high MFI or the samples with low MFI were incorrect and that more tests were needed to verify the result. The individual experiments were analyzed again and from this it was found that the samples with the higher values seemed to have a higher degree of lymphocyte concentration and a low percentage of target cells. A follow-up test was also done by another person in the group including 11 new control samples. The results from this experiment supported as well the assumption that the lower MFI values were correct. Therefore the conclusion that could be drawn from this experiment needed to be reconsidered to immature moDC from patients being more active in endocytosis than immature moDC from healthy controls. Figure 4.2B shows corrected result. In this graph it seems like the immature moDC from controls take up even less dextan than the mature moDC from patients (controls immature moDC M= 1384,73 vs. patients mature moDC M= 1857,63) and that mature moDC from patient have much higher dextran uptake than mature moDC from controls (patients M= 1857,63 vs. controls M= 479,96). This is most likely not real, but a result of the control populations having very few samples. In the follow-up study the difference between the immature moDC from patients and control was not as large, and there was no difference between the mature moDC populations.

The reason why several of the samples from the healthy controls had such high fluorescence intensity can have several different explanations, but most likely it has a connection with the fact that those were the very first samples that I analyzed, while the four samples that had very low values were the last. It is therefore highly possible that some mistake was made in the very beginning of the experiment.

The reason for higher dextan uptake in immature moDC from patients could possibly be the result of an upregulation of mannose receptors (MR) in immature moDC from SS patients.

These receptors are highly regulated and have been found to be upregulated in response to anti-inflammatory cytokines. For instance is IL-4, which is used in generation of DC together with GM-CSF, one important regulator (66, 67).and it has been found in experiments using macrophages and a murine fetal skin dendritic cell line (FCDC) that IL4 and IL10 are important in transcriptional control of MR (68, 69). Increased endocytosis due to upregulation of MR in moDC has also been reported in patients with psoriasis vulgaris (70).
The hypothesis of the thesis suggested that elevated levels of IFNα/β from pDC could stimulate the immature DC. Lately IFNα has been studied both in DC generation (71-74) and in DC maturation (75-77). It is therefore plausible that immature moDC from SS patients have an altered maturation status leading to increased antigen uptake.

The result of the chemotaxis assay may also support the hypothesis.
A recent study on DC maturation examined the addition of IFNα to a maturation cocktail. They got the result of an upregulation of CD38 which recently have been linked to migration (77). In the chemotaxis experiment performed in this thesis the result suggests that mature moDC from patients had a higher chemotaxis than mature moDC from controls.
If it is the case that the mature moDC from patients have higher chemotaxis due to upregulation of CD38, it could explain why there is a difference in spite of the fact that the migration was not specific towards CCL19.
The control experiment which was performed showed that the chemotaxis of the moDC was not specific towards CCL19 but rather spontaneous since moDC migrated equally towards medium with and without CCL19 addition. Why the moDC do not migrate towards CCL19 when this is a known chemokine receptor ligand pathway can have multiple reasons. Primarily this is a very complex pathway and it has been observed that the upregulation of CCR7 simply by maturation stimuli is insufficient for moDC migration. Additional stimuli are required like for instance prostaglandin E2 (PGE_2) which has been found to increase moDC CCR7 mediated migration (42, 78, 79). It has also been suggested that complement C1q accumulated at the inflammatory site upregulates CCR7 and thereby increase chemotaxis (80).

The analysis of Ro52 expression showed that its expression was significantly reduced in mature moDC from patients compared to controls. As seen in figure 4.6 there seems to be a slight difference between the immature moDC as well. The median of the SS patient population was a bit higher than the median of the control population.

It is interesting that the results seem to show opposite trends. Within the patient group the expression of Ro52 in moDC decreases upon stimulation with CL097 and in the control groups the stimulation with CL097 leads to an increase in Ro52 expression. The role Ro52 has in the cell and its target substrates for ubiquitination is still illusive, but it has been suggested that it plays a role in proliferation and cell death (51). So far IRF-3 and IRF-8have been discovered as targets for ubiquitination by Ro52 leading to degradation and possibly regulation of IRF-3 and IRF-8 (55, 56). In the immature moDC from SS patients it seems like Ro52 is slightly upregulated. This can be explained by findings that have been published earlier suggesting that Ro52 is upregulated due to pro-inflammatory stimuli (81-83) and also as a result of IFNα stimuli (82).
Ro52 expression in mature moDC from controls seems to be upregulated in response to CL097. In SS patients on the other hand it seems to be downregulated instead. An explanation for this is difficult since none of the above studies of Ro52 expression is done on DC, but it could be the result of a dysregulation in a pathway, possibly as a result of an improper early maturation, leading to autoubiquitination and degradation. Overexpression has also been suggested to increase cell death which then might mean that lower expression de-sensitize the cell to death stimuli.

Furthermore, the result of the Western blot analyzing IRF-8 expression show that in the immature moDC the expression of IRF-8 is lower in the patients than in the controls. Considering that IRF-8 is a transcription factor which is induced by IFNγ it was though that maybe the IRF-8 expression would be higher in immature moDC from SS patients and in mature moDC. This seems not to be the case as seen from the results (figure 4.5). The analysis gave the opposite result suggesting a downregulation of IRF-8 from SS patients and mature moDC. The reason behind this could be a possible downregulation or degradation as a result of activation, or as a result of a conformational change upon activation. It would in this case be interesting to analyze nuclear extracts to examine the localization of IRF-8, which would indicate whether the protein had been activated. The fact that I analyzed the expression after 48 hours could also make a difference. It is of course possible that sooner after stimulation the expression could be higher. This result though suggests that IRF-8 expression in immature moDC from patients is closer to the result of mature moDC than immature moDC from healthy controls.
The result also shows that after stimulation the IL-12p70 production is clearly increased both in patients and in controls. Between the patients and controls it is a bit unclear if there is a

difference. It seems like the mature moDC have produced a bit less IL-12p70 than the controls. The possible explanation for this could be also be contributed to a dysfunction in the moDC from SS patient.

The ELISA showed a very low level of IL-12p70 produced by the mature moDC both from patients and controls. This could be explained with the knowledge that TLR7 stimulation does not lead to secretion of high amounts of IL-12, The stimulation of TLR7/8 with R-848, of which CL097 is a derivative, has also been found to lead to a relatively low secretion of IL-12p70 (84) (85).

5.1 Technical issues

While working with the experiments there were some problems that occurred. During the isolation of the moDC there is a washing step to remove non adherent cells like B and T lymphocytes which also are a part of PBMC. If this wash is not done well enough the samples get contaminated with lymphocytes which can possibly influence the moDC population. In the beginning the populations seemed to not be washed well enough, this could be seen in the flow cytometry analysis where two different populations could be seen and there was a low percentage of the target population. It could be possible that lymphocytes could have secreted cytokines inducing upregulation of MR which could lead highly improved endocytic capacity. This could possible explain the high endocytic capacity of the immature moDC from some of the control samples.

During harvesting there was a problem during several of the experiments which resulted in fewer cells than calculated which was distributed between the different experiments. This resulted in too few cells being distributed, which gave an unknown number of moDC used in the migration assay and difficulty in acquiring 10 000 cells in the gate during the flow cytometry assay. The problem was found to stem from an issue with the harvesting. After counting and distributing the cells between different tubes for the experiments they were washed two more times with PBS which resulted in the loss of many cells and possibly some cells died due to prolonged suspension in PBS. After changing the protocol and immediately after counting, resuspending the cells in medium this problem was solved. The prolonged suspension in PBS that the cells in the majority of the experiments were exposed for is in itself a source of error since it could possibly interfere with moDC viability and function.

Concerning the dextran assay it has already been mentioned that in some of the experiments with immature moDC from controls the values were most likely too high due to some error. This would also possibly affect the mature moDC from the same patients since there was a correlation with higher MFI values from immature DC and higher MFI values of mature moDC from the same person. Another possibility is that could be the source of the high MFI values was that a different aliquot of FITC-dextran was used for the first experiments, which potentially could have given more fluorescence than the new aliquot. As was mentioned in the result section, several experiments where performed with different incubation lengths, 16, 5, and 4 hours where tried out. There seemed to be no difference between these experiments since the migration most likely occurs during the first few hours of incubation. The reason that these experiments are not documented is that there seemed to be too few cells added to the top chamber due to the problem during harvesting. This resulted in possibly different numbers of cells added to top chamber of migration plate at different experiments, making them difficult to compare.

A source of error concerning the Western blot is the fact that the experiments were done on several different blots where there may have been small differences in the gels and the way they were handled. To minimize these errors there was a standard which was applied to all blots and actin was used as a loading control. The standard in itself may be used as a source for error since it was used several times and was due to this thawed and frozen several times. It is of course possible that some of the proteins started degrading and would in this way change the expression level on the blot.

A source of error in relation to the ELISA could be a possible effect on IL-12p70 in cell supernatant to prolonged time between harvesting and collection of cell supernatant until it was frozen at -20ºC. If this took too long it could possibly result in degradation of IL-12p70. Secretion has also been found to be time limited, in example R-848 has the highest secretion after 12 hours (84). We matured the moDC for 48 hours and this could lead to lower levels of secreted IL-12p70.

5.2 Conclusion

The conclusion of this study and the most important part of the results is the fact that they show that there are indeed functional differences between moDC from SS patients and moDC from healthy controls. This indicates that the moDC in SS patients are dysfunctional compared to moDC in healthy controls. One explanation for this dysfunction could possibly be an inappropriate activation of the immature DC as suggested in the hypothesis that was the basis for this study.

The results from this master thesis also emphasize the need to continue the studies of examining the possible role of DC in SS.

5.3 Future perspectives

The potential role of DC in SS is of yet a largely unexplored subject and these experiments are just a part of a large study. With this in mind and that these experiments suggest that there in fact are functional differences between moDC from patients with SS and healthy controls, it supports the need to continue the studies. Since there were some uncertainties about some of the experiments more samples are needed to verify these results.

Other experiments that will be interesting to perform in the near future are the analysis of MR expression on the different moDC populations to see if the increased endocytic capacity is indeed a result of upregulation. Nuclear extracts was also was collected from most of the samples, and these will be interesting to analyze for expression of IRF-8 and Ro52 and also other possible signaling molecules. Many other proteins may also be interesting to analyze by Western blot.

It would also be interesting to analyze secretion of several other cytokines and chemokines. The migration assay is interesting to repeat using different chemokine ligands than CCL19 or maybe adding additional stimuli like PGE2 or the complement Cq1 which may upregulate the CCR7 receptor. Since it could be a possibility that a difference in CD38 expression is the cause of higher chemotaxis in mature moDC from SS patients it is also interesting to analyze CD38 expression in the different populations.

Antigen presentation and stimulation of T cells is an important role of the DC, therefore it could be exiting to analyze the T cell stimulatory capacity of the different moDC populations.

These different experiments are just naming a few interesting studies that could be performed in the near future. Since this is still a wildly undiscovered field the possibilities are immense and discoveries will hopefully shed some new light on the mechanisms behind the development of SS. Any new insight could possibly contribute to development of better medication and help that will in the end benefit the patients.

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