Tackling the Achilles heel of spondyloarthritis

T.S. Van Kempen

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Cover story: This photo was taken by Eugenie Heesen-Leemans at a salvage yard. She is my great-aunt. Eugenie had a sixth sense for objects, and used her camera to meticulously render details into an abstract art composition. She translated the series of photographs into a narrative. She was honoured that this photograph was chosen as my cover. She passed away in 2018.

Tackling the Achilles heel of spondyloarthritis

Is it an autoinflammatory or autoimmune disease?

Aanpak van de Achilleshiel van spondyloarthritis

Is het een auto-inflammatoire of auto-immuunziekte?
(met een samenvatting in het Nederlands)

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"The first principle of science is that you must not fool yourself– and you are the easiest person to fool. So you have to be very careful about that. After you've not fooled yourself, it's easy not to fool other scientists and laymen."
- Richard P. Feynman-

Voor Vincent, Julius en Maud

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Chapter 1

Introduction

Progress in science is made with key discoveries, proper data analyses, and discussion about scientific ideas. Scientific controversies are inevitable since time immemorial. The battle between the "cellularists" and the "humoralists" in the late 19th century is a classic example of disputes within the immunology community. The development of the small pox vaccine by Edward Jenner and the attenuated vaccine by Louis Pasteur were one of the first observations that confirmed that certain diseases are caused by micro-organisms (1, 2). The mechanism of how vaccination protects the host towards a second exposure was uncertain, but it was not much later that Elie Metchnikoff described the existence of *phagocytic cells* in invertebrates (1). He extrapolated his findings to animal models using anthrax bacillus and detected the bacilli in phagocytic cells after intraperitoneal injections (2). These cells, now known as *macrophages* in vertebrate animals, were thought to function as key players in immunity as these cells digest pathogens. The concept *cellular immunity* was proposed. At the same time, Emil Behring and Shibasaburo Kitasato discovered that mice immunized with diphtheria and tetanus toxoid had humoral factors in their serum that neutralized toxins. These findings were corroborated by Paul Ehrlich, who administered plant toxins ricin and abrin orally to mice and rabbits (2). Here, serum that was transferred passively was able to induce resistance to a bacterial insult in previously unexposed mice (1, 2). In this experimental setup, phagocytic cells were dispensable players in the protection against toxins. The hypothesis was that all immunity is made by humoral factors and *humoral immunity* became the centre of attention. A scientific battle between the cellularist and humoralists arose and undermined recognition of the contribution of cells in immunity. Experiments with cells were discouraged by the scientific community. It was only after World War II that cellular immunology revived (2). *Plasma cells* and *B cells* were discovered and defined as the producers of antibodies (3-5). Another key discovery was made by Steinman and Cohn, who described a cell that had tree-like processes and was constantly forming and retracting. They named them *dendritic cells* (DC) (6). Studies on DCs have shown that these cells process antigens and present the antigen-derived peptides to *T cells*, which in turn activate B cells. B cells differentiate into plasma cell that produce antibodies (7-9). After a century of research, the DC formed the link between the cellularists and humoralists. This case shows that the scientific community can be rather conservative and reluctant to change once ideas are rooted in their mind. However, perseverance can stimulate new experiments to come closer to the truth.

In the 21th century, scientific controversies are still apparent. There is still no consensus reached on the aetiology of the disease spondyloarthritis (SpA). SpA encompasses a heterogeneous spectrum of rheumatic diseases and comprises of ankylosing spondylitis (AS), psoriatic arthritis (PsA), reactive arthritis (ReA) and SpA related to inflammatory bowel disease (SpA-IBD) (10). SpA is an inflammatory disease that manifests with articular and extra-articular involvement. AS mainly affects the axial skeleton and the dramatic rigidity

seen in these patients is caused by enthesitis of the sacroiliac and apophyseal joints and has the propensity for sacroiliac joint and spinal fusion (10). PsA have more peripheral manifestations and can be subdivided in five groups, namely symmetrical polyarthritis, asymmetrical oligoarthritis, distal interphalangeal (DIP) joint involvement, axial disease, and arthritis mutilans (11, 12). Furthermore, SpA can also show extra-articular manifestations at epithelial barriers, including the skin (psoriasis), and at sites with high biomechanical load, including the gut (terminal ileum, near the ileocecal/Bauhin's valve) ciliary bodies in the eye, nail root, aortic root and aortic valve (13-16).
Although patients can present with clinically distinct symptoms, SpA can be considered to have some shared immunopathogenic mechanisms. Firstly, SpA show linkage at genome-wide significance to chromosome 6p21, which harbours the polymorphic human leukocyte antigen (HLA) class I and class II genes (17, 18). The frequency of HLA-B27 in AS is over 85% and HLA-B07, -08, -B27, B38 and -B39 are risk factors for PsA (19-21). Secondly, the IL-17-inflammatory pathway plays a predominant role in the pathogenesis of SpA as the blocking of IL-17 by antibodies improves clinical outcome (22-24). Of note, the blockage of IL23p19 in peripheral SpA showed encouraging effects on inhibiting skin and peripheral joint symptoms as shown in a phase II clinical trial, but the beneficial effect were not seen in axial SpA (25, 26). This suggests that IL-23 does not directly translates to IL-17, and the IL-23-IL-17 axis contributes differently in PsA versus AS. Thirdly, as described above, the clinical features of SpA localizes at the interface with the external environment (intestinal mucosa and skin) and to sites with physical stress (enthesis organ) (13).

Two hypotheses were developed to explain the pathogenicity of SpA. The first hypothesis is based on uncontrolled immune responses towards an intrinsic or extrinsic factor and vindicate the *autoinflammatory concept*. Local factors at disease-prone sites, including biomechanical stress and aberrant HLA-B27 molecules on the cell surface, determines the uncontrolled activation of innate immune cells. The second hypothesis covers the *autoimmune concept*, which is based on the canonical role of HLA class I molecules in presenting peptides to CD8+ T cells. The breach of tolerance to autoantigens leads to the development of adaptive immune reactivity towards native antigens.

Before I will highlight the research supporting the two hypotheses, self-directed inflammation, the anatomical structures that dissipate mechanical stress (called the 'enthesis organ') and the antigen presentation pathways will first be explained.

Self-directed inflammation: autoinflammation versus autoimmunity

The immune system is composed of two main branches, namely the innate and adaptive immune system. Innate immune cells are comprised, though not exclusively, of the phagocyte-type cells that Elie Metchnikoff described: monocytes, macrophages and DCs. Adaptive immunity comprises defense mechanisms mediated by cellular immunity (T- and B cells) and humoral immunity (for example antibodies). Innate immune cells express receptors that recognize pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) (27). It is a semantic discussion whether innate immune responses are specific or non-specific to PAMPs and DAMPs, but it initiates the first reaction to the invader or danger signal. As part of the immune response, innate immune cells control the inflammatory responses by instructing adaptive immune cells. Adaptive immune cells express recognition receptors that are exquisitely specific to any endogenously or exogenously expressed molecules. The immune response time of T- and B cells takes days to weeks to establish, but is characterized by its capacity to develop immune memory, the process that vaccination is based upon (27).

Inappropriate control of this defence mechanism leads to progression to a chronic disease state, including self-directed inflammation. Two concepts are defined, namely autoinflammation and autoimmunity.

Autoinflammation is defined as a self-directed tissue inflammation, where local factors at certain predisposed sites exacerbate immune responses. Innate immune cells are the drive behind the pathogenesis of autoinflammation and adaptive immune cells are negligible in the disease. Autoimmunity is also defined as self-directed tissue inflammation, but adaptive immune cells are key players in the pathogenesis of the disease. The breach of tolerance to self-antigens leads to immune reactivity towards native proteins (28). Of note, in contrast to monogenic autoimmune diseases, the aetiology of polygenic autoimmune diseases lies not in the adaptive immune system itself, but in affected tissue from which danger signals arise.

Anatomy of the enthesis organ

Entheses take part of the muscle-tendon-enthesis-bone or ligament-enthesis-bone complex, which play an important role in transmitting contractile forces between different musculoskeletal systems and reducing mechanical forces to minimize tissue damage (in the case of tendons), and in providing stability (in the case of ligaments) (29). Entheses are mainly localized outside the joint capsule, and are either in close proximity with the synovial membrane (synovio-enthesial complex; such as the Achilles tendon, Figure 1) or inserting into periarticular bone (such as the flexor tendon insertions of the phalanges) (30). There are a few exceptions to this rule. Tendons that join a joint capsule, for example sternoclavicular, sacroiliac and acromioclavicular joints, are articular. All the above listed examples are sites that are prone to entheseal inflammation in SpA (31).

At the microscopic level, enthesis is composed of fibrous or fibrocartilaginous connective tissue (29). Fibrous entheses are typically found at the diaphyses and metaphyses of long bones covered with periosteum, whereas fibrocartilage entheses are characteristic for epiphyses and apophyses of long bones that lack periosteum, short bones in hand and feet, and certain ligaments located in the spine (32). The location of fibrocartilage correlates with areas of high sheared or compressive forces (33). Indeed, enthesitis associated with SpA is mainly a disease of fibrocartilaginous entheses. Of note, fibrocartilaginous entheses are avascular and reflect the lack of direct interaction between blood vessels present in bone marrow and healthy enthesis (32). The enthesis is further composed of tenocytes (fibroblast-like stromal cells) and chondrocytes. Enthesis-resident immune cells are scarce, but type 3 innate lymphoid cells (ILC3), γδT cells and myeloid cells have been reported in healthy human tissue (34-36).

The enthesis is supported by adjacent structures, including fad-pad, bursa and synovium, which assist in dissipating mechanical stress load at the enthesis. The array of anatomical structures that dissipate mechanical stress is called the enthesis organ (37). Findings of magnetic resonance and ultrasound imaging show more diffuse changes with the involvement of adjacent structures rather than solely changes at the focal enthesis attachment site in SpA (37). These data support the concept that the inflammation site in SpA is not restricted to the enthesis itself, but also includes adjacent anatomical structures that support the reduction of stress at the enthesis (38, 39).

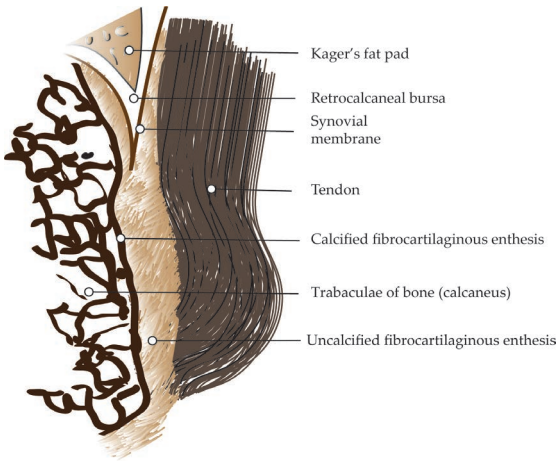

Figure 1 | A sagittal drawing of the enthesis organ of the Achilles heel. Modified from (29).

Antigen presentation pathway

The antigen presentation pathway involves the display of protein/antigen-derived peptides in human leukocyte antigen (HLA) molecules on cells. HLA class I and HLA class II present peptides to CD8+ T cells and CD4+ T cells, respectively. HLA class I is expressed by all nucleated cells, whereas the expression of HLA class II is under non-inflammatory circumstances usually limited to antigen presenting cells (APCs), including DCs, monocytes and macrophages.

The peptide ligands for HLA/peptide complexes that can be displayed to patrolling T cells are generated via distinct intracellular pathways. HLA class I molecules can bind peptides from nuclear or intracellular origin. The antigens that contain the peptide cargo for presentation can be first degraded by nuclear or cytosolic proteasomes. Peptides with a length of 8 to 16 amino acids are next transported to the endoplasmatic reticulum (ER) via transporter associated with antigen presentation (TAP). The transported peptides can be further trimmed by ER amino peptidase 1 (ERAP1) or ERAP2 to a size that fit in HLA class I molecules (40). ERAP1 preferentially cleaves hydrophobic amino acids, whereas ERAP2 preferentially cleaves basic residues (41). In the ER, HLA class I molecules are assembled with a heavy chain and a light chain, called β2 microglobulin, and stabilized by the chaperones tapasin, ERp57 and calreticulin, which forms the peptide-loading complex (PLC). Tapasin regulates the loading of a diverse repertoire of peptides in class I (42). Accordingly, the HLA class I-peptide complex leaves the ER to allow display at the cell surface, for inspection by HLA-restricted T cells.

HLA class II molecules present peptides derived from exogenous protein antigens and are processed in the endosomal pathway. In contrast to HLA class I molecules for which loading occurs in the ER, peptide loading in HLA class II molecules occurs in the endosomal compartment. The intracellular trafficking and sorting of HLA class II molecules towards endosomal compartments require its association with the chaperone molecule CD74 (also known as the invariant chain (Ii)). CD74 is a type II transmembrane protein that functions as a trimeric scaffold that allows for the assembly of three HLA αβ heterodimers as a nine subunit complex during biosynthesis in the ER (43). The N-terminus of CD74 contains signals that ensure the trafficking of class II/peptide complexes from the ER and Golgi apparatus to late endosomal/lysosomal compartments. The loading of HLA class II molecules with antigenic peptides requires proteolysis of both internalized protein antigens and CD74 in endosomal compartments by several known proteases (44-48). CD74 processing requires the proteases cathepsin S and signal peptide peptidase-like 2A (SPPL2a) (49, 50). Cathepsin S is responsible for cleaving the N-terminal side of CD74 to produce class II-associated invariant chain peptide (CLIP), which occludes the HLA class II peptide binding cleft and thereby prevents premature peptide loading. SPPL2a is probably the last

enzyme in the cascade, which cleaves the CD74 transmembrane remnants (49-51). CLIP is exchanged for a peptide, usually with the help of HLA-DM (47, 52). Eventually, the class II/peptide complex is transported to the cell surface, again to allow inspection by HLA-restricted T cells.

The third antigen presentation pathway constitutes a link between the HLA class I and HLA class II presentation pathways and is called *cross-presentation*. This pathway is important in initiating an immune reaction to cancer cells and to viruses that do not infect antigen presenting cells. Two routes have been described on how HLA class I molecules get access to peptides from exogenous material, namely the vacuolar and endosome-to-cytosol pathway (Figure 2C).
The vacuolar pathway has an analogy with the HLA class II presentation pathway. In the vacuolar pathway, exogenous antigens that are sequestered in the endosomal pathway are cleaved by proteases, such as cathepsin S, and proteasomes present in vacuoles (47, 53). The mechanisms by which HLA class I molecules are transported to the endosomes and maintained in their peptide-receptive state is incompletely understood. The newly formed peptide/HLA class I complex transit to the plasma membrane for recognition by CD8+ T cells (54). In the endosome-to-cytosol pathway, sequestered antigens in the phagosomal pathway are transported from the phagosomal lumen to the cytosol. The antigenic transport mechanisms are incompletely understood. Proteasomes present in the cytosol degrade the antigen and the proteasome-derived peptides enter either the classic HLA class I presentation pathway or the endosomal pathway. In the classical pathway, proteasome-derived peptides are transported to the ER, trimmed by ERAP and loaded onto MHC class I molecules. Peptides imported in the phagosome are trimmed by insulin-responsive aminopeptidase (IRAP) and loaded onto HLA class I molecules residing in the endosomal membrane (55-58). The molecules are trafficked to the cell surface.

HLA class I polymorphisms
HLA class I molecules, encoded by HLA-A, HLA-B and HLA-C genes, are highly polymorphic. Polymorphic class I molecules have different peptide-binding grooves owing to peptide-binding motif variations that affects peptide binding (59). Post-translational modifications, such as phosphorylated ligands, affect peptide binding to HLA class molecules (60, 61). Polymorphic molecules also behave differently. HLA-B molecules do not associate with the PLC complex, and the transport is faster to the cell surface compared to HLA-A and HLA-C (62-65). HLA-C is even more selective to peptide binding than HLA-A and HLA-B and dissociate less efficient from TAP and retain longer in the ER (66). The occupancy of HLA molecules is also distinct in that most HLA-B molecules are loaded with peptide, whereas only a fraction of HLA-A and HLA-C are occupied with peptide (30-70%) (62). Furthermore, for example, the biochemical properties of HLA-B27 is unique in a sense that

it presents more stable peptides (67). The overall cell surface expression of HLA molecules is limited to receptivity to the peptide repertoire and peptide/molecules stability and by microRNA regulation (66, 68-70). Overall, the biochemical properties of the polymorphic HLA molecules determine the cell surface expression and its stability, which in turn influences the CD8+ T cell response.

Autoinflammatory hypothesis – uncontrolled immune responses to microinjury and aberrant protein expression

The sites affected in SpA are prone to mechanical stress (enthesis organ) and to exterior stressors (skin, mucosa). There is compelling evidence that physical injury can act as a trigger for inflammation, as for example observed in psoriasis patients. Trauma to the skin following injury stimulates the formation of psoriatic lesions, which is known as the Koebner phenomenon (71). Trauma to the musculoskeletal system, known as "deep" Koebner phenomenon, can also induce joint inflammation and clinical manifestations, and is for example seen in digital pulley system of the fingers (72). In addition to this phenomena, several epidemiological studies have shown that there is an association between biomechanical trauma and SpA, including physically demanding occupations and sport trauma (73-77). Also HLA-B27 carriage has been linked to inflammation of enthesis-like structures, which are found in the heart valves, aortic root, plantar fasciitis and ciliary body in the eye (78, 79). These anatomical sites are continuously exposed to mechanical strain and confers a risk for SpA. The observations that mechanical stress induce SpA is a concept that is also supported by in vivo data. Exposure to mechanical stress at the tendon insertion site is sufficient to induce a SpA-like phenotype in mice, including new bone formation and enthesitis (80, 81).

Upon mechanical strain, tendon cells (tenocytes) can produce various cytokines and signalling molecules, such as IL-6, IL-8, TNF, inflammasome-induced IL-1β and prostaglandin E2 (PGE2) (Figure 2A) (33). CCL2 is also produced by mechanically stressed tendons, as observed in mice that systemically overexpress TNF (an effector arthritis model that partly mimics SpA) and in mice with collagen antibody-induced arthritis (81). In these in vivo models, CCL2 recruits classical monocytes to the affected site and differentiate into osteoclasts. Hindlimb unloading, to relieve mechanical stress, prevented enthesitis. The influx of classical monocytes in inflamed tendons was further confirmed in humans using Achilles tendon of patients with chronic tendinopathy. Of note, mechanical stress-induced arthritis was still observed in RAG knock out mice, which have no mature T and B cells, and suggests that adaptive immune cells are dispensable for initiating enthesitis in this model (80, 81). Overall, biomechanical stress-induced inflammation in tendon-entheseal tissue can mediate uncontrolled inflammation and degradation of entheses and its associated structures by promoting the influx of immune cells and inducing connective tissue changes in an autoinflammatory fashion (33).

Another hypothesis is that the free cysteine (cys67, cys164 and cys101) in HLA-B27 heavy chain can form disulphide bond-mediated dimers, which renders the heavy chain incapable to form a complex with β2-microglobulin (Figure 2B) (82). These dimers have an open conformation and might induce pathogenic immune responses towards the antigen. HLA-B27 homodimers are expressed on peripheral mononuclear cells (PBMCs) and synovial mononuclear cells (SFMCs) from AS patients, but not on healthy control cells (83). The expression of HLA-B27 homodimers is dependent on the B27 dosage and peptide supply, as shown in HLA-B27 transgenic rats (84). AS is associated with ERAP1 and HLA-B27 as explained in the next paragraph. Studies show that ERAP1 controls the expression of HLA-B27 in its open conformation, as evidenced by ERAP1 gene suppression assays and stratification analysis on different ERAP1 variants (85-87).
Killer-cell immunoglobulin-like receptors (KIR) are proposed to interact with HLA-B27 homodimers. KIR recognizes conformational changes of HLA and are expressed on natural killer (NK) cells and specific T cell subsets. HLA-B27 homodimer is a ligand for KIR3DL1 and KIR3DL2 (88). In vitro studies have shown that the interaction of KIR3DL2-expressing CD4+ T cells with HLA-B27 homodimer-expressing cells stimulate these CD4+ cells to differentiate into Th17 cells (89). Also the functional inhibition of KIR3DL2 and HLA-B27 homodimer interactions prevents the production of pro-inflammatory cytokines by PBMCs, including IL-17 (83). So, the interaction between KIR and HLA-B27 homodimers might induce tissue inflammation. However, it should be noted that KIR3DL2 is a framework gene (which means that it is expressed by all individuals with a very few exceptions) and is therefore not specific for AS.

Autoimmune hypothesis - breach of tolerance to native proteins
The reason why HLA class I molecules are linked to SpA is not completely understood, but the observations that certain HLA class I alleles and haplotypes confer the risk of developing particular clinical features in SpA supports a role for HLA molecules in the pathogenesis of the disease (17, 18). In PsA, HLA-B27 is associated with dactylitis, enthesitis and symmetrical sacroiliitis, whereas HLA–B8 is associated with joint fusion and deformities, asymmetrical sacroiliitis and skin disease (17). In axial spondyloarthritis, HLA-B27 is associated with younger disease onset, familial aggregation and syndesmophytes (calcification of ligaments), whereas HLA-B27 negative cases were associated with peripheral and extra-articular manifestations (90, 91). The association between HLA and specific anatomical patterns in SpA might indicate that tissue-specific antigens are recognized as an autoantigen (Figure 2C).
In addition to HLA, genetic studies have shown that ERAP is a susceptible factor to induce SpA. The highly active form of ERAP1 and highly expressed form of ERAP2 is association with disease severity in AS, whereas the loss-of-function of ERAP1 and loss-of expression of ERAP2 is protective in

AS (18, 92, 93). ERAP1 is in epistasis with HLA-B27 and HLA-B40 (18, 94), whereas ERAP2 is independently associated with HLA class I molecules (95). The finding that ERAP1 is in epistasis with HLA-B27 and HLA-B40 indicate that these molecules act in the same pathway and mediate disease susceptibility. In addition, these observations also suggest that different mechanisms are involved in the pathogenesis of AS. Indeed, ERAP1 and ERAP2 have a significant and distinct effect on the HLA-B27 peptidome in terms of altered peptide availability, peptide affinity and HLA class I stability; factors that all help steer adaptive immune responses (41).

The medical and scientific community still describe SpA as seronegative arthritis. However, there is accumulating evidence that sera from patients with SpA contain autoantibodies. For example, AS is associated with the presence of anti-CD74 and NAD-dependent protein deacetylase sirtuin-1 (SIRT1) autoantibodies (96-98). In PsA, autoantibodies have been detected in sera that are directed towards antigens present in skin cells (ADAMTSL5, α6-integrin, fibrillin 3, and desmocollin 3) and enthesis (nebulin-related anchoring protein (N-RAP)) (99-101). The presence of circulating autoantibodies indicates that humoral responses are activated in SpA. The involvement of adaptive immune cells in the pathogenesis of SpA is further supported by the presence of adaptive immune cells at the inflammatory sites. The subsynovial site of joints are positive for plasma cells, CD4+ T cells and CD3+ T cells (39, 102, 103). Furthermore, bone marrow oedema is a hallmark for SpA, and the bone marrow component of the enthesis showed CD3+ lymphocyte aggregates, predominantly composed of CD8+ T cells, and plasma cells (104, 105). The identification of autoantibodies and adaptive immune cells in SpA might suggest an autoimmune origin.

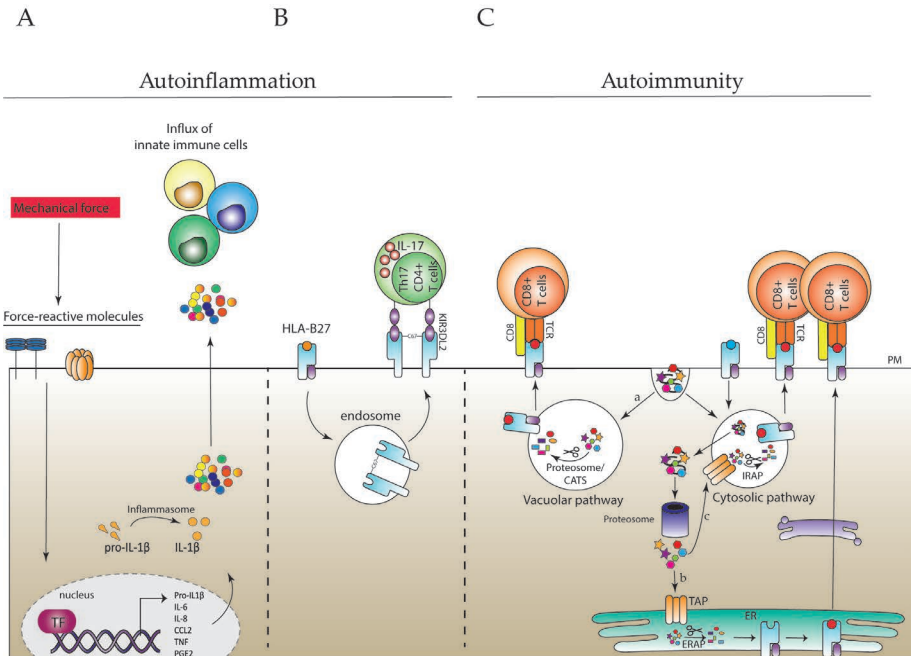

Figure 2 | The autoinflammatory and autoimmune hypotheses for SpA pathogenesis. (A) In response to mechanical force, tenocytes produce various inflammatory mediators that attract innate immune cells to the enthesis organ. (B) Endosomal recycling of folded HLA-B27 complexes leads to the formation of disulphide bond HLA-B27 heavy chain dimers, which return to the plasma membrane (PM). The homodimers can be recognized by killer cell immunoglobulin-like receptors (KIR) that are expressed by T cells and NK cells, which cause an inflammatory response. For example, KIR3DL2-expressing CD4+ T cells respond to the homodimers by producing IL-17. (C) HLA class I molecules present peptides that are recognized by T cell receptors (TCRs) present on the cell surface of autoreactive CD8+ T cells. The activation of CD8+ T cells contribute to tissue damage. The figure depicts cross-presentation pathways. In the vacuolar pathway (a), antigen is taken up into a phagosome, processed by cathepsin S (CATS) or phagosomal proteasomes, and the antigen-derived peptides are loaded onto HLA class I molecules present in the endosomal membrane and transported to the PM. In the cytosolic pathway, extracellular antigens are taken up into phagosomes, transported to the cytosol and degraded by the cytosolic proteasome. The generated peptides either enter the (b) classical HLA class I presentation pathway or transported back into the (c) phagosomal lumen by transporter associated with antigen presentation (TAP). In the case of the classical pathway, peptides are imported into the endoplasmic reticulum (ER) by TAP, trimmed by ER aminopeptidases (ERAP), loaded onto nascent HLA class I molecules and transported to the PM. Peptides that are imported into the phagosomes by TAP are trimmed by insulin-responsive aminopeptidase (IRAP), loaded onto endosomal-resident (recycled) HLA class I molecules and transported to the cell surface.

Aim and outline of this thesis

The studies presented in this thesis were designed with the intention to explore whether SpA is driven by antigen-specific immunity, and to identify molecular mechanisms that contribute to the exposure of self-antigens.

In **Chapter 2** we review the current knowledge on the immune pathogenesis of archetypical autoimmune diseases, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), and autoinflammatory disorders. We describe plausible underlying causes and discriminating factors between autoimmune and autoinflammatory diseases. My contribution to this chapter includes the literature search and the writing of the manuscript.

In **Chapter 3**, we evaluate the function of the CD74 processing enzyme SPPL2a in monocytes from SpA and RA patients. We further explored the consequences of aberrant SPPL2a function and their possible associations with the pathogenesis of AS. My contributions to this chapter included conceiving of and designing the study with T.R. and M.B, performing cellular biological assay, Western blot, flow cytometry, confocal experiment, cell fractionation, and immunoprecipitation, analyzing data, making figures, and contributing to the writing of the manuscript with input from all authors.

In **Chapter 4**, we examined the hypothesis that PsA is an antigen-driven disease, using an in vitro model to identify autoantigen-reactive CD8+ T cell in blood and skin from patients with PsA. My contributions to this chapter included conceiving of and designing the study with T.R. and M.B, including developing and executing a new cellular assay, analyzing flow cytometry data and making figures, and writing the manuscript with input from all authors.

In **Chapter 5**, we explored whether innate lymphoid cells (ILC) were present in blood and synovial fluid from patients with PsA and RA. My contribution to the chapter included sample collection, discussing the study design with E.L. and giving input on the manuscript.

In the **Discussion**, I discuss the main research findings of this thesis and offer a broad perspective on future research into the pathogenesis of SpA.

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Chapter 2

Perception of self: distinguishing autoimmunity from autoinflammation

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Abstract | Rheumatic disease can be divided into two groups, autoinflammatory and autoimmune diseases. The clinical presentation of both disorders overlap, but pathological pathways underlying autoimmunity and autoinflammation are distinct and are currently subject of investigation. There are numerous of ways in which the diseases differ from each other in terms of disease mechanisms and therapeutic responses. First, autoinflammatory diseases are driven by endogenous danger signals, metabolic mediators and pro-inflammatory cytokines, whereas autoimmunity involves the activation of B- and T-lymphocytes that require V-(D)-J recombination of receptor chain gene segments. Second, the efficacy of therapeutic biologicals directed against pro-inflammatory cytokines (for example IL-1β and TNF) trace similar discriminatory lines between autoinflammatory and autoimmune processes. Finally, whereas autoinflammatory diseases are mostly driven by inflammasome-induced IL-1β and IL-18 production, autoimmune diseases are associated with type I IFN signatures in blood. In this Review, we provide an overview of intracellular pathways within monocytes that drive autoinflammation and autoimmunity. We convey recent findings on how type I IFN can interfere with the IL-1β signaling pathways (and vice versa) and discuss why IL-1β autoinflammatory diseases do not perpetuate into autoimmune disease. The origins of intracellular autoantigens in autoimmune diseases are also discussed. Finally, we provide a perspective on how new mechanistic knowledge should help to improve treatment strategies to benefit patient care.

Introduction

Rheumatic diseases can be stratified into autoinflammatory and autoimmune disorders. Although certain autoinflammatory diseases can phenocopy autoimmune diseases - and do sometimes serve as prelude to autoimmunity – the underlying pathological mechanisms are mostly distinct. Autoinflammatory diseases are innate immune disorders triggered by exogenous or endogenous components and are characterized by systemic inflammation (1). By contrast, autoimmune diseases result from aberrant responses to self-constituents by both innate and adaptive immune cells. Activation of autoreactive T- and B-cells ultimately causes the collapse of immune tolerance, resulting in chronic inflammation and local tissue destruction (1).

Differences in effectiveness of biological agents underscore the mechanistic divide between autoimmunity and autoinflammation. In rheumatoid arthritis (RA), the introduction of TNF neutralizing agents revolutionized treatment outcomes, whereas more limited results were obtained in polygenic autoinflammatory diseases such as in systemic-onset juvenile idiopathic arthritis (sJIA) and systemic-onset adult Stills disease (sAOSD)) (2-4). Conversely, IL-1β receptor antagonist is an effective treatment for patients with sJIA and sAOSD, yet ineffective for most patients with RA (2-5). Genetic fingerprinting approaches used to examine various rheumatic diseases using gene expression profiling and genotyping have emerged to understand disease etiologies. High expression levels of type I interferon (IFN) genes (known as type I IFN signatures) were discovered in systemic lupus erythematosus (SLE) and confirmed in additional autoimmune disorders, including primary Sjögrens syndrome (SS), systemic sclerosis (SSc) and RA (6,7). Autoinflammatory diseases that persist as such, without perpetuating into autoimmunity, show mainly an IL-1β/IL-18 signature (Figure 1) (8). A paradigm has emerged that supports the concept that autoimmune diseases involve type I IFN pathways, whereas polygenic autoinflammatory disorders reside with dysregulated IL-1β/IL-18 pathways.

Symptom flares in either autoinflammatory or autoimmune diseases are only rarely linked to bacterial or viral infections, suggesting the existence of intrinsic triggers for inflammation. Intracellular organelles are sources of "danger signals", and particularly mitochondria are suspect of harboring such signals. This Review focuses on monocytes and cells derived thereof – that is macrophages and dendritic cells - owing to their central roles in both autoinflammation and autoimmunity and their capacity to produce large amounts of IL-1β/IL-18 or type I IFN (9, 10). Neutrophils serve perhaps equally relevant roles in rheumatoid diseases, but were recently covered elsewhere (11). Here, we discuss autoinflammatory diseases and the autoimmune diseases RA and SLE, highlighting the roles of mitochondria and the recent developments in our understanding of intracellular mechanisms whereby autoantigens are produced.

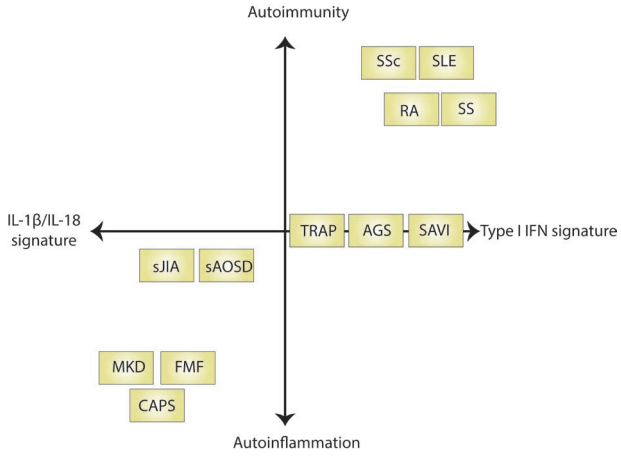

Figure 1 | Stratification of autoimmune and autoinflammatory diseases based on cytokine signature. The horizontal axis depicts the IL-1β/IL-18 and type I IFN cytokine signatures. The vertical axis stratifies the diseases in terms of their basis as autoinflammatory or autoimmune diseases. Diseases placed on the cytokine signature axis have both autoimmune and autoinflammatory components. Abbreviations: ACP5, ACP5-related disease; AGS, Aicardi-Goutières syndrome; CAPS, cryopyrin-associated autoinflammatory syndrome; IFN, interferon; MKD, mevalonate kinase deficiency; sAOSD systemic adult onset Still disease; SAVI, STING-associated vasculopathy with onset in infancy; sJIA, systemic juvenile idiopathic arthritis; SLE, systemic lupus erythematosus; pSS, primary Sjögren syndrome; SSc, systemic sclerosis; RA, rheumatoid arthritis.

Autoinflammation: type I IFN and IL-1β

IL-1β and type I IFN are inflammatory mediators with diametrically opposed immune consequences. A disturbance in the balance between these mediators appears pivotal to drive innate immune cells to trigger disease. For example, the treatment of sJIA patients with IL-1 blockade turns their IL-1β hallmark signature towards a type I IFN signature (5). Also, lupus-prone mice that produce less mature IL-1β and IL-18, owing to a deletion in the NLRP3 gene express upregulated type I IFN responsive genes compared to their NLRP3-sufficient control (12). In patients with SLE, expression of type I IFN-induced Myxovirus resistance 1 (MX1) correlates with suppressed NLRP3 mRNA expression (13). Clarification of how innate immune cells are primed for the hyper-production of IL-1β and how such production might be directed towards type I IFN secretion or vice versa appears imperative.

DAMPS—endogenous danger signals

The search for intrinsic Danger Associated Molecular Patterns (DAMPs) that provoke IL-1β or type I IFN in monocytes/macrophages has resulted in the identification of several candidates, including reactive oxygen species production by mitochondria, tricarboxylic acid (TCA) metabolites, RNA and mitochondrial oxidized DNA (14-18). But why would mitochondrial organelles be enriched for such DAMPs? And when considering human

disease: do mitochondrial DAMPs contribute to type I IFN or IL-1β hypersecretion? To address these questions, we revisit the endosymbiotic theory, which centers on the ancestry of mitochondria as prokaryotes (19). The historical occurrence of proto-mitochondria being engulfed by unicellular organisms laid the foundation for the existence of the eukaryotic cell. This mitochondrial intrusion brought along proton pumps and electron carriers, which made it possible to increase the cellular capacity for ATP synthesis by oxidative phosphorylation. This benefit, however, came at a price. Owing to the great similarity between prokaryotic and eukaryotic mitochondrial polypeptides, metabolites and DNA motifs, leakage of mitochondrial components into the circulation inevitably become recognized by immune cells as DAMPs (20). Recent work shows that mitochondrial N-formylated peptides and CpG DNA repeats are found in the plasma of patients with trauma and these evolutionary conserved agents stimulate polymorphonuclear neutrophils via formyl-peptide receptor-1 (FPR1) and TLR9, respectively (21). Furthermore, the regulatory pathways of NLRP3 inflammasome complex activation by bacterial infection likely has an overlap with those activated by mitochondrial injury. Indeed, selective antibiotics, such as nigericin, similarly interfere with mitochondrial signalling and affect the activation status of NLRP3 inflammasome (22). Another indication of the relationship between mitochondrial injury and immune activation is the mitochondrial toxicity of certain antibiotics. Treatment of macrophages with linezolid causes the exposure of the inner mitochondrial lipid cardiolipin, which subsequently triggers NLRP3-driven inflammation (23). Mitochondrial damage can thus function as a causative factor for autoinflammatory disease flares.

Acute inflammatory pathways

IL-1β and type I IFN production

IL-1β is synthesized in its biologically inactive form pro-IL-1β and requires processing by caspase-1 to achieve its mature form. The activation of caspase-1 is regulated through inflammasome assembly, comprised of a sensor molecule, the adaptor molecule hASC (apoptosis-associated speck-like protein containing a CARD) and caspase-1. DAMPs or pathogen-associated molecular patterns (PAMPs) bind to the receptor - such as Toll like receptors (TLRs), NOD-like receptors (NLRs) and ATP-gated cation channel (P2X) receptors - and initiate the transcription of pro-IL-1β and NLRP3 in a nuclear factor κB (NFκB)-dependent fashion, termed signal 1 (10, 24). "Signal 2" involves hASC recruitment and inflammasome activation as a consequence of DAMP or PAMP binding to the inflammasome sensor molecule NLRP3 (Figure 2a) (16,23). NLRP3 is the most studied sensor component of inflammasomes.
The synthesis of type I IFN is regulated at the transcriptional level. The transcriptional regulation of type I IFN is orchestrated by specific transcription factors, including interferon regulatory factor 3 (IRF3) and IRF7, which are activated by TLR family members and cytosolic RIG-I-like RNA helicase receptors (RLHs) and cyclic GMP-AMP synthase (cGAS) (25). Candidate

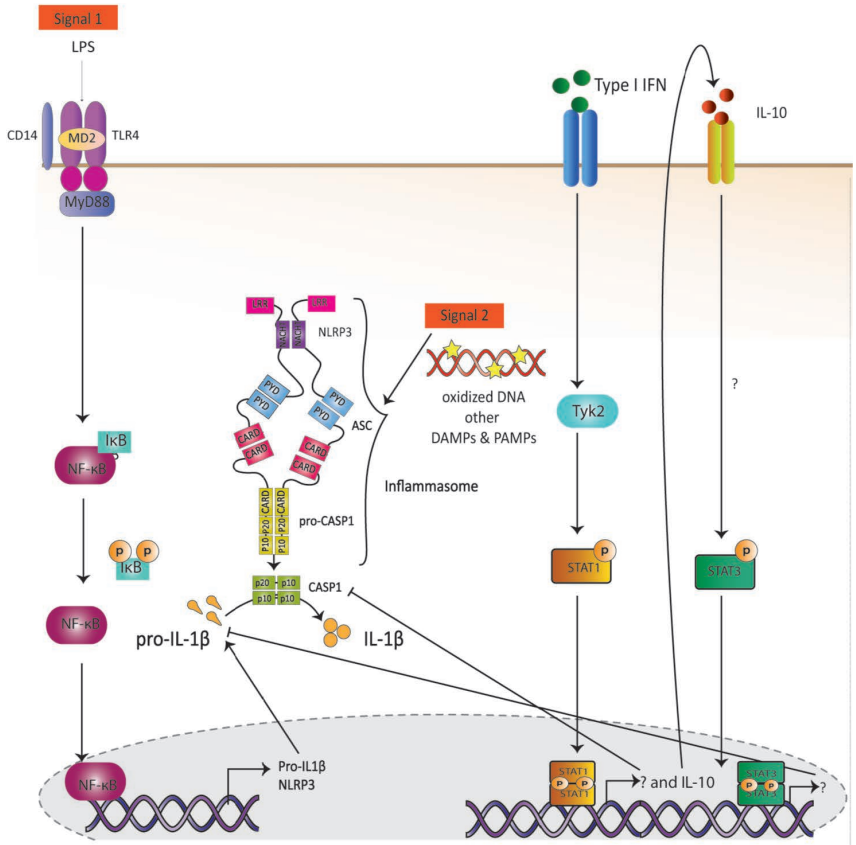

Figure 2 | NLRP3 inflammasome activation and the reciprocally interference of type I IFN on acute inflammasome-dependent IL-1β production (signal 1 and 2). The first signal is the binding of DAMPs or PAMPs to receptors, such as TLR4, which induces the phosphorylation of IκB and allows the translocation of NF-κB to the nucleus following the dissociation from phospho-IκB. Here, NF-κB initiates the transcription of NLRP3 and pro-IL-1β. The second signal induces the recruitment of hASC in order to fulfill the complete function of the NLRP3 inflammasome. The functional inflammasome converts pro-caspase-1 (pro-CASP1) into active caspase-1, which in turn induces the conversion of pro-IL-1β into mature IL-1β. Type I IFN can interfere with the acute phase of inflammasome activation by inhibiting post-translational production of pro-IL-1β via TYK2-STAT1-IL-10-STAT3-induced gene transcript. Furthermore, caspase-1 activation is inhibited by a STAT1-induced gene product. Abbreviations: ASC, apoptosis-associated speck-like protein containing a CARD; CASP1, caspase 1; CARD, caspase recruitment domain; DAMP, damage-associated molecular pattern; IkB, NFκB inhibitor β; IFN, interferon; LPS, lipopolysaccharide; LRR, leucine-rich repeat; MD2, lymphocyte antigen 96; MyD88, myeloid differentiation primary response protein MyD88; NACHT, NAIP (neuronal apoptosis inhibitor protein), C2TA (MHC class 2 transcription activator), HET-E (incompatibility locus protein from Podospora anserina) and TP1 (telomerase-associated protein); p, phosphorylation; PAMP, pathogen-associated molecular pattern; PYD, pyrin domain; NLRP3, NACHT, LRR and PYD domains-containing protein 3; STAT1, signal transducer and activator of transcription 1-α/β; STAT3, signal transducer and activator of transcription 3; TLR4, Toll-like receptor 4.

mechanisms that intersect here will be discussed below, as they appear relevant to propagation of autoinflammation into autoimmune disease. The synthesis of new pro-IL-1β protein is opposed by the presence of type I IFN. Here, type I IFN-mediated translational inhibition of NLRP3-containing inflammasomes is regulated via the STAT1 transcription factor. The phosphorylation of STAT1 by tyrosine kinase 2 (Tyk2) promotes nuclear translocation of STAT1 (signal transducer and activator of transcription 1), for induction of IL-10 expression (26, 27). IL-10, in turn, triggers phosphorylation of STAT3, ultimately inhibiting pro-IL-1β production at the translational level. Furthermore, caspase-I activation is also inhibited in a STAT1-dependent manner (26). IL-1β-dependent upregulation of the eicosanoid lipid mediator prostaglandin E2 is, however, important for antagonizing the type I IFN pathway and thereby control bacterial growth in macrophages (28).

Nucleic acids

Nucleic acids present in pathogens and mitochondria serve as ligands for both the cytosolic RLHs (such as RIG-I, MDA5, LGP2) and the NLRP3 inflammasome and, thereby, drive production of type I IFN and IL-1β (30, 31). The canonical RLH signalling cascade moreover has similarities with the NLRP3 signalling pathway (Figure 3). Elicited by direct contact with double-stranded RNA (dsRNA), RLH undergo conformational changes whereby the caspase-recruitment domain (CARD) of RIG-I is exposed (32). As a consequence, the E3 ubiquitin/ISG15 ligase TRIM25 can bind to the CARD domain of RIG-I, and mediate lysine 63 (K63)-linked polyubiquitination of RIG-I (33, 34). Considering NLRP3, the upstream component DExD/H-box helicase 33 (DHX33) senses and binds dsRNA, interacts with NLRP3 and induces conformation. The Pyrin domain (PYD) within NLRP3 is now revealed (31, 35).The K63 ubiquitin chains on NLRP3 need to be removed by the deubiquitinase enzyme BRCC3 before NLRP3 oligomerization and activation can occur (36, 37). Both oligomerized NLRP3 and RLH can bind to the mitochondrial antiviral signaling adaptor MAVS (also known as IPS-1, Cardif or VISA) (38, 39). Here, oligomerization is possibly the impetus to initiate MAVS conversion into a detergent-resistant multimeric complex on the outer membrane of the mitochondria (38, 40, 41), from where it recruits signaling molecules that facilitate the production of either type I IFN or IL-1β. The oligomerization of NLRP3 additionally induces the polymerization of linear ubiquitinated hASC, stimulating CARD-CARD interaction and thereby the recruitment of caspase-1 for cleavage of pro-IL-1β into mature IL-1β (Figure 3a) (38, 42). With respect to type I IFN production, MAVS recruits the ubiquitin E3 ligases TRAF2, TRAF5 and TRAF6 and the adaptor protein NEMO (NFκB essential modulator) which, through activation of IKK and TBK1 respectively, transduces signals towards NFκB and IRF3-responsive genes (Figure 3b) (40, 43).

How do these mechanistic changes relate to patient's clinical features? Serum autoantibodies against Ro68 (also known as TRIM68) are found in patients

with SLE and Sjögren syndrome. Anti-Ro68 autoantibodies may activate the RLH signalling cascade and increase type I IFN production by inactivating TRIM68, thereby impeding lysosomal degradation of TRK-fused gene (TFG) and eventually TRIM25 degradation (44). Also, MAVS aggregation was observed in peripheral blood molecular cells (PBMCs) from patients with SLE, where 82% of the patients positive for MAVS aggregation had anti-Ro antibodies compared to 40% of the patients negative for MAVS-aggregation (45). MAVS aggregation was not observed in RA patients. The RLH-MAVS signalling pathway may thus be particularly important in SLE and Sjögren syndrome pathogenesis. Direct competition of NLRP3 with RLHs was shown for the interaction with MAVS: the cellular overexpression of NLRP3 impedes MAVS-induced transcription of type I IFN in response to Sendai virus (a RNA virus) or PolyI:C, while the opposite was observed when NLRP3 was depleted in macrophages (41). Both RLH and NLRP3 inflammasome signaling may thus expedite rheumatic disease features.

Chronic inflammatory pathways

TCA metabolites

Repeated stimulation of monocytes/macrophages with inflammasome activation signals 1 and 2 leads to a tolerogenic cellular state (46, 47). However, this tolerogenic state can be overcome. For instance, simultaneous ligation of the A2A receptor by adenosine can trigger inflammasome activation through the transcription factor hypoxia-inducible factor-1α (HIF1α; Figure 4) (47). Also intracellular components, for example the metabolites succinate and citrate of the TCA cycle, can enable chronic activation of NLRP3 inflammasomes by promoting HIF1α responsive genes (17, 48). Accordingly, after TLR4 stimulation, innate immune cells suppress mitochondrial oxidative phosphorylation and mainly rely on ATP synthesis by aerobic glycolysis, also known as the Warburg effect (49, 50). Such metabolic remodeling is driven by a change in expression and activity of TCA cycle enzymes and glycolytic enzymes in monocytes/macrophages (17, 49). For example, adenosine increases the glycolytic flux by enhancing fructose 2,6-bisphosphate enzyme expression (also known as 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3) (51). This remodeling ultimately causes citrate and succinate to accumulate. Indeed, 20 out of 105 metabolites tested in synovial fluids were found discriminative between patients with RA and patients without RA, with succinate being one of the distinctive metabolites (52). This may suggest a commitment to aerobic glycolysis in cells present in the synovial fluid. HIF1α expression is moreover upregulated in RA synovial tissue and macrophages (53, 54). Succinate and citrate in this regard can inhibit HIF prolyl-4-hydroxylases (HIF-P4H) and HIF asparaginyl hydroxylase (FIH), respectively, to allow HIF1α to escape proteasomal degradation and dimerize with HIF1β (55). The resultant HIF1α complex is able to bind to HIF-responsive elements and induce hypoxia-regulated target genes, including pro-IL-1β (17). Since HIF1α-mediated upregulation of pro-IL-1β is distinct from the NF-κB pathway initiated by signal 1,

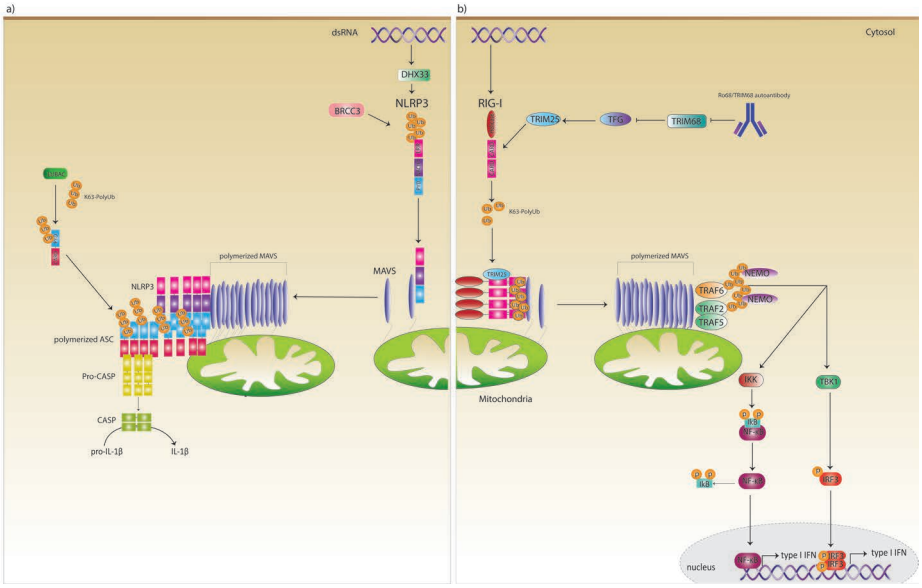

Figure 3 | MAVS in the NLRP3 inflammasome and the RIG-I signalling cascade. (a) IL-1β production by NLRP3 inflammasome. The upstream DHX33 recognizes dsRNA and activates NLRP3 by the removal of the K63 ubiquitin chain on NLRP3 by BRCC3. Simultaneously, ASC is ubiquitinated by linear ubiquitin assembly complex (LUBAC). Conformational changed and activated NLRP3 binds to MAVS, which functions as a signal for MAVS polymerization and hASC recruitment. Polymerized hASC and ultimately activated caspase-1, converts pro-IL-1β into its bioactive form. (b) The entrance of dsRNA into the cytosol is sensed by RIG-I. The recognition of dsRNA by RIG-I leads to a conformational change and facilitates the recruitment of TRIM25 and K63 polyubiquitin. The RIG-I/TRIM25/K63 polyubiquitin complex binds to MAVS and induces the polymerization of MAVS. MAVS recruits polyubiquitinated TRAF2, TRAF5 and TRAF6 and NEMO, which in turn phosphorylates and activates NF-κB and IRF3. TRIM68 autoantibodies (anti-Ro68), found in serum of SLE and SS patients, may activate the RIG-I/type I IFN pathway by impeding TRIM68 activity and thereby inhibiting the degradation of TRK-fused gene (TFG). Abbreviations: ASC, apoptosis-associated speck-like protein containing a CARD; BRCC3, lys-63-specific deubiquitinase BRCC36; dsRNA, double-stranded RNA; DHX33, putative ATP-dependent RNA helicase DHX33; LUBAC, linear ubiquitin assembly complex; MAVS, mitochondrial antiviral-signalling protein; NACHT, NAIP (neuronal apoptosis inhibitor protein), C2TA (MHC class 2 transcription activator), HET-E (incompatibility locus protein from Podospora anserina) and TP1 (telomerase-associated protein); NEMO, NFκB essential modulator; NLRP3, NACHT, LRR and PYD domains-containing protein 3; RIG-I, retinoic acid-inducible gene I protein; TFG, TRK-fused gene; TRAF2, TNF receptor-associated factor 2; TRIM25, E3 ubiquitin/ISG15 ligase TRIM25; Ub, ubiquitin.

such complementation of different mitochondrial inflammatory stimuli may reinforce the severity and the duration of inflammation (Figure 4). In the setting of RA, HIF1α-STAT3 signaling is activated in the synovium and promotes pro-inflammatory cytokine production (53). Further support to the importance of STAT3 signaling in RA synovium is demonstrated in a Clinical Phase II Trial using the JAK-inhibitor tofacitinib (56). JAK1/TYK2 are responsible for the integration of signals from type I IFN. The oral administration of the drug leads to a decrease in type I-interferon induced chemokines and a decline in p-STAT3 and p-STAT1 in the synovium, which was highly correlated with clinical response (56). This suggests that hypoxia may regulate both IL-1β and type I IFN signaling.

Sterol synthesis

How does the accumulation of metabolites relate to inflammasome activation and IL-1β hypersecretion? The monogenic metabolic autoinflammatory disease mevalonate kinase deficiency (MKD) is characterized by the accumulation of mevalonic acid and may provide mechanistic insights. The recurring fever episodes in MKD patients are triggered by injury and stress and NLRP3 activation is central to this disease (57). The release of DAMPs into the blood circulation may switch the metabolism in innate immune cells to aerobic glycolysis by triggering pattern recognition receptors, including TLR4.

In addition to a switch in the ATP synthesis pathway, TLR4 stimulation also leads to the remodeling of the lipidome (58). The cholesterol synthesis pathway and TCA cycle are integrated by metabolizing Acetyl-coenzyme A (acetyl-CoA) to acetoacetate-CoA and eventually to acetoacetate. Acetoacetate can be transported into the cytosol and further converted to acetoacetate-Coa and Acetyl-CoA and finally into 3-hydroxy-30methylglutaryl (HMG)-CoA in extrahepatic tissue (Figure 4) (59). If this same pathway occurs in innate immune cells, HMG-CoA can be used to produce cholesterol and its precursors, which has been described to have anti-inflammatory properties under physiological circumstances (60). Furthermore, type I IFN can interfere with the HMG-CoA-driven cholesterol synthesis pathway: the activation of the type I IFN receptor upregulates the gene cholesterol 25-hydroxylase (CH25h). CH25h converts cholesterol into oxysterol 25-hydroxycholesterol, which in turn inhibits the sterol response element–binding protein-1 (SREBP-1) and consequently, reduce the transcription of pro-IL-1β (Figure 4)(60). Indeed, macrophages deficient in CH25h overproduce pro-IL-1β and show dysregulated caspase-1 activity (61). This suggests that this oxysterol can modulate the magnitude whereby innate immune cells respond to danger signals. It is not known how SREBP upregulates pro-IL1β transcription, as no direct interaction with SREBP and the IL-1β promoter is reported (62). Overall, the interplay between the TCA cycle and sterol pathway guides inflammasome signaling.

Autoimmunity: immunogenic self-peptides

Gene mutations causing dysregulation in the NLRP3 inflammasome signalling usually do not perpetuate into autoimmune diseases. Instead, gene mutations in type I IFN signalling pathways (for example, Aicardi-Goutières syndrome, STING-associated vasculopathy with onset in infancy and ACP5-related disease) can trigger autoimmune disorders, such as the production of anti-nuclear antibodies (63-65).

The role of NLRP3 in the regulation of adaptive immune activation is incompletely understood. NLRP3 relates to adaptive immunity through regulation of antigen presentation: the NLRP3-mediated activation of caspase-1 regulates antigen presentation by acidifying the phagosome, thereby interfering with cross-presentation to CD8+ T-cells, in vivo (12, 66, 67). Additionally, adjuvants that are positively charged (for example alum) in vaccine formulations can activate NLRP3 following lysosomal rupture and release of cathepsin B, for stimulation of CD4+ T-cell responses (68, 69). However, the role of NLRP3 in T-helper-cell activation needs further clarification (69). The stimulation of type I IFN in immune cells increases the expression of genes that selectively link innate and adaptive immunity in response to infections and cancer and promote antibody production and generation of memory T cells (70).

Break in tolerance

In addition to the priming of antigen-presenting cells (APCs) by innate stimuli, the presence of immunogenic self-antigen is required for the induction of autoimmunity. Several hypotheses were proposed that explain how normally tolerated native proteins can turn immunogenic. The first hypothesis is "molecular mimicry", which states that microbial antigens share similarities in epitopes with human self-proteins (71). Despite considerable effort, little evidence exists that supports molecular mimicry as cause for autoimmune disease (72). Instead, a recent new hypothesis emerged that focuses on antigen mimicry at the host level, namely "immunoediting" (73). This hypothesis was first proposed in the cancer field and was extrapolated to autoimmune settings. The theory holds that neoantigens are derived from somatically mutated genes and provoke an effective immunological reaction towards cancer cells, which in turn cross-react with native proteins (73).

The antigen presentation of so-called "cryptic epitopes" is now assigned to have a contributory role in autoimmunity. Sir Karl Landsteiner, the 1930 Nobel Prize winner in Physiology or Medicine, was the first to show the hapten-carrier concept. He demonstrated that the injection of the hapten aminobenzoic acid isomers coupled to the carrier serum albumin, but not the uncoupled hapten, is highly immunogenic and induced an antibody response in rabbits. The antibodies produced could distinguish between the aminobenzoic acid isomers (73). The main implication of this finding was that conformational changes of the epitope can induce an antibody response in vivo. This hypothesis has been supported by other studies using the immunogenic proteins β2-glycoproteins and myelin oligodendrocyte glycoproteins (75, 76).

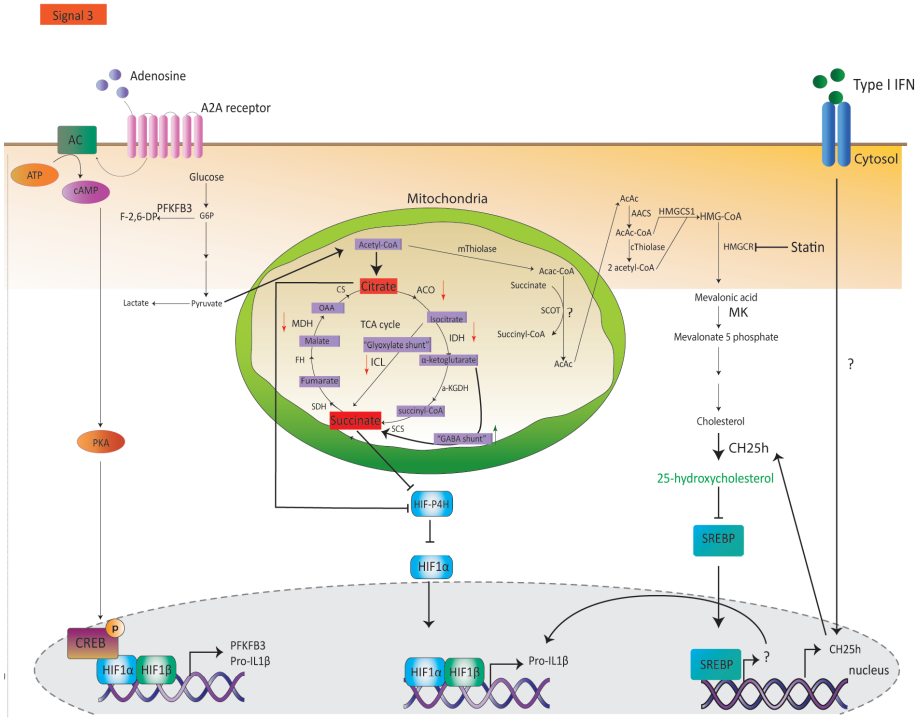

Figure 4 | NLRP3 inflammasome activation and the reciprocal interference of type I IFN on chronic inflammasome-dependent IL-1β production (signal 3). The binding and activation of the A-2A receptor by the extrinsic factor adenosine leads to the upregulation of pro-IL1β and the glycolytic enzyme PFKFB3 in a HIF1α-dependent fashion. Furthermore, upon TLR4 activation, the switch from oxidative phosphorylation to aerobic glycolysis results in the accumulation of both citrate and succinate, which suppresses FIH and HIF-P4H, respectively, and prevents the degradation of HIF1α and co-activator recruitment. The dimerization of HIF1 allows the transcription of pro-IL-1β. Succinate can accumulate following 1) the conversion of succinyl-CoA to succinate by SCS, via the 2) "GABA shunt" and 3) "glyoxylate shunt". Whereas the GABA shunt is upregulated, the glyoxylate shunt needs to be downregulated before NLRP3 becomes activated (17, 49). The cholesterol pathway and the TCA cycle can be integrated by the conversion of 2 Acetyl-CoA and Acac-CoA to HmG-CoA by HMGCS1. Type I IFN interfere with the chronic inflammasome activation status by the upregulation of the enzyme CH25h, which prevents the transcription of pro-IL1β via the inhibition of transcription factor SREBP. Abbreviations: AACS, acetoacetyl-CoA synthetase; AC, adenylate cyclase; ACO, aconitase; cAMP, cyclic AMP; Ch25h, cholesterol 25-hydroxylase; CS, citrate synthase; F-2,6-DP: fructose 2,6-diphosphate; FH, fumarate hydratase; G6P, glucose-6-phosphate; ICL; isocitrate lyase; IDH, isocitrate dehydrogenase; α-KGDH, α-ketoglutarate-dehydrogenase; FIH, HIF asparaginyl hydroxylase; HIF, hypoxia-inducible factor; HIF-P4H, HIF prolyl-4-hydroxylases; HMGCR, HMG-CoA reductase; HMGCS1, 3-hydroxy-3-methylglutaryl-CoA synthase 1; MDH, malate dehydrogenase; MK, mevalonate kinase; NACHT, NAIP (neuronal apoptosis inhibitor protein), C2TA (MHC class 2 transcription activator), HET-E (incompatibility locus protein from Podospora anserina) and TP1 (telomerase-associated protein); OAA, oxaloacetate; PFKFB3, 6-phosphofructo-2-kinase; PKA, protein kinase A; SCOT, succinyl-CoA:3-ketoacid CoA transferase; SCS, succinyl-CoA synthetase; SDH, succinate dehydrogenase; SREBP-1, sterol response element–binding protein-1; TCA, tricarboxylic acid; TGFβ, transforming growth factor β; TLR4, Toll-like receptor 4.

Furthermore, conformational changes in proteins determine the immunogenicity of T-cell responses, suggesting altered antigen processing within APCs, and modification in the displayed peptide/MHC repertoire (75).

Self-antigens

Less than 1-2% of all transcribed human proteins are estimated to account for the induction of an autoantibody response (77). So, what intrinsic features does a protein require to become an autoantigen? There is some evidence that contribute to this process, such as longer exon length and protein motifs such as the conserved sequence motif ELR, granzyme B cleavage sites, highly repetitive sequences and coiled-coil arrangements (78, 79). In addition to data supporting the immunoediting hypothesis, we describe experimental evidence of the contribution of distinct cellular organelles to the generation of cryptic epitopes.

Nucleus

A large fraction of proteins fold spontaneously into globular structures in vitro, suggesting that the amino acid sequence drives the folding of polypeptide into native conformations. The alteration of a single nucleotide pair (SNP) in a gene, however, can cause amino acid substitution (called nonsynonymous SNPs) and thereby alter protein folding. Interestingly in this respect is that human genes encoding autoantigens tend to have more SNP variation compared to other human genes, and thereby may contribute to the development of a humoral antibody response towards self-proteins (78, 80). In addition to SNPs, somatic mutations may likely predisposes to the development of autoimmunity. Somatic mutations can result from DNA damage caused by free radicals, metabolites, cytokines and chemokines that are released under chronic inflammatory conditions. In patients with RA, synovial tissue contains random mutations in tumour suppressor gene TP53, which may suggest that the chronic inflammatory burden on synovial tissue accelerates genomic instability (81, 82). Indeed, both fibroblast-like synoviocytes (FLS) in patients with RA and low-density granulocytes in patients with SLE show loss of heterozygosity, copy number alterations, somatic mutations and microsatellite instability, which are signs of genomic instability (83, 84). Furthermore, patients with RA or SLE have an increased inherent risk (unrelated to treatment) for developing certain cancers, including lymphomas (85, 86). In accordance, serum autoantibodies against TP53 can be found in patients with SLE or RA (87, 88).

Recently, the identification of antibodies that targeted cancer cells harbouring a missense mutation in the polymerase III polypeptide A (POLR3A) gene showed cross-reactivity against the native POLR3A-encoded RNA polymerase III subunit (RPC1) expressed in cells from patients with SSc (73). Although the antibodies towards RCP1 could not discriminate between the mutated and the native form, the CD4+ T-cells did not show cross-reactivity towards mutated or wild-type peptide fragments. Finally, certain cell-penetrating anti-DNA

antibodies found in patients with SLE target intracellular DNA and interfere with DNA repair in BRCA2-deficient human cancer cells and eventually induce senescence (89). Autoimmune diseases may therefore possibly even be the result of induced immune responses towards newly exposed self-antigens in (pre-)malignant cells.

Ribosome products

Defective ribosomal products (DRiPs) may account for output of cryptic epitopes (90). Here, newly synthesized yet misfolded polypeptides with half-lives of <10 minutes serve as a source of immunogenic antigens (91). Degradation of correctly folded mature proteins can also expose cryptic epitopes. Strands of mRNA that contain not one but two reading frames, which occurs in ~1% of the expressed genome, are one such source (92). One of the mechanisms whereby alternative proteins are produced is by programmed ribosomal frameshifting (PRF). PRFs can indeed supply autoantigenic peptides in rheumatic disease (93), as was deduced after successful isolation of CD8+ T-cells autoreactive towards IL-10 from synovial fluid of patients with reactive arthritis. The IL-10 peptide sequence analysis revealed that the fusion product of open reading frame 1 (ORF1) and ORF2 was presented, as a consequence of a +1 frameshift. The observation that cryptic epitopes can be derived from ribosomal slippage is further supported by studies with viruses. Bypassing the conventional reading frame of the thymidine kinase gene of the herpes simplex virus and genes of human immunodeficiency virus can produce alternative translational products which are recognized by CD8+ T-cells in vitro and in vivo (94, 95).

Endoplasmic reticulum

The pool of self-proteins that can become immunogenic seems enriched for proteins directed towards transmembrane proteins or those involved in secretion (78, 79). This category of proteins is destined for entry into the ER lumen where the oxidizing redox environment facilitates the N-linked glycosylation and the formation of disulphide bonds. However, protein assembly is inefficient and slow in the ER lumen (96). Misfolded polypeptides are toxic and can be removed by the ER-associated degradation process (ERAD). This process facilitates the transfer of misfolded proteins into the cytosol for eventual degradation by the ubiquitin-proteasome system (96). Amongst co-factors, the chaperone machinery plays a crucial role in protein assembly and breakdown of nascent polypeptides. The ER chaperone glucose-regulated protein 78 (GRP78, also known as BiP) assists in the folding-pathway by binding to hydrophobic parts in the nascent polypeptide. The targets of GRP78 include unshielded hydrophobic transmembrane (TM) domains present in membrane receptors. Unembedded TM domains of the T-cell receptor α-chain are, for example, sequestered by GRP78 within the ER lumen and retrotranslocated into the cytosol, for destruction by ERAD (97). The unshielded hydrophobicity of misfolded proteins thus signals for

degradation, and generates potential substrates for antigen presentation (98). The ER can also influence antigen processing and presentation by post-translational modification of the substrate via redox reactions. The harsh oxidizing environment within the ER promotes polypeptide oxidation and some amino acids, such as methionine and tyrosine, have increased sensitivity to oxidation by free radicals (99). So, when oxidized misfolded proteins are retrotranslocated from the ER into the cytosol, it may be processed differently compared to proteins derived from the cytosol. In support of this hypothesis, proteins targeted in the ER yield a different epitope output in MHC class I (100, 101). Furthermore, the nitration of tyrosine in peptide/MHC complexes can trigger immune responses towards model antigens, suggesting that oxidized peptides have increased immunogenicity (102). In the case of SLE, it has been demonstrated that these patients have higher levels of oxidized proteins in serum compared to healthy controls: a significant correlation was found between the presence of autoantibodies directed against protein oxidation products and SLEDAI score (103). This finding may indicate a pathogenic role of oxidized proteins and their antibodies in SLE. Of note, autoinflammatory diseases associated with ER stress, such as TNF receptor-associated periodic syndrome (TRAPS), do not develop into autoimmunity (104), suggesting that the regulation of adaptive immunity or disease penetrance into autoimmunity differs in patients with this disease compared to type I interferonopathies.

Conclusion

In this Review, we discussed molecular pathways that distinguish between autoinflammatory and autoimmune diseases, and provided insight on how these pathways can influence the function and fate of various cell subsets culminating in the activation of both innate and adaptive immune branches. Mutations that cause dysregulation of the type I IFN signaling pathway remain on the borderline of autoinflammation and autoimmunity, and clarifications are needed to understand why certain patients progress towards autoimmunity and others do not. However, studies of these diseases have emphasized on the role of aberrant type I IFN production in autoimmunity. It should be noted that within the page restrains we were unable to discuss exhaustively immune regulatory systems. In particular, future work should focus on unravelling the role of the microbiome and other (non)immune regulatory pathways to clarify further the difference between autoinflammation and those autoinflammatory diseases that perpetuate towards autoimmunity. Although the rheumatology field was revolutionized by the introduction of biologics, and more recently also small molecule inhibitors, treatment is usually not yet curative. It is our hope for the near future that the pathways highlighted here will pave the way to a better understanding of autoimmune and autoinflammatory diseases, and bring the rheumatology research community one step closer to personalized medicine.

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Chapter 3

Impaired proteolysis by SPPL2a causes CD74 fragment accumulation that can be recognized by anti-CD74 autoantibodies in human ankylosing spondylitis

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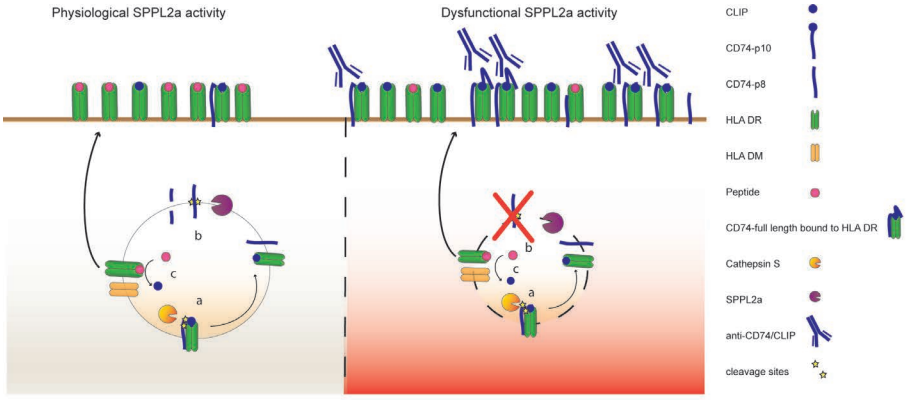
Abstract | Ankylosing spondylitis (AS) is associated with autoantibody production to Class II MHC-associated invariant chain peptide, CD74/CLIP. In this study, we considered that anti-CD74/CLIP autoantibodies present in sera from AS might recognize CD74 degradation products that accumulate upon deficiency of the enzyme signal peptide peptidase-like 2A (SPPL2a). We analyzed monocytes from healthy controls (n=42), psoriatic arthritis (n=25), rheumatoid arthritis (n=16) and AS patients (n=15) for SPPL2a enzyme activity and complemented the experiments using SPPL2a-sufficient and deficient THP-1 cells. We found defects in SPPL2a function and CD74 processing in a subset of AS patients, which culminated in CD74 and HLA class II display at the cell surface. These findings were verified in SPPL2a-deficient THP-1 cells, which showed expedited expression of MHC class II, total CD74 and CD74 N-terminal degradation products at the plasma membrane upon receipt of an inflammatory trigger. Furthermore, we observed that IgG anti-CD74/CLIP autoantibodies recognize CD74 N-terminal degradation products that accumulate upon SPPL2a defect. In conclusion, reduced activity of SPPL2a protease in monocytes from AS predisposes to endosomal accumulation of CD74 and CD74 N-terminal fragments, which, upon IFNγ-exposure, is deposited at the plasma membrane and can be recognized by anti-CD74/CLIP autoantibodies.

Graphical abstract



Introduction

Spondyloarthritis (SpA) and rheumatoid arthritis (RA) are chronic inflammatory rheumatic diseases. The spondyloarthropathies encompass a spectrum of rheumatic diseases subdivided in axial and peripheral SpA that include both ankylosing spondylitis (AS) and psoriatic arthritis (PsA) (1). SpA is often considered an autoinflammatory condition – caused by repetitive trauma and subsequent inflammatory responses – whereas RA is considered an archetypical autoimmune disease (2, 3). Although RA and SpA can share features such as destructive and chronic inflammation of the joints, the overall clinical presentation of these diseases is quite distinct. The latter suggests that these conditions share final common pathological pathways, but at the same time have unique molecular mechanisms causative to their specific phenotypes. At the molecular level, both SpA and RA are similar in showing linkage at genome-wide significance to chromosome 6p21, which harbors the polymorphic human leukocyte antigen (HLA) class I and class II genes (4, 5). AS is strongly associated with the HLA class I molecule B27, while RA associated with the HLA class II molecule DRB1. The propensity of this genetic background implies that the antigen presentation pathway or the type of T cell response elicited, is important to the pathogenesis of both diseases.

The initiation of all adaptive immune responses requires the display of antigenic fragments by professional antigen-presenting cells (APCs) to T cells and B cells. As part of the MHC class II antigen presentation pathway, CD74/MHC class II complexes are sorted from the endoplasmic reticulum to late endosomal compartments. Cathepsin S is an important protease in this process, cleaving the N-terminal side of CD74 to produce class II-associated invariant chain peptide (CLIP), which occludes the HLA class II peptide binding cleft and thereby prevents premature peptide loading. A defect in cathepsin S proteolysis leads to the accumulation of CD74-p10, retention of the sorting signals in the N-terminus, interference with peptide loading and a block in surface-directed HLA class II transport (6-9). Cathepsin S deficiency furthermore shows aberrant endosomal architecture in B cells (7). Finally, the transmembrane part of CD74 is processed by intramembrane cleavage by the protease signal peptide peptidase-like 2A (SPPL2a). Disturbed SPPL2a proteolysis triggers the accumulation of CD74-p8 fragments, enlargement of endosomes and, in contrast to cathepsin S deficiency, increased plasma membrane display of full-length CD74 and HLA class II (10-12). The rate of CD74 proteolysis, as contributed by cathepsin S and presumably SPPL2a, thereby imparts control over endosomal architecture and surface-directed transport of CD74 and HLA class II molecules.

IgG autoantibodies against the CD74/CLIP domain are associated with early AS (13-16). However, mechanisms contributing to the production of CD74 autoantigens are unknown. We here tested the hypothesis that anti-CD74 autoantibodies recognize residential products from CD74 that accumulate upon impaired SPPL2a function.

Materials and methods

Ethics: Written informed consent was obtained in all cases before participation, and the study was conducted in accordance with the Helsinki principles. Ethical approval was obtained from the institutional review board in the University Medical Center Utrecht (Study ID NTR4626; 13-696-M). Clinical parameters and blood samples were collected from a cohort of patients with AS, RA and PsA at the outpatient clinic of the Department of Rheumatology and Clinical Immunology. Healthy control (HC) blood samples were collected within the UMCU.

Reagents: (Z-Leu-Leu-NHCH2)2CO, 1,3-Di-(N-Carboxybenzoyl-L-leucyl-L-leucyl)amino Acetone, 2,2'-(2-Oxo-1,3-propanediyl)bis[N-[(phenylmethoxy)carbonyl]-L-leucyl-L-leucinamide) ((Z-LL_2)-ketone) was purchased from Santa Cruz (cat# sc-311559), N-Morpholinurea–leucine–homophenylalanine–vinylsulfone-phenol (LHVS) was synthesized in the laboratory of Dr. C. Driessen, Department of Oncology and Hematology, Cantonal Hospital St Gallen, St Gallen, Switzerland, following protocols as published in (27). Recombinant IFNγ was purchased from eBioscience (cat# 34-8319-82). The following antibodies were used: mouse anti-human LAMP-1 PE (BD; clone H4A3; cat# 555801), mouse anti-human CLIP FITC (BD; clone CerCLIP; cat# 555981) mouse anti-human CD74 PE (Biolegend; clone LN2; cat# 326807), mouse anti-human CD14 Pacific blue (Biolegend; clone M5E2; cat# 301815), mouse anti-human HLA ABC PE-Cy7 (BD; clone G46-2.6; cat# 561349), mouse anti-human HLA DR PerCP (Biolegend; clone L243; cat# 560896), mouse anti-human CD74 N-terminal (Abcam; clone 2D1B3; cat# ab181465), goat anti-human β actin (Santa Cruz; clone I-19; cat# SC1616), goat anti-mouse AF647 (Thermofisher Scientific; cat# A28181), rabbit anti-human Na-K ATPase (cell signalling; cat# 3010s), mouse anti-human LAMP1 (BD, clone H4A3; cat# 555798), Rabbit Anti-mouse-HRP antibody, (Dako; cat# P0161), Rabbit anti-goat HRP antibody (Dako; cat# P0160).

Monocyte isolation: Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-isopaque (GE Healthcare) density gradient centrifugation. CD14+ monocytes were isolated by magnetic cell sorting using mouse anti-human CD14 magnetic beads and autoMACS Pro Separator (Miltenyi biotec; cat# 130-050-201) according to manufacturer's instructions.

Cell culture: Human monocytic leukemia cell line (THP-1) and CD14+ monocytes were cultured in RPMI-1640 (Gibco) supplemented with GlutaMAX (Fisher Scientific), 100 IU/ml penicillin and 100 μg/ml streptomycin, 20mM HEPES (Gibco) and 10% fetal calf serum (FCS; Biowest). Of note, cultures contained no L-glutamine, because of its known interference with endosomal acidification and CD74 processing(28). To overcome the basification of endosomes, the cells were cultured in medium containing Glutamax in all experiments. 293T human embryonic kidney (HEK293T) cells

were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated FCS, Glutamax, Penicillin and Streptomycin. Mycoplasma infection was tested and ruled on a two-monthly basis.

Lentiviral production: HEK293T cells were transfected with the CRISPR/Cas9 vector, pMD.2G, pRSV-REV, and pMDlg/p-RRE in order to produce VSVg-pseudotyped lenti-CRISPR virions. Viral supernatants were harvested after 48h, filtered through a 0.45μm filter and used to transduce THP-1 cells by infection in the presence of 6 μg/ml polybrene. Cells were centrifuged for 1 h at 700g. Transduced cells were selected with 5 μg/ml puromycin (Sigma-Aldrich) three days post transduction for 48h. The clones were validated using DNA sequencing. The sequences were analyzed using CRISPR-ID (29). The clonally expanded cells were subjected to western blotting to test for their functional defects.

Transduction of THP-1 cells using the CRISPR-Cas9 lentiviral system: A lentiviral CRISPR/Cas9 vector (pSicoR-CRISPR-PuroR) is used which is described previously (30). The gRNAs were designed based on the reference human genome database. The gene-specific RNA sequence cloned was: SPPL2a KO: 5'- GCCGGGGCCGCCCTACTCT -3'. HLA-A2/A3: 5'-GACCTGCGCTCTTGGACCG-3'. For control KO cells we used an empty vector.

Protein extraction and Western blot: For the detection of CD74, $2x10^6$ CD14+ monocytes were cultured with the vehicle control DMSO, 1μM $(Z\text{-}LL)_2$- ketone or 20nM LHVS for 24h at 37°C. $1x10^6$ CRISPRed THP-1 were either incubated with or without 1,000 U/ml IFN-γ for 24 h at 37°C. CD14+ monocytes and THP-1 cells were washed twice in PBS, lysed in Laemmli buffer containing 5% β-mercaptoethanol and boiled for 5 minutes. The samples were stored at -20°C until further use. The protein concentration was determined using BCA protein assay kit (Thermo Fisher Scientific),separated on a 4-20% polyacrylamide gradient gel (Biorad) andtransferred on 0.2μm PVDF-PSQ (Thermo fisher scientific) membrane by wet immunoblotting. Membranes were blocked with 5% filtered bovine serum albumin (BSA) followed by primary antibody incubation (overnight 4°C in 0.5% BSA/TBS-T), and stained with secondary antibody (RT, 1h in 0.5% BSA/TBS-T). Between each step, the membranes were washed 3 times in TBS-T. For the detection of LAMP-1 and Na-K ATPase and CD74-NT in endosomes, endosomes were resuspended in Laemmli buffer without reducing agent and separated on 10% polyacrylamide gel. Protein was transferred on 0.45μm PVDF-P membrane and blocked with 5% dried non-fat milk. Primary and secondary antibody steps were performed as described above. The blot was developed using enhanced chemiluminescence (ECL)-prime (GE healthcare; cat# RPN2236) and detected in the Bio-Rad Chemi-doc. To confirm equal protein loading, antibody against β-actin was used. The bands were quantified using ImageLab, Bio-Rad.

Immunofluorescence: $2.5x10^5$ THP-1 cells were seeded in a 24-well plate and stimulated with 1,000U/ml IFNγ for 0, 3 or 24h at 37°C. Furthermore, THP-1 cells were incubated with 20nM LHVS for 24h at 37°C. After 23.5 h, 20μM Hoechst was added to each well. 8-well Lab-Tek® II Chamber Slides (ThermoFischer scientific) were pre-coated with 0.001% Poly-L-lysine for 30 minutes at RT and washed twice with PBS. The Hoechst-stained THP-1 cells were plated in the chamber slides and centrifuged for 30 seconds at 300g in order to let the cells adhere to the slides. The cells were fixed with cytofix/cytoperm (BD biosciences) for 30 minutes at RT. Cytofix/cytoperm was removed and the cells were blocked with 2% goat serum in permeabilization buffer (eBioscience) for 30 minutes at RT, followed by anti-LAMP-1 incubation (45 min, RT) and goat anti-mouse AF647 secondary antibody (ThermoFisher scientific; 45 min, RT). Between each step, the slides were washed once with permeabilization buffer. Coverslips were mounted with Mowiol solution. All images were obtained with 1.3x optical zoom using "Plan-Apochromat" 63x 1.40 oil DIC M27 objective on a Zeiss LSM710, and processed using Zen 2009 software (Zeiss Enhanced Navigation). LAMP-1-positive endosome perimeter was analyzed using the ImageJ plug-in Squassh (31).

Flow cytometry: $1x10^6$ THP-1 cells and peripheral blood mononuclear cells (PBMC) from HC, PsA and AS were stained with the viability dye efluor® 780 (eBioscience; cat# 65-0865-14) in PBS for 30 min at 4°C, washed in FACS buffer (PBS supplemented with 1% BSA and 0.1% sodium azide), stained for antibodies directed against CD14, CD74, CLIP, HLA-DR and HLA-ABC plus 10% mouse serum for 15 min at 4°C. Cells were washed and acquired on BD Fortessa flow cytometer. Analysis was done with FlowJo. Gating strategies used to examine the expression levels of the markers are illustrated in the Supporting Information Figure 5. The flow cytometry experiments were performed according to the guidelines (32).

Endosome and plasma membrane isolation by percoll density gradient centrifugation: Endosome and plasma membrane isolation was performed as described previously with minor modifications (33). Briefly, $50x10^6$ THP-1 cells were incubated with 500U/ml IFNγ for 24h at 37°C, harvested and washed twice in PBS. Cells were resuspended in Homogenization Buffer (HB; 250 mM sucrose, 25 mM Tris-HCl pH 7.4 and 1 mM EDTA and complete protease inhibitor) and disrupted mechanically by 5 passages through a 25 gauge syringe needle. A centrifugation step of 1,000g for 10 minutes was performed to obtain a post-nuclear supernatant. In a 13 ml tube, 8.7 ml of 20% Percoll solution in HB was under layered with 0.5ml of 65% (w/v) sucrose in Tris-HCl, pH7.4. On top, 1 ml of post-nuclear supernatant was added. Finally, the tubes were topped off to a volume of 12 ml with PBS. Centrifugation was performed in centrifuge Optima L-90K and LE-80K (Beckman Coulter Optima) in SW40 Ti rotor at 40,500g for 60 minutes at 4°C. Fractions of 500μl were collected from top (fraction 1) to bottom (fraction 24). The fractions were

topped off to a volume of 5 ml with PBS and centrifuged in a SW60 Ti rotor (Beckman Coulter) at 100,000g for 1 h 5 min. The pellet was resuspended in Laemmli buffer and loaded on Western blot.

Immunoprecipitation: $5x10^6$ THP-1 cells were cultured with 500U/ml IFNγ for 24h at 37°C, washed in PBS and lysed in CHAPS buffer (1% CHAPS, 30 mM Tris-HCL pH 8.0, 150 mM NaCl) for 45 minutes on ice and spun down at 13,000g for 15 minutes. THP-1 lysates were pre-cleared using empty Protein G-agarose beads (Roche; PROTGA-RO) for 30 minutes under constant agitation at 4°C. Protein G-agarose beads were incubated with human serum for 2 h under constant agitation at 4°C in order to bind to IgG. Next, beads were washes twice in CHAPS buffer and incubated with pre-cleared THP-1 lysate for 16 h under constant agitation at 4°C. Beads were separated from lysates by centrifugation, washed twice in CHAPS buffer and resuspended in Laemmli buffer The samples were boiled and loaded on Western blot.

Statistics: Cohorts of healthy donors and patients investigated for CD74-p8 accumulation and cell surface marker expression were analyzed using two-tailed unpaired t tests. Spearman's rank correlation was used to correlate disease activity parameters and full-length expression of CD74 to CD74-p8 accumulation. Confocal microscopy results were analyzed using one-way ANOVA, followed by a Tukey-Kramer test. Biological replicates for each data point are included in the figure legends. Statistical analyses were performed using IBM SPSS statistics 23 (Statistical Package for the Social Sciences). Data were considered statistically significant at p value of <0.05.

Results

Monocytes from patients with AS have decreased SPPL2a enzyme activity. We first took a pharmacologic inhibitor-based approach by culturing monocytes from HC in the presence or absence of the SPP/SPPL inhibitor $(Z\text{-}LL)_2$-ketone for 24 hours at the published IC50 dose (1μM (17-19)). CD74-p8 accumulation was tested on Western blot. As a control, we treated monocytes from HC with cathepsin S inhibitor LHVS (20nM). LHVS blocks the endosomal proteolysis of CD74-p10 to CLIP (6). Monocytes were still viable at the concentrations $(Z\text{-}LL)_2$-ketone and LHVS used (data not shown). Confirming earlier results, the SPPL2a inhibitor-treated monocytes showed N-terminal fraction (NTF) accumulation of 8 kDa (CD74-p8) (10-12), and the cathepsin S inhibitor-treated monocytes revealed accumulation of CD74-p10 (6) (Figure 1A). Thus, both enzymes promote proteolysis of CD74 in human monocytes.

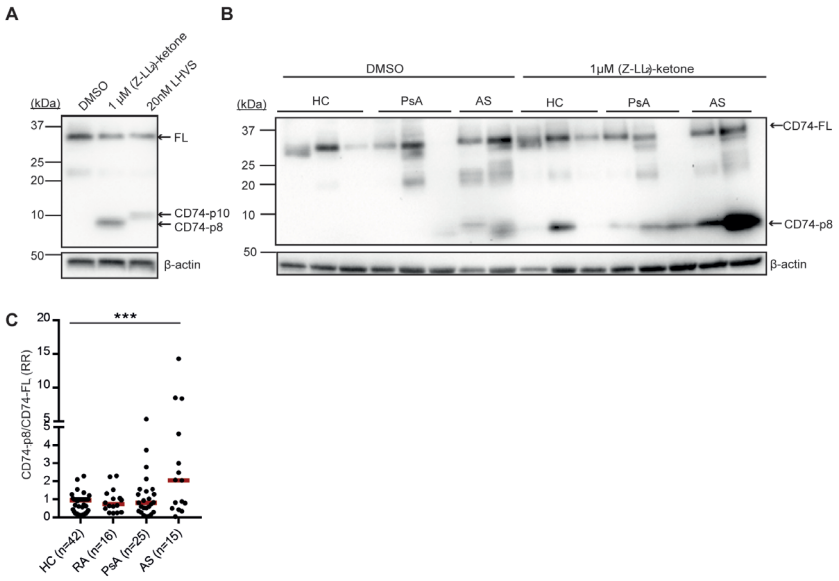


Figure 1 | Monocytes from AS patients show an increase in CD74-p8 accumulation. (A–C) CD14+ monocytes were isolated from PBMCs from HC, RA, PsA and AS by CD14 magnetic beads. (A, B) CD14+ monocytes were incubated with the vehicle control DMSO, 1 μM (Z-LL)2 ketone, or 20nM LHVS for 24 h. At least two HCs were included on each Western blot. The protein lysates were tested for CD74 using an antibody directed against the N-terminus (clone: 2D1B3). The blot was stripped and reprobed for β-actin as a loading control. Representative blots. (C) CD74-p8 was normalized to CD74-p33/p35 (CD74-FL) following normalization to a HC. Data combined from 16 independent experiments with seven to eight samples per experiment. The median is represented in the dot plot. ***$p < 0.001$; unpaired t test.

We next examined the SPPL2a enzyme function in monocytes from HC donors and patients diagnosed with PsA, RA and AS (characteristics of the study population are described in Table 1). Lysates from monocytes were tested for CD74-p8 accumulation on Western blot. Without pharmacologic inhibition (baseline), some CD74-p8 accumulation could be detected, but only in patients (Figure 1B). We first quantified and normalized the full-length of CD74 (33/35kDa) and CD74-p8 to β-actin in $(Z\text{-}LL)_2$-ketone-treated monocytes and assessed whether full-length CD74 correlated with CD74-p8 accumulation. In RA, full-length CD74 strongly correlated with CD74-p8 accumulation (r=0.78, p=<0.001) (Supporting Information Figure 1B). In HC and PsA, we also found a correlate between full-length CD74 and CD74-p8 (r=0.44, p=0.004 and r=0.58, p=0.03, respectively) (Supporting Information Figure 1A and C). In AS, however, we did not observe a correlation between the two measurements (r=0.23, p=0.42) (Supporting Information Figure 1D). This suggests that the increased accumulation of CD74-p8 in monocytes from HC, RA and PsA was caused by increased substrate availability.

Table 1 | Clinical and demographic characteristics of the study group.

Variable	HC (N=42)	RA (N=16)	PsA (N=25)	AS (N=15)
Age in years - median (range)	45 (23-65)	61 (26-73)	54 (31-73)	42 (19-65)
Female sex - No. (%)	20 (48)	12 (75)	6 (24)	4 (27)
Disease duration in years - median (range)	n.a.	10 (1-28)	11 (1-28)	9 (0.5-30)
Age of disease onset in years - median (range)	n.a.	46 (14-69)	44 (24-66)	33 (13-57)
HLA-B27+ - No. (%)	n.a.	n.a.	0 (0)	12 (80)
RF+ - No. (%)	n.a.	11 (69)	1 (4)	0 (0)
Axial involvement - No. (%)	n.a.	n.a.	1 (4)	15 (100)
ESR in mm/hour - median (range)	n.a.	7 (5-47)	8 (1-42)	13 (1-42)
TJC, of 76 - median (range)	n.a.	4 (0-13)	1 (0-26)	0 (0-4)
SJC, of 78 - median (range)	n.a.	3 (0-13)	0 (0-13)	0 (0-4)
Medication[a] – No. (%)	n.a.	16 (100)	18 (72)	3 (20)

RF: rheumatoid factor; ESR: erythrocyte sedimentation rate; TJR: tender joint count; SJC: swollen joint count; [a]Systemic immunomodulatory medication consisted of methotrexate (MTX; 10 patients with RA, 16 patients with PsA, 1 patient with AS) leflunomide (2 patients with PsA) , hydroxychloroquine (HCQ; 1 patient with RA), prednisone (1 patient with PsA) and sulfalazine (SSZ; 2 patients with AS), MTX + HCQ (5 patients with RA) and SSZ + prednisone (1 RA patient).

In AS, CD74-p8 accumulation is independent of substrate availability. Due to this observation, CD74-p8 was normalized to full-length CD74. After full-length CD74 correction, monocytes from patients with AS showed significantly more CD74-p8 accumulation upon SPPL2a inhibition compared to HC (Figure 1C). Thus, CD74 proteolysis by SPPL2a is affected in monocytes from patients with AS.
It appears that there are two groups of patients with AS, namely those who have a low accumulation of CD74-p8 and those who have a high accumulation of CD74-p8. We examined whether there is a correlation between the accumulation of CD74-p8 and clinical parameters. No correlation was found between CD74-p8 and the inflammatory marker erythrocyte sedimentation rate (ESR), Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), disease duration, sex or systemic immunomodulatory medication (Supporting Information Table 1, Supporting Information Figure 2A).

Knockout of SPPL2a results in retention of CD74-p8 in response to IFN-γ
To understand the function of SPPL2a in monocytes, we created SPPL2a-deficient cells using CRISPR-based whole genome editing in a human monocytic cell line THP-1. Additionally, we considered that interferon-gamma (IFN-γ) plays an important role in the antigen presentation pathway and CD74 processing (20-22), and therefore tested CD74-p8 accumulation in SPPL2a wildtype (WT), empty vector (E.V.) control, and SPPL2a knock out (KO) THP-1 cells, in both the absence and presence of IFN-γ. We found that both untreated and IFN-γ-treated SPPL2a KO cells yield CD74 accumulation (Figure 2A and B). However, IFN-γ-treated SPPL2a KO cells showed a higher expression of CD74-p8. In order to confirm that the NTF of CD74 in IFN-γ-stimulated SPPL2a KO cells corresponds to CD74-p8, we compared the size of the NTF with $(Z\text{-}LL)_2$-ketone-treated THP-1 cells. The size of the accumulated NTF in SPPL2a KO THP-1 cells resembled that of the accumulated NTF in SPPL2a-inhibited THP-1cells, confirming the SPPL2a KO. The SPPL2a KO cells are further confirmed by DNA sequencing (Supporting Information Figure S3A). These data suggest that the inflammatory signal IFN-γ increases CD74-p8 accumulation in THP-1 cells and we continued our experiments using IFN-γ- stimulated SPPL2a KO THP-1 cells.

SPPL2A KO THP-1 cells show compressed LAMP-1+ endosomes in response to IFN-γ.
To test if the endosomal accumulation of CD74-p8 drives morphological changes of late endosomes, we measured the average size of endosomes of SPPL2a KO and WT THP-1 cells. We included IFN-γ stimulation as a prerequisite condition to examine SPPL2a dysfunction on endosomal morphology in THP-1 cells. While both WT and E.V. control THP1 cells showed similarly sized endosomes in the steady state, IFN-γ stimulation triggered endosomal enlargement after 24 h (Supporting Information Figure 4A, B). Pre-treatment with cathepsin S inhibitor LHVS resulted in endosomal enlargement

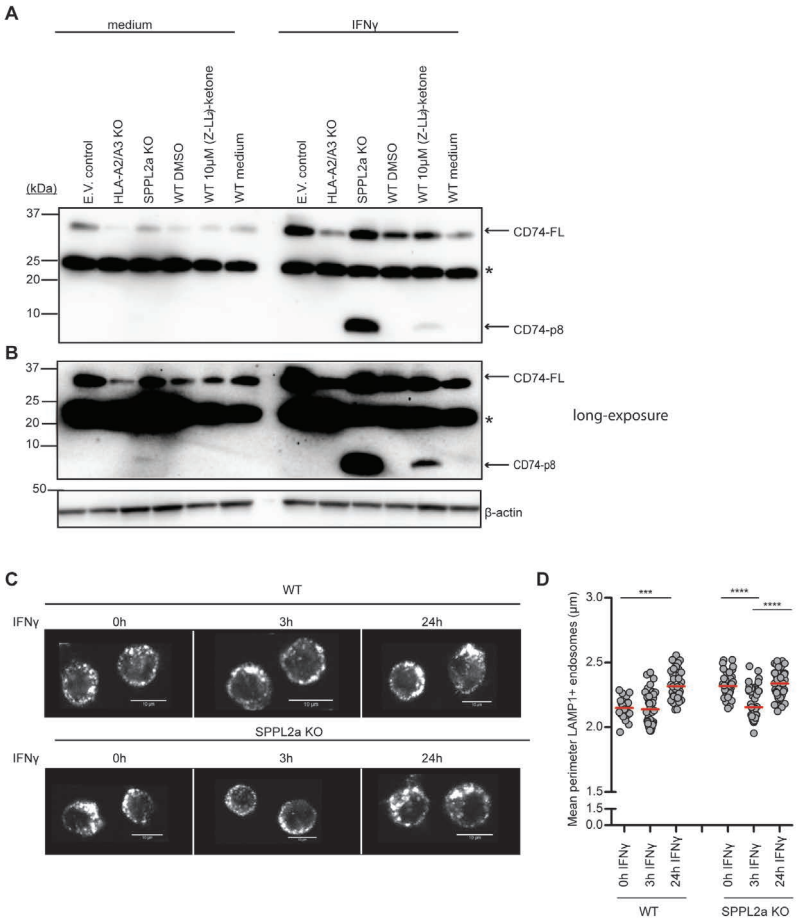


Figure 2 | SPPL2a KO cells show compression in endosomal perimeter size upon IFN-γ stimulation. For the generation of a SPPL2a KO cell line, THP-1 was depleted for the targets using CRISPR/Cas9 combined with lentiviral transduction technology. Empty vector and HLA-A2/A3 KO were used as CRISPR controls.(A,B) The effectof CRISPR gene destruction in THP-1 cells was evaluated on Western blot.The CRISPRed cells were treated with IFN-γ for 24 h. WT cells were treated with vehicle control DMSO or 10 μM $(Z\text{-}LL)_2$-ketone in the presence or absence of IFN-γ for 24 h. Western blot lysates were blotted for CD74 N-terminus (clone:2D1B3). Blots were stripped and reprobed for β-actin as a loading control. Blot is representative of three independent experiments. (B) In the long exposure, CD74-p8 can be detected in the untreated SPPL2a KO THP-1 lysates. The asterisk (*) represents nonspecific protein binding by the primary antibody. (C) Representative immunofluorescence images of LAMP-1+ endosomes of WT and SPPL2a KO THP-1 cells that were treated with or without IFN-γ for 0, 3, or 24 h. Image captured at 63× magnification. Scale bars: 10 μm. (D) Dot plot represents quantification of the perimeter of LAMP-1+endosomes. Eight to nine pictures with at least 20 cells from each condition were taken. Each dot represents the mean endosomal LAMP1+perimeter from one confocal image, analyzed by the Image J plug-in Squassh. The data are combined from five independent experiments with one sample condition/experiment. ***$p < 0.001$; ****$p < 0.0001$; one-way ANOVA, Tukey's test.

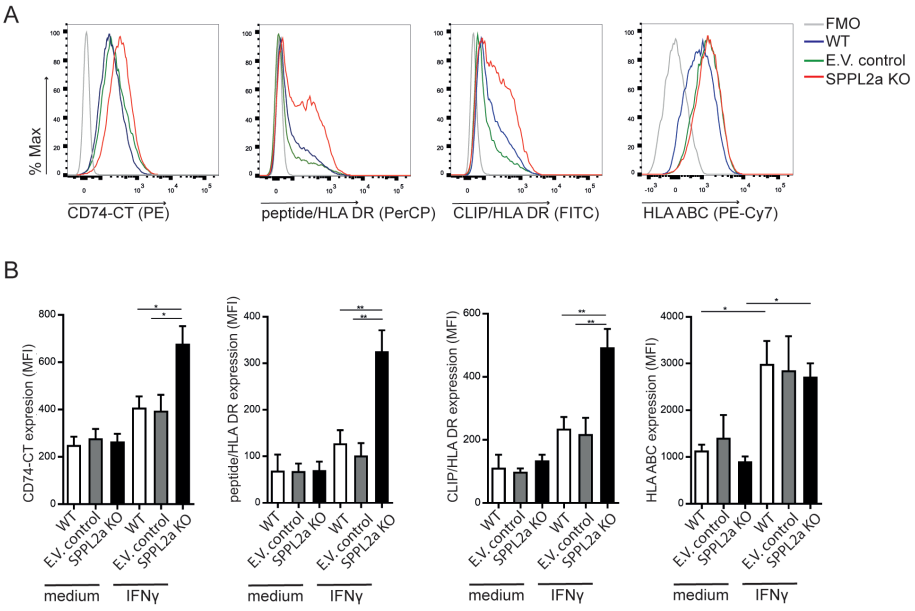


Figure 3 | SPPL2a KO cells exhibit increased surface display of full-length CD74, peptide-bound HLA-DR, and CLIP-bound HLA-DR upon IFN-γ stimulation. (A) Representative histogram of flow cytometry analysis of expression of full-length CD74 C-terminal (CT; LN2), CLIP-bound to HLADR (CerCLIP), peptide-bound HLA-DR (L243), and HLA-ABC (G46-2.6) on WT, E.V. control, or SPPL2a KO THP-1 cells, incubated in the presence of IFN-γ for 24 h. (B) Mean fluorescence intensity (MFI) of CD74, CLIP-bound to HLA-DR, peptide-bound to HLA-DR, and HLA-ABC on THP-1 cells from (A). The gating strategies are shown in Supporting Information Fig. 5A. (B) Error bars show mean and SEM from four independent experiments with one sample condition/experiment. The mean is represented in the dot plot.
*$p < 0.05$; **$p < 0.01$; unpaired t test.

(7), which did not enlarge further upon IFNγ stimulation. Similarly, SPPL2a KO cells revealed enlarged endosomes at basal level (12) with no additional changes induced after 24 h IFN-γ stimulation (Supporting Information Figure 4A, B). However, SPPL2a KO cells showed compressed endosomes after 3 h IFN-γ stimulation that return to the original size by 24h, which is not observed in WT cells (Figure 2C, D). Overall, these data may suggest that SPPL2a KO cells can resolve the endosomally accumulated CD74 fragments by IFN-γ-induced traffic to the surface, after which endosome size reverses to basal level.

SPPL2A KO THP-1 cells exhibit increased surface display of HLA-DR and CD74 in response to IFN-γ.

To test if SPPL2a deficiency might enhance plasma membrane expression of CD74 and possibly other endosome-derived molecules, we stimulated THP-1 cells with IFN-γ and assessed the cell surface expression of full-length CD74, peptide-bound HLA-DR, CLIP-bound HLA-DR and HLA-ABC by flow cytometry. Under unstimulated conditions, we observed low levels of CLIP,

HLA-DR, full-length CD74 and HLA-ABC in WT, E.V. control and SPPL2a KO THP-1 (Figure 3B). Upon 24h IFNγ treatment, SPPL2a KO THP-1 cells showed significantly increased expression of CD74, HLA-DR and CLIP-bound HLA-DR compared to controls (Figure 3A, B). We observed no difference in HLA-ABC surface expression between IFN-γ-exposed control and SPPL2a KO cells, confirming that IFN-γ-induced HLA-ABC surface display is not contributed significantly by late endosomal stores (23). Therefore, the compressed endosomal perimeter in SPPL2a KO associates with increased surface-directed transport of full-length CD74, HLA-DR and CLIP-bound HLA-DR.

Monocytes from patients with AS have increased expression of full-length CD74 and HLA-DR.

We asked whether defective SPPL2a function in CD14+ monocytes from patients with AS relates to increased cell surface markers. We observed a significant accumulation of full-length CD74 and peptide-bound HLA-DR on the cell surface of monocytes from patients with AS, but not in PsA (Figure 4A, B). CLIP-bound HLA-DR and HLA-ABC were similarly expressed throughout all individuals investigated (Figure 4A, B). Thus, CD14+ monocytes from patients with AS were more prone to increased cell surface expression of HLA class II and full-length CD74.

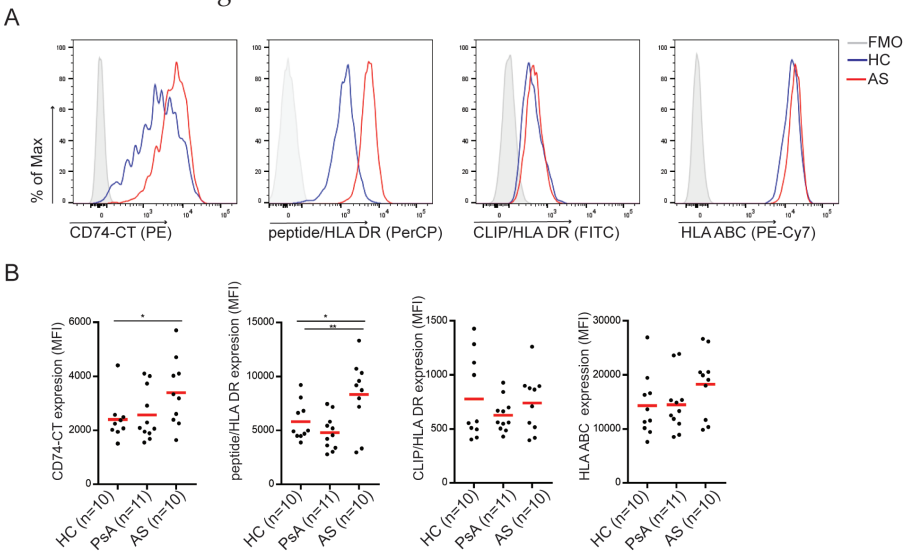


Figure 4 | Monocytes from AS patients have increased full-length CD74 and peptide-bound HLA-DR expression. (A) Representative histogram of flow cytometry analysis of expression of full-length CD74 C-terminal (CT;LN2), CLIP-bound to HLA-DR (CerCLIP), peptide-bound HLA-DR (L243), and HLA-ABC (G46-2.6) on CD14+ monocytes from HC or AS patient. The gating strategies are shown in Supporting Information Fig. 5B. (B) Dot plot represents the mean fluorescence intensity (MFI) of the cell surface molecules measured from each donor. The mean is represented in the dot plot. Data combined from three independent experiments with three to four HC, PsA, or AS samples per experiment. *p < 0.05; **p < 0.01; unpaired t test.

IFN-γ treatment triggers increased surface display of CD74-p8 NTF in SPPL2a KO THP1 cells.

To investigate whether NTF (CD74-p8) can also accumulate on the plasma membrane, we stimulated THP-1 cells with IFN-γ for 24h and isolated subcellular fractions by discontinuous Percoll-sucrose density gradient, and subjected to Western blot, probing for CD74, LAMP1 and Na+/K+ ATPase. LAMP1 and Na+/K+ ATPase were included as markers for late endosome/lysosome and plasma membrane respectively. As positive controls, whole lysates showed expression of LAMP1, Na+/K+ ATPase, and both full-length CD74 and CD74-p8 (two lanes on the right side of Figure 5A and B). Both E.V. control and SPPL2a KO cells showed co-localization of full-length CD74 with the plasma membrane marker Na+/K+ ATPase (fraction 8-10, Figure 5A). E.V. control did not reveal CD74-p8 accumulation. However, in SPPL2a KO, CD74-p8 was enriched in both the LAMP-1, late endosome, fraction (fraction 22, Fig. 5B), and the Na+/K+ ATPase and LAMP-1 cell surface fraction (fraction 8-10 Fig. 5A). LAMP-1 is present in transport vesicles, directed to fuse with the plasma membrane (24). These data suggest that NTF (CD74-p8) is transported to the plasma membrane from the late endosomes/lysosomes.

IgG present in sera from AS recognize CD74 fragments that accumulate in SPPL2a KO THP-1 cells.

We finally investigated whether sera from patients with AS contain IgG antibodies directed against CD74 fragments, using immunoprecipitation experiments. We selected sera from four patients with AS showing high CD74-p8 accumulation, and one HC as control. We used lysates from IFN-y-treated E.V. control and IFNy-treated SPPL2a KO THP-1 cells as a source of CD74 protein fragments, and screened for the presence of anti-CD74 antibodies by preincubation of protein G agarose beads with patient or control serum. Immunoprecipitated CD74 fragments, as a measure for the presence of anti-CD74 antibodies, were examined using Western blot. CD74 fragments were not detected in E.V. control lysates (Figure 5C). However, we observed anti-CD74 IgG antibodies in sera from three patients with AS, as the antibodies present in patient sera detected CD74 fragments in lysates from SPPl2a KO cells (Figure 5C). Antibodies that were present in serum samples from HC and one patient with AS did not detect CD74 fragments. The molecular weight of the detected CD74 fragments is higher compared to CD74-p8. This corroborates the finding that autoantibodies recognize CLIP, which is not present in CD74-p8 (13, 15). Together, these data support that decreases in SPPL2a function might contribute to the generation of CD74 fragments, which can be recognized by anti-CD74 autoantibodies.

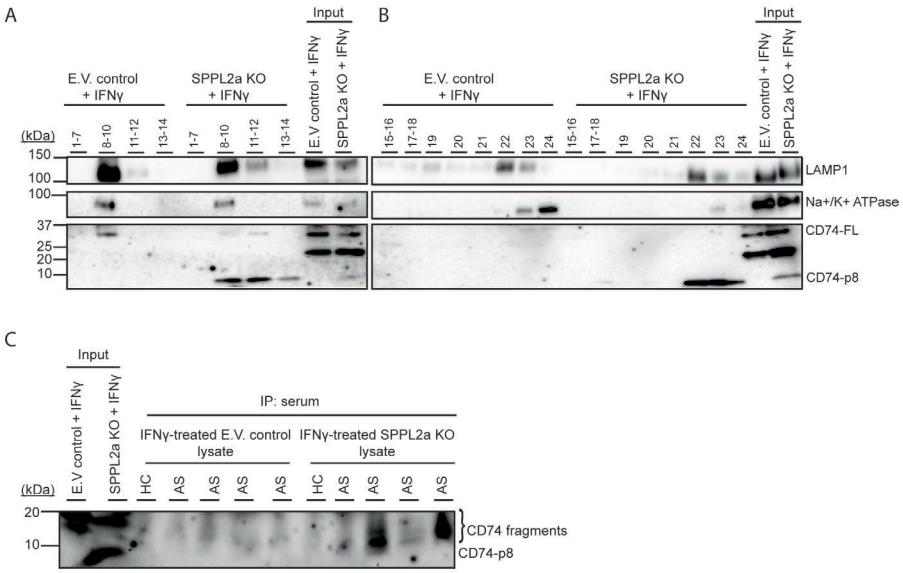


Figure 5 | Sera from patients with AS contain IgG CD74-autoantibodies that recognize accumulated CD74 degradation products in SPPL2a KO THP-1 cells. (A, B) E.V. control and SPPL2a KO THP-1 cells were stimulated with IFN-γ for 24 h and lysed in homogenization buffer (HB) and subcellularly fractionated using a Percoll-density gradient.Twelve fractions were collected with a volume of 500 μL(top fraction 1,bottom fraction 24).Fractions 1–7, 8–10, 11–12, 13–14, 15–16, 17–18, 19–20, 21, 22, 23, and 24 were collected and centrifuged to pellet the organelles and lysed in Laemmli buffer and analyzed for LAMP-1,CD74 N-terminus, and Na+/K+ ATPase expression usingWestern blot. (C) Protein-G agarose beads were coated with sera from four patients with AS and one HC. IFN-y-treated E.V. control and SPPL2a KO THP-1 cells were lysed and proteins were immunoprecipitated using serum-coated beads. Immunoprecipitates were examined using Western blot and analyzed for CD74 fragments. (A–C) Blots are representative of two independent experiments.

Discussion

In this study, we considered that anti-CD74 autoantibodies present in sera from AS might recognize CD74 degradation products that accumulate upon SPPL2a deficiency. We found defects in SPPL2a function and CD74 processing in a subset of AS patients, which culminated in increased CD74 and HLA class II display at the cell surface. These findings were corroborated by the observations in SPPL2a deficient THP-1 cells, which showed compressed late endosomes upon receipt of an inflammatory trigger. This feature coincided with increased expression of MHC class II and total CD74 at the plasma membrane. Furthermore, the increase in accumulation of CD74 degradation products were recognized by anti-CD74 autoantibodies present in sera from patients with AS. These data reveal a clear distinction between SPPL2a and cathepsin S, as in cathepsin S KO cells the MHC class II molecule expression on the cell surface is impeded and late endosomes are enlarged in size (7, 10-12). These results support that cathepsin S proteolysis facilitates MHC class II transport, whereas SPPL2a proteolysis prevents premature MHC class II surface-directed transport.

It is unclear why there is considerable variation amongst AS, in terms of CD74-p8 accumulation. No correlation was found when comparing ESR, disease activity and duration with CD74-p8 accumulation. In addition, it is unclear why CD14+ monocytes from patients with PsA did not reveal a significant SPPL2a dysfunction and the presence of anti-CLIP antibodies, considering that PsA shares some immunologic and phenotypic overlap with AS (13). Important to note in this respect is that although the heterogeneity in SPPL2a dysfunction is seen in monocytes from patients with AS, it supports that causation of the disease and/or the chronic inflammatory state are not singular, and might evolve during the course of the disease.

To our knowledge, this is the first study to describe the increased display of CD74-p8 at the plasma membrane in response to a defect in SPPL2a activity upon exposure to IFNγ. Based on this assertion, the trafficking of MHC class II molecules to the plasma membrane can be accompanied by CD74 remnants. The accumulation of CD74-NTF at the plasma membrane, which under healthy circumstances would remain hidden from the humoral immune system, might be recognized by autoantibodies directed towards CD74/CLIP and contribute to inflammatory responses in AS (13, 15). Recently, a potential oral bioavailable inhibitor has been described to significantly and selectively inhibit SPPL2a in mice and rats and as a consequence resulted in a decrease in B cell and mDC numbers (25, 26). SPPL2a inhibition may suppress autoimmune features, but with the new knowledge that the inhibition of SPPL2a can increase CD74 remnants on the cell surface, the drug can also worsen pathology in patients with AS who are positive for anti-CD74 autoantibodies.

A limitation of our study is the technical challenge to detect IgG anti-CD74 antibodies in the serum from patients with AS by ELISA, which we attribute to the incompatibility of detergents we used to generate THP-1 cell lysates as source of CD74 protein fragments, with the antibodies and substrates for ELISA. For this reason, we resorted to immunoprecipitation-based detection, which allowed for detection of anti-CD74 reactivity in AS sera and not control sera.
A further limitation is that we did not investigate the cause of the production of anti-CD74 autoantibodies in AS. In future studies, it would be interesting to examine whether the accumulation of CD74 remnants on the cell surface of SPPL2a deficient cells provoke the immune system to produce autoantibodies to CD74, in analogy to viral particles that are kept hidden from the humoral immune system until displayed at the cell surface.

In summary, our data support that a dysfunction in the protease SPPL2a contributes to dysregulation of CD74 processing in AS. Accordingly, exposure to inflammatory stimuli triggers the surface-directed transport of late endosomal cargo for increased display at the plasma membrane of CD74/CLIP. Our research provides a mechanistic explanation for the production of CD74 autoantigens that can be recognized by CLIP domain-specific IgG that is seen in 67-85% of patients with AS (13, 15).

Acknowledgements
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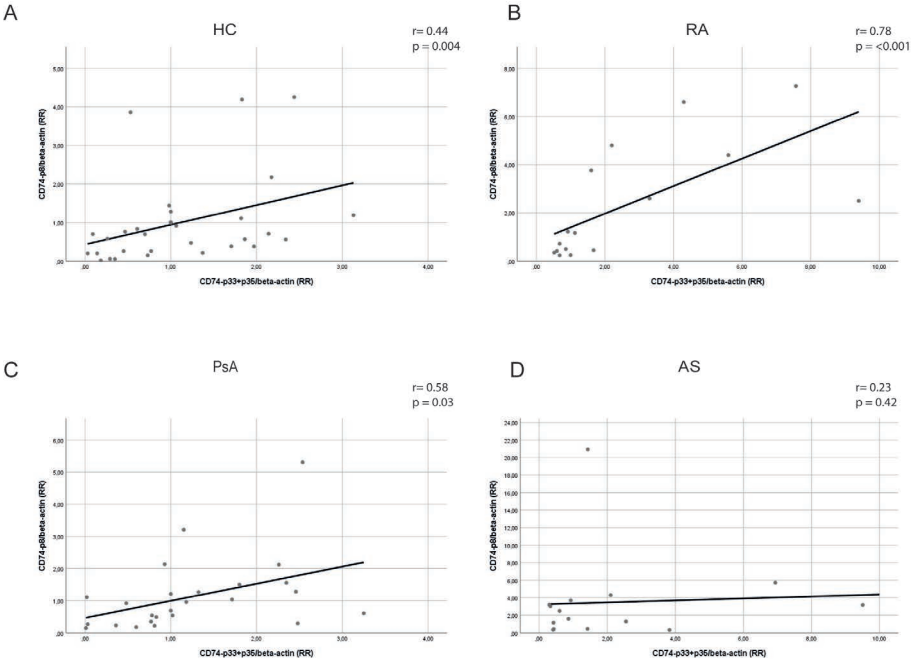
Supplementary information

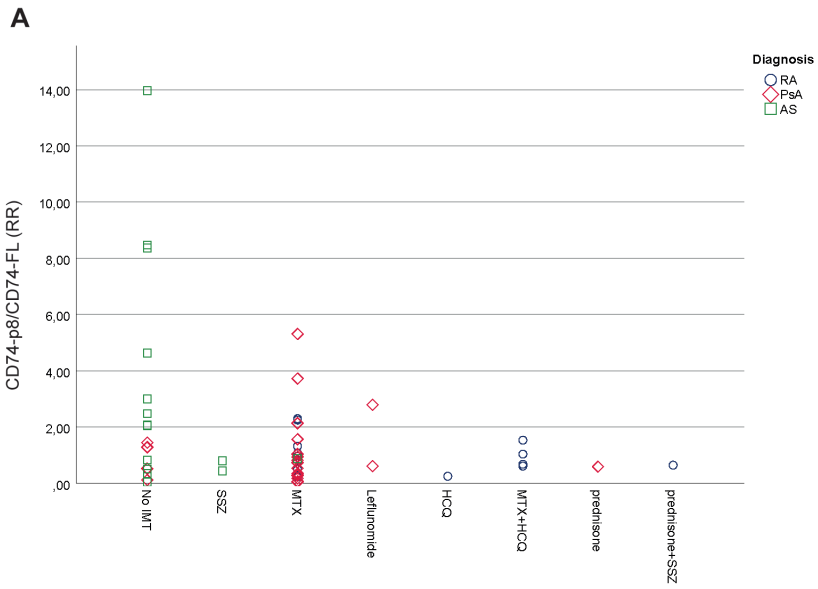


Supporting information Figure S1 | CD74-p8 and full-length CD74 (CD74-p33/35) protein expression correlation. (A-D) CD14+ monocytes were isolated from PBMCs from HC, RA, PsA and AS by CD14 magnetic cell sorter beads. CD14+ monocytes were incubated with 1μM $(Z\text{-}LL)_2$-ketone for 24 h. At least 2 HCs were included on each western blot. The protein lysates were analyzed for the presence of CD74 protein using an antibody directed against de N-terminus (clone: 2D1B3). The blot was stripped and reprobed for β-actin as a loading control (sixteen independent experiments). Full-length CD74 and CD74-p8 were normalized to β-actin following normalization to a HC. Full-length CD74 and CD74-p8 were correlated in monocytes from (A) HC, (B) RA, (C) PsA and (D) AS. Spearman's rank correlation (r) and p-value are shown in the figure.

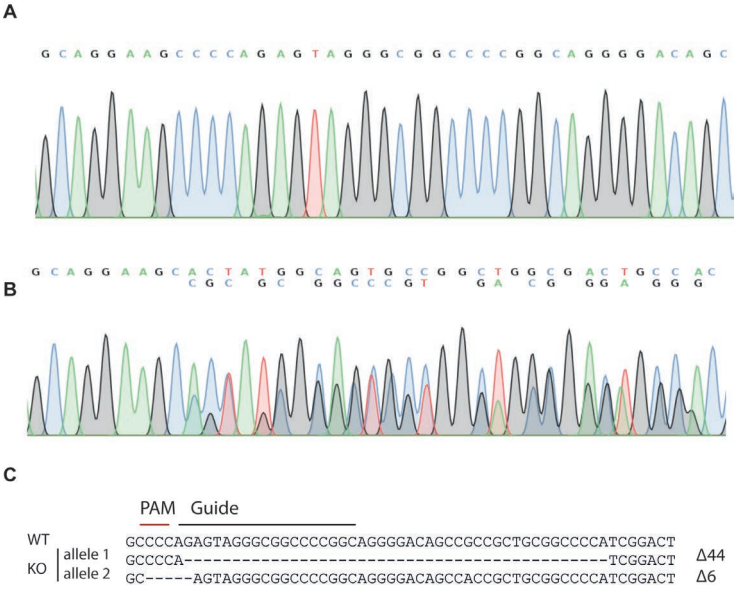
Supplemental information Table 1 | Correlation between patient characteristics and laboratory data with CD74-p8.

Clinical variable	*unit*	*N (diagnosis)*	*value*	*p-value*
ESR	mm/hour	14 (AS)	r: 0.24	0.41[a]
Sex	Female/male	15 (AS)	U: 17	0.34[b]
BASDAI	-	11 (AS)	r: 0.43	0.29[a]
Disease duration	Years	15 (AS)	r: 0.12	0.45[a]
Systemic immunomodulatory drugs	-	55 (AS, PsA, RA)	χ^2: 6.282	0.51[c]

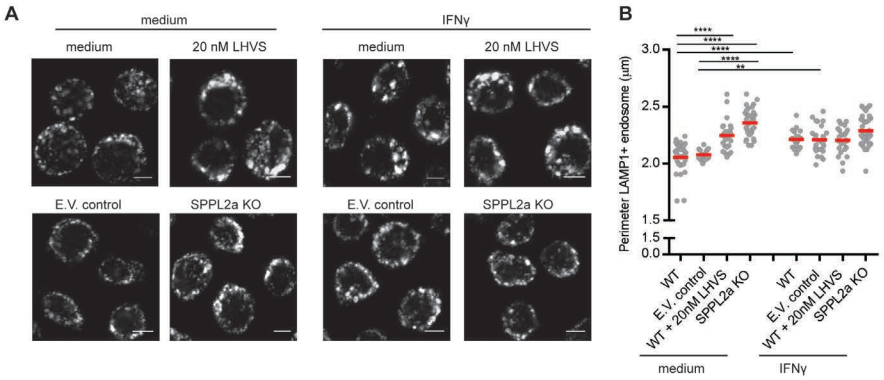
a Spearman's rank correlation
b Mann-Whitney U test
c Kruskal Wallis H test
ESR: erythrocyte sedimentation rate; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index.



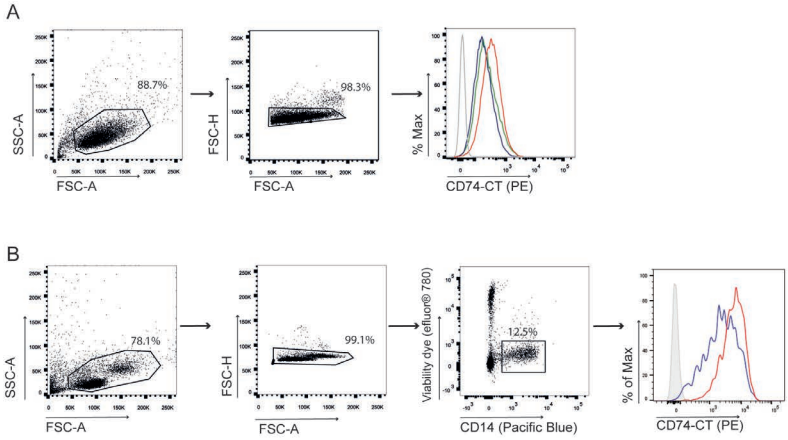
Supporting information Figure S2 | (A) CD74-p8 analysis stratified by the usage of systemic immunomodulatory medication. IMT = immunomodulatory therapy, MTX = methotrexate, HCQ = hydroxychloroquine, SSZ = sulfalazine.



Supporting information Figure S3 | Sequence analysis of SPPL2a KO cells. Genomic DNA was amplified by PCR and subjected to sequencing. Representative sequencing images of (A) WT and (B) SPPL2a KO cells upon CRISPR/Cas9 targeting. The gRNA-target site and PAM sequence is underlined in the figure. (C) Sequence variants of both alleles are depicted.



Supporting information Figure S4 | SPPL2a KO cells have enlarged late endosomes at baseline. (A) Representative immunofluorescence images of LAMP-1+ endosomes of WT, E.V. control and SPPL2a KO THP-1 cells that were treated with or without IFN-γ or with 20 nM LHVS. Image captured at 63x magnification. Scale bars: 10µm. (B) Dot plot represents quantification of the perimeter of LAMP-1+ endosomes. Eight to nine pictures with at least 20 cells from each condition were taken. Each dot represents the mean endosomal LAMP1+ perimeter from one confocal image, analyzed by the Image J plug-in Squassh. The dot plot shows the mean from five independent experiments. *p<0.05; **p<0.01;*** p<0.001; **** p<0.0001; one-way ANOVA, Tukey's test.



Supporting Information Figure S5 | Gating strategy for FACS analysis of THP-1 cells and CD14+ monocytes. (A - B) monocytes and THP-1 cells are processed and cultured according to Materials and Methods. (A) THP-1 cells were gated on size and singlets. (B) PBMCs were gated on size, singlets, live and CD14+.

Chapter 4

Identification of shared T cell receptor sequences in psoriatic arthritis

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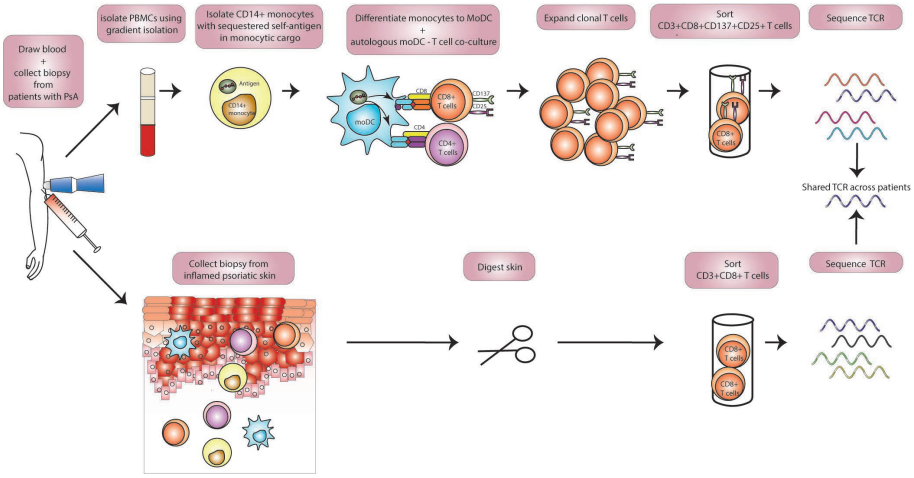
Submitted for publication

Abstract | Psoriatic arthritis (PsA) is a chronic inflammatory disease with HLA class I gene association. It is unknown whether PsA is driven by unrestricted inflammation or also by (auto)antigens. In this study, we tested the hypothesis that PsA is an autoantigen-driven disease. We developed an autologous co-culture system using human peripheral blood mononuclear cells in which we enforced the expansion of antigen-specific CD8+ T cells (n=28 PsA, n=22 healthy controls (HC)). Considering that monocytes can store antigens for days, we differentiated CD14+ monocytes from patients with PsA into monocyte-derived dendritic cells (moDCs). MoDCs were co-cultured with autologous CD3+ T cells, and assayed for antigen cross-presentation-induced T-cell expansion. We first established that combined expression of CD137 (upregulated upon T-cell receptor (TCR)-mediated antigen recognition) and CD25 (activation marker) hallmarked an enriched pool of antigen-reactive CD8+ T cells (CD25+CD137+CD8+ T cells). In PsA, we observed a significantly higher expansion of CD25+CD137+CD8+ T cells in vitro, as compared to HC. Finally, we identify TCRs by sequencing of T cells in the co-culture system, which were shared between patients with PsA and could be traced back to psoriatic skin, suggesting the existence of antigen-mediated stimulation of TCRs as correlate with PsA pathogenesis.

Graphical abstract



Introduction

Psoriatic arthritis (PsA) is considered a chronic inflammatory disease that clinically manifests as the combination of psoriasis and arthritis. Since inflammatory triggers, such as trauma and obesity, can increase the risk of developing PsA, the disease may appear as non-antigen-driven (1-3). Other evidence, however, does suggest that PsA may also be caused by autoantigen specific T cells. The oligoclonal expansion of CD8+ T cells that is observed in skin and synovium from PsA patients supports a role for T cells in the disease process (4-6). Further, the primary genetic susceptibly for PsA maps to the major histocompatibility complex (MHC) region and includes HLA class I alleles, such as HLA-B27, supporting a role for CD8+ T cell mediated pathology (7). Indeed, several class I alleles are linked with distinct clinical features of the disease (8-10). For example, HLA B*27:05 is associated with enthesitis, dactylitis and syndesmophyte symmetry, whereas HLA-B*08:01 is associated with joint fusion and joint deformity in PsA (8, 10).

The observation that certain HLA class I alleles and haplotypes confer a risk for developing clinical features in PsA might support that fragments of tissue-specific (auto)antigens predominantly bind to the HLA peptide-binding pockets encoded by risk alleles (8). However, the evidence for T cell autoimmunity in PsA remains mostly circumstantial and proof for direct involvement of antigen-driven CD8+ T cells is scarce.

In order to investigate whether PsA has a disease associated autoantigen-specific T cell involvement, we developed an CD14+ monocyte-based experimental strategy to find potential autoreactive T cell receptors (TCRs) expressed on CD8+ T cell cells in blood from patients. Monocytes can sequester antigens by sampling the tissue environment or by cell-associated antigen uptake, and migrate back to the circulation (11-13). Furthermore, monocytes are well known to store sampled antigens for days and, after differentiation to monocyte-derived dendritic cells (moDCs), are able to efficiently cross-present these as part of their antigenic cargo via peptide/HLA-class I complexes to CD8+ T cells (11-13). Exploiting this strategy, we assessed whether the in vitro-expanded autoantigen-reactive T cells, upon the interaction with autologous moDCs, express TCRβ chains that are uniquely shared between patients and are traced back to the affected psoriatic skin.

Materials and methods

Study approval: Written informed consent was obtained in all cases before participation, and the study was conducted in accordance with the Helsinki principles. Ethical approval was obtained from the institutional review board in the University Medical Center Utrecht (UMCU; study ID #15-429; #13-696-M). Clinical parameters, blood samples and skin biopsies were collected from a cohort of patients with PsA at the outpatient clinic of the Department of Rheumatology and Clinical Immunology. Healthy control (HC) blood samples were collected within the UMCU.

Antibodies: The following antibodies were used for flow cytometry. mouse-anti human CD45 APC (BD; clone 2D1, Cat#340910), mouse anti-human CD137 PE (Biolegend; clone 4B4-1, Cat# 309804), mouse anti-human CD137 APC (BD, clone 4B4-1, Cat#561702), mouse anti-human CD25 FITC (BD; clone M-A251, Cat# 555431), mouse anti-human CD25 APC (BD; clone M-A251, Cat# 555434), mouse anti-human CD45Ra BV711 (Biolegend; clone HI100, Cat# 304137), mouse anti-human CD4 APC-efluor780 (eBioscience; clone RPA-T4, Cat# 47-0049-42), mouse anti-human CD3 Pacific blue (Biolegend; clone UCHT1, Cat# 300431), mouse anti-human CD3 PE-Cy7 (Biolegend, clone UCHT1, Cat# 300420), mouse anti-human CD8 V500 (BD; clone RPA-T8, Cat# 560774), mouse anti-human CCR7 Pe-Cy7 (Biolegend; clone G043H7, Cat# 353225), 7-AAD (eBioscience, Cat# 00-6993-50).

Cell isolation: Lithium-heparinized blood samples were retrieved from HCs and patients with PsA. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-isopaque (GE Healthcare) density gradient centrifugation. CD14+ monocytes and untouched CD3+ T cells were isolated by magnetic cell sorting using mouse anti-human CD14 magnetic beads (Miltenyi Biotec, Cat#130-050-201) or antibodies coupled to magnetic beads from the pan CD3+T cell isolation kit (Miltenyi Biotech, Cat# 130-096-53), respectively, according to Manufacturer's instructions. AutoMACS Pro Separator (Miltenyi biotec) was used for cell isolation.

Autologous T cell activation assay: after cell isolation, CD3+ T cells were stored at -196°C until further use. CD14+ monocytes were cultured in RPMI-1640 supplemented with GlutaMAX (Fisher Scientific), 100IU/ml penicillin and 100μg/ml streptomycin (both from Life Technologies), 20mM HEPES (Gibco) and 10% fetal calf serum (FCS; Biowest Cat S1810). moDCs were generated by culturing CD14+ cells in a 6-well plate in the presence of 800U/ml GM-CSF (R&D Systems Cat# 215-GM-500) and 500U/ml IL-4 (R&D Systems, Cat# 204-IL) for six days. At days 3, fresh GM-CSF and IL-4 were added. At day six, moDCs were harvested and seeded in a 96-well round bottom plate at a density of 30.000 cells in CTL medium (RPMI-1640 GlutaMAX, penicillin, streptomycin, HEPES and 10% human AB serum). The cells were kept overnight at 37°C, 5% CO2. At day 7, autologous CD3+ T cells were thawed and resuspended in

CTL medium. 60.000 CD3+ T cells were added to the wells with moDCs. In addition, 60.000 T cells were stimulated with 50ng/ml IL-15 in a 96 well round bottom plate without moDCs in culture. The co-cultures were incubated for 5 days at 37°C, 5%CO2. Cells were stained according to the FACS cell sorting protocol and activation of CD8+ T cells were assessed by flow cytometer BD LSRFortessaTM.

HCMV pp65 T cell activation assay: CD14+ cells and CD3+ T cells were isolated from PBMCs from HLA-A2 positive donors (as described in Cell isolation). CD3+ T cells were stored at -196°C. MoDCs were differentiated from monocytes, harvested and seeded in a 96 well plate (as described in Autologous T cell activation assay). At day 7, 10µg HCMV recombinant pp65 protein (Miltenyi Biotec, Cat# 130-091-824) was added to the wells. After 24h, CD3+ T cells were thawed, resuspended in CTL medium, and added to each well with pp65 protein-loaded moDCs, and cultured for 5 days. Cells were stained according to the FACS cell sorting protocol and measured by flow cytometer BD LSRFortessaTM.

Skin digestion: Skin biopsies from psoriatic lesional skin were retrieved from patients with PsA and were processed to allow for cell sorting by using the whole skin dissociation kit (Miltenyi Biotec, Cat# 130-101-540) according to Manufacturer's instructions. Cells were transferred to a gentleMACS™ C Tube (Miltenyi Biotec) and the skin biopsy was minced using gentleMACS™ Dissociator (Miltenyi Biotec). Cells were filtered with a 70µm filter and centrifuged for 10 min at 300g. Cells were stained according to the FACS cell sorting protocol and the cells were sorted on BD FACSAriaTM III.

FACS cell sorting: Co-cultures containing autologous CD3+ T cells and moDCs were harvested, resuspended in FACS buffer (PBS complemented with 1% BSA (w/v; Sigma-Aldrich)) and 0.1% sodium azide (w/v; Sigma-Aldrich)) and stained with antibodies directed against CD3, CD137, CCR7, CD45Ra, CD8, CD25 and CD4 for 15 minutes at 4°C. Simultaneously, non-specific binding was prevented by incubating the cells in 10% mouse serum. 7-AAD was added to discriminate between live and dead cells. For the co-cultures that contain HCMV recombinant pp65, cells were additionally stained with a R-PE labelled Pro5 MHC pentamer A*02:01 NLVPMVATV (Proimmune). Cells that were harvested after skin digestion were stained with antibodies directed against CD45, CD3, CD137, CCR7, CD45Ra, CD8, CD4 and CD25. Cells were washed and 7-AAD was added to discriminate between live and death cells. Cells were acquired and sorted in RTL+ buffer (Qiagen). Flow cytometry analysis was done with FACS DIVA software and FlowJo.
RNA isolation: RNA from T cells was isolated using the RNAeasy microkit according to Manufacturer's instructions (Qiagen).

HLA-typing: High-resolution HLA typing was performed on PBMC using Next generation Sequencing technology (MiSeq; Illumina).

TCR sequencing: TCR amplification was performed according to a protocol described by Mamedov et al. (30). Briefly, total RNA was reverse transcribed by RACE using a primer directed to the constant region. Thirteen nucleotide long unique molecular identifiers (UMIs) were incorporated during cDNA synthesis. Subsequently, two-stage semi-nested and barcoded PCR amplification was performed including a size selection/ agarose gel purification step after the first PCR (30). Next, the TCR amplicons were subjected to high-throughput sequencing according to the instructions of the manufacturers using the Ovation Low Complexity Sequencing System kit from NuGEN (San Carlos, CA, USA) and the Illumina MiSeq (San Diego, CA, USA) using indexed paired end 300 cycle runs.

TCR analysis: Raw Illumina paired-end reads were assembled using Paired-End reAd mergeR (PEAR, (31)). The assembled read files were then subjected to barcode correction using the 'umi_group_ec' command from the Recover T Cell Receptor (RTCR) pipeline (32), allowing 0 mismatches in the barcode seed sequence for UMI detection. Subsequently, the barcode corrected samples were subjected to TCR recovery analysis using the RTCR pipeline. Identification of shared TCR sequences was performed using the tcR R package (33).

Statistics: Cohorts of healthy donors and patients investigated for cell surface marker expression were analyzed using Mann-Whitney test. Statistical analyses were performed using Prism 7 (GraphPad Software Inc.). The generation probabilities were computed using Optimized Likelihood estimate of immunoGlobulin Amino-acid sequences (OLGA) (26).

Results and Discussion

Co-expression of CD25 and CD137 on CD8+ T cells can be used as a surrogate marker to identify antigen-specific TCRs.

CD137 has been described to be transiently upregulated on the cell surface of CD8+ T cells upon TCR-mediated antigen recognition (14, 15). We first asked if upregulation of CD137 could be used as a marker to identify antigen-specific CD8+ T cells. Additionally, we included CD25 as an early activation marker for T cells, which is upregulated upon TCR ligation and correlates with antigen dose and affinity (16, 17). We therefore compared CD137 and CD25 expression on activated CD8+ T cells, which were cultured in presence or absence of HCMV antigen-loaded moDCs. We considered that CD4+ T helper cells are essential for generating effective CD8+ T cell responses, and therefore added unstimulated CD3+ T cells to the moDC-T cell co-culture for 5 days (Figure 1A) (18, 19). A fluorescently labeled HLA-A*02*01/NLVPMVATV pentamer was used to identify the pp65-specific T cell population using multicolor flow cytometry.

We first examined whether pp65 peptide-specific CD8+ T cells can be identified in the total CD8+ T cell population. We found that the HCMV seronegative donor did not show pp65 peptide-specific CD8+ T cells in neither the untreated nor pp65-treated condition (Figure 1B). The HCMV seropositive donor exhibited 0.08% of pp65 peptide-specific CD8+ T cells in the medium control condition. However, pp65-loaded moDCs significantly induced the proliferation of HCMV-reactive CD8+ T cells (14.2%; Figure 1B). In agreement, we observed an expansion of CD8+ T cells in the HCMV seropositive donor after administration of pp65 compared to medium control, but hardly any proliferation in the HCMV seronegative donor (Figure 1B). Thus, pp65 recombinant protein-loaded moDCs activate CD8+ T cells in donors that are chronically infected with HCMV, but not in HCMV negative donors.

Next, we asked whether the expression of CD137 and CD25 on CD8+ T cells can function as surrogate markers for antigen-specific CD8+ T cells. To this end, we subdivided CD8+ T cells into CD25-CD137-, CD25+CD137- and CD25+CD137+, and examined the presence of pentamer-reactive CD8+ T cells in these populations. In the HCMV seropositive donor, only a small fraction of the CD25-CD137- and CD25+CD137- CD8+ T-cells were found to be reactive to the pp65 peptide (0.16% and 2% respectively; Figure. 1D). However, we found 22.5% of the T cells to be responsive to pp65 peptide in the CD25+CD137+CD8+ T cell population. In the HCMV seronegative donor, there were no pentamer-positive CD8+ T cells found in any of the subpopulations.

These data show that using combined expression of CD25 and CD137 allows for enrichment of antigen-reactive CD8+ T cells using a single peptide/HLA class I based strategy.

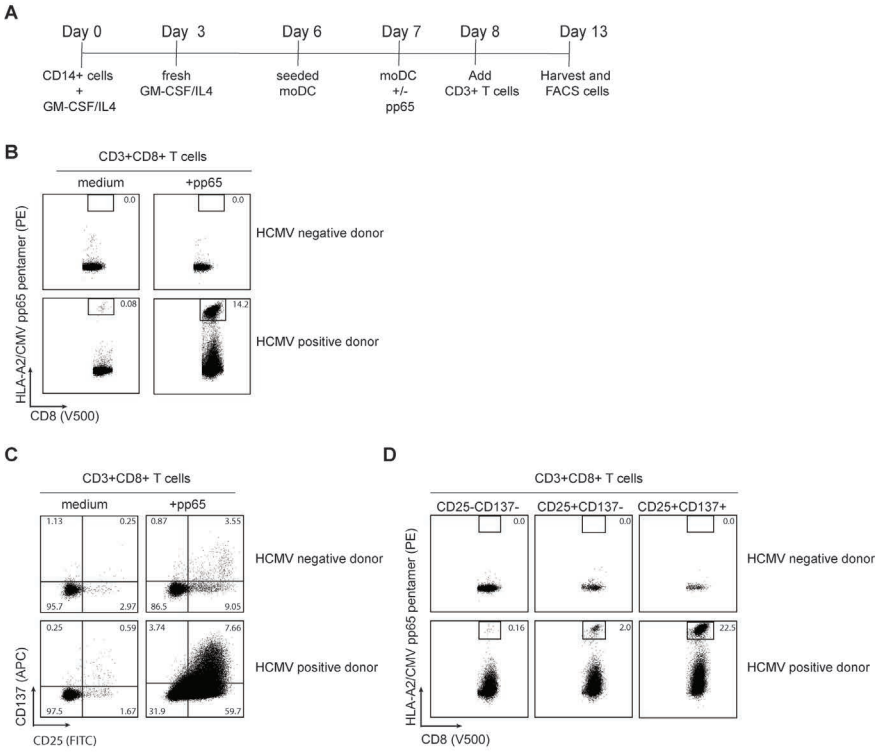


Figure 1 | CD25 and CD137 are enrichment markers for antigen-specific CD8+ T cells. (A) Timeline of the culture experiment. CD14+ monocytes and CD3+ T cells were isolated from PBMCs from HLA-A2 positive donors, who were seronegative or seropositive for HCMV. CD14+ cells were differentiated to moDCs with GM-CSF and IL-4 in 6 days. moDCs were rested for 24 h and loaded with 10μg pp65 for an additional 24 h and co-cultured with thawed autologous CD3+ T cells in 1:2 moDC:T cell ratio for an additional 5 days. Cells were stained with CD3, CD8, CD4, CD25, CD137, 7-AAD and fluorescent HLA-A*02*01/NLVPMVATV pentamer and measured on flow cytometer. (B) Representative flow cytometry analysis plot of HLA-A*02*01/NLVPMVATV pentamer reactive CD8+ T cells present after co-culture with untreated and pp65-treated moDCs. Gates were set on lymphocyte gate, singlets, live cells and CD3+CD8+CD4-. The gating strategies are shown in Supporting Information Figure 3A. (C-D) Representative flow cytometry analysis plot of CD8+ T cells subdivided into CD137-CD25-, CD137-CD25+ and CD137+CD25+ and examined for HLA-A*02*01/NLVPMVATV pentamer reactive CD8+ T cells. Representative from 2 separate experiments.

Autologous moDC-T cell co-culture derived from patients with PsA shows expansion of CD25+CD137+CD8+ T cells.

Considering that monocytes are phagocytes that are abundantly present in the circulation and constantly engulf, process and store antigens for a longer period of time (after blood withdrawal), we hypothesized that blood-derived CD14+ monocytes would be a significant source of in vivo-captured (auto) antigens (11-13). CD14+ monocytes were isolated and differentiated into moDCs from peripheral blood samples of 28 patients with PsA and

22 HCs and cultured with CD3+ T cells for 5 days (clinical features are shown in Table 1). CD8+ T cells were examined for CD137 and CD25 expression by flow cytometry (Figure 2A). Cultures with IL-15-stimulated CD3+ T cells or unstimulated (no moDC or IL-15) were included as a control. Unstimulated CD8+ T cells from HCs and patients with PsA did not exhibit double-positivity for CD25 and CD137 (left panels) (Figure 2B). In the moDC-T cell co-cultures, the percentage of CD25+CD137+CD8+ T cells in PsA was significantly higher than in HC co-cultures (Figure 2C). Of note, at baseline, we did not observe a difference in CD137 expression on blood CD8+ T cells between HC and patients with PsA (Supplemental information Figure 1A/B). We also found that the moDC-T cell co-culture induced a broad range of CD25+CD137+CD8+ T cells expansion between the different patients with PsA (range 0.1% - 7.8%).

We examined whether there was a correlation between the percentage of CD25+CD137+ CD8+ T cell and clinical measures of inflammatory activity, including erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), tender joint count (TJC), swollen joint count (SJC), but no significant correlation was found (Supplemental Table 1). However, we did observe significantly higher expansion of CD25+CD137+CD8+ T cells in patients who received disease-modifying anti-rheumatic drugs (DMARDs) compared to naive patients (Supplemental Figure 2A). Patients with an active disease state are treated with DMARDs, and this observation may indicate that DMARDs do not completely block the pathogenic immune response in PsA.

Table 1 | Clinical and demographic features of the study group.

Variable	HC (N=22)	PsA (N=28)
Age in years - median (range)	52 (31-68)	42 (18-68)
Sex female – %	57	50
PsA duration in years - median (range)	n.a.	2 (0-45)
ESR in mm/hour - median (range)	n.a.	5 (1 - 58)
CRP in mg/L -median (range)	n.a.	3 (0.3-84.0)
TJC, of 76 – median (range)	n.a.	1 (0-11)
SJC, of 78 - median (range)	n.a.	0 (0-11)
DMARD use – %	n.a.	36
NSAID use – %	unknown	57

ESR: erythrocyte sedimentation rate; TJR: tender joint count; SJC: swollen joint count. NSAID: Non-steroidal anti-inflammatory drugs. Disease-modifying anti-rheumatic drugs (DMARD) consisted of methotrexate, leflunomide, hydroxychloroquine, predinisone, and sulfalazine.

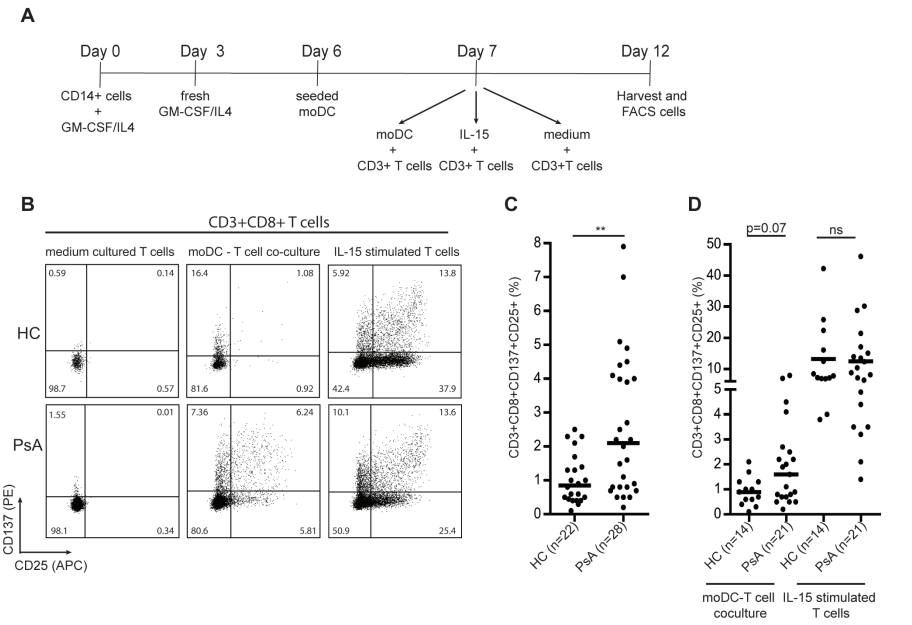


Figure 2 | moDCs from patients with PsA expand CD25+CD137+ CD8+ T cells in autologous moDC-T cell co-culture
(A) Timeline of the culture experiment. CD14+ monocytes and CD3+ T cells were isolated from PBMCs from HCs and patients with PsA. CD14+ cells were differentiated to moDCs with GM-CSF and IL-4 in 6 days. (B to C) moDCs were rested for 24 h and co-cultured with thawed autologous CD3+ T cells in 1:2 moDC:T cell ratio for an additional 5 days or (B and D) CD3+ T cells only were cultured with IL-15 for 5 days. Cells were stained with 7-AAD, CD3, CD8, CD4, CD25, CD137 and 7-AAD and examined on flow cytometer. (B) Representative flow cytometry analysis plot of CD8+ T cells expressing CD25 and CD137 after co-culture with moDCs or IL-15. Gates were set on lymphocyte gate, singlets, live cells and CD3+CD8+CD4-. The gating strategies are shown in Supporting Information Figure 3B. (C) Percentage of CD8+ T cells within the CD8+ T cell pool that are double positive for CD25 and CD137 after moDC-T cell co-culture in HCs and PsA. Data combined from twenty independent experiments with 2-4 samples per experiment. (D) CD3+ T cells from HCs and patients with PsA were stimulated with IL-15. Percentage of CD25+CD137+ CD8+ T cells were calculated and plotted. Data combined from sixteen independent experiments with 2-4 samples per experiment.

To evaluate if the CD8+ T cells from PsA are more prone to increase CD25 and CD137 upon non-specific stimuli, we cultured CD3+ T cells with IL-15 for 5 days (n=14 HC; n=21 PsA), which was selected from the sample pool of samples (20, 21). These experiments showed that CD8+ T cells from both HC and PsA respond in a comparable manner to IL-15, reaching on average 13% of CD137+ CD25+ positive cells in the total fraction of CD8+ T cells (Fig. 2B and D). Thus, we considered that the increase in CD25/CD137 double-positive CD8+ T cell fraction in PsA may include antigen-reactive T cells, which is a possibility that we pursued next.

Expanded CD25+CD137+CD8+ T cells express TCRβ sequences that are shared between patients with PsA

We next investigated whether the expanded CD25+CD137+CD8+ T cell fraction expresses certain TCRβ sequences that are shared between patients and can be traced back to lesional skin, which supports a role for an antigen-specific component in the disease process of PsA. TCR sequencing was performed on CD25+CD137+CD8+ T cells obtained after 5 days from moDC-T cell co-cultures and on CD8+ T cells obtained from lesional skin samples (patient characteristics are described in Supplemental information Table 2). TCR sequences of CD25+CD137+CD8+ T cells (3.641 reads) were compared with sequences derived from lesional skin (489 reads). We identified two TCR sequences that were observed in both the co-culture and psoriatic skin.

The first sequence was present in two co-culture samples as well as in one PsA skin biopsy sample (Figure 3A). This CD8+ T cell sequence was the most immunodominant in co-culture from patient 3 (23%, 199/878 reads). In patient 1, the TCR was less frequent in both the co-culture and skin (0.002%, 1/496 reads and 0.003%; 1/365 reads, respectively). The CD8+ T cell was endowed with Vβ9 chain. The second sequence we identified was found in co-culture from patient 4 and skin biopsy from patient 1. In patient 4, this sequence was in the top three of the most frequent T cell sequences in co-culture (9%, 24/268 reads). In patient 1, we found one sequence out of 365 sequences (0.003%, 1/365 reads) in the skin biopsy. The CD8+ T cell expressed the Vβ13 chain. Prior studies reported that psoriasis patients also have a preference for TCR Vβ13 usage, which recognizes a melanocyte-expressed autoantigen (22-24). Furthermore, CD8+ T cells that express the Vβ9 chain are oligoclonally expanded in psoriatic skin from patients with psoriasis and mainly produce IL-17 (24); a cytokine known to play an important role in the pathogenesis of PsA (25). These observations indicate that psoriatic arthritis share common pathogenic mechanisms with psoriasis in psoriatic skin.

To exclude the possibility that the two TCRs that are shared between psoriatic skin samples and cultured cells are identified by chance, we computed the generation probability of the CDR3 sequences using Optimized Likelihood estimate of immunoGlobulin Amino-acid sequences (OLGA, (26)). A higher generation probability of a given TCR may indicate a higher chance of finding this sequence in any given individual (26). Generation probabilities between 10^{-5} and 10^{-10} are typically considered as "high" (26). The TCR found in patient 1 and 4 had a generation probability close to 10^{-10} ($4.67*10^{-10}$) (Figure 3B). The TCR that is shared between patient 1 and patient 3 has a generation probability of $2.35*10^{-12}$. We additionally typed the HLA class I loci and observed that patient 1 and 3 do not show an overlap in HLA loci. A low generation probability and no overlap in HLA class I loci suggests that the given TCR is not just shared by chance.

However, patient 1 and 4 share the same HLA-A loci (HLA-A*24:02:01; Supplemental Table 2). Prior studies have shown that unrelated individuals with overlapping TCR share common HLA haplotypes, but sharing of TCRs were only significantly increased with higher HLA similarities (27, 28). Since patient 1 and patient 4 shared only one HLA allele, the generation of the overlapping TCR sequence is likely unrelated to HLA sharing, but cannot be formally excluded.

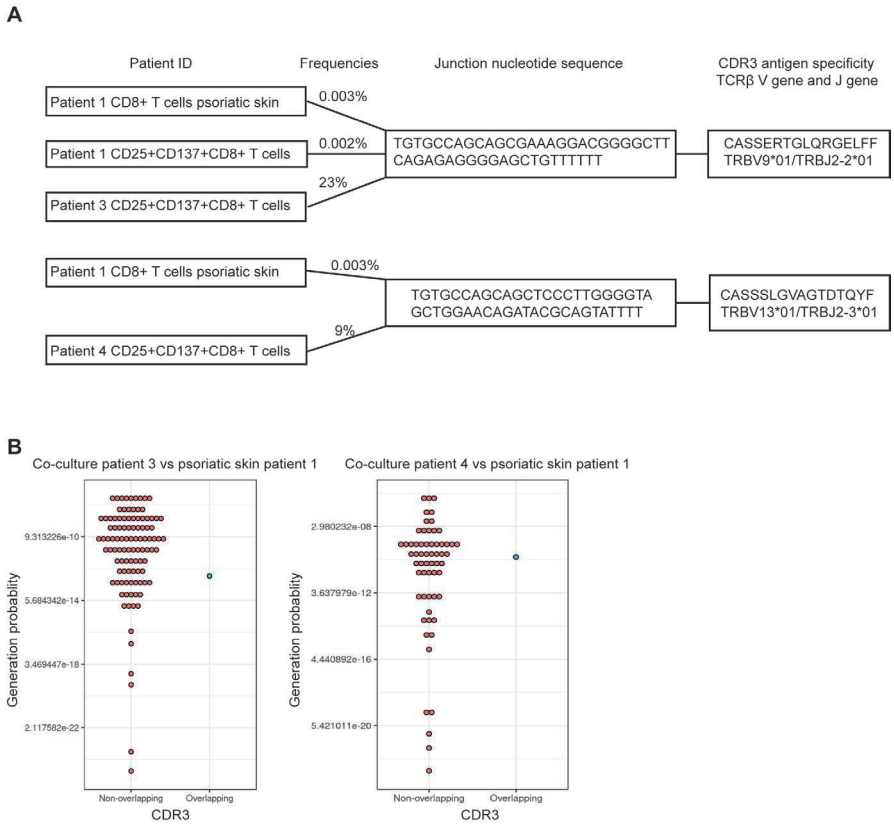


Figure 3 | Putative pathogenic T cell clones are found in PsA
(A) Bulk sorted CD8+T cells from lesional skin and CD25+CD137+CD8+ T cells from co-culture were sequenced for Vβ genes and CDR3 antigens. CD8+ T cells with identical CDR3 nucleotide sequences were found in co-culture samples and skin biopsy. The gating strategies are shown in Supporting Information Figure 2C.

We further queried the CDR3 sequences in the VDJ database that comprises more than 39.000 CDR3 sequences published in various diseases, including viral infections and cancer (29). Unfortunately, we did not find any prior epitope associated with these two TCRs. This suggests that we have selected for TCR candidates, endowed with Vβ13*01 and Vβ9*01 chains, with an unknown reactivity.

In this study, only 4 patients were sequenced for TCRβ and it remains to be determined whether the two shared TCRs are public TCRs (i.e. TCRs frequently observed in PsA). Future studies should examine whether these TCRβ are able to confer overt autoantigen-reactivity in PsA, which falls beyond the scope of our current study. Overall, the data here support that PsA has an antigen-specific component.

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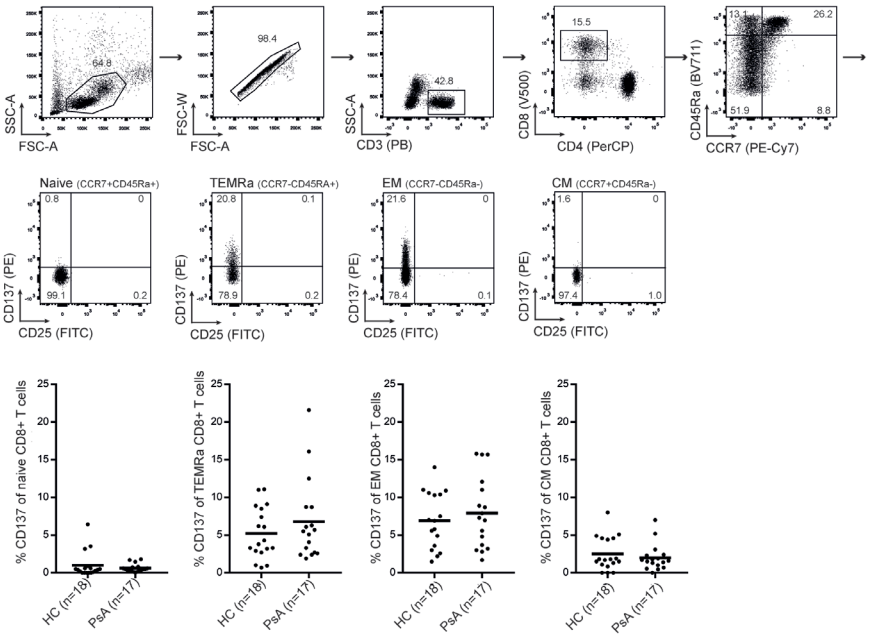
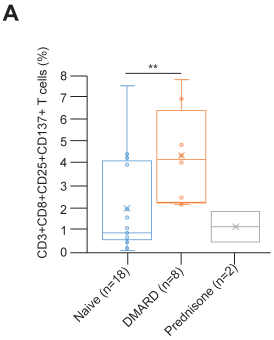
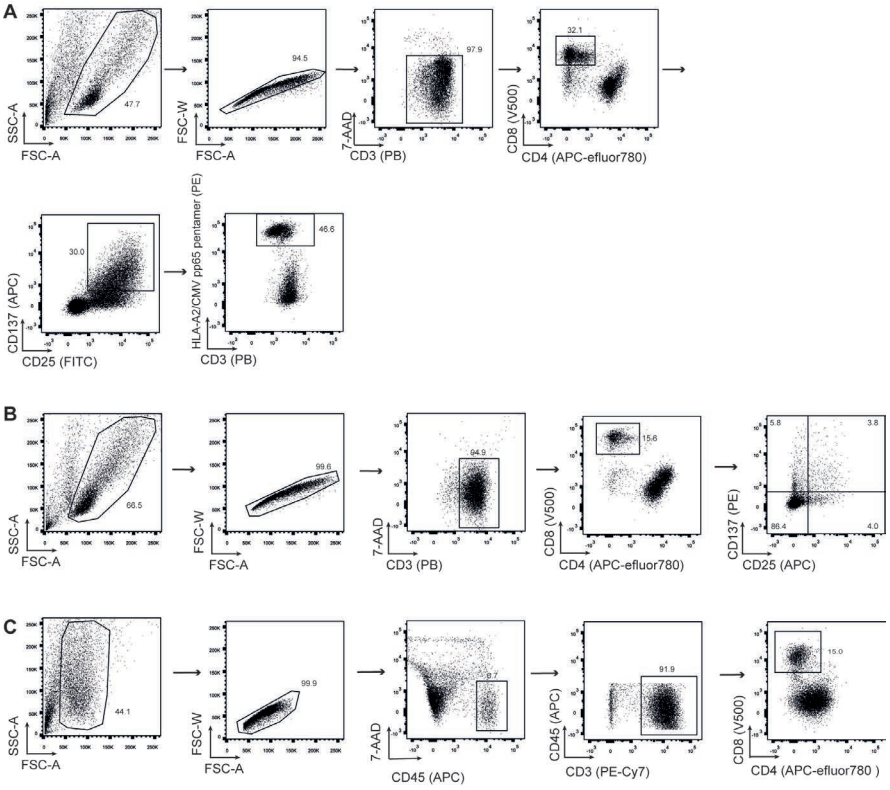
Supplementary information



Supplemental Figure S1 | Expression levels of CD137 and CD25 on subsets of CD8+ T cells in HCs and PsA at baseline. (A) Gating strategy used for phenotyping CD8+ T cells in PBMCs. (B) Percentage of CD25+CD137+CD8+ T cells subdivided into naïve, effector memory T cells CD45Ra–positive (TEMRA), effector memory (EM) and central memory (CM) were calculated and plotted in a dot plot with the mean represented in the graph.



Supplemental Figure S2 | moDCs from DMARD-treated PsA patients show significant increased expansion of CD25+CD137+CD8+ T cells in autologous moDC-T cell co-culture compared to naive patients. (A) percentage of CD25+CD137+CD8+ T cell expansion in PsA patients stratified by drug use. Data is plotted in a box and wisker plot. Systemic immunomodulatory medication consisted of methotrexate (MTX; n=4) leflunomide (Lef; n=1) , MTX + hydroxychloroquine (HCQ; n=1), sulfalazine (n=1), MTX + HCQ (n=1) and prednisone (n=2). **p<0.01; Mann-Whitney U test.



Supplemental Figure S3 | Gating strategy for FACS analysis of co-culture and skin biopsy. Gating strategy used for phenotyping CD8+ T cells that were co-cultured with (A) pp65-loaded moDCs or (B) unstimulated moDCs from PsA patient. (C) Gating strategy used for cell sorting of intralesional skin CD8+ T cells.

Supplemental Table 1 | Correlation between patient characteristics and percentage of CD25+CD137+CD8+ T cells.

Clinical variable	unit	value	p-value
ESR	mm/hour	r: -0.11	0.64[a]
CRP	mg/L	r:0.22	0.36[a]
Tender joint count, of 76	counts	r:0.17	0.42[a]
Swollen joint count, of 78	counts	r: 0.04	0.84[a]

a Spearman's rank correlation; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein.

Supplementary Table 1 | Patient information.

Patient	Patient ID	Sex	Age	Psoriatic plaque location	PASI category	TJC	TJC category	SJC	SJC category	ESR	CRP	duration of PsO (years)	duration of PsA (years)	NSAID	DMARD	Remark	HLA genotype
1	EXUP16	m	52	leg	moderate	3	oligo	3	oligo	4	1.1	7	<1	no	no		A*01:01:01:01, A*24:02:01 B*08:01:01:01, B*45:01:01 C*07:01:01, C*16:01:01:01
2	EXUP19	f	41	n.a.	n.a.	11	poly	11	poly	28	12.0	31	21	yes	yes	twin sister EXUP20	A*02:01:01, A*74:01:01:01 B*07:05:01, B*18:01:01 C*06:02:01, C*07:04:01
3	EXUP20	f	41	n.a.	n.a.	6	poly	7	poly	20	2,8	32	22	yes	yes	twin sister EXUP19	A*02:01:01, A*74:01:01:01 B*07:05:01, B*18:01:01 C*06:02:01, C*07:04:01
4	EXUP21	f	28	back	low	0	remission	0	remission	2	1,4	5	<1	no	no		A*24:02:01, A*32:01:01 B*27:02:01:01, B*44:02:01 C*02:02:02:03, C*05:01:01:02

Chapter 5

Enrichment of activated group 3 innate lymphoid cells in psoriatic arthritis synovial Fluid

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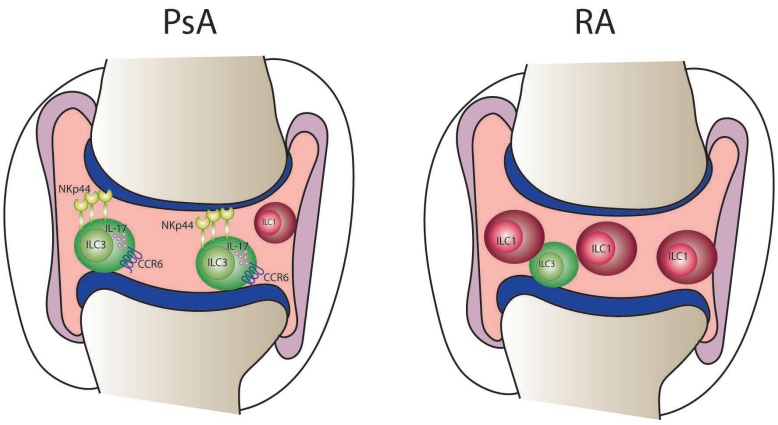
Abstact | Innate lymphoid cells (ILCs) are a recently discovered group of cells that are essential to epithelial homeostasis and are implicated in psoriasis pathogenesis, yet they have never been reported in psoriatic arthritis (PsA).
Methods. ILC classes and subsets were characterized in the peripheral blood (PB) of healthy controls, patients with psoriasis, and patients with PsA and in the synovial fluid (SF) of patients with PsA and patients with rheumatoid arthritis (RA). Cell surface marker expression and intracellular cytokine production following stimulation were analyzed using flow cytometry.
Results. ILCs were identified in the SF and were four-fold more abundant in PsA SF than in PsA PB. Fewer CCR6+ ILCs were found in PsA PB than in healthy control PB, while PsA SF was enriched for CCR6+ ILCs compared to PsA PB and RA SF. Natural cytotoxicity receptor NKp44+ group 3 ILCs were rare in PB and RA SF, but abundant in PsA SF. Increased numbers of interleukin-17A (IL-17A)–producing ILCs were present in PsA SF compared to RA SF. CCR6, NKp44, and melanoma cell adhesion molecule (MCAM) were expressed on the cell surface of SF ILCs that produced IL-17A. The number of circulating NKp44+, CCR6+, and MCAM+ ILCs in blood was inversely correlated with PsA disease activity.
Conclusion. Our findings indicate that PsA SF is enriched for group 3 ILCs that express CCR6 and NKp44, which distinguishes the synovial compartment from RA. The increased IL-17A production by SF ILCs indicates a novel role for ILCs in PsA.

Graphical abstract



Introduction

The interleukin-23 (IL-23)/IL-17 cytokine axis is well established in the pathogenesis of both psoriasis (PsO) and psoriatic arthritis (PsA). The production of IL-17 is mainly considered a T cell phenomenon, although innate lymphoid cells (ILCs) produce it as well. ILCs are a rare immune cell population defined by their lymphoid morphology, lack of T cell receptor, and absence of lineage markers (e.g., markers of dendritic cells, monocytes, stem cells, or mast cells) (1). Conventional natural killer cells are prototypical ILCs, yet more ILCs were recently discovered and are classified into group 1, group 2, or group 3. Based on transcriptional and functional characteristics, these subsets relate to the Th1, Th2, and Th17 T helper cell classifications, respectively (2). For instance, group 1 ILCs and Th1 cells share the transcription factor T-bet and produce interferon-γ, whereas group 3 ILCs and Th17 cells share the transcription factor retinoic acid receptor–related orphan nuclear receptor γt and produce IL-17A. ILCs have gained interest in the field of immunology for their roles in balancing immunity and inflammation in epithelial disease (1). They are an established source of IL-17A in Crohn's disease (3), and an IL-17A–producing subset of ILCs, group 3 ILCs, which express the natural cytotoxicity receptor NKp44, are increased in the skin and blood of patients with PsO (4–6). Group 3 ILCs express IL-23 receptor and rely on IL-23 for their full phenotypic development. Although ILCs are a rare subset in peripheral blood (PB), at epithelial sites they are responsible for the first wave of cytokine production and have been shown to be necessary for the development of psoriatic skin lesions (7,8).

We postulate that ILCs migrate not only to skin, but also to the joint, where they may function as rapid innate immune responders that propagate arthritis in PsA. To our knowledge, this is the first study to document the presence, phenotype, and functional capacities of novel ILC groups in the synovial compartment of patients with PsA.

Materials and Methods

Patient population. PB and synovial fluid (SF) samples were obtained in accordance with local Institutional Review Board approval (Trial ID NTR4626; 13-696-M). All SF samples were collected from the knee joint as part of routine clinical care. Patients with PsA fulfilled Classification of Psoriatic Arthritis Study Group criteria. A rheumatologist (E.F.A.L or J.M.R.M-vA) took the medical history and performed physical examinations of patients with PsO in order to exclude concomitant PsA. One subject with PsA donated a matched PB and SF sample; the remainder of samples from patients with PsA were unmatched. PB samples were collected from 12 healthy controls (mean ± SEM age 44 ± 2.5 years), 8 patients with psoriasis (age 57 ± 5.3 years; disease duration 12 ± 6.6 years), and 13 patients with PsA (age 46 ± 2.8 years; disease duration 7.7 ± 2.4 years). SF samples were collected from 11 patients with PsA (mean ± SEM age 51 ± 3.8 years; mean ± SEM disease duration 9.5 ± 3.1 years) and 12 patients with RA (age 53 ± 4.2 years; disease duration 16 ± 2.6 years). In patients with PsA, axial involvement was present in 3 patients, enthesitis in 4 patients, and dactylitis in 2 patients. The frequency of erosive disease was lower in the PsA SF group (1 of 11 patients) than in the RA SF group (11 of 12 patients) ($P < 0.05$). None of the other clinical or demographic features differed between the groups (see Supplementary Table 1).

Experimental procedure. PB mononuclear cells and SF mononuclear cells were isolated according to standard Ficoll gradient procedure. Seven million isolated mononuclear cells were stained with the antibody cocktail in the presence of BD Horizon Brilliant stain buffer (catalog no. 563794). ILCs were defined as cells within the lymphocyte gate on the scatter plot that were single cells, lineage negative, CD45 positive, and CD127 positive. See Supplementary Figure 1 for an example of the gating strategy and Supplementary Table 2 for the list of antibodies used. ILCs were subsequently classified as group 1 ILCs, group 2 ILCs, or group 3 ILCs based on the markers CD117 (c-Kit) and CD294 (chemoattractant receptor–like molecule expressed on Th2 cells) (Figure 1A). We specifically excluded the population of conventional NKs from our ILC population (using CD94 and CD16, as previously proposed (1)) to focus on the novel subgroups of ILCs, and our data on ILCs should be interpreted as referring to "ILCs minus conventional NKs."

In order to detect intracellular cytokine production by ILCs, SF mononuclear cell samples were thawed and stimulated for 4 hours at 37°C in culture medium (RPMI 1640 medium [Gibco] with 10% fetal calf serum, 1% penicillin/streptomycin, and 1% glutamine) containing phorbol myristate acetate (PMA; 50 ng/ml), ionomycin (1 mg/ml), and GolgiStop (catalog no. 554724) followed by extracellular and intracellular antibody staining. ILC-specific cytokine production was also compared to the expression of cell surface markers (before and after stimulation). Samples were measured on an LSRFortessa analyzer. Dead cells were excluded using a viability dye. Cutoff values were determined using fluorescence minus one. SF samples were handled and

stored the same way, with subsequent experiments being performed pairwise (i.e., PsA samples matched with RA samples) using identical protocols to avoid potential confounders.

Statistical analysis. Our primary objective was to compare PB ILCs between healthy controls, patients with PsO, and patients with PsA and to compare SF ILCs from patients with PsA to SF ILCs from patients with RA and PB ILCs from patients with PsA. Our secondary objective was to evaluate the correlation between PB ILC classes or subsets and the clinical features of the patients with PsO or PsA. Normally distributed data are presented as the mean ± SEM and were analyzed by Student's t-test and Pearson correlation. Non-normally distributed data are presented as the median (interquartile range [IQR]) and were analyzed by Mann-Whitney U test and Spearman's test. P values less than 0.05 were considered significant. FACSDiva version 7.0 and FlowJo version 10 software were used for fluorescence activated cell sorting (FACS) data analysis and FACS graphs. Data were analyzed using GraphPad Prism version 6.

Results

Composition of ILC classes in PB and SF.

Patients and healthy controls had comparable frequencies of ILCs in PB, which was ~0.1% of circulating lymphocytes. Subdividing PB ILCs into group 1 ILCs, group 2 ILCs, or group 3 ILCs resulted in an ~1:1:1 ratio (Figure 1A), without skewing in patients versus controls (Figures 1B–D). We readily identified ILCs in SF samples using the same gating strategy used to identify PB ILCs (see Supplementary Figure 1). Moreover, ILCs were 4-fold more abundant in PsA SF (mean ± SEM 0.55 ± 0.14%) than in PsA PB (0.12 ± 0.015%) ($P < 0.05$) (Figure 1B). There was a trend towards a greater percentage of ILCs in PsA SF than in RA SF (0.27 ± 0.11%), but this difference did not reach significance ($P = 0.13$). Compared to PB, group 1 ILCs were expanded in the SF of both PsA and RA (Figure 1B) ($P < 0.05$), whereas group 3 ILCs were specifically expanded in PsA SF (38 ± 6.6%) compared to RA SF (13 ± 2.7%) ($P < 0.05$).

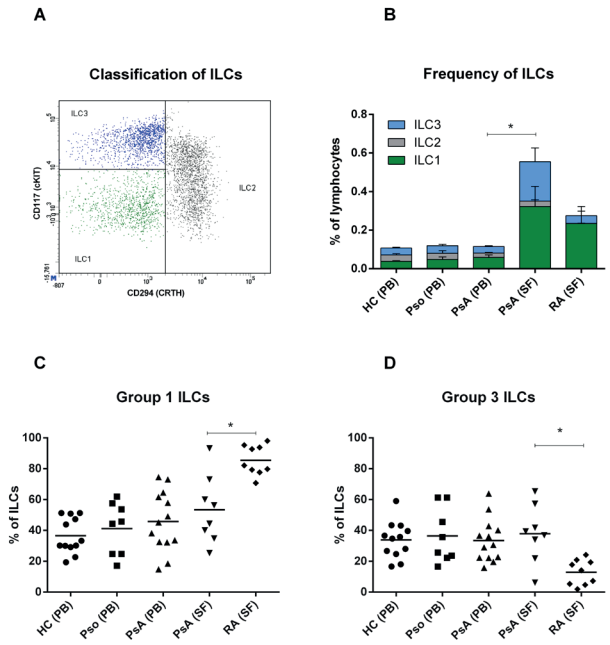


Figure 1 | Increased abundance of innate lymphoid cells (ILCs) in psoriatic arthritis (PsA) synovial fluid (SF). (A) A representative fluorescence activated cell sorter plot of a PsA peripheral blood (PB) sample, demonstrating the gating strategy that classified ILCs as either group 1 ILCs (ILC1) (CD117-CD294-), group 2 ILCs (CD294+), or group 3 ILCs (CD117+CD294-). (B) frequency of groups 1, 2, and 3 ILCs in PB from healthy controls (HCs), patients with psoriasis (Pso), and patients with PsA and in SF from patients with PsA and patients with rheumatoid arthritis (RA), presented as a percentage of the total number of lymphocyte events measured. Bars show the mean ± SEM. (C) and (D), Distribution of ILC classes as a percentage of the entire ILC population. Symbols represent individual patients; horizontal lines show the median. *=$P < 0.05$.

Group 2 was the least common class of ILCs found in SF, yet group 2 ILCs were more common in PsA SF (9.2 ± 2.9%) than in RA SF (1.9 ± 0.89%) ($P < 0.05$) (Figures 1B–D). Activation and homing markers on ILCs in PB and SF. The percentages of CCR6+ ILCs in the PB of patients with PsA (median 24% [IQR 20–32%]) were reduced compared to healthy controls (median 41% [IQR 31–45%]) ($P < 0.05$). A greater percentage of CCR6+ ILCs was found in PsA SF (median 45% [IQR 35–62%]) than in PsA PB or RA SF (median 22% [IQR 6.9–32%]) (both $P < 0.05$) (Figure 2A). NKp44+ group 3 ILCs were rare in PB overall but were more common in the PB of patients with PsO (0.90% [IQR 0.30–1.4%]) and the PB of patients with PsA (0.50% [IQR 0.40–1.2%]) than in the PB of healthy controls (0.20% [IQR 0.10–0.40%]) (both $P < 0.05$). In contrast, NKp44+ group 3 ILCs represented a large proportion of SF ILCs, specifically in PsA SF (12% [IQR 3.0–19%]), but not RA SF (0.99% [IQR 0.29–2.4%]) ($P < 0.05$) (Figure 2B). NKp44+ group 3 ILCs uniformly coexpressed CCR6.

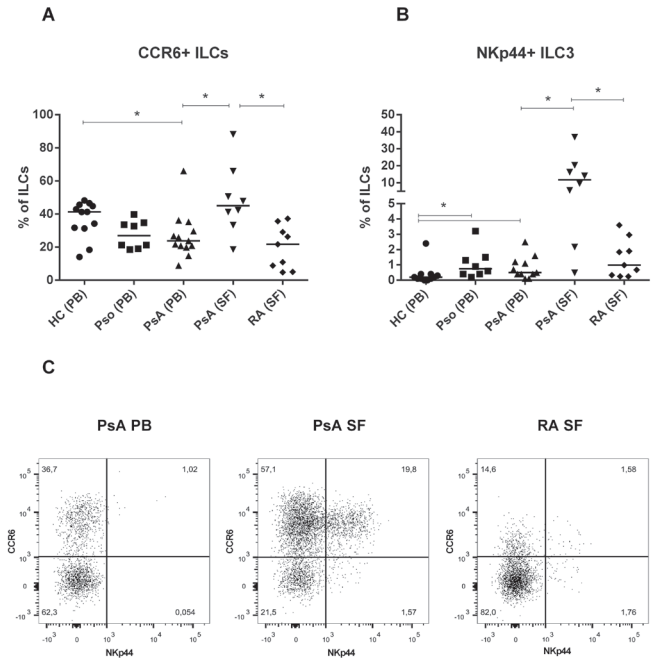


Figure 2 | Increased presence of CCR6- and NKp44-expressing innate lymphoid cells (ILCs) in psoriatic arthritis (PsA) synovial fluid (SF). (A - B) Levels of CCR6+ ILCs (A) and NKp44+ group 3 ILCs (ILC3) (B), expressed as a percentage of the total number of ILC events measured. Symbols represent individual patients; horizontal lines show the median. *= $P < 0.05$. HC= healthy control; Pso = psoriasis. (C) Matched peripheral blood (PB) and SF from a patient with PsA, showing that PsA PB contains relatively few CCR6+ ILCs and that NKp44+ ILCs are very scarce in PsA PB, whereas PsA SF is enriched for CCR6+ ILCs and contains a clear NKp44+ (CCR6+) ILC population. A representative rheumatoid arthritis (RA) SF sample is shown to illustrate that CCR6 and NKp44 expression was specific for PsA SF. *=$P < 0.05$.

Analysis of one matched, freshly isolated sample of PB and SF from a patient with PsA showed a shift toward a predominant CCR6+ ILC population in SF, of which a robust proportion express NKp44 (Figure 2C). When examining other activation markers, we found that ILCs in the SF also expressed more melanoma cell adhesion molecule (MCAM) and CD56, but these markers did not differentiate PsA SF from RA SF (see Supplementary Figure 2). However, CCR6 was coexpressed on the CD56+ and MCAM+ ILCs specifically in PsA SF (see Supplementary Figure 3). There were no differences between groups in PB ILC expression of CXCR3, HLA–DR, or common leukocyte antigen.

Production of IL-17A by SF ILCs and relationship of PB ILCs to disease activity.

Upon in vitro stimulation of SF ILCs, IL-17A production by SF ILCs was observed (see Supplementary Figure 4). Increased percentages of IL-17A–producing SF ILCs were observed in PsA (median 2.4% [IQR 1.3–2.8%]) compared to RA (median 0.77% [IQR 0.40–1.2%]) ($P < 0.05$) (Figure 3A). There was no difference in IL-22–producing ILCs in PsA SF (median 0.70% [IQR 0.28–1.7%]) versus RA SF (1.4% [IQR 0.30–3.2%]). IL-17A–producing ILCs coexpressed CCR6, NKp44, and MCAM, but did not coexpress CD56 (Figure 3B). Consistent with these findings, the frequency of IL-17A+ ILCs after stimulation was also correlated with the frequency of CCR6+ ILCs (r=0.68), NKp44+ group 3 ILCs (r=0.82), and MCAM+ ILCs (r=0.59) present in the SF prior to stimulation. There was no cell surface marker that could specifically identify all IL-17A+ ILCs. Upon in vitro stimulation of PB ILCs, no production of IL-17A or IL-22 was observed. Interestingly, high disease activity was correlated with reduced frequencies of PB ILCs classified as CCR6+ group 3 ILCs (r=-0.63 when disease activity was measured by erythrocyte sedimentation rate [ESR]) (Figure 3C), NKp44+ (r=-0.81 when disease activity was measured by Disease Activity Score in 28 joints using the C-reactive protein level [DAS28-CRP]) (Figure 3D), and MCAM1 (r=-0.79 when disease activity was measured by DAS28-CRP and r =-0.90 when disease activity was measured by Disease Activity for Psoriatic Arthritis) ($P < 0.05$). No correlation between other ILC subsets and disease activity or other clinical features of disease was observed.

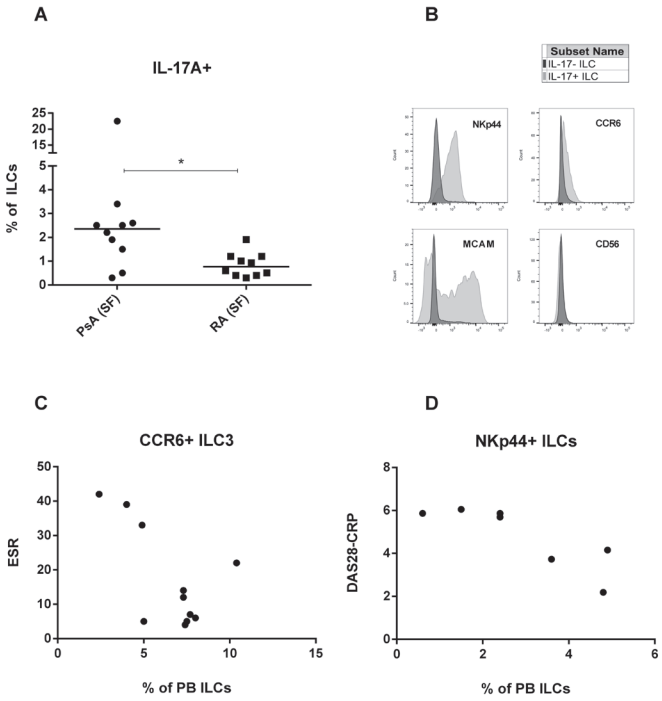


Figure 3 | Increased levels of interleukin-17A (IL-17A)–producing innate lymphoid cells (ILCs) in psoriatic arthritis (PsA) synovial fluid (SF) and inverse relationship between IL-17A–producing subsets in peripheral blood (PB) and disease activity. (A) Levels of IL-17A+ ILCs in PsA and rheumatoid arthritis (RA) SF mononuclear cells that were thawed and stimulated for 4 hours in the presence of phorbol myristate acetate/ ionomycin/GolgiStop. The percentage of ILCs that stained positive for IL-17A is shown as a percentage of the total number of ILC events measured. Symbols represent individual patients; horizontal lines show the median. *=$P < 0.05$. (B) Expression of NKp44, CCR6, melanoma cell adhesion molecule (MCAM), and CD56 in IL-17A+ SF ILCs and IL-17A- SF ILCs from a representative PsA SF sample. (C) Inverse relationship between the erythrocyte sedimentation rate (ESR) in patients with PsA and the percentage of PB ILCs classified as CCR6+ group 3 ILCs (ILC3) ($r=-0.63$, $P < 0.05$). (D) Inverse relationship between the Disease Activity Score in 28 joints using the C-reactive protein level (DAS28- CRP) in patients with PsA and the percentage of PB ILCs classified as NKp44+ ($r=-0.81$, $P < 0.05$).

Discussion

This is the first study to systematically characterize all 3 groups of ILCs in SF and PB and compare 2 different rheumatic diseases. Recent reports indicate that NKp44+ group 3 ILCs are involved in the development of PsO. Our study establishes that NKp44+ group 3 ILCs also accumulate in the joints of patients with PsA.

ILCs were significantly increased in PsA SF compared to PB, which is similar to increased numbers of ILCs in psoriatic skin compared to blood (4,5). Our ILC data are best interpreted as the net effect of ILC differentiation, combined with influx/efflux within the compartment analyzed (SF or PB). Environmental factors are known to steer the developmental fate of ILCs, and plasticity between classes has been reported. Of relevance to our results, the presence of IL-1β and IL-23 can direct ILCs from the NKp44- phenotype toward the NKp44+ phenotype, at least under culture conditions (4,9,10). The actual presence of and genetic susceptibility to such a milieu might explain why NKp44+ group 3 ILCs were specifically abundant in PsA SF, but not in RA SF. The present study is exploratory in nature and included relatively few subjects, which restricts interpretation regarding a causal relationship between specific SF ILCs and disease pathogenesis or clinical phenotype. However, a recent study confirmed the presence of NKp44+ ILCs in the SF of patients with ankylosing spondylitis, a disease similarly marked by a strong IL-23/IL-17 pathway (11).

Interestingly, patients with PsA had fewer CCR6+ ILCs in PB. Reciprocally, CCR6 was up-regulated on SF ILCs from patients with PsA, which suggests that this chemokine receptor may contribute to the extravasation of ILCs into the joints of patients with PsA. Our group has previously identified an increase in CCL20, the CCR6 ligand, in PsA SF (see Supplementary Figure 5), but functional studies are needed to determine whether ILC homing to the joint is CCL20/CCR6 mediated. ILCs from the SF of patients with PsA also expressed high levels of MCAM, an integrin linked to IL-17A–producing T cells and expressed in ILCs (12), and high levels of CD56, which is associated with the effector phenotype of ILCs (13).

We showed that IL-17A is produced by ILCs in the joints of patients with PsA and is produced by more than one ILC subset. Our results indicate that the "Th17-like" label of group 3 ILCs is an oversimplification and are consistent not only with what is already known in T cells, but also with the findings of a recent study on the transcriptome of different ILC classes, which found that IL-17A was not group 3 ILC restricted (14). SF samples were obtained at a stage in the disease when other cells have massively homed to the inflamed joint, thus when the absolute contribution of ILC-produced cytokines is relatively low. However, the established function of ILCs is their rapid (innate) production of cytokines prior to the production by T cells, and our results point to ILCs as candidate cells capable of initiating an inflammatory cascade in the joint.

Beyond the cytokine effector function of ILCs, little is known about their function in humans, and future studies should evaluate the interaction between ILCs and other cells in the joint (13). It is unclear if the presence of NKp44 on SF ILCs only signifies an activated status or if this receptor itself induces activation of ILCs (13,15). Tonsil-derived NKp44 ILCs have been shown to produce tumor necrosis factor following activation of their NKp44 receptor (15). In addition, conventional NKs also express NKp44, which recognizes the NKp44 ligand on normal human chondrocytes (16) and fibroblast-like synoviocytes (17) to directly cause (cytotoxic) activity. It will be of interest to determine whether these ligands can similarly activate NKp44+ SF ILCs in the joint early in the disease course.

We conclude that distinct classes of ILCs are present in the joints of patients with rheumatic diseases. Group 1 ILCs are predominant in RA SF, whereas PsA SF is characterized by CCR6- and NKp44-expressing group 3 ILCs with increased capacity to produce IL-17A. These findings underscore the critical role played by the IL-23/IL-17 pathway in PsA and ILCs as potential instigators of inflammation in the joints of patients with PsA.

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Supplementary information

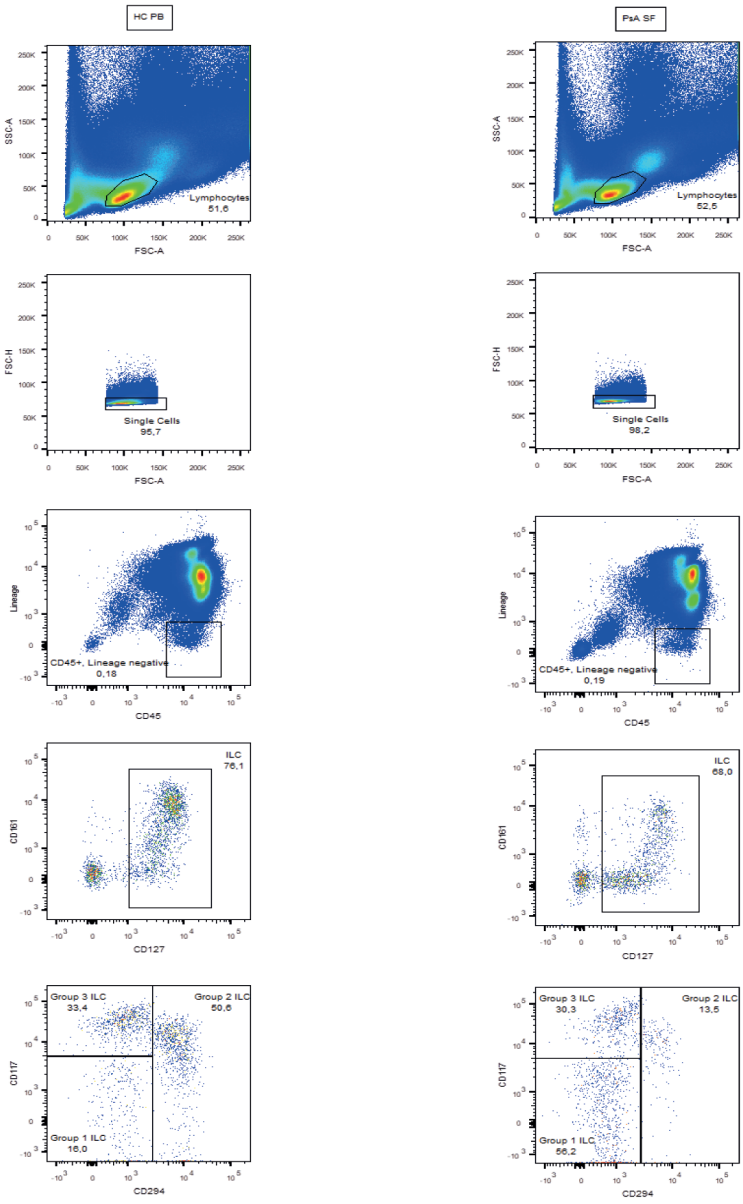
Table 1 | Demographics and disease activity of study participants

		HC PB	Pso PB	PsA PB	PsA SF	RA SF
Samples						
	Total samples (n)	12	8	13	11	12
	Fresh samples (n)	12	6	13	2	3
	Frozen samples (n)	0	2	0	9	9
Demographics						
	Age (y)	44 ± 2.5	57 ± 5.3	46 ± 2.8	51 ± 3.8	53 ± 4.2
	Female	9/12	4/8	5/13	5/11	4/12
	Pso duration (y)	.	12 ± 6.6	20 ± 4.8	12 ± 4.6	.
	PsA/RA duration (y)	.	.	7.7 ± 2.4	9.5 ± 3.1	16 ± 2.6
Therapy						
	Prednisone (oral)	.	0/8	0/13	2/11	6/12
	Methotrexate	.	1/8	5/13	6/11	5/12
	TNF-inhibitor	.	0/8	0/13	0/11	2/12
Disease activity						
	PASI	.	3.3 (1.2-6.1)	2.1 (0.75-2.9)	1.4 (0.3-2.4)	.
	28 TJC (n)	.	.	5.5 ± 1.4	2.8 ± 0.75	6.0 ± 3.0
	28 SJC (n)	.	.	5.3 ± 1.5	3.0 ± 0.91	5.6 ± 2.6
	ESR (mm/hr)	.	8.5 (3.5-15)	7 (4.5-28)	m	m
	CRP (mg/L)	.	3.9 ± 1.9	10 ± 2.7	m	m
	DAS28-ESR score	.	.	4.1 ± 0.61	m	m
	DAS28-CRP score	.	.	4.2 ± 0.57	m	m
	DAPSA score	.	.	39 ± 8.5	m	.
	Dactylitis present	.	.	1/13	1/11	.
	Axial involvement	.	.	3/13	0/11	.
	Enthesitis present	.	.	4/13	m	.
	Erosive disease	.	.	6/13	1/11	11/12*
	RF/aCCP positive	.	.	0/13	0/11	10/12

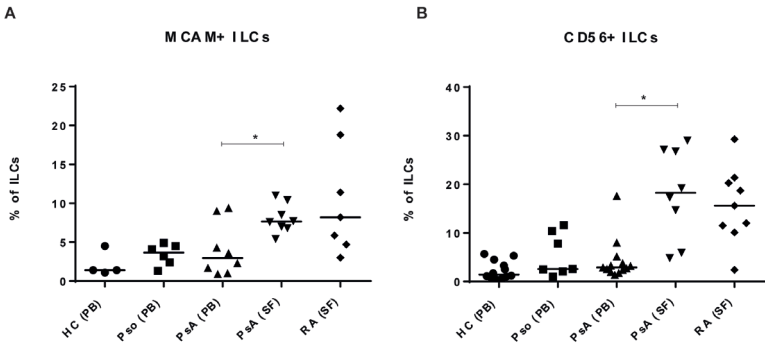
Data shown as mean ± SEM or median (IQR). One patient with PsA had donated a matched PB and SF sample, the remainder of samples were unmatched. There were significantly more patients with erosive disease in the RA SF group compared to the PsA SF group (fishers exact test, $p<0.05$). No other variables differed between the study groups. SF samples were collected during synovial fluid aspiration as part of standard care, when other disease activity parameters were typically not assessed by the treating physician resulting in some missing clinical data (m) in the SF groups.

Table 2 | List of antibodies used

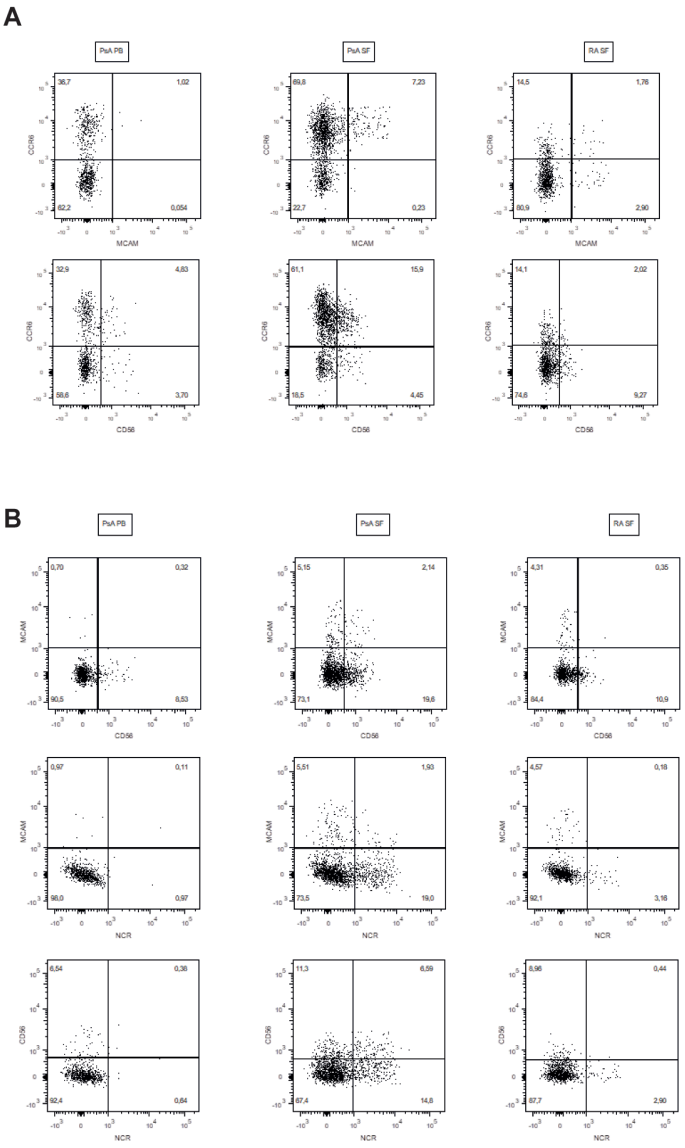
Antibody	Description	Fluorochrome	Company	Catalog #
CD3	Lineage: T-cell	FITC	BD Horizon	345763
CD19	Lineage: B-cell	FITC	BD Horizon	555412
CD14	Lineage: Monocyte	FITC	BD Horizon	555397
CD16	Lineage: NK	FITC	BD Horizon	555406
CD94	Lineage: NK	FITC	eBioscience	11-0949-41
CD11c	Lineage: mDC	FITC	Biolegend	337214
CD141	Lineage: mDC	FITC	Miltenyi	130-090-513
CD34	Lineage: stem cell	FITC	Biolegend	343603
BDCA2	Lineage: pDC	FITC	Miltenyi	130-090-51
CD123	Lineage: pDC	FITC	eBioscience	11-1239-42
FCeR1	Lineage: mast cell	FITC	eBioscience	11-5899-41
CD127	IL-7Rα	AF647	Biolegend	351318
CD161	KLRB1	BV510	Biolegend	339921
CD45	Leucocytes	PE-Cy7	BD Horizon	557748
CD294	CRTH2	PE-CF594	BD Horizon	563501
CD117	cKIT	BV605	Biolegend	313217
CD336	NKp44	PerCP-eF710	eBioscience	46-3369-41
CD56	NCAM	AF700	BD Horizon	557919
CD146	MCAM	BV711	BD Horizon	563186
CD196	CCR6	PE	BD Horizon	559562
CD196	CCR6	PerCP-Cy5.5	BD Horizon	560467
HLA-DR	MHC II	BV711	BD Horizon	563696
CD183	CXCR3	PB	Biolegend	353723
CLA	Skin homing	PB	Biolegend	321307
IL-22	IL-22	eF450	eBioscience	48-7229-41
IL-17A	IL-17A	PE	eBioscience	12-7179-42
Viability Dye	Live cells	eF506	eBioscience	65-0866-14



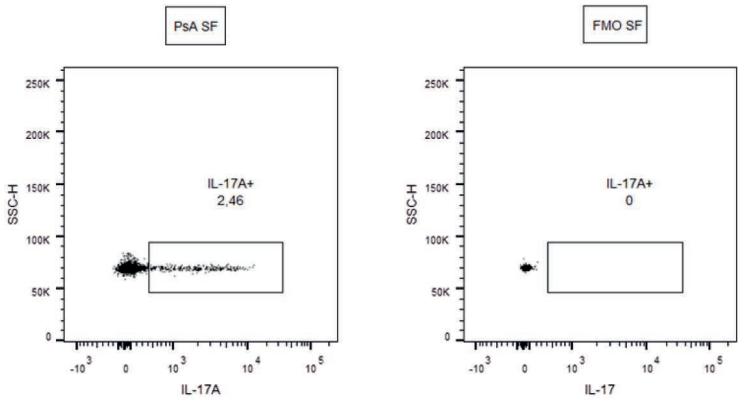
Supplementary figure S1 | ILC gating strategy. Representative gating strategy for analysis of ILCs from freshly isolated PBMC (HC) and SFMC (PsA) sample is shown. ILCs were defined as falling within the lymphocyte gate, singles, lineage negative, CD45+, CD127+ (as expected, CD161 was not uniformly expressed by ILCs). ILC1 are identified as CD117- and CD294-; ILC2 as CD294+; ILC3 as CD117+ and CD294-.



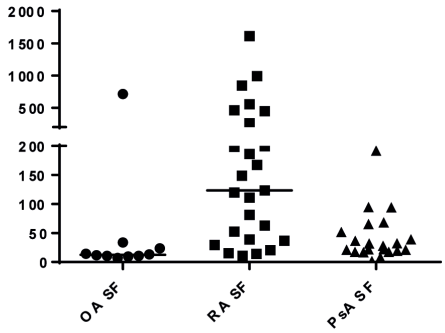
Supplementary figure S2 | Increased intra-articular frequencies of MCAM-expressing and CD56-expressing ILCs. (A) In PB, there was no difference in MCAM expression between HC, Pso, and PsA. In PsA SF, increased percentages of ILCS expressing MCAM as compared to PsA PB were observed ($p<0.05$). There was no difference in the expression of MCAM between PsA SF and RA SF. (B) In PB, there was no difference in CD56 expression between HC, Pso, and PsA. In PsA SF, there were more ILCs expressing CD56 than in PsA PB ($p<0.05$). There was no difference in the expression of CD56 between PsA SF and RA SF. Bars represent the median per group. * indicates $p<0.05$



Supplementary figure S3 | Representative MCAM and CD56 expression in PsA PB, PsA SF, and RA SF. (A) MCAM+ ILCs and CD56+ ILCs in PsA SF typically co-expressed CCR6, whereas this was not the case for MCAM+ or CD56+ ILCs in RA SF. (B) The co-expression pattern of NKp44 (NCR), MCAM, and CD56 is shown. As can be appreciated, NKp44+, MCAM+, and CD56+ ILCs are distinct ILC subsets: not typically co-expressed on the same ILC.



Supplementary figure S4 | The gating strategy to identify IL-17A+ ILCs, based on the corresponding negative control of that sample (fluorescence minus one, 'FMO'). Stimulated PB ILCs did not produce IL-17A or IL-22, neither in patients or heatlhy controls (not shown).



Supplementary figure S5 | Increased CCL20 concentrations in joints of patients with RA and PsA. In a previous cohort we have quantified the concentration of CCL2, the known ligand of CCR6, in SF samples collected from patients with osteoarthritis (OA, n=10), rheumatoid arthritis (RA, n=25), and psoriatic arthritis (PsA, n=28). Both RA patients and PsA patients had elevated levels of CCL20 in SF compared to OA, without a difference between RA and PsA. The bars represent median; * $p<0.05$, Mann-Whitney test.

Chapter 6

Discussion

The emergence of transgenic mouse models, in vitro culture systems and (multi)–omics technology, as disciplines in immunological research has given insights into the immunopathogenic mechanisms of SpA. Knowledge that was available prior to this thesis work suggested that SpA is a systemic inflammatory disease and the IL-23-IL-17 pathway plays an important role in active inflammation. This knowledge contributed to the development of immunological therapeutic interventions (biologicals), which benefit SpA patients by reducing clinical manifestations. Biologicals are now widely used in the clinic. However, a subset of SpA patients does not respond to biologicals, even though they take part in the same spectrum of disease. For example, Ustekinumab (anti-IL-12p40) and Risankizumab (anti-IL-23p19), both targeting IL-23, were found to be effective in PsA (1-3), but not in AS (4, 5). Also within the same disease group, not all patients benefit from the same treatment. This suggests that the ability to describe the features of a disease state does not equal how that state arose or how it responds in one way or another. The challenge that my thesis work involved, was to unravel pathogenetic mechanisms in SpA, with a focus on antigen-induced inflammation.

In **chapter 3** of this thesis, we show that CD14+ monocytes from patients with AS have decreased signal peptide peptidase-like 2a (SPPL2a) activity. Furthermore, anti-CD74 autoantibodies, present in sera from patients with AS but not in peripheral PsA, recognize CD74 degradation products that accumulate upon impaired SPPL2a function in human monocytes.
The reason why monocytes from patients with AS have reduced enzyme activity is unknown. Genome-wide association studies (GWAS) did not reveal SPPL2a as a disease susceptibility gene in AS. However, the sample sizes of GWAS that have been performed on AS are relatively small compared to other cohorts, such as inflammatory bowel disease and multiple sclerosis (6). Large scale GWAS studies can give better insight in associated signals that might be mapped on causal variants, including SPPL2a. Unexpectedly, PsA showed a linkage disequilibrium with SPPL2a (7). We did not find impaired enzyme function in PsA, which suggests that the GWAS nominated a cluster of single nucleotide polymorphisms (SNP) in linkage disequilibrium on chromosome 15q21 that either identified a neutral variant in linkage and might not have a direct effect on the disease or the SNP is associated with other molecular pathways (7). Another potential cause is that cells can produce natural inhibitors that compromise enzyme activity (8). Lastly, a decrease in SPPL2a activity might be caused by post-translational modifications. For example, the N-terminal site of SPPL2a contains multiple N-glycan sites, which are important for proper protein folding and enzyme function (9).

The canonical role of CD74 in the MHC class II-associated antigen presentation pathway is relatively established (10). However, the role of CD74 in modulating endosome morphology and function in the context of antigen

presentation is incompletely understood. In this chapter, we found that a defect in SPPL2a leads to enlarged LAMP1+ endosomes and, upon inflammation, to restoration of enlarged LAMP1+endosomes back to normal size. In addition, changes in endosomal size upon inflammation coincided with increased full-length CD74, CD74-p8, the proteolytic cleavage product of SPPL2a, and HLA class II molecules at the cell surface. The exact relationship between endosomal size and increase in molecule display at the cell surface is uncertain, but might be due to an aberrant delivery/retrieval of molecules to/from the multivesicular bodies (MVBs).
Upon a maturation signal, CD74-MHC class II complexes traffic via the trans-Golgi network to MHC class II compartments (MIIC), which comprise of late endosomes and/or lysosomes(11). MIIC can mature into true MVBs and the transition from MVBs to tubular endolysosomes is required to allow efficient trafficking of peptide-loaded MHC to the plasma membrane for presentation and allow inspection by appropriately MHC-restricted T cells. In the absence of an appropriate trigger, MIIC do not migrate towards the cell surface (11). MVBs harbouring MHC class II and CD74 fragments can either fuse directly with the plasma membrane or indirectly by budding and fission of limiting membrane of MVB followed by fusion with the plasma membrane (11, 12). Considering that we observed compressed LAMP1+ endosomes in IFNγ-treated SPPL2a KO cells, it is more likely that we detected LAMP1+ endosomes that were derived as a result of budding and fission from MVBs. While we were the first to show a role for SPPL2a in regulation of the endosomal versus cell surface presence of CD74, CD74-p8 and HLA Class II molecules, the role of SPPL2a and/or the accumulation of CD74 NTFs on endosomal modulation remains to be determined.

We further demonstrated that anti-CD74 autoantibodies recognized accumulated CD74 fragments in SPPL2a KO lysates. The detected fragments by autoantibodies are larger in size compared to CD74-p8. This observation confirms previous findings that anti-CD74 autoantibodies recognize CD74 fragments that contain the MHC class II associated peptide (CLIP) domain (13, 14), as CD74-p8 lack the CLIP domain (15). Antibodies recognize antigens that are present at the cell surface and initiate the clearance of pathogens or (diseased) cells via complement activation or opsonisation (16). We showed that CD74-p8 accumulate on the cell surface upon an inflammatory trigger using density-gradient centrifugation followed by Western blotting using antibody to CD74. As an attempt to address the question if larger fragments can also accumulate at the plasma membrane, we deployed a different approach, involving the use of membrane permeable and impermeable biotin labelling techniques followed by immunoprecipitation analysis. Larger CD74 fragments on Western blot were detected in the lysate that was labelled with cell-impermeable biotin (data not shown). However, the lysate also contained RAB7 protein, which is mainly described to be present intracellularly (17).

A limitation of our study is therefore that we did not provide unequivocal proof that larger fragments can also accumulate on the plasma membrane. In future studies, other techniques, such as liquid chromatography-mass spectrometry, need to be assessed to evaluate if larger CD74 fragments can indeed be found on the plasma membrane.

A critical question is whether impaired SPPL2a enzyme activity and the sequential accumulation of CD74 fragments is a cause or coincidence of anti-CD74 autoantibody formation in AS. The presence of circulating autoantibodies reflects breach of tolerance of central and/or peripheral tolerance mechanisms in AS. CD74 fragments are normally hidden from the immune system (reminiscent of viral evasion of immune reactivity) and the release of these cryptic antigens in SPPL2a-impaired cells might activate autoreactive T and B cells. However, experimental autoimmune models have shown that both Freund's adjuvant and microbes are required to induce an immune response towards self-antigens (18). This perspective has also been incorporated in the "danger model", proposed by Polly Matzinger. She argues that the immune system is trained to respond to danger/alarm signals from injured tissue rather than to self/non-self discrimination (19). Indeed, a correlate between autoantibody formation and danger signal release is also found in patients with brain trauma or myocardial infarction, and patients who had surgery on the heart (20, 21). So, the initiation of an immune response towards CD74 fragments requires additional inflammatory signals and the newly exposed cryptic antigen is not sufficient itself. It is considered that biomechanical trauma provokes a pathogenic inflammatory response in AS. Biomechanical trauma as the inflammatory trigger is less likely, since biomechanical stress does not lead to increased autoantibody formation, as reflected by unchanged autoantibody titres with or without mechanical loading in collagen-antibody induced arthritis mice (22). Thus, it remains to be determined which pathogenic triggers induce danger signals and stimulate the production of anti-CD74 autoantibodies in AS.

We further queried whether (auto)antigen-reactive T cells are present in patients with SpA. In **chapter 4** of this thesis, shared T cell receptors (TCR) were identified in patients with PsA. In this experimental design, we established a "clean" cell culture model in which ex vivo monocyte-derived dendritic cells (moDCs) from patients with PsA were used as an (auto)antigen source (23-25). moDCs were cultured with CD3+ T cells to expand antigen-reactive CD8+ T cells. We used CD25 and CD137 as enrichment markers for CD8+ T cells that were activated in the moDC-T cell co-culture system. In PsA patients, we observed increased expansion of CD25+CD137+CD8+ T cells compared to HCs. The expansion of CD25+CD137+CD8+ T cells can be explained by the presence of antigens that are sequestered in the antigenic cargo of moDCs from PsA patients, which are sampled from the tissue microenvironment. moDCs cross-present these antigens as part of

their antigenic cargo via peptide/HLA-class I complexes to CD8+ T cells. As described above, the outcome of antigen presentation by DCs is dependent on the presence of danger signals. Previous studies reported that compartmentalization of danger signals together with self-antigens in phagosomes control the generation of TCR ligands and makes them immunogenic (26, 27). The observation that moDCs from almost half of the patients with PsA did not stimulate expansion of CD25+CD137+CD8+ T cells might be due to the absence of danger signals and/or antigens in the antigenic cargo of monocytes. Interestingly, disease modifying anti-rheumatic drugs (DMARD)-treated PsA patients show increased percentage of CD25+CD137+CD8+ T cells compared to naive patients. This suggests that DMARD do not completely block the antigen presentation pathway. Indeed, 75% of PsA patients who were in remission and discontinued DMARD treatment showed relapse within two months and the severity of disease increased the likelihood of disease recurrence (28). These culture-based data support that pathogenic immune cells might perpetuate pathology while still present at sites of inflammation, which corroborates the observation that residual populations of T cells are present in clinically resolved synovial tissue lesions (29). Compared to DMARD-naive patients with active joint disease, DMARD-treated patients with PsA revealed oligoclonality of the human T cell repertoire in resolved synovial tissue (29). Also residual populations of T cells were found in psoriatic skin in patients with psoriasis, supporting a possibility that also in psoriasis local T cells contribute to the pathological sequelae (30).

CD25+CD137+CD8+ T cells from four patients with PsA were sorted and sequenced for their TCRβ repertoire. Furthermore, CD8+ T cells from two psoriatic skin biopsies were sequenced. Although the sample size is modest in this study, we believe that it is encouraging that two shared TCRs were found, as proxy for clonally expanded T cells, that we succeeded to trace back in psoriatic lesional skin. Of course, the relevance of these findings remains to be confirmed and expanded, by determining whether these TCR sequences can be repeatedly found in multiple patients diagnosed with PsA. Another limitation of this study is the lack of data on the TCR repertoire under baseline conditions. We therefore cannot say if the identified TCRs are preferentially found at baseline in some patients. However, the observation that unstimulated CD3+ T cells did not express CD137 or CD25 following 5 days in vitro culture, supports our proposition that expansion of these particular TCR-bearing T cells required the encounter of a moDC-derived antigenic stimulus.

Further studies should be conducted to understand which (auto)antigens or cells that express these (auto)antigens are targeted by these TCRs. An unbiased assay has been described that identifies T cell antigens that are recognized by CD8+ T cells (31). The identification of the T cell target antigen gives insight into which cells are attacked by the pathogenic CD8+ T cell and, in the context of PsA, whether the antigen is shared between skin and joints.

This knowledge might contribute to the understanding why certain patients with psoriasis develop PsA. Overall, **chapter 4** revealed evidence that shows the existence of an antigen-specific component in PsA.

We also investigated whether patients with PsA have a distinct abundant population of innate lymphoid cells (ILCs) in peripheral blood and synovial fluid. ILCs are primarily tissue-resident cells, present at barrier sites and exhibit heterogeneous phenotypes across different tissues (32, 33). Because of their strategic locations, ILCs rapidly react to pathogens or danger signals by producing cytokines. Three subtypes of ILCs have been grouped into various entities: ILC1, ILC2 and ILC3 which produce similar, but not exclusively, cytokines to those produced by T helper cell subsets Th1, Th2 and Th17/22, respectively (32, 34). In **chapter 5** of this thesis, we identified ILC3s in synovial fluid of patients with PsA. Compared to RA, PsA showed increased frequencies of ILC3s that were double-positive for CCR6 and NKp44 in synovial fluid. Furthermore, upon phorbol myristate acetate (PMA)/ionomycin stimulation, NKp44+CCR6+ ILC3 cells produced IL-17. Both the presence of NKp44+ILC3 and IL-17-secreting ILC3 in inflamed tissue have been reported in synovial tissue from SpA patients, psoriatic skin from patients with psoriasis, and in bone marrow, synovial fluid and ileum from patients with AS (35-39). It should be noted that only a small percentage of ILC3s express IL-17 in SpA, which is also corroborated by others (37, 39, 40). ILC3s are not the main source for IL-17. However, IL-22 and GM-CSF are predominantly expressed in synovial tissue-derived ILC3s in SpA (39, 40).
There is growing evidence that T cells can produce multiple cytokines simultaneously (39, 41, 42). For example, polyfunctional CD4+ T cells derived from synovial tissue express GM-CSF+TNFα+IL-17A+ or/IFN-γ+, which positively associates with disease activity in PsA (42). Furthermore, polyfunctional T cells are more abundant at inflammatory sites and are associated with the pathogenicity of disease (39, 43). ILCs mirror T cell function and phenotype and it is tempting to speculate that ILCs can also produce multiple cytokines simultaneously. Interestingly, the combined activation of NKp44 receptor and cytokine receptors acts synergistically to induce polyfunctional IL-22+/GM-CSF+or/TNFα ILC3 (44). It would be interesting to further explore whether ILCs also associate with polyfunctional cytokine expression and whether the tissue microenvironment determines the cytokine polyfunctionality in ILCs.

The question remains: Do ILC3s play a pro-inflammatory or an anti-inflammatory role in SpA? ILCs are scarce in normal tissue and the frequencies increases in inflamed tissue (45). In situ mapping revealed that ILC3s are in close proximity with T cells and can mature into antigen presenting cells that can shape T cell immune responses, as evidenced by experiments using mice and human material (45-48). ILC3s activate or suppress T cells. For example, in contrast to the gut, where ILC3s are efficient T cell suppressors, the spleen

microenvironment activates the antigen-presenting capacity of ILC3s (46, 49). As such, the tissue inflammatory milieu can orchestrate the functional plasticity of ILC3s, which determines the fate of local T cell responses with potential implications for SpA. The effect of the entheseal inflammatory milieu in synovial joints on ILC3 antigen presentation capacity remains to be determined in SpA.

In the context of the disease, how do autoantibodies, T cells and ILC3s contribute to the pathogenesis of SpA? Enthesopathy is a key manifestation in SpA. However, one of the unsolved challenges of the pathology of SpA is its tendency to wax and wane in parallel with skin and gut lesions (50). Except for the joints, extra-articular sites usually heal without any evidence of residual sequelae. The joints probably suffer as a result of their distinct anatomical structure in having no blood vessels and lymphatics (51). Consequently, enthesis has a limited natural healing capacity and are susceptible for progressive calcification and mineralized scaring (enthesophytes and syndesmophytes) (52).
The lack of vessels grants the enthesis an unique immune-privileged status (38, 53, 54). In contrast to immune tolerance, which is systemically regulated by regulatory cells, immune privilege is a local phenomenon, acting locally to prevent overt inflammation and attenuate collateral damage so that non-renewable tissue remains protected. Immune privilege in the enthesis is maintained via distinct mechanisms. Firstly, diffusion of antibodies to entheseal site is obstructed due to increased interstitial hydrostatic pressure(55). Secondly, healthy entheses have minimal numbers of antigen presenting myeloid cells, which are less responsive to pathogen-associated molecular patterns (PAMPs) compared to myeloid cells present in the peri-enthesis (53). Thirdly, entheses harbour tissue-resident cells, including ILC3 and γδ T cells, which might play a role in controlling inflammation and wound healing (38, 54). A break of privilege by an unknown factor is essential to induce a vicious cycle of inflammation causing neoangio- and neolymphogenesis and sequential influx of inflammatory immune cells in entheseal tissue. Indeed, immature, tortuous and obliterated blood vessels with disorganized branching patterns in the enthesis, peri-enthesis and synovium are characteristic for SpA (56-58). These sprouting vessels within the enthesis, peri-enthesis and synovium are connected with bone marrow via cortical vessel channels and are an anatomical location for bone damage (59, 60). Interestingly the bone marrow part of the enthesis has the highest frequency of immune infiltrate in patients with longstanding disease (61). Accordingly, the cortical vessels present between bone marrow and (peri-)enthesis fuel the soft bone tissue with inflammatory T cell, B cell, plasma cell and innate immune cells. A vicious cycle of inflammation and entheseal tissue healing with progressive scarring occurs. Whether the inflammation start in the bone marrow and spreads to the peri-entheseal site or the other way around needs to be examined. Also the question why lesions in SpA come and go in the disease course needs further investigation.

When considering the potential clinical implications of the results presented in this thesis, it should be understood why there appears to be a dichotomy in response. For example, we showed in **chapter 3** that patients with AS can either have low accumulation of CD74-p8 or high accumulation of CD74-p8 in monocytes. We also observed that only half of the patients with PsA have CD8+ T cells in the circulation that expanded in the moDC-T cell co-culture system (**chapter 4** of this thesis). It remains to be determined whether the observed dichotomy represents disease heterogeneity, with some cases being autoinflammatory or autoimmune in origin, or represents identical etiopathogenic mechanism but involves distinct molecular drivers that leads to different SpA subtypes. These differences have consequences for efforts towards personalized medicine. However, evidence presented in this thesis supports that SpA have an autoimmune component and unearth new opportunities to treat SpA. The use of pharmacological means to disrupt adaptive immune signalling might be effective. Indeed, abatacept treatment, a CD80/CD86 co-stimulation modulator, have proven beneficial in the treatment of PsA (62) and interference with antigen presentation pathways might be a new therapeutic strategy in SpA. However, the disadvantage of targeting general immunity increases the risk for infection (63). A more specific strategy includes the delivery of SpA relevant peptide-MHC class I complexes to induce dominant T- and B-cell tolerance. However, this approach might only have beneficial therapeutic efficacy in early disease (possibly even before clinical manifestations), as the development of autoimmune diseases coincides with epitope spread that results from chronic tissue damage (64). An alternative approach would be to target cells that initiate the inflammatory response in SpA. Cells can die via different pathways, and each pathway unequally guide the immune response and tissue micro-environment (65, 66). For example, the mechanisms whereby tumours hijack cell death pathways to restrict pro-inflammatory responses can be used as pharmacological targets, so that therapies causes a response that mimics tumour defence mechanisms (65, 66). This might cause dying cells to release tolerogenic molecules that inhibit an immune response and restore tissue homeostasis.

Progress in science is a long-winded affair and dissension in science can be very conservative once ideas are rooted in researcher's minds. My PhD research and the resulting thesis has led me to argue that adaptive immunity plays a role in the pathogenesis of SpA. However, the presented studies did not answer the question whether aberrant activation of adaptive immunity is a cause or corollary of SpA. At this stage neither of the proposed hypotheses can be excluded, although new insights were yielded. As techniques become more advanced, more data will be gathered to fill up the gaps in our understanding of the development of SpA and renders chosen hypotheses more or less probably. And maybe, as was the case for the celluralist-humoralist battle, new evidence, possibly also what is provided in this thesis, will form the bridging gap between the autoimmune and autoinflammatory hypotheses.

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6

Epilogue

Nederlandse samenvatting
Dankwoord
Curriculum vitae

Nederlandse samenvatting

Spondyloartritis (SpA) is een verzamelnaam voor een groep reumatische aandoeningen die wordt onderverdeeld in axiale en perifere SpA. Axiale SpA wordt gekenmerkt met ontstekingen van de gewrichten van het axiale skelet, terwijl de ontsteking bij perifere SpA meer in de perifere gewrichten zit. De meest voorkomende vorm van axiale SpA is de ziekte van Bechterew (spondylitis ankylopoëtica (SA)) en van perifere SpA artritis psoriatica (PsA). De ontsteking bij de ziekte van SpA is gelokaliseerd in de aanhechting van pezen en ligamenten aan bot (enthesis) en omliggende structuren van de enthesis ("enthesis orgaan"). Naast gewrichtsontstekingen kan SpA gepaard gaan met extra-articulaire symptomen, waaronder psoriasis, uveitis en inflammatoire darmziekten. Ondanks dat het SpA-spectrum verschillende klinische symptomen vertoont, is er een overlap in de immunopathologische mechanismen. Ten eerste is SpA geassocieerd met HLA klasse I eiwitten. Bijvoorbeeld, 85% van de patiënten met SA zijn drager van het HLA-B27 eiwit. Ten tweede speelt de cytokine interleukine (IL)-17 een belangrijke rol in de pathogenese van SpA. Ten derde zijn de klinische verschijnselen gelokaliseerd op plekken die hoge fysieke belasting verdragen zoals enthesis, of continue blootgesteld worden aan het externe milieu (huid en darm).

De etiologie van SpA is echter onbekend en verschillende hypotheses zijn opgesteld die de mogelijke oorzaak van de ziekte kunnen verklaren. De eerste hypothese is gebaseerd op een ongecontroleerde ontstekingsreactie (auto- inflammatoire ziekte) in de entheses en geassocieerde anatomische structuren in reactie op biomechanische stress en/of afwijkende HLA-B27 eiwit op het celoppervlak. De ongecontroleerde inflammatie wordt gedreven door immuun cellen van het aangeboren immuunsysteem (zoals monocyten en macrofagen) en niet door cellen van het verworven immuun system (T- en B cellen). De tweede hypothese is gebaseerd op de gebruikelijke functie van HLA klasse I eiwitten, namelijk het presenteren van peptiden door antigeen presenterende cellen (APCs) aan T- en B cellen. Het immuunsysteem reageert op lichaamseigen antigenen (auto-immuun ziekte) die onder andere aanwezig zijn in het gewricht en veroorzaakt een vicieuze ontstekingsreactie die onder andere gemedieerd wordt door T- en B cellen.

Het doel van mijn promotieonderzoek, en daarmee dit proefschrift, is de kennis over de immunopathogenese van SpA te vergroten en met name de betrokkenheid van de verworven immuniteit in de ziekte te onderzoeken.
In **hoofdstuk 1** introduceer ik het onderwerp en beschrijf ik de wetenschappelijke studies die de verschillende hypotheses van het ontstaan van SpA onderbouwen.
Hoofdstuk 2 is een literatuurstudie over verschillende aspecten van autoinflammatoire en auto-immuunziekte. De auto-immuunziekten reumatoïde artritis (RA) en systemische lupus erythematodus (SLE) worden vergeleken met monogenetische auto-inflammatoire aandoeningen en de verschillen tussen

de ziekten worden op moleculair niveau beschreven. Daarnaast wordt de mogelijke oorsprong van antigenen besproken die autoimmuunreacties kunnen opwekken.
In **hoofdstuk 3** hebben we naar de functie van het enzym Signal Peptide Peptidase-Like 2a (SPPL2a) in CD14+ monocyten gekeken in relatie tot de ziekte SA. Voorafgaand onderzoek had aangetoond dat anti-CD74 autoantilichamen aanwezig zijn in SA. Deze autoantilichamen herkennen het class II-associated li chain peptide (CLIP) wat zich bevindt in het CD74 eiwit. CD74 speelt een belangrijk rol in de HLA klasse II presentatie route en het coördineren van de endosomale route. SPPL2a knipt het gedeelte van CD74 dat zich in het membraan bevindt (CD74-p8). In dit onderzoek hebben we geobserveerd dat SPPL2a niet optimaal functioneert in monocyten van sommige patiënten met SA vergeleken met monocyten van gezonden mensen en patiënten met PsA en RA. In dit onderzoek is er gebruik gemaakt van de monocyten cellijn THP1. Met behulp van CRISPR-Cas9 techniek is SPPL2a uit de cel verwijderd (SPPL2a knock-out (KO)). Een verhoogde cel oppervlakte expressie van HLA-DR en CD74 was gemeten in IFNγ-gestimuleerde SPPL2a KO cellen. Daarnaast kon ook het substraat van SPPL2a, CD74-p8, op het oppervlak gedetecteerd worden. De verhoogde expressie van HLA-DR en CD74 is ook bevestigd in niet-gestimuleerde monocyten van patiënten met SA. Ook zagen we dat LAMP1+ endosomen verkleinen in reactie op IFNγ en uiteindelijk dezelfde grootte weer aannamen als niet gestimuleerde THP-1 cellen. Gezien de verhoogde expressie van CD74 fragmenten en HLA klasse II eiwitten op het celoppervlak, vroegen we ons af of anti-CD74 autoantilichamen afbraakproducten herkennen die ophopen in SPPL2a KO cellen. IgG antilichamen die aanwezig waren in sera van patiënten met SA herkenden CD74 afbraakproducten. Deze resultaten geven een verband weer tussen een verslechterde SPPL2a functie en de aanwezigheid van anti-CD74 autoantilichamen in patiënten met SA.
In **hoofdstuk 4** onderzochten we of patiënten met PsA autoreactieve CD8+ T cellen hebben in hun lichaam. Voorgaande studies hebben laten zien dat monocyten monsters nemen van ontstoken weefsel en deze met zich mee dragen naar de circulatie. Monocyten die differentiëren naar dendritische cellen (moDCs) hebben het vermogen om T cellen te activeren via presentatie van HLA klasse/peptide complex op het celoppervlak. Deze kennis hebben we omgezet in een in vitro experiment. In dit experiment hebben we autologe CD3+ T cellen en monocyten geïsoleerd uit bloed. Monocyten zijn gedifferentieerd tot moDCs en samen met CD3+ T cellen in kweek gezet. In dit experiment was een expansie van geactiveerde CD8+ T cellen gemeten in de co-culturen van PsA. Deze populatie was gesorteerd en geanalyseerd betreffende hun T cel receptor beta (TCRβ) gebruik. Iedere T cel draagt vele kopieën van één TCR variant en herkent specifieke HLA klasse/peptide complexen. Het TCR repertoire van geëxpandeerde CD8+ T cellen werden vergeleken met het TCR repertoire van CD8+ T cellen die aanwezig waren in psoriasis laesies (aangedane huid) van patiënten met PsA. De TCRs die

in frequenties het meeste voorkwamen in het in vitro experiment (wat wijst op geëxpandeerde CD8+ T cellen) werden ook terug gevonden in de huid van een andere patiënt met PsA. Dit experiment geeft onderbouwing dat de gevonden CD8+ T cellen een antigen herkennen in huid en mogelijk bedraagt aan de ontstekingsreactie in PsA.
In **hoofdstuk 5** hebben we gekeken naar de aanwezigheid van aangeboren lymphoide cellen (ILCs) in synoviaal vloeistof van patiënten met PsA. ILCs zijn verwant aan T cellen. In tegenstelling tot T cellen, dragen ze geen TCRs op hun celoppervlak, maar reageren ze snel op een trigger via de productie van cytokines. In dit onderzoek zagen we een verhoogd percentage ILC3s in synoviaal vloeistof in vergelijking met RA. Daarnaast waren de ILC3s positief voor de receptoren NKp44 en CCR6. Deze populatie produceerde IL-17.
Tot slot wordt in **hoofdstuk 6** de inhoud van het proefschrift bediscussieerd. In de discussie worden de onderzoeken geplaatst in de context van de bevindingen uit literatuuronderzoek en tegelijkertijd worden voorstellen voor vervolgonderzoek gedaan.

De onderzoeken die beschreven zijn in dit proefschrift wijzen erop dat het verworven immuunsysteem bijdraagt aan het ziekteproces van SpA en dat SpA mogelijk een auto-immuun component heeft. Dit is informatie die eerder nog niet bekend was. Verder onderzoek is nu nodig om te bepalen of SpA daadwerkelijk wordt veroorzaakt door autoantigen herkenning. De kennis van de immunologische mechanismen die ten grondslag liggen aan het ziekteproces van SpA zal uiteindelijk leiden tot een beter begrip van het ziektebeeld en kunnen mogelijk gebruikt worden voor het verbeteren van de behandelingen.

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Yeah! Het einde is in zicht. Dit is het allerlaatste hoofdstuk wat ik ga schrijven voor mijn proefschrift en dan is het af! Dit zou niet gelukt zijn zonder alle hulp van collega's, vrienden en familie. Ik wil hierbij iedereen bedanken die mijn promotietijd tot een onvergetelijke ervaring hebben gemaakt.

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Curriculum vitae

Tessa Stephanie Van Kempen graduated secondary school from Rythovius college in Eersel, The Netherlands. She earned her BSc in Biology and Medical Laboratory Sciences at Avans University of Applied Sciences, Breda, The Netherlands. During her bachelor, she performed an internship working on the symbiotic biochemical interactions between the animal host and its intracellular algae in the lab of Dr. Adrienne Grant, University of Sydney, Australia. She performed another internship working on the effect of hepatitis B virus on the phenotype and function of monocyte-derived dendritic cells in the lab of Dr. Andrea Woltman, Erasmus Medical Center, Rotterdam, The Netherlands. After completing her undergraduate study, she earned her MSc in Biomedical Sciences at Leiden University, Leiden, The Netherlands. During her master, she worked on the development of an in vitro model to study indirect antigen presentation after organ transplantation in the lab of Prof. Dr. Cees van Kooten, Leiden University Medical Center, Leiden, The Netherlands. She performed an additional internship working on the effect of fluctuations in oxygenation on trophoblast function in the lab of Prof. Dr. Graham Burton, University of Cambridge, Cambridge, United Kingdom. After completing her Master's degree, she worked on the role of NADPH oxidases in cardiac disease and ageing in the lab of Prof. Dr. Harald Schmidt, University of Maastricht, Maastricht, The Netherlands. She completed her graduate studies with a PhD in Translational Immunology working on the immunopathogenesis of spondyloarthritis in the lab of Prof. Dr. Timothy Radstake and Dr. Marianne Boes at University Medical Center Utrecht, Utrecht, The Netherlands. She next performed a Post-Doctoral Fellowship in the group of Prof. Dr. Hendrik Stunnenberg working on the transcriptome of monocytes from patients with sepsis at the Radboud University, Nijmegen, The Netherlands. She is currently a lecturer for the Bachelor of Nursing at the Fontys University of Applied Sciences, Eindhoven, The Netherlands, where she teaches anatomy and physiology.

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