TSLP-driven activation of CD1c myeloid dendritic cells and T cell activity in rheumatoid arthritis

Fréderique Marie Moret

This research was performed within the framework of CTMM, the Center for Translational Molecular Medicine (www.ctmm.nl), and the Dutch Arthritis Foundation, project TRACER (grant 04I-202).

Printing of this thesis was financially supported by CTMM, the Center for Translational Molecular Medicine (www.ctmm.nl), and the Dutch Arthritis Foundation, project TRACER (grant 04I-202) and Infection & Immunity Utrecht.

Cover design by Thamara van Meeteren
Lay-out by Marieke Vianen-van den Bosch
Printed by Ipskamps Drukkers

ISBN: 978-94-6259-147-9

TSLP-driven activation of CD1c myeloid dendritic cells and T cell activity in rheumatoid arthritis

TSLP-gedreven activatie van CD1c myeloïde dendritische cellen en T cel activiteit in reumatoïde artritis
(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op donderdag
8 mei 2014 des middags te 12.45 uur

door

Fréderique Marie Moret

geboren op 11 juni 1985 te Tiel

Promotoren: Prof. dr. F.P.J.G. Lafeber
Prof. dr. J.W.J. Bijlsma

Copromotor: Dr. J.A.G. van Roon

CONTENTS

1

Introduction

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disorder characterized by chronic synovial inflammation and swelling of the joints resulting in joint damage that leads to joint deformities and dysfunction. Although the aetiology of RA remains unknown, genetic as well as environmental factors contribute to the development of RA (1).

In around 70% of the RA patients autoantibodies can be detected against citrullinated proteins, anti-citrullinated protein antibody (ACPA), and against the Fc portions of immunoglobulin (rheumatoid factor (RF)) (2, 3). Already many years prior to the onset of RA these antibodies can appear in the serum. The formation of these antibodies might be triggered by environmental factors, like smoking, infectious agents (e.g. Epstein-Barr virus and bacteria like porphyromonas gingivalis), or even the gastrointestinal microbiome, in a certain genetic context (1). RA patients, positive for ACPA have a less favorable prognosis and tend to have a more aggressive disease course compared to ACPA-negative RA patients (1, 2). An interaction effect for developing ACPA-positive or RF-positive RA was previously observed between human leukocyte antigen (HLA)-DRB1 shared epitope (SE) alleles and smoking, showing that smokers carrying single or double SE alleles display an increased risk for developing ACPA-positive or RF-positive RA, with the highest risk for carriers of double SE alleles (4, 5). The genetic risk factors for ACPA-negative RA also seem to be as important as those for ACPA-positive RA. However, these factors are less well established and appear to involve different genes (1, 2), complicating our understanding of these factors to the development of ACPA-negative RA.

T cells

Multiple genes are associated with genetic predisposition to RA, with in particular the HLA locus accounting for 30% to 50% of the overall genetic susceptibility to RA (1, 2). The HLA locus encodes for the HLA molecules that play an important role in the induction of T cell tolerance and T cell immunity by the presentation of self-antigens and nonself-antigens, respectively. The loss of tolerance to self-peptides, like the development of ACPA, can lead to autoimmunity (2).

HLA molecules display a remarkable degree of polymorphism (6). RA is strongest associated with HLA-DR genes encoding HLA class II molecules that present antigens to CD4 T cells (1, 2, 7). In particular *HLA-DRB1* SE alleles are associated with RA susceptibility and are proposed to induce autoimmunity by the selection of potential autoreactive arthritogenic T cells in the thymus, the presentation of arthritogenic peptides to those autoreactive T cells in the periphery, and the failure to generate appropriate regulatory T cells to control T cell activation (2).

Several other genes (among others *PTPN22, AFF3, STAT4*) associated with RA susceptibility are involved in T cell biology and can impact T cell activation through effects

on T cell selection, antigen presentation, threshold for T cell activation, and T cell differentiation into T-helper type (Th)-1 and Th17 cells (1, 2). Autoreactive CD4 T cells producing Th1 and Th17 cytokines (interferon gamma (IFNγ) and interleukin (IL)-17, respectively) are indicated to importantly contribute to the initiation and perpetuation of immune responses in RA (8-11).

Large numbers of CD4 T cells as well as B cells and myeloid cells (macrophages and dendritic cells) are present in inflamed joints of RA patients and their activity have been indicated to correlate with clinical symptoms (8, 12-15). The development of therapeutic agents based on the biologic characteristics of these cells and cell activities has made enormous progress in the reduction of RA-related clinical symptoms. Among others, T cell activity is targeted by CTLA4-Ig (abatacept), B cells are targeted by anti-CD20 (rituximab), and tumour necrosis factor alpha (TNFα) production by myeloid cells is targeted by anti-TNFα (e.g. adalimumab) (13, 16, 17).

Among the most potent activators of CD4 T cells are the classical or myeloid dendritic cells (mDCs). Despite the fact that mDCs have been extensively studied in immune disorders in mice and man and that they have been suggested to play an important role in the pathogenesis of RA (14), functional data on naturally occurring mDCs in RA are scarce. In this thesis the capacities of one of the subtypes of mDCs, the naturally occurring CD1c mDCs, have been subject of study.

Naturally occurring CD1c myeloid dendritic cells

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that instruct T cells to develop a proper immune response during infections by uptake and presentation of antigens and the delivery of costimulatory signals and cytokines. Additionally, DCs have the ability to instruct T cells to induce self-tolerance by presenting self-antigens to T cells and induce deletion or inactivation (anergy) of self-reactive T cells in the periphery (18). In addition to HLA gene predisposition (7), in the periphery external agents such as cytokines, tissue-derived factors, pathogen-derived antigens and organic molecules may alter the balance between tolerogenic and immunogenic functions of DCs and may initiate autoimmune disease (19, 20).

Several types of DCs are described in human blood characterized by high expression of HLA class II molecules and the absence of lineage markers (CD3, CD19, CD14, CD20, CD56 and glycophorin A). Human blood DCs can be divided into at least 3 subtypes, plasmacytoid dendritic cells and two types of myeloid or classical dendritic cells (mDC1 and mDC2) (21, 22), based on the expression of blood-derived dendritic cell antigen (BDCA)-molecules (23, 24). BDCA-1 (CD1c) identifies the mDC1 subset, which are potent activators of CD4 T cells, whereas mDC2 cells, identified by expression of BDCA-3 (CD141), more potently activate CD8 T cells (22-24). In this thesis, mDC1 cells were studied because of their strong potential to activate CD4 T cells. In this respect, it is important to notice that the characterisation of mDC1

cells by CD1c is more specific than the previously used identification and more broadly expressed marker CD11c (22, 23).

CD1c is an antigen-presenting molecule related to the major histocompatibility complex (MHC) or HLA molecules. In contrast to HLA molecules presenting peptides, CD1c is a cell surface glycoprotein that presents lipid and glycolipid, self-antigens and nonself-antigens and restricts responses to lipid antigen-specific T cells in a HLA-unrestricted manner (25). However, CD1c mDCs also have a strong capacity to induce HLA-dependent antigen-driven allogeneic mixed lymphocyte reactions (26). Human circulating CD1c mDCs have been suggested to represent immature DCs that express CD86 and respond rather to microbial products than to inflammatory stimuli like TNFα (27). Recently, CD1c mDCs were described to have an immunoregulatory function in response to certain microbial triggers (28, 29).

Previous studies on mDCs in RA were based on CD33/CD14 expression (30) describing a larger mDC population as the recently defined CD1c mDCs, since CD33 is not only expressed on CD1c mDCs, but also on $CD16^+$ and BDCA-3^+ DC subpopulations (26). Only a small percentage of CD1c mDCs express CD14 and the function of these double positive mDCs is still unknown (23). In RA, mDCs are increased in the joints compared with the circulation and express costimulatory molecules (31, 32). However, a detailed analysis of the capacity of *ex vivo* cultured CD1c mDCs from RA patients to produce inflammatory mediators and to activate autologous CD4 T cells has not been performed. In addition, although many triggers of DC activation are described in literature, knowledge on the specific triggers that activate these cells in the inflamed joints of RA patients is limited. Recent data suggest that thymic stromal lymphopoietin (TSLP), an IL-7-related cytokine, may be a pivotal driver of mDC activation in RA joints (33).

Thymic stromal lymphopoietin and mDC activation

TSLP is a member of the IL-2 family and accomplishes its biologic activities via the low affinity TSLP receptor (TSLPR) in conjunction with the IL-7 receptor-alpha chain (IL-7Rα) that is shared with IL-7 (34, 35). Human TSLPR has been shown to be expressed by DCs, monocytes, pre-activated T cells and mast cells (36-38). mDCs express the highest levels of TSLPR and are indicated to be the main target cell population for TSLP (36, 39). In line with this, TSLP does not induce proliferation of mononuclear cells (40), but has been described as a potent trigger for mDCs, identified by the expression of CD11c, to induce Th2-mediated immune responses. TSLP is suggested to be an important proinflammatory cytokine in allergic diseases both in mice and man (39, 41, 42).

TSLP potently activates mDCs to strongly upregulate the expression of antigen-presenting (HLA-DR) and costimulatory molecules (CD40, CD80, and CD86) and to secrete Th2 cell-attracting chemokines including thymus and activation-regulated chemokine (TARC/CCL17) and macrophage-derived chemokine (MDC/CCL22) (39, 43). As a consequence, TSLP-activated mDCs induce strong proliferation of allogeneic naive CD4 T

cells which is associated with the production of Th2 cytokines (IL-4, IL-5, and IL-13) (39). The induction of Th2 activity is demonstrated to be dependent on the expression of OX40 ligand (43).

Furthermore, TSLP has been shown to play a pivotal role in homeostatic T cell expansion (44). Human CD11c mDCs that are activated by TSLP induce a robust expansion of autologous CD4 T cells, which depends on the presentation of self-peptides by HLA class II molecules and costimulation. These CD4 T cells activated by autologous TSLP-activated mDCs mainly produce Th1 cytokines (IL-2, IFNγ, and TNFα) in addition to modest levels of Th2 cytokines (44).

Although TSLP is well known to be an important proinflammatory cytokine in Th2-mediated allergic diseases (39, 41, 42), TSLP is also indicated to be involved in Th1 and Th17-mediated (rheumatoid) arthritis. Although no robust data were provided, increased TSLP levels were observed in synovial fluid (SF) of RA patients compared with osteoarthritis (OA) patients (a joint disease with mild to no inflammation) (45). In addition, RA synovial fibroblasts are shown to produce TSLP upon Toll-like receptor and TNFα triggering (45, 46). Moreover, recently, it is demonstrated that TSLP and its receptor enhance Th17-driven arthritis and tissue destruction in experimental arthritis models (33). Considering the potency of TSLP to induce mDC-driven T cell activation, its activities on mDCs from RA patients were studied in this thesis. In addition to this, effects of IL-7, which directly targets T cells and induces T cell-driven DC activation were studied.

Interleukin-7 and T cell activation

IL-7 is also a member of the IL-2 family and signals through IL-7Rα in conjunction with the common gamma chain (γc) that is shared by receptors for IL-2, IL-4, IL-9, IL-15, and IL-21. The majority of mature T cells express the IL-7Rα, although IL-7Rα is also expressed on immature T and B cells (47, 48) as well as on DCs and activated monocytes (36).

IL-7 is demonstrated to play a prominent role in naïve and memory CD4 T cell development and homeostasis by the provision of signals for proliferation, growth and survival of both developing and mature T cells (47, 49, 50). Apart from the function of IL-7 in naïve and memory CD4 T cell development and homeostasis (49), IL-7 also stimulates several effector functions of T cells as well as other cells of both the innate and adaptive immune system. In the absence of APCs, IL-7 augments survival and proliferation of naïve CD4 T cells (51). In the presence of APCs such as DCs or monocytes/macrophages, IL-7-induced CD4 T cell activation is far more efficient (52). This enhanced CD4 T cell activation is associated with T cell-dependent activation of APCs resulting in upregulation of (co)stimulatory molecules both on CD4 T cells (like lymphocyte function associated antigen (LFA-1) and CD69) and on myeloid cells (CD40, CD80, CD86) (52). Apart from the capacity of IL-7 to upregulate costimulatory molecules on CD4 T cells, IL-7 is also demonstrated to downregulate the programmed death 1 (PD-1) receptor that negatively regulates T cell activation by binding to

its ligands PD-L1 and PD-L2 (53, 54). In addition, IL-7 is shown to overcome PD-1 mediated inhibition of T cells by STAT5 activation that induces T cell activation (55). These mechanisms could all contribute to the enhancement of IL-7 induced T cell activation. Most *in vitro* studies demonstrated that IL-7 primarily induces Th1 and Th17 cytokine secretion with only limited Th2 cytokine secretion (56). IL-7 induced T cell activation is not limited to CD4 T cells, since IL-7 also stimulates cytokine secretion and cytotoxic activity of CD8 T cells (57, 58).

In addition to the effects of IL-7 on T cells and T cell-dependent activation of myeloid cells, IL-7 can also directly activate myeloid cells. However, high, possibly supra-physiologic levels of IL-7 (50-100 ng/ml) are required to induce cytokine secretion by human monocytes (57, 59, 60). This is approximately 100- to 1000-fold higher than what is required for activation of T cells (52, 56, 57) and might be related to the much lower to absent IL-7Rα surface expression on these cells compared with T cells (61).

Strongly elevated IL-7 levels are observed in SF (up to 480 pg/ml) and synovial tissue (ST) biopsies of RA patients compared with OA patients (52). In SF and ST of RA patients the majority of CD4 T cells express the IL-7Rα (40, 56), with a significantly higher IL-7Rα expression in RA ST than in OA ST (40). In addition, a substantial percentage of B cells and macrophages from RA SF and ST also express IL-7Rα, although less prominently than T cells (40). Responder T cells are characterized by high IL-7Rα expression, whereas regulatory T cells that control immune activation largely lack the IL-7Rα (40, 62). This indicates that IL-7 rather may act on responder cells than on regulatory T cells. In line with this, IL-7 is shown to induce a pronounced loss of regulatory T cell mediated suppression of responder T cells (62), despite increased numbers of regulatory T cells in SF with enhanced suppressive activity compared with these cell in the peripheral blood (63, 64). This suggests that IL-7 can significantly contribute to joint inflammation in RA patients by activating predominantly T cells, but also B cells and macrophages. Soluble human IL-7Rα is indicated to inhibit IL-7Rα-mediated immune activation *in vitro* (40).

The role of IL-7 in mediating arthritis is confirmed in experimental mice models showing that IL-7 and its receptor exacerbate arthritis severity and joint destruction, associated with increasing Th1 and Th17 activity (65). Furthermore, blockade of IL-7Rα in these mice potently inhibited joint inflammation and destruction, associated with specific reductions in T cell numbers, T cell-associated cytokines, and inflammatory mediators (66).

Targeting T cell/antigen-presenting cell interactions in RA

Currently, several strategies are applied in rheumatic diseases, including RA, to inhibit T cell activation. These include drugs that target T cell signalling, such as tofacitinib, and those that target the interaction of T cells with APCs, including DCs, such as abatacept. Abatacept (CTLA4-Ig) is a recombinant human fusion protein consisting of the extracellular domain of human CTLA4 combined with a modified Fc region of IgG1. CTLA4 is normally expressed on conventional activated T cells to act as a negative regulator for T cell activation (67). CD4 T

cell activation by APCs is accomplished after T cell receptor (TCR) activation and CD28 ligation by antigen-presentation in HLA class II molecules and CD80/86 costimulation, respectively. CTLA4 and CD28 both interact with CD80/86 molecules on APCs although CTLA4 interacts with a higher affinity and avidity to these ligands. While competition between CTLA4 and CD28 for ligand binding has previously been described as the most essential role for CTLA4, recent data suggested that CTLA4 inhibits T cell activation by mediating trans-endocytosis of CD80 and CD86 thereby inducing T cell anergy (67, 68). Accordingly, the best-described mechanism of action for abatacept is preventing T cell activation by blocking CD80/86 costimulation, although mechanisms independent of CD28 ligation and directly dependent on CD80/86 signaling have been described for CTLA4-Ig as well (69). Several clinical trials have demonstrated the efficacy and safety of abatacept (CTLA4-Ig) for the treatment of RA patients (70-73). However, not all patients benefit from this therapy and the reasons for this are largely unexplained. Baseline numbers of circulating CD28-negative T cells recently were suggested to predict clinical response to abatacept in patients with RA (74). In addition, the number of T cells at the site of inflammation as well as local concentrations of the drug may be influencing its efficacy, although to our knowledge this has not been demonstrated. In this thesis we provide evidence for a mechanism that may be detrimental for abatacept efficacy.

Thesis outline

In view of the previously reported role of TSLP in activation of mDCs and its potent immunostimulatory role in models for experimental arthritis, the **aim of this thesis was to investigate the inflammatory potential of TSLP to activate natural occurring CD1c myeloid dendritic cells and subsequently the regulation of CD4 T cell responses in rheumatoid arthritis (RA) as well as the capacity of T cell targeting therapies to influence TSLP driven immunopathology in RA**.

We hypothesized that TSLP plays an important role in inflamed joints of RA patients by activating CD1c myeloid dendritic cells and subsequently enhancing Th1 and Th17 cell activity in addition to the previously reported induction of Th2 cell activity by TSLP-activated CD11 mDCs. Abatacept is an effective treatment in a considerable number of RA patients and prevents T cell activation by blocking T cell costimulation and is able to prevent TSLP-mDC driven T cell activation. However, we hypothesized that prevention of costimulation by abatacept is less beneficial in an inflammatory environment with high levels of T cell-activating cytokines like IL-7.

To investigate our hypotheses, first we examined the numbers and potential of natural occurring CD1c myeloid dendritic cells (mDCs) from the peripheral blood in comparison with mDCs from the SF of RA patients to secrete inflammatory mediators and to activate

autologous CD4 T cells (**chapter 2**). TSLP is demonstrated to enhance experimental arthritis and is suggested to be present in joints of RA patients. Since TSLP is suggested to primarily target mDCs, we investigated the potential of TSLP to specifically activate CD1c mDCs and to regulate autologous CD4 T cell responses in RA patients (**chapter 3**).

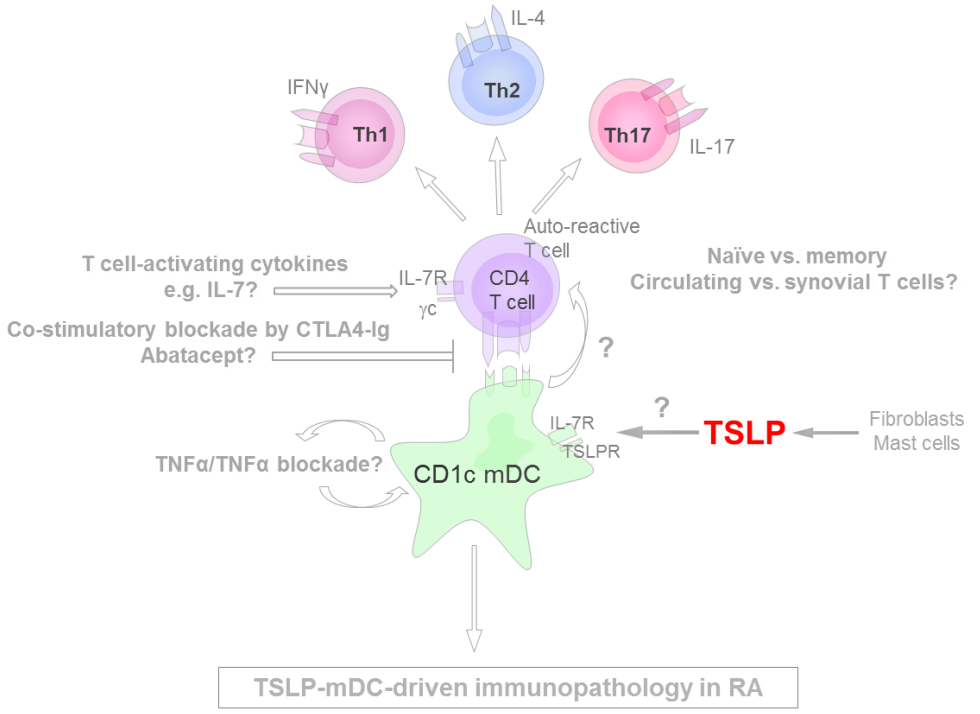
Since Th2 activity is virtually absent in RA joints and TSLP is known to trigger Th2 responses in many conditions, we also tried to define whether certain mediators might counteract Th2 induction induced by TSLP. In **chapter 4** we assessed the capacity of TSLP to regulate TNFα production by natural occurring CD1c mDCs and the effects of TSLP-induced TNFα production on TSLP-mediated mDC and subsequent CD4 T cell activation by blocking TNFα.

Since not all RA patients benefit from abatacept treatment, we investigated whether T cell-activating cytokines like IL-7 could have the potential to overrule abatacept efficacy using an *in vitro* assay of TSLP-activated CD1c mDCs in coculture with CD4 T cells. Since TSLP-activated mDCs are extremely potent T cell activators with high expression levels of CD80 and CD86, these mDCs were considered ideal T cell-activating cells to study abatacept efficacy (**chapter 5**).

In contrast to the strong activation of peripheral blood-derived T cells by TSLP-primed mDCs, SF-derived T cells were hyporesponsive upon TSLP-primed mDC stimulation. In **chapter 6** we investigated the role of PD-1/PD-L interactions in this SF T cell hyporesponsiveness and whether IL-7 was able to overcome this hyporesponsiveness as IL-7 previously was shown to downregulate PD-1 expression on T cells and because of the potent T cell stimulatory capacity of IL-7.

The capacity of IL-7 to mediate inflammation can be reduced by soluble IL7 receptor α (sIL-7Rα) both in mice and man. In **chapter 7** we discussed that levels of the naturally occurring sIL-7Rα are strongly increased in SF of RA patients compared to OA patients and that these levels correlate with markers of inflammation in RA.

In **chapter 8** the results of these studies are summarized and discussed in a broader perspective including implications for future research and therapeutic strategies.



Schema summarizing the investigations performed in this thesis. The numbers and functional properties of CD1 mDCs were studied in the joints of RA patients. In addition, the level and potential of TSLP to activate natural occurring CD1c myeloid dendritic cells (mDC) and subsequently the regulation of CD4 T cell responses in rheumatoid arthritis (RA) were studied. Also, the capacity of T cell targeting (CTLA4-Ig, abatacept) and TNFα blocking drugs to influence TSLP-driven immunopathology were investigated. Finally, the capacity of activated mDCs to stimulate T cells and the effect of T cell-activating cytokines like IL-7 to influence DC-driven immune responses were investigated.

REFERENCES

1. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. N Engl J Med 2011;365(23):2205-19.
2. Imboden JB. The immunopathogenesis of rheumatoid arthritis. Annu Rev Pathol 2009;4:417-34.
3. Nishimura K, Sugiyama D, Kogata Y, Tsuji G, Nakazawa T, Kawano S, et al. Meta-analysis: diagnostic accuracy of anti-cyclic citrullinated peptide antibody and rheumatoid factor for rheumatoid arthritis. Ann Intern Med 2007;146(11):797-808.
4. Lundstrom E, Kallberg H, Alfredsson L, Klareskog L, Padyukov L. Gene-environment interaction between the DRB1 shared epitope and smoking in the risk of anti-citrullinated protein antibody-positive rheumatoid arthritis: all alleles are important. Arthritis Rheum 2009;60(6):1597-603.
5. Padyukov L, Silva C, Stolt P, Alfredsson L, Klareskog L. A gene-environment interaction between smoking and shared epitope genes in HLA-DR provides a high risk of seropositive rheumatoid arthritis. Arthritis Rheum 2004;50(10):3085-92.
6. Trowsdale J, Knight JC. Major histocompatibility complex genomics and human disease. Annu Rev Genomics Hum Genet 2013;14:301-23.
7. Taneja V, David CS. Role of HLA class II genes in susceptibility/resistance to inflammatory arthritis: studies with humanized mice. Immunol Rev 2010;233(1):62-78.
8. Morita Y, Yamamura M, Kawashima M, Harada S, Tsuji K, Shibuya K, et al. Flow cytometric single-cell analysis of cytokine production by CD4+ T cells in synovial tissue and peripheral blood from patients with rheumatoid arthritis. Arthritis Rheum 1998;41(9):1669-76.
9. Chabaud M, Durand JM, Buchs N, Fossiez F, Page G, Frappart L, et al. Human interleukin-17: A T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. Arthritis Rheum 1999;42(5):963-70.
10. Cornelissen F, van Hamburg JP, Lubberts E. The IL-12/IL-23 axis and its role in Th17 cell development, pathology and plasticity in arthritis. Curr Opin Investig Drugs 2009;10(5):452-62.
11. van Roon JA, van Roy JL, Duits A, Lafeber FP, Bijlsma JW. Proinflammatory cytokine production and cartilage damage due to rheumatoid synovial T helper-1 activation is inhibited by interleukin-4. Ann Rheum Dis 1995;54(10):836-40.
12. Namekawa T, Wagner UG, Goronzy JJ, Weyand CM. Functional subsets of CD4 T cells in rheumatoid synovitis. Arthritis Rheum 1998;41(12):2108-16.
13. Edwards JC, Szczepanski L, Szechinski J, Filipowicz-Sosnowska A, Emery P, Close DR, et al. Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. N Engl J Med 2004;350(25):2572-81.
14. Thomas R, MacDonald KP, Pettit AR, Cavanagh LL, Padmanabha J, Zehntner S. Dendritic cells and the pathogenesis of rheumatoid arthritis. J Leukoc Biol 1999;66(2):286-92.
15. Burmester GR, Stuhlmuller B, Keyszer G, Kinne RW. Mononuclear phagocytes and rheumatoid synovitis. Mastermind or workhorse in arthritis? Arthritis Rheum 1997;40(1):5-18.

16. Kremer JM, Westhovens R, Leon M, Di Giorgio E, Alten R, Steinfeld S, et al. Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig. N Engl J Med 2003;349(20):1907-15.

17. Olsen NJ, Stein CM. New drugs for rheumatoid arthritis. N Engl J Med 2004;350(21):2167-79.

18. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392(6673):245-52.

19. Coquerelle C, Moser M. DC subsets in positive and negative regulation of immunity. Immunol Rev 2010;234(1):317-34.

20. Torres-Aguilar H, Blank M, Jara LJ, Shoenfeld Y. Tolerogenic dendritic cells in autoimmune diseases: crucial players in induction and prevention of autoimmunity. Autoimmun Rev 2010;10(1):8-17.

21. Satpathy AT, Wu X, Albring JC, Murphy KM. Re(de)fining the dendritic cell lineage. Nat Immunol 2012;13(12):1145-54.

22. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. Annu Rev Immunol 2013;31:563-604.

23. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of monocytes and dendritic cells in blood. Blood 2010;116(16):e74-80.

24. Dzionek A, Fuchs A, Schmidt P, Cremer S, Zysk M, Miltenyi S, et al. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. J Immunol 2000;165(11):6037-46.

25. Beckman EM, Melian A, Behar SM, Sieling PA, Chatterjee D, Furlong ST, et al. CD1c restricts responses of mycobacteria-specific T cells. Evidence for antigen presentation by a second member of the human CD1 family. J Immunol 1996;157(7):2795-803.

26. MacDonald KP, Munster DJ, Clark GJ, Dzionek A, Schmitz J, Hart DN. Characterization of human blood dendritic cell subsets. Blood 2002;100(13):4512-20.

27. Piccioli D, Tavarini S, Borgogni E, Steri V, Nuti S, Sammicheli C, et al. Functional specialization of human circulating CD16 and CD1c myeloid dendritic-cell subsets. Blood 2007;109(12):5371-9.

28. Kassianos AJ, Hardy MY, Ju X, Vijayan D, Ding Y, Vulink AJ, et al. Human CD1c (BDCA-1)+ myeloid dendritic cells secrete IL-10 and display an immuno-regulatory phenotype and function in response to Escherichia coli. Eur J Immunol 2012;42(6):1512-22.

29. Qian C, Cao X. Naturally occurring CD1c+ human regulatory dendritic cells: immunoregulators that are expanded in response to E. coli infection. Eur J Immunol 2012;42(6):1388-92.

30. Thomas R, Davis LS, Lipsky PE. Rheumatoid synovium is enriched in mature antigen-presenting dendritic cells. J Immunol 1994;152(5):2613-23.

31. Smolewska E, Stanczyk J, Brozik H, Biernacka-Zielinska M, Cebula B, Robak T, et al. Distribution and clinical significance of blood dendritic cells in children with juvenile idiopathic arthritis. Ann Rheum Dis 2008;67(6):762-8.

32. Jongbloed SL, Lebre MC, Fraser AR, Gracie JA, Sturrock RD, Tak PP, et al. Enumeration and phenotypical analysis of distinct dendritic cell subsets in psoriatic arthritis and rheumatoid arthritis. Arthritis Res Ther 2006;8(1):R15.

33. Hartgring SA, Willis CR, Dean CE, Jr., Broere F, van Eden W, Bijlsma JW, et al. Critical proinflammatory role of thymic stromal lymphopoietin and its receptor in experimental autoimmune arthritis. Arthritis Rheum 2011;63(7):1878-87.

34. Pandey A, Ozaki K, Baumann H, Levin SD, Puel A, Farr AG, et al. Cloning of a receptor subunit required for signaling by thymic stromal lymphopoietin. Nat Immunol 2000;1(1):59-64.

35. Park LS, Martin U, Garka K, Gliniak B, Di Santo JP, Muller W, et al. Cloning of the murine thymic stromal lymphopoietin (TSLP) receptor: Formation of a functional heteromeric complex requires interleukin 7 receptor. J Exp Med 2000;192(5):659-70.

36. Reche PA, Soumelis V, Gorman DM, Clifford T, Liu M, Travis M, et al. Human thymic stromal lymphopoietin preferentially stimulates myeloid cells. J Immunol 2001;167(1):336-43.

37. Rochman I, Watanabe N, Arima K, Liu YJ, Leonard WJ. Cutting edge: direct action of thymic stromal lymphopoietin on activated human CD4+ T cells. J Immunol 2007;178(11):6720-4.

38. Allakhverdi Z, Comeau MR, Jessup HK, Yoon BR, Brewer A, Chartier S, et al. Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. J Exp Med 2007;204(2):253-8.

39. Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. Nat Immunol 2002;3(7):673-80.

40. Hartgring SA, van Roon JA, Wenting-van Wijk M, Jacobs KM, Jahangier ZN, Willis CR, et al. Elevated expression of interleukin-7 receptor in inflamed joints mediates interleukin-7-induced immune activation in rheumatoid arthritis. Arthritis Rheum 2009;60(9):2595-605.

41. Al-Shami A, Spolski R, Kelly J, Keane-Myers A, Leonard WJ. A role for TSLP in the development of inflammation in an asthma model. J Exp Med 2005;202(6):829-39.

42. Zhou B, Comeau MR, De Smedt T, Liggitt HD, Dahl ME, Lewis DB, et al. Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. Nat Immunol 2005;6(10):1047-53.

43. Ito T, Wang YH, Duramad O, Hori T, Delespesse GJ, Watanabe N, et al. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. J Exp Med 2005;202(9):1213-23.

44. Watanabe N, Hanabuchi S, Soumelis V, Yuan W, Ho S, de Waal Malefyt R, et al. Human thymic stromal lymphopoietin promotes dendritic cell-mediated CD4+ T cell homeostatic expansion. Nat Immunol 2004;5(4):426-34.

45. Koyama K, Ozawa T, Hatsushika K, Ando T, Takano S, Wako M, et al. A possible role for TSLP in inflammatory arthritis. Biochem Biophys Res Commun 2007;357(1):99-104.

46. Ozawa T, Koyama K, Ando T, Ohnuma Y, Hatsushika K, Ohba T, et al. Thymic stromal lymphopoietin secretion of synovial fibroblasts is positively and negatively regulated by Toll-like receptors/nuclear factor-kappaB pathway and interferon-gamma/dexamethasone. Mod Rheumatol 2007;17(6):459-63.

47. Fry TJ, Mackall CL. Interleukin-7: from bench to clinic. Blood 2002;99(11):3892-904.

48. Armitage RJ, Ziegler SF, Beckmann MP, Idzerda RL, Park LS, Fanslow WC. Expression of receptors for interleukin 4 and interleukin 7 on human T cells. Adv Exp Med Biol 1991;292:121-30.

49. Kondrack RM, Harbertson J, Tan JT, McBreen ME, Surh CD, Bradley LM. Interleukin 7 regulates the survival and generation of memory CD4 cells. J Exp Med 2003;198(12):1797-806.

50. Seddon B, Tomlinson P, Zamoyska R. Interleukin 7 and T cell receptor signals regulate homeostasis of CD4 memory cells. Nat Immunol 2003;4(7):680-6.

51. Webb LM, Foxwell BM, Feldmann M. Putative role for interleukin-7 in the maintenance of the recirculating naive CD4+ T-cell pool. Immunology 1999;98(3):400-5.

52. van Roon JA, Verweij MC, Wijk MW, Jacobs KM, Bijlsma JW, Lafeber FP. Increased intraarticular interleukin-7 in rheumatoid arthritis patients stimulates cell contact-dependent activation of CD4(+) T cells and macrophages. Arthritis Rheum 2005;52(6):1700-10.

53. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 2000;192(7):1027-34.

54. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat Immunol 2001;2(3):261-8.

55. Bennett F, Luxenberg D, Ling V, Wang IM, Marquette K, Lowe D, et al. Program death-1 engagement upon TCR activation has distinct effects on costimulation and cytokine-driven proliferation: attenuation of ICOS, IL-4, and IL-21, but not CD28, IL-7, and IL-15 responses. J Immunol 2003;170(2):711-8.

56. van Roon JA, Glaudemans KA, Bijlsma JW, Lafeber FP. Interleukin 7 stimulates tumour necrosis factor alpha and Th1 cytokine production in joints of patients with rheumatoid arthritis. Ann Rheum Dis 2003;62(2):113-9.

57. Alderson MR, Tough TW, Ziegler SF, Grabstein KH. Interleukin 7 induces cytokine secretion and tumoricidal activity by human peripheral blood monocytes. J Exp Med 1991;173(4):923-30.

58. Mehrotra PT, Grant AJ, Siegel JP. Synergistic effects of IL-7 and IL-12 on human T cell activation. J Immunol 1995;154(10):5093-102.

59. Standiford TJ, Strieter RM, Allen RM, Burdick MD, Kunkel SL. IL-7 up-regulates the expression of IL-8 from resting and stimulated human blood monocytes. J Immunol 1992;149(6):2035-9.

60. Ziegler SF, Tough TW, Franklin TL, Armitage RJ, Alderson MR. Induction of macrophage inflammatory protein-1 beta gene expression in human monocytes by lipopolysaccharide and IL-7. J Immunol 1991;147(7):2234-9.

61. van Roon J, Wijngaarden S, Lafeber FP, Damen C, van de Winkel J, Bijlsma JW. Interleukin 10 treatment of patients with rheumatoid arthritis enhances Fc gamma receptor expression on monocytes and responsiveness to immune complex stimulation. J Rheumatol 2003;30(4):648-51.

62. van Amelsfort JM, van Roon JA, Noordegraaf M, Jacobs KM, Bijlsma JW, Lafeber FP, et al. Proinflammatory mediator-induced reversal of CD4+,CD25+ regulatory T cell-mediated suppression in rheumatoid arthritis. Arthritis Rheum 2007;56(3):732-42.

63. van Amelsfort JM, Jacobs KM, Bijlsma JW, Lafeber FP, Taams LS. CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. Arthritis Rheum 2004;50(9):2775-85.

64. Walter GJ, Evans HG, Menon B, Gullick NJ, Kirkham BW, Cope AP, et al. Interaction with activated monocytes enhances cytokine expression and suppressive activity of human CD4+CD45ro+CD25+CD127(low) regulatory T cells. Arthritis Rheum 2013;65(3):627-38.

65. Hartgring SA, Willis CR, Bijlsma JW, Lafeber FP, van Roon JA. Interleukin-7-aggravated joint inflammation and tissue destruction in collagen-induced arthritis is associated with T-cell and B-cell activation. Arthritis Res Ther 2012;14(3):R137.

66. Hartgring SA, Willis CR, Alcorn D, Nelson LJ, Bijlsma JW, Lafeber FP, et al. Blockade of the interleukin-7 receptor inhibits collagen-induced arthritis and is associated with reduction of T cell activity and proinflammatory mediators. Arthritis Rheum 2010;62(9):2716-25.

67. Walker LS, Sansom DM. The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses. Nat Rev Immunol 2011;11(12):852-63.

68. Qureshi OS, Zheng Y, Nakamura K, Attridge K, Manzotti C, Schmidt EM, et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. Science 2011;332(6029):600-3.

69. Deppong CM, Bricker TL, Rannals BD, Van Rooijen N, Hsieh CS, Green JM. CTLA4Ig inhibits effector T cells through regulatory T cells and TGF-beta. J Immunol 2013;191(6):3082-9.

70. Schiff M, Keiserman M, Codding C, Songcharoen S, Berman A, Nayiager S, et al. Efficacy and safety of abatacept or infliximab vs placebo in ATTEST: a phase III, multi-centre, randomised, double-blind, placebo-controlled study in patients with rheumatoid arthritis and an inadequate response to methotrexate. Ann Rheum Dis 2008;67(8):1096-103.

71. Schiff M, Weinblatt ME, Valente R, van der Heijde D, Citera G, Elegbe A, et al. Head-to-head comparison of subcutaneous abatacept versus adalimumab for rheumatoid arthritis: two-year efficacy and safety findings from AMPLE trial. Ann Rheum Dis 2013.

72. Bathon J, Robles M, Ximenes AC, Nayiager S, Wollenhaupt J, Durez P, et al. Sustained disease remission and inhibition of radiographic progression in methotrexate-naive patients with rheumatoid arthritis and poor prognostic factors treated with abatacept: 2-year outcomes. Ann Rheum Dis 2011;70(11):1949-56.

73. Genovese MC, Schiff M, Luggen M, Becker JC, Aranda R, Teng J, et al. Efficacy and safety of the selective co-stimulation modulator abatacept following 2 years of treatment in patients with rheumatoid arthritis and an inadequate response to anti-tumour necrosis factor therapy. Ann Rheum Dis 2008;67(4):547-54.

74. Scarsi M, Ziglioli T, Airo P. Baseline numbers of circulating CD28-negative T cells may predict clinical response to abatacept in patients with rheumatoid arthritis. J Rheumatol 2011;38(10):2105-11.

2

Intra-articular CD1c-expressing myeloid dendritic cells from rheumatoid arthritis patients express a unique set of T cell-attracting chemokines and spontaneously induce Th1, Th17 and Th2 cell activity

Frederique M. Moret, Cornelis E. Hack, Kim M.G. van der Wurff-Jacobs, Wilco de Jager, Timothy R.D.J. Radstake, Floris P.J.G. Lafeber, Joel A.G. van Roon

Arthritis Res Ther. 2013 Oct 20;15(5):R155

ABSTRACT

Objective. Myeloid dendritic cells (mDCs) are potent T cell-activating antigen-presenting cells that have been suggested to play a crucial role in the regulation of immune responses in many disease states, including rheumatoid arthritis (RA). Despite this, studies that have reported on the capacity of naturally occurring circulating mDCs to regulate T cell activation in RA are still lacking. This study aimed to evaluate the phenotypic and functional properties of naturally occurring CD1c (BDCA-1)$^+$ mDCs from synovial fluid (SF) compared to those from peripheral blood (PB) of RA patients.

Methods. CD1c$^+$ mDC numbers and expression of costimulatory molecules were assessed by fluorescence-activated cell sorting (FACS) analysis in SF and PB from RA patients. *Ex vivo* secretion of 45 inflammatory mediators by mDCs from SF and PB of RA patients was determined by multiplex immunoassay. The capacity of mDCs from SF to activate autologous CD4$^+$ T cells was measured.

Results. CD1c$^+$ mDC numbers were significantly increased in SF versus PB of RA patients (mean 4.7% vs. 0.6%). mDCs from SF showed increased expression of antigen-presenting (human leukocyte antigen (HLA) class II, CD1c) and costimulatory molecules (CD80, CD86 and CD40). Numerous cytokines were equally abundantly produced by mDCs from both PB and SF (including IL-12, IL-23, IL-13, IL-21). SF mDCs secreted higher levels of interferon γ-inducible protein-10 (IP-10), monokine induced by interferon γ (MIG), and thymus and activation-regulated chemokine (TARC), but lower macrophage-derived chemokine (MDC) levels compared to mDCs from PB. mDCs from SF displayed a strongly increased capacity to induce proliferation of CD4$^+$ T cells associated with a strongly augmented IFNγ, IL-17, and IL-4 production.

Conclusions. This study suggests that increased numbers of CD1c$^+$ mDCs in SF are involved in the inflammatory cascade intra-articularly by the secretion of specific T cell-attracting chemokines and the activation of self-reactive T cells.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disorder characterized by persistent joint inflammation resulting in progressive destruction of the joint tissues [1]. $CD4^+$ T cells producing T-helper type (Th)-1 (interferon gamma (IFNγ)) and Th17 cytokines (interleukin (IL)-17) [2-5], as well as B cells of the adaptive immune system and macrophages and dendritic cells (DCs) of the innate immune system all contribute to joint inflammation and immunopathology of RA.

DCs are the professional antigen-presenting cells involved in the coordination of adaptive immune responses during infections and against tumour cells. DCs instruct T cells to develop a proper immune response by uptake and presentation of antigens and the provision of costimulatory signals and cytokines. In addition, DCs have the ability to instruct T cells to induce self-tolerance by presenting self-antigens to T cells and subsequent deletion or inactivation of self-reactive T cells [6]. External agents to DCs such as cytokines, tissue-derived factors, pathogen-derived antigens and organic molecules may alter the balance between tolerogenic and immunogenic activity of DCs and induce autoimmune disease [7, 8].

Human blood DCs are divided into several phenotypically and functionally different subpopulations including myeloid dendritic cells (mDCs) [9]. mDCs express CD11c and are subdivided into three subsets, of which $CD1c^+$ ($BDCA\text{-}1^+$) mDCs are the most abundant population [10-12]. Since CD1c, apart from mDCs, is only expressed by a subset of B cells, this marker can be used to indentify and isolate this unique subset of human mDCs [13, 14]. CD1c is a major histocompatibility complex class I-like cell surface glycoprotein that presents lipid and glycolipid self-antigens and nonself-antigens, so $CD1c^+$ mDCs can activate restricted lipid antigen-specific T cells [15]. However, these mDCs also have a strong capacity to induce a major histocompatibility complex-dependent antigen-driven allogeneic mixed lymphocyte reaction [11]. $CD1c^+$ mDCs in the circulation have been suggested to represent immature DCs that express CD86 and respond to microbial products rather than to inflammatory stimuli (such as tumour necrosis factor alpha) [12]. Recently, $CD1c^+$ mDCs were described to have an immunoregulatory function in response to certain microbial triggers [16, 17].

Despite the fact that mDCs have been extensively studied in immune disorders in mice and man and that they have been suggested to play an important role in the pathogenesis of RA [18], functional data on naturally occurring mDCs in RA, including those expressing CD1c, are scarce. Previous studies on mDCs in RA were based on CD33/CD14 expression, describing a larger mDC population than the recently defined $CD1c^+$ mDCs [19], since CD33 is not only expressed on $CD1c^+$ mDCs but also on $CD16^+$ and $BDCA\text{-}3^+$ DC subpopulations [11]. Only a small percentage of $CD1c^+$ mDCs express CD14 and the function of these double-positive mDCs is still unknown [20]. In RA, mDCs are increased in the joints as

compared with the circulation and express costimulatory molecules [21, 22]. However, a detailed analysis of the capacity of *ex vivo* cultured CD1c$^+$ mDCs from RA patients to produce inflammatory mediators and activate T cells has not been performed.

In the present study, the function of CD1c$^+$ mDCs (also referred to as mDCs) from peripheral blood (PB) and synovial fluid (SF) of RA patients was examined. The capacity of mDCs to secrete T cell-differentiating cytokines (including IL-12, IL-33, IL-23), chemokines (including CCL17/thymus and activation-regulated chemokine (TARC), CXCL9/monokine induced by interferon-gamma (MIG), CXCL10/ interferon-gamma inducible protein-10 (IP-10)) and proinflammatory cytokines (including IL-1β, IL-6) was studied in combination with their capacity to induce autologous T cell proliferation and cytokine production (IFNγ, IL-17 and IL-4).

MATERIALS AND METHODS

Patients

The patients included in this study all met the American College of Rheumatology criteria for RA [23]. Demographic and clinical data of these patients are presented in Table 1. SF and PB samples were collected from the patients. Ethical approval for this study was given by the medical ethical committee of the University Medical Center Utrecht for the collection of patient samples in compliance with the Helsinki Declaration. All patients gave their informed consent.

Table 1. Demographic and clinical characteristics of rheumatoid arthritis patients

	PB	SF	Paired PB + SF
Age (years)	51 ± 11	44 ± 6	48 ± 19
Gender (male/female)	4/7	0/5	8/10
Disease duration (years)	15 ± 10	16 ± 2	13 ± 8
Rheumatoid factor (positive/negative)	6/5	2/3	6/9[a]
Erythrocyte sedimentation rate (mm/hour)	16 ± 19	26 ± 22	34 ± 22
C-reactive protein (mg/l)	3 ± 2[b]	14 ± 7	39 ± 61[b]
Treatment (NSAID/Corticosteroids/DMARD/Biological)	0/0/9/2	0/0/2/3	1/4/5/5[a]

Data presented as mean ± standard deviation or number. DMARD, disease-modifying anti-rheumatic drugs; NSAID,

Flow cytometry

The number and characteristics of CD1c$^+$ mDCs present in mononuclear cell (MNC) fractions of paired PB and SF samples of RA patients were analysed by fluorescence-activated cell sorting (FACS) analysis using a FACS CANTO II flow cytometer (BD Biosciences, San Jose, CA, USA). Cells were stained for CD1c-PE (Biolegend, San Diego, CA, USA), CD19-PERCP-Cy5.5 (BD Biosciences) and CD14-APC (BD Pharmingen) and were identified as CD19-negative and

CD1c-positive. Cell surface marker expression on $CD1c^+$ mDCs was studied with the following reagents: IgG isotype FITC/PE (Immunotech, Marseille, France), HLA-DR/DP/DQ-FITC (BD Pharmingen), CD80-PE, CD86-PE, CD40-PE and CD19-PERCP-Cy5.5 (all BD Biosciences), and CD1c-Pacific Blue (Biolegend). All data were analysed using FlowJo software (Tree Star, Ashland, OR, USA). To compare mean fluorescence intensity (MFI) of human leukocyte antigen (HLA) class II, CD80, CD86 and CD40 expression on mDCs from PB and SF, the intensity of autofluorescence assessed using isotype controls from PB and SF mDCs was subtracted from the MFI of the stainings to reveal true expression differences.

Cell isolation

MNCs from lithium-heparinised PB and SF were isolated by density centrifugation using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). Prior to MNC isolation, PB or SF was diluted 1:1 with RPMI 1640 medium (Gibco, Life Technologies, New York, USA) containing penicillin (100U/ml), streptomycin (100µg/ml), and L-glutamine (2 mM) (all PAA Laboratories, Pasching, Austria). $CD1c^+$ mDCs from PB and SF and $CD4^+$ T cells from PB were isolated from MNC fractions by magnetic-activated cell sorting using $CD1c^+$ ($BDCA\text{-}1^+$) DC and $CD4^+$ T cell isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively, according to the manufacturer's instructions.

Cell cultures

Cells were cultured in RPMI glutamax (Gibco) supplemented with penicillin (100U/ml), streptomycin (100µg/ml) (both PAA Laboratories), and 10% human AB serum (v/v, GemCell, West Sacramento, CA, USA). Isolated $CD4^+$ T cells were seeded in round-bottomed 96-well plates (NUNC, Roskilde, Denmark) at a concentration of $0.5x10^6$ cells/ml and stored at 37°C before co-culturing with mDCs. The viable mDCs were counted and equal amounts of live cells were either cultured alone or cocultured with $CD4^+$ T cells. To measure cytokine production, isolated mDCs from PB and SF of RA patients were cultured at a concentration of 0.5×10^6 cells/ml for 20 hours at 37°C. Supernatants were harvested and tested for multiple cytokines with multiplex immunoassay. To determine the effect of PB-derived and SF-derived mDCs (≤10,000 cells/well) on autologous $CD4^+$ T cells (50,000 cells/well), mDCs derived from PB or SF were added to autologous peripheral $CD4^+$ T cells in triplicate at increasing DC:T cell ratios in the absence of additional stimuli. To determine the effect of CD80/86-dependent costimulation in SF mDC/T cell cocultures, cells were cultured in the presence of CTLA4-Ig (10 µg/ml, Bristol-Myers Squibb, New York, NY, USA). To study whether the observed differences between PB and SF mDCs were related to mDC maturation, as a control we activated mDCs from PB with thymic stromal lymphopoietin (TSLP, 20 ng/ml for 20 hours preceding coculture; R&D systems, Minneapolis, MN, USA). Cells were cocultured in round-bottomed 96-well plates for 6 days and subsequently

proliferation and cytokine production were measured. Proliferation was measured by ^{3}H-thymidine incorporation assay at the end of the culture period. ^{3}H-thymidine (1 µCi/well; PerkinElmer, Waltham, MA, USA) was added during the last 18 hours of the culture period. T cell cytokine production was measured in supernatants of cocultured cells upon short-term restimulation with 500 ng/ml ionomycin and 50 ng/ml phorbol myristate acetate (both Sigma-Aldrich, St. Louis, MO, USA) during the last 24 hours of the culture period.

Table 2. Cytokines assessed in supernatants of cultured CD1c^{+} myeloid dendritic cells derived from PB and SF of rheumatoid arthritis patients

	PB (pg/ml)	SF (pg/ml)
Proinflammatory cytokines		
IL-1α	409 (150)	344 (343)
IL-1β	258 (65.5)	298 (280)
IL-1RA	2561 (1034)	2769 (3531)
IL-6	41.0 (13.3)	29.6 (22.6)
IL-6R	324 (100)	343 (199)
IL-18	149 (47.9)	180 (165)
Tumour necrosis factor alpha	99.1 (95.5)	170 (165)
Interferon alpha	153 (69.5)	157 (168)
Thymic stromal lymphopoietin	< LDL	< LDL
T cell-differentiating and T cell-activating cytokines		
IL-12p70	17.6 (11.1)	18.9 (18.5)
IL-23	444 (354)	420 (393)
IL-33	260 (101)	336 (391)
IL-13	857 (387)	1054 (1426)
IL-10	112 (49.9)	126 (63.1)
Macrophage migration inhibitory factor	1872 (704)	2948 (1960)
IL-7	25.8 (6.9)	29.2 (29.7)
IL-9	3087 (1195)	3280 (3758)
IL-15	21.7 (8.7)	29.4 (27.3)
IL-21	5114 (1834)	6343 (4710)
IL-22	19.2 (6.1)	29.0 (27.3)
IL-25	1140 (526)	1239 (1310)
Chemokines		
IL-16	239 (101)	633 (581)†
CCL2/MCP1	29.7 (18.5)	24.6 (25.5)
CCL3/MIP1α	1268 (1847)	2776 (3954)
CCL5/RANTES	< LDL	< LDL
CCL17/TARC	1.2 (0.5)	26.4 (23.6)**
CCL19/MIP3β	28.4 (11.5)	57.5 (51.5)
CCL22/MDC	4397 (1627)	2456 (1023)*
CXCL9/MIG	23.9 (16.1)	90.4 (67.9)**
CXCL10/IP-10	54.0 (70.3)	247 (310)*

Table 2. Cytokines assessed in supernatants of cultured CD1c$^+$ myeloid dendritic cells derived from PB and SF of rheumatoid arthritis patients *(Continued)*

	PB (pg/ml)	SF (pg/ml)
Growth factors/others		
Oncostatin M	28.0 (21.7)	23.5 (17.8)
Fibroblast growth factor basic	4046 (1647)	4777 (5559)
Nerve growth factor	63.0 (23.2)	76.7 (90.2)
Hepatocyte growth factor	118 (46.8)	151 (143)
Granulocyte-macrophage colony-stimulating factor	650 (283)	827 (859)
Macrophage colony-stimulating factor	484 (188)	571 (730)
Vascular endothelial growth factor	191 (110)	202 (125)
IL-11	49.6 (20.1)	53.6 (63.9)
Soluble intracellular adhesion molecule-1	34067 (13911)	37260 (46225)
Osteopontin	11192 (4471)	15528 (10471)
Matrix metalloproteinase-8	2237 (1594)	2742 (4634)
Tissue inhibitor of metalloproteinases-1	2689 (1126)	2455 (2169)
Cathepsin B	4204 (1554)	6283 (4948)
Cathepsin L	215 (101)	594 (731)
Cathepsin S	133 (55.3)	159 (130)

Data presented as mean (standard deviation). IP-10, interferon-gamma inducible protein-10; LDL, lower detection limit; MCP-1, monocyte chemotactic protein-1; MDC, macrophage-derived chemokine; MIG, monokine induced by interferon-gamma; MIP, macrophage inflammatory protein; PB, peripheral blood; RANTES, regulated upon activation, normally T cell-expressed and presumably secreted; SF, synovial fluid; TARC, thymus and activation-regulated chemokine; IL, interleukin. *P <0.05; **P <0.01; $^{\dagger}P$ = 0.10.

Cytokine assessment

Cytokines and chemokines in supernatants of cultured mDCs derived from PB and SF were assessed by a multiplex immunoassay as described elsewhere [24]. The cytokines measured and their expression levels are presented in Table 2. T cell cytokines produced by CD4$^+$ T cells cocultured with CD1c$^+$ mDCs were analysed upon restimulation with ionomycin/phorbol myristate acetate. IFNγ, IL-4 (Invitrogen, Life Technologies, New York, NY, USA) and IL-17 (R&D systems) were measured by enzyme-linked immunosorbent assay according to the manufacturer's instructions.

Statistical analysis

Paired-sample evaluation was performed using the nonparametric Wilcoxon signed-rank test. Unpaired data analysis was performed using the nonparametric Mann-Whitney U test. Data analysis was performed using SPSS software (IBM, Armonk, NY, USA). Data were considered statistically significant at P <0.05.

RESULTS

CD1c$^+$ mDCs have an increased frequency and activated phenotype in RA synovial fluid

mDCs were characterised by the expression of CD1c and the absence of CD19 in MNC fractions of paired PB and SF samples (n = 10) of RA patients (representative dot plot; Figure 1A).

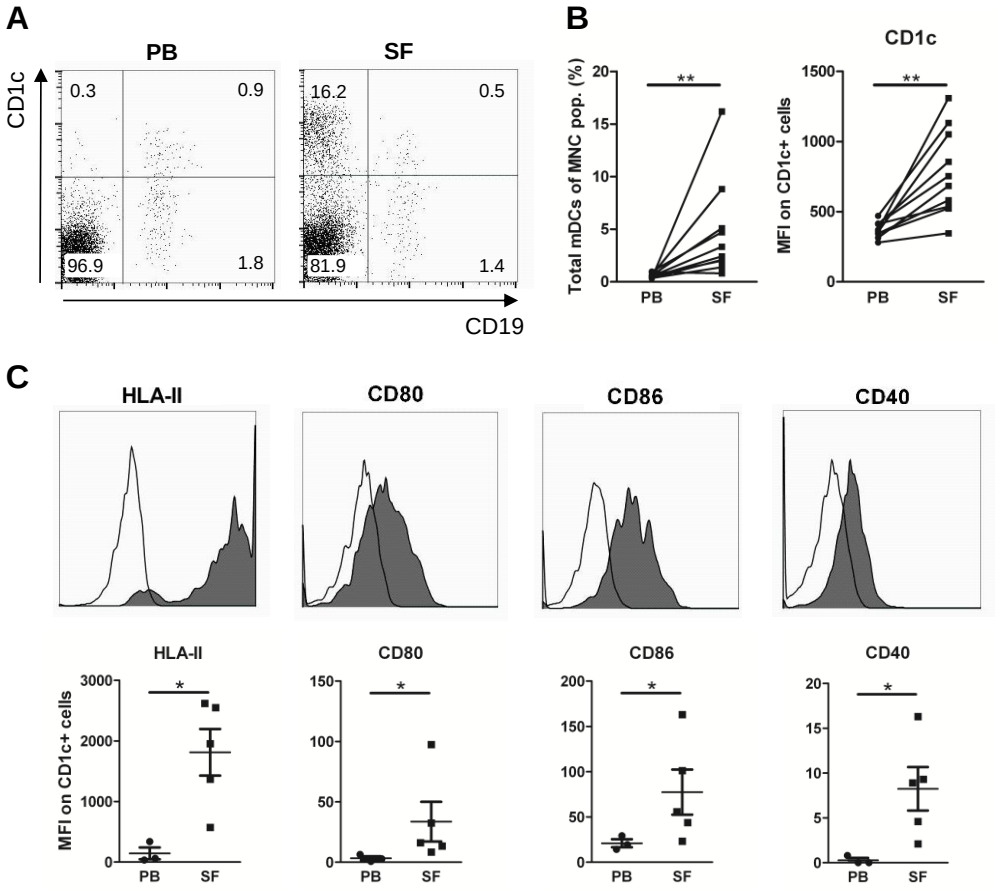


Figure 1. CD1c$^+$ myeloid dendritic cells are abundantly present in joints of rheumatoid arthritis patients and express increased levels of antigen-presenting and costimulatory molecules. (A) Representative fluorescence-activated cell sorting (FACS) dot plot of CD1c-expressing myeloid dendritic cells (mDCs) and CD19$^+$ cells in the peripheral blood (PB) and synovial fluid (SF) of a rheumatoid arthritis (RA) patient. (B) mDC numbers are increased in SF versus PB of RA patients (n = 10), and SF mDCs express higher CD1c levels. Percentages (%) of total mononuclear cell (MNC) population and mean fluorescence intensity (MFI) of CD1c expression are given. (C) mDCs derived from SF (n = 5) express enhanced levels of antigen-presenting (human leukocyte antigen class II (HLA-II)) and costimulatory molecules (CD80, CD86 and CD40) as compared with PB-derived mDCs (n = 3). Representative histograms of isotype control (open) and HLA-II, CD80, CD86 and CD40 (filled) expression and mean are shown (MFI corrected for isotype fluorescence). Statistically significant differences of *P <0.05 and **P <0.01.

The number of mDCs, determined as a percentage of total MNCs, was significantly increased in SF versus PB (mean ± standard error of the mean: 4.7 ± 1.5% vs. 0.6 ± 0.1%, respectively, *P* <0.01) as well as the CD1c expression (MFI: 778 ± 97 vs. 372 ± 18, respectively, *P* <0.01; Figure 1B). Cell surface markers on mDCs derived from PB and SF of RA patients were also studied (*n* = 3 and *n* = 5, respectively). mDCs derived from SF compared with those from PB expressed increased levels of antigen-presenting molecules HLA class II and costimulatory molecules CD80, CD86 and CD40 (representative histograms and MFI; Figure 1C). In addition to the MFI, the percentage of SF mDCs expressing all these activation markers was also significantly increased compared with PB mDCs (mean ± standard error of the mean % positive cells in PB vs. SF, respectively: HLA class II, 39 ± 10% vs. 90 ± 3%, *P* <0.05; CD80, 4 ± 1% vs. 35 ± 10%, *P* <0.05; CD86, 30 ± 7% vs. 63 ± 10%, *P* = 0.07; CD40, 0.2 ± 0% vs. 11 ± 3%, *P* <0.05).

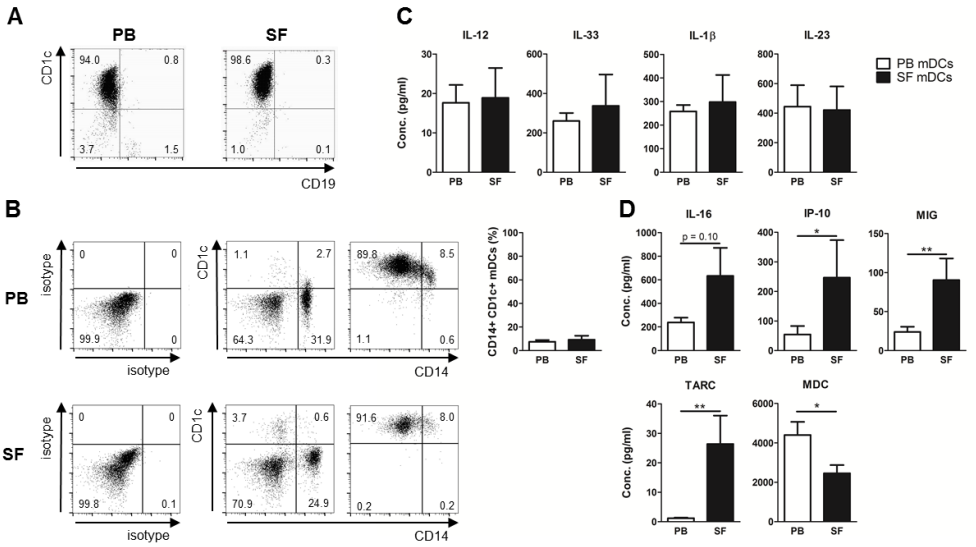


Figure 2. CD1c$^+$ myeloid dendritic cells from synovial fluid of rheumatoid arthritis patients produce increased chemokine levels but equal amounts of T cell-differentiating cytokines compared with those from peripheral blood. (A) Representative dot plot of isolated CD1c-expressing myeloid dendritic cells (mDCs) from peripheral blood (PB) and synovial fluid (SF) of a rheumatoid arthritis (RA) patient. (B) Representative dot plots of isotype (left), CD1c and CD14 expression on CD19$^-$ mononuclear cells (middle) and on isolated mDCs (right plot) from PB and SF. A small percentage of mDCs from PB and SF (*n* = 6, paired samples) expresses CD14 (bar graph). (C) PB mDCs and SF mDCs (*n* = 6) produced comparable levels of T-helper type (Th)-1, Th17 and Th2-differentiating cytokines interleukin (IL)-12, IL-23, IL-33. (D) Production of several chemokines by SF mDCs was significantly upregulated and macrophage-derived chemokine (MDC) significantly downregulated as compared with PB mDCs. Apart from the T cell-differentiating cytokines, only inflammatory mediators that showed *P* ≤0.10 are shown. Statistically significant differences of **P* <0.05 and ***P* <0.01. IP-10, interferon-gamma inducible protein-10; MIG, monokine induced by interferon-gamma; TARC, thymus and activation-regulated chemokine.

Synovial fluid-derived CD1c$^+$ mDCs from RA patients secrete higher levels of specific cytokines compared with peripheral blood mDCs

To study the functional properties of CD1c$^+$ mDCs these were isolated from PB MNCs and SF MNCs of RA patients (n = 6; representative dot plot, Figure 2A). A small subset of CD1c$^+$ mDCs has been described to express CD14. Therefore, the percentage of CD14-expresssing cells was assessed in CD19$^-$ MNC fractions and in isolated CD1c$^+$ cells. Only a small proportion of isolated CD1c$^+$ cells expressed CD14, to a similar extent on CD1c$^+$ mDCs from PB and SF (n = 6; representative dot plots and mean of paired PB and SF samples, Figure 2B).

Isolated CD1c$^+$ cells were cultured for 20 hours in the absence of stimuli and cytokine production was assessed (Table 2). Production of T cell-differentiating cytokines IL-12p70, IL-33, IL-1ß, and IL-23 by mDCs from SF was not significantly different from that of PB mDCs (all P >0.10; Figure 2C). The secretion of the chemoattractive mediators IP-10, MIG, and TARC by SF mDCs was significantly increased as compared with PB mDCs. Macrophage-derived chemokine (MDC) production was significantly decreased by SF mDCs compared with PB mDCs (Figure 2D). The production of the T cell attractant IL-16 by SF mDCs was also elevated compared with PB mDCs, although this did not reach statistical significance (P = 0.10; Figure 2D).

Inflammatory mediators that were under the detection limit included RANTES (regulated upon activation, normally T cell expressed and presumably secreted) and TSLP. All other mediators were secreted in equal amounts by PB and SF mDCs (all P >0.10) (Table 2).

Intra-articular CD1c$^+$ mDCs spontaneously induce proliferation and cytokine secretion of autologous CD4$^+$ T cells in RA patients.

Since mDCs from SF express increased levels of antigen-presenting and costimulatory molecules and produced abundant amounts of T cell-differentiating cytokines as well as increased levels of several inflammatory mediators compared with mDCs from PB, we investigated the capacity of PB-derived and SF-derived mDCs (n = 11 and n = 5, respectively) to activate autologous CD4$^+$ T cells. CD4$^+$ T cells cocultured with SF mDCs showed a robust increase in the induction of T cell proliferation compared with PB mDCs (PB mDCs vs. SF mDCs:CD4 T cells (1:5), 1,503 ± 443 vs. 26,935 ± 7,543 counts/minute, P <0.01, respectively; Figure 3A). Similar increased T cell activation was observed when paired PB and SF mDCs from the same donor were analysed (Figure 3B). The increase in the induction of T cell proliferation was also associated with a robust increase in the production of T cell cytokines, measured after restimulation with ionomycin/phorbol myristate acetate. The production of IFNγ, IL-17 and IL-4 was higher by SF mDCs versus PB mDCs (Figure 3C). To test whether enhanced SF mDC-induced T cell activation was dependent on CD80/86 upregulation, CD80/86 costimulation was blocked by CTLA4-Ig. SF mDC-induced T cell

activation was completely blocked by CTLA4-Ig (Figure 3D). To study whether the observed differences between PB and SF mDCs were related to DC maturation, as a control, we activated mDCs from PB with TSLP, previously shown to activate CD11c$^+$ DCs and implicated to play a proinflammatory role in RA [25, 26]. TSLP-stimulated CD1c$^+$ mDCs induced robust proliferation of autologous CD4$^+$ T cells (unstimulated vs. TSLP-stimulated mDCs:CD4 T cells (1:5), proliferation: from 1,000 ± 284 to 14,206 ± 2,426 counts/minute, P = 0.01, respectively; n = 8 from PB of healthy controls).

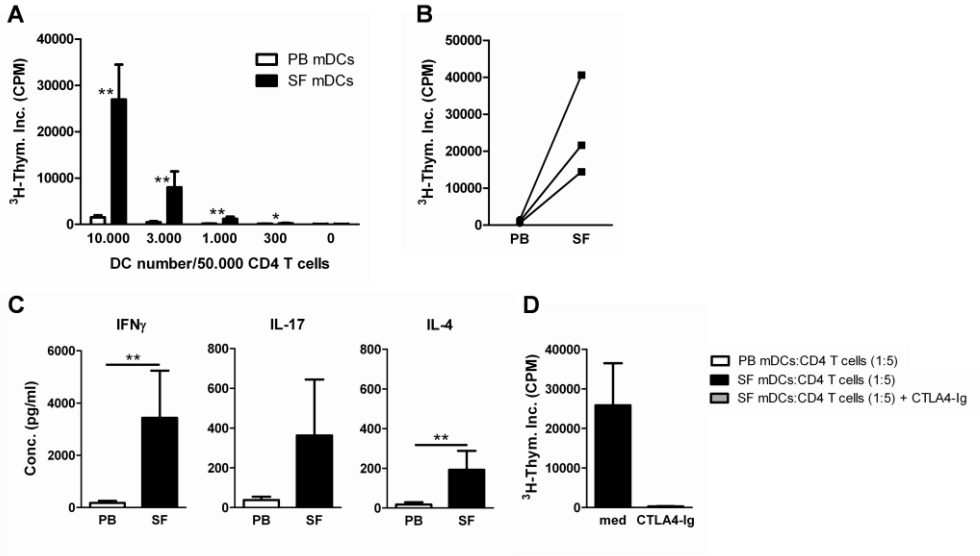


Figure 3. Intra-articular CD1c$^+$ myeloid dendritic cells induce strong proliferation of CD4$^+$ T cells associated with a strong increase in proinflammatory T cell cytokine production. (A) Inflammatory myeloid dendritic cells (mDCs) from synovial fluid (SF) when added to autologous CD4$^+$ T cells induced a strong spontaneous proliferation of CD4$^+$ T cells of rheumatoid arthritis (RA) patients (n = 5), significantly higher compared with mDCs from peripheral blood (PB; n = 11). (B) Paired analysis of mDCs from PB and SF from the same donors (n = 3, 10,000 DCs/50,000 CD4$^+$ T cells) showed a similar robust induction of autologous T cell proliferation by SF mDCs. (C) mDCs from SF (n = 5) induced a strong production of proinflammatory cytokines (interferon gamma (IFNγ), interleukin (IL)-17 and IL-4) compared with PB mDCs (n = 11). (D) SF mDC-induced T cell proliferation was strongly dependent on CD80/86 costimulation as it was completely blocked in the presence of CTLA4-Ig. Statistically significant differences of *P <0.05 and **P <0.01. CPM, counts per minute.

DISCUSSION

Hitherto, functional data on CD1c$^+$ (BDCA-1)$^+$ mDCs from RA patients are scarce. Here we demonstrate that in RA patients naturally occurring CD1c$^+$ mDCs are present in higher frequency in SF and that these mDCs have an activated phenotype and secrete increased

levels of a unique set of chemokines in comparison with mDCs from PB. To our knowledge this is also the first study that reports $CD1c^+$ mDCs from SF of RA patients to compellingly cause autologous T cell activation.

The majority of T cells present in SF of RA patients were previously shown to express the CXC chemokine receptor 3 (CXCR3), with a higher percentage of T cells expressing CXCR3 in SF than in PB of RA patients [27, 28]. CXCR3 is mainly expressed on Th1 cells and binds to its ligands CXCL10/IP-10 and CXCL9/MIG [29, 30]. Elevated IP-10 and MIG levels are observed in SF of RA patients compared with control SF from patients with osteoarthritis or traumatic joint injury [27], and the present study shows that *in vivo*-activated $CD1c^+$ mDCs from RA joints secrete high levels of IP-10 and MIG. Furthermore, these mDCs secrete higher CCL17/TARC levels compared with mDCs derived from PB. TARC is a selective ligand for the CC chemokine receptor 4 (CCR4) that is expressed by Th2 cells [29, 30], Th17 cells [31], and regulatory T cells [32], suggesting that TARC contributes to attraction of these T cells into the inflamed joints. Secretion of CCL22/MDC by SF mDCs, another CCR4 ligand, was significantly lower compared with that by PB mDCs. To what extent this differential expression of CCR4 ligands influences the chemotaxis of Th2, Th17 and regulatory T cells remains to be demonstrated. Recently, it was demonstrated that MDC levels in SF of RA patients are elevated compared with osteoarthritis patients [33]. This may indicate that other cell types could contribute to these enhanced levels. Alternatively, the increased number of $CD1c^+$ mDCs (~5-fold higher) in the joint compared with the circulation of RA patients could contribute to elevated intra-articular levels.

Apart from enhancing immune activation, DCs induced under different conditions can also induce tolerance – by increasing regulatory T cell activity, among other effects [7, 8]. Our data demonstrate that, in line with the enhanced expression of antigen-presenting and costimulatory molecules, SF-derived $CD1c^+$ mDCs spontaneously increased proliferation and Th1, Th17 and Th2 cell cytokine production of autologous $CD4^+$ T cells. Although we did not measure regulatory T cell function, our data demonstrate that mDCs from SF overrule suppression by regulatory T cells and indicate that *in vivo*-activated $CD1c^+$ mDCs from the joint have a strong capacity to induce T cell expansion of infiltrating T cells without strongly skewing the T cell balance. In line with this observation SF and PB mDCs secreted equal amounts of T cell-differentiating cytokines. Since in RA joints a predominance of Th1 and Th17 activity is observed, our data suggest that in joints of RA patients further Th1 and Th17 cell skewing is facilitated by local inflammatory mediators (for example, IL-12) produced, for example, by macrophages.

Recently, Frankenberger and colleagues showed via gene expression analysis that $CD14^{++}CD16^-$ as well as $CD16^+$ monocytes are both distinct from the $CD1c^+$ blood DCs [34]. In the present study CD14-expressing cells that lacked CD1c were not present or were hardly present in the isolated DC fractions (~0.5%); however, a small percentage of the $CD1c^+$ mDCs coexpressed CD14, similar to previous reports for healthy controls [20]. Our data

demonstrate that activated mDCs from SF of RA patients show a robust homogenic upregulation of HLA class II, CD80, CD86, CD40 and CD1c compared with their circulating counterparts. This corresponds to the robust T cell stimulatory capacity of these SF mDCs and contradicts the assumption that a subpopulation of $CD14^+$ cells is responsible for the observed effects. In addition, the percentages $CD14^+CD1c^+$ mDCs between PB and SF did not differ. These latter suggestions are in line with the much lower T cell stimulatory capacity of total $CD14^+$ monocytes, either isolated from SF or from PB (unpublished data). Recently, however, $CD1c^+$ DCs expressing CD14 were suggested to represent human inflammatory DCs, present in inflammatory environments such as ascites and RA joints, and potent inducers of Th17 cells [35]. Although individual data from RA SF cannot be deduced from this latter study, CD14 seems to be expressed at higher levels on inflammatory $CD1c^+$ mDCs versus blood $CD1c^+$ mDCs [35]. Differences in this latter and the present study, showing low and comparable percentages of $CD14^+CD1c^+$ mDCs between PB and SF, might be related to the site of inflammation studied (SF vs. ascites), but this remains to be established.

Although a wide range of triggers might activate $CD1c^+$ mDCs, the predominant triggers that activate these cells in RA joints remain to be demonstrated [36]. Jongbloed and colleagues investigated the capacity of SF mDCs to respond to toll-like receptor-2 agonism. As compared with healthy PB mDCs, mDCs from the joints of RA patients produced equal amounts of tumour necrosis factor alpha and increased amounts of IL-10 in response to activation by *Staphylococcus aureus* peptidoglycan [22]. Based on this study on toll-like receptor triggering, mDCs from the joints of RA patients were suggested not to be activated and to display a semi-mature phenotype [22]. In line with these data the present study also failed to detect any robust differences between PB and SF mDCs in secretion of cytokines (including IL-1, IL-6, tumour necrosis factor alpha, IFNα) typically induced upon toll-like receptor-2 or other toll-like receptor triggering. However, the present data clearly reveal an activated status of SF mDCs and a powerful stimulation of T cells by these SF mDC, which suggests that stimuli other than toll-like receptor ligands contribute to the enhanced activity of these cells *in vivo,* associated with a unique set of secreted chemokines. Recently, increased levels of TSLP in SF of RA patients versus osteoarthritis patients were documented [37, 38]. TSLP strongly induced TARC production by mDCs from PB and SF of RA patients [38]. Furthermore, mDCs from the peripheral blood, activated by TSLP, showed phenotypical and functional similarities with mDCs derived from the joints of RA patients, suggesting that TSLP might be an important trigger for mDC activation in RA joints [38].

CONCLUSIONS

There is clear evidence that multiple subsets of DCs exist, which display specialised functions although the tissue microenvironment and the stage of maturation can influence their

phenotype and function. The present study suggests that accumulating $CD1c^+$ mDCs in the joints of RA patients importantly contribute to inflammation by inducing secretion of a unique set of T cell-attracting chemokines and by spontaneously activating $CD4^+$ T cells to proliferate and secrete proinflammatory cytokines. The identification of the factors that control the capacity of mDCs to elicit Th1, Th17 and Th2 development in RA is critical to envisage new approaches to manipulate the immune system to the benefit of these patients.

ACKNOWLEDGEMENT

This research was performed within the framework of CTMM, the Center for Translational Molecular Medicine (www.ctmm.nl), and the Dutch Arthritis Foundation, project TRACER (grant 04I-202).

REFERENCES

1. Feldmann M, Brennan FM, Maini RN: Rheumatoid arthritis. *Cell* 1996, 85(3):307-310.
2. Morita Y, Yamamura M, Kawashima M, Harada S, Tsuji K, Shibuya K, Maruyama K, Makino H: Flow cytometric single-cell analysis of cytokine production by CD4+ T cells in synovial tissue and peripheral blood from patients with rheumatoid arthritis. *Arthritis Rheum* 1998, 41(9):1669-1676.
3. Chabaud M, Durand JM, Buchs N, Fossiez F, Page G, Frappart L, Miossec P: Human interleukin-17: A T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. *Arthritis Rheum* 1999, 42(5):963-970.
4. Cornelissen F, van Hamburg JP, Lubberts E: The IL-12/IL-23 axis and its role in Th17 cell development, pathology and plasticity in arthritis. *Curr Opin Investig Drugs* 2009, 10(5):452-462.
5. van Roon JA, van Roy JL, Duits A, Lafeber FP, Bijlsma JW: Proinflammatory cytokine production and cartilage damage due to rheumatoid synovial T helper-1 activation is inhibited by interleukin-4. *Ann Rheum Dis* 1995, 54(10):836-840.
6. Banchereau J, Steinman RM: Dendritic cells and the control of immunity. *Nature* 1998, 392(6673):245-252.
7. Coquerelle C, Moser M: DC subsets in positive and negative regulation of immunity. *Immunol Rev* 2010, 234(1):317-334.
8. Torres-Aguilar H, Blank M, Jara LJ, Shoenfeld Y: Tolerogenic dendritic cells in autoimmune diseases: crucial players in induction and prevention of autoimmunity. *Autoimmun Rev* 2010, 10(1):8-17.
9. Steinman RM, Inaba K: Myeloid dendritic cells. *J Leukoc Biol* 1999, 66(2):205-208.
10. Dzionek A, Fuchs A, Schmidt P, Cremer S, Zysk M, Miltenyi S, Buck DW, Schmitz J: BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. *J Immunol* 2000, 165(11):6037-6046.
11. MacDonald KP, Munster DJ, Clark GJ, Dzionek A, Schmitz J, Hart DN: Characterization of human blood dendritic cell subsets. *Blood* 2002, 100(13):4512-4520.
12. Piccioli D, Tavarini S, Borgogni E, Steri V, Nuti S, Sammicheli C, Bardelli M, Montagna D, Locatelli F, Wack A: Functional specialization of human circulating CD16 and CD1c myeloid dendritic-cell subsets. *Blood* 2007, 109(12):5371-5379.
13. Small TN, Knowles RW, Keever C, Kernan NA, Collins N, O'Reilly RJ, Dupont B, Flomenberg N: M241 (CD1) expression on B lymphocytes. *J Immunol* 1987, 138(9):2864-2868.
14. Delia D, Cattoretti G, Polli N, Fontanella E, Aiello A, Giardini R, Rilke F, Della Porta G: CD1c but neither CD1a nor CD1b molecules are expressed on normal, activated, and malignant human B cells: identification of a new B-cell subset. *Blood* 1988, 72(1):241-247.
15. Beckman EM, Melian A, Behar SM, Sieling PA, Chatterjee D, Furlong ST, Matsumoto R, Rosat JP, Modlin RL, Porcelli SA: CD1c restricts responses of mycobacteria-specific T cells. Evidence for antigen presentation by a second member of the human CD1 family. *J Immunol* 1996, 157(7):2795-2803.

16. Kassianos AJ, Hardy MY, Ju X, Vijayan D, Ding Y, Vulink AJ, McDonald KJ, Jongbloed SL, Wadley RB, Wells C *et al*: Human CD1c (BDCA-1)+ myeloid dendritic cells secrete IL-10 and display an immuno-regulatory phenotype and function in response to Escherichia coli. *Eur J Immunol* 2012, 42(6):1512-1522.

17. Qian C, Cao X: Naturally occurring CD1c+ human regulatory dendritic cells: immunoregulators that are expanded in response to E. coli infection. *Eur J Immunol* 2012, 42(6):1388-1392.

18. Thomas R, MacDonald KP, Pettit AR, Cavanagh LL, Padmanabha J, Zehntner S: Dendritic cells and the pathogenesis of rheumatoid arthritis. *J Leukoc Biol* 1999, 66(2):286-292.

19. Thomas R, Davis LS, Lipsky PE: Rheumatoid synovium is enriched in mature antigen-presenting dendritic cells. *J Immunol* 1994, 152(5):2613-2623.

20. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, Leenen PJ, Liu YJ, MacPherson G, Randolph GJ *et al*: Nomenclature of monocytes and dendritic cells in blood. *Blood* 2010, 116(16):e74-80.

21. Smolewska E, Stanczyk J, Brozik H, Biernacka-Zielinska M, Cebula B, Robak T, Smolewski P: Distribution and clinical significance of blood dendritic cells in children with juvenile idiopathic arthritis. *Ann Rheum Dis* 2008, 67(6):762-768.

22. Jongbloed SL, Lebre MC, Fraser AR, Gracie JA, Sturrock RD, Tak PP, McInnes IB: Enumeration and phenotypical analysis of distinct dendritic cell subsets in psoriatic arthritis and rheumatoid arthritis. *Arthritis Res Ther* 2006, 8(1):R15.

23. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS *et al*: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988, 31(3):315-324.

24. de Jager W, te Velthuis H, Prakken BJ, Kuis W, Rijkers GT: Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. *Clin Diagn Lab Immunol* 2003, 10(1):133-139.

25. Reche PA, Soumelis V, Gorman DM, Clifford T, Liu M, Travis M, Zurawski SM, Johnston J, Liu YJ, Spits H *et al*: Human thymic stromal lymphopoietin preferentially stimulates myeloid cells. *J Immunol* 2001, 167(1):336-343.

26. Hartgring SA, Willis CR, Dean CE, Jr., Broere F, van Eden W, Bijlsma JW, Lafeber FP, van Roon JA: Critical proinflammatory role of thymic stromal lymphopoietin and its receptor in experimental autoimmune arthritis. *Arthritis Rheum* 2011, 63(7):1878-1887.

27. Patel DD, Zachariah JP, Whichard LP: CXCR3 and CCR5 ligands in rheumatoid arthritis synovium. *Clin Immunol* 2001, 98(1):39-45.

28. Qin S, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, Koch AE, Moser B, Mackay CR: The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J Clin Invest* 1998, 101(4):746-754.

29. Bonecchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, Sozzani S, Allavena P, Gray PA, Mantovani A *et al*: Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J Exp Med* 1998, 187(1):129-134.

30. Sallusto F, Lenig D, Mackay CR, Lanzavecchia A: Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J Exp Med* 1998, 187(6):875-883.

31. Leipe J, Grunke M, Dechant C, Reindl C, Kerzendorf U, Schulze-Koops H, Skapenko A: Role of Th17 cells in human autoimmune arthritis. *Arthritis Rheum* 2010, 62(10):2876-2885.

32. Baatar D, Olkhanud P, Sumitomo K, Taub D, Gress R, Biragyn A: Human peripheral blood T regulatory cells (Tregs), functionally primed CCR4+ Tregs and unprimed CCR4- Tregs, regulate effector T cells using FasL. *J Immunol* 2007, 178(8):4891-4900.

33. Flytlie HA, Hvid M, Lindgreen E, Kofod-Olsen E, Petersen EL, Jorgensen A, Deleuran M, Vestergaard C, Deleuran B: Expression of MDC/CCL22 and its receptor CCR4 in rheumatoid arthritis, psoriatic arthritis and osteoarthritis. *Cytokine* 2010, 49(1):24-29.

34. Frankenberger M, Hofer TP, Marei A, Dayyani F, Schewe S, Strasser C, Aldraihim A, Stanzel F, Lang R, Hoffmann R *et al*: Transcript profiling of CD16-positive monocytes reveals a unique molecular fingerprint. *Eur J Immunol* 2012, 42(4):957-974.

35. Segura E, Touzot M, Bohineust A, Cappuccio A, Chiocchia G, Hosmalin A, Dalod M, Soumelis V, Amigorena S: Human inflammatory dendritic cells induce Th17 cell differentiation. *Immunity* 2013, 38(2):336-348.

36. Leslie DS, Vincent MS, Spada FM, Das H, Sugita M, Morita CT, Brenner MB: CD1-mediated gamma/delta T cell maturation of dendritic cells. *J Exp Med* 2002, 196(12):1575-1584.

37. Koyama K, Ozawa T, Hatsushika K, Ando T, Takano S, Wako M, Suenaga F, Ohnuma Y, Ohba T, Katoh R *et al*: A possible role for TSLP in inflammatory arthritis. *Biochem Biophys Res Commun* 2007, 357(1):99-104.

38. Moret FM, Hack CE, van der Wurff-Jacobs KMG, Lafeber FPJG, van Roon JAG: TSLP: a novel potent proinflammatory mediator that activates myeloid dendritic cells to stimulate Th1 and Th17 activity in rheumatoid arthritis [abstract]. *Ann Rheum Dis* 2011, 70:A43-A44. [conditionally accepted Arthritis Rheum 2013].

3

Thymic stromal lymphopoietin (TSLP): a novel proinflammatory mediator in rheumatoid arthritis that potently activates CD1c^{+} myeloid dendritic cells to attract and stimulate T cells

Frederique M. Moret, Cornelis E. Hack, Kim M.G. van der Wurff-Jacobs,
Timothy R.D.J. Radstake, Floris P.J.G. Lafeber, Joel A.G. van Roon

Arthritis Rheum. 2014 Jan 8 [Epub ahead of print]

ABSTRACT

Objectives. To determine TSLP levels and numbers of TSLPR-expressing CD1c (BDCA-1)$^+$ myeloid dendritic cells (mDCs) in joints of rheumatoid arthritis (RA) patients versus peripheral blood (PB) and the capacity of TSLP to induce mDC-dependent T cell activation.

Methods. TSLP was measured in synovial fluid (SF) of RA and osteoarthritis (OA) patients. mDC numbers in PB and SF from RA patients and TSLPR expression on these cells were assessed by FACS analysis. PB mDCs and SF mDCs of RA patients were stimulated with TSLP and cytokine production was measured by multiplex immunoassay. TSLP-primed mDCs were co-cultured with autologous CD4$^+$ T cells in the absence of additional stimuli and subsequently proliferation and cytokine production were measured.

Results. TSLP levels were significantly increased in SF of RA versus OA patients. TSLPR-expressing mDC numbers in SF were significantly increased versus PB from RA patients and SF mDCs displayed increased TSLPR levels. TSLP selectively stimulated production of TARC and MIP1α by CD1c$^+$ mDCs. TSLP-primed mDCs from PB and SF potently stimulated proliferation of autologous CD4$^+$ T cells compared to unstimulated mDCs. Enhanced proliferation was associated with increased production of IFNγ, IL-17 and IL-4.

Conclusions. These data support an inflammatory mechanism in which increased intra-articular TSLP in RA potently activates TSLPR-expressing CD1c$^+$ mDCs in the joints to secrete chemokines causing chemotaxis and subsequent activation of CD4$^+$ T cells. In addition to the demonstrated inflammatory potential of TSLP in experimental arthritis, this suggests that TSLP and TSLPR-expressing mDCs could both play a pivotal role in the immunopathology of RA.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disorder characterized by persistent joint inflammation resulting in progressive tissue destruction and functional impairment (1). This joint inflammation is strongly dependent on $CD4^+$ T cells producing Th1 (IFNγ) and Th17 cytokines (IL-17) (2-5). Activation and differentiation of $CD4^+$ T cells to become Th1 or Th17 cells can be strongly regulated by antigen-presenting cells, like dendritic cells, that present antigens via human leukocyte antigen (HLA) class II molecules and provide costimulation by molecules like CD80 and CD86. Myeloid dendritic cells (mDCs) are considered as the most potent T cell-activating antigen-presenting cells in the body and these cells are abundantly present in joints of RA patients (6-9).

Several types of dendritic cells are described in human blood characterized by high expression of HLA class II molecules and the absence of lineage markers (CD3, CD19, CD14, CD20, CD56 and glycophorin A). Human blood dendritic cells are divided into plasmacytoid dendritic cells and two types of myeloid dendritic cells (mDC1 and mDC2), also called classical dendritic cells (10, 11). Currently, the blood-derived dendritic cell antigen (BDCA)-molecules are the most important markers to distinguish blood dendritic cells from each other with great accuracy (12, 13). BDCA-1 (CD1c) identifies the mDC1 subset, which are potent activators of $CD4^+$ T cells, whereas mDC2 cells, identified by expression of BDCA-3 (CD141) more strongly activate $CD8^+$ T cells (11-13). In this respect, it is important to notice that the characterization of mDC1 cells by CD1c is more specific than the more broadly expressed marker CD11c (11, 13). Because of the prominent role of $CD4^+$ T cells in arthritic processes and the recently demonstrated strong capacity of synovial fluid-derived $CD1c^+$ mDCs from RA patients to activate $CD4^+$ T cells (9), the present study focuses on a potential trigger for $CD1c^+$ mDCs called thymic stromal lymphopoietin (TSLP).

TSLP is shown to be a potent activator of mDCs, identified by the expression of CD11c, to induce Th2-mediated immune responses and is suggested to be an important proinflammatory cytokine in allergic diseases both in men and mice (14-16). TSLP accomplishes its biologic activities via the low affinity TSLP receptor (TSLPR) in conjunction with the IL-7 receptor-alpha chain (IL-7Rα) that is shared with IL-7 (17, 18). Human TSLPR is mainly expressed on mDCs expressing CD11c, indicating that these cells are a main target cell population for TSLP (14, 19). Indeed, TSLP activates these mDCs to strongly upregulate the expression of antigen-presenting and costimulatory molecules and to secrete Th2 cell-attracting chemokines including CCL17/TARC (thymus and activation-regulated chemokine) and CCL22/MDC (macrophage-derived chemokine) (14, 20). As a consequence, TSLP-activated mDCs induce strong proliferation of allogeneic naive $CD4^+$ T cells at very low numbers (DC:T cell ratio 1:150), which is associated with the production of Th2 cytokines (14).

TSLP also has been shown to play a pivotal role in homeostatic T cell expansion (21). Human $CD11c^+$ mDCs that are activated by TSLP induce a robust expansion of autologous $CD4^+$ T cells, which depends on the presentation of self-peptides by HLA class II molecules and costimulation, and these $CD4^+$ T cells activated by autologous TSLP-activated mDCs mainly produce Th1 cytokines (IL-2, IFNγ and TNFα) in addition to modest levels of Th2 cytokines (21).

Recently, TSLP was also implicated to play a role in Th1 and Th17-mediated arthritis. It is demonstrated that TSLP and its receptor enhance Th17-driven arthritis and tissue destruction in experimental arthritis (22). In addition, in synovial fluid of RA patients, TSLP levels are increased in comparison to osteoarthritis patients (23). Moreover, RA synovial fibroblasts were shown to produce TSLP upon Toll-like receptor and TNFα triggering (23, 24).

In this study the potential of TSLP to drive $CD1c^+$ mDC-dependent T cell activation was studied. We measured TSLP in the joints of a large group of RA patients and evaluated the number of TSLPR-expressing $CD1c^+$ mDCs in synovial fluid (SF) compared to cells in the peripheral blood (PB). Furthermore, we investigated cytokine production by TSLP-activated mDCs derived from PB and SF of RA patients and the capacity of TSLP-primed mDCs to activate autologous $CD4^+$ T cells and drive Th1 and Th17 activity.

MATERIALS AND METHODS

Patients

Table 1 shows the demographic and clinical characteristics of the RA and osteoarthritis (OA) patients included in this study. Patients with RA were classified according to the American College of Rheumatology criteria (25). Synovial fluid (SF) samples were obtained from patients attending our outpatient clinic. Collection of samples was approved by the medical ethical committee of the University Medical Center Utrecht. All patients were asked to give and gave their informed consent.

Cytokine assessment

TSLP levels in SF and blood samples were measured with an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D systems, Minneapolis, USA). Levels were measured in SF samples of 50 RA and 24 OA patients. In addition, in these samples soluble IL-7Rα was measured as described elsewhere (26) and IL-6, IL-8, TNFα were measured using commercial assays (Invitrogen, Life Technologies, New York, USA). Prior to analysis, SF samples were treated with hyaluronidase (20U/ml; type IV, Sigma-Aldrich, St. Louis, USA).

Table 1. Demographic and clinical characteristics of RA and OA patients

	SF TSLP analysis		RA PB and SF samples	
	RA	OA	PB	SF
Age (mean ± SD years)	58 ± 13	63 ± 10	52 ± 14	53 ± 15
Sex nr. (male/female)	14/36	9/15	15/18	8/11
Disease duration (mean ± SD years)	15 ± 13	-	14 ± 8	14 ± 6
RF nr. (positive/negative)	29/18	-	18/13	9/8
ESR (mean ± SD mm/hour)	55 ± 44	n.a.	24 ± 28	34 ± 33
CRP (mean ± SD mg/liter)	48 ± 43	n.a.	17 ± 22	20 ± 22

RA = rheumatoid arthritis, OA = osteoarthritis, PB = peripheral blood, SF= synovial fluid, RF = rheumatoid factor, ESR = erythrocyte sedimentation rate, CRP = C-reactive protein. n.a. = not available, - = not applicable.

Supernatants of cultured $CD1c^+$ mDCs, either or not stimulated with recombinant TSLP at 20ng/ml (R&D systems), were analyzed for 44 cytokines including proinflammatory cytokines (IL-1α, IL-1ß, IL-1RA, IL-6, IL-6R, IL-18, IFNα), T cell-differentiating and activating cytokines (IL12p70, IL-23, IL-33, IL-13, IL-10, MIF, IL-7, IL-9, IL-15 IL-21, IL-22, IL-25), chemokines (IL-16, CCL2/MCP1, CCL3/MIP1α, CCL5/RANTES, CCL17/TARC, CCL19/MIP3β, CCL22/MDC, CXCL8/IL-8, CXCL9/MIG, CXCL10/IP-10) and growth factors (OSM, FGF basic, NGF, HGF, GM-CSF, M-CSF, VEGF, IL-11, sICAM-1, OPN, MMP8, TIMP-1, Cathepsin B, Cathepsin L, Cathepsin S) by multiplex immunoassay as described elsewhere (27). Cell supernatants of co-cultured $CD1c^+$ mDCs and $CD4^+$ T cells were analyzed for IFN-γ, IL-4 (assays obtained from Invitrogen) and IL-17 (R&D systems) by ELISA according to the manufacturer's instructions. IL-5 and IL-13 were determined by multiplex immunoassay as described elsewhere (27).

Flow cytometry

TSLPR expression by intra-articular and circulating $CD1c^+$ mDCs was analyzed by isolating mononuclear cells (MNC) from paired peripheral blood (PB) and SF samples from 9 RA patients. TSLP mediated regulation of CD1c+ mDCs surface receptors related to activation (CD80, CD86) and Th2 skewing (OX40L) was analyzed from thawed PB and SF samples from 5 and 3 RA patients, respectively. Expression was measured with fluorescence-activated cell sorting (FACS) analysis using a FACS CANTO II flow cytometer (BD Biosciences, San Jose, USA). Cells were stained with CD1c-PE (Biolegend, San Diego, USA), CD19-PERCP-Cy5.5 (BD Biosciences), and TSLPR-APC (Biolegend) or OX40L-PE (Biolegend), CD80-PE, CD86-PE, CD19-PERCP-Cy5.5 (all BD Biosciences) and CD1c-APC (eBioscience, San Diego, USA). $CD1c^+$ mDCs were gated as CD19 negative and CD1c positive. $CD1c^+$ mDCs were expressed as the percentage of the total MNC population. Unlabelled cells were used as a control. All samples were analyzed using FlowJo software (Tree Star, Ashland, USA).

Cell isolation

Mononuclear cells were isolated from lithium-heparinized PB and SF by density centrifugation using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). Prior to MNC isolation, PB or SF was diluted 1:1 with RPMI 1640 medium (Gibco, Life Technologies, New York, USA) containing penicillin (100U/ml), streptomycin (100µg/ml), and glutamine (2 mM) (all PAA Laboratories, Pasching, Austria). $CD1c^+$ mDCs and $CD4^+$ T cells were isolated from MNC of PB and SF (n=11 and n=5 respectively) by magnetic-activated cell sorting (MACS) using CD1c (BDCA-1)$^+$ dendritic cell and $CD4^+$ T cell isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

Cell cultures

Cells were cultured in RPMI glutamax (Gibco) supplemented with penicillin, streptomycin, and 10%, v/v, human AB serum (GemCell, West Sacramento, USA). Isolated $CD4^+$ T cells were seeded in round-bottomed 96-well plates at a concentration of $0.5x10^6$ cells/ml and stored at 37°C before co-culturing with mDCs. To assess the effect of TSLP on isolated $CD1c^+$ mDCs from PB and SF of RA patients (n=6), $CD1c^+$ mDCs ($0.5x10^6$ cells/ml) were stimulated with 20ng/ml recombinant TSLP (R&D systems) in Sarstedt tubes at 37°C. DC culture supernatants were collected at 20 hours and frozen at -80° until analysis.

To determine the effect of TSLP-primed $CD1c^+$ mDCs (≤10.000 cells/well) on autologous $CD4^+$ T cells (50.000 cells/well), washed TSLP-primed mDCs and unstimulated mDCs derived from PB or SF were added to autologous peripheral blood $CD4^+$ T cells in triplicate at increasing DC:T cell ratios in the absence of additional stimuli. To determine the effect of antigen presentation or costimulation upon TSLP activation, cells were cultured in the presence of anti-HLA-DR mAb (BD Biosciences) or CTLA4-Ig (Bristol-Myers Squibb, New York, USA) respectively. Cells were co-cultured for 6 days and subsequently proliferation and cytokine production were measured. Proliferation was measured by ^{3}H-thymidine incorporation assay at the end of the culture period. ^{3}H-thymidine (1µCi/well; PerkinElmer, Waltham, USA) was added during the last 18 hours of the culture period. In separate cultures, supernatants of co-cultured T cells were re-stimulated with ionomycin (500ng/ml) and phorbol myristate acetate (50ng/ml) (both from Sigma-Aldrich) during the last 24 hours of the culture period. Subsequently, T cell cytokine production was measured by ELISA.

Statistical analysis

The relationship between parameters was evaluated after log-transformation using Pearson rank correlation analysis. Paired sample evaluation was performed using the nonparametric Wilcoxon signed rank test. Unpaired data analysis was performed using the nonparametric Mann-Whitney U test. The analysis was done using SPSS software (IBM, Armonk, USA). Data were considered statistically significant at $p<0.05$.

RESULTS

Increased TSLP concentrations in synovial fluid of RA patients versus OA patients

TSLP concentrations were significantly increased in SF of RA patients compared to OA patients (Figure 1A). Next, it was evaluated whether TSLP levels were associated with disease duration and systemic markers of disease activity (ESR, CRP) or with local inflammatory markers (TNFα, IL-8, IL-6 en soluble IL-7Rα). Intra-articular TSLP levels did not correlate with disease duration and ESR, CRP or intra-articular levels of TNFα, IL-8 or IL-6. However, TSLP significantly correlated with intra-articular levels of soluble IL-7Rα (Figure 1B, r=0.39 Pearson correlation coefficient, $p<0.01$). In addition, we compared intra-articular TSLP concentrations in patients with different medication. Generally, DMARD and biological-treated patients showed lower TSLP concentrations in SF compared to patients who did not receive DMARDs, which was only significant in the case of patients that were on TNFα blockers (Figure 1C). Disease duration or other systemic disease activity markers between these latter groups did not significantly differ.

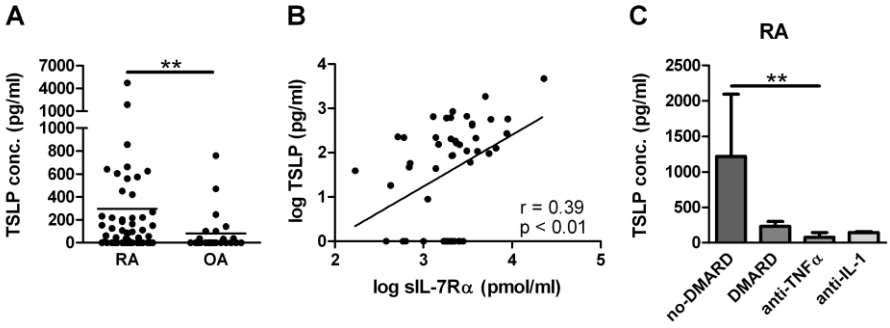


Figure 1. TSLP concentrations are increased in synovial fluid of rheumatoid arthritis patients, correlate with soluble IL-7Rα levels and are influenced by medication. (A) TSLP concentrations were measured in synovial fluid (SF) of rheumatoid arthritis (RA) patients (n=50) compared to osteoarthritis (OA) patients (n=24). (B) TSLP levels significantly correlated with levels of soluble IL-7Rα. Pearson correlation coefficient (r) and p-value are given. (C) TSLP concentrations (mean ± standard error of the mean) in SF of RA patients differed between patients not using DMARDs (no-DMARD, n=5) and treatment groups DMARD (n=28), anti-TNFα (n=8), and anti-IL-1 (n=3). Untreated patients had higher TSLP concentrations compared to DMARD- or biological-treated RA patients. Statistically significant difference of **p<0.01. DMARD, disease-modifying antirheumatic drug; sIL-7Rα, soluble interleukin-7 receptor alpha; TNFα, tumor necrosis factor alpha; TSLP, thymic stromal lymphopoietin.

TSLPR-expressing CD1c$^+$ mDCs are abundantly present in RA synovial fluid

Since mDCs are a major target of TSLP, the number of CD1c$^+$ mDCs in paired samples of SF and PB from RA patients was determined. The number of mDCs, expressed as the percentage of the total MNC population, was significantly increased in SF compared to PB of RA patients (n=9) (Figure 2A). Additionally, mDCs were stained for TSLPR and the percentage of TSLPR-expressing mDCs as well as the mean fluorescent intensity (MFI) of TSLPR expression by these mDCs were determined (Figure 2B). TSLPR was expressed by a majority of mDCs present in PB and SF (Figure 2C). In addition, TSLPR expression level by SF-derived mDCs was significantly higher than that by PB-derived mDCs (Figure 2C).

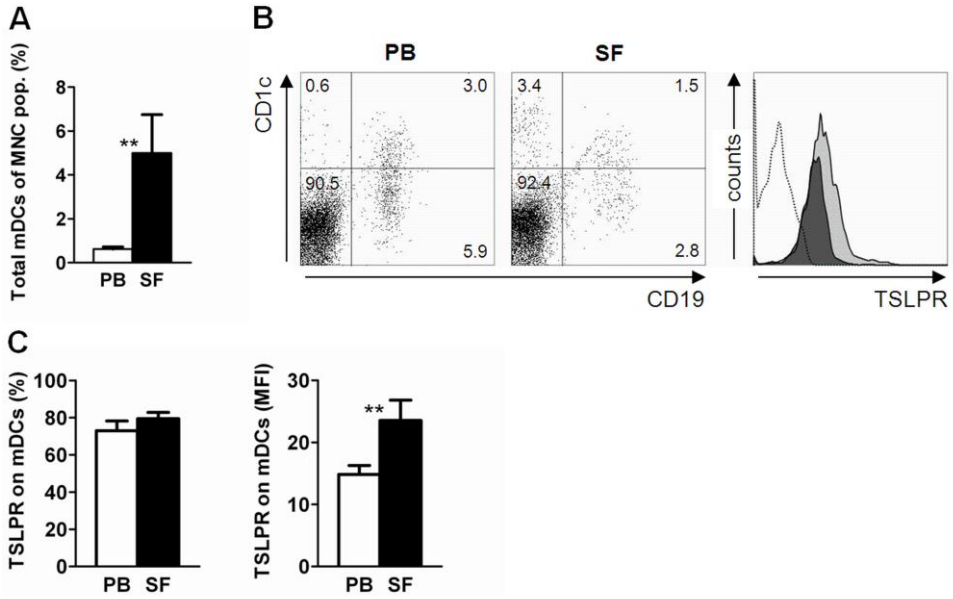


Figure 2. CD1c$^+$ myeloid dendritic cells expressing enhanced levels of TSLPR are increased in synovial fluid compared with peripheral blood of RA patients. (A) CD1c$^+$ myeloid dendritic cell (mDC) numbers are significantly increased in synovial fluid (SF) versus peripheral blood (PB) (n=9, mean ± standard error of the mean). (B) Representative histogram of TSLPR expression on CD1c$^+$ mDCs from PB (filled dark grey) and SF (filled light grey) of an individual RA patient. Dashed line respresents autofluorescence. (C) Percentage TSLPR-expressing CD1c$^+$ mDCs are similar in PB and SF. Mean fluorescent intensity (MFI) of TSLPR expression on CD1c$^+$ mDCs is significantly increased on SF mDCs compared to PB mDCs. Statistically significant difference of **p<0.01. MNC, mononuclear cell; TSLPR, thymic stromal lymphopoietin receptor.

TSLP selectively enhances TARC and MIP1α secretion by CD1c$^+$ mDCs from RA patients

Apart from a significantly increased IL-10 production by PB mDCs, TSLP had no consistent effect on the production of T cell-differentiating cytokines (IL-12, IL-13, and IL-23) by both

PB and SF mDCs (Figure 3A). Nevertheless, TSLP selectively and significantly increased the secretion of CCL17/TARC by both PB and SF mDCs (Figure 3B). Similarly, the secretion of CCL3/MIP1α (macrophage inflammatory protein-1-alpha) was significantly increased upon TSLP stimulation (Figure 3B). In contrast to previous reports, TSLP did not increase the production of MDC and IL-8 (Figure 3B). Apart from upregulating chemokine expression, TSLP only significantly enhanced the secretion of oncostatin M (OSM) by mDCs from PB, but not from SF (PB: mean from 28 to 69 pg/ml, $p<0.05$, SF: from 24 to 46 pg/ml, not significant). In addition, the production of other cytokines was not affected by TSLP as measured by multiplex assay (data not shown).

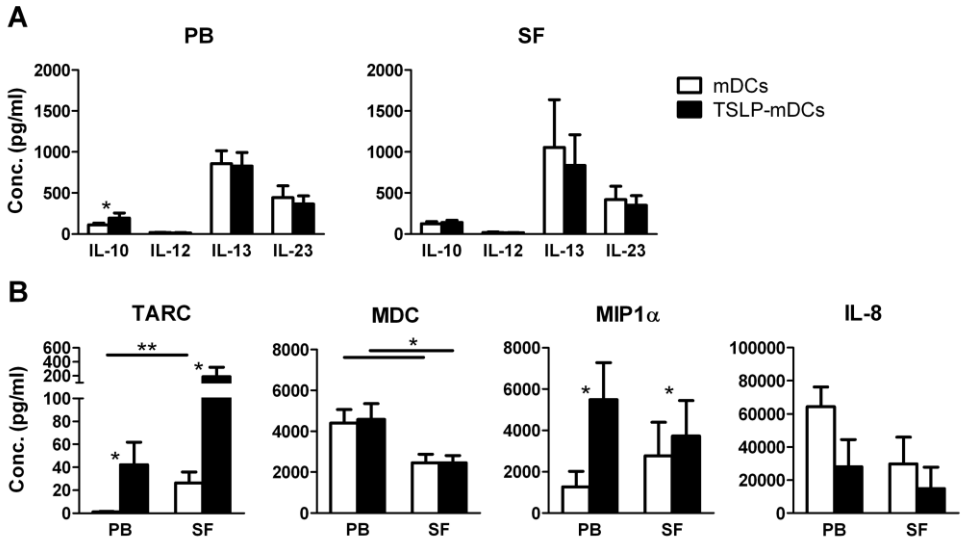


Figure 3. TSLP selectively increases TARC and MIP1α production by peripheral blood and synovial fluid myeloid dendritic cells, but hardly alters the production of other chemokines or T cell-differentiating cytokines. Production of T cell-differentiating cytokines (A) and chemokines (B) by unstimulated $CD1c^+$ myeloid dendritic cells (mDCs) versus TSLP-activated (20ng/ml) $CD1c^+$ mDCs from peripheral blood (PB) and synovial fluid (SF) ($5x10^5$/ml, n=6) was measured after 20 hours of culture (mean ± standard error of the mean). Statistically significant differences of *$p<0.05$ and **$p<0.01$. MDC, macrophage-derived chemokine; MIP1α, macrophage inflammatory protein 1 alpha; TARC, thymus and activation-regulated chemokine; TSLP, thymic stromal lymphopoietin.

TSLP-primed $CD1c^+$ mDCs induce Th1 and Th17 activity in RA patients in addition to Th2 activity

Next, the capacity of TSLP-primed $CD1c^+$ mDCs to activate autologous circulating $CD4^+$ T cells in RA patients was investigated. Compared to PB mDCs, SF mDCs expressed significantly increased levels of CD80 and CD86 costimulatory molecules as well as OX40L,

previously shown to be critical in Th2 skewing (Figure 4). TSLP priming of mDCs resulted in an increased expression of CD80 and CD86 which was most strongly observed on SF mDCs (MFI, CD80: average from 169 to 435, $p<0.05$, and CD86: average from 1443 to 2259, $p<0.05$). SF mDC expression of OX40L was hardly and insignificantly affected by TSLP (MFI, average from 152 to 164, not significant) (Figure 4). Recently, we confirmed these data on the RNA level by microarray analysis showing statistically significant upregulation by TSLP of CD80 and CD86 but not of OX40L (n=5, data not shown).

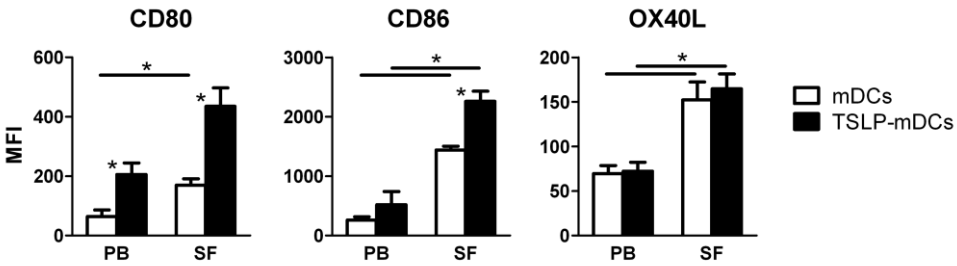


Figure 4. TSLP increases expression of the costimulatory molecules CD80 and CD86, but hardly changes expression of OX40L on CD1c$^+$ myeloid dendritic cells from peripheral blood and synovial fluid. Whereas CD80, CD86 as well as OX40L expression is significantly increased on synovial fluid (SF)-derived myeloid dendritic cells (mDCs) (n=3) compared with peripheral blood (PB)-derived mDCs (n=5), TSLP only upregulates CD80 and CD86 expression. Statistically significant difference of *$p<0.05$. MFI, mean fluorescence intensity; OX40L, OX40 ligand; TSLP, thymic stromal lymphopoietin.

TSLP-primed mDCs from PB and SF potently activated peripheral blood CD4$^+$ T cells compared to unstimulated mDCs (Figure 5A). To study the dependency of T cell activation on antigen presentation and CD80/86 costimulation the effects of HLA class II blockade and CTLA4-Ig were investigated. Activation of CD4$^+$ T cells was completely inhibited in the presence of HLA class II blockade (anti-HLA-DR mAb) or CTLA4-Ig (Figure 5B).

Proliferation was associated with a strong induction of T cell cytokine secretion (Figure 5C). CD4$^+$ T cells activated by TSLP-primed mDCs versus unstimulated mDCs derived from PB significantly produced more IFNγ (mean ± standard error of the mean, from 179 ± 79 to 655 ± 177 pg/ml, $p<0.01$) and IL-17 (from 39 ± 17 to 353 ± 182 pg/ml, $p<0.05$), in addition to IL-4 (from 17 ± 12 to 246 ± 162 pg/ml, $p<0.01$), IL-5 (from 257 ± 251 to 758 ± 332 pg/ml, $p<0.05$) and IL-13 (from 270 ± 229 to 838 ± 334 pg/ml, $p<0.05$). Moreover, peripheral blood CD4$^+$ T cells activated by TSLP-primed mDCs derived from SF produced higher cytokine levels and secreted significantly more IFNγ, IL-17, IL-5 and IL-13 compared to unstimulated mDCs (IFNγ: from 601 ± 342 to 1867 ± 1154 pg/ml, $p<0.05$; IL-17: from 363 ± 282 to 1382 ± 1034 pg/ml, $p<0.05$; IL-5: from 205 ± 145 to 1023 ± 525 pg/ml, $p<0.05$; IL-13: from 341 ±

182 to 1384 ± 626 pg/ml, p<0.05). The production of IL-4 was enhanced, however not statistically significant (from 193 ± 96 to 775 ± 557 pg/ml, not significant) (Figure 5C).

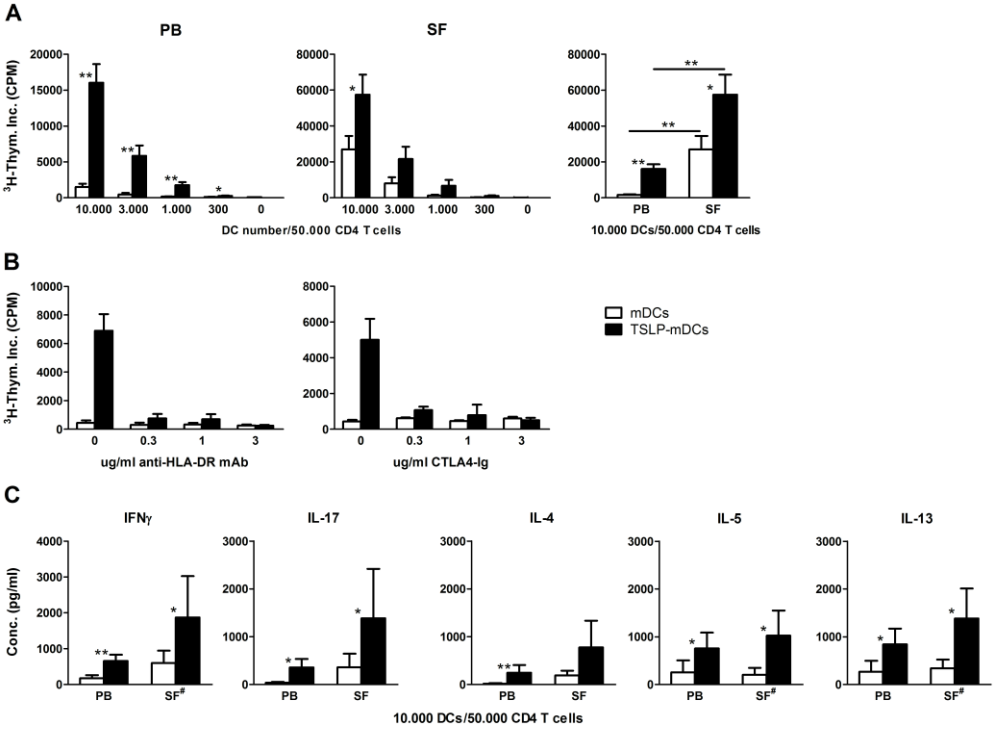


Figure 5. Robust TSLP-primed CD1c$^+$ myeloid dendritic cell-driven CD4$^+$ T cell activation in rheumatoid arthritis is critically dependent on antigen presentation and costimulation. (A) TSLP-primed CD1c$^+$ myeloid dendritic cells (mDCs) derived from peripheral blood (PB; n=11) and synovial fluid (SF; n=5) strongly activate autologous circulating CD4$^+$ T cells. (B) CD4$^+$ T cell activation is blocked in the presence of anti-HLA-DR mAb or CTLA4-Ig (DC:T cell ratio 1:10). (C) IFNγ, IL-17, IL-4, IL-5 and IL-13 are highly produced by peripheral blood CD4$^+$ T cells activated by TSLP-primed mDCs compared with unstimulated mDCs from PB (n=11, with n=6 for IL-5 and IL-13). CD4$^+$ T cells activated by TSLP-primed mDCs derived from SF produced higher cytokine levels (n=5). Statistically significant differences of *p<0.05 and **p<0.01. # indicates the condition 3000 DCs/50.000 CD4$^+$ T cells. Interferon gamma (IFNγ) production at 10.000 DCs plateaued: 3428 ± 1809 and 4327 ± 1874 pg/ml medium versus TSLP, respectively. IL, interleukin; TSLP, thymic stromal lymphopoietin.

DISCUSSION

In this study, it is demonstrated that concentrations of TSLP in SF of RA patients are elevated and that TSLP activates CD1c (BDCA-1)$^+$ mDCs that express enhanced levels of TSLPR. TSLP-induced activation of CD1c$^+$ mDCs, in particular those from SF, is associated by a selective increased production of T cell-attracting chemokines and a strongly increased capacity to activate CD4$^+$ T cells secreting Th1 and Th17 cytokines, in addition to Th2 cytokines.

In agreement with a previous study showing increased TSLP levels in SF in a small number of RA patients, the present study confirms that TSLP levels are significantly increased in the inflamed joints of RA patients compared to levels in OA patients (23). A further analysis of the present data revealed some patients with remarkably high TSLP concentrations in SF, whereas others showed lower TSLP concentrations. We did not find significant correlations between intra-articular TSLP and disease duration or systemic markers of inflammation (ESR, CRP). However, our data suggest that TSLP concentrations might be influenced by treatment and/or local inflammation. Indeed intra-articular TSLP levels were higher in patients that did not receive DMARDs compared to patients on DMARDs or biologicals. Since proinflammatory mediators such as TNFα can induce TSLP (23), downregulation of these proinflammatory cytokines by medication could explain reduced TSLP levels. We did not find a correlation between intra-articular TSLP and local concentrations of several proinflammatory cytokines (including TNFα, IL-6 and IL-8). TSLP concentrations, however, did correlate with intra-articular concentrations of soluble IL-7Rα, an antagonist of IL-7, which was recently shown to be increased in RA patients and to correlate with fibroblast activation which is regulated by multiple inflammatory cytokines (26, 28).

Activation of CD1c$^+$ mDCs from PB and SF of RA patients by TSLP resulted in a significant increased and selective secretion of TARC, which secretion was more pronounced by SF mDCs. These data are in line with the reported increased production of TARC by healthy TSLP-activated CD11c$^+$ mDCs (14, 20). Intra-articular TARC production might facilitate recruitment of Th2 cells (29), Th17 cells (30), and regulatory T (Treg) cells (31) to the joint, since all express CC chemokine receptor 4 (CCR4), which is the receptor for TARC. To our knowledge the present study is also the first to report TSLP-induced increase in production of MIP1α by mDCs. Increased levels of MIP1α (a ligand of the CCR5 receptor) as well as an accumulation of the CCR5 positive Th1 target cells are detected in SF of RA patients (32). Together this suggests that increased TSLP mediates attraction of Th1, Th2, and Th17 cells as well as Treg cells to the joints by the stimulation of chemokine secretion by intra-articular CD1c$^+$ mDCs. In contrast to TARC and MIP1α, no induction of MDC and IL-8 by TSLP-activated CD1c$^+$ mDCs from PB and SF of RA patients was observed. This differs from others that showed MDC and IL-8 expression upon TSLP stimulation of mDCs from PB. Differences might be due to the fact that CD11c$^+$ mDCs were used in this latter study (20), which consist

of both $CD1c^+$ and $CD1c^-$ mDC subsets. Whether these mDC subsets respond differently to TSLP with respect to MDC and IL-8 expression remains to be demonstrated.

RA is characterized by increased numbers of activated proliferating T cells and a predominance of Th1 and Th17 cells intra-articularly (33). We therefore investigated whether increased TSLP levels in the joints of RA patients could contribute to the induction of Th1 and Th17 cell activity in addition to the previously reported Th2 cell activation. The present study shows that in the absence of additional stimuli TSLP-primed $CD1c^+$ mDCs from PB of RA patients have a remarkable capacity to induce proliferation of autologous $CD4^+$ T cells. Moreover, $CD4^+$ T cell proliferation induced by TSLP-primed $CD1c^+$ mDCs from SF was much stronger than that induced by TSLP-primed $CD1c^+$ mDCs from PB. Even more remarkable, $CD1c^+$ mDCs derived from SF stimulated $CD4^+$ T cells to produce an array of proinflammatory cytokines such as IFNγ and IL-17. Considering the capacity of TSLP to activate $CD1c^+$ mDCs, this suggests that TSLP could be an important trigger for natural occurring intra-articular mDCs.

The TSLP-enhanced proliferation that was associated with an increased secretion of IFNγ and IL-17 implies that TSLP-primed mDCs promote Th1 and Th17 activity, in addition to Th2 activity (indicated by an increased IL-4, IL-5 and IL-13 secretion). These data are in line with the finding that TSLP is involved in homeostatic expansion of $CD4^+$ T cells by activation of autologous $CD11c^+$ mDCs (21). Similar to the latter study, the T cell expansion in our study appears to be dependent on the presentation of self-peptides and costimulation as T cell activation was completely prevented by HLA class II blockade and prevention of CD80/86-mediated costimulation by CTLA4-Ig. Furthermore, the expansion was not associated by skewing of the T cell response, in line with the fact that TSLP did not change secretion of T cell-differentiating cytokines by $CD1c^+$ mDCs. Based on the presented data, we hypothesize that TSLP in joints of RA patients has the capacity to expand autoreactive $CD4^+$ T cells via its effects on $CD1c^+$ mDCs. The local inflammatory environment in RA joints likely mediates the skewing that results in Th1 and Th17 predominance. Recent studies support this notion and demonstrated that the skewing of T cell responses towards Th1 activity by TSLP-primed $CD11c^+$ mDCs is critically dependent on IL-12 (20, 34). In addition, TSLP and Toll-like receptor 3-mediated signalling were shown to promote Th17 activity (35).

In summary, we demonstrated that increased intra-articular TSLP concentrations in RA can potently activate TSLPR-expressing $CD1c^+$ mDCs from SF to secrete enhanced levels of T cell-attracting chemokines and to potently induce Th1 and Th17 activity, in addition to Th2 activity. Together with the previously demonstrated critical role of TSLP in experimental Th17-driven arthritis this suggests that TSLP and its receptor could play an essential role in the immunopathology of RA patients.

ACKNOWLEDGEMENT

This research was performed within the framework of CTMM, the Center for Translational Molecular Medicine (www.ctmm.nl), and the Dutch Arthritis Foundation, project TRACER (grant 04I-202). We acknowledge the MultiPlex Core Facility of the Laboratory for Translational Immunology (University Medical Center Utrecht) for performing the multiplex immunoassays, which were developed and validated in-house.

REFERENCES

1. Feldmann M, Brennan FM, Maini RN. Rheumatoid arthritis. Cell 1996;85(3):307-10.
2. Morita Y, Yamamura M, Kawashima M, Harada S, Tsuji K, Shibuya K, et al. Flow cytometric single-cell analysis of cytokine production by CD4+ T cells in synovial tissue and peripheral blood from patients with rheumatoid arthritis. Arthritis Rheum 1998;41(9):1669-76.
3. Chabaud M, Durand JM, Buchs N, Fossiez F, Page G, Frappart L, et al. Human interleukin-17: A T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. Arthritis Rheum 1999;42(5):963-70.
4. Cornelissen F, van Hamburg JP, Lubberts E. The IL-12/IL-23 axis and its role in Th17 cell development, pathology and plasticity in arthritis. Curr Opin Investig Drugs 2009;10(5):452-62.
5. van Roon JA, van Roy JL, Duits A, Lafeber FP, Bijlsma JW. Proinflammatory cytokine production and cartilage damage due to rheumatoid synovial T helper-1 activation is inhibited by interleukin-4. Ann Rheum Dis 1995;54(10):836-40.
6. Thomas R, Davis LS, Lipsky PE. Rheumatoid synovium is enriched in mature antigen-presenting dendritic cells. J Immunol 1994;152(5):2613-23.
7. Cavanagh LL, Boyce A, Smith L, Padmanabha J, Filgueira L, Pietschmann P, et al. Rheumatoid arthritis synovium contains plasmacytoid dendritic cells. Arthritis Res Ther 2005;7(2):R230-40.
8. Jongbloed SL, Lebre MC, Fraser AR, Gracie JA, Sturrock RD, Tak PP, et al. Enumeration and phenotypical analysis of distinct dendritic cell subsets in psoriatic arthritis and rheumatoid arthritis. Arthritis Res Ther 2006;8(1):R15.
9. Moret FM, Hack CE, van der Wurff-Jacobs KM, de Jager W, Radstake TR, Lafeber FP, et al. Intra-articular CD1c-expressing myeloid dendritic cells from rheumatoid arthritis patients express a unique set of T cell-attracting chemokines and spontaneously induce Th1, Th17 and Th2 cell activity. Arthritis Res Ther 2013;15(5):R155.
10. Satpathy AT, Wu X, Albring JC, Murphy KM. Re(de)fining the dendritic cell lineage. Nat Immunol 2012;13(12):1145-54.
11. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. Annu Rev Immunol 2013;31:563-604.
12. Dzionek A, Fuchs A, Schmidt P, Cremer S, Zysk M, Miltenyi S, et al. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. J Immunol 2000;165(11):6037-46.
13. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of monocytes and dendritic cells in blood. Blood 2010;116(16):e74-80.
14. Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. Nat Immunol 2002;3(7):673-80.
15. Al-Shami A, Spolski R, Kelly J, Keane-Myers A, Leonard WJ. A role for TSLP in the development of inflammation in an asthma model. J Exp Med 2005;202(6):829-39.

16. Zhou B, Comeau MR, De Smedt T, Liggitt HD, Dahl ME, Lewis DB, et al. Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. Nat Immunol 2005;6(10):1047-53.

17. Pandey A, Ozaki K, Baumann H, Levin SD, Puel A, Farr AG, et al. Cloning of a receptor subunit required for signaling by thymic stromal lymphopoietin. Nat Immunol 2000;1(1):59-64.

18. Park LS, Martin U, Garka K, Gliniak B, Di Santo JP, Muller W, et al. Cloning of the murine thymic stromal lymphopoietin (TSLP) receptor: Formation of a functional heteromeric complex requires interleukin 7 receptor. J Exp Med 2000;192(5):659-70.

19. Reche PA, Soumelis V, Gorman DM, Clifford T, Liu M, Travis M, et al. Human thymic stromal lymphopoietin preferentially stimulates myeloid cells. J Immunol 2001;167(1):336-43.

20. Ito T, Wang YH, Duramad O, Hori T, Delespesse GJ, Watanabe N, et al. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. J Exp Med 2005;202(9):1213-23.

21. Watanabe N, Hanabuchi S, Soumelis V, Yuan W, Ho S, de Waal Malefyt R, et al. Human thymic stromal lymphopoietin promotes dendritic cell-mediated CD4+ T cell homeostatic expansion. Nat Immunol 2004;5(4):426-34.

22. Hartgring SA, Willis CR, Dean CE, Jr., Broere F, van Eden W, Bijlsma JW, et al. Critical proinflammatory role of thymic stromal lymphopoietin and its receptor in experimental autoimmune arthritis. Arthritis Rheum 2011;63(7):1878-87.

23. Koyama K, Ozawa T, Hatsushika K, Ando T, Takano S, Wako M, et al. A possible role for TSLP in inflammatory arthritis. Biochem Biophys Res Commun 2007;357(1):99-104.

24. Ozawa T, Koyama K, Ando T, Ohnuma Y, Hatsushika K, Ohba T, et al. Thymic stromal lymphopoietin secretion of synovial fibroblasts is positively and negatively regulated by Toll-like receptors/nuclear factor-kappaB pathway and interferon-gamma/dexamethasone. Mod Rheumatol 2007;17(6):459-63.

25. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31(3):315-24.

26. Badot V, Durez P, Van den Eynde BJ, Nzeusseu-Toukap A, Houssiau FA, Lauwerys BR. Rheumatoid arthritis synovial fibroblasts produce a soluble form of the interleukin-7 receptor in response to pro-inflammatory cytokines. J Cell Mol Med 2011;15(11):2335-42.

27. de Jager W, te Velthuis H, Prakken BJ, Kuis W, Rijkers GT. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. Clin Diagn Lab Immunol 2003;10(1):133-9.

28. Moret FM, Badot V, Lauwerys BR, van Roon JA. Intraarticular soluble interleukin-7 [corrected] receptor levels are increased in patients with rheumatoid arthritis and correlate with local mediators of inflammation: comment on the article by Pickens et al [letter] [published erratum appears in Arthritis Rheum 2012;64:1302]. Arthritis Rheum 2012;64:594–5.

29. Sallusto F, Lenig D, Mackay CR, Lanzavecchia A. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. J Exp Med 1998;187(6):875-83.

30. Leipe J, Grunke M, Dechant C, Reindl C, Kerzendorf U, Schulze-Koops H, et al. Role of Th17 cells in human autoimmune arthritis. Arthritis Rheum 2010;62(10):2876-85.

31. Baatar D, Olkhanud P, Sumitomo K, Taub D, Gress R, Biragyn A. Human peripheral blood T regulatory cells (Tregs), functionally primed CCR4+ Tregs and unprimed CCR4- Tregs, regulate effector T cells using FasL. J Immunol 2007;178(8):4891-900.

32. Suzuki N, Nakajima A, Yoshino S, Matsushima K, Yagita H, Okumura K. Selective accumulation of CCR5+ T lymphocytes into inflamed joints of rheumatoid arthritis. Int Immunol 1999;11(4):553-9.

33. van Roon JA, Bijlsma JW, Lafeber FP. Diversity of regulatory T cells to control arthritis. Best Pract Res Clin Rheumatol 2006;20(5):897-913.

34. Yokoi T, Amakawa R, Tanijiri T, Sugimoto H, Torii Y, Amuro H, et al. Mycobacterium bovis Bacillus Calmette-Guerin suppresses inflammatory Th2 responses by inducing functional alteration of TSLP-activated dendritic cells. Int Immunol 2008;20(10):1321-9.

35. Tanaka J, Watanabe N, Kido M, Saga K, Akamatsu T, Nishio A, et al. Human TSLP and TLR3 ligands promote differentiation of Th17 cells with a central memory phenotype under Th2-polarizing conditions. Clin Exp Allergy 2009;39(1):89-100.

4

TNFα downregulates the capacity of TSLP-activated CD1c^{+} myeloid dendritic cells from rheumatoid arthritis patients to promote Th2 activity

Frederique M. Moret, Timothy R.D.J. Radstake, Janneke Tekstra,
Johannes W.J. Bijlsma, Floris P.J.G. Lafeber, Joel A.G. van Roon

Submitted for publication

ABSTRACT

Objective. To assess the capacity of thymic stromal lympopoietin (TSLP)-induced TNFα production by $CD1c^+$ myeloid dendritic cells (mDCs) on TSLP-mediated mDC and $CD4^+$ T cell activation.

Methods. mDCs from RA patients were activated with TSLP for 20 hours with or without anti-TNFα mAb. Cytokine production was measured by multiplex immunoassay. The T cell-activating potential of these mDCs was assessed by adding them to autologous T cells and analysing proliferation and cytokine production.

Results. TSLP significantly stimulated TNFα secretion by mDCs from blood and synovial fluid. TNFα blockade during TSLP stimulation of mDCs did not affect T cell-differentiating cytokine secretion, but significantly decreased MIP1α and slightly enhanced TARC production, significantly altering the ratio of these chemokines. Compared to TSLP-mDCs, anti-TNFα treated TSLP-mDCs did not affect IFNγ and IL-17 production of co-cultured T cells, but significantly increased IL-4 production and reduced the IFNγ:IL-4 ratio.

Conclusion. TNFα produced by TSLP-mDCs inhibits Th2 responses by favouring Th1-attracting chemokine production and preventing T cells to secrete Th2 cytokines, contributing to TSLP-induced immunopathology in RA. As such, stimulation of TSLP-mediated Th2 activity by TNFα blockade might facilitate inhibition of disease activity.

INTRODUCTION

Tumor necrosis factor α (TNFα) is shown to be an important inflammatory mediator in rheumatoid arthritis (RA) and targeting this cytokine has become a successful strategy in the treatment of a considerable number of RA patients (1). The activation of immune cells like monocytes, mast cells, fibroblasts, and T cells by inflammatory mediators in joints of RA patients can all contribute to the production of TNFα (1). TNFα displays a wide array of inflammatory functions including leukocyte and endothelial activation, induction of cytokine and chemokine production, suppression of regulatory T cells and synovial fibroblast activation (1).

Triggering of RA synovial fibroblast by proinflammatory cytokines like TNFα and IL-1β and toll-like receptor ligands is shown to result in increased production of thymic stromal lymphopoietin (TSLP) (2). TSLP is in general strongly associated with Th2-mediated diseases via activation of $CD11c^+$ myeloid dendritic cells (mDCs) (3). However, TSLP has recently been considered a potent inflammatory mediator in patients with RA. TSLP levels as well as $CD1c^+$ mDCs, a subset of $CD11c^+$ mDCs, are both significantly increased in joints of RA patients (4). In addition, TSLP robustly activates $CD1c^+$ mDCs from RA patients to produce T cell-attracting chemokines and strongly increases Th1, Th17 and Th2 activity (4). In support of a role in RA, abrogation of TSLP-induced responses in experimental arthritis driven by cells of innate (neutrophils and macrophages) as well as acquired immunity (Th17, germinal center B cells) was shown to prevent inflammation and immunopathology (5).

TNFα is indicated to play an essential role in dendritic cell (DC) activation and DC survival (6). In vitro blockade of TNFα during lipopolysaccharide stimulation of monocyte-derived-DCs results in increased apoptosis and impaired DC maturation (6, 7). In the present study, we assessed the capacity of TSLP to regulate TNFα production by natural occurring $CD1c^+$ mDCs from RA patients. By blocking autocrine TNFα, the influence of TNFα in regulating TSLP-mediated mDC activation and the consequence for autologous $CD4^+$ T cell responses was investigated.

MATERIALS AND METHODS

Patients

Synovial fluid (SF) was obtained from 6 RA patients during effusion of the knee. From an additional 23 RA patients that did not receive anti-TNFα treatment 70cc peripheral blood (PB) was collected. RA was classified according to the American College of Rheumatology criteria. Collection of blood samples was approved by the medical ethical committee of the University Medical Center Utrecht. All patients gave their written informed consent.

Cell isolation

Mononuclear cells (MNC) were isolated from PB and SF by density centrifugation using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). Prior to MNC isolation, PB or SF was diluted 1:1 with RPMI 1640 medium (Gibco, Life Technologies, NY, USA) containing penicillin (100U/ml), streptomycin (100µg/ml), and glutamine (2 mM) (all PAA Laboratories, Pasching, Austria). PB- and SF-derived $CD1c^+$ mDCs and PB-derived $CD4^+$ T cells were isolated from the MNC fraction by magnetic-activated cell sorting (MACS) using CD1c (BDCA-1)$^+$ dendritic cell and $CD4^+$ T cell isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

Cell cultures

All cells were cultured in RPMI glutamax (Gibco) supplemented with penicillin, streptomycin and 10% (v/v) human AB serum (GemCell, West Sacramento, USA). The mDCs were cultured at a cell concentration of $0.5x10^6$ cells/ml with or without recombinant TSLP at 20 ng/ml (R&D systems) and with or without anti-TNFα mAb (adalimumab, 10 µg/ml) for 20 hours in Sarstedt tubes at 37°C. mDC culture supernatants were collected at 20 hours and frozen at -80° until analysis.

The effects of the differently cultured PB-derived mDC conditions were assessed on PB-derived autologous $CD4^+$ T cells. For this, isolated T cells were seeded in round-bottomed 96-well plates at a concentration of $0.25x10^6$ cells/ml and stored at 37°C in full culture medium before co-culturing with the mDCs. TSLP-activated mDCs washed twice with or without anti-TNFα mAb and unstimulated mDCs were added to the autologous T cells (mDC:T cell ratio 1:20, unless indicated otherwise) in triplicate in the absence of additional stimuli and co-cultured for 6 days (n=5). Subsequently, proliferation was measured by ^3H-thymidine incorporation (1µCi/well added during the last 18 hours of a culture period; PerkinElmer, Waltham, USA) and in separate co-cultures T cell cytokine production was measured by ELISA.

Cytokine assessment

In the first series of experiments supernatants of cultured mDCs from PB and SF of RA patients (n=6), stimulated with or without TSLP for 20 hours, were analysed for TNFα production. Secondly, supernatants of cultured mDCs from PB of RA patients (n=7), stimulated with or without TSLP in the presence or absence of anti-TNFα, were analysed for 12 cytokines by multiplex immunoassay as described in detail elsewhere (8). Cytokines comprised proinflammatory cytokines (IL-1ß, IL-6, TNFα), T cell differentiating and activating cytokines (IL-12p70, IL-23, IL-33, IL-13, IL-10, IL-25), and chemokines (CCL3/MIP1α, CCL17/TARC).

TNFα, IFN-γ, IL-4 (assays from Invitrogen, Life Technologies, NY, USA) and IL-17 (R&D systems) production was measured by ELISA in cell supernatants of co-cultured mDCs with T cells re-stimulated with ionomycin (500 ng/ml) and phorbol myristate acetate (50 ng/ml) (both from Sigma-Aldrich) during the last 24 hours of the culture period. Assays were performed according to the manufacturer's instructions.

Flow cytometry

Regulation of mDCs surface receptors related to activation (CD40, CD83, CD80) and Th2 skewing (OX40L) was analysed from at least 6 RA patients by flow cytometry. Fluorescence-activated cell sorting (FACS) analysis was performed using a FACS CANTO II flow cytometer (BD Biosciences, San Jose, USA). Cultured mDCs were stained with CD40-PE (BD Biosciences), OX40L (Biolegend, San Diego, USA), CD19-PERCP (Biolegend), and CD1c-APC (eBioscience, San Diego, USA). mDCs were gated as CD19 negative and CD1c positive. Isotype antibodies were used as controls. All samples were analyzed using FlowJo software (Tree Star, Ashland, USA).

Statistical analysis

Differences between conditions were assessed by paired statistics using the nonparametric Wilcoxon signed rank test using SPSS software version 20.0 (IBM, Armonk, USA). Data were considered statistically significant at $p<0.05$.

RESULTS

Blockade of TNFα produces bij TSLP activated mDCs decreases MIP1α: TARC ratio's

Activation of isolated mDCs from PB and SF of RA patients by TSLP significantly increased TNFα production (Figure 1A). The influence of this TSLP-induced TNFα on the activation of mDCs was investigated by blocking secreted TNFα during TSLP stimulation of mDCs from PB of RA patients. In this set of experiments, TSLP increased TNFα production by mDCs to comparable levels as observed in the initial experiment and TNFα production measured by Luminex was totally blocked in the presence of anti-TNFα mAb (Figure 1B). TSLP stimulation of mDCs did not affect the production of Th1 (IL-12), Th2 (IL-10, IL-13, IL-33, IL-25) and Th17 (IL-1β, IL-6, IL-23) differentiating cytokines except for an upregulation of IL-1β, although levels were very low (Figure 1B). Addition of anti-TNFα mAb also did not affect these cytokines. On the other hand, the production of TARC (CCR4 ligand; Th2 cell attractant) and MIP1α (CCR5 ligand; Th1 cell attractant) was significantly increased upon TSLP stimulation of

mDCs (Figure 1C). TARC was modestly enhanced and MIP1α was strongly downregulated upon TNFα blockade; as a consequence the ratio MIP1α:TARC was robustly and significantly decreased.

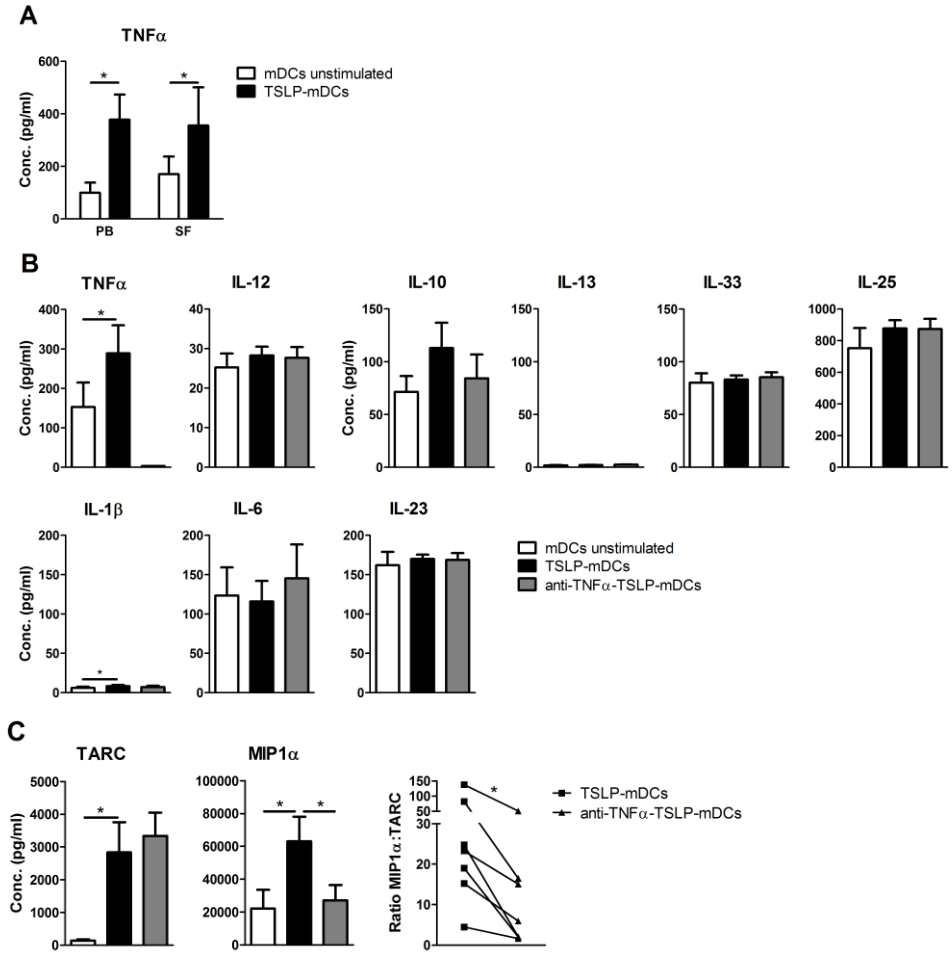


Figure 1. TNFα blockade decreases the ratio MIP1α:TARC during TSLP stimulation of peripheral blood myeloid dendritic cells from rheumatoid arthritis patients. (A) TSLP-activated myeloid dendritic cells (mDCs) from peripheral blood (PB) and synovial fluid (SF) of rheumatoid arthritis patients (n=6) produced significantly increased TNFα levels. (B) Blockade of TNFα during TSLP stimulation of mDCs does not affect Th1(IL-12), Th17 (IL-1b, IL-6, IL-23) or Th2 (IL-10, IL-13, IL-33, IL-25) differentiating cytokine production (n=7). (C) The ratio MIP1α:TARC is decreased by blocking TNFα production during TSLP stimulation of mDCs. Statistically significant difference of *p<0.05. IL, interleukin; MIP1α, macrophage inflammatory protein 1 alpha; TARC, thymus and activation-regulated chemokine; TNFα, tumor necrosis factor alpha; TSLP, thymic stromal lymphopoietin.

TNFα blockade during TSLP stimulation of mDCs enhances their Th2-inducing potential

TSLP stimulation of mDCs significantly increased their capacity to induce $CD4^+$ T cell proliferation (Figure 2A). TNFα blockade during TSLP stimulation of mDCs did not significantly affect this T cell stimulatory capacity (Figure 2A). The enhanced IFNγ (Th1 activity) and IL-17 (Th17 activity) production upon TSLP-mDC activation were also not affected by blocking TNFα during TSLP stimulation of mDCs (Figure 2B). However, the TSLP-enhanced IL-4 production was significantly increased upon TNFα blockade during TSLP-mDC stimulation (Figure 2B). Consequently, TNFα blockade during mDC stimulation with TSLP altered the T cell balance between IFNγ and IL-4 cytokine secretion, in favour of IL-4, decreasing the IFNg:IL4 ratio (Figure 2C).

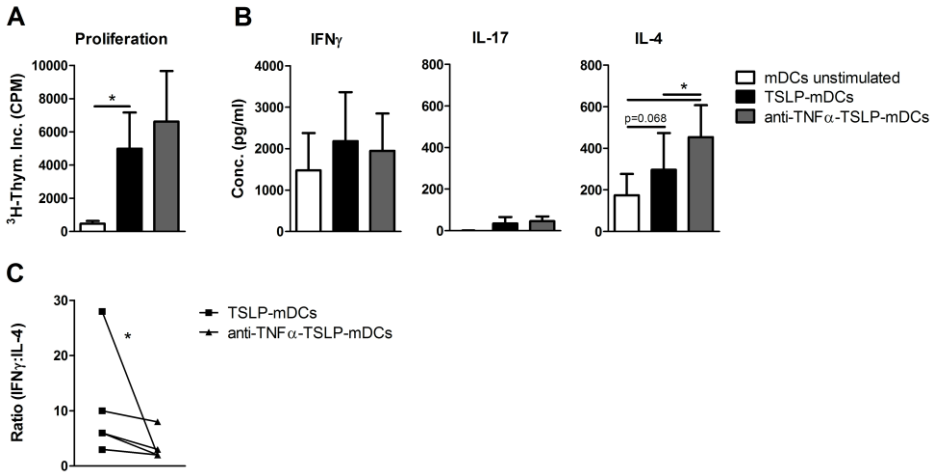


Figure 2. Blockade of TNFα enhances the Th2 priming potential of TSLP-activated myeloid dendritic cells. (A) Anti-TNFα monoclonal antibodies slightly but insignificantly enhances TSLP-mDC-induced $CD4^+$ T cell proliferation (n=5). (B) TNFα blockade during TSLP-priming of mDCs significantly increases IL-4 production without affecting IFNγ and IL-17 production (n=5) resulting in a significantly decreased (C) IFNγ:IL-4 ratio upon TNFα blockade. Statistically significant difference of *$p<0.05$. IFNγ, interferon gamma; mDCs, myeloid dendritic cells; TNFα, tumor necrosis factor alpha; TSLP, thymic stromal lymphopoietin.

TNFα blockade during TSLP stimulation of mDCs decreases CD40, CD83 and OX40L expression

Previously, upregulation of OX40 ligand (OX40L) was demonstrated to be involved in enhanced IL-4 production by $CD4^+$ T cells upon activation by TSLP-stimulated $CD11c^+$ mDCs (3). Regulation of OX40L, in addition to activation markers CD40, CD83 and CD80, by TNFα

blockade during TSLP stimulation of mDCs was determined to understand the observed Th2 skewing response (Figure 3A, representative histograms). TSLP-activated-mDCs significantly increased the percentage positive cells for CD40, CD83 and CD80, but did not affect OX40L expression (Figure 3B). This was also observed for expression levels (mean fluorescence intensity, Figure 3C). TNFα blockade during TSLP stimulation of mDCs decreased the percentage of CD40 and CD83 positive cells (Figure 3B) and significantly decreased the expression levels of OX40L, CD40, CD83 and CD80 although the latter only modestly (Figure 3C).

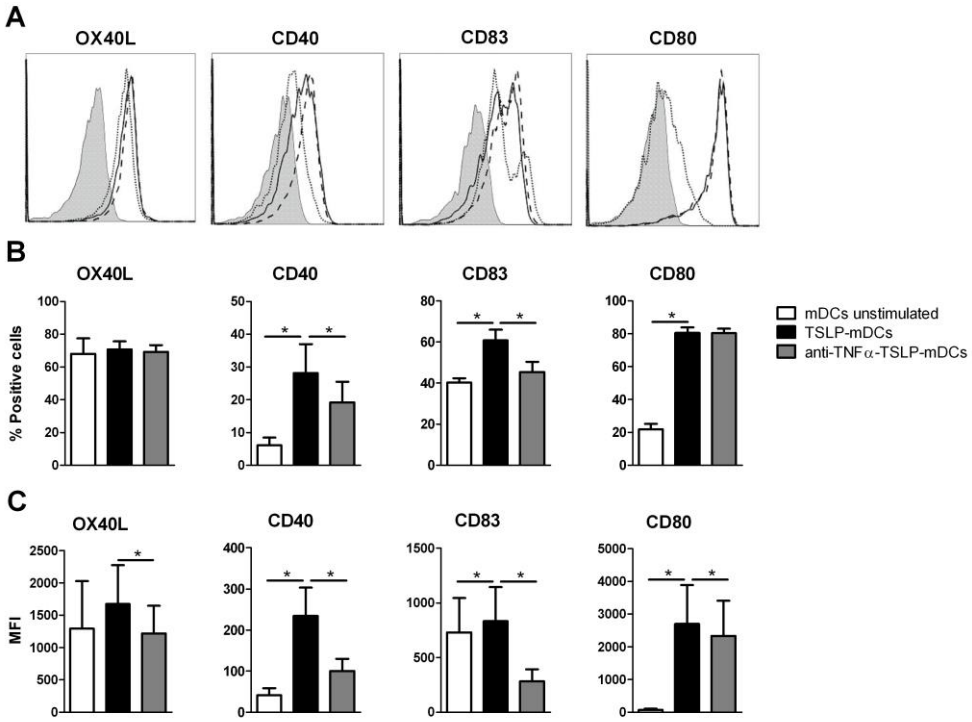


Figure 3. TNF blockade during TSLP stimulation of peripheral blood myeloid dendritic cells from rheumatoid arthritis patients specifically decreases the expression of costimulatory molecule CD40 and maturation marker CD83. (A) Representative histograms of isotype control (filled histogram) OX40L (n=8), CD40 (n=8), CD83 (n=6) and CD80 (n=6) expression. Expression of unstimulated myeloid dendritic cells (mDCs) (dotted line) and TSLP-activated mDCs in the absence (dashed line) or presence of TNF blockade (solid line) are indicated. (B) TSLP increases the number of positive mDCs for CD40, CD83, and CD80, without affecting the number of mDCs expressing OX40L. TNFα blockade during TSLP stimulation of mDCs decreases the number of CD80 and CD83 positive cells, but hardly affects the number of mDCs expressing OX40L and CD80. (C) TSLP stimulation of mDCs increases CD40, CD83 and CD80 expression and slightly increases OX40L expression. Blockade of TNFα during TSLP stimulation of mDCs decreases OX40L, CD40, CD83 and CD80 expression, although CD80 expression is minimally affected. Statistically significant difference of *$p<0.05$. MFI, mean fluorescence intensity; OX40L, OX40 ligand; TNFα, tumor necrosis factor alpha; TSLP, thymic stromal lymphopoietin.

DISCUSSION

The present study demonstrates that TNFα may limit TSLP-induced Th2 responses in RA patients. Blockade of autocrine TNFα produced by TSLP-activated mDCs of RA patients skews the mDC chemokine secretion towards the Th2-attracting chemokine TARC and stimulates co-cultured T cells towards IL-4 production. The increased induction of Th2 activity upon TNFα blockade of TSLP-activated mDCs is associated with downregulation of OX40L, CD40, and CD83 expression.

Recently, several studies indicated a proinflammatory role for TSLP in both animal and human in vitro arthritis models (4, 5). In these studies, TSLP induced vigorous T cell activation via PB and SF-mDCs from RA patients at a 1:5 DC/T cell ratio and this was associated by strong IFNγ (Th1) and IL-17 (Th17) cytokine secretion in addition to Th2 cytokine secretion (IL-4, IL-5, IL-13). This was somewhat unexpected since in atopic patients TSLP-mDCs induce IL-4 secretion more prominently. In the present study, TSLP specifically increases TNFα secretion next to TARC and MIP1α by $CD1c^+$ mDCs derived from RA patients. Previous studies investigating PB-derived $CD11c^+$ mDCs did not observe an induction of TNFα upon TSLP stimulation (3). This suggests that TSLP-induced TNFα production by $CD1c^+$ mDCs could be specific for $CD1c^+$ mDCs or restricted to mDCs derived from RA patients. In view of this and the presumed role of TNFα in DC function, the capacity of TNFα to regulate DC function was investigated.

The present study shows that autocrine TNFα production induced by TSLP facilitates MIP1α production, since TNFα blockade resulted in significantly decreased MIP1α levels. In contrast, TNFα blockade slightly increases TARC levels, significantly altering the MIP1α/TARC balance in favour of TARC. Given the fact that TARC is a CCR4 ligand that mediates attraction of Th2 cells as well as regulatory T (Treg) cells (9, 10), and MIP1α mediates attraction of CCR5-expressing Th1 cells (11), this suggests that TNFα blockade favours migration of Th2 and Treg cells induced by TSLP-activated $CD1c^+$ mDCs. Since TNFα blockade in RA patients is demonstrated to enhance Treg cell function and to diminish Th1 and Th17 cell numbers (12), the favoured attraction of Th2 and Treg cells to joints of RA patients could be an additional beneficial mechanism of TNFα blockade in RA patients (12, 13). In addition to shifting the chemokine balance, TNFα blockade of TSLP-activated-mDCs favours IL-4 secretion by co-cultured autologous $CD4^+$ T cells and is associated by a decreased IFNγ:IL-4 ratio. These data are in line with a previous study demonstrating that the activation of allogeneic $CD3^+$ T cells by LPS-activated monocyte-derived DCs in the presence of anti-TNFα increased their IL-4 and IL-10 production (6). Since Th2 activity, in particular that induced by IL-4, is shown to inhibit numerous other proinflammatory responses in RA (13), this effect could contribute to the beneficial effects of TNFα blockade in RA.

TSLP is well described to induce Th2 activity via upregulation of OX40L on $CD11c^+$ mDCs (3), which defines a less clear mDC population than $CD1c^+$ mDCs (14). Our data demonstrate

that TSLP-activated CD1c$^+$ mDCs from RA patients increase Th2 cell activity, while OX40L upregulation on TSLP-activated mDCs is hardly found. TNFα blockade did not upregulate and even modestly inhibited OX40L expression on RA CD1c$^+$ mDCs. Therefore, the increased Th2 response seems to be OX40L independent. By contrast, downregulation of CD40 and CD83 seem to indicate reduced maturation of the mDCs that is associated with increased IL-4 secretion. In this respect the less affected CD80 as well as CD86 expression (data not shown), which are both strongly upregulated by TSLP, could explain the maintained T cell stimulatory capacity as witnessed by T cell proliferation. In line with the present data, in vivo administration of anti-TNFα in RA patients is shown to result in a reduced CD83 expression on circulating CD11c$^+$ DCs associated with clinical improvement of the patients (15).

In conclusion, the present study demonstrates that TNFα prevents TSLP-induced Th2 responses. TNFα blockade in RA patients could have a beneficial effect by modulating TSLP-activated CD1c$^+$ mDCs to increase the chemotaxis of Th2 cells to the joints and to skew the T cell response towards Th2 activity that is related to a reduced mDC maturation status. Apart from preventing TNFα-induced TSLP production, this could add to the anti-inflammatory potential of TNFα blockade.

ACKNOWLEDGEMENT

This research was performed within the framework of CTMM, the Center for Translational Molecular Medicine (www.ctmm.nl), and the Dutch Arthritis Foundation, project TRACER (grant 04I-202). We acknowledge the MultiPlex Core Facility of the Laboratory for Translational Immunology (University Medical Center Utrecht) for performing the multiplex immunoassays, which were developed and validated in-house.

REFERENCES

1. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. N Engl J Med 2011;365(23):2205-19.
2. Ozawa T, Koyama K, Ando T, Ohnuma Y, Hatsushika K, Ohba T, et al. Thymic stromal lymphopoietin secretion of synovial fibroblasts is positively and negatively regulated by Toll-like receptors/nuclear factor-kappaB pathway and interferon-gamma/dexamethasone. Mod Rheumatol 2007;17(6):459-63.
3. Ito T, Liu YJ, Arima K. Cellular and molecular mechanisms of TSLP function in human allergic disorders--TSLP programs the "Th2 code" in dendritic cells. Allergol Int 2012;61(1):35-43.
4. Moret FM, Hack CE, van der Wurff-Jacobs KM, Radstake TR, Lafeber FP, van Roon JA. Thymic stromal lymphopoietin (TSLP): a novel proinflammatory mediator in rheumatoid arthritis that potently activates CD1c myeloid dendritic cells to attract and stimulate T cells. Arthritis Rheum 2014; doi: 10.1002/art.38338. [Epub ahead of print].
5. Hartgring SA, Willis CR, Dean CE, Jr., Broere F, van Eden W, Bijlsma JW, et al. Critical proinflammatory role of thymic stromal lymphopoietin and its receptor in experimental autoimmune arthritis. Arthritis Rheum 2011;63(7):1878-87.
6. Baldwin HM, Ito-Ihara T, Isaacs JD, Hilkens CM. Tumour necrosis factor alpha blockade impairs dendritic cell survival and function in rheumatoid arthritis. Ann Rheum Dis 2010;69(6):1200-7.
7. van Lieshout AW, Barrera P, Smeets RL, Pesman GJ, van Riel PL, van den Berg WB, et al. Inhibition of TNF alpha during maturation of dendritic cells results in the development of semi-mature cells: a potential mechanism for the beneficial effects of TNF alpha blockade in rheumatoid arthritis. Ann Rheum Dis 2005;64(3):408-14.
8. de Jager W, te Velthuis H, Prakken BJ, Kuis W, Rijkers GT. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. Clin Diagn Lab Immunol 2003;10(1):133-9.
9. Perros F, Hoogsteden HC, Coyle AJ, Lambrecht BN, Hammad H. Blockade of CCR4 in a humanized model of asthma reveals a critical role for DC-derived CCL17 and CCL22 in attracting Th2 cells and inducing airway inflammation. Allergy 2009;64(7):995-1002.
10. Faustino L, da Fonseca DM, Takenaka MC, Mirotti L, Florsheim EB, Guereschi MG, et al. Regulatory T cells migrate to airways via CCR4 and attenuate the severity of airway allergic inflammation. J Immunol 2013;190(6):2614-21.
11. Patel DD, Zachariah JP, Whichard LP. CXCR3 and CCR5 ligands in rheumatoid arthritis synovium. Clin Immunol 2001;98(1):39-45.
12. Nie H, Zheng Y, Li R, Guo TB, He D, Fang L, et al. Phosphorylation of FOXP3 controls regulatory T cell function and is inhibited by TNF-alpha in rheumatoid arthritis. Nat Med 2013;19(3):322-8.
13. van Roon JA, van Roy JL, Duits A, Lafeber FP, Bijlsma JW. Proinflammatory cytokine production and cartilage damage due to rheumatoid synovial T helper-1 activation is inhibited by interleukin-4. Ann Rheum Dis 1995;54(10):836-40.
14. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of monocytes and dendritic cells in blood. Blood 2010;116(16):e74-80.

15. Balanescu A, Radu E, Nat R, Regalia T, Bojinca V, Ionescu R, et al. Early and late effect of infliximab on circulating dendritic cells phenotype in rheumatoid arthritis patients. Int J Clin Pharmacol Res 2005;25(1):9-18.

5

The efficacy of abatacept to reduce synovial T cell activation by CD1c myeloid dendritic cells is overruled by T cell-activating cytokines

Frederique M. Moret, Johannes W.J. Bijlsma, Floris P.J.G. Lafeber, Joel A.G. van Roon

Submitted for publication

ABSTRACT

Objective To investigate the potential of abatacept to inhibit the vigorous CD1c myeloid dendritic cell (mDC)-driven activation of naïve and memory T cells in the presence of T cell-activating cytokines like IL-7.

Methods Naïve (Tn), central memory (Tcm), and effector memory (Tem) T cell subsets from peripheral blood (PB) of healthy controls (HC) and from PB and synovial fluid (SF) of rheumatoid arthritis (RA) patients were isolated. T cell subsets were cocultured with autologous TSLP-primed CD1c mDCs with or without abatacept (CTLA4-Ig) and/or IL-7 and subsequently T cell proliferation and cytokine production were measured.

Results Percentages of CD4 T cell subsets from the circulation of HC and RA patients were comparable and mainly consisted of Tn and Tcm cells, whereas SF of RA patients mainly consisted of Tcm and Tem cells. Activation of these T cell subsets by TSLP-primed mDCs from PB was completely blocked by abatacept. IL-7 strongly increased T cell activation and overruled the inhibitory capacity of abatacept. This IL-7-induced reversal was associated with robust induction of IFNγ, TNFα, and IL-17 secretion. Similarly, CD4 T cell proliferation induced by TSLP-primed mDCs from SF of RA patients was strongly blocked by abatacept and also vigorously overruled by IL-7.

Conclusion Our data indicate that the presence of T cell-activating cytokines, like IL-7, as present in joints of RA patients, reduce the inhibitory capacity of abatacept on mDC-driven T cell activation. This mechanism adds to explaining the partial and sometimes absence response to abatacept therapy in a subset of patients.

INTRODUCTION

The introduction of biological agents for the treatment of rheumatoid arthritis (RA) has advanced treatment of RA patients with an inadequate response to conventional disease-modifying anti-rheumatic drugs (DMARDS) including methotrexate (MTX). After TNF inhibitors, many other biological agents became available including those that target interactions of T cells and antigen presenting cells, like abatacept (CTLA4-Ig). Trials comparing anti-TNF treatment (adalimumab or infliximab) to abatacept treatment have demonstrated similar clinical efficacy for both treatments in RA patients with an inadequate response to MTX (1, 2). Additionally, several clinical trials have demonstrated the efficacy and safety of abatacept on background MTX for the treatment of RA patients with an inadequate response to MTX alone or to anti-TNF therapy (1-4).

Abatacept is a recombinant human fusion protein consisting of the extracellular domain of human CTLA4 combined with a modified Fc region of IgG1. CTLA4 is normally expressed on T cells upon activation acting as a negative regulator for T cell activation (5). CD4 T cell activation is accomplished after T cell receptor (TCR) and CD28 ligation induced by human leukocyte antigen (HLA) class II molecules, presenting antigen to the TCR and CD80/86 interaction with CD28 as costimulation by the antigen-presenting cell (APC). CTLA4 and CD28 both interact with CD80/86 molecules on APCs, but CTLA4 interacts with a higher affinity and avidity to these ligands. While competition between CTLA4 and CD28 for ligand binding has previously been described as the most essential role for CTLA4, recent data suggested that CTLA4 inhibits T cell activation by mediating trans-endocytosis of CD80 and CD86 thereby inducing T cell anergy (5, 6). Accordingly, the mechanism of action of abatacept is preventing T cell activation by blocking CD80/86 costimulation, although mechanisms independent of CD28 ligation and directly dependent on CD80/86 signaling have been described for CTLA4-Ig as well (7).

To prevent self-reactive T cell responses, the activation of naïve CD4 T cells is strictly regulated and strongly dependent on CD80/86 costimulation in combination with antigen presentation (8). The role for costimulation in the activation of memory CD4 T cells is less prominent, however optimal activation of memory T cells is shown to also require CD80/86 costimulation, since memory T cells are susceptible to CTLA4-Ig mediated inhibition (9). Memory T cells can be subdivided into central memory and effector memory T cells, based on the expression of CD27 (10). Effector memory T cells, lacking CD27, are shown to respond to TCR-triggering also in the absence of CD80/86 costimulation, while central memory T cells need this costimulation (10). Abatacept effectiveness in RA patients reflects the need for costimulation in the activation of naïve and at least part of the memory T cells (9).

Thymic stromal lymphopoietin (TSLP) and its family member IL-7 are both elevated in synovial fluid (SF) of RA patients compared to controls and are shown to contribute to immunopathology of experimental arthritis models (11-14). Recently, we have demonstrated

that TSLP potently activates CD4 T cells via the activation of CD1c myeloid dendritic cells (mDCs), which depends on antigen presentation and CD80/86 costimulation (11). CD1c mDCs are professional and extremely potent APCs that are abundantly present in joints of RA patients. These CD1c mDCs are strongly activated by TSLP to induce T cell attraction and T cell activation (11, 15).

Interleukin-7 (IL-7) targets naïve as well as memory T cells and has the capacity to enhance T cell homeostatic expansion and survival of especially memory CD4 T cells (16, 17). IL-7 directly acts on T cells and strongly induces T cell-dependent activation of APCs (12). Recently, homeostatic cytokines like IL-7 and IL-15 were shown to decrease the signalling threshold for TCR activation and thereby caused T cell proliferation induced by recognition of auto-antigens. This cytokine-driven homeostatic T cell expansion induced by recognition of auto-antigens drives naïve T cells to differentiate into memory cells that have a diminished requirement for CD28 costimulation and favours differentiation into inflammatory Th1 pathways rather than differentiation into Th2 pathways (18, 19). As both IL-7 and IL-15 cytokines are increased in joints of RA patients and can exacerbate inflammation, this could be an important disease mechanism in RA leading to continuous activation of autoreactive T cells (12, 18, 20).

Blocking CD80/CD86 costimulation by abatacept to prevent T cell activation in RA patients has been an effective treatment in a considerable number of patients. However, not all patients benefit to the same extent from this therapy and some are even non-responders (1-4). To investigate potential mechanisms responsible for the (partial) unresponsiveness to this treatment, the potential of abatacept to inhibit the vigorous CD1c mDC-driven activation of autologous naïve and memory CD4 T cells in the presence and absence of IL-7 was examined. Activation of autologous CD4 T cells that occurs in joints of RA patients was mimicked by TSLP-primed CD1c mDCs as both TSLP and (CD1c) mDCs (21) were shown to be increased in RA joints and were shown to be critical in T cell-driven arthritis.

MATERIALS AND METHODS

Patients

Peripheral blood (PB) was collected from 8 healthy controls (HC) and 7 RA patients. Additionally, synovial fluid (SF) samples were obtained from 12 RA patients attending our outpatient clinic. RA was classified according to the American College of Rheumatology criteria (22). Sample collection was according to ethical regulations of the University Medical Center Utrecht. All patients gave their informed consent.

Cell isolation

Mononuclear cells (MNC) were isolated from lithium-heparinized PB and SF by density centrifugation using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). Prior to MNC isolation, PB or SF was diluted 1:1 with RPMI 1640 medium (Gibco, Life Technologies, New York, USA) containing penicillin (100U/ml), streptomycin (100µg/ml), and glutamine (2 mM) (all PAA Laboratories, Pasching, Austria). CD1c mDCs and CD4 T cells were isolated from MNC of PB and SF by magnetic-activated cell sorting (MACS) using CD1c (BDCA-1)$^+$ dendritic cell and CD4$^+$ T cell isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. In addition, CD45RO/CD27-defined CD4 T cell subsets were sorted by FASC-sorting as described below.

Cell cultures

All cells were cultured in RPMI glutamax (Gibco) supplemented with penicillin, streptomycin, and 10% (v/v) human AB serum (GemCell, West Sacramento, USA). The CD1c mDCs were cultured at a cell concentration of $0.5x10^6$ cells/ml and stimulated with recombinant TSLP at 20 ng/ml (R&D systems, Minneapolis, USA) at 37°C. In triplicate, isolated autologous CD4 T cells and their subsets were added to the TSLP-primed CD1c mDCs (mDC:T cell ratio 1:10, unless indicated otherwise).
Abatacept efficacy (10 µg/ml or as indicated otherwise, Bristol-Myers Squibb, New York, USA) was assessed in TSLP-primed mDC/CD4 T cell cocultures in the absence and presence of IL-7 (10 ng/ml or as indicated otherwise, Peprotech). Cells were cocultured for 6 days and subsequently proliferation was measured by ^{3}H-thymidine incorporation (1µCi/well added during the last 18 hours of a culture period; PerkinElmer, Waltham, USA).

Cytokine assessment

TNFα, IFN-γ, IL-4 (assays obtained from Invitrogen, Life Technologies, NY, USA), and IL-17 (R&D systems) release was measured in cell supernatants of cocultured mDCs and CD4 T cells re-stimulated with ionomycin (500 ng/ml) and phorbol myristate acetate (50 ng/ml) (both from Sigma-Aldrich) during the last 24 hours of the culture period. Culture supernatants were collected at day 6 and frozen at -80°C until analysis by ELISA according to the manufacturer's instructions.

Flow cytometry

Fluorescence-activated cell sorting (FACS) analysis was performed using a FACSAria flow cytometer (BD Biosciences, San Jose, USA) for sorting T cells and a FACSCanto II flow cytometer (BD Biosciences) was used for analysing cell surface marker expression. Human PB-derived CD4 T cells can be divided into naïve and memory T cells based on CD45RO expression and memory T cells can be further subdivided into central memory and effector

memory T cells based on CD27 expression (9, 10). To sort and analyse naïve T cells (CD27+CD45RO-; Tn), central memory T cells (CD27+CD45RO+; Tcm) and effector memory T cells (CD27-CD45RO+; Tem), CD4 T cells were stained with CD4-PERCP (Biolegend, San Diego, USA), CD27-APC (Immunotools, Friesoythe, Germany), and CD45RO-FITC (BD Biosciences). Isotype controls were used as controls. All samples were analysed using FlowJo software (Tree Star, Ashland, USA).

Statistical analysis

Paired sample evaluation was performed by the nonparametric Wilcoxon signed rank test. Non-paired sample evaluation was performed by the nonparametric Mann-Whitney U test. The analysis was done using SPSS software version 20.0 (IBM, Armonk, USA). Data were considered statistically significant at $p<0.05$.

RESULTS

Abatacept-inhibited naïve and memory CD4 T cell activation is completely abrogated by IL-7

CD4 T cells activated by autologous TSLP-primed CD1c mDCs from PB of RA patients in different ratio's proliferate strongly and this T cell proliferation was completely blocked in the presence of 10 µg/ml abatacept (Figure 1A, representative RA patient and Figure 1B mean of 5 donors at DC/T cell ratio 1:5). Abatacept almost completely blocked this proliferation already at a concentration of 0.1 µg/ml (data not shown) illustrating the strong capacity of this drug to block T cell costimulation.

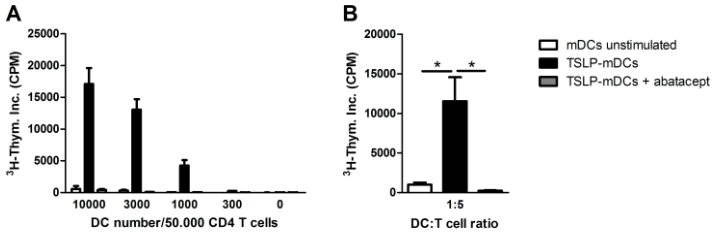


Figure 1. TSLP-primed myeloid dendritic cell driven CD4 T cell proliferation is completely blocked by abatacept (CTLA4-Ig). (A) In contrast to unstimulated myeloid dendritic cells (mDCs), thymic stromal lymphopoietin (TSLP)-primed mDCs (20 ng/ml TSLP stimulation for 20 hours) potently activate CD4 T cells from the peripheral blood of rheumatoid arthritis (RA) patients at DC/T cell ratios as low as 1:50. Proliferation induced by TSLP-mDCs is completely blocked by abatacept (10 µg/ml). Representative RA patient is shown with error bars indicating triplicates. (B) TSLP-primed mDCs strongly activate CD4 T cell proliferation at a DC/T cell ratio 1:5 that is completely blocked in the presence of abatacept. Mean ± standard error of the mean of healthy controls and RA patients (n=5). Statistically significant difference of *$p<0.05$.

The composition of circulating CD4 T cells from HC and RA patients is comparable and consist mainly of naïve T (Tn) cells and central memory T (Tcm) cells with a low percentage effector memory T (Tem) cells (Figure 2A). To investigate the efficacy of abatacept to inhibit activation of these T cell subsets in the presence and absence of T cell-activating cytokines like IL-7, the CD4 T cell subsets were sorted by their distinct CD27 and CD45RO expression (Figure 2B). Activation of Tn, Tcm, and Tem cells by TSLP-primed mDCs was totally inhibited by abatacept (Figure 2C). However, IL-7 strongly overruled the capacity of abatacept to inhibit Tn activation as well as Tcm and Tem cell activation (Figure 2C). Tcm and Tem cell activation in the presence of IL-7, despite the presence of abatacept, was even significantly increased versus Tcm and Tem cell activation that was induced by TSLP-primed mDCs (Figure 2C). Associated with the induction of proliferation, IFNγ and TNFα production by activated Tn, Tcm, and Tem cells was inhibited by abatacept, but strongly enhanced by the addition of IL-7 despite the presence of abatacept (Figure 2D).

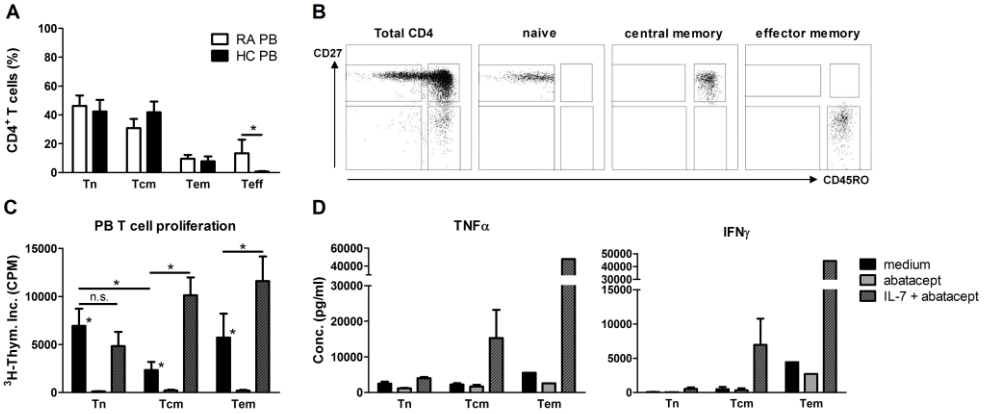


Figure 2. Abatacept-induced inhibition of T cell activation by TSLP-primed myeloid dendritic cells from peripheral blood is overruled by IL-7. (A) T cell subsets from the circulation of RA patients versus healthy controls (HC) are comparable (n=5), except for T effector (Teff) cells. (B) Representative dot plots of sorted T cell subsets based on CD27 and CD45RO expression from peripheral blood (PB) of HC. (C) Thymic stromal lymphopoietin (TSLP)-primed myeloid dendritic cell-induced proliferation of naïve (Tn), central memory (Tcm) as well as effector memory (Tem) T cells from PB of HC (n=5) is completely blocked by abatacept, whereas interleukin-7 (IL-7) robustly increased T cell proliferation in the presence of abatacept. (D) The production of tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) by Tn, Tcm, and Tem cells activated by TSLP-primed myeloid dendritic cells from PB of HC is strongly increased by IL-7 even despite the presence of abatacept (Tn and Tcm n=3, Tem n=1, paired samples). Statistically significant difference of *$p<0.05$.

Effector T cells negative for CD45RO and CD27 (data not shown) were only cultured twice for all conditions due to low cell numbers. These T cells showed a lack of response to TSLP-primed mDCs (220 ± 116 counts per minute (cpm)), and subsequently in the presence of abatacept the proliferation was low (67 ± 26 cpm). However, the presence of IL-7 robustly increased the T cell proliferation of these effector T cells (12864 ± 406 cpm), which was only slightly affected by abatacept (10794 ± 3975 cpm).

Although the IL-7 concentrations (10 ng/ml) in our experimental conditions may exceed those measured in the RA joint (1 ng/ml) it is generally appreciated that such concentrations are present in the cellular microenvironment in the RA synovial tissue. The efficacy of abatacept on naïve and memory T cells stimulated with TSLP-primed mDCs was also tested case wise in the presence of lower IL-7 concentrations. Similar results were observed at 1 ng/ml although the stimulatory capacity was somewhat lower (data not shown). Increasing the abatacept concentration to reported plasma concentration in patients treated with abatacept (30 µg/ml) did not increase the inhibitory capacity of abatacept (data not shown).

IL-7 robustly enhances synovial fluid CD4 T cell activation that is hardly affected by abatacept

T cell composition based on CD27 and CD45RO expression differs significantly between PB and SF of RA patients (Figure 3A). CD4 T cells derived from SF of RA patients hardly contain naïve T cells and consist mainly of memory T cells with Tcm as the largest population (Figure 3A). Activation of SF-derived CD4 T cells by TSLP-primed SF-derived mDCs is significantly inhibited in the presence of abatacept and robustly increased in the presence of IL-7 (Figure 3B). Although abatacept reduced SF-derived T cell activation in the presence of IL-7, the T cell stimulatory capacity of IL-7 in the presence of abatacept remained strong and significantly increased compared to the absence of IL-7. Th1, Th17, and Th2 cytokine production by activated SF-derived CD4 T cells was only observed in the presence of IL-7 with a high induction of IFNγ, TNFα, and IL-17 and an insignificant IL-4 production (Figure 3C). The robust IL-7-induced cytokine production by SF-derived CD4 T cells was hardly affected by the presence of abatacept (Figure 3C).

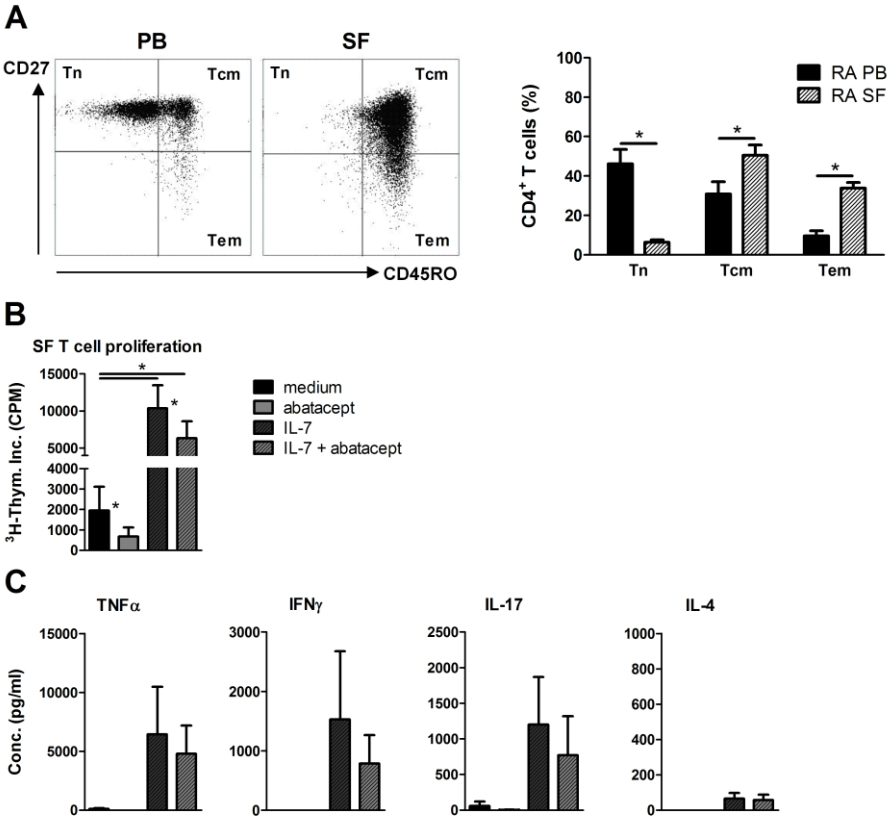


Figure 3. The capacity of abatacept to inhibit TSLP-primed myeloid dendritic cell driven T cell activation from synovial fluid of rheumatoid arthritis patients is much less effective in the presence of IL-7 leaving IL-7-induced T cell activation largely unaffected. (A) Representative dot plot of CD4 T cells comprising of CD27+CD45RO- naïve (Tn), CD27+CD45RO+ central memory (Tcm), and CD27-CD45RO+ effector memory (Tem) T cell subsets in peripheral blood (PB) and synovial fluid (SF) of a rheumatoid arthritis (RA) patient, showing that CD4 T cells from PB of RA patients mainly consist of Tn and Tcm cells, whereas CD4 T cells from SF of RA patients predominantly contain Tcm and Tem cells (n=5). (B) Blockade of TSLP-primed myeloid dendritic cell-induced CD4 T cell proliferation from SF of RA patients by abatacept is overruled by interleukin-7 (IL-7) (n=7). (C) TSLP-primed myeloid dendritic cells hardly trigger proinflammatory T cell cytokine production by SF T cells, however this is robustly driven by IL-7, which in its turn is minimally affected by abatacept (n=3). Statistically significant difference of *$p<0.05$.

DISCUSSION

The present study demonstrates that abatacept potently blocks TSLP-primed mDC driven naïve and memory T cell activation. The capacity of abatacept to completely block PB-derived T cell activation from healthy controls is abrogated by the presence of the T cell-activating cytokine IL-7, most strongly affecting central memory and effector memory T cells. Abatacept inhibition of T cells derived from SF of RA patients that mainly express a memory phenotype was also strongly overruled by IL-7.

The activation of autologous CD4 T cells by TSLP-primed mDCs is demonstrated to be dependent on presentation of self-antigen via HLA class II molecules and CD80/86 costimulation (11, 23). Also, in conditions where T cell receptor (TCR)-HLA class II interactions occur it is shown that memory T cells have a higher threshold for CD80/86 costimulation compared to naïve T cells to become activated (8). This is explained by the expression of CTLA-4 induced upon CD4 and HLA class II molecule interactions, which continuously recycles from intracellular stores to the cell surface in memory T cells, but not in naive T cells. Consequently, low TCR engagement with HLA class II molecules does prevent memory T cell activation reducing the risk for inappropriate responses (8). In line with these observations the present study demonstrates that in response to TSLP-primed mDCs, Tcm cells showed a significantly lower T cell proliferation compared to Tn cells. However, Tem cell activation induced by TSLP-primed mDCs resulted in a stronger proliferation comparable to naïve T cells. This is in line with previous observations showing that Tem cells are shown to respond to TCR-triggering by anti-CD3 monoclonal antibody in the absence of CD80/86 costimulation and have a high capacity to respond to antigen recall responses when compared to Tn and Tcm cells (10). Irrespective of the strength of T cell activation induced by TSLP-primed mDCs, the presence of abatacept completely blocked Tn, Tcm as well as Tem proliferation suggesting that the TSLP-primed mDC induced T cell activation was clearly dependent on CD80/86 costimulation. The effector function of Tem cells was observed by their ability to produce high IFNγ and TNFα cytokine levels in the absence and presence of IL-7.

Recently, it is shown that IL-7 decreases the signaling threshold for TCR activation and thereby enabling T cell responses to respond to self-antigens resulting in increased proliferation and preferential Th1 cell differentiation (18). The observed reduced capacity of abatacept to prevent T cell activation in the presence of IL-7 suggests that IL-7-induced T cell activation reduces the threshold for CD80/86 costimulation as well, in particular for memory T cells. This is in line with the capacity of IL-7 to induce homeostatic T cell expansion that drives naïve T cells to differentiate into memory cells that have a diminished requirement for CD80/86 costimulation (18). As T cells derived from the joints of RA patients mainly consist of memory T cells, IL-7 strongly activated these synovial fluid T cells to proliferate and to induce Th1 and Th17 cytokine production instead of Th2 cytokine

production. In this context, the presence of IL-7 overruled the inhibitory capacity of abatacept to prevent T cell activation. In addition to IL-7, similar results were observed for IL-15-induced T cell activation mediated by TSLP-primed mDCs (data not shown). Therefore, these data suggest that T cell-activating cytokines in general may similarly affect abatacept efficacy, but this remains to be demonstrated.

In conclusion, our data suggest that the presence of T cell-activating cytokines, like IL-7, in joints of RA patients reduces the inhibitory capacity of abatacept on mDC-driven T cell activation. As a subset of RA patients does not respond sufficiently or have an absent response to this therapy, our data suggest that this might be attributed to the overexpression of proinflammatory cytokines like IL-7, overruling abatacept responses. The identification of RA patients that are characterized by cytokine driven T cell-activation would require other treatment strategies, for instance those that target specific cytokines or cytokine-induced signaling pathways.

ACKNOWLEDGEMENT

This research was performed within the framework of CTMM, the Center for Translational Molecular Medicine (www.ctmm.nl), and the Dutch Arthritis Foundation, project TRACER (grant 04I-202).

REFERENCES

1. Schiff M, Keiserman M, Codding C, Songcharoen S, Berman A, Nayiager S, et al. Efficacy and safety of abatacept or infliximab vs placebo in ATTEST: a phase III, multi-centre, randomised, double-blind, placebo-controlled study in patients with rheumatoid arthritis and an inadequate response to methotrexate. Ann Rheum Dis 2008;67(8):1096-103.
2. Schiff M, Weinblatt ME, Valente R, van der Heijde D, Citera G, Elegbe A, et al. Head-to-head comparison of subcutaneous abatacept versus adalimumab for rheumatoid arthritis: two-year efficacy and safety findings from AMPLE trial. Ann Rheum Dis 2014;73(1):86-94.
3. Genovese MC, Schiff M, Luggen M, Becker JC, Aranda R, Teng J, et al. Efficacy and safety of the selective co-stimulation modulator abatacept following 2 years of treatment in patients with rheumatoid arthritis and an inadequate response to anti-tumour necrosis factor therapy. Ann Rheum Dis 2008;67(4):547-54.
4. Bathon J, Robles M, Ximenes AC, Nayiager S, Wollenhaupt J, Durez P, et al. Sustained disease remission and inhibition of radiographic progression in methotrexate-naive patients with rheumatoid arthritis and poor prognostic factors treated with abatacept: 2-year outcomes. Ann Rheum Dis 2011;70(11):1949-56.
5. Walker LS, Sansom DM. The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses. Nat Rev Immunol 2011;11(12):852-63.
6. Qureshi OS, Zheng Y, Nakamura K, Attridge K, Manzotti C, Schmidt EM, et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. Science 2011;332(6029):600-3.
7. Deppong CM, Bricker TL, Rannals BD, Van Rooijen N, Hsieh CS, Green JM. CTLA4Ig inhibits effector T cells through regulatory T cells and TGF-beta. J Immunol 2013;191(6):3082-9.
8. Metz DP, Farber DL, Taylor T, Bottomly K. Differential role of CTLA-4 in regulation of resting memory versus naive CD4 T cell activation. J Immunol 1998;161(11):5855-61.
9. Ndejembi MP, Teijaro JR, Patke DS, Bingaman AW, Chandok MR, Azimzadeh A, et al. Control of memory CD4 T cell recall by the CD28/B7 costimulatory pathway. J Immunol 2006;177(11):7698-706.
10. Schiott A, Lindstedt M, Johansson-Lindbom B, Roggen E, Borrebaeck CA. CD27- CD4 memory T cells define a differentiated memory population at both the functional and transcriptional levels. Immunology 2004;113(3):363-70.
11. Moret FM, Hack CE, van der Wurff-Jacobs KMG, Lafeber FPJG, van Roon JAG. TSLP: a novel potent proinflammatory mediator that activates myeloid dendritic cells to stimulate Th1 and Th17 activity in rheumatoid arthritis. Arthritis Rheum 2014; doi: 10.1002/art.38338. [Epub ahead of print].
12. van Roon JA, Verweij MC, Wijk MW, Jacobs KM, Bijlsma JW, Lafeber FP. Increased intraarticular interleukin-7 in rheumatoid arthritis patients stimulates cell contact-dependent activation of CD4(+) T cells and macrophages. Arthritis Rheum 2005;52(6):1700-10.
13. Hartgring SA, Willis CR, Dean CE, Jr., Broere F, van Eden W, Bijlsma JW, et al. Critical proinflammatory role of thymic stromal lymphopoietin and its receptor in experimental autoimmune arthritis. Arthritis Rheum 2011;63(7):1878-87.

14. Hartgring SA, Willis CR, Alcorn D, Nelson LJ, Bijlsma JW, Lafeber FP, et al. Blockade of the interleukin-7 receptor inhibits collagen-induced arthritis and is associated with reduction of T cell activity and proinflammatory mediators. Arthritis Rheum 2010;62(9):2716-25.

15. Moret FM, Hack CE, van der Wurff-Jacobs KM, de Jager W, Radstake TR, Lafeber FP, et al. Intra-articular CD1c-expressing myeloid dendritic cells from rheumatoid arthritis patients express a unique set of T cell-attracting chemokines and spontaneously induce Th1, Th17 and Th2 cell activity. Arthritis Res Ther 2013;15(5):R155.

16. Churchman SM, Ponchel F. Interleukin-7 in rheumatoid arthritis. Rheumatology (Oxford) 2008;47(6):753-9.

17. Kondrack RM, Harbertson J, Tan JT, McBreen ME, Surh CD, Bradley LM. Interleukin 7 regulates the survival and generation of memory CD4 cells. J Exp Med 2003;198(12):1797-806.

18. Deshpande P, Cavanagh MM, Le Saux S, Singh K, Weyand CM, Goronzy JJ. IL-7- and IL-15-mediated TCR sensitization enables T cell responses to self-antigens. J Immunol 2013;190(4):1416-23.

19. van Roon JA, Glaudemans KA, Bijlsma JW, Lafeber FP. Interleukin 7 stimulates tumour necrosis factor alpha and Th1 cytokine production in joints of patients with rheumatoid arthritis. Ann Rheum Dis 2003;62(2):113-9.

20. McInnes IB, al-Mughales J, Field M, Leung BP, Huang FP, Dixon R, et al. The role of interleukin-15 in T-cell migration and activation in rheumatoid arthritis. Nat Med 1996;2(2):175-82.

21. Fujikado N, Saijo S, Yonezawa T, Shimamori K, Ishii A, Sugai S, et al. Dcir deficiency causes development of autoimmune diseases in mice due to excess expansion of dendritic cells. Nat Med 2008;14(2):176-80.

22. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31(3):315-24.

23. Watanabe N, Hanabuchi S, Soumelis V, Yuan W, Ho S, de Waal Malefyt R, et al. Human thymic stromal lymphopoietin promotes dendritic cell-mediated CD4 T cell homeostatic expansion. Nat Immunol 2004;5(4):426-34.

6

Preventing PD-1/PD-L1 interactions reverses hypo-responsiveness of synovial CD4 T cells in response to synovial fluid-derived CD1c myeloid dendritic cells

Frederique M. Moret, Kim M.G. van der Wurff-Jacobs, Dirk van Leenen, Marian J.A. Groot Koerkamp, Johannes W.J. Bijlsma, Floris P.J.G. Lafeber, Joel A.G. van Roon

Submitted for publication

ABSTRACT

Objectives To investigate PD-1/PD-L1 involvement in the hypo-responsiveness of rheumatoid arthritis (RA) synovial fluid (SF) CD4 T cells upon stimulation by (TSLP-primed) CD1c myeloid dendritic cells (mDCs).

Methods Expression of PD-1 on naïve (Tn), central memory (Tcm), and effector memory (Tem) CD4 T cell subsets was assessed by flow cytometry. PD-L1 expression and its regulation upon TSLP stimulation of mDCs from peripheral blood (PB) and SF of RA patients were investigated by microarray analysis and flow cytometry. The involvement of PD-1/PD-L1 interactions in SF T cell hypo-responsiveness upon (TSLP-primed) mDC activation was determined by cell culture in the presence of PD-1 blocking antibodies, with or without IL-7 as recognized suppressor of PD-1 expression.

Results PD-1 expression was increased on CD4 T cells derived from SF compared to PB of RA patients. TSLP increased PD-L1 mRNA expression in both PB and SF mDCs. PD-L1 protein expression was increased on SF mDCs compared to PB mDCs and was associated with T cell hypo-responsiveness. Blockade of PD-1, as well as IL-7 stimulation, during cocultures of memory T cells and (TSLP-primed) mDCs from RA patients significantly recovered T cell proliferation.

Conclusion SF T cell hypo-responsiveness upon (TSLP-primed) mDC stimulation in RA joints is partially dependent on PD-1/PD-L1 interactions as PD-1 and PD-L1 are both highly expressed on SF T cells and mDCs, respectively and inhibiting PD-1 availability restores T cell proliferation. The potential of IL-7 to robustly reverse this hypo-responsiveness suggest that such proinflammatory cytokines in RA joints strongly contribute to memory T cell activation.

Introduction

Rheumatoid arthritis (RA) is characterized by progressive joint inflammation resulting in tissue damage (1). This is strongly dependent on CD4 T cells producing Th1 (IFNγ) and Th17 cytokines (IL-17) (2-5). Activation and differentiation of CD4 T cells to become Th1 or Th17 cells is strongly regulated by antigen-presenting cells such as dendritic cells (DCs) (6). Several types of DCs are described to circulate in human blood, characterized by high expression of HLA class II molecules and the absence of lineage markers (CD3, CD19, CD14, CD20, CD56 and glycophorin A). Human blood DCs can be divided into at least 3 subtypes, plasmacytoid DCs and two types of myeloid or classical DCs (mDC1 and mDC2) (7, 8), based on the blood-derived DC antigen (BDCA)-molecules (9, 10). BDCA-1 (CD1c) identifies the mDC1 subset, which are potent activators of CD4 T cells, whereas mDC2 cells, identified by expression of BDCA-3 (CD141) more potently activate CD8 T cells (7, 9, 10). In this respect, it is important to notice that the characterisation of mDC1 cells by CD1c is more specific than the previously used and more broadly expressed marker, CD11c (7, 9).

CD1c mDCs are abundantly present in joints of RA patients and these synovial fluid-derived mDCs are recently demonstrated to have an extremely strong capacity to activate autologous peripheral blood-derived CD4 T cells (11). Thymic stromal lymphopoietin (TSLP) is recently considered as a potential trigger to activate CD1c mDCs in joints of RA patients. TSLP cytokine levels are significantly increased in synovial fluid of RA patients compared to synovial fluid of osteoarthritis patients (12, 13). TSLP is demonstrated to potently activate TSLPR-expressing CD1c mDCs from synovial fluid to secrete enhanced levels of T cell-attracting chemokines and to strongly activate peripheral blood-derived CD4 T cells to induce Th1, Th17 and Th2 activity (13). In addition, recently, TSLP and its receptor were also shown to enhance Th1 and Th17-mediated experimental arthritis and tissue destruction (14).

Because of the prominent role of CD4 T cells in arthritic processes and the potential of synovial fluid (SF)-derived mDCs and TSLP-primed mDCs to activate autologous peripheral blood (PB)-derived CD4 T cell, in this study we investigated the potential of these mDCs to activate autologous SF-derived CD4 T cells. An evident hypo-responsiveness of SF-derived CD4 T cells upon mDC or TSLP-primed mDC activation was observed. Several observations led us to investigate the role of programmed cell death-1 (PD-1) and its ligand interactions in this hypo-responsiveness, since ligation of PD-1 by PD-L1 and PD-L2 leads to inhibition of T cell proliferation (15, 16). First, analyzing gene expression profiles of TSLP-primed mDCs from RA patients revealed significant and specific upregulation of PD-L1. In addition, preliminary data had shown us that PD-L1 was upregulated on SF mDCs of RA patients. Third, data from previous studies (17) and ours indicated overexpression of PD-1 on synovial CD4 T cells of RA patients. Since recently IL-7 was shown to downregulate PD-1 expression on T cells (18) and because of the potent T cell stimulatory capacity of IL-7 (19), we also

examined the role of IL-7 in the regulation of PD-1/PD-L interactions in hypo-responsiveness of synovial T cells. Our data demonstrate that hypo-responsiveness of synovial CD4 T cells in response to SF-derived CD1c mDCs is dependent on PD-1/PD-L1 interactions and is robustly reversed by IL-7.

MATERIALS AND METHODS

Patients

Synovial fluid (SF) was obtained from 13 rheumatoid arthritis (RA) patients during effusion of the knee and 70cc heparinized peripheral blood (PB) was collected from 16 RA patients. RA was classified according to the American College of Rheumatology criteria (20). Patient material collection was according to ethical regulations of the University Medical Center Utrecht and patients gave written informed consent.

Cell isolation

Mononuclear cells (MNC) were isolated from PB and SF by density centrifugation using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). Prior to MNC isolation, PB or SF was diluted 1:1 with RPMI 1640 medium (Gibco, Life Technologies, NY, USA) containing penicillin (100U/ml), streptomycin (100µg/ml), and glutamine (2 mM) (all PAA Laboratories, Pasching, Austria). PB and SF-derived CD1c mDCs and CD4 T cells were isolated from the MNC fraction by magnetic-activated cell sorting (MACS) using CD1c (BDCA-1)$^+$ dendritic cell and CD4$^+$ T cell isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany). Peripheral blood memory T cells were isolated from the CD4 T cell fraction by their lack of CD45RA expression using the CD45RA$^+$ isolation kit (Miltenyi Biotec). Isolations were performed according to the manufacturer's instructions.

Cell cultures

All cells were cultured in RPMI glutamax (Gibco) supplemented with penicillin, streptomycin, and 10% (v/v) human AB serum (GemCell, West Sacramento, USA). The mDCs were cultured at a cell concentration of $0.5x10^6$ cells/ml with or without recombinant TSLP at 20 ng/ml (R&D systems, Minneapolis, USA) for 20 hours in Sarstedt tubes at 37°C.

The functional capacities of PB and SF-derived mDCs were assessed by measuring activation of autologous CD4 T cells derived from PB and SF. For this purpose, isolated T cells were seeded in round-bottomed 96-well plates at a concentration of $0.25x10^6$ cells/ml and kept at 37°C in full culture medium before co-culturing with the (TSLP-primed) mDCs. Washed TSLP-activated mDCs and unstimulated mDCs were added to the autologous T cells (mDC:T cell ratio 1:5 or 1:10) in triplicate in the absence of additional stimuli and cocultured for 6 days.

To test the effects of PD-1 blockade or IL-7 effects in cocultures of mDCs and memory T cells from PB and SF, anti-PD-1 mAB (1 µg/ml, Biolegend, San Diego, USA) or IL-7 (10 ng/ml, Peprotech, Rocky Hill, USA) was added at the start of the cocultures. Proliferation was measured by ^{3}H-thymidine incorporation (1µCi/well added during the last 18 hours of a culture period; PerkinElmer, Waltham, USA).

Flow cytometry

PD-1 expression on CD4 T cells and PD-L1 expression on CD1c mDCs of RA patients and healthy controls was analysed by flow cytometry using a FACSCanto II flow cytometer (BD Biosciences, San Jose, USA). Ex vivo or cultured mDCs were stained with CD1c-PE (BD Biosciences), CD19-PERCP (Biolegend), and CD274(PD-L1)-APC (Biolegend). mDCs were gated as CD1c positive and CD19 negative. Ex vivo CD4 T cells were stained with CD45RO-FITC (DAKO, Glostrup, Denmark), CD27-APC (Invitrogen, Life Technologies, Carlsbad, USA), CD279-PE and CD4-PERCP (Biolegend) using isotype antibodies or autofluorescence as controls. All samples were analysed using FlowJo software (Tree Star, Ashland, USA). To compare mean fluorescence intensity (MFI) values, the autofluorescence intensity was subtracted from the MFI of the stainings to reveal true expression values.

Microarray gene expression analysis

To examine TSLP-mediated gene regulation with regard to PD-1 ligands and costimulatory molecules in isolated CD1c mDCs from PB and SF of RA patients (n=5), CD1c mDCs ($0.5x10^{6}$ cells/ml) were stimulated with 20 ng/ml recombinant TSLP (R&D systems) for 20 hours in Sarstedt tubes at 37°C. mDCs were collected and lysed in RLT buffer (Qiagen, Venlo, Netherlands) prior to RNA isolation. Total RNA was isolated using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. RNA quantity was measured by a NanoDrop Spectrophotometer (NanoDrop technologies) and RNA quality was assessed on a 2100 BioAnalyzer (Aligent Technologies).

Universal Human Reference RNA (Stratagene) was used as a common reference. All patient samples were labeled with cy5, reference cRNA was labeled with cy3. Microarrays used were human whole genome gene expression microarrays V1 (Agilent, Belgium) representing 41000 *H. sapiens* 60-mer probes in a 4x44K layout.

cDNA synthesis, cRNA double amplification, labeling, quantification, quality control and fragmentation were performed with an automated system (Caliper Life Sciences NV/SA, Belgium), starting with 70 ng total RNA from each sample, all as previously described in detail (21, 22). Microarray hybridization and washing was performed with a HS4800PRO system with QuadChambers (Tecan, Benelux) using 500 ng, 1-2% Cy5/Cy3 labeled cRNA per channel as described elsewhere (22). Slides were scanned on an Agilent G2565BA scanner at 100% laser power, 100% PMT. After automated data extraction using Imagene 8.0

(BioDiscovery), Loess normalization was performed on mean spot-intensities (23). Data were further analysed by paired MANOVA (24), modeling sample, array and dye effects in a fixed effect analysis. P-values were determined by a permutation F2-test, in which residuals were shuffled 10000 times globally. Gene probes with $p<0.05$ after false discovery rate determination (FDR by Benjamini-Hochberg) were considered significantly different.

Statistical analysis

Apart from the microarray data, differences between conditions were assessed by paired-sample evaluation using the nonparametric Wilcoxon signed-rank test. Unpaired data analysis was performed using the nonparametric Mann-Whitney *U* test. Data analysis was performed using SPSS software version 20.0 (IBM, Armonk, USA. Data were considered statistically significant at $p<0.05$.

RESULTS

Synovial CD4 T cells from RA patients are hypo-responsive to stimulation by (TSLP-primed) CD1c myeloid dendritic cells

Myeloid DCs derived from SF of RA patients have a strong capacity to activate autologous PB-derived CD4 T cells. In addition, TSLP-primed mDCs (TSLP-mDCs) from PB and SF strongly activate PB-derived T cells. In contrast, SF-derived CD4 T cells were hardly responsive to these in vivo and in vitro activated mDCs (Figure 1A). TSLP-primed mDCs from SF have a stronger capacity to activate PB-derived CD4 T cells than TSLP-primed mDCs from PB (Figure 1A and (13)), TSLP-primed mDCs from SF had a poor capacity to activate SF-derived CD4 T cells. This activating potential was significantly reduced compared to TSLP-primed mDCs from PB to activate PB-derived CD4 T cells (Figure 1B).

Several recent observations by others and us indicated that programmed cell death-1 (PD-1)/PD ligand 1 (PD-L1) interactions could play a role in regulation of synovial T cell hypo-responsiveness to (TSLP-primed) mDCs from RA patients. First, PD-L1 was the only significantly upregulated inhibitory ligand that was induced upon TSLP activation (Figure 3) in addition to upregulated costimulatory and antigen presenting molecules such as CD80, CD86, MHC class II and CD1c (data not shown). In addition, preliminary data had shown us that PD-L1 was upregulated on SF mDCs of RA patients. Therefore, we set out to investigate the expression levels of PD1 on T cells, PD-L1 on mDCs from PB and SF of RA patients and the capacity that this interaction has to regulate T cell responsiveness.

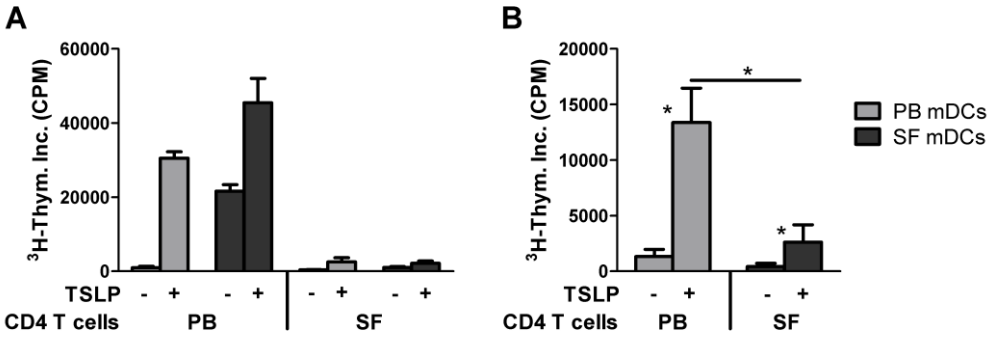


Figure 1. CD4 T cells derived from synovial fluid of rheumatoid arthritis patients are hypo-responsive upon (TSLP-primed) myeloid dendritic cell stimulation in contrast to peripheral blood-derived CD4 T cells. TSLP-primed myeloid dendritic cells (mDCs) from peripheral blood (PB) as well as both mDCs and TSLP-primed mDCs from synovial fluid (SF) of rheumatoid arthritis (RA) patients strongly activate PB-derived CD4 T cells, whereas SF-derived CD4 T cells are hyporesponsive upon (TSLP-primed) mDC activation. (A) mDC:T cell ratio 1:5, paired analysis of CD4 T cells and mDCs of a representative donor is shown and (B) mDC:T cell ratio 1:10, n=5 unpaired donors. Statistically significant difference of *$p<0.05$. CPM, counts per minute; TSLP, thymic stromal lymphopoietin.

Memory CD4 T cells from synovial fluid express robustly increased PD-1 levels

CD4 T cell subsets can be divided into naïve, central memory, and effector memory T cells (Tn, Tcm, and Tem cells, respectively) based on the expression of CD27 and CD45RO. In RA patients, PB-derived T cells mainly consist of naive and central memory T cells, whereas SF-derived T cells hardly contain naïve T cells and mainly consist of central memory and effector memory T cells (Figure 2A). Since synovial fluid memory T cell hypo-responsiveness upon TSLP-mDC stimulation can be caused by enhanced expression of PD-1 interactions, we measured the expression of this inhibitory receptor on the CD4 T cell subsets derived from PB and SF of RA patients (Figure 2B, representative histograms). Naïve T cells from PB and SF expressed the lowest levels of PD-1, although naïve CD4 T cells from SF had strongly upregulated levels of PD-1 compared to naïve CD4 T cells from PB (Figure 2C). However, considering the different expression levels of CD45RO on these "naïve" SF T cells compared to those from PB, we are reluctant to characterize these cells as 'naïve' (Figure 2A). Mainly memory T cell subsets expressed PD-1 and a robustly increased percentage of Tcm and Tem cells from SF expressed PD-1 compared to those from PB (Figure 2C).

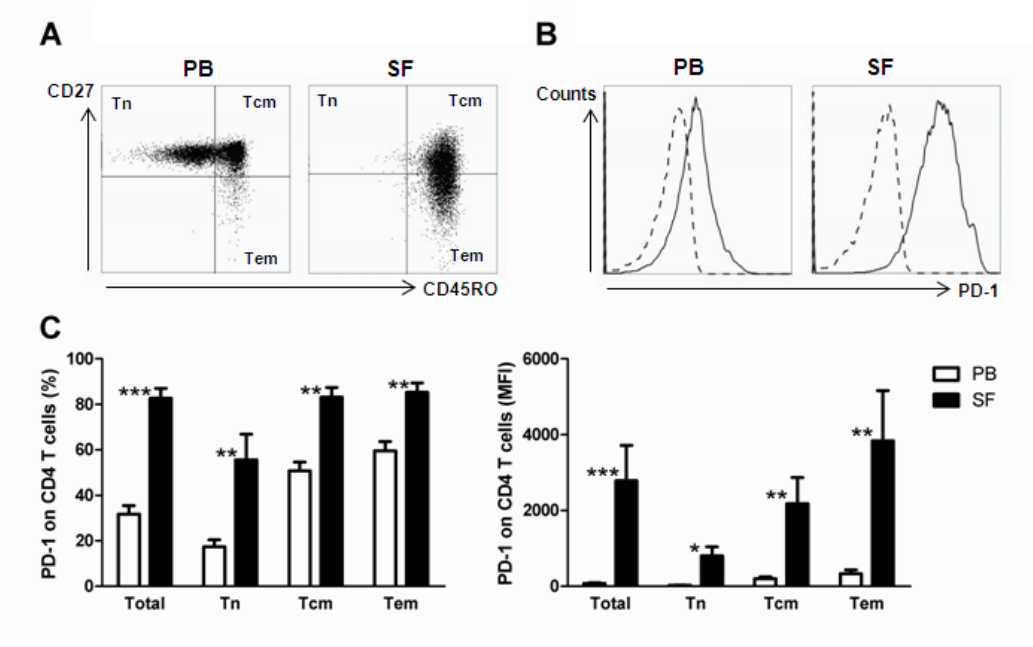


Figure 2. Memory CD4 T cells, within particular synovial fluid-derived T cell subsets, express increased PD-1 levels. (A) T cells derived from synovial fluid (SF) of rheumatoid arthritis (RA) patients hardly contain naïve T (Tn) cells and express increased PD-1 levels. (B) Representative histogram of one donor is shown. (C) PD-1 expression is increased on central memory (Tcm) and effector memory (Tem) T cells in comparison with naïve T cells (Tn cells) from peripheral blood (PB; n=9, both $p<0.01$) and SF (n=7, both $p<0.05$). PD-1 expression was strongly upregulated on all T cell subsets derived from SF versus PB of RA patients. Statistically significant differences of *$p<0.05$, **$p<0.01$, and ***$p<0.001$. MFI, mean fluorescence intensity; PD-1, programmed cell death-1.

PD-L1 is upregulated by TSLP and highly expressed on synovial fluid CD1c mDCs

PD-L1 and PD-L2 are both ligands for PD-1. The capacity of TSLP to regulate the expression of among others PD-L1 and PD-L2 on mDCs from PB and SF was determined by microarray analysis. TSLP selectively and significantly upregulated the mRNA expression of PD-L1, but not that of PD-L2 in PB and SF-derived mDCs (Figure 3A). In addition, (unstimulated) SF mDCs had a higher PD-L1 mRNA expression compared to PB mDCs, although this did not reach statistical significance ($p=0.08$, Figure 3A). The PD-L1 mRNA upregulation upon TSLP stimulation of mDCs was confirmed by an increased PD-L1 surface expression (Figure 3B). Since PD-L1 mRNA levels were affected by TSLP and very low PD-L2 mRNA levels were observed in CD1c mDCs, we focused on the extracellular PD-L1 protein expression on ex vivo CD1c mDCs from PB and SF of RA patients (Figure 3C). The number of mDCs expressing PD-L1 was significantly increased in SF compared to PB and SF mDCs had a higher intensity of PD-L1 expression compared to mDCs derived from PB (Figure 3D).

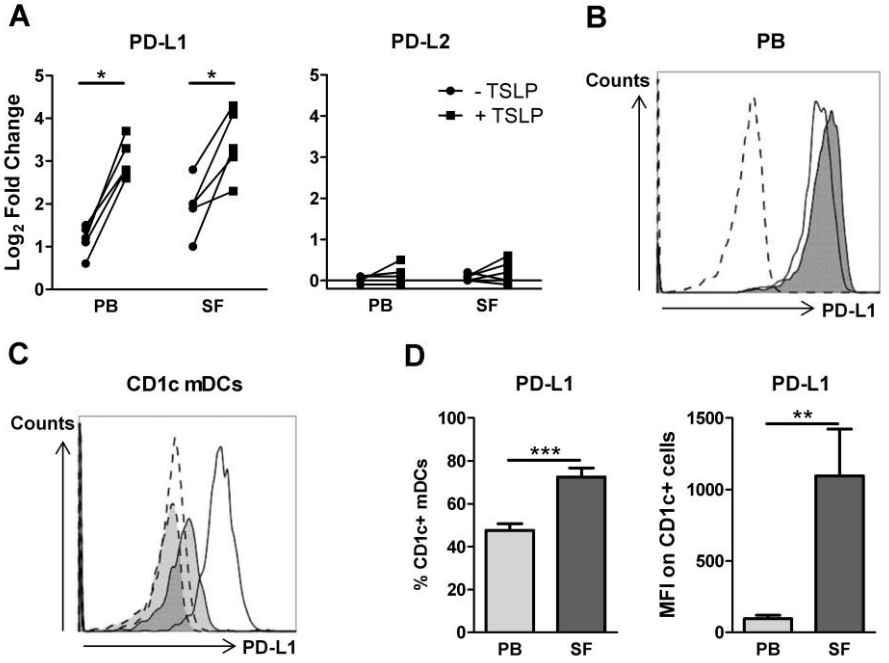


Figure 3. PD-L1 is upregulated by TSLP and highly expressed on synovial CD1c myeloid dendritic cells. (A) TSLP stimulation of myeloid dendritic cells (mDCs) significantly upregulates PD-L1 mRNA expression (peripheral blood (PB)-derived and synovial fluid (SF)-derived mDCs, n=5, PD-L2 not significant) as well as (B) PD-L1 protein expression, representative donor of PD-L1 expression on TSLP-stimulated mDCs (filled dark gray) and unstimulated mDCs (transparant) from PB. Dashed line represents autofluorescence and numbers indicate mean fluorescence intensities (MFI). (C, D) PD-L1 is expressed to a higher extent on CD1c mDCs derived from SF (n=8) compared to PB (n=9) of RA patients. (C) Representative histogram of PD-L1 expression on PB mDCs (filled light gray; solid line) and SF mDCs (transparent; solid line). Dashed line respresents autofluorescence for PB mDCs (filled light gray) and SF mDCs (transparant). (D) Mean percentages and MFI of PD-L1 expression of PB and SF mDCs. Statistically significant differences of *p<0.05, **p<0.01, and ***p<0.001. PD-L, programmed cell death-ligand; TSLP, thymic stromal lymphopoietin.

TSLP-mDC driven memory T cell hypo-responsiveness is partially dependent on PD-1/PD-L1 interaction

Next, we examined whether the hypo-responsiveness of SF memory CD4 T cells was caused by their increased expression of PD-1 in combination with the increased PD-L1 expression on SF-derived mDCs. For this purpose we cocultured memory CD4 T cells, either derived from PB or SF of RA patients with TSLP-activated mDC from PB or SF, respectively, in the presence and absence of PD-1 blocking antibodies. Both memory CD4 T cells from PB and SF showed a similar reduced capacity to respond to TSLP-activated mDCs (data not shown). Blocking PD-1 interactions in cocultures of memory T cells activated by mDCs resulted in a significant increase of the T cell proliferation (Figure 4A), which was similar for memory T

cells from SF and PB (data not shown). Stimulation of mDCs by TSLP resulted in an upregulated memory T cell proliferation, which was also elevated by blocking PD-1 ligation (Figure 4A).

Previously it has been demonstrated that synovial CD4 T cells are hyper-responsive to IL-7 and that IL-7 has the capacity to downregulate PD-1 expression (18, 19). Therefore, in another way to regulate PD-1 signaling we assessed the functional consequence of IL-7 addition. IL-7 strongly enhanced memory T cell proliferation activated by mDCs or TSLP-mDCs. However, although we observed that IL-7 already inhibits PD-1 expression after 20 hours, PD-1 downregulation was apparently not complete as IL-7-induced T cell proliferation was further enhanced upon prevention of PD-1 signaling by anti-PD-1 antibody (Figure 4B).

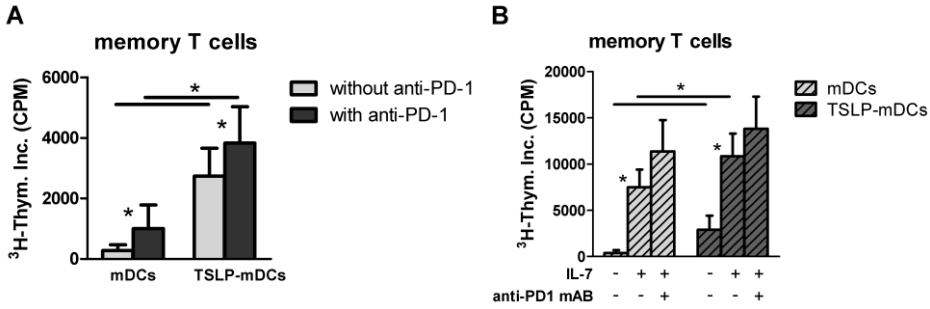


Figure 4. Hypo-responsiveness of memory T cells from rheumatoid arthritis patients upon TSLP-primed myeloid dendritic cell stimulation is at least partially reversed by PD-1 blockade. (A) Blockade of the PD-1/PD-L1 interaction by anti-PD-1 antibody (1 µg/ml) in (TSLP-primed) myeloid dendritic cell (mDC) driven memory T cell activation partially restores the T cell proliferative capacity (n=8). (B) Memory T cell hypo-responsiveness is strongly reversed by interleukin-7 (IL-7), a cytokine that largely downregulates PD-1. Additional PD-1 blockade by anti-PD-1 antibody further reversed the downregulated proliferation (n=5). Statistically significant difference of *p<0.05. PD-1, programmed cell death-1; PD-L1, programmed cell death-ligand 1; TSLP, thymic stromal lymphopoietin.

DISCUSSION

In the present study it is demonstrated that in contrast to CD4 T cells from PB, CD4 T cells derived from SF are hypo-responsive upon activation by (TSLP-primed) CD1c mDCs. SF-derived CD4 T cells mainly consist of memory T cells, which express elevated levels of PD-1, while SF-derived CD1c mDCs abundantly express its ligand PD-L1, which is upregulated by TSLP. Memory T cell hypo-responsiveness is partially dependent on PD-1/PD-L1 interaction, since blockade of this by anti-PD-1 mAb partially restores the T cell proliferation. Importantly, IL-7, an inhibitor of PD-1 expression, also overcomes hypo-responsiveness and

robustly activates synovial memory T cells in the context of activated (TSLP-primed) mDCs, which still could be further enhanced by blocking PD-1.

Similar to TSLP-primed mDCs from PB, SF-derived mDCs have a strongly enhanced capacity to activate T cells derived from PB that is even upregulated upon TSLP stimulation (present data and (13)). Associated with this enhanced T cell stimulatory capacity, enhanced expression of antigen-presenting molecules including CD1c, HLA class II, and costimulatory molecules including CD80, CD86 and CD40 have been demonstrated (11, 13). In support of this, activation of PB-derived T cells by (TSLP-primed) mDCs is strongly dependent on costimulation and antigen presentation, as blockade of HLA-class II molecules or CD80/86 costimulation blocked T cell activation. However, despite the abundance of antigen-presenting and costimulatory molecules the activity of T cells derived from SF of RA patients activated by autologous TSLP-primed mDCs is strongly hampered. Although several mechanisms might be involved in the reduced capacity of SF-derived T cells in response to activated mDCs, the role of PD-1/PD-L1 interactions, which negatively regulates T cell activation (15), was investigated for obvious reasons, such as overexpression of PD-L1 by activated mDCs from SF and selective induction of PD-L1 by TSLP. Blockade of PD-1/PD-L1 interactions has been shown to result in increased T cell activity (25) and PD-1 deficiency as well as PD-L1 deficiency is shown to play a critical role in the develop of autoimmune disorders in mice (26, 27).

In line with our results showing increased PD-1 expression on SF-derived memory T cells compared to PB-derived T cell, PD-1 expression was previously described to be increased on SF-derived CD4 T cells (17). These T cells were previously suggested to be resistant to PD-1 mediated suppression as PD-L1 expression on SF-derived CD14+ monocytes and macrophages was not sufficiently high to effectively downregulate T cell activation (17). In contrast to this latter suggestion we observed hypo-responsiveness of synovial fluid T cells upon activation by in vivo activated synovial mDCs and in vitro activated TSLP-mDCs, which in contrast expressed high levels of PD-L1. As PD-L2 mRNA expression levels were low and not significantly upregulated by TSLP, this suggested that mainly PD-1/PD-L1 interactions could be involved in mDC-T cell interactions and cause control of synovial T cells. PD-1 blocking antibodies, blocking interaction with PD-L1, resulted in an increase in the T cell proliferation indicating that SF-derived T cell hypo-responsiveness is at least partially dependent of PD-1/PD-L interactions. However, based on the strong expression of both PD-1 on T cells and PD-L1 on activated mDCs more robust inhibition was expected. Lack of response might be due to the fact that PD-1 is also expressed on regulatory T cells preventing immunosuppression by these Tregs (28). Since previous studies including those of our group have demonstrated increased presence of regulatory T cells in the synovial fluid (29, 30), blockade of PD-1, next to increased activation of effector T cells, might enhance function of regulatory T cells, resulting in a somewhat more limited reversal of T cell inhibition. An alternative explanation is that activated T cells express PD-L1, which was

previously shown to prevent T cell activation (27). Blockade of PD-1 may therefore also prevent downregulation of T cell activity.

Hypo-responsiveness of SF-derived T cells upon mDC or TSLP-mDC stimulation was robustly overruled by IL-7, which is in line with previous data demonstrating vigorous activation and even hyper-responsiveness of synovial CD4 T cells in the context of monocytic cells (19). Recently, IL-7 was shown to prevent shutdown of T cell activation by downregulation of PD-1 expression (18). We also found PD-1 downregulation upon IL-7 stimulation of CD4 T cells in this study (data not shown). Apart from the capacity of IL-7 to downregulate PD-1 expression (18), IL-7 is also shown to overcome PD-1 mediated inhibition of T cells by STAT5 activation that is regulated by IL-2 and family members like IL-7 and IL-15 (31, 32). IL-7 and IL-15 are both abundantly present in joints of RA patients (19, 33) and this suggests that the inflammatory environment in RA joints could play a critical role in overcoming PD-1 mediated inhibition of T cells. Although PD-1-mediated inhibition is likely a crucial mechanism whereby IL-7 overcomes hypo-responsiveness, other mechanisms induced by IL-7 could be involved as well, such as upregulation of (co)stimulatory molecules that were previously found to be induced by IL-7, both on T cells (e.g. lymphocyte function associated antigen (LFA-1) and CD69) and on myeloid cells (CD40, CD80, CD86) (19).

The present study demonstrates that PD-1/PD-L1 interactions between SF-derived T cells and in vivo activated SF mDCs or in vitro TSLP-primed mDCs contribute to T cell hypo-responsiveness. The potential of IL-7 to robustly overrule this hypo-responsiveness suggest that such cytokines in the joints of RA patients might strongly contribute to the activation of memory T cells, in addition to the potential of SF-derived mDCs to activate autologous peripheral (naïve) CD4 T cells attracted to the joint.

REFERENCES

1. Feldmann M, Brennan FM, Maini RN. Rheumatoid arthritis. Cell 1996;85(3):307-10.
2. Morita Y, Yamamura M, Kawashima M, Harada S, Tsuji K, Shibuya K, et al. Flow cytometric single-cell analysis of cytokine production by CD4+ T cells in synovial tissue and peripheral blood from patients with rheumatoid arthritis. Arthritis Rheum 1998;41(9):1669-76.
3. van Roon JA, van Roy JL, Duits A, Lafeber FP, Bijlsma JW. Proinflammatory cytokine production and cartilage damage due to rheumatoid synovial T helper-1 activation is inhibited by interleukin-4. Ann Rheum Dis 1995;54(10):836-40.
4. Cornelissen F, van Hamburg JP, Lubberts E. The IL-12/IL-23 axis and its role in Th17 cell development, pathology and plasticity in arthritis. Curr Opin Investig Drugs 2009;10(5):452-62.
5. Chabaud M, Durand JM, Buchs N, Fossiez F, Page G, Frappart L, et al. Human interleukin-17: A T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. Arthritis Rheum 1999;42(5):963-70.
6. Thomas R, Davis LS, Lipsky PE. Rheumatoid synovium is enriched in mature antigen-presenting dendritic cells. J Immunol 1994;152(5):2613-23.
7. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. Annu Rev Immunol 2013;31:563-604.
8. Satpathy AT, Wu X, Albring JC, Murphy KM. Re(de)fining the dendritic cell lineage. Nat Immunol 2012;13(12):1145-54.
9. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of monocytes and dendritic cells in blood. Blood 2010;116(16):e74-80.
10. Dzionek A, Fuchs A, Schmidt P, Cremer S, Zysk M, Miltenyi S, et al. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. J Immunol 2000;165(11):6037-46.
11. Moret FM, Hack CE, van der Wurff-Jacobs KM, de Jager W, Radstake TR, Lafeber FP, et al. Intra-articular CD1c-expressing myeloid dendritic cells from rheumatoid arthritis patients express a unique set of T cell-attracting chemokines and spontaneously induce Th1, Th17 and Th2 cell activity. Arthritis Res Ther 2013;15(5):R155.
12. Koyama K, Ozawa T, Hatsushika K, Ando T, Takano S, Wako M, et al. A possible role for TSLP in inflammatory arthritis. Biochem Biophys Res Commun 2007;357(1):99-104.
13. Moret FM, Hack CE, van der Wurff-Jacobs KMG, Lafeber FPJG, van Roon JAG. TSLP: a novel potent proinflammatory mediator that activates myeloid dendritic cells to stimulate Th1 and Th17 activity in rheumatoid arthritis. Arthritis Rheum 2014; doi: 10.1002/art.38338. [Epub ahead of print].
14. Hartgring SA, Willis CR, Dean CE, Jr., Broere F, van Eden W, Bijlsma JW, et al. Critical proinflammatory role of thymic stromal lymphopoietin and its receptor in experimental autoimmune arthritis. Arthritis Rheum 2011;63(7):1878-87.

15. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 2000;192(7):1027-34.

16. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat Immunol 2001;2(3):261-8.

17. Raptopoulou AP, Bertsias G, Makrygiannakis D, Verginis P, Kritikos I, Tzardi M, et al. The programmed cell death 1/programmed cell death ligand 1 inhibitory pathway is up-regulated in rheumatoid synovium and regulates peripheral T cell responses in human and murine arthritis. Arthritis Rheum 2010;62(7):1870-80.

18. Penaranda C, Kuswanto W, Hofmann J, Kenefeck R, Narendran P, Walker LS, et al. IL-7 receptor blockade reverses autoimmune diabetes by promoting inhibition of effector/memory T cells. Proc Natl Acad Sci U S A 2012;109(31):12668-73.

19. van Roon JA, Verweij MC, Wijk MW, Jacobs KM, Bijlsma JW, Lafeber FP. Increased intraarticular interleukin-7 in rheumatoid arthritis patients stimulates cell contact-dependent activation of CD4(+) T cells and macrophages. Arthritis Rheum 2005;52(6):1700-10.

20. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31(3):315-24.

21. Roepman P, Wessels LF, Kettelarij N, Kemmeren P, Miles AJ, Lijnzaad P, et al. An expression profile for diagnosis of lymph node metastases from primary head and neck squamous cell carcinomas. Nat Genet 2005;37(2):182-6.

22. van Wageningen S, Kemmeren P, Lijnzaad P, Margaritis T, Benschop JJ, de Castro IJ, et al. Functional overlap and regulatory links shape genetic interactions between signaling pathways. Cell 2010;143(6):991-1004.

23. Yang YH, Dudoit S, Luu P, Lin DM, Peng V, Ngai J, et al. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res 2002;30(4):e15.

24. Wu J, Kendrick KM, Feng J. A novel approach to detect hot-spots in large-scale multivariate data. BMC Bioinformatics 2007;8:331.

25. Brown JA, Dorfman DM, Ma FR, Sullivan EL, Munoz O, Wood CR, et al. Blockade of programmed cell death-1 ligands on dendritic cells enhances T cell activation and cytokine production. J Immunol 2003;170(3):1257-66.

26. Ansari MJ, Salama AD, Chitnis T, Smith RN, Yagita H, Akiba H, et al. The programmed cell death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. J Exp Med 2003;198(1):63-9.

27. Latchman YE, Liang SC, Wu Y, Chernova T, Sobel RA, Klemm M, et al. PD-L1-deficient mice show that PD-L1 on T cells, antigen-presenting cells, and host tissues negatively regulates T cells. Proc Natl Acad Sci U S A 2004;101(29):10691-6.

28. Sage PT, Francisco LM, Carman CV, Sharpe AH. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. Nat Immunol 2013;14(2):152-61.

29. Walter GJ, Evans HG, Menon B, Gullick NJ, Kirkham BW, Cope AP, et al. Interaction with activated monocytes enhances cytokine expression and suppressive activity of human CD4+CD45ro+CD25+CD127(low) regulatory T cells. Arthritis Rheum 2013;65(3):627-38.

30. Walter GJ, Evans HG, Menon B, Gullick NJ, Kirkham BW, Cope AP, et al. Interaction with activated monocytes enhances cytokine expression and suppressive activity of human CD4+CD45ro+CD25+CD127(low) regulatory T cells. Arthritis Rheum;65(3):627-38.

31. Bennett F, Luxenberg D, Ling V, Wang IM, Marquette K, Lowe D, et al. Program death-1 engagement upon TCR activation has distinct effects on costimulation and cytokine-driven proliferation: attenuation of ICOS, IL-4, and IL-21, but not CD28, IL-7, and IL-15 responses. J Immunol 2003;170(2):711-8.

32. Leonard WJ, Lin JX. Cytokine receptor signaling pathways. J Allergy Clin Immunol 2000;105(5):877-88.

33. McInnes IB, al-Mughales J, Field M, Leung BP, Huang FP, Dixon R, et al. The role of interleukin-15 in T cell migration and activation in rheumatoid arthritis. Nat Med 1996;2(2):175-82.

7

Intra-articular soluble interleukin-7 receptor levels are increased in patients with rheumatoid arthritis and correlate with local mediators of inflammation: comment on the article by Pickens et al.

Frederique M. Moret, Valerie Badot, Bernard R. Lauwerys, Joel A.G. van Roon

Arthritis Rheum. 2012 Feb;64(2);594-5

Pickens et al. (1) documented increased expression of IL-7 and IL-7Rα in the synovial tissue of patients with rheumatoid arthritis (RA), supporting previous studies (2, 3). In addition, they demonstrated a simultaneous increased IL-7 and IL-7Rα expression in circulating monocytes, synovial fluid macrophages, endothelial cells and fibroblasts of RA patients as compared to healthy controls. Also they demonstrated the upregulation of IL-7 and IL-7Rα mRNA expression by macrophages, fibroblasts and endothelial cells upon stimulation by TolI-like receptor ligands (LPS) and pro-inflammatory cytokines. In line with expressed IL-7Rα, exogenously added IL-7 was shown to induce angiotensin-1 secretion by macrophages and endothelial cells. These data strengthen the role for IL-7/IL-7Rα in immunopathology of RA.

Recently, we have documented increased numbers of IL-7Rα+ cells in synovial tissue and fluid of RA patients (2). The most abundant expression in these compartments was found on (CD4) T cells, to a lesser extent on B cells and, comparable to the present study, substantially on macrophages. These findings matched our immunohistochemical stainings, however, this was only observed after critical selection of a monoclonal antibody specifically recognizing membrane-bound IL-7Rα. When other antibodies were tested, similar staining patterns were observed as previously described and as reported in the present study, characterized by an abundant staining of non-immune cells in both lining and sublining of RA synovium (1, 4). Recently, a fascinating observation suggests that a new aspect should be considered when examining IL-7Rα expression. Cells from RA synovial tissue produce large amounts of soluble IL-7Rα (5). This naturally occurring IL-7Rα lacks the exon coding for the transmembrane region and is secreted by RA synovial fibroblasts upon stimulation by monokines such as TNFα, IL-1・ and T cell cytokines such as IL-17. In support of this, we observed strongly increased levels of sIL-7Rα in synovial fluid of RA patients as compared to osteoarthritis (OA) patients (Figure 1A). Soluble IL-7Rα levels correlate with intra-articular proinflammatory cytokine levels (IL-6, Figure 1B) and systemic disease activity markers (ESR, $r=0.391$ $p<0.05$, data not shown). Recently, fibroblasts were shown to be a major source of sIL-7Rα, expressing it to a higher extent than CD4 T cells. This was in contrast to activated CD8 T cells and B cells, which expressed full-length IL-7Rα but not sIL-7Rα (5). Recently, circulating naturally occurring sIL-7Rα was found to significantly block IL-7Rα-mediated immune activation in HIV-infected patients (6). In addition, recombinant sIL-7Rα inhibits Th1 cell activity in cultures of mononuclear cells from blood and synovial fluid of RA patients and strongly prevents proliferation of cocultures of CD4 T cells and fibroblasts from these patients (2, 5). Interestingly, sIL-7Rα in DMARD-resistant RA patients are significantly associated with poor response to TNFα blockade (infliximab) and vice versa (5). Hence, it was indicated that sIL-7Rα, reflecting fibroblast activation (e.g. caused by exposure to proinflammatory cytokines), could serve as a factor to predict response to anti-TNFα therapy. In addition, since sIL-7Rα seems to represent a homeostatic mechanism to counteract IL-7, increased sIL7Rα might reflect an increased IL-7-driven immune activation. In support of this notion persistence of IL-7 levels was also associated with lack of response

to anti-TNFα as well as MTX therapy in RA (7, 8). Finally, genetic predisposition may contribute to altered levels of sIL-7Rα in RA, since haplotypes of the IL-7Rα influence the balance of expression of soluble and membrane-bound forms of this receptor and are associated with susceptibility to multiple sclerosis (9).

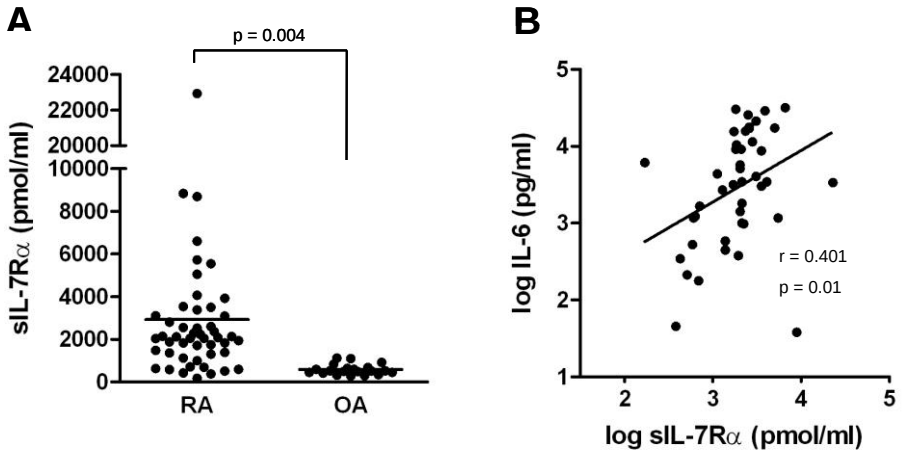


Figure 1. Soluble IL-7Rα concentrations are increased in the synovial fluid of rheumatoid arthritis (RA n=50) patients as compared to osteoarthritis (OA, n=20) patients (A) and correlate with intraarticular IL-6 concentrations (B). Soluble IL7Rα and IL-6 were measured by ELISA as previously described (ref 5). P values (p) and pearson correlation coefficient (r) are given.

In conclusion the above-mentioned data reveal an important regulation of IL-7/IL-7Rα-mediated immune activation by production of soluble IL-7Rα, not only in RA patients but also other inflammatory (autoimmune) diseases. In most of the experiments in the paper by Pickens et al. it is uncertain which IL-7Rα isoform was expressed. In view of the strong capacity of sIL-7Rα to prevent IL-7-mediated cell signaling it is imperative to examine both expression of soluble and native IL-7Rα.

REFERENCES

1. Pickens SR, Chamberlain ND, Volin MV, Pope RM, Talarico NE, Mandelin AM et al. Characterization of IL-7 and IL-7R in the pathogenesis of Rheumatoid Arthritis. Arthritis Rheum 2011;10.
2. Hartgring SA, van Roon JA, Wenting-van WM, Jacobs KM, Jahangier ZN, Willis CR et al. Elevated expression of interleukin-7 receptor in inflamed joints mediates interleukin-7-induced immune activation in rheumatoid arthritis. Arthritis Rheum 2009; 60(9):2595-2605.
3. van Roon JA, Verweij MC, Wijk MW, Jacobs KM, Bijlsma JW, Lafeber FP. Increased intraarticular interleukin-7 in rheumatoid arthritis patients stimulates cell contact-dependent activation of CD4(+) T cells and macrophages. Arthritis Rheum 2005; 52(6):1700-10.
4. Nzeusseu TA, Galant C, Theate I, Maudoux AL, Lories RJ, Houssiau FA et al. Identification of distinct gene expression profiles in the synovium of patients with systemic lupus erythematosus. Arthritis Rheum 2007; 56(5):1579-88.
5. Badot V, Durez P, Van den EB, Nzeusseu-Toukap A, Houssiau F, Lauwerys B. Rheumatoid arthritis synovial fibroblasts produce a soluble form of the interleukin-7 receptor in response to pro-inflammatory cytokines. J Cell Mol Med 2010;10-4934.
6. Crawley AM, Faucher S, Angel JB. Soluble IL-7R alpha (sCD127) inhibits IL-7 activity and is increased in HIV infection. J Immunol 2010; 184(9):4679-87.
7. van Roon JA, Hartgring SA, Wenting-van WM, Jacobs KM, Tak PP, Bijlsma JW et al. Persistence of interleukin 7 activity and levels on tumour necrosis factor alpha blockade in patients with rheumatoid arthritis. Ann Rheum Dis 2007; 66(5):664-9.
8. van Roon JA, Jacobs K, Verstappen S, Bijlsma J, Lafeber F. Reduction of serum interleukin 7 levels upon methotrexate therapy in early rheumatoid arthritis correlates with disease suppression. Ann Rheum Dis 2008; 67(7):1054-55.
9. Hoe E, McKay F, Schibeci S, Heard R, Stewart G, Booth D. Interleukin 7 receptor alpha chain haplotypes vary in their influence on multiple sclerosis susceptibility and response to interferon Beta. J Interferon Cytokine Res 2010; 30(5):291-98.

8

Summary and Discussion

Frederique M. Moret, Timothy R.D.J Radstake, Joel A.G. van Roon

Parts of this chapter have been published in Ann Rheum Dis. 2014 Mar 1;73(3):477-9

SUMMARY

Myeloid dendritic cells: potent orchestrators of rheumatoid inflammation

Rheumatoid arthritis (RA) is an autoimmune disorder characterized by chronic joint inflammation resulting in progressive destruction of joint tissues (1). This is strongly dependent on CD4 T cells producing T-helper type (Th)-1 (interferon gamma (IFNγ)) and Th17 cytokines (interleukin (IL)-17) (2-5). Activation and differentiation of CD4 T cells to become Th1 or Th17 cells is strongly regulated by antigen presenting cells (6). Dendritic cells (DCs) are the professional antigen presenting cells (APCs) involved in the coordination of adaptive immune responses during infections and against tumour cells. DCs instruct T cells to develop a proper immune response by uptake and presentation of antigens and the provision of costimulatory signals. In addition, DCs have the ability to instruct T cells to induce self-tolerance by presenting self-antigens to T cells and subsequent deletion or inactivation of self-reactive T cells (7). External agents such as cytokines, tissue-derived factors, pathogen-derived antigens, and organic molecules may alter the balance between tolerogenic and immunogenic activity of DCs and with that induce autoimmune disease (8, 9).

Of the human blood DCs, the type 1 myeloid (or classical) dendritic cells (mDC1) (10, 11), expressing blood-derived dendritic cell antigen (BDCA)-1 (12, 13), were studied because of their strong potential to activate CD4 T cells. Despite the fact that mDCs have been extensively studied in immune disorders in mice and man and that they have been suggested to play an important role in the pathogenesis of RA (14), functional data on naturally occurring mDCs in RA, including those expressing CD1c, were scarce. In RA, mDCs are increased in the joints as compared with the circulation and express costimulatory molecules (15, 16). However, a detailed analysis of the capacity of *ex vivo* cultured CD1c mDCs from RA patients to produce inflammatory mediators and to activate CD4 T cells has not been performed. This is crucial to understand how these cells mediate inflammation and immunopathology and to understand how to regulate them. Hence, we set out **to investigate the presence, phenotype, and the functional capacity of naturally occurring CD1c mDCs to regulate T cell activity in RA patients and to study how inflammatory stimuli and currently used drugs might influence the activity of CD1c mDCs.**

In this thesis **chapter 2** provides data to conclude that *"Intra-articular CD1c-expressing myeloid dendritic cells from rheumatoid arthritis patients express a unique set of T cell-attracting chemokines and spontaneously induce Th1, Th17 and Th2 cell activity"*. CD1c mDC numbers were about 8-fold higher in synovial fluid (SF) versus peripheral blood (PB) of RA patients. mDCs from SF showed increased expression of antigen presenting (human leukocyte antigen (HLA) class II and CD1c) and costimulatory molecules (CD80, CD86, and CD40). Numerous cytokines were equally abundantly produced by mDCs from both PB and SF (including IL-12, IL-23, IL-13, IL-21),

but SF mDCs secreted higher levels of CXCL10/interferon γ-inducible protein-10 (IP-10), CXCL9/monokine induced by interferon γ (MIG), and CCL17/thymus and activation-regulated chemokine (TARC), and lower CCL22/macrophage-derived chemokine (MDC) levels compared with mDCs from PB. mDCs from SF displayed a strongly increased capacity to induce proliferation of autologous CD4 T cells associated with a strongly augmented IFNγ, IL-17, and IL-4 production. This suggests that increased numbers of CD1c mDCs in SF are involved in the inflammatory cascade intra-articularly by the secretion of specific T cell-attracting chemokines and the activation of self-reactive T cells.

Previous data demonstrated a pivotal role of thymic stromal lymphopoietin (TSLP) in experimental arthritis and several proinflammatory triggers are known to induce TSLP expression by synovial tissue fibroblasts. Therefore, we set out to investigate whether TSLP is a factor that could play a role in activation of mDCs, as TSLP previously was shown to specifically target CD11c mDCs. In **chapter 3** it is demonstrated that *TSLP is a novel proinflammatory mediator in RA that potently activates CD1c myeloid dendritic cells to attract and stimulate T cells*. TSLP levels were significantly increased in SF of RA compared with SF of osteoarthritis patients. TSLP receptor (TSLPR)-expressing CD1c mDC numbers in SF were significantly increased compared with PB from RA patients and SF mDCs displayed increased levels of TSLPR. TSLP selectively stimulated production of TARC and CCL3/macrophage inflammatory protein-1-alpha (MIP1α) by CD1c mDCs. TSLP-primed mDCs from PB and SF potently stimulated proliferation of autologous CD4 T cells compared to unstimulated mDCs. Enhanced proliferation was associated with increased production of IFNγ, IL-17, and IL-4. These data point towards an inflammatory mechanism in which increased intra-articular TSLP in RA potently activates TSLPR-expressing CD1c mDCs in the joints to secrete chemokines causing chemotaxis and subsequent activation of CD4 T cells. In addition to the demonstrated inflammatory potential of TSLP in experimental arthritis, our data suggest that TSLP and TSLPR-expressing mDCs could both play a pivotal role in the immunopathology of RA.

In **chapter 4** it was shown that *"TNFα downregulates the capacity of TSLP-activated CD1c myeloid dendritic cells from rheumatoid arthritis patients to promote Th2 activity"*. TSLP significantly stimulated tumour necrosis factor alpha (TNFα) secretion by CD1c mDCs from PB and SF. TNFα blockade during TSLP stimulation of mDCs did not affect T cell-differentiating cytokine secretion, but significantly decreased MIP1α (a ligand of CC chemokine receptor 5 (CCR5)-expressing Th1 cells) and slightly enhanced TARC production (a ligand of CCR4-expressing Th2 cells), significantly altering the ratio of these chemokines. Compared to TSLP-mDCs, anti-TNFα treated TSLP-mDCs did not affect IFNγ and IL-17 production of cocultured T cells, but significantly increased IL-4 production and reduced the IFNγ:IL-4 ratio. These data show that TNFα produced by TSLP-mDCs inhibits Th2 responses by favouring Th1-attracting chemokine production and preventing T cells to secrete Th2 cytokines that might contribute to

TSLP-induced immunopathology in RA. As such, shifting the balance of TSLP-mediated Th1/Th17 activity towards TSLP-mediated Th2 activity by TNFα blockade may contribute to the inhibition of disease activity.

In **chapter 5** it is shown that "*Efficacy of abatacept to reduce synovial T cell activation by CD1c myeloid dendritic cells is overruled by T cell-activating cytokines*". Our data demonstrated that percentages of CD4 T cell subsets from the circulation of healthy controls and RA patients were comparable and mainly consisted of naive and central memory T cells, whereas SF of RA patients mainly consisted of central memory and effector memory T cells. Activation of these T cell subsets by TSLP-primed mDCs from PB was completely blocked by abatacept. IL-7 strongly increased T cell activation and overruled the inhibitory capacity of abatacept. This IL-7-induced reversal was associated with robust induction of IFNγ, TNFα, and IL-17 secretion. Similarly, CD4 T cell proliferation induced by TSLP-primed mDCs from SF of RA patients was strongly blocked by abatacept and vigorously overruled by IL-7. Our data therefore indicate that the presence of T cell-activating cytokines, like IL-7, in joints of RA patients reduce the inhibitory capacity of abatacept on mDC-driven T cell activation. This mechanism could explain the unresponsiveness as well as partial response to abatacept in subsets of patients.

Because of the prominent role of CD4 T cells in arthritic processes and the potential of SF-derived mDCs and TSLP-primed mDCs to activate autologous PB-derived CD4 T cell, in **chapter 6** we investigated the potential of these mDCs to activate autologous SF-derived CD4 T cells. An evident hypo-responsiveness of SF-derived CD4 T cells upon mDC or TSLP-primed mDC activation was observed. Several observations led us to investigate the role of programmed cell death-1 (PD-1) and PD ligand (PD-L) interactions in this hypo-responsiveness, since ligation of PD-1 by PD-L1 and PD-L2 was previously shown to lead to inhibition of T cell proliferation (17, 18). Apart from investigating the role of PD-1 blockade in hypo-responsiveness of synovial T cells, we also investigated the role of IL-7, since this cytokine was recently shown to downregulate PD-1 expression on T cells (19), and because of its potent T cell stimulatory capacity (20). In **chapter 6** it is demonstrated that "*preventing PD-1/PD-L1 interactions reverses hypo-responsiveness of synovial CD4 T cells in response to synovial fluid-derived CD1c myeloid dendritic cells*". PD-1 expression was increased on CD4 T cells derived from SF, mainly consisting of memory T cells, compared with PB of RA patients. TSLP increased PD-L1 mRNA, but not PD-L2 mRNA expression in both PB and SF mDCs. PD-L1 protein expression was increased on SF mDCs compared with PB mDCs. Blockade of PD-1, during cocultures of memory T cells and (TSLP-primed) mDCs from RA patients significantly enhanced but not fully recovered T cell proliferation where IL-7 stimulation strongly augmented T cell proliferation. These data suggest that synovial T cell hypo-responsiveness upon (TSLP-primed) mDC stimulation in RA joints is at least partially dependent on PD-1/PD-L1 interactions as PD-1 and PD-L1 are both highly expressed on synovial T cells and mDCs, respectively. The potential of IL-7 to overrule this hypo-

responsiveness suggests that such proinflammatory cytokines in RA joints strongly contribute to memory T cell activation.

In previous studies and in chapters 5 and 6 the powerful capacity of IL-7 to activate T cells in the context of mDCs from PB and SF of RA patients was shown. In **chapter 7** we described that levels of the naturally occurring soluble IL-7Rα (sIL-7Rα) were strongly increased in SF of RA patients compared with osteoarthritis patients and these levels correlated with intra-articular proinflammatory cytokine levels (IL-6) and the erythrocyte sedimentation rate, a systemic disease activity marker. This naturally occurring IL-7Rα lacks the exon coding for the transmembrane region and is secreted by RA synovial fibroblasts upon stimulation by monokines such as TNFα, IL-1β, and T cell cytokines such as IL-17. Early studies suggested that secretion of sIL-7Rα seems to represent a homeostatic mechanism to counteract IL-7 activity. However, a recent paper (21) suggests that native sIL-7Rα at certain ratios can potentiate the activity of IL-7 and promotes autoimmunity. In addition, increased sIL-7Rα enhanced levels of IL-7, indicating that increased sIL-7Rα might facilitate and be associated with an increased IL-7-driven immune activation. In support of this notion persistent IL-7 levels as well as sIL-7Rα are associated with lack of response to anti-TNFα as well as methotrexate therapy in RA (22-24).

DISCUSSION

Therapeutic strategies aimed at dendritic cells to influence immunopathology of RA

This thesis contributes to the understanding of the role of CD1c mDCs in the immunopathology of RA. These mDCs are abundantly present in joints of RA patients and are implicated to play an important role in RA by their *in vitro* capacity to secrete high levels of T cell-attracting chemokines and spontaneously induce PB-derived T cell activation in the absence of *in vitro* added stimuli, this in contrast to mDCs from PB. The abundance of DCs and their enhanced activation state is implicated to cause autoimmunity in mice deficient for the inhibitory DC immunoreceptor (DCIR). DCIR is mainly expressed on DCs and transduces negative signals into the cells to negatively regulate DC expansion. DCIR-/- mice are shown to have a markedly increased susceptibility and severity to collagen-induced arthritis associated with the expansion of CD11c myeloid DCs and their increased capacity to enhance T cell and B cell activity, associated with autoantibodies typical of autoimmune diseases such as RA and primary Sjögren's syndrome (25). Together this suggests that targeting activated CD1c mDCs in RA could be a therapeutic strategy to reduce arthritis severity.

Several strategies could be attempted to reduce mDC activity. Depletion of mDCs by depleting antibodies, as is achieved for example by anti-CD20 (rituximab) to deplete B cells, might be successful since depletion of mDCs has been shown to prevent T cell-driven inflammation (26, 27). Other strategies causing inhibition of mDC activity, for example by antibodies cross-linking DCIR, may be effective as well. Previous studies have shown that this successfully inhibits activity of mDCs activated by Toll-like receptor (TLR)-8 ligands (28). In humans, this inhibitory activity may not be limited to mDCs as plasmacytoid dendritic cells also express DCIR and upon cross-linking of DCIR induction of interferon alpha (IFNα) was prevented (29). However, these approaches need further study. Another approach that can be successful to treat RA is making use of the potential of mDCs to induce tolerogenic immune responses and cause immunosuppression by immunotherapy. Tolerogenic dendritic cells (TolDC) have become a promising immunotherapeutic tool for restoring immune tolerance in RA. Recently, findings from human *in vitro* and mouse *in vivo* studies have been translated into clinical trials (30, 31). Currently, a study is initiated to look at safety, feasibility and acceptability of *in vitro* generated TolDC, derived from monocytes, which will be injected into the knee joints of RA patients (31). The efficacy of this strategy needs to be awaited.

The activated phenotype of SF-derived mDCs suggested that triggers in the inflammatory environment were responsible for the capacity of SF mDCs to spontaneously activate PB-derived T cells. Several triggers such as TLR stimulation or cytokines like TNFα can cause mDC activation in inflamed joints of RA patients. However, TLR-2 triggering of these mDCs did not result in SF-derived mDC activation (16). In addition, we also failed to detect

differences between PB and SF mDCs in secretion of cytokines (including IL-1, IL-6, TNFα, and IFNα) typically induced upon TLR triggering. This thesis demonstrates that CD1c mDCs from the joint express enhanced levels of a specific set of chemokines, one of them being TARC. We demonstrate that TSLP is a factor that could play a role in activation of mDCs, as TSLP specifically targets mDCs and upregulates TARC expression. In addition, increased TSLP levels were observed in joints of RA patients. Although TSLP was previously mainly associated with Th2-mediated immune responses, in this thesis TSLP is indicated to play an important role in Th1/Th17 mediated arthritis as well. Below it is discussed that TSLP is one of the examples to illustrate that mediators previously Th2-typified represent targets in not only RA, but also in several other rheumatic autoimmune diseases. Furthermore, TSLP is a factor that is involved in induction of several Th2-typified mediators, such as the activation of mast cells (32). As such, TSLP and Th2-typified immune responses are discussed in a broader perspective.

Targeting Th2-typified immune responses to prevent immunopathology in rheumatic diseases: belittled therapeutic strategies?

Although Th2-associated immune responses have been well defined in atopic diseases and parasitic infections the potential contribution to immunopathology in rheumatic diseases has hardly been recognized. This is likely related to the clear predominance of Th1/Th17 over Th2 cells in a number of rheumatic diseases, such as RA, psoriatic arthritis (PsA), and primary Sjogren's syndrome (pSS) (33-35). Also, whereas a clear role of Th1/Th17 activity in immunopathology has been clearly demonstrated in these diseases, Th2-related phenomena such as IgE production are hardly detected. In fact, Th2-associated atopic conditions as well as Th2 cytokines like IL-4 and IL-10 were shown to inhibit Th1-induced inflammatory responses in these diseases (36, 37). As a consequence the understanding of Th2-associated mediators, other than IL-4 and IgE, in these and other rheumatic conditions and the potential to target these have been under appraised. However, recent data demonstrate that immune activation by typical Th2-associated pathways, such as mast cell activation, and histamine-induced responses or Th2-typified key regulatory molecules, such as TSLP, IL-33, and IL-13, contribute to rheumatoid inflammation and immunopathology in several rheumatic diseases.

Mast cells and pathways driving histamine release: targets in rheumatic diseases

Classically, mast cells have been shown to play a detrimental role in allergic reactions and parasitic infections. In addition, mast cells have been shown to be increased at inflammatory

sites in a plethora of rheumatic conditions including RA, pSS, systemic sclerosis (SSc), spondyloarthropathies (SpA), dermatomyositis, and eosinophilia-myalgia syndromes (38-43). Mast cells also have been shown to be important mediators in different experimental inflammatory (arthritis) models by secretion of proinflammatory mediators like TNFα, IL-6, IL-8, and IL-17. In addition, inhibitors of histamine release such as salbutamol and cromolyn were shown to prevent joint destruction in immune complex-mediated arthritis (44, 45). Recent data suggest that targeting of mast cell-associated pathways prevents inflammatory responses driven by the innate and acquired immune system. One such pathway that was recently suggested may be prevention of immune activation through blockade of the histamine H_4 receptor (H_4R). The human H_4R expression is largely restricted to cells of the human immune system (e.g. mast cells, eosinophils, monocytes, dendritic cells, and T cells) and mediates several proinflammatory effects on these cell types, including cytokine secretion and chemotaxis (46). Recently, a study (47) added new insights into the mechanism of this receptor, demonstrating that specific targeting of the H_4R by an orally administered receptor antagonist results in prevention of joint inflammation and inflammation-induced destruction of cartilage and bone. Using different experimental arthritis models they demonstrate that innate effector cells, such as neutrophils and monocytes (collagen antibody-induced arthritis model), as well as acquired effector cells, like Th17 cells (collagen-induced arthritis model), play critical roles. Previous studies using antagonists of the H_1 and H_2 receptors have not been successful in treating autoimmune diseases; however, these antagonists do not affect the H_4R (48). These findings indicate new therapeutic opportunities in RA, but also in many other rheumatic diseases associated with increases in histamine and (upregulated) expression of H_4R, in particular given its restricted expression to immune cells (48).

Histamine levels have been shown to be enhanced at inflammatory sites in several (rheumatic) autoimmune diseases, including multiple sclerosis, inflammatory bowel's disease, RA, ankylosing spondylitis, PsA, and SSc. Triggers of histamine release by mast cells include IgE-dependent but also many IgE-independent pathways. Increased IgE complexes and total serum IgE levels in various autoimmune diseases such as RA, pSS, and systemic lupus erythematosus (SLE) are present in only a minority of patients (~10-30% of the patients). To what extent antigen-specific IgE antibodies trigger mast cell activation and histamine release in rheumatic diseases remains to be demonstrated but this could play a role in at least a subgroup of patients. Activation of mast cells by cross-linking of the high-affinity IgE receptor results in high TSLP and IL-33 secretion by these cells (49, 50). Both cytokines are upregulated in several rheumatic diseases and induce activation of mast cells (32, 38), potentially contributing to a positive feed-back loop in chronic inflammation by potentiating release of histamine and other proinflammatory mediators, like TNFα and IL-17. IgE-independent mechanisms that have been described to trigger degranulation and histamine release include Ig-complexes, complement, and free-light chains (45, 51). Also,

TLR-mediated histamine release has been suggested although the data are not consistent (52), which is in contrast to data on numerous inflammatory mediators that are released upon TLR-induced mast cell activation (for instance interferons and TNFα) (53). Despite the inconsistency on direct histamine release by TLR ligation, TLR-induced activation of fibroblasts, either directly or indirectly through release of proinflammatory cytokines (including IL-1β and TNFα), induces release of cytokines that potentiate histamine release like IL-33 and TSLP (54-56). In addition, recently it was demonstrated that TLR-induced activation of neutrophils promotes histamine production via a PI3 kinase dependent mechanism (57). Likewise, it has been demonstrated that cell types such as dendritic cells contribute to increased histamine levels during inflammation, albeit that these cells possibly induce lower levels (48). Of interest in this respect is that activation of cells through triggering of the H_4R requires much lower levels of histamine as compared to the $H_{1\text{-}3}$ receptors (~100 to 1000-fold) (48). Collectively, the increased numbers of mast cells, enhanced levels of histamine and numerous triggers to activate these and other cells to release histamine suggests that new approaches targeting histamine-associated pathways and including inhibition of mast cell activation, hold promise in various rheumatic diseases.

TSLP, IL-33, and IL-13: other examples of targetable "Th2-typified" amplifiers in immunopathology of rheumatic diseases

Recently in (models for) RA (including this thesis), SpA, SSc, and SLE, other pathways that have been typified as atopy/Th2-associated immune responses, including those driven by TSLP, IL-33, and IL-13, have been identified as targets for treatment in rheumatic diseases. Strongly enhanced levels of these cytokines facilitating proinflammatory responses are present in a much higher percentage of patients than is for example the case for IgE.

In this thesis, increased TSLP levels were reported in synovial fluid of RA as compared with osteoarthritis patients (58). TSLP was shown to activate enhanced numbers of CD1c-expressing mDCs to robustly induce Th1 and Th17 activity in addition to Th2 activity (58, 59). In support of a role in RA, TSLP in mice enhanced severity of collagen-induced arthritis, associated with enhanced joint inflammation and destruction of cartilage and bone. Oppositely, mice deficient for TSLPR, displayed strongly reduced arthritis severity and immunopathology, associated with strong inhibition of evident Th17 activity and modest Th2 activity (60). In analogy to histamine, this illustrates that TSLP may be a target not only in atopic diseases, but also in autoimmune disease like RA.

Mediators like TSLP were also shown to play a role in rheumatic diseases apart from RA in which Th2/Th17 responses seem to be more prominent. Recently, it was demonstrated that in addition to epithelial cells and fibroblasts, mast cells in human SSc skin and lung fibrosis and in the bleomycin model of scleroderma overexpressed TSLP (56, 61), which in its

turn can activate mast cells (32). In cultured skin fibroblasts, TSLP expression was induced upon TLR triggering. TSLP in the skin of patients with SSc induced profibrotic genes and intracellular signaling that overlap with those induced by IL-13 and transforming growth factor (TGF) β, both pivotal mediators in SSc (61). In addition, TSLP was demonstrated to induce TGFβ-dependent fibrosis induction. Furthermore, in TSLPR-deficient mice, bleomycin-induced fibrosis was significantly reduced in parallel with significantly reduced local expression of IL-13 (56). Finally, TSLP could also be a link to IL-33 expression, which induces IL-13-dependent cutaneous fibrosis (62) and whose levels are raised in SSc patients correlating with the extent of skin sclerosis and severity of pulmonary fibrosis (63). Since TSLP has the capacity to enhance Th2 activity associated with IL-33 and IL-13 production in protease-allergen-induced airways inflammation (64), this indicates a potential TSLP-induced axis in SSc, contributing to fibrosis.

Other rheumatic diseases in which enhanced expression of IL-33 and IL-13 has been demonstrated include pSS, SpA, and RA (54, 65, 66). Serum levels of IL-33 were significantly elevated in pSS patients, especially in patients with interstitial lung disease. IL-33 significantly correlated with rheumatoid factor and anti-SSB autoantibody levels in pSS (65). Increased levels of IL-33 and IL-13 were also detected in the joints of RA patients and have been indicated to contribute to synovial fibroblast hyperplasia (54).

The beneficial effects of blockade of the H_4R to prevent innate and acquired immune responses and thereby immunopathology in experimental arthritis have been demonstrated (47). Furthermore, recent data demonstrate that targeting of other Th2-associated mediators, like TSLP, IL-33, and IL-13 prevents inflammation and immunopathological processes such as fibrosis. Collectively these data underscore that targets previously considered relevant in Th2-associated diseases, should be evaluated to prevent specific immunopathological processes in rheumatic diseases such as SSc, RA, pSS, SpA and SLE. Clinical trials will be required to document how such strategies could fit in the current armamentarium to treat these diseases.

Is prevention of costimulation by dendritic cells the (complete) answer to T cell-driven arthritis?

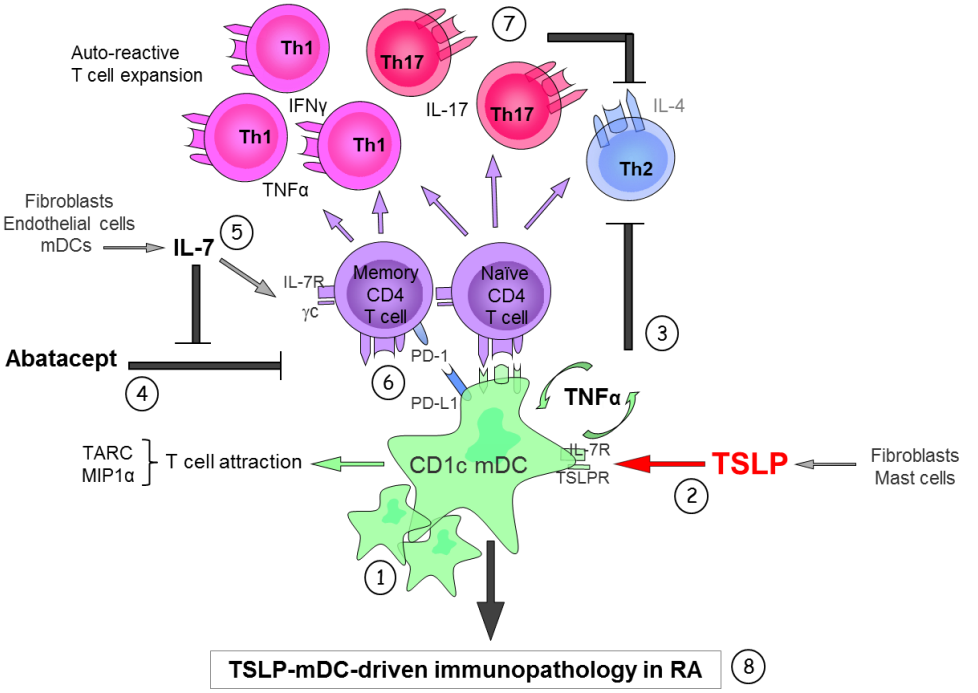
Two observations made in this thesis revealed that blockade of costimulation by drugs like abatacept (CTLA4-Ig) may not be completely successful to prevent T cell activation in RA. First, T cell-activating cytokines might overrule abatacept efficacy. As a consequence of TSLP driven activation of mDCs, T cell-attracting chemokines are secreted and T cell activity is induced. Mainly PB-derived T cells are potently activated by mDCs from the joint suggesting that T cells attracted from the blood to the joint or to the secondary lymphoid organs are most prone for (TSLP-primed) mDC driven activation. Targeting TSLP-mDC driven T cell

activation by blocking CD80/86 costimulation using abatacept is extremely effective; abatacept almost completely prevented activation of circulating naïve and memory T cells. However, high levels of T cell-activating cytokines like IL-7, IL-15, and IL-18 are demonstrated in joints of RA patients and strongly activate T cells (20, 67, 68). This thesis demonstrates that the inhibitory capacity of abatacept to prevent TSLP-mDC driven T cell activation is overruled by IL-7 and potentially other cytokines. This suggests that patients with high local levels of T cell-activating cytokines have a partial or no response to abatacept therapy and would rather benefit from other treatment strategies like targeting signaling pathways induced by such cytokines. Along the same line we did a second observation that indicates that abatacept may be unsuccessful to inhibit T cell activation as synovial T cells are hypo-responsive to activated mDCs. Since synovial T cells show many signs of activation, this indicates that these cells are likely activated in other ways. In this thesis we show that cytokines like IL-7 can completely reverse hypo-responsiveness that is minimally prevented by abatacept. Together this suggests that abatacept may primarily target cells that will enter the sites of inflammation, but may leave synovial T cells in the joints of RA patients unaffected. Such patients would be in need of other T cell-targeting strategies or mDC-targeted strategies that result in tolerance induction.

Concluding remarks

In this thesis it is demonstrated that *in vivo* activated CD1c mDCs can play an important role in the initiation and perpetuation of rheumatoid arthritis by the attraction and activation of T cells inducing Th1, Th17 as well as Th2 activity. TSLP is identified as one of the potential drivers of mDC activation. The inflammatory environment likely plays an important role in skewing these T cell responses towards Th1 and Th17 activity as production of TNFα by TSLP-stimulated mDCs favored a more pronounced Th1 activity. Furthermore, our data demonstrate that the presence of T cell-activating cytokines, like IL-7, in joints of RA patients reduce the inhibitory capacity of abatacept on mDC-driven T cell activation and reverses the tolerogenic signals that induce synovial T cell hypo-responsiveness.

Prevention of TSLP- and IL-7-driven mDC/T cell activation, which in animal models has demonstrated almost complete prevention of inflammation and immunopathology, seem to have therapeutic potential. However, other ways to prevent activation of CD1c mDCs and induce tolerogenic activity may also have therapeutic potential and should be unraveled and exploited.



Schema summarizing the findings of the current thesis. Increased numbers of CD1c myeloid dendritic cells (mDCs) show an activated phenotype associated with enhanced production of a unique set of chemokines and increased capacity to activate self-reactive T cells (1). Increased levels of TSLP are present in joints of rheumatoid arthritis patients and *in vitro* TSLP-activated mDCs from peripheral blood mimick *ex vivo*-activated CD1c mDCs from synovial fluid in their phenotype, chemokine secretion and activation of CD4 T cells (2). TNFα produced by (TSLP)-activated mDCs prevents attraction of Th2 cells and favours skewing towards Th1 cells (3), partially explaining the seemingly reduced induction of Th2 activity by TSLP in rheumatoid arthritis patients. Blockade of costimulation by CTLA4-Ig (abatacept) robustly prevents activation of CD4 T cells by TSLP-activated mDCs (4). T cell-activating cytokines, like IL-7, robustly reverse the inhibition induced by abatacept and contributes to Th1 and Th17 activation and TNFα production (5). Synovial fluid T cells, mainly memory cells, are hypo-responsive to activated mDCs, which is associated with strong up regulation of PD-1 on T cells and PD-L1 on mDCs (6). Ways that block PD-1/PD-L1 interaction, by an PD-1 blocking antibody and by addition of IL-7 that downregulates PD-1, reverse the hypo-responsiveness. The net result of increased and activated CD1c mDCs, secretion of TNFα and T cell-activating factors, like IL-7, may result in predominance of Th1/Th17 cells (7) that play an important role in the initiation and perpetuation of rheumatoid arthritis as well induction of immunopathology (8).

REFERENCES

1. Feldmann M, Brennan FM, Maini RN. Rheumatoid arthritis. Cell 1996;85(3):307-10.
2. Morita Y, Yamamura M, Kawashima M, Harada S, Tsuji K, Shibuya K, et al. Flow cytometric single-cell analysis of cytokine production by CD4+ T cells in synovial tissue and peripheral blood from patients with rheumatoid arthritis. Arthritis Rheum 1998;41(9):1669-76.
3. van Roon JA, van Roy JL, Duits A, Lafeber FP, Bijlsma JW. Proinflammatory cytokine production and cartilage damage due to rheumatoid synovial T helper-1 activation is inhibited by interleukin-4. Ann Rheum Dis 1995;54(10):836-40.
4. Cornelissen F, van Hamburg JP, Lubberts E. The IL-12/IL-23 axis and its role in Th17 cell development, pathology and plasticity in arthritis. Curr Opin Investig Drugs 2009;10(5):452-62.
5. Chabaud M, Durand JM, Buchs N, Fossiez F, Page G, Frappart L, et al. Human interleukin-17: A T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. Arthritis Rheum 1999;42(5):963-70.
6. Thomas R, Davis LS, Lipsky PE. Rheumatoid synovium is enriched in mature antigen-presenting dendritic cells. J Immunol 1994;152(5):2613-23.
7. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392(6673):245-52.
8. Coquerelle C, Moser M. DC subsets in positive and negative regulation of immunity. Immunol Rev 2010;234(1):317-34.
9. Torres-Aguilar H, Blank M, Jara LJ, Shoenfeld Y. Tolerogenic dendritic cells in autoimmune diseases: crucial players in induction and prevention of autoimmunity. Autoimmun Rev 2010;10(1):8-17.
10. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. Annu Rev Immunol 2013;31:563-604.
11. Satpathy AT, Wu X, Albring JC, Murphy KM. Re(de)fining the dendritic cell lineage. Nat Immunol 2012;13(12):1145-54.
12. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of monocytes and dendritic cells in blood. Blood 2010;116(16):e74-80.
13. Dzionek A, Fuchs A, Schmidt P, Cremer S, Zysk M, Miltenyi S, et al. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. J Immunol 2000;165(11):6037-46.
14. Thomas R, MacDonald KP, Pettit AR, Cavanagh LL, Padmanabha J, Zehntner S. Dendritic cells and the pathogenesis of rheumatoid arthritis. J Leukoc Biol 1999;66(2):286-92.
15. Smolewska E, Stanczyk J, Brozik H, Biernacka-Zielinska M, Cebula B, Robak T, et al. Distribution and clinical significance of blood dendritic cells in children with juvenile idiopathic arthritis. Ann Rheum Dis 2008;67(6):762-8.
16. Jongbloed SL, Lebre MC, Fraser AR, Gracie JA, Sturrock RD, Tak PP, et al. Enumeration and phenotypical analysis of distinct dendritic cell subsets in psoriatic arthritis and rheumatoid arthritis. Arthritis Res Ther 2006;8(1):R15.
17. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 2000;192(7):1027-34.
18. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat Immunol 2001;2(3):261-8.

19. Penaranda C, Kuswanto W, Hofmann J, Kenefeck R, Narendran P, Walker LS, et al. IL-7 receptor blockade reverses autoimmune diabetes by promoting inhibition of effector/memory T cells. Proc Natl Acad Sci U S A 2012;109(31):12668-73.

20. van Roon JA, Verweij MC, Wijk MW, Jacobs KM, Bijlsma JW, Lafeber FP. Increased intraarticular interleukin-7 in rheumatoid arthritis patients stimulates cell contact-dependent activation of CD4(+) T cells and macrophages. Arthritis Rheum 2005;52(6):1700-10.

21. Lundstrom W, Highfill S, Walsh ST, Beq S, Morse E, Kockum I, et al. Soluble IL7Ralpha potentiates IL-7 bioactivity and promotes autoimmunity. Proc Natl Acad Sci U S A 2013;110(19):E1761-70.

22. van Roon JA, Hartgring SA, Wenting-van Wijk M, Jacobs KM, Tak PP, Bijlsma JW, et al. Persistence of interleukin 7 activity and levels on tumour necrosis factor alpha blockade in patients with rheumatoid arthritis. Ann Rheum Dis 2007;66(5):664-9.

23. van Roon JA, Jacobs K, Verstappen S, Bijlsma J, Lafeber F. Reduction of serum interleukin 7 levels upon methotrexate therapy in early rheumatoid arthritis correlates with disease suppression. Ann Rheum Dis 2008;67(7):1054-5.

24. Badot V, Durez P, Van den Eynde BJ, Nzeusseu-Toukap A, Houssiau FA, Lauwerys BR. Rheumatoid arthritis synovial fibroblasts produce a soluble form of the interleukin-7 receptor in response to pro-inflammatory cytokines. J Cell Mol Med 2011;15(11):2335-42.

25. Fujikado N, Saijo S, Yonezawa T, Shimamori K, Ishii A, Sugai S, et al. Dcir deficiency causes development of autoimmune diseases in mice due to excess expansion of dendritic cells. Nat Med 2008;14(2):176-80.

26. Wilson J, Cullup H, Lourie R, Sheng Y, Palkova A, Radford KJ, et al. Antibody to the dendritic cell surface activation antigen CD83 prevents acute graft-versus-host disease. J Exp Med 2009;206(2):387-98.

27. Markey KA, Banovic T, Kuns RD, Olver SD, Don AL, Raffelt NC, et al. Conventional dendritic cells are the critical donor APC presenting alloantigen after experimental bone marrow transplantation. Blood 2009;113(22):5644-9.

28. Meyer-Wentrup F, Cambi A, Joosten B, Looman MW, de Vries IJ, Figdor CG, et al. DCIR is endocytosed into human dendritic cells and inhibits TLR8-mediated cytokine production. J Leukoc Biol 2009;85(3):518-25.

29. Meyer-Wentrup F, Benitez-Ribas D, Tacken PJ, Punt CJ, Figdor CG, de Vries IJ, et al. Targeting DCIR on human plasmacytoid dendritic cells results in antigen presentation and inhibits IFN-alpha production. Blood 2008;111(8):4245-53.

30. Stoop JN, Robinson JH, Hilkens CM. Developing tolerogenic dendritic cell therapy for rheumatoid arthritis: what can we learn from mouse models? Ann Rheum Dis 2011;70(9):1526-33.

31. Hilkens CM, Isaacs JD. Tolerogenic dendritic cell therapy for rheumatoid arthritis: where are we now? Clin Exp Immunol 2013;172(2):148-57.

32. Allakhverdi Z, Comeau MR, Jessup HK, Yoon BR, Brewer A, Chartier S, et al. Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. J Exp Med 2007;204(2):253-8.

33. Leung S, Liu X, Fang L, Chen X, Guo T, Zhang J. The cytokine milieu in the interplay of pathogenic Th1/Th17 cells and regulatory T cells in autoimmune disease. Cell Mol Immunol 2010;7(3):182-9.

34. van Woerkom JM, Kruize AA, Wenting-van Wijk MJ, Knol E, Bihari IC, Jacobs JW, et al. Salivary gland and peripheral blood T helper 1 and 2 cell activity in Sjogren's syndrome compared with non-Sjogren's sicca syndrome. Ann Rheum Dis 2005;64(10):1474-9.

35. Nguyen CQ, Hu MH, Li Y, Stewart C, Peck AB. Salivary gland tissue expression of interleukin-23 and interleukin-17 in Sjogren's syndrome: findings in humans and mice. Arthritis Rheum 2008;58(3):734-43.

36. Verhoef CM, van Roon JA, Vianen ME, Lafeber FP, Bijlsma JW. The immune suppressive effect of dexamethasone in rheumatoid arthritis is accompanied by upregulation of interleukin 10 and by differential changes in interferon gamma and interleukin 4 production. Ann Rheum Dis 1999;58(1):49-54.

37. Ghoreschi K, Thomas P, Breit S, Dugas M, Mailhammer R, van Eden W, et al. Interleukin-4 therapy of psoriasis induces Th2 responses and improves human autoimmune disease. Nat Med 2003;9(1):40-6.

38. Kashiwakura J, Yanagisawa M, Lee H, Okamura Y, Sasaki-Sakamoto T, Saito S, et al. Interleukin-33 synergistically enhances immune complex-induced tumor necrosis factor alpha and interleukin-8 production in cultured human synovium-derived mast cells. Int Arch Allergy Immunol 2013;161 Suppl 2:32-6.

39. Konttinen YT, Tuominen S, Segerberg-Konttinen M, Jungell P, Malmstrom MJ, Gronblad M, et al. Mast cells in the labial salivary glands of patients with Sjogren's syndrome: a histochemical, immunohistochemical, and electron microscopical study. Ann Rheum Dis 1990;49(9):685-9.

40. Hugle T, Hogan V, White KE, van Laar JM. Mast cells are a source of transforming growth factor beta in systemic sclerosis. Arthritis Rheum 2011;63(3):795-9.

41. Noordenbos T, Yeremenko N, Gofita I, van de Sande M, Tak PP, Canete JD, et al. Interleukin-17-positive mast cells contribute to synovial inflammation in spondylarthritis. Arthritis Rheum 2012;64(1):99-109.

42. Shrestha S, Wershil B, Sarwark JF, Niewold TB, Philipp T, Pachman LM. Lesional and nonlesional skin from patients with untreated juvenile dermatomyositis displays increased numbers of mast cells and mature plasmacytoid dendritic cells. Arthritis Rheum 2010;62(9):2813-22.

43. Seidman RJ, Kaufman LD, Sokoloff L, Miller F, Iliya A, Peress NS. The neuromuscular pathology of the Eosinophilia-Myalgia syndrome. J Neuropathol Exp Neurol 1991;50(1):49-62.

44. Kneilling M, Hultner L, Pichler BJ, Mailhammer R, Morawietz L, Solomon S, et al. Targeted mast cell silencing protects against joint destruction and angiogenesis in experimental arthritis in mice. Arthritis Rheum 2007;56(6):1806-16.

45. Lee DM, Friend DS, Gurish MF, Benoist C, Mathis D, Brenner MB. Mast cells: a cellular link between autoantibodies and inflammatory arthritis. Science 2002;297(5587):1689-92.

46. Walter M, Kottke T, Stark H. The histamine H(4) receptor: targeting inflammatory disorders. Eur J Pharmacol 2011;668(1-2):1-5.

47. Cowden JM, Yu F, Banie H, Farahani M, Ling P, Nguyen S, et al. The histamine H4 receptor mediates inflammation and Th17 responses in preclinical models of arthritis. Ann Rheum Dis 2013.

48. Stegaev V, Sillat T, Porola P, Hanninen A, Falus A, Mieliauskaite D, et al. Brief report: first identification of H(4) histamine receptor in healthy salivary glands and in focal sialadenitis in Sjogren's syndrome. Arthritis Rheum 2012;64(8):2663-8.

49. Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. Nat Immunol 2002;3(7):673-80.

50. Hsu CL, Bryce PJ. Inducible IL-33 expression by mast cells is regulated by a calcium-dependent pathway. J Immunol 2012;189(7):3421-9.

51. Redegeld FA, van der Heijden MW, Kool M, Heijdra BM, Garssen J, Kraneveld AD, et al. Immunoglobulin-free light chains elicit immediate hypersensitivity-like responses. Nat Med 2002;8(7):694-701.

52. Varadaradjalou S, Feger F, Thieblemont N, Hamouda NB, Pleau JM, Dy M, et al. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human mast cells. Eur J Immunol 2003;33(4):899-906.

53. Lappalainen J, Rintahaka J, Kovanen PT, Matikainen S, Eklund KK. Intracellular RNA recognition pathway activates strong anti-viral response in human mast cells. Clin Exp Immunol 2013;172(1):121-8.

54. Hu F, Shi L, Mu R, Zhu J, Li Y, Ma X, et al. Hypoxia-inducible factor-1alpha and interleukin 33 form a regulatory circuit to perpetuate the inflammation in rheumatoid arthritis. PLoS One 2013;8(8):e72650.

55. Ozawa T, Koyama K, Ando T, Ohnuma Y, Hatsushika K, Ohba T, et al. Thymic stromal lymphopoietin secretion of synovial fibroblasts is positively and negatively regulated by Toll-like receptors/nuclear factor-kappaB pathway and interferon-gamma/dexamethasone. Mod Rheumatol 2007;17(6):459-63.

56. Usategui A, Criado G, Izquierdo E, Del Rey MJ, Carreira PE, Ortiz P, et al. A profibrotic role for thymic stromal lymphopoietin in systemic sclerosis. Ann Rheum Dis 2013;72(12):2018-23.

57. Smuda C, Wechsler JB, Bryce PJ. TLR-induced activation of neutrophils promotes histamine production via a PI3 kinase dependent mechanism. Immunol Lett 2011;141(1):102-8.

58. Moret FM, Hack CE, van der Wurff-Jacobs KM, Radstake TR, Lafeber FP, van Roon JA. Thymic stromal lymphopoietin (TSLP): a novel proinflammatory mediator in rheumatoid arthritis that potently activates CD1c myeloid dendritic cells to attract and stimulate T cells. Arthritis Rheum 2014; doi: 10.1002/art.38338. [Epub ahead of print].

59. Moret FM, Hack CE, van der Wurff-Jacobs KM, de Jager W, Radstake TR, Lafeber FP, et al. Intra-articular CD1c-expressing myeloid dendritic cells from rheumatoid arthritis patients express a unique set of T cell-attracting chemokines and spontaneously induce Th1, Th17 and Th2 cell activity. Arthritis Res Ther 2013;15(5):R155.

60. Hartgring SA, Willis CR, Dean CE, Jr., Broere F, van Eden W, Bijlsma JW, et al. Critical proinflammatory role of thymic stromal lymphopoietin and its receptor in experimental autoimmune arthritis. Arthritis Rheum 2011;63(7):1878-87.

61. Christmann RB, Mathes A, Affandi AJ, Padilla C, Nazari B, Bujor AM, et al. Thymic stromal lymphopoietin is up-regulated in the skin of patients with systemic sclerosis and induces profibrotic genes and intracellular signaling that overlap with those induced by interleukin-13 and transforming growth factor beta. Arthritis Rheum 2013;65(5):1335-46.

62. Rankin AL, Mumm JB, Murphy E, Turner S, Yu N, McClanahan TK, et al. IL-33 induces IL-13-dependent cutaneous fibrosis. J Immunol 2010;184(3):1526-35.

63. Yanaba K, Yoshizaki A, Asano Y, Kadono T, Sato S. Serum IL-33 levels are raised in patients with systemic sclerosis: association with extent of skin sclerosis and severity of pulmonary fibrosis. Clin Rheumatol 2011;30(6):825-30.

64. Togbe D, Fauconnier L, Madouri F, Marchiol T, Chenuet P, Rouxel N, et al. Thymic Stromal Lymphopoietin Enhances Th2/Th22 and Reduces IL-17A in Protease-Allergen-Induced Airways Inflammation. ISRN Allergy 2013;2013:971036.

65. Zhao L, Yao L, Yuan L, Xia L, Shen H, Lu J. Potential contribution of interleukin-33 to the development of interstitial lung disease in patients with primary Sjogren's Syndrome. Cytokine 2013;64(1):22-4.

66. Theoharides TC, Zhang B, Kempuraj D, Tagen M, Vasiadi M, Angelidou A, et al. IL-33 augments substance P-induced VEGF secretion from human mast cells and is increased in psoriatic skin. Proc Natl Acad Sci U S A 2010;107(9):4448-53.

67. McInnes IB, al-Mughales J, Field M, Leung BP, Huang FP, Dixon R, et al. The role of interleukin-15 in T-cell migration and activation in rheumatoid arthritis. Nat Med 1996;2(2):175-82.

68. Dai SM, Shan ZZ, Xu H, Nishioka K. Cellular targets of interleukin-18 in rheumatoid arthritis. Ann Rheum Dis 2007;66(11):1411-8.

9

Addendum

Nederlandse samenvatting
Dankwoord
Curriculum vitae
List of publications

NEDERLANDSE SAMENVATTING

Introductie

Alhoewel het immuunsysteem er op gericht is om het eigen lichaam te beschermen tegen ziekteverwekkers komt het wel eens voor dat cellen van het immuunsysteem bovenmatig gaan reageren tegen lichaamseigen componenten, cellen en eiwitten. Hierdoor ontstaat een auto-immuunziekte waarbij het eigen lichaam wordt aangevallen en er schade aan gezond weefsel ontstaat.

Reumatoïde artritis (RA) is een auto-immuunziekte die voorkomt bij ongeveer 1% van de bevolking waarvan het merendeel vrouw is. RA wordt gekenmerkt door chronische ontsteking van de binnenbekleding van een gewricht (het synovium), waardoor het gewricht opzwelt, pijnlijk is en het gewricht beschadigd raakt. Schade aan het gewricht kan leiden tot vervormingen en een verminderde functie van het gewricht. Verschillende typen cellen van het immuunsysteem spelen een rol in RA, waaronder T cellen en B cellen. B cellen produceren antistoffen die gericht zijn tegen specifieke eiwitten. In een groot deel (70%) van de RA patiënten worden antistoffen aangetoond tegen lichaamseigen antistoffen (Reumafactor) en tegen gemodificeerde eiwitten, de zogenaamde gecitrullineerde eiwitten die zich in het ontstoken synovium bevinden (ACPA antistoffen). Deze antistoffen kunnen vaak al aangetoond worden voordat de ziekte zich klinisch manifesteert en zijn zeer specifiek voor RA. De aanleiding voor de ontwikkeling van RA is tot op heden niet bekend. Wel is het bekend dat zowel genetische- als omgevingsfactoren een rol spelen.

Genen die sterk geassocieerd worden met de ontwikkeling van RA coderen met name voor eiwitten die een belangrijke rol spelen in het activeren van T cellen door antigenen (eiwit fragmenten) te presenteren. Dit zijn de humane leukocyt antigenen (HLA)-moleculen, die zich bevinden op professionele antigeen-presenterende cellen, waaronder de dendritische cellen. Dendritische cellen spelen een belangrijke rol in de coördinatie van de immuunrespons tegen ziekteverwekkers. Dit doen zij door de ziekteverwekkers op te nemen en te presenteren in een HLA-molecuul, zodat deze te herkennen zijn door specifieke T cellen. Daarnaast hebben dendritische cellen ook een belangrijke rol in het presenteren van lichaamseigen eiwitten om te zorgen dat de T cellen tolerant worden voor lichaamseigen eiwitten. T cellen die lichaamseigen eiwitten te sterk herkennen worden normaliter uit het repertoire van T cellen verwijderd in de thymus, zodat zij geen schade aan kunnen richten, maar het komt wel eens voor dat deze T cellen toch ontsnappen. Ook kunnen normale T cellen die een mate van zelfherkenning hebben ontsporen. Het immuunsysteem is echter uitgerust met extra beschermingsmechanismen om te voorkomen dat deze T cellen geactiveerd worden. Een voorbeeld hiervan is dat T cellen naast het herkennen van een

stukje eiwit ook nog costimulatie nodig hebben om daadwerkelijk geactiveerd te raken. Dendritische cellen kunnen costimulatie aan de T cel geven nadat ze zelf geactiveerd zijn. Een DC kan geactiveerd worden door receptoren op zijn oppervlakte die ziekteverwekkers herkennen of door cytokines (boodschappermoleculen die door cellen worden uitgescheiden), zoals thymic stromal lymphopoietin (TSLP).

TSLP staat in de literatuur bekend voor het induceren van een bepaald type T cel respons, de CD4 T helpercel 2 (Th2) respons, die onder andere een belangrijke rol hebben in allergieën. TSLP induceert de Th2 respons met name via de activatie van myeloïde dendritische cellen (mDCs) en deze Th2 respons kan herkend worden aan de productie van onder andere interleukine-4 (IL-4). Dendritisch cellen kunnen onderverdeeld worden in verschillende typen, waaronder de mDCs, en deze kunnen geïdentificeerd worden aan de hand van verschillende markers. Veel onderzoek is gedaan naar mDCs die geïdentificeerd zijn aan de hand van de marker CD11c. Echter, recent onderzoek heeft aangetoond dat deze marker niet heel nauwkeurig is voor mDCs, omdat CD11c ook tot expressie komt op andere cellen zoals monocyten. Een betere marker voor het identificeren van mDCs is het het blood-derived dendritic cell antigen (BDCA)-molecuul type 1 (BDCA-1) oftewel CD1c. CD1c mDCs zijn erg goed in het activeren van CD4 T cellen.
In RA spelen twee andere typen CD4 T cellen een prominente rol, de Th1 en Th17 cellen. Th1 cellen zijn te herkennen aan de productie van de cytokine interferon gamma (IFNγ) en Th17 cellen aan de productie van IL-17.
Ondanks dat TSLP in de literatuur erg bekend staat voor het induceren van Th2 activiteit dat de werking van Th1 en Th17 activiteit kan verminderen, hebben eerder gepubliceerde data van onze groep laten zien dat TSLP een belangrijke rol speelt in de verergering van de artritis in muizenmodellen. Daarnaast kunnen verschillende ontstekingsmediatoren leiden tot de productie van TSLP door fibroblasten (weefselcellen) uit het synovium van gewrichten van RA patiënten.

Dit proefschrift

Het doel van dit proefschrift was om te onderzoeken hoe TSLP de natuurlijk voorkomende CD1c mDCs activeert en hoe deze vervolgens de CD4 T-cel responsen reguleren in RA. Een ander doel was om te onderzoeken hoe abatacept, een anti-reumatisch middel dat de T cel costimulatie blokkeert, deze TSLP-gedreven immunopathologie in RA kan beïnvloeden.

Hoofdstuk 2 laat zien dat de CD1c mDC aantallen 8-keer zo hoog zijn in de synoviale vloeistof (de gewrichtsvloeistof) van RA patiënten ten opzichte van de aantallen in het bloed. De CD1c mDCs uit de gewrichtsvloeistof hebben ook een geactiveerd uiterlijk (fenotype); zij vertonen een hogere expressie van antigeen-presenterende moleculen en costimulatoire

moleculen en zijn in staat om spontaan autologe CD4 T cellen te activeren, waardoor Th1, Th17 en Th2 cel activiteit ontstaat. CD1c mDCs uit het bloed zijn echter niet in staat om spontaan CD4 T cellen te activeren. De inductie van deze typen T cel responsen ligt niet aan de productie van cytokines die de T cel respons in een bepaalde richting kunnen sturen, want er is geen verschil tussen CD1c mDCs uit het bloed en uit de gewrichtsvloeistof in de productie van T cel-differentiërende cytokines. Wel produceren CD1c mDCs uit de gewrichtsvloeistof een hogere concentratie van een unieke set van chemokines (stofjes die cellen aantrekken; in dit geval T cellen) specifiek voor T cellen. Deze data suggereren dat het verhoogde aantal CD1c mDCs in de gewrichtsvloeistof van RA patiënten betrokken kunnen zijn bij het ontstekingsproces door het aantrekken en activeren van T cellen.

In **hoofdstuk 3** hebben we onderzocht of TSLP zou kunnen bijdragen aan de activatie van mDCs en daardoor T cellen. In dit hoofdstuk laten we zien dat TSLP zeer potent CD1c mDCs activeert om T cellen aan te trekken en te activeren. De levels van TSLP waren significant verhoogd in de gewrichtsvloeistof van RA patiënten ten opzichte van artrose patiënten (een aandoeningen van het gewricht met een milde of geen ontsteking). De CD1c mDC aantallen die de TSLP receptor tot expressie brachten waren ook significant verhoogd in de gewrichtsvloeistof ten opzichte van het bloed van RA patiënten en de mDCs uit de gewrichtsvloeistof hadden een hogere TSLP receptor expressie per cel. De stimulatie van mDCs met TSLP in celkweken zorgde voor een hogere productie van de chemokines TARC en MIP1α, die specifiek T cellen aantrekken, en het resulteerde in een sterk verhoogde activiteit van deze TSLP-gestimuleerde mDCs om autologe T cel proliferatie te induceren ten opzichte van ongestimuleerde mDCs. De T cel proliferatie was geassocieerd met een verhoogde productie van IFNγ, IL-17 en IL-4. Deze data laten een ontstekingsmechanisme zien waarin verhoogde TSLP levels de CD1c mDCs die de TSLP receptor tot expressie brengen potent kunnen activeren in het gewricht van RA patiënten om T cel aantrekkende chemokines uit te scheiden en vervolgens T cellen te activeren. In toevoeging op de eerder beschreven rol van TSLP in de verergering van artritis in muizen suggereren deze data dat TSLP en de mDCs die de TSLP receptor tot expressie brengen beide een belangrijke rol kunnen spelen in de immunopathologie van RA.

Naast dat TSLP-gestimuleerde CD1c mDCs een verhoogde concentratie van specifieke chemokines produceren ten opzichte van ongestimuleerde mDCs, induceert TSLP ook de productie van TNFα door CD1c mDCs uit zowel bloed als gewrichtsvloeistof van RA patiënten. TNFα is een zeer sterk ontstekingsbevorderend cytokine en therapieën die deze factor remmen in RA patiënten behoren op dit moment tot de belangrijkste anti-reumatische middelen. Om het effect van deze TSLP-geïnduceerde productie van TNFα te bestuderen in de activatie van CD1c mDCs en vervolgens T cellen hebben we gedurende de TSLP stimulatie de geproduceerde TNFα geblokkeerd door middel van een TNFα-blokkerend

antilichaam. In **hoofdstuk 4** is aangetoond dat TNFα de capaciteit van TSLP-geactiveerde CD1c mDCs van RA patiënten om Th2 activiteit te promoten vermindert. Dit is aangetoond doordat TNFα blokkade gedurende de TSLP stimulatie van mDCs niet de productie van T cel-differentiërende cytokines beïnvloedde, maar wel de productie van MIP1α (een ligand voor de CC chemokine receptor 5 (CCR5) die tot expressie komt op Th1 cellen) significant verlaagde en de productie van TARC (een ligand voor de CCR4 die tot expressie komt op Th2 cellen) licht verhoogde. Vergeleken met TSLP-mDCs hadden anti-TNF behandelde TSLP-mDCs geen effect op de IFNγ en IL-17 productie van samen gekweekte CD4 T cellen, maar verhoogde ze wel significant de productie van IL-4 en verminderde ze de IFNγ:IL-4 ratio. Deze data laten zien dat TNFα, geproduceerd door TSLP-geactiveerde mDCs, de Th2 respons remt door het induceren van het Th1-aantrekkende chemokine MIP1α en het voorkomen van T cellen om IL-4 te produceren. Beide processen kunnen mogelijk een bijdrage leveren aan de TSLP-geïnduceerde immunopathologie in RA. Daarbij kan de verschuiving in de balans van TSLP-gemedieerde Th1/Th17 activiteit naar TSLP-gemedieerde Th2 activiteit door het therapeutisch blokkeren van TNFα een mogelijke bijdrage leveren aan het verminderen van de ziekteactiviteit.

In **hoofdstuk 5** is gekeken of abatacept, een ander voornaam anti-reumatisch middel dat T cel activatie voorkomt door het blokkeren van de costimulatie, effectief is om TSLP-mDC gedreven T cel activatie te voorkomen. Dit is getest in de aan- en afwezigheid van cytokines die verhoogd geproduceerd worden in gewrichten van RA patiënten en die direct T cellen kunnen activeren, zoals IL-7. Dit hoofdstuk laat zien dat CD4 T cellen in het bloed met name bestaan uit T cellen die nog niet eerder geactiveerd zijn geweest (naïeve T cellen) en T cellen die al opgeleid zijn om eiwitten te herkennen (memorie T cellen). T cellen die voorkomen in de gewrichtsvloeistof bestaan met name uit memorie T cellen en nauwelijks uit naïeve T cellen. De activatie van naïeve en memorie T cellen door TSLP-gestimuleerde mDCs uit het bloed werd compleet geblokkeerd door abatacept. Echter, in de aanwezigheid van IL-7 deelden deze T cellen sterk en scheidden grote hoeveelheden cytokines (IFNγ, TNFα en IL-17) uit ondanks de aanwezigheid van abatacept. Op een vergelijkbare manier werd de T cel proliferatie geïnduceerd door TSLP-gestimuleerde mDCs uit de gewrichtsvloeistof van RA patiënten geblokkeerd door abatacept en sterk geactiveerd in de aanwezigheid van IL-7. Concluderend laten deze data zien dat de aanwezigheid van cytokines die direct T cellen kunnen activeren, zoals IL-7, in de gewrichtsvloeistof van RA patiënten de werking van abatacept verminderen in het geval van mDC-gedreven T cel activiteit. Dit mechanisme zou een mogelijke reden kunnen zijn waarom een deel van de RA patiënten niet of nauwelijks reageert op abatacept therapie.

Omdat CD4 T cellen een grote rol spelen in het onstekingsproces in de gewrichten van RA patiënten en omdat mDCs uit de gewrichtsvloeistof erg potent zijn in het activeren van

autologe T cellen uit het bloed, hebben we in **hoofdstuk 6** onderzocht hoe goed deze mDCs autologe T cellen uit de gewrichtsvloeistof kunnen activeren. De T cellen uit de gewrichtsvloeistof reageerden echter nauwelijks op activatie door mDCs of TSLP-gestimuleerde mDCs, de synoviale T cellen waren dus hypo-responsief. Verschillende observaties leidden er toe om de rol van de receptor programmed cell death-1 (PD-1) te onderzoeken in deze hypo-responsiviteit. PD-1 wordt geactiveerd door binding met zijn ligand programmed cell death ligand 1 of 2 (PD-L1 of PD-L2, respectievelijk) en deze interactie zorgt ervoor dat de T cel activatie geremd wordt. De rol van PD-1 hebben we onderzocht door deze receptor te blokkeren en daarnaast hebben we ook gekeken naar de rol van IL-7, omdat is aangetoond dat IL-7 de receptor expressie van PD-1 remt en omdat IL-7 zeer potent T cellen kan activeren. In dit hoofdstuk is aangetoond dat het voorkomen van PD-1/PD-L1 interacties de hypo-responsiviteit van synoviale T cellen omkeert die ontstaat door activatie met CD1c mDCs uit het gewricht. De expressie van PD-1 was zeer hoog op CD4 T cellen uit de gewrichtsvloeistof van RA patiënten ten opzichte van deze cellen uit het bloed. Stimulatie van mDCs met TSLP verhoogde de mRNA levels (stukjes afgeschreven gen dat codeert voor een eiwit, in dit geval PD-L1) van PD-L1 maar niet van PD-L2 mRNA in mDCs uit zowel bloed als gewrichtsvloeistof en dit gold ook voor PD-L1 op het eiwitniveau. Het blokkeren van PD-1 gedurende de kweken met memorie T cellen en (TSLP-gestimuleerde) mDCs van RA patiënten verhoogde significant de T cel proliferatie, maar herstelde dit niet volledig terwijl het stimuleren met IL-7 voor een sterke T cel proliferatie zorgde. Deze data suggereren dat de hypo-responsiviteit van T cellen uit het gewricht die ontstaat door activatie met (TSLP-gestimuleerde) mDCs uit het gewricht van RA patiënten voor een gedeelte afhankelijk is van PD-1/PD-L1 interacties. De capaciteit van IL-7 om deze hypo-responsiviteit te doorbreken suggereert dat zulke ontstekingsbevorderende cytokines in de gewrichten van RA patiënten een belangrijke bijdrage kunnen leveren aan het activeren van de T cellen in het gewricht.

In eerdere studies en in hoofdstuk 5 en 6 is aangetoond dat IL-7 erg potent is in het activeren van T cellen in de context van mDCs uit het bloed en de gewrichtsvloeistof van RA patiënten. In **hoofdstuk 7** is beschreven dat de hoeveelheden van de natuurlijk voorkomende soluble IL-7Rα (sIL-7Rα), de niet membraan-gebonden receptor voor IL-7, erg sterk verhoogd zijn in de gewrichtsvloeistof van RA patiënten ten opzichte van artrose patiënten en dat deze levels gerelateerd zijn aan de ontstekingsbevorderende cytokine IL-6 en de bezinkingssnelheid (een maat voor ontsteking dat gemeten kan worden a.d.h.v. het bloed). Eerdere studies hebben aangetoond dat de productie van sIL-7Rα door bijvoorbeeld fibroblasten een mechanisme zou zijn om de werking van IL-7 tegen te gaan, maar recent onderzoek suggereert echter dat sIL-7Rα in bepaalde hoeveelheden de werking van IL-7 kan bevorderen en daarmee dus juist ook de ontsteking en de auto-immuniteit.

Conclusie

Dit onderzoek laat zien dat *in vivo* geactiveerde CD1c mDCs een belangrijke rol kunnen spelen in RA door het aantrekken en activeren van T cellen, waardoor Th1, Th17 en Th2 activiteit wordt verhoogd. TSLP is geïdentificeerd als een potentiële trigger voor het activeren van mDCs in het gewricht van RA patiënten. Het ontstekingsmilieu in het gewricht speelt hoogstwaarschijnlijk een belangrijke rol in het sturen van de T cel responsen naar Th1 en Th17 activiteit, aangezien de productie van TNFα door TSLP-geactiveerde mDCs een hogere Th1 activiteit induceerde. Verder laten onze data zien dat de aanwezigheid van T cel-activerende cytokines in het gewricht van RA patiënten, zoals IL-7, de therapeutische werking van abatacept op mDC-gedreven T cel activatie kunnen verminderen en de hypo-responsiviteit van synoviale T cellen teniet kunnen doen. Het voorkomen van TSLP- en IL-7-gedreven mDC/T cel activiteit zijn potentiële therapeutische strategieën voor RA zoals eerder door onze groep in diermodellen is aangetoond. Andere mogelijkheden om CD1c mDC activatie te voorkomen of tolerantie van deze cellen te induceren zijn ook veelbelovende strategieën.

DANKWOORD

Het is zover, ik mag mijn dankwoord schrijven! Mijn dank is groot aan een ieder die betrokken is geweest bij de totstandkoming en voltooiing van dit proefschrift; of het nu een grote of een kleine bijdrage is geweest.

Met veel plezier heb ik gewerkt aan mijn promotieonderzoek op de afdeling Reumatologie & Klinische Immunologie. Natuurlijk had ik zo af en toe mijn ups en downs, maar ik kijk terug op een hele leuke en mooie periode wat ertoe geleid heeft dat ik mijn doctorstitel heb kunnen behalen.

Mijn grootste dank gaat uit naar Joël, mijn copromotor. Als mijn dagelijkse begeleider ben jij van A tot Z betrokken geweest bij dit proefschrift en heb je ook de grootste bijdrage geleverd aan mijn vorming van PhD student tot PhD. Bedankt voor al je inzet en begeleiding die je me hebt gegeven om mezelf te ontwikkelen in het doen, opschrijven en presenteren van wetenschappelijk onderzoek. Ik heb veel van je geleerd! Ik bewonder je enorme kennis en je hart voor het onderzoek en ik hoop dat er uit de veelheid van jouw ideeën nog vele mooie publicaties gaan komen! Het was erg leuk en fijn om met je samen te werken. Ik vond het leuk om met je te brainstormen over lastige immunologische kwesties en daarnaast ben je heel grappig! Ook wist je me altijd gerust te stellen als ik dat nodig had, dat was erg fijn! Dankjewel Joël voor dit alles!

Ook gaat veel van mijn dank uit naar Floris en prof. Bijlsma, mijn promotoren, jullie zijn ook heel belangrijk geweest bij dit proefschrift en in mijn carrière tot PhD.

Floris, bedankt dat ik binnen jouw leuke afdeling wetenschappelijk onderzoek mocht doen. Ik heb het erg naar mijn zin gehad en ik ben blij met het eindresultaat! Jij wist altijd goed de druk op de ketel te houden. Dat heb ik erg gewaardeerd! Dankjewel voor al je inzet en begeleiding die je me gegeven hebt voor het succesvol afronden van mijn proefschrift!

Prof. Bijlsma, u ook zeer bedankt voor uw betrokkenheid en aandeel in mijn proefschrift. Vanuit de kliniek wist u mijn onderzoek goed aan te vullen en uw positieve woorden hebben mij altijd erg goed gedaan!

Graag wil ik alle reumapatiënten die hebben meegewerkt aan dit onderzoek heel hartelijk bedanken. Ook aan alle artsen en (research-)verpleegkundigen, hartelijk dank voor jullie inzet en medewerking!

Marjolein, de datamanager, dankjewel voor alle leuke gesprekken die ik met je heb gehad, je gezelligheid en inzit wat betreft de bloedaanvragen!

De analisten, Arno, Katja, Kim, Marion en oud-analisten Angelique, Dorien en Karin, heel hartelijk dank voor al jullie praktische hulp en ondersteuning en ook voor jullie gezelligheid!

Kim, jij bent heel belangrijk geweest voor mijn kennis en ervaring in het lab. Veel praktische technieken heb ik van jou geleerd en van jou heb ik ook de meest praktische hulp gekregen. Heel erg bedankt voor deze ondersteuning, al je antwoorden op mijn vragen (want dat zijn er veel geweest) en voor je gezelligheid op het lab!

Arno, 'je zit toch niet te knoeien, he?'. Haha, ik hoor het je nog zeggen! Bedankt Arno voor al je hulp en je bereidheid om te helpen daar waar het kon! En natuurlijk voor alle lol die ik met je heb gehad op het lab! Blijven zingen (ja, ik heb het gehoord) en dansen Arno ☺!

Katja, ik verbaas me nog steeds hoe snel jij werkt in het lab! Ga zo door! Bedankt voor al je hulp en gezelligheid!

Marion, jou wil ik vooral bedanken voor alle leuke en fijne gesprekken die ik met je heb gehad. Ik kan altijd met je lachen en ook serieuze gesprekken met je voeren. Bedankt voor je interesse en je persoonlijke betrokkenheid!

Dan wil ik mijn leuke mede OIO-collega's bedanken voor de leuke tijd die ik met jullie heb gehad op de afdeling reumatologie & klinische immunologie!

Angela, Erwin, Femke, Jos, Karen, Laurens, Margot, Marije, Marlies, Monique, Nathalie, Sarita en Tineke jullie zijn momenteel geen OIO-collega meer, maar dat neemt niet weg dat ik jullie toch nog wil bedanken voor de handige tips & trucs die ik als beginnend OIO van jullie mocht leren en niet te vergeten voor de leuke gesprekken, de ontspannende koffie/thee momentjes en de leuke OIO-etentjes en uitjes!

En dan nu mijn huidige OIO-collega's die ik jammer genoeg ga verlaten; Bart, Huub, Lize, Maarten, Maartje, Maud, Sandhya en Thijmen, jullie ook ontzettend bedankt voor de leuke tijd die ik met jullie heb gehad!

Maarten, mijn immuno-maatje, geen gevecht meer om materiaal, it's all yours! Veel succes met alle DC isolaties die je nog gaat doen en ik hoop dat er mooie resultaten uit gaan komen! En… ik ben natuurlijk heel benieuwd naar het filmpje waar je het al zo lang over hebt ☺!

Lize, ik ben nog steeds heel sexy in mijn shirt, haha! Jammer dat je niet bij mijn promotie en het feestje aanwezig kunt zijn; veel plezier en succes in Melbourne! Ook veel succes met al je lopende projecten, dat gaat helemaal goed komen! En ik kom graag langs als jullie huisje helemaal af is!

Maartje, jammer dat ik niet meer (zo vaak) naar je kan zwaaien, koffie kan drinken en dinerdates met je kan hebben. Het lekkerbekje is toch wel het beste menu van de brink ☺! Ik

hoop dat er nog veel naar je gezwaaid wordt en dat je lekker jezelf blijft op het lab en daarbuiten! Veel succes met je onderzoek!

Thijmen, leuk om jou nog als collega te hebben leren kennen. Ik hoop echt dat de plannen die je met je distractieframe hebt helemaal gaan lukken, maar met jouw technische kennis en kunde moet dat wel goed komen! Veel succes met je onderzoek en met de begeleiding van je TG-studenten!

Huub, het was leuk om je gezelschap te hebben in de laatste weken van mijn onderzoek en in de begin weken van jouw onderzoek! Die hitjesradio heeft ons er goed doorheen geholpen! Succes met alle mooie plannen die je hebt!

Mijn OIO-collega's van Q:

Sandhya, mijn CTMM-maatje, leuk om door jou de Indiase cultuur te leren kennen en te proeven en leuk om jou kennis te laten maken met de Nederlandse taal en gewoontes. Dat schaatsen was erg leuk en ook de reis ernaartoe ☺!

Maud, gezellig dat je vanuit Q altijd even kwam buurten op D om te vragen hoe het ging. Ons congresbezoekje in Berlijn was ook een toppertje! Je was een uitstekende gids en een goede steun, want het was je niet ontgaan dat ik best zenuwachtig was.. ☺!

Bart, leuk dat je promotieonderzoek bent gaan doen op het biological-cohort. Ik hoop echt dat er mooie dingen uit gaan komen en dat je naast je promotieonderzoek genoeg tijd over houdt om lekker sportief bezig te zijn!

Heel veel succes jullie allen met jullie onderzoeken en mooie toekomstplannen!

Ook wil ik alle studenten, TGers en niet-TGers, die de afgelopen jaren stage hebben gelopen op de afdeling bedanken voor hun gezelschap en voor sommigen hun bijdrage aan mijn onderzoek!

De postdocs, Anne Karien, Paco en Simon, een ieder van jullie wil ik bedanken voor jullie bijdrage aan mijn onderzoek. Bedankt voor jullie kennis en ondersteuning die jullie mij hebben gegeven vanuit jullie eigen vakgebied, de behulpzaamheid en jullie leuke gezelschap!

I also would like to thank all the people of the Laboratory of Translational Immunology (LTI). Thank you for your input, your help, and your nice company! Good luck with all the beautiful plans you have!

Tim, het hoofd van het LTI, jou wil ik graag bedanken voor je bijdrage aan en betrokkenheid bij dit proefschrift. Het was leuk om je te leren kennen en onderdeel te mogen zijn van je groep. Ik zie uit naar mooie resultaten uit al jouw plannen en investeringen!

In de beginfase van mijn onderzoek heb ik ook regelmatig overleggen gehad met het ontstekingslab onder leiding van Erik Hack. Ook jullie, en met name Erik, wil ik graag bedanken voor jullie input, hulp en gezelligheid.

Dit boekje had er nooit zo mooi uit gezien zonder de hulp van Marieke en Thamara.

Thamara, wat heb jij er een prachtige cover van gemaakt! Je bent echt goed in je werk! Ik ben je heel dankbaar voor dit mooie ontwerp, je creativiteit en je behulpzaamheid!

Marieke, heel erg bedankt voor het lay-outen van mijn proefschrift! Fijn dat je me hier zo goed bij geholpen hebt! Het is echt mooi geworden; helemaal naar mijn zin!

Dan wil ik natuurlijk ook heel graag bedanken; al mijn lieve vriendinnen en vrienden uit de kerk, van orkest, van de goede oude schooltijd en daarbuiten en mijn vioollerares Arwen! Bedankt voor jullie steun en van sommige ook gebed, de ontspanning die jullie mij gaven en de leuke tijd die jullie mij nu geven!

En als laatste, maar zeker niet als minste wil ik mijn lieve familie, verloofde en aanstaande schoonfamilie bedanken.

Lieve papa en mama, dankzij jullie ben ik zover gekomen en heb ik mijzelf zover kunnen ontwikkelen! Ik ben jullie hier héél erg dankbaar voor! Dankbaar ben ik voor het feit dat ik altijd alles heb kunnen doen wat ik graag wilde doen en dat jullie zo goed voor mij hebben gezorgd! Bedankt voor de enorme liefde en steun die ik van jullie krijg! Ik hou van jullie!

Lieve broers, zussen, zwagers, schoonzussen, neefjes & nichtjes, het zijn er nog al veel, daarom schrijf ik niet al jullie namen voluit, maar doe ik het even zo. Wat ben ik blij met jullie en hou ik van jullie! Het is zo fijn om zo'n familie om je heen te hebben! Ik wil jullie ook heel graag bedanken voor de liefde die ik van jullie krijg, voor jullie steun & bemoedigingen en voor jullie ontzettend leuke gezelschap! Cathérine en Marie-José, jullie willen mijn paranimfen zijn en daar ben ik heel blij mee! Dankjulliewel daarvoor!

Lieve Jaap, mijn liefste liefste, in het derde jaar van mijn promotieonderzoek ben jij in mijn leven gekomen en vanaf dat moment ben jij erbij betrokken geweest. De laatste loodjes wegen toch wel het zwaarst en dat heb jij ook gemerkt. Ondanks dat is jouw liefde voor mij niet minder geworden, integendeel! Ik ben je dankbaar dat je naast me staat, me steunt, me helpt bij de dingen die ik moeilijk vind, in mij gelooft en trots op me bent! Ik hou heel veel van je en ben heel blij dat we dit jaar gaan trouwen; dat JIJ, Jaap van Vliet, mijn man wordt en ik er een hele lieve en leuke schoonfamilie bij krijg!

Fréderique

CURRICULUM VITAE

Fréderique Marie Moret was born on the 11th of June 1985 in Tiel, the Netherlands. In 2004 she finished secondary school at the R.S.G. Lingecollege in Tiel.
In September 2004 Fréderique started the study Biomedical Sciences at the University of Utrecht and in September 2009 she obtained her Master of Science (MSc) degree. For her master Immunity & Infection, Fréderique performed her major internship at the department of Rheumatology & Clinical Immunology at the University Medical Center (UMC) Utrecht under the supervision of dr. J.A.G. van Roon. During this internship she studied IL-7Rα+ and IL-7Rα- T cells in patients with primary Sjögren's Syndrome. For her minor internship, Fréderique studied the survival mechanisms of Campylobacter jejuni in Acanthamoeba polyphaga at the department of Infectious Diseases and Immunology of the faculty of veterinary medicine, Utrecht University, under the supervision of dr. N.M.C. Bleumink-Pluym and prof. dr. J.P.M. van Putten. A literature study on pattern recognition molecules and innate immune evasion was written under the supervision of dr. K.P. van Kessel from the department of Medical Microbiology at the UMC Utrecht.
From September 2009 until December 2009 Fréderique worked as a junior investigator at the department of Rheumatology & Clinical Immunology at the UMC Utrecht. In January 2010 Fréderique started the research for this thesis at the same department under the supervision of dr. J.A.G. van Roon, prof. dr. F.P.J.G. Lafeber, and prof. dr. J.W.J. Bijlsma.

LIST OF PUBLICATIONS

FM Moret, KMG van der Wurff-Jacobs, D van Leenen, MJA Groot Koerkamp, JWJ Bijlsma, FPJG Lafeber, JAG van Roon. Preventing PD-1/PD-L1 interactions reverses hypo-responsiveness of synovial CD4 T cells in response to synovial fluid-derived CD1c myeloid dendritic cells. *Submitted for publication.*

FM Moret, JWJ Bijlsma, FPJG Lafeber, JAG van Roon. The efficacy of abatacept to reduce synovial T cell activation by CD1c myeloid dendritic cells is overruled by T cell-activating cytokines. *Submitted for publication.*

FM Moret, TRDJ Radstake, J Tekstra, JWJ Bijlsma, FPJG Lafeber, JAG van Roon. TNFα downregulates the capacity of TSLP-activated $CD1c^+$ myeloid dendritic cells from rheumatoid arthritis patients to promote Th2 activity. *Submitted for publication.*

FM Moret, TRDJ Radstake, JAG van Roon. Targeting Th2-typified immune responses to prevent immunopathology in rheumatic diseases: belittled therapeutic strategies? *Ann Rheum Dis. 2014 Mar 1;73(3):477-9.*

FM Moret, CE Hack, KMG van der Wurff-Jacobs, FPJG Lafeber, JAG van Roon. Thymic stromal lymphopoietin (TSLP): a novel proinflammatory mediator in rheumatoid arthritis that potently activates $CD1c^+$ myeloid dendritic cells to attract and stimulate T cells. *Arthritis Rheum. 2014 Jan 8 [Epub ahead of print].*

FM Moret, CE Hack, KMG van der Wurff-Jacobs, W de Jager, TRDJ Radstake, FPJG Lafeber, JAG van Roon. Intra-articular CD1c-expressing myeloid dendritic cells from rheumatoid arthritis patients express a unique set of T cell-attracting chemokines and spontaneously induce Th1, Th17 and Th2 cell activity. *Arthritis Res Ther. 2013 Oct 20;15(5):R155.*

A Bikker**, FM Moret**, AA Kruize, JWJ Bijlsma, FPJG Lafeber, JAG van Roon. IL-7 drives Th1 and Th17 cytokine production in patients with primary SS despite an increase in CD4 T cells lacking the IL-7Rα. *Rheumatology (Oxford). 2012 Jun;51(6):996-1005.*

FM Moret, V Badot, BR Lauwerys, JAG van Roon. Intra-articular soluble interleukin-7 receptor levels are increased in patients with rheumatoid arthritis and correlate with local mediators of inflammation: Comment on the article by Pickens et al. *Arthritis Rheum. 2012 Apr;64(4):1302.*