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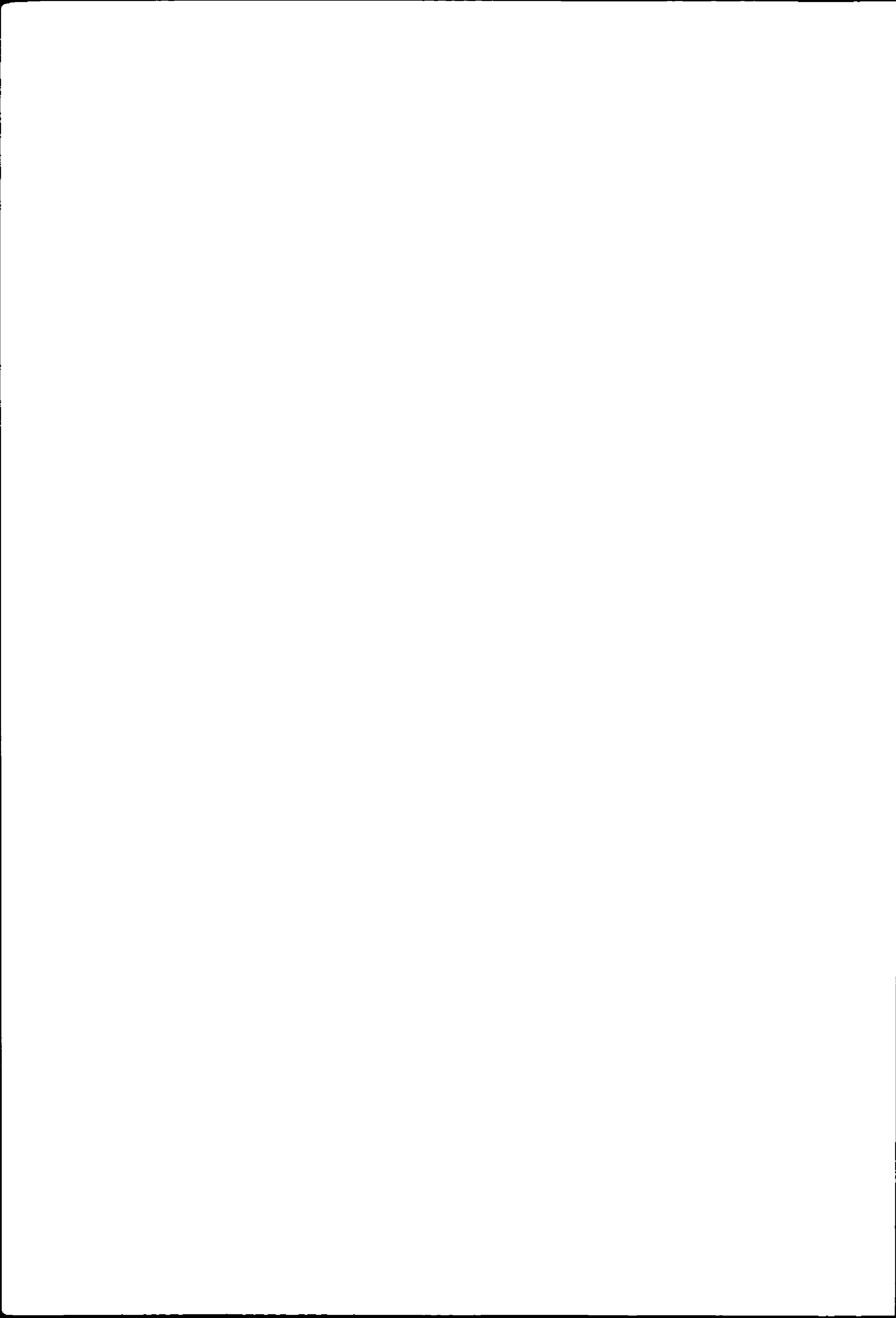
T CELL SUBSETS IN
AUTOIMMUNE DISEASES

Wiebo van der Graaff

T Cell Differentiation In Autoimmune Diseases



T Cell Differentiation in Autoimmune Diseases

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. mr. P.F. van der Heijden
ten overstaan van een door het college voor promoties
ingestelde commissie, in het openbaar te verdedigen
in de Aula der Universiteit

woensdag 23 april 2003, te 12.00 uur

door

Wiebo Leendert van der Graaff

Geboren te Leiden

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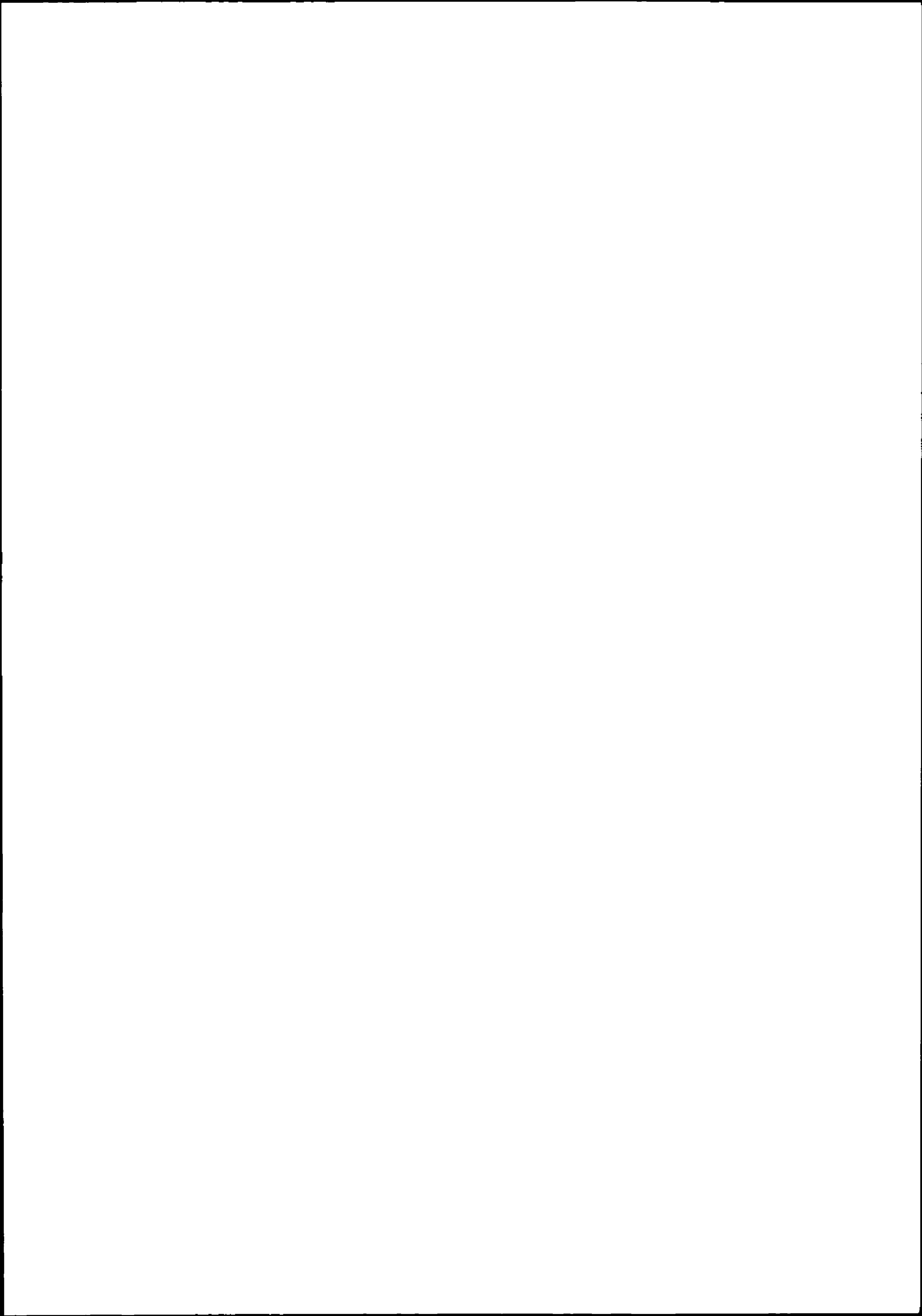
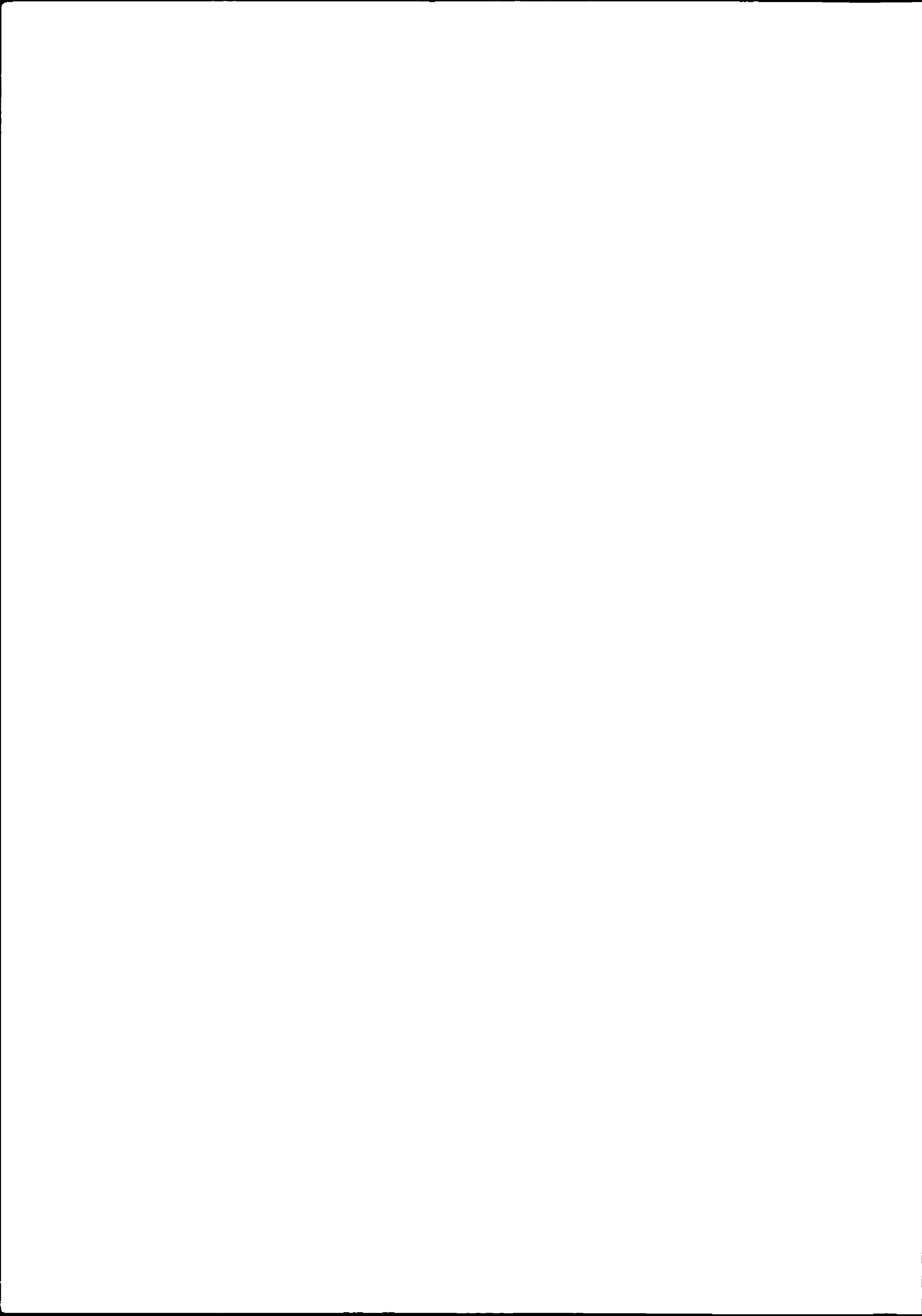
The studies described in this thesis were performed at the Department of Immunobiology, CLB, Sanquin Blood Supply Foundation and Dr Jan van Breemen Institute, Amsterdam, The Netherlands. This research project was financially supported by the Dutch League Against Rheumatism.

The printing of this thesis was financially supported by:

CLB/Sanquin Blood Supply Foundation

The University of Amsterdam





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Chapter 1

Introduction

Immunity

The human immunesystem consists of specific and non-specific subsystems that interact to defend the host against pathogens. The non-specific (innate) immunesystem consists of (1) phagocytes like macrophages, monocytes and neutrophilic granulocytes, (2) natural killer cells and (3) soluble components such as the molecules of the complement system. After pathogens have managed to cross the barriers of the human body (skin, mucous membranes), the innate immune system is able to act directly by opsonizing the pathogen with complement. This leads either to lysis of the microbe or to apoptosis of the intruder by recruited phagocytes. Additionally, pathogens can trigger pathogen recognition receptors on cells of the innate immune system by pathogen-associated molecular patterns. This induces endocytosis and, after binding to members of the Toll like receptor family, production of cytokines and membrane-bound co-stimulatory molecules that augments the innate immune response and activates the adaptive immune system.[1]

The specific (adaptive) immune system consists of lymphocytes subdivided in T cells and B cells.
B cells can produce antibodies (immunoglobulins, Ig) that have direct effects like neutralizing bacterial toxins, interfering with bacterial adhesion molecules and preventing viruses to enter host cells. Antibodies bound to the pathogen can amplify the effect of the non-specific immune system by activation of the complement system and by binding to immunoglobulin receptors on phagocytes, thereby improving phagocytosis. Furthermore, through surface Ig, B cells can ingest antigens, which after intracellular protein processing, leads to presentation of antigenic peptides to T cells. [2,3]

T cells can be divided in CD4 positive and CD8 positive cells. To participate in the defence mechanism they need to be recruited by the non-specific immune system. T cells can be activated if their specific T cell receptors (TCR) recognize antigen-derived peptides but only if they are presented to them by antigen presenting cells (APC) in HLA class I (CD8 cells)-, or HLA class II (CD4 cells) molecules. Once activated the response of T-cells depends on an interplay of:

1. The quality and quantity of antigen specific signal.
2. The quality and quantity of additional signals from cells of the non-specific immune system or B cells.
3. The stage of differentiation of the T cell.

Ad 1. Naïve T cells are only activated if their TCR has sufficient affinity for the particular peptide MHC complex and the density of peptides on APC has reached a threshold for activation

Ad 2. Activation of naïve T cells by the T cell receptor (TCR) alone will not initiate productive T cell activation but rather leads to cell-death, anergy or ignorance. However simultaneous triggering of for instance CD27[4,5], CD28[6,7], or LFA-1[18-10] by their specific ligands on APC results in T cell proliferation and differentiation.

Ad 3. During T cell differentiation the cellular activation requirements change. A repeated trigger of the TCR is sufficient for activation, and a co-stimulatory signal is less crucial. In addition activation of naïve T cells results in proliferation and IL-2 production, while reactivation of differentiated T cells results in the execution of effector functions.

Differentiated CD8 positive T cells have principally a cytotoxic effect on tissue cells presenting foreign antigen-derived peptides in HLA class I molecules. The presence of these peptides reveals that these cells are infected by pathogens. Although CD8 cells may develop in cytotoxic effector cells in the absence of CD4 positive T cells, they have been shown to be more effective when supported by CD4 positive T cells[11].

Differentiated CD4 positive T cells have predominantly immunomodulating effects. Dendritic cells presenting antigen to CD8 cells can do this more effectively when they have first had an interaction with specific CD4 T cells. In the interaction between CD4+ T cells and dendritic cells the CD40-CD40ligand interaction plays an essential role[12,13].

In addition, differentiated CD4 T cells provide help for B cells to produce antibodies with a higher affinity and induce a shift from production of IgM to IgG, A and E.

Apart from these modulating effects on the specific immune system CD4 effector cells can activate cells of the non-specific immune system like macrophages via the production of interferon gamma (IFN-γ) or via direct cell contact (e.g. CD40-CD40ligand, ICAM-LFA1)[14,15]

Observations by Mosmann and Coffman in 1986[16] revealed that different functions of murine CD4+ T helper cells seem to be executed by different T cell subsets. These are distinguished on the basis of the production of different sets of cytokines.

So-called type-1 helper cells produce IL-2, interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), while type-2 CD4+ cells produce IL-4, IL-5 and IL-10. Later similar subsets were found in humans as well[17]. Prototypically human type-1 T cells produce IFN-γ while type-2 cells produce IL-4. Functional studies have shown that type 2 cells are more effective in providing B cell help, while both type-1 and type-2 cells have a regulatory effect on cells of the non-specific immune system. Type-1 cells are proinflammatory as they can enhance the activation of macrophages and their forerunners monocytes, resulting in more effective killing of ingested bacteria as well as the production of proinflammatory cytokines like TNF-α, IL-1 and IL-6[18-20]

The different compartments of T cell subsets

Pathogens usually enter the body through the skin or mucous membranes. Locally an inflammatory response is generated by the innate immune system. If the pathogen is unknown there will be no production of specific effector T cells. To generate these, antigens have to be presented to naïve T cells by APC. Naïve human T cells can be identified by the expression of the CD45RA isoform and are receptive to costimulatory signals as they express CD27 and CD28. After their generation in the thymus they recirculate between the blood and lymphoid tissue using their homing receptors CD62L and CCR7.

Professional APC are present both in peripheral tissues as well in T cell areas of the lymphoid tissue. The differentiation of pathogen-specific T cell effectors from naïve T cell takes place in these T cell areas when antigen is drained from the site of entry by the blood or lymph and is filtered by the lymph nodes or spleen, respectively when professional APC like dendritic cells pick up pathogens/antigens in the peripheral tissue and migrate to the nearest lymph node. Circulating naïve T cells will be trapped in the lymph node if their T cell receptor recognizes specific peptide/MHC complexes present on the APC. When adequately activated by the APC by additional costimmulatory signals these cells will expand and differentiate into effector T cells. During this process the CD45RA isotype is downregulated while the CD45R0 isotype is upregulated. In parallel, molecules responsible for recirculation into the lymphnodes are diminished, while adhesion molecules and chemokine receptor molecules involved in homing to inflamed tissues are upregulated: alpha1, 2 integrins, CCR5 and CXCR-3 on type-1[21-23], CCR3 and CCR8 on type-2 T cells[24,25].

Whether T cells develop into a type-1 or type-2 phenotype depends on signals like IL-12 and IL-18 for type-1, and IL-4 for type-2 T cells. In turn whether APC produce type-1 or type-2 directing signals depends on properties of the pathogen i.e. binding to particular PRR as well as the innate defence mechanism of the host. Positive and negative feedback loops exist in T cell differentiation. Type-1 cells can amplify type-1 responses by producing IFN-γ and inhibit type-2 responses, whereas type-2 T cells can do the opposite.

Rheumatoid Arthritis

Rheumatoid arthritis is a syndrome of symmetric polyarthritis mostly involving the small joints of hands and feet. Although spontaneous remission can occur, it has the tendency to become chronic resulting in progressive damage to the cartilage and underlying bone structures of the joints. It can be concluded from incidence numbers that both genetic and environmental factors are involved in the pathogenesis. In the general population rheumatoid arthritis is found in 0.5% to 1% of individuals[26-30] while it can be found in 2% to 4% of people who have a sibling with rheumatoid arthritis[31,32]. That also environmental factors are needed for the disease to develop can be suspected from the fact that it is only found in 12% to 15% of people who have an identical twin with rheumatoid arthritis[33-35].

Clues for the pathogenesis

The underlying mechanisms resulting in development of rheumatoid arthritis are largely unknown. It can be considered that rheumatoid arthritis is an autoimmune disease since constant or intermittent inflammation is present in the synovium without evidence of an initiating pathogen. However an autoantigen at which the autoimmune reaction is directed has never been established.

A clue for the involvement of the specific immune system in the pathogenesis of RA is the association of the disease with the "shared epitope". The shared epitope is a homologues sequence of amino acids in the antigen binding groove of several MHC class II molecules that are associated with the disease[36].

Clinical responses of the disease to TNF-α neutralizing therapies imply that TNF-α plays a substantial role in generation of the symptoms[37]. TNF-α in the synovium is predominantly produced by synoviocytes and macrophages. Still it is not known what factors are initiating and sustaining the unbalanced production of this pro-inflammatory cytokine.

Differentiated T cells are capable of regulating inflammatory responses. Type-1 T cells which are abundantly found in the synovium can activate macrophages to produce inflammatory cytokines like TNF-α, while Type-2 cells can inhibit this effect. IL-4 producing cells are only found in small amounts in the joint, which has lead to the hypothesis that an imbalance between type-1 and type-2 T cells can contribute to the excessive TNF-α production in the synovium. Since it is not known whether an autoantigen is involved in the pathogenesis of rheumatoid arthritis, uncertainty exists about whether synovial T cells are antigen specific or just bystanders that are trapped by the abundance of TNF-α induced adhesion molecules and chemokines.

Aim of the thesis

In analogy to what has been found in animal models, it has been hypothesized that the balance between pro-inflammatory $T_{HELPER(H)}1$ and anti-inflammatory T_H2 cells plays an important role in (1) sensitivity for and (2) clinical course of autoimmune disease in humans. The experiments described in this thesis aimed to scrutinize this hypothesis in Rheumatoid Arthritis. For this a cohort study was performed in which T cell functions were analyzed longitudinally in patients who visited the Early Arthritis Clinic of the *Jan van Breemen Institute.*

The studies have focussed on five separate aspects:

1. Compartmentalization of T_H1 and T_H2 cells in inflamed synovium of RA patients (Chapter 2).
2. Markers of T cell differentiation in patient groups with other autoimmune diseases, i.e. SLE (Chapter 3) and M. Bechterew (Chapter 4).

3. Effects of DMARD's and anti-TNFα treatment on the T_H1 and T_H2 balance in the circulation (Chapters 5 and 6).
4. Relation of disease activity with markers of T-cell differentiation (Chapter 2, 8).
5. Predictive value of the T_H1 and T_H2 balance for disease progression (Chapter 7, 8).

A summary and conclusion of the experimental chapters will be given in Chapter 9.

References

1. Janeway CAJ, Medzhitov R. Innate immune recognition. Annu.Rev.Immunol 2002.;20:197-216
2. Chesnut RW, Colon SM, Grey HM. Antigen presentation by normal B cells, B cell tumors, and macrophages: functional and biochemical comparison. J Immunol 1982;128:1764-1768
3. Constant S, Schweitzer N, West J, Ranney P, Bottomly K. B lymphocytes can be competent antigen-presenting cells for priming CD4+ T cells to protein antigens in vivo. J Immunol 1995;155:3734-3741
4. Hintzen RQ, Lens SM, Lammers K, Kuiper H, Beckmann MP, van Lier RA. Engagement of CD27 with its ligand CD70 provides a second signal for T cell activation. J Immunol 1995;154:2612-2623
5. Akiba H, Oshima H, Takeda K, et al. CD28-independent costimulation of T cells by OX40 ligand and CD70 on activated B cells. J Immunol 1999;162:7058-7066
6. Harding FA, McArthur JG, Gross JA, Raulet DH, Allison JP. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. Nature 1992;356:607-609
7. Gimmi CD, Freeman GJ, Gribben JG, Gray G, Nadler LM. Human T-cell clonal anergy is induced by antigen presentation in the absence of B7 costimulation. Proc.Natl.Acad.Sci.U.S.A. 1993;90:6586-6590
8. van Noesel C, Miedema F, Brouwer M, de Rie MA, Aarden LA, van Lier RA. Regulatory properties of LFA-1 alpha and beta chains in human T-lymphocyte activation. Nature 1988;333:850-852
9. Van Seventer GA, Shimizu Y, Horgan KJ, Shaw S. The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T cell receptor-mediated activation of resting T cells. J Immunol 1990;144:4579-4586
10. Gaglia JL, Greenfield EA, Mattoo A, Sharpe AH, Freeman GJ, Kuchroo VK. Intercellular adhesion molecule 1 is critical for activation of CD28-deficient T cells. J Immunol 2000;165:6091-6098
11. Bourgeois C, Rocha B, Tanchot C. A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. Science 2002;297:2060-2063

12. Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature 1998;393:478-480

13. Schoenberger SP, Toes RE, van d, V, Offringa R, Melief CJ. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature 1998;393:480-483

14. Alderson MR, Armitage RJ, Tough TW, Strockbine L, Fanslow WC, Spriggs MK. CD40 expression by human monocytes: regulation by cytokines and activation of monocytes by the ligand for CD40. J Exp Med 1993;178:669-674

15. Kiener PA, Moran-Davis P, Rankin BM, Wahl AF, Aruffo A, Hollenbaugh D. Stimulation of CD40 with purified soluble gp39 induces proinflammatory responses in human monocytes. J Immunol 1995;155:4917-4925

16. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986;136:2348-2357

17. Wierenga EA, Snoek M, de Groot C, et al. Evidence for compartmentalization of functional subsets of CD2+ T lymphocytes in atopic patients. J Immunol 1990;144:4651-4656

18. Collart MA, Belin D, Vassalli JD, de Kossodo S, Vassalli P. Gamma interferon enhances macrophage transcription of the tumor necrosis factor/cachectin, interleukin 1, and urokinase genes, which are controlled by short-lived repressors. J Exp Med 1986;164:2113-2118

19. Eden E, Turino GM. Interleukin-1 secretion by human alveolar macrophages stimulated with endotoxin is augmented by recombinant immune (gamma) interferon. Am.Rev.Respir.Dis. 1986;133:455-460

20. Gifford GE, Lohmann-Matthes ML . Gamma interferon priming of mouse and human macrophages for induction of tumor necrosis factor production by bacterial lipopolysaccharide. J Natl.Cancer Inst. 1987;78:121-124

21. 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;187:875-883

22. Loetscher P, Uguccioni M, Bordoli L, et al. CCR5 is characteristic of Th1 lymphocytes. Nature 1998;391:344-345

23. Bonecchi R, Bianchi G, Bordignon PP, et al. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. J Exp Med 1998;187:129-134

24. Zingoni A, Soto H, Hedrick JA, et al. The chemokine receptor CCR8 is preferentially expressed in Th2 but not Th1 cells. J Immunol 1998;161:547-551

25. Gerber BO, Zanni MP, Uguccioni M, et al. Functional expression of the eotaxin receptor CCR3 in T lymphocytes co-localizing with eosinophils. Curr.Biol 1997;7:836-843

26. Alarcon GS. Epidemiology of rheumatoid arthritis. Rheum.Dis.Clin North Am. 1995;21:589-604

27. Hochberg MC. Epidemiology of rheumatoid arthritis in developed countries. J Rheumatol Suppl. 1983;10:7-10

28. Hochberg MC, Spector TD. Epidemiology of rheumatoid arthritis: update. Epidemiol.Rev. 1990;12:247-52

29. Gabriel SE. The epidemiology of rheumatoid arthritis. Rheum.Dis.Clin North Am.2001; 27:269-281

30. Kaipiainen-Seppanen O, Aho K, Isomaki H, Laakso M. Incidence of rheumatoid arthritis in Finland during 1980-1990. Ann.Rheum.Dis. 1996;55:608-611

31. del Junco D, Luthra HS, Annegers JF, Worthington JW, Kurland LT. The familial aggregation of rheumatoid arthritis and its relationship to the HLA-DR4 association. Am.J Epidemiol. 1984;119:813-829

32. Jones MA, Silman AJ, Whiting S, Barrett EM, Symmons DP. Occurrence of rheumatoid arthritis is not increased in the first degree relatives of a population based inception cohort of inflammatory polyarthritis. Ann.Rheum.Dis. 1996;55:89-93

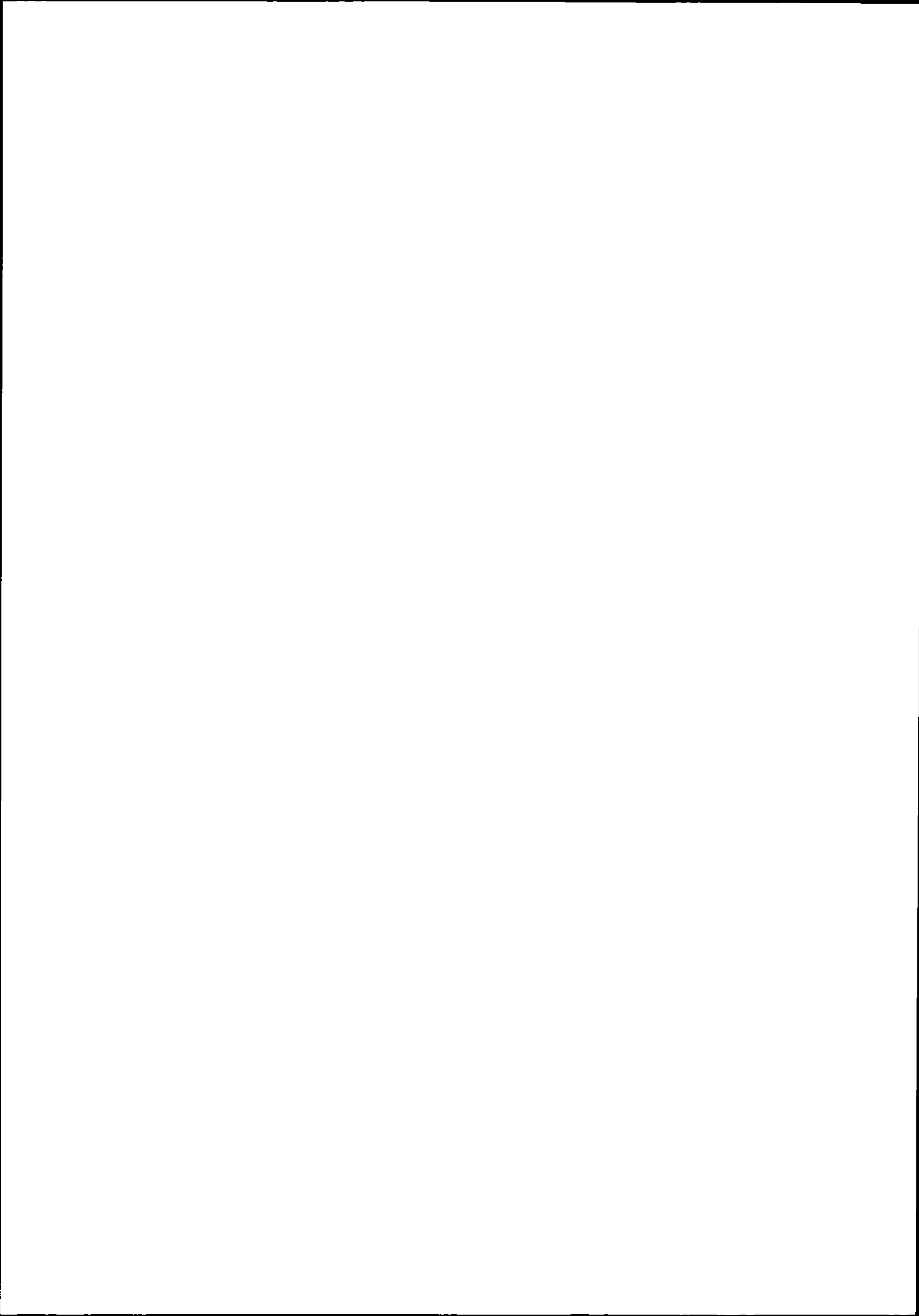
33. Aho K, Koskenvuo M, Tuominen J, Kaprio J. Occurrence of rheumatoid arthritis in a nationwide series of twins. J Rheumatol 1986;13:899-902

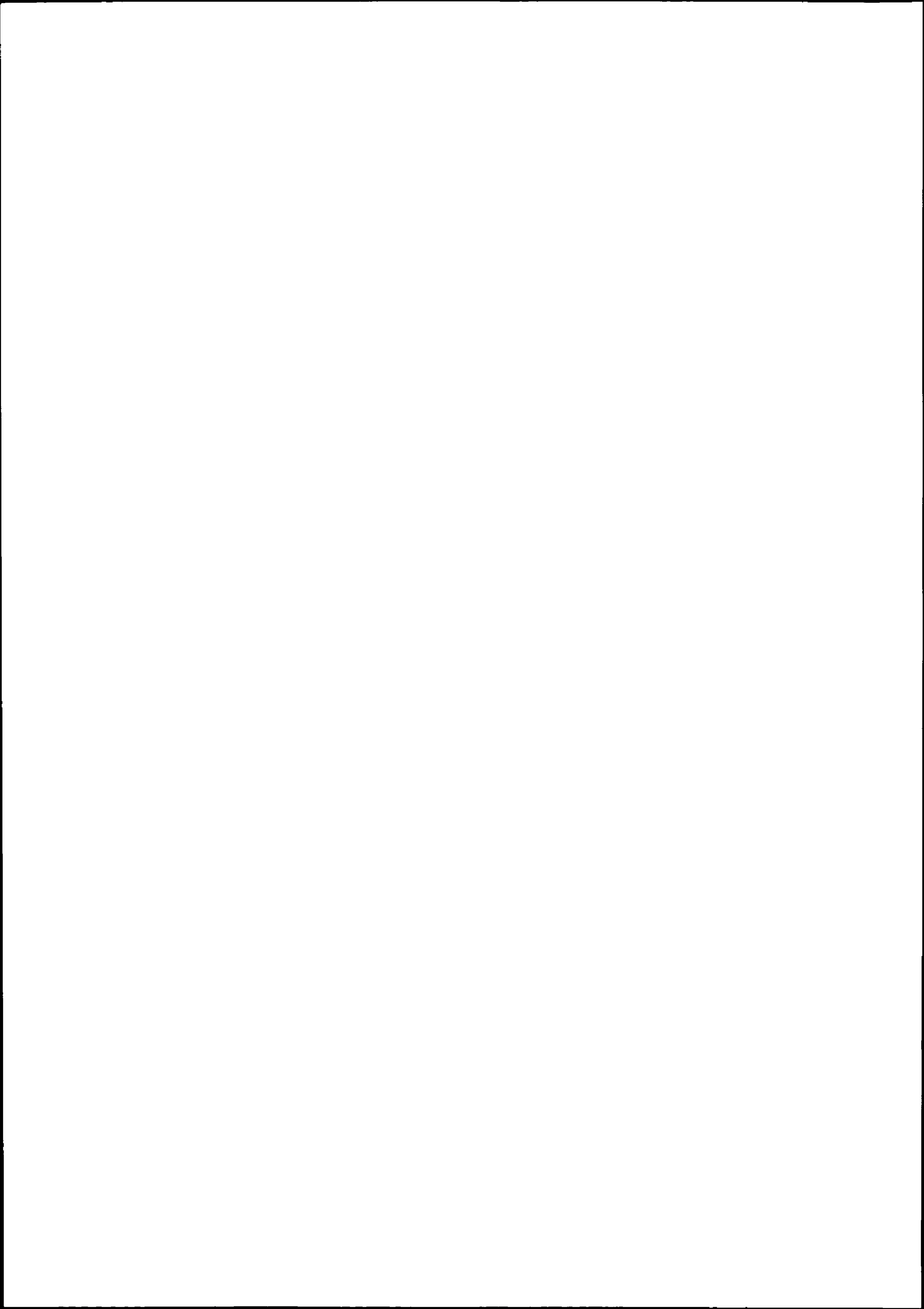
34. Silman AJ, MacGregor AJ, Thomson W, et al. Twin concordance rates for rheumatoid arthritis: results from a nationwide study. Br.J Rheumatol 1993;32:903-907

35. Bellamy N, Duffy D, Martin N, Mathews J. Rheumatoid arthritis in twins: a study of aetiopathogenesis based on the Australian Twin Registry. Ann.Rheum.Dis. 1992;51:588-593

36. Gregersen PK, Silver J, Winchester RJ. The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum. 1987;30:1205-1213

37. Elliott MJ, Maini RN, Feldmann M, et al. Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumor necrosis factor alpha. Arthritis Rheum. 1993;36:1681-1690





Chapter 2

Quantitation of interferon gamma and interleukin-4-producing T cells in synovial fluid and peripheral blood of arthritis patients

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Rheumatology 38:214–220,1999

Quantitation of interferon gamma- and interleukin-4-producing T cells in synovial fluid and peripheral blood of arthritis patients

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Abstract

Objective. The balance between T cells able to produce interferon gamma (IFN-γ) (type 1) and interleukin-4 (IL-4) (type 2) is considered to be important in the development of autoimmunity. In this study, we quantitated the percentage of both cell types in synovial fluid (SF) and peripheral blood (PB) of rheumatoid arthritis (RA) patients, non-rheumatoid arthritis patients and healthy controls.

Methods. After short-term stimulation of synovial mononuclear cells with phorbol ester and ionomycin, cytokine-producing cells were quantitated using an intracellular staining technique and flow cytometric analysis.

Results. Although no significant differences in CD8+ cells were found, significantly higher percentages of IFN-γ-producing CD4+ (Th1) and IL-4-producing CD4+ (Th2) cells were found in the peripheral blood of RA patients in comparison with healthy controls. However, the Th1/Th2 ratio was not different between the two groups. Comparative studies between PB and SF showed that in both RA and non-RA patients, percentages of Th1 cells were higher in SF than in PB, while Th2 cells were preferentially found in the PB, resulting in a higher Th1/Th2 ratio in the SF. The Th1/Th2 ratio in the SF correlated with disease activity as estimated by the erythrocyte sedimentation rate.

Conclusion. These results are in agreement with the hypothesis that Th1 cells preferentially home to inflamed joints in both RA and non-RA patients, but show that this does not result in an altered Th1/Th2 ratio in the PB of RA patients.

KEY WORDS: Rheumatoid arthritis, Th1/Th2 cells, Cytokines.

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting primarily synovial tissue in multiple joints. A marked hyperplasia of synoviocytes and blood vessels in the synovium, and a mononuclear cellular infiltrate consisting of macrophages, T and B cells, are found. There is controversy on the issue of which cell is crucial in the inflammatory process [1, 2]. Nevertheless, CD4+ T-helper cells (Th) appear to play an important role, since susceptibility to RA is specifically associated with class II MHC alleles possessing a shared epitope [3]. CD4+ T cells can differentiate into two distinct subsets designated Th1 and Th2 type cells, which are characterized by different cytokine production profiles and effector functions. Th1 cells produce interleukin-2 (IL-2) and interferon gamma (IFN-γ), support macrophage activation and are involved in delayed-type hypersensitivity responses. Th2 cells, on the other hand, secrete IL-4, IL-5 and IL-13, and provide efficient help for B-cell activation, antibody production and down-modulate the production of pro-inflammatory cytokines by macrophages. From animal experiments, it has become clear that balances between Th1 and Th2 cells, or their cytokines, are important in the induction or prevention of organ-specific autoimmune disease [4]. Several reports have been published on the detection of Th1 and Th2 cytokines in RA [5–13]. Using either immunohistochemistry or ELISPOT techniques, evidence was obtained for an increased IFN-γ/IL-4 ratio in synovial fluid (SF) compared to peripheral blood (PB).

Submitted 15 January 1998; revised version accepted 14 October 1998.

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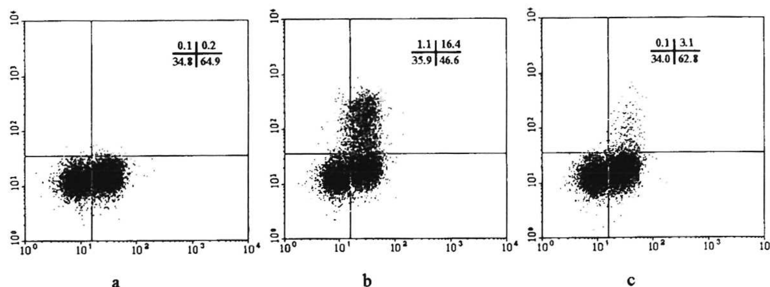


FIG. 1. Flow cytometric analysis after simultaneous staining with anti-CD95 and negative control antibody (a), IFN-γ (b) or IL-4 (c) of CD4+ gated PBMC of an RA patient.

To obtain information on the phenotype of the cytokine-producing T cells in this study, three-colour immunofluorescence analysis with intracellular staining was used to quantify cytokine-producing T cells in PB and SF of arthritis patients. Our findings show that in both arthritis patients and age-matched healthy controls, IFN-γ- and IL-4-secreting T cells are contained within the memory (defined as CD95 +) compartment. Furthermore, comparison between PB and SF showed that higher percentages of IFN-γ-secreting, but not of IL-4-producing cells, are found in the joint, resulting in a relatively high local Th1/Th2 ratio.

Materials and methods

Isolation of mononuclear cells

SF, PB, or both, were obtained in heparinized tubes. SF and PB were diluted in phosphate-buffered saline (PBS) containing 10% fetal calf serum (FCS) and 5% trisodium citrate. Mononuclear cells were isolated by density centrifugation on a Ficoll-Hypaque gradient ($d = 1.079$). Cells were suspended in Iscove's modified Dulbecco's medium (IMDM) containing 30% FCS and 10% dimethylsulphoxide, and stored in liquid nitrogen until use. Peripheral blood mononuclear cells (PBMC) of a healthy donor were processed in the same way and used as standard control cells.

Immunofluorescence

T cells producing IFN-γ and IL-4 were detected by intracellular staining with flow cytometric analysis as described before [14–16]. Briefly, 1.2 million thawed viable mononuclear cells were stimulated in 1 ml IMDM/10% FCS with 1 ng phorbol myristate acetate (PMA; Sigma Chemicals) and 1 μM ionomycin (Sigma Chemicals). Monensin (1 μM; Sigma Chemicals) was added to prevent excretion of the newly synthesized cytokines. After 4 h, cells were washed in PBS/0.5% bovine serum albumin (BSA) and stained with directly labelled monoclonal antibodies (mAb) against surface molecules for 20 min. After two washing steps with PBS/BSA and one with PBS, cells were fixed with 4% paraformaldehyde during 10 min. Fixation was stopped by washing the cells three times in PBS. Cells were permeabilized with 0.1% saponin in PBS/BSA supplemented with 10% pooled human serum (HPS) to block aspecific binding of mAb. Phycoerythrin- or biotin-labelled mAb directed against IFN-γ or IL-4, and an irrelevant IgG1 murine mAb as a negative control, were diluted in PBS/BSA/saponin to a concentration of 5 μg/ml and added to the cells in aliquots of 50 μl for 25 min at 4°C. After binding of streptavidin–RED670 to the biotinylated antibodies, cells were analysed on a FACScan (Becton Dickinson). IFN-γ- and IL-4-producing T cells were identified by setting a marker on the sample containing the negative control antibody (Fig. 1a). To correct for daily variation, standard control PBMC of one healthy donor were used. The relative amounts of cytokine-producing cells in the PB and SF were defined as the percentage positive cells measured in study samples divided by that of the standard control PBMC. By way of this normalization procedure, results of individual patients analysed in different sets of experiments could be compared. In experiments that compared results from SFMC and PBMC from the same patient, percentages of CD8 – and CD8+ cytokine-positive cells were first divided by the percentage CD95+ cells. Percentages of CD95 + CD4+ and CD95+ CD8+ cells were determined by flow cytometric analysis after staining of mononuclear cells with directly labelled antibodies.

Antibodies

Phycoerythrin (PE)–anti-CD3, CD4, CD8, IFN-γ, IL-4, fluorescein isothiocyanate (FITC)–anti-CD8 and PerCP–anti-CD4 were purchased from Becton Dickinson. Biotinylated anti-IFN-γ (MD1, IgG1) was obtained from Dr P. van der Meide, TNO, Rijswijk, The Netherlands. Biotinylated anti-IL-4 (5A4, IgG1) was kindly provided by Dr T. van der Pouw-Kraan, CLB, Amsterdam, The Netherlands. Binding of biotinylated antibodies was visualized by binding of streptavidin-coupled RED670 (Dako, Denmark). Anti-CD4–PE cyanin 5.1 and anti-CD95–FITC were purchased from

Immunotech, Marseille, France. As negative control antibodies for the intracellular staining, PE-labelled anti-KLH (Becton Dickinson) and biotin-labelled anti-*Aspergillus niger* (Dako, Denmark) were used.

Patients

Fifty-three patients with arthritis were recruited for this study. Thirty-nine of them fulfilled the 1987 ACR criteria [17] for RA. Of the non-RA patients, four had reactive arthritis, three were classified as suffering from undifferentiated monoarthritis, two from psoriatic arthritis and five from undifferentiated oligoarthritis. In 12 patients (seven RA, five non-RA), SF and PB were withdrawn simultaneously, in 18 patients (nine RA, nine non-RA) only SF was obtained and in 23 RA patients only PB was obtained. All patients used non-cytotoxic anti-inflammatory drugs, except for two RA patients (methotrexate, azathioprine) who donated only SF. No steroids were prescribed.

Statistical analysis

Data were analysed using non-parametric methods: Mann–Whitney *U*-test, Spearman rank correlation coefficient and Wilcoxon's signed rank test.

Results

Comparison of IFN-γ- and IL-4-producing T cells in PB of RA patients and healthy controls

IFN-γ- and IL-4-producing cells were determined in the PB of 23 RA patients with early disease (<6 months) and 23 healthy controls. Patients and controls were matched for gender and age (both groups 10 males, 13 females, median age 49 yr, range 37–67 yr). After correction for the daily control (see Materials and methods), IFN-γ- and IL-4-producing cells were calculated in CD4+CD95+, CD4+CD95−, CD8+CD95+ and CD8+CD95− subsets. Within the CD4+ and CD8+ subset, the vast majority of cytokine producers were CD95+ (Table 1). RA patients had more CD95+ cells in both CD4+ and CD8+ subsets; however, only the difference in the CD4+ compartment reached statistical significance. Within the CD4+ subset, the percentages of both IFN-γ- and IL-4-producing cells were significantly higher in the RA patients in comparison with the healthy controls (Fig. 2a and b). However, within the cytokine-enriched CD4+CD95+ subset, no significant difference was found (Fig. 2d and e). The IFN-γ/IL-4 ratios within the CD4+ or in the CD4+CD95+ subset (Th1/Th2 ratio) were not different between patients and controls (Fig. 2c and f). Within the CD8+ subset, neither the relative percentages of IFN-γ- and IL-4-producing cells nor their ratio differed between patients and controls.

TABLE 1. Percentage of CD95+ cells within different T-cell subsets in the peripheral blood of RA patients and healthy controls. The medians are given with the range in parentheses

	RA ($n = 23$)	Controls ($n = 23$)	P
CD4+	54.2 (39.3–77.4)	48.1 (24.6–81.2)	0.02
CD8+	62.2 (46.0–79.6)	55.9 (25.4–82.29)	0.27
CD4+IFN+	91.4 (62.9–98.8)	83.4 (56.7–96.2)	0.15
CD4+IL-4+	95.0 (80.4–98.8)	92.9 (73.7–100.0)	0.05
CD8+IFN+	83.3 (39.2–96.9)	81.4 (53.2–96.1)	0.98
CD8+IL-4+	94.2 (59.7–100.0)	95.2 (78.1–100.0)	0.75

Comparison of IFN-γ- and IL-4-producing cells between SF and PB in arthritis patients

To analyse the relationship between IFN-γ- and IL-4-producing T cells in the PB and SF, both cell types were determined in paired samples from 12 patients (seven RA, five non-RA). Since the production of IFN-γ and IL-4 by T cells is highly enriched in the CD95+ subset (Fig. 1 and Table 1), differences in percentages of CD95+ cells between SF and PB affect the comparison of IFN-γ- and IL-4-producing T cells between these two compartments. Therefore, IFN-γ- and IL-4-producing cells were expressed as percentage of CD95+ cells determined by three-colour flow cytometry. Again, results were expressed as relative amounts compared with PBMC from a healthy control. Both in CD8− and CD8+ cells, IFN-γ-positive cells were found in higher percentages in the SF, while CD8-IL-4-positive cells were overexpressed in the PB (Fig. 3a, b and d). Therefore, a higher Th1/Th2 ratio was found in the SF (Fig. 3c). This finding was consistent in both RA and non-RA patients. Comparison of the values for cytokine-producing cells between SF and PB yielded a significant correlation for Th1 cells, but not for Th2 cells or the Th1/Th2 ratio (Table 2).

Correlation between IFN-γ- and IL-4-producing T cells in SF and clinical parameters

IFN-γ- and IL-4-positive CD8− T cells were determined in SF samples of 30 arthritis patients (16 RA and 14 non-RA). Correlations were calculated between the relative percentages of cytokine producers and clinical parameters of all arthritis patients. No significant correlation could be found between age or disease duration and cytokine producers. However, a significant inverse correlation was observed between the erythrocyte sedimentation rate (ESR) and IL-4 producers in the SF, which resulted in a positive correlation between the ESR and Th1/Th2 ratio as well (Table 2).

Discussion

The role of T cells in RA is still controversial. Several attempts have been made to detect Th1 and Th2 cytokines or their encoding mRNAs in blood [8, 12, 13], synovium [5–7, 9, 11, 18] or SF [7, 9, 10, 12] of RA patients and, depending on the methodology used, both Th1 and Th2 dominance have been reported. Two

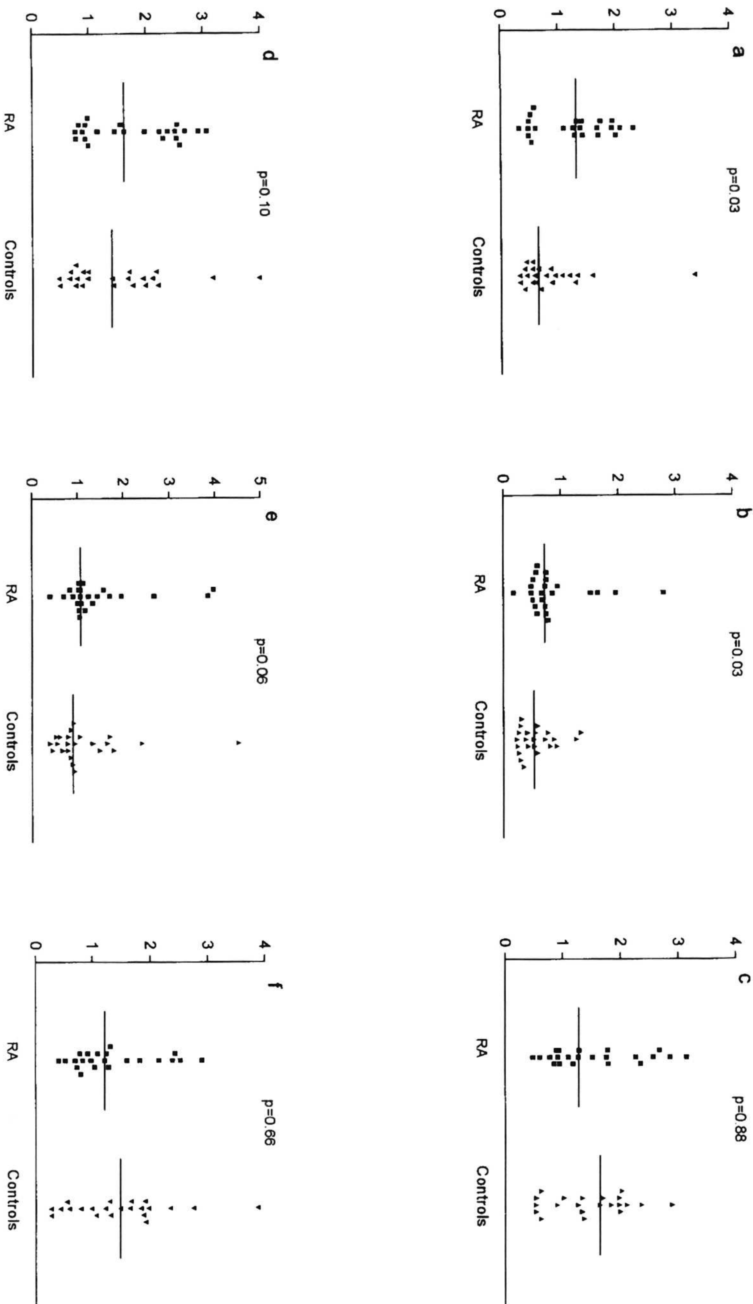


FIG. 2. Comparison of IFN-γ-positive cells (a and d), IL-4-positive cells (b and e) and IFN-γ/IL-4 ratio in CD4+ (a–c) and CD4+CD95+ (d–f) gated cells in the PB between RA patients and healthy controls. Relative amounts were found by dividing the results of individual experiments by that of standard control PBMC. Statistical significance (P) between the patient groups was calculated by a non-parametric test (Mann–Whitney).

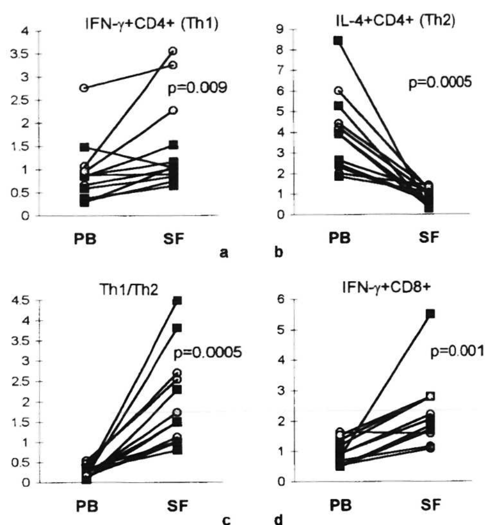


FIG. 3. Comparison of the relative amounts of CD8−IFN-γ+ (Th1) (a), CD8−IL−4+ (Th2) (b), Th1/Th2 ratio (c) and IFN-γ-producing CD8+ T cells between PB and SF of rheumatoid (■) and non-rheumatoid (○) arthritis patients. Two dots joined by a line represent the values for SF and PB of the same patient. Statistical significance (*P*) between the compartments was calculated by a non-parametric test (Wilcoxon's signed rank test).

reports have been published on the paired quantitation of IFN-γ- and IL-4-producing cells in PB and SF of RA patients. Significant increases in IFN-γ-producing cells in the SF [19, 20], a significant increase in IL-4-producing cells in unstimulated cells from the SF [20] and no difference in IL-4 producers in stimulated cells [19, 20] were found. Importantly, in previous studies, no attempts were made to correlate cytokine-producing capacity with membrane phenotype. Nevertheless, this is likely to be relevant since the T cells present in the rheumatoid SF are largely CD4+CD45RO+CD95+ 'memory' cells, while in the PB both naive (CD45RO−CD95−) and memory cells are found [21]. In order to differentiate between naive and memory type cells, we used an intracellular cytokine staining technique combined with cell surface phenotype analysis. This flow cytometric analysis was first described by Jung *et al.* [22] and has since then been modified and used for different purposes [14–16, 23–25]. After short (4 h) stimulation with PMA and ionomycin, both types of cells could be detected in CD4+, CD3+CD8− and CD8+ cells of every individual patient. We found that both IFN-γ-and IL-4-producing CD4+ cells were largely CD95+ in PB of RA patients and healthy controls (Table 1); moreover, >95% of all SF CD4+ cells were CD95+ (data not shown).

In all PBMC and SFMC of arthritis patients tested, more IFN-γ- than IL-4-producing T cells were found, which was not different from results with PBMC of healthy donors. However, significantly more IFN-γ- and IL-4-producing CD4+ T cells were found in RA patients in comparison with healthy controls. Since the percentage of CD95+ cells within the CD4+ populations is higher in RA patients (Table 1), cytokine producers were also determined within the CD95+ subset. No significant difference could be seen in the percentages of IFN-γ or IL-4 producers within the CD4+CD95+ subset. Therefore, it can be concluded that part of the higher percentages of cytokine-producing CD4+ cells in the PB of RA patients can be ascribed to higher percentages of CD95+ cells. We could not find a difference in the IFN-γ/IL-4 ratio either within the CD4+ or in the CD8+ subsets when patients and healthy controls were compared. These findings are in agreement with those of Ronnelid *et al.* [20] who used an ELISPOT technique without any *in vitro* stimulation and did not find a difference in the IFN-γ/IL-4 ratio of total PBMC. Van Roon *et al.* [26], using an *in vitro* culture system, reported higher IFN-γ/IL-4 ratios in RA compared to controls. Differences in the various studies may possibly be explained by the fact that the latter study depends on long-term *in vitro* culture.

In RA patients, IFN-γ-producing T cells are reported to be found in higher percentages in the SF than in PB [12, 19]. This study shows that when only the subset of PB T cells is considered that is present in the SF as well (CD95+), this finding can be supported for both RA and non-RA patients, and shows in addition that there is an even more striking difference in IL-4-positive CD8− T cells (considered CD4+) between SF and PB. In all patients tested, lower percentages were found in the SF, resulting in significantly higher Th1/Th2 ratios

TABLE 2. Spearman rank correlation coefficients between cytokine producers in SF, PB and clinical data of both RA and non-RA patients

	SFMC IFN−CD4+	SFMC IL4+CD4+	SFMC Th1/Th2	PBMC IFN+CD4+	PBMC IL4+CD4+	PBMC Th1/Th2
Age	−0.11	−0.34	0.25	−0.15	0.10	−0.18
Disease duration	−0.33	−0.26	−0.10	−0.11	−0.03	0.08
ESR	−0.26	−0.56**	0.48*	0.25	−0.07	0.58
SFMC IFN+CD4+				0.66*	0.10	0.64*
SFMC IL4+CD4+				0.31	0.34	0.63*
SFMC Th1/Th2				0.50	0.55	0.08

*$P < 0.05$; **$P < 0.01$.

in the SF. This finding may be largely due to different homing properties of Th1 and Th2 cells since murine Th1 cells are shown to home better to inflamed synovial tissue than Th2 cells [27]. In addition, we have shown that human Th1 cells express higher levels of CD49d (Maurice *et al.*, submitted), which is used in the binding to activated endothelium as well as adhesion to fibronectin, which is abundantly expressed in the rheumatoid synovium [28]. Remarkably, the percentage of Th1 cells in the SF correlates with that in the PB, while no correlation between those two compartments could be found for Th2 cells. This might point to the possibility that, apart from different homing properties, Th2 cells are modulated in the synovium while Th1 cells are not. It could very well be that Th2 cells entering the synovium are downregulated by the pro-inflammatory cytokines present since IL-1 is known to inhibit IL-4 expression [29, 30]. This would explain the inverse correlation observed between ESR and Th2 cells in the SF since pro-inflammatory cytokines are major contributors to elevation of the ESR. Alternatively, it could be hypothesized that in patients with low numbers of Th2 cells within the synovium, Th1 cells can amplify the release of macrophage-derived pro-inflammatory cytokines, which results in a higher ESR. The results of this study are in agreement with the hypothesis that Th1 cells preferentially home to inflamed joints in both RA and non-RA patients, but show that this does not result in an altered Th1/Th2 ratio in the PB of RA patients.

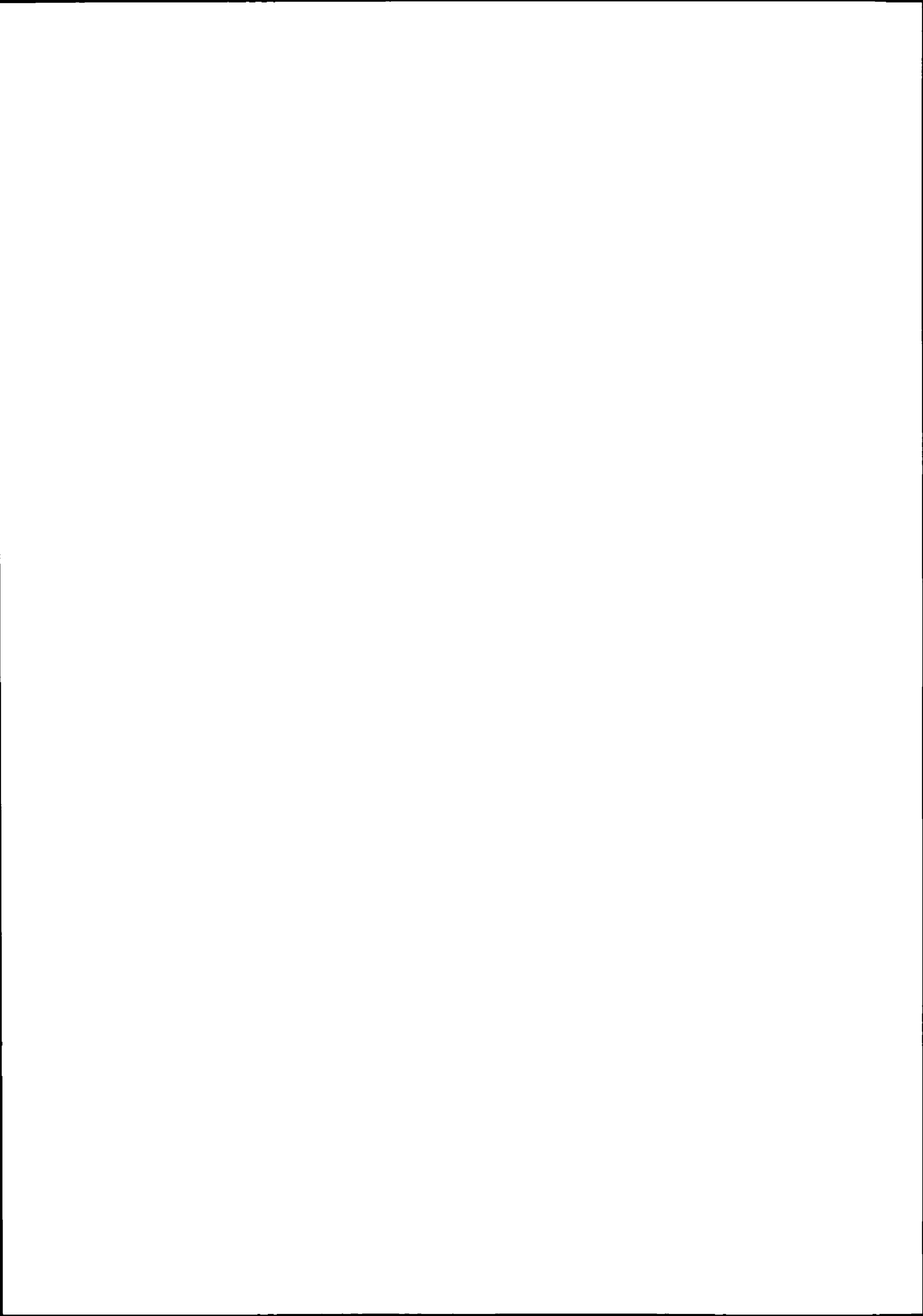
Acknowledgement

This study was supported by the Dutch League Against Rheumatism.

References

1. Firestein GS, Zvaifler NJ. How important are T cells in chronic rheumatoid synovitis? Arthritis Rheum 1990;33:768–73.
2. Panayi GS, Lanchbury JS, Kingsley GH. The importance of the T cell in initiating and maintaining the chronic synovitis of rheumatoid arthritis. Arthritis Rheum 1992;35:729–37.
3. Gregersen PK, Silver J, Winchester RJ. The shared epitope hypothesis: an approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum 1987;30:1205–13.
4. Liblau RS, Singer SM, McDevitt HO. Th1 and Th2 CD4+ T cells in the pathogenesis of organ specific auto immune diseases. Immunol Today 1995;16:34–8.
5. Miltenburg AM, van Laar JM, de Kuiper R, Daha MR, Breedveld FC. T cells cloned from human rheumatoid synovial membrane functionally represent the Th1 subset. Scand J Immunol 1992;35:603–10.
6. Cohen SB, Katsikis PD, Chu CQ *et al.* High level of interleukin 10 production by the activated T cell population within the rheumatoid synovial membrane. Arthritis Rheum 1995;38:946–52.
7. Quayle AJ, Chomarat P, Miossec P, Kjeldsen Kragh J, Forre O, Natvig JB. Rheumatoid inflammatory T cell clones express mostly Th1 but also Th2 and mixed (Th0 like) cytokine patterns. Scand J Immunol 1993;38:75–82.
8. Schulze Koops H, Lipsky PE, Kavanaugh AF, Davis LS. Elevated Th1 or Th0 like cytokine mRNA in peripheral circulation of patients with rheumatoid arthritis. Modulation by treatment with anti ICAM 1 correlates with clinical benefit. J Immunol 1995;155:5029–37.
9. Chen E, Keystone EC, Fish EN. Restricted cytokine expression in rheumatoid arthritis. Arthritis Rheum 1993;36:901–10.
10. Schlaak JF, Pfers I, Meyer zum Buhenfelde KH, Marker Hermann E. Different cytokine profiles in the synovial fluid of patients with osteoarthritis, rheumatoid arthritis and seronegative spondylarthropathies. Clin Exp Rheumatol 1996;14:155–62.
11. Simon AK, Seipelt E, Sieper J. Divergent T cell cytokine patterns in inflammatory arthritis. Proc Natl Acad Sci USA 1994;91:8562–6.
12. Bucht A, Larsson P, Weisbrot L *et al.* Expression of interferon gamma (IFN gamma), IL 10, IL 12 and transforming growth factor beta (TGF beta) mRNA in synovial fluid cells from patients in the early and late phases of rheumatoid arthritis (RA). Clin Exp Immunol 1996;103:357–67.
13. al Janadi N, al Dalaan A, al Balla S, Raziuddin S. CD4+ T cell inducible immunoregulatory cytokine response in rheumatoid arthritis. J Rheumatol 1996;23:809–14.
14. Hamann D, Hilkens CM, Grogan JL *et al.* CD30 expression does not discriminate between human Th1 and Th2 type T cells. J Immunol 1996;156:1387–91.
15. Meyaard L, Hovenkamp E, Keet IP *et al.* Single cell analysis of IL 4 and IFN gamma production by T cells from HIV infected individuals: decreased IFN gamma in the presence of preserved IL 4 production. J Immunol 1996;157:2712–8.
16. Rep MH, van Oosten BW, Roos MT, Ader HJ, Polman CH, van Lier RA. Treatment with depleting CD4 monoclonal antibody results in a preferential loss of circulating naive T cells but does not affect IFN gamma secreting TH1 cells in humans. J Clin Invest 1997;99:2225–31.
17. Arnett FC, Edworthy SM, Bloch DA *et al.* The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
18. Kotake S, Schumacher HR Jr, Yarboro CH *et al. In vivo* gene expression of type 1 and type 2 cytokines in synovial tissues from patients in early stages of rheumatoid, reactive, and undifferentiated arthritis. Proc Assoc Am Physicians 1997;109:286–301.
19. Dolhain RJ, van der Heiden AN, ter Haar NT, Breedveld FC, Miltenburg AM. Shift toward T lymphocytes with a T helper 1 cytokine secretion profile in the joints of patients with rheumatoid arthritis. Arthritis Rheum 1996;39:1961–9.
20. Ronnelid J, Berg L, Rogberg S, Nilsson A, Albertsson K, Klareskog L. Production of T cell cytokines at the single cell level in patients with inflammatory arthritides: enhanced activity in synovial fluid compared to blood. Br J Rheumatol 1998;37:7–14.
21. Pitzalis C, Kingsley GH, Murphy J, Panayi GS. Abnormal distribution of the helper inducer and suppressor inducer T lymphocyte subsets in the rheumatoid joint. Clin Immunol Immunopathol 1987;45:252–8.
22. Jung T, Schauer U, Heusser C, Neumann C, Rieger C.

Detection of intracellular cytokines by flowcytometry. J Immunol Methods 1993;159:197–207.
23. Picker LJ, Singh MK, Zdraveski Z *et al.* Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. Blood 1995;86:1408–19.
24. Elson LH, Nutman TB, Metcalfe DD, Prussin C. Flow cytometric analysis for cytokine production identifies T helper 1, T helper 2, and T helper 0 cells within the human CD4+CD27 lymphocyte subpopulation. J Immunol 1995;154:4294–301.
25. Prussin C. Cytokine flow cytometry: understanding cytokine biology at the single cell level. J Clin Immunol 1997;17:195–204.
26. van Roon JA, Verhoef CM, van Roy JL *et al.* Decrease in peripheral type 1 over type 2 T cell cytokine production in patients with rheumatoid arthritis correlates with an increase in severity of disease. Ann Rheum Dis 1997;56:656–60.
27. Austrup F, Vestweber D, Borges E *et al.* P and E selectin mediate recruitment of T helper 1 but not T helper 2 cells into inflammed tissues. Nature 1997;385:81–3.
28. Pitzalis C. Role of adhesion mechanisms in the pathogenesis of chronic synovitis. Br J Rheumatol 1996;35:1198–215.
29. Sandberg CI, Imfeld KL, Zaldivar F Jr, Wang Z, Buckingham BA, Berman BA. IL 4 expression in human T cells is selectively inhibited by IL 1 alpha and IL 1 beta. J Immunol 1995;155:5206–12.
30. Satoskar AR, Okano M, Connaughton S, Raisanen Sokolwski A, David JR, Labow M. Enhanced Th2 like responses in IL 1 type 1 receptor deficient mice. Eur J Immunol 1998;28:2066–74.



Chapter 3

Low percentages of IFN-γ producing T cells in Systemic Lupus Erythematosus

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Abstract

It has been suggested that the balance between IFN-γ (type-1) and IL-4 (type-2) producing T cells plays a role in the induction and perpetuation of autoimmunity. This balance was reported to be altered in the peripheral blood of SLE patients due to a low percentage of IFN-γ producing T cells. The purpose of this study was to quantitate this balance and investigate whether alterations in the type1/type-2 ratio are related to changes in the composition of the circulating T-cell compartment.

Methods: Peripheral blood was obtained from SLE patients and healthy matched controls. IFN-γ and IL-4 producing T cells (CD3+) were enumerated after short stimulation using an intracellular staining technique and flowcytometric analysis. Next to this the differentiation state of the peripheral blood T cells was evaluated.

Results: Both CD8- and CD8+ T cells of SLE patients contained lower percentages of IFN-γ producing cells than age- and sex matched healthy controls. Since percentages of IL-4 positive CD8- cells did not differ between both groups the IFN-γ/IL-4 (Th1/Th2) ratio was significantly lower in SLE patients. After in culture of PBMC with anti-CD2 and anti-CD28 antibodies, IFN-γ producers stayed significantly lower in patients, but after addition of exogenous IL-12 (1 ng/ml) the difference disappeared. Phenotypic analysis of freshly isolated T cells revealed that low percentages of IFN-γ producing T cells in SLE patients could not be ascribed to differences in memory typed CD45RA-CD95+ cells between the two groups. Interestingly, significantly lower percentages of terminally differentiated CD4+CD27- cells were found in SLE patients.

Conclusion: Low percentages of IFN-γ producing CD8- and CD8+ T cells were found in the peripheral blood of SLE patients in comparison with healthy controls. Circulating SLE T cells showed signs of diminished differentiation but could still be directed into IFN-γ producing cells when stimulated in an IL-12 rich environment.

Introduction

Systemic Lupus Erythematosus (SLE) is a chronic remitting-relapsing disease, which can affect virtually any organ. The origin is believed to be multifactorial resulting in systemic B-cell hyperactivity and production of pathogenic autoantibodies. T cells appear to play a role since specific clones can be isolated from patients that can drive B cells to produce pathogenic anti-DNA autoantibodies [1,2].
Furthermore intrinsic T cell abnormalities have been described in SLE patients[3-6].
Special attention has been paid to the balance between the interferon-γ (IFN-γ) producing type-1 and interleukin-4 (IL-4) producing type-2 T cells in SLE. An imbalance between these two subsets is believed to play a role in the development and progression of autoimmunity in general[7]. Several reports suggest that in SLE there is an imbalance in favour of the type-2 cells since there is an impaired IFN-γ production by peripheral blood T lymphocytes[8-11]. The differentiation of T cells into either the type-1 or type-2 direction depends on many factors but the production of cytokines by antigen presenting cells (APC)

appears to play a role. APC derived IL-12 is a potent inducer of IFN-γ production while IL-10 gives rise to type-2 T cells partly by antagonizing effects on IL-12 production. Several reports have shown that in SLE there are increased IL-10 levels[12-14]which could explain the suppression of IFN-γ production.
Despite the low production by peripheral blood cells, IFN-γ has been shown to be a contributing factor in the development of tissue damage in murine models[15-17] while IFN-γ treatment can exacerbate human SLE[18,19]. This paradox could be explained by the assumption that peripheral blood T cells of SLE patients can still differentiate into pathogenic type-1 cells after entering the tissues. Therefore we investigated the differentiation state of peripheral blood T cells of SLE patients in relation to the type-1/type-2 balance. We found that decreased percentages of IFN-γ producing CD4+ T cells in the peripheral blood of SLE patients may be related to an underrepresentation of terminally differentiated CD4+ cells lacking the CD27 antigen[20] in the blood. Moreover, both CD4+ and CD8+ SLE T cells were capable of differentiating into adequate IFN-γ producing cells in the presence of IL-12 in vitro.

Materials and methods

Study population. Of 8 SLE patients and 8 healthy controls peripheral blood (PB) was obtained in heparinized tubes and diluted in phosphate buffered saline (PBS) containing 10% fetal calf serum (FCS) and 5% trisodiumcitrate. Mononuclear cells were isolated by density centrifugation on a Ficoll-Hypaque gradient (d=1.079). Cells were suspended in Iscove 's modified Dulbecco 's medium (IMDM) containing 30% FCS and 10% dimethylsulphoxide and stored in liquid nitrogen until use.

Intracellular staining technique. Cells positive for IFN-γ and IL-4 were determined by flowcytometry. In short, mononuclear cells were stimulated with 1 ng phorbol mystirate acetate (PMA) (Sigma chemicals) and 1μM Ionomycin (Sigma chemicals). 1 μM Monensin (Sigma chemicals) was added to prevent excretion of the newly synthesized cytokines. After 4 hours of incubation cells were stained with PE-anti CD3 and FITC-anti CD8 monoclonal antibodies (mAb) (both Becton and Dickinson, Mountain View, CA). Cells were fixed with 4% paraformaldehyde. Cells were stored overnight in PBS at 4°C. Permeabilisation of the cells was done with 0.1% saponin supplemented with 10% pooled human serum (HPS) to block aspecific binding of mAb. Directly phycoerythrin (PE) labeled anti-IFN-γ, anti-IL-4 and a negative control (all Becton Dickinson) were diluted in PBS/BSA/Saponin to a concentration of 5μg/ml and added to the cells in aliquots of 50ml for 25 minutes at 4°C. Cells were analyzed on a FACScan (Becton Dickinson). IFN-γ and IL-4 containing CD8- (defined as CD4+) and CD8+ T cells were identified by gating for CD3 postivity and setting a marker using the sample containing the negative control antibody. Cytokine producers were expressed as percentages of CD3+CD4+ and CD3+CD8+ T cells.

Induction of IFN-γ producing T cells by IL-12. 0.4 million PBMC were stimulated with 1:1000 ascites containing anti-CD2 antibodies (CLB T11.1/1, CLB T11.2/1, Hik-27, CLB, Amsterdam, The Netherlands) and anti CD28 (CLB-CD28/1, CLB, Amsterdam, The Netherlands) in the presence or absence of recombinant IL-12 (1 ng/ml) for four days. Then cells were restimulated with PMA and Ionomycin for 4 hours in the presence of Monensin and stained for intracellular IFN-γ as described above.

Determination of the differentiation antigens on peripheral blood T cells. Three-color flowcytometric analysis was performed on 0.2 million freshly isolated mononuclear cells with combinations of anti CD95-FITC (Immunotech), anti CD45RA (2H4, Coulter), anti CD27-FITC (3A12, CLB, Amsterdam, The Netherlands), anti CD4-PerCP and anti CD8-PerCP (both Becton Dickinson) in 100 μl PBS/0.05% BSA. Cells were incubated for 20 minutes at 4°C.

Production of monokines. Whole blood of patients and controls was diluted in LPS free IMDM (1:10). 100 pg/ml lipooligosacharide (LOS) was added with thromboliquine. After incubation for 24 hours cells and supernatant were stored at -20°C. IL-12 (p40), IL-10 and IL-6 were determined in supernatant with ELISA (CLB, Amsterdam, The Netherlands).

Results

Clinical features

Eight patients with SLE with low disease activity and eight healthy controls were included in this study. All patients fulfilled the 1982 ARA criteria for SLE. Demographic data of the patients included in the study are presented in Table 1. Three patients were using prednisone (3, 4 and 30 mg/day). In addition one of them used 200mg azathioprine daily.

Table 1. Characteristics of population studied

	SLE	Controls
Female/Male	7/1	7/1
Age* (years)	37.9 ± 12.7	41.1 ± 10.5
Disease duration* (years)	7.9 ± 5.7	
Disease activity* (SLEDAI)	3.8 ± 2.9	

*= Mean ± standard deviation

Quantitation of IFN-γ and IL-4 producing T cells

To determine whether the percentages of type-1 and type-2 cells in the peripheral blood of SLE patients were different from healthy controls, intracellular cytokine staining experiments were performed and presented in Figure 1. In agreement with previous reports,

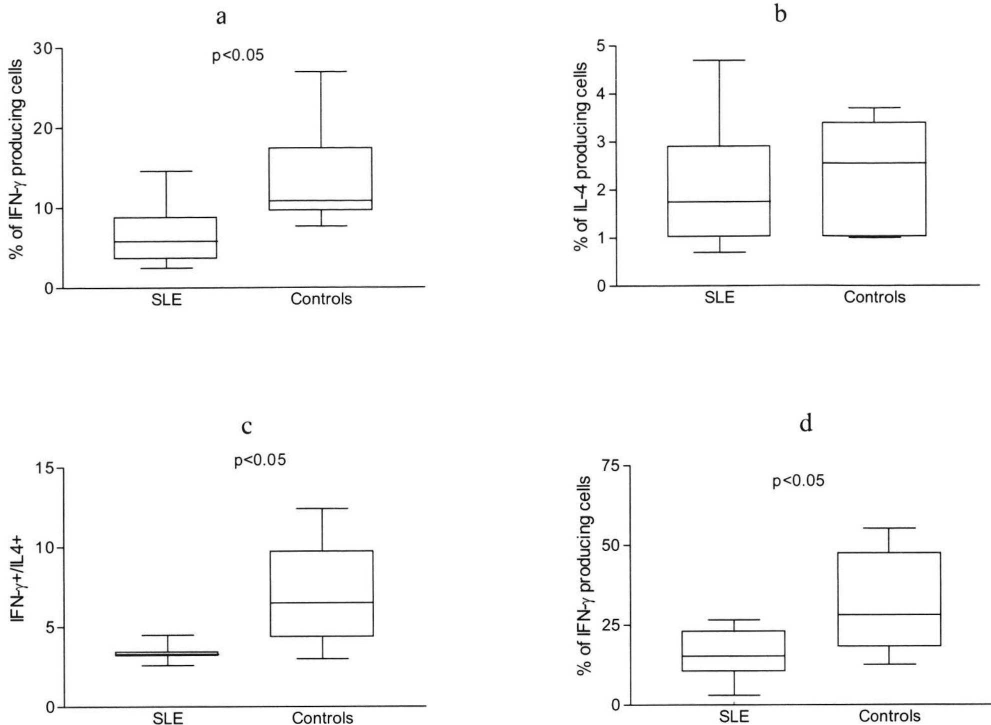


Figure 1. Comparison of the percentages IFN-γ (a+d) and IL-4 (b) producing cells as well as their ratio (c) in CD8+ (d) and CD8- (a-c) circulating CD3+ cells between SLE patients and healthy controls. The box indicates the median while the whiskers show the range.

lower percentages of IFN-γ producing CD3+CD8- T cells (Th1) were found in SLE patients in comparison with healthy controls. However IL-4 producing CD3+CD8- (Th2) cells were not significantly different between the two groups. The Th1/Th2 ratios were therefore lower in SLE patients. Within the CD8+ T cell compartment lower percentages of IFN-γ producing cells were found as well. IL-4 positive CD8+ T cells were only detectable in 2 SLE patients and 3 healthy controls.

Phenotypic analysis of T cells in patients and controls

To determine whether the decreased percentage of IFN-γ producing cells could be explained by the differentiation state of T cells in SLE patients, phenotyping for the differentiation antigens CD45RA, CD95 and CD27 was performed with simultaneous analysis of CD4 and CD8 expression. After gating for CD4+ and CD8+ cells, percentages of "naive" CD45RA+ CD95-, CD45RA+CD95+ and "memory" CD45RA-CD95+ cells were not different between SLE patient and healthy controls. However significant lower percentages of terminally differentiated CD4+CD27- cells were found in SLE patients than

in healthy controls (table 2). The percentage of CD27- cells however was not different for CD8+ cells.

Differentiation of Type-1 cells in vitro

To investigate whether peripheral blood T cells of SLE patients were able in vitro to enhance IFN-γ (type 1) production after prolonged activation, mononuclear cells of patients and controls were cultured in vitro in the presence and absence of recombinant IL-12 (table 3). After four days of culture still lower percentages of IFN-γ producing cells were found in SLE patients in both CD8- and CD8+ subsets. However in the presence of exogenous IL-12 no significant difference between SLE patients and controls could be found any more after 4 days.

Production of monokines in whole blood

Since the capacity to differentiate in type-1 cells of SLE peripheral blood T cells seems to be normal in the presence of IL-12, low IFN-γ production could be caused by a diminished production of IL-12 or an overproduction of IL-10 which is antagonizing IL-12. Therefore we investigated whether the capacity of T cells to produce IFN-γ was related to the capacity to produce IL-12 or IL-10 in these SLE patients. Diluted whole blood was stimulated with LOS and production of the cytokines IL-12 (p40), IL-10 and IL-6 was determined in he supernatant after 24 hours. As presented in table 4, no significant differences were found for either of these cytokines between SLE patients and controls nor did they correlate with IFN-γ production.

Table 2. Percentages of cells positive for differentiation markers in the peripheral blood of SLE patients and matched healthy controls

		CD4+		*CD8+*	
		Median	Range	Median	Range
SLE	CD45RA+CD95-	50.3	24.9 - 75.7	43.8	20.6 - 74.6
Controls		40.3	5.1 - 70.3	52.2	10.1 - 83.4
SLE	CD45RA+CD95+	14.0	6.7 - 39.5	45.7	21.7 - 59.5
Controls		17.1	9.7 - 22.4	32.5	13.6 - 56.8
SLE	CD45RA-CD95+	23.6	16.9 - 42.4	11.4	1.6 - 19.7
Controls		40.3	17.7 - 75.0	14.3	2.6 - 32.5
SLE	CD27-	2.1	1.6 - 10.0	8.3	1.3 - 31.3
Controls		6.7*	1.8 - 14.0	9.1	2.7 - 22.2

* $p<0.05$ by Mann-Whitney test for SLE *vs.* controls

Table 3. Percentage of IFN-γ positive cells in cultured PBMC stimulated with anti-CD3 and anti-CD28 antibodies in the absence or presence of IL-12 for 4 days

		CD4+		*CD8+*	
		Median	range	Median	range
Medium	SLE	12.3	6.9 - 22.5	25.5	11.7 - 60.1
	Controls	19.8*	12.8 - 43.9	48.7*	24.3 - 72.9
IL-12	SLE	43.5	26.7 - 62.1	62.4	39.2 - 86.4
	Controls	55.6	39.0 - 61.9	77.4	69.0 - 93.2

*p<0.05 with the Mann-Whitney test for SLE vs. Controls

Table 4. Production of monokines by LOS stimulated wholeblood of SLE patients and healthy matched controls

		Median	Range
SLE	IL-10	141	4 - 403
Controls		81	1 - 221
SLE	IL-12 (p40)	414	100 - 2490
Controls		574	12 - 952
SLE	IL-6	1711	962 - 5407
Controls		1548	1 - 5168

Discussion

In the pathogenesis of SLE a dysbalance in the type-1/type-2 T cells is suggested. In the peripheral blood of SLE patients decreased IFN-γ production has been documented in previous reports[8-10] suggesting a Th2 dominance in the pathogenic process. We here confirm these results in patients with low disease activity, both in the CD8- and CD8+ T cell compartments. Although three SLE patients were taking immunosuppressive drugs these were not responsible for this finding. It has been suggested that the lower percentage of IFN-γ producing T cells in the circulation of SLE could result from low production of

IL-12[10,21-23] or by overproduction of by IL-10[8,13,14,23-25]during active disease. IL-10 is a well-known downregulator of IL-12 p40 production[26,27]. In our patient group, with low disease activity, both IL-10 and IL-12 p40 production as well as their ratio were not different in comparison with healthy controls. In addition no significant correlation was observed between IL-12 p40, IL-10 production or their ratio and Th1/Th2 ratio measured. This suggests that the lower percentage of IFN-γ producing T cells in these patients is not caused by an ongoing altered production of IL-12 or IL-10. However, it could that during periods of disease activity high production of IL-10 can interfere with the development of type-1 T cells.

Both IFN-γ and IL-4 are produced by differentiated "memory" (CD45RA-CD45RO+CD95+) T cells. SLE patients are reported to have a higher proportion of memory type T cells than healthy controls especially during active disease[28]. Therefore the observation of low percentage of IFN-γ could be due to low percentages of memory T cells. Phenotypic analysis however showed that SLE patients in this study had no significant different proportion of CD45RA- T cells in both CD8- and CD8+ compartments than healthy controls, probably due to low disease activity. Neither were any differences found in CD95+ cells between patients. This was a surprising observation since CD4+CD45RA+ cells in Japanese SLE patients are reported to be CD95 positive[29]. This discrepancy could be caused by a selection of different patients considering that we found high percentages of CD45RA+CD95+ cells in a patient with primary Sjögren syndrome (data not shown).

Despite normal percentages of memory typed cells, significant lower percentages of CD4+CD27- cells were found in SLE patients. CD4+CD27- cells are highly differentiated since they are generated after prolonged stimulation of CD4+CD45RO+CD27+ cells in vitro[20,30]. Within the CD4+CD27- subset more IFN-γ and IL-4 producers are found than in the CD4+CD27+ subset[31]. Lower percentages of CD4+CD27- cells in this patient group could therefore suggest that CD4+ cells in inactive SLE patients are less differentiated than that of healthy controls and could explain the low percentages of IFN-γ producing cells. Both with respect to homing receptor and chemokine receptor expression CD4+CD27- T cells are distinct from recirculating CD4+CD45RO+CD27+ memory T cells[32]. Therefore the low abundance of CD4+CD27- T cells in the circulation of SLE patients could be comparable with an increased representation of these cells in affected tissue.

Although there is a Th1 deficiency in the peripheral blood of SLE patients in murine SLE studies it has been shown that there is a local production of IL-12 in the kidney[33] and that IFN-γ can accelerate the development of clinical symptoms[34]. This suggests that the local induction of type-1 cells can be involved in the development of the process of tissue damage in SLE. Since the IFN-γ deficient T cells in the circulation of SLE patients are not differentiated into type-2 effector cells as shown here by normal percentages of IL-4 producing cells, the capacity to produce IFN-γ after they leave the circulation could be important for a local Th1 dominated reaction. In this study we could demonstrate that after prolonged stimulation in vitro, the increase in IFN-γ producing cells is still lower in SLE patients than in healthy controls. However activation in the presence of IL-12 achieves percentages of IFN-γ producing CD8+ and CD8- T cells that are not significantly different between SLE patients and healthy controls.

Conclusion

Low IFN-γ production by mononuclear cells of SLE patients in vitro is often explained by the hypothesis that T cells are exhaustingly stimulated in vivo. Our results suggests that peripheral blood T cells in SLE despite low production of IFN-γ can be induced to yield higher percentages of IFN-γ when activated in an IL-12 rich environment. Therapeutic use of IL-12 might therefore result in an increase in IFN-gamma producing cells in the peripheral blood. However it could on the other hand amplify a localized type-1 response leading to increased tissue damage in affected organs.

References

1. Shivakumar S, Tsokos GC, Datta SK. T cell receptor alpha/beta expressing double-negative (CD4-/CD8-) and CD4+ T helper cells in humans augment the production of pathogenic anti- DNA autoantibodies associated with lupus nephritis. J Immunol 1989;143:103-112

2. Desai-Mehta A, Mao C, Rajagopalan S, Robinson T, Datta SK. Structure and specificity of T cell receptors expressed by potentially pathogenic anti-DNA autoantibody-inducing T cells in human lupus. J Clin Invest. 1995;95:531-541

3. Liossis SN, Ding XZ, Dennis GJ, Tsokos GC. Altered pattern of TCR/CD3-mediated protein-tyrosyl phosphorylation in T cells from patients with systemic lupus erythematosus. Deficient expression of the T cell receptor zeta chain. J Clin Invest. 1998;101:1448-1457

4. Vassilopoulos D, Kovacs B, Tsokos GC. TCR/CD3 complex-mediated signal transduction pathway in T cells and T cell lines from patients with systemic lupus erythematosus. J Immunol 1995;155:2269-2281

5. Kammer GM. Impaired T cell capping and receptor regeneration in active systemic lupus erythematosus. Evidence for a disorder intrinsic to the T lymphocyte. J Clin Invest. 1983;72:1686-1697

6. Desai-Mehta A, Lu L, Ramsey-Goldman R, Datta SK. Hyperexpression of CD40 ligand by B and T cells in human lupus and its role in pathogenic autoantibody production. J Clin Invest. 1996;97:2063-2073

7. Charlton B, Lafferty KJ. The Th1/Th2 balance in autoimmunity. Curr.Opin.Immunol 1995;7:793-798

8. Hagiwara E, Gourley MF, Lee S, Klinman DM. Disease severity in patients with systemic lupus erythematosus correlates with an increased ratio of interleukin-10:interferon-gamma-secreting cells in the peripheral blood. Arthritis.Rheum. 1996;39:379-385

9. Funauchi M, Ikoma S, Enomoto H, Horiuchi A. Decreased Th1-like and increased Th2-like cells in systemic lupus erythematosus. Scand.J Rheumatol 1998;27:219-224

10. Horwitz DA, Gray JD, Behrendsen SC, et al. Decreased production of interleukin-12 and other Th1-type cytokines in patients with recent-onset systemic lupus erythematosus. Arthritis Rheum. 1998;41:838-844

11. Amel-Kashipaz MR, Huggins ML, Lanyon P, Robins A, Todd I, Powell RJ. Quantitative and qualitative analysis of the balance between type 1 and type 2 cytokine-producing CD8(-) and CD8(+) T cells in systemic lupus erythematosus. J Autoimmun.2001; 17:155-163

12. al Janadi M, al Dalaan A, al Balla S, al Humaidi M, Raziuddin S. Interleukin-10 (IL-10) secretion in systemic lupus erythematosus and rheumatoid arthritis: IL-10-dependent CD4+CD45RO+ T cell-B cell antibody synthesis. J.Clin.Immunol. 1996;16:198-207

13. Park YB, Lee SK, Kim DS, Lee J, Lee CH, Song CH. Elevated interleukin-10 levels correlated with disease activity in systemic lupus erythematosus. Clin Exp Rheumatol 1998;16:283-288

14. Houssiau FA, Lefebvre C, Vanden Berghe M, Lambert M, Devogelaer JP, Renauld JC. Serum interleukin 10 titers in systemic lupus erythematosus reflect disease activity. Lupus. 1995;4:393-395

15. Balomenos D, Rumold R, Theofilopoulos AN. Interferon-gamma is required for lupus-like disease and lymphoaccumulation in MRL-lpr mice. J Clin Invest. 1998;101:364-371

16. Seery JP, Carroll JM, Cattell V, Watt FM. Antinuclear autoantibodies and lupus nephritis in transgenic mice expressing interferon gamma in the epidermis. J Exp Med 1997;186:1451-1459

17. Schwarting A, Wada T, Kinoshita K, Tesch G, Kelley VR. IFN-gamma receptor signaling is essential for the initiation, acceleration, and destruction of autoimmune kidney disease in MRL- Fas(lpr) mice. J Immunol 1998;161:494-503

18. Graninger WB, Hassfeld W, Pesau BB, Machold KP, Zielinski CC, Smolen JS. Induction of systemic lupus erythematosus by interferon-gamma in a patient with rheumatoid arthritis. J Rheumatol 1991;18:1621-1622

19. Machold KP, Smolen JS. Interferon-gamma induced exacerbation of systemic lupus erythematosus. J Rheumatol 1990;17:831-832

20. de Jong R, Brouwer M, Hooibrink B, Van der Pouw-Kraan T, Miedema F, van Lier RA. The CD27- subset of peripheral blood memory CD4+ lymphocytes contains functionally differentiated T lymphocytes that develop by persistent antigenic stimulation in vivo. Eur.J.Immunol. 1992;22:993-999

21. Liu TF, Jones BM, Wong RW, Srivastava G. Impaired production of IL-12 in systemic lupus erythematosus. III: deficient IL-12 p40 gene expression and cross-regulation of IL-12, IL- 10 and IFN-gamma gene expression. Cytokine. 1999;11:805-811

22. Liu TF, Jones BM. Impaired production of IL-12 in system lupus erythematosus. II: IL-12 production in vitro is correlated negatively with serum IL-10, positively with serum IFN-gamma and negatively with disease activity in SLE. Cytokine. 1998;10:148-153

23. Liu TF, Jones BM. Impaired production of IL-12 in systemic lupus erythematosus. I. Excessive production of IL-10 suppresses production of IL-12 by monocytes. Cytokine. 1998;10:140-147

24. Llorente L, Richaud P, Wijdenes J, et al. Spontaneous production of interleukin-10 by B lymphocytes and monocytes in systemic lupus erythematosus. Eur.Cytokine.Netw. 1993;4:421-427

25. Llorente L, Richaud P, Fior R, et al. In vivo production of interleukin-10 by non-T cells in rheumatoid arthritis, Sjogren's syndrome, and systemic lupus erythematosus. A potential mechanism of B lymphocyte hyperactivity and autoimmunity. Arthritis.Rheum. 1994;37:1647-1655

26. Du C, Sriram S. Mechanism of inhibition of LPS-induced IL-12p40 production by IL-10 and TGF-beta in ANA-1 cells. J Leukoc.Biol 1998;64:92-97

27. Aste-Amezaga M, Ma X, Sartori A, Trinchieri G. Molecular mechanisms of the induction of IL-12 and its inhibition by IL- 10. J Immunol 1998;160:5936-5944

28. Gordon C, Matthews N, Schlesinger BC, et al. Active systemic lupus erythematosus is associated with the recruitment of naive/resting T cells. Br.J.Rheumatol. 1996;35:226-230

29. Amasaki Y, Kobayashi S, Takeda T, et al. Up-regulated expression of Fas antigen (CD95) by peripheral naive and memory T cell subsets in patients with systemic lupus erythematosus (SLE): a possible mechanism for lymphopenia. Clin.Exp.Immunol. 1995;99:245-250

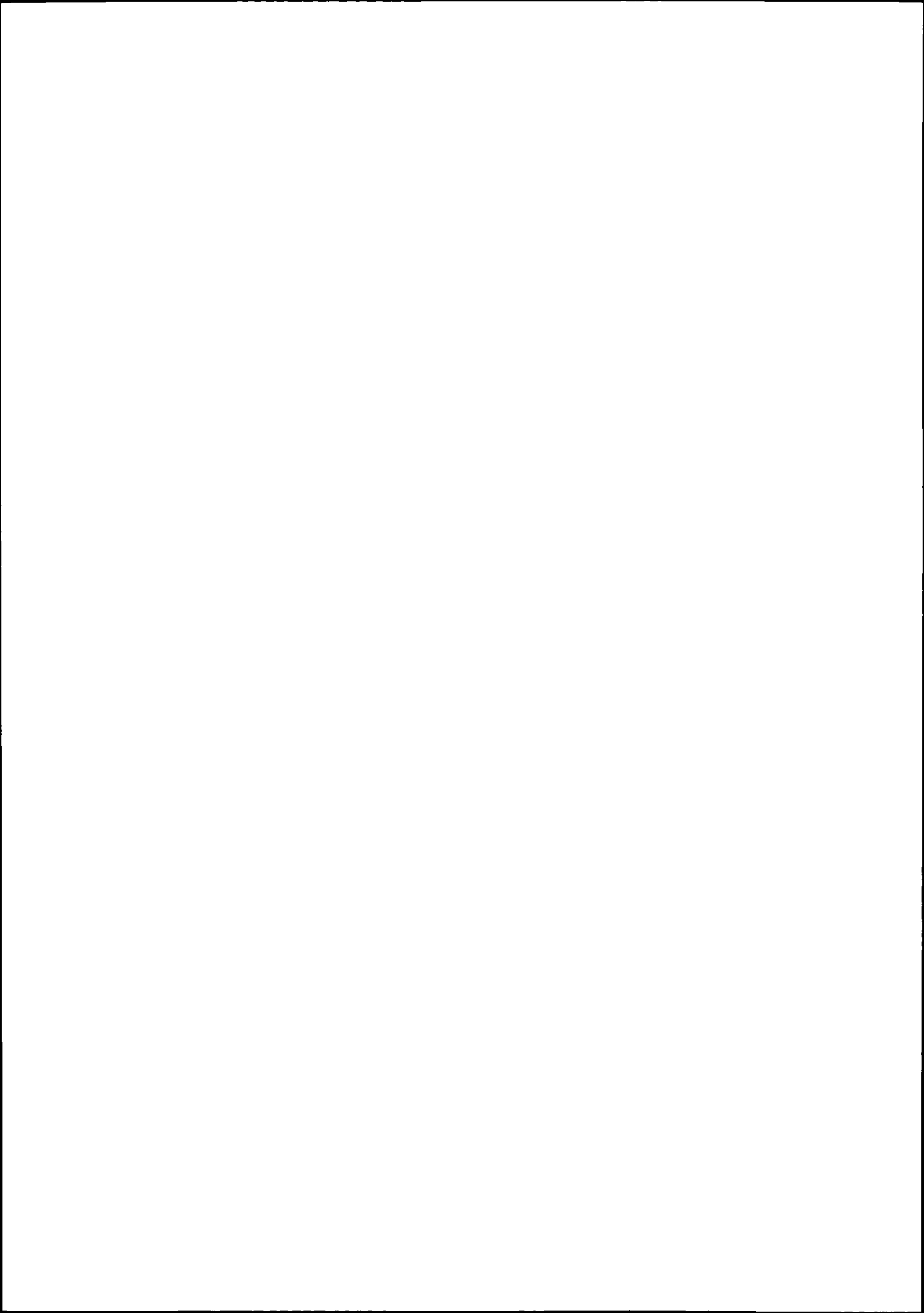
30. Hintzen RQ, de Jong R, Lens SM, Brouwer M, Baars P, van Lier RA. Regulation of CD27 expression on subsets of mature T-lymphocytes. J Immunol 1993;151:2426-2435

31. Elson LH, Nutman TB, Metcalfe DD, Prussin C. Flow cytometric analysis for cytokine production identifies T helper 1, T helper 2, and T helper 0 cells within the human CD4+CD27- lymphocyte subpopulation. J.Immunol. 1995;154:4294-4301

32. Baars PA, Maurice MM, Rep M, Hooibrink B, van Lier RA. Heterogeneity of the circulating human CD4+ T cell population. Further evidence that the CD4+CD45RA-CD27- T cell subset contains specialized primed T cells. J Immunol 1995;154:17-25

33. Fan X, Wuthrich RP. Upregulation of lymphoid and renal interferon-gamma mRNA in autoimmune MRL-Fas(lpr) mice with lupus nephritis. Inflammation 1997;21:105-112

34. Masutani K, Akahoshi M, Tsuruya K, et al. Predominance of Th1 immune response in diffuse proliferative lupus nephritis. Arthritis Rheum.2001;44:2097-2106



Chapter 4

T cell subsets in the peripheral blood of ankylosing spondylitis patients

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Abstract

Ankylosing spondylitis (AS) is an HLA-B27 related autoimmune disease in which T cells are believed to play a role in the pathogenesis. Since the balance between type-1, IFN-gamma producing, and type-2, IL-4 producing, T cells is believed to be involved in the development of autoimmunity, these two cell types were determined in the peripheral blood of AS patients.

Methods: Percentages of IFN-γ and IL-4 producing CD4+ and CD8+ T cells were determined after short stimulation in vitro and subsequently intracellular staining and flowcytometric analysis. Next to this, the differentiation state of the peripheral T cells was evaluated and related to clinical data.

Results: In AS patients no significant differences were found in IFN-γ and IL-4 producing peripheral blood T cells or in their ratio as compared to age- and sex matched healthy controls.
Within the CD4+ T cell subset AS patients showed a higher percentage of IL-2 producing cells and a lower percentage of differentiated CD27- cells. The percentage of IL-2 producing cells was related to disease severity as determined by the Bath Ankylosing Spondylitis metrology index (BASMI), but not to erythrocyte sedimentation rate or disease duration.

Conclusion: The balance in type-1 and type-2 T cells is not altered in peripheral blood of AS patients. CD4+ T cells of AS patients contain higher percentages of IL-2 producing cells which is correlated to disease severity.

Introduction

Ankylosing spondylitis (AS) is one of the spondylathropathies and is characterized by arthritis of the sacroiliac joints and the spinal ligaments, ascending spinal stiffening and spinal ligament calcification. Peripheral arthritis, enthesitis and extra-articular manifestations, including, pulmonary, cardiac and ocular involvement can be found as well. Clinically the disease can vary from intermittent episodes of back pain to severe chronic disease leading to progressive spinal restriction.
Ankylosing Spondylitis is like other spondylathropathies strongly associated with the presence of HLA-B27[1]. Since the primary function of this molecule is to present antigenic peptides to CD8+ T cells, researchers have focused on HLA-B27 restricted cytotoxic T-cells. CD8+ clones directed against arthritogenic bacteria, viral peptides or peptides derived from the HLA-B27 molecule itself have been described in AS patients[2-4].
Alternatively CD4 positive T lymphocytes have been implicated in the pathogenesis as they can directly react with HLA B27[5].
Involvement of T cells has been suggested in the development of different autoimmune diseases and multiple abnormalities have been described in T cell phenotypes and cytokine

profiles. Previously we have shown that the balance between type-1 en type-2 T cells in the peripheral blood rheumatoid arthritis is not significantly different from healthy controls[6], while in the peripheral blood of systemic lupus erythematosus patients there are significantly less T cells with a type-1 phenotype[7]. To make a comparison with RA and SLE, in this study percentages of different T cells were estimated in the peripheral blood of AS patients. This was done by analyzing expression of membrane differentiation markers as well as cytokine production capacity of CD4+ and CD8+ T lymphocytes.

Materials and methods

Study population. Peripheral blood from 20 patients with AS as defined by the modified New
York criteria[8] and healthy controls was obtained in heparinized tubes and diluted in phosphate buffered saline (PBS) containing 10% fetal calf serum (FCS) and 5% trisodiumcitrate. Mononuclear cells were isolated by density centrifugation on a Ficoll-Hypaque gradient (d=1.079). Cells were suspended in Iscove 's modified Dulbecco 's medium (IMDM) containing 30% FCS and 10% dimethylsulphoxide and stored in liquid nitrogen until use.

Intracellular staining technique. Cells positive for IFN-γ, IL-4 and IL-2 were determined by flowcytometry. In short, mononuclear cells were stimulated with 1 ng phorbol mystirate acetate (PMA) (Sigma chemicals) and 1μM Ionomycin (Sigma chemicals). 1 μM Monensin (Sigma chemicals) was added to prevent excretion of the newly synthesized cytokines. After 4 hours of incubation at 37°C cells were stained with FITC-anti T cell receptor α/β and PerCP-anti CD8 monoclonal antibodies (mAb) (both Becton and Dickinson, Mountain View, CA). Cells were fixed with 4% paraformaldehyde. Cells were stored overnight in PBS at 4°C. Permeabilisation of the cells was done with 0.1% saponin supplemented with 10% pooled human serum (HPS) to block aspecific binding of mAb. Directly phycoerythrin (PE) labeled anti-IFN-γ, anti-IL-4, anti-IL-2 mAb and a negative control mAb (all Becton Dickinson) were diluted in PBS/BSA/Saponin to a concentration of 5 μg/ml and added to the cells in aliquots of 50ml for 25 minutes at 4°C.
Cells were analyzed on a FACScan (Becton Dickinson). IFN-γ and IL-4 containing CD8- and CD8+ T cells were identified by gating for TCR α/β postivity and setting a marker using the sample containing the negative control antibody. Cytokine producers were expressed as percentages of TCRα/β +CD8- and TCRα/β+CD8+ T cells.

Determination of differentiation antigens on peripheral blood T cells.
Three color flowcytometric analysis was performed on 0.2 million freshly isolated mononuclear cells with combinations of anti CD95-FITC (Immunotech), anti CD45RA (2H4, Coulter), anti CD27-FITC (3A12, CLB, Amsterdam, The Netherlands), anti CD4-PerCP and anti CD8-PerCP (both Becton Dickinson) in 100 μl PBS/0.05% BSA. Cells were incubated for 20 minutes at 4°C.

Results

Clinical features

20 patients with AS, recruited from the Jan van Breemen Institute (Amsterdam, The Netherlands) and matched healthy controls selected from a donor pool of laboratory workers were included in this study. On withdrawal of blood the Bath Ankylosing Spondylitis Metrology Index (BASMI)[9] was determined to obtain data referring to restriction of the joints. The Bath Ankylosing Spondylitis Patient Global Score (BAS-G)[10] was determined to get a parameter for disease activity since this score reflects the effect of AS on the patient's well-being relative to the last six months. BASMI and BAS-G were available for 14 patients. None of the patients had signs of peripheral arthritis. Clinical data of the patients included in the study are presented in Table 1. No cytotoxic drugs or steroids were used; twelve patients took nonsteroidal anti-inflammatory drugs.

Table 1. Clinical data of ankylosing spondylitis patients participating in this study

	Median (range)
Gender	18 male, 2 female
Age	48 (35-66) years
ESR	22 (2-64) mm
Disease duration	20 (4-45) years
BASMI	4 (0.4-7)
BAS-G score	0 (-3.5 – 7)

Quantitation of cytokine positive T lymphocytes

Percentages of IFN-gamma and IL-4 positive T cells and their ratio in the peripheral blood were determined for CD4+ and CD8+ cells after short stimulation ex vivo. No significant differences were found for these subsets between AS patients and healthy controls. However the percentage of IL-2 positive cells was significantly higher in the AS patients but only for CD4+ cells (Table 2)

Table 2. Median percentages of cytokine positive cells in the peripheral blood of ankylosing spondylitis (AS) patients and healthy controls

		AS patients		Healthy controls	
		Median	*Range*	*Median*	*Range*
CD4+	IFN-γ	11.9	7.1-30.6	13.5	5.9-21.3
	IL-4	5.3	1.9-15.2	5.0	2.8-7.9
	IL-2	18.8*	9.2-31.2	13.7	5.8-17.3
	IFN-γ /IL-4	3.4	1.6-6.0	3.0	1.5-8.5
CD8+	IFN-γ	24.3	8.4-53.9	20.3	12.8-36.8
	IL-4	4.1	0.8-22.3	4.3	0.4-9.8
	IL-2	17.8	8.4-60.0	18.7	15.4-27.7
	IFN-γ /IL-4	7.4	1.4-34.0	7.3	2.4-29.0

* Significantly different from healthy controls p=0.02 (Mann-Whitney)

Phenotypic analysis of T cells in patients and controls

Phenotyping for the differentiation antigens CD45RA, CD95 and CD27 was performed with simultaneous analysis of CD4 and CD8 expression. After gating for CD4+ and CD8+ cells, the percentages of primed "memory" typed cells as determined by CD45RA negativity or CD95 positivity, were not different between AS patient and healthy controls. However significant lower percentages of terminally differentiated CD4+CD27- cells were found in AS patients compared to healthy controls (Table 3). The percentage of CD27- cells however was not different for CD8+ cells.

To investigate whether the abnormal distribution of CD27-negative and Il-2-positive CD4 T lymphocytes in the peripheral blood of AS patients was related to clinical parameters, Spearman Rank correlations were calculated between them in 14 patients. As shown in Table 4 the percentage of CD27 negative cells was not correlated to any of the parameters investigated, while a significant correlation could be found between the BASMI and the percentage of IL-2 positive CD4 T lymphocytes (see also figure 1). No correlation with the BAS-G score was found. This might suggests that high percentages of IL-2 producers are related to disease progression rather than activity.

Table 3 Median percentages of cells expressing differentiation markers within peripheral blood T cells of ankylosing spondylitis (AS) patients and healthy controls

		AS patients		Healthy controls	
		Median	*Range*	*Median*	*Range*
CD4+	%CD45RA-	36.4	24.2-53.7	41.2	22.5-60.8
	%CD27-	9.8*	4.0-18.5	13.3	9.5-26.6
	%CD95+	52.0	41.2-59.6	45.9	38.8-50.3
CD8+	%CD45RA-	18.6	6.2-28.9	20.0	7.3-32.1
	%CD27-	16.0	9.9-24.3	20.1	12.0-36.3
	%CD95+	27.6	24.7-42.6	28.8	20.2-45.0

* Significantly different from healthy controls $p<0.05$ (Mann-Whitney)

Table 4 Spearman-Rank correlation coefficients between CD4 cell phenotype and clinical parameters of ankylosing spondylitis patients

	$CD4^{pos}CD27^{neg}$		$CD4^{pos}IL2^{pos}$	
	Correlation	p	Correlation	p
BASMI	-0.21	0.15	0.63	0.02*
BAS-G	-0.12	0.65	-0.35	0.21
ESR	-0.04	0.89	0.46	0.10
Disease duration	-0.01	0.96	0.26	0.36

* Significantly different from healthy controls $p<0.05$ (Mann-Whitney)

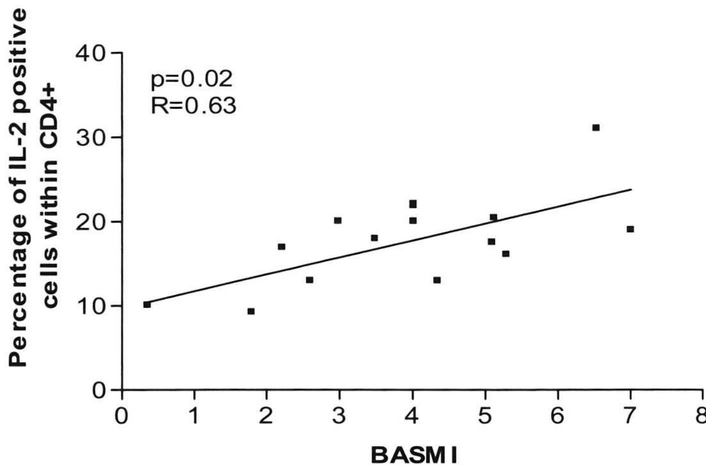


Fig 1. Correlation between the percentages of IL-2 positive CD4+ T lymphocytes in the peripheral blood of ankylosing spondylitis patients and the Bath Ankylosing Spondylitis Metrology Index. R= Spearman-Rank correlation coefficient

Discussion

The balance in cytokine producing T cells is believed to play a role in the initiation and persistence of autoimmunity. In the synovium of arthritis patients predominantly type-1 interferon-gamma producing T cells can be found while type-2 IL-4 producing cells are sporadic[11,12]. Ankylosing spondylitis is one of the spondylarthropaties in which it has been shown that although interferon-gamma producing T cells are dominant in the synovium the ratio between interferon and IL-4 is lower as compared with rheumatoid arthritis[13,14]. Previously we found no difference in the ratio between type-1 and type-2 T cells in the peripheral blood of rheumatoid arthritis patients compared to healthy controls[6]. In this study we do not find significant alterations in the peripheral blood Thl/Th2 ratio in ankylosing spondylitis patients either. This could mean that a relative lower type-1/type-2 ratio in the synovium of spondylarthopathy in comparison with RA is not resulting from a lower type-1/type-2 set point but could result from distinct migration of primed T cells to the synovium possibly as a result from expression of certain homing receptors or chemokines. Alternatively, excess type-1 promoting factors in the rheumatoid synovium like IL-12 and IL-18 can be an additional explanation for the high Thl/Th2 ratio in RA. Possibly these factor are less abundantly expressed in the AS synovium[15].

In contrast to the typical type-1 and type-2 cytokines we found a significant higher percentage of IL-2 producing CD4+ cells in SA patients than in healthy controls . It has

been published that in the synovium of spondylarthropathy patients in contrast to rheumatoid arthritis synovium both messenger RNA as well as IL-2 protein can be found[16,17]. Since we could not find a significant difference in IL-2 producing cells in the peripheral blood of rheumatoid arthritis patients compared with matched healthy controls (unpublished results), IL-2 producing CD4+ T lymphocytes might be participants in the pathogenic process of ankylosing spondylitis. The finding that the percentage of IL-2 producing CD4+ cells was correlated to the BASMI may be taken in support of this. We did not find a correlation with the BAS-G or erythrocyte sedimentation rate (ESR). Since the BASMI is measuring the mobility of the spine and is largely dependent on disease progression while the BAS-G an ESR are more related to disease activity, it might be that the presence of high percentages of IL-2 positive CD4+ cells correlates with more severe, progressive disease.

As we have found previously in systemic lupus erythematosus (SLE) (chapter 3), AS patients had a lower percentage of CD27- CD4 T cells. CD27- CD4+ cells are differentiated effector cells and are formed from CD4+CD27+ cells by prolonged stimulation[18,19]. Since differentiation of CD4+CD27+ into CD27- cells is coinciding with the loss of IL-2 producing capability, high IL-2 production in AS patients could also reflect an impaired ability to generate CD4+ CD27- cells. Why this would predispose to the development of AS is not clear at this moment. It has recently been shown that CD4+CD27- cells can execute cytolysis[20] therefore a shortage of CD4+CD27- cells could result in an inability to deal with intracellular bacteria resulting in chronic infection. Alternatively cytolytic CD4+ cells may be involved in terminating immune responses by removing activated antigen presenting cells, this may also contribute to the pathogenesis of AS.

References

1. Schlosstein L, Terasaki PI, Bluestone R, Pearson CM. High association of an HL-A antigen, W27, with ankylosing spondylitis. N.Engl.J Med 1973;288:704-706

2. Hermann E, Yu DT, Meyer zBK, Fleischer B. HLA-B27-restricted CD8 T cells derived from synovial fluids of patients with reactive arthritis and ankylosing spondylitis. Lancet 1993;342:646-650

3. Fiorillo MT, Maragno M, Butler R, Dupuis ML, Sorrentino R. CD8(+) T-cell autoreactivity to an HLA-B27-restricted self-epitope correlates with ankylosing spondylitis. J Clin Invest.2000;106:47-53

4. Marker-Hermann E, Meyer zBK, Wildner G. HLA-B27-derived peptides as autoantigens for T lymphocytes in ankylosing spondylitis. Arthritis Rheum. 1997;40:2047-2054

5. Boyle LH, Goodall JC, Opat SS, Gaston JS. The recognition of HLA-B27 by human CD4(+) T lymphocytes. J Immunol 200;167:2619-2624

6. van der Graaff WL, Prins AP, Niers TM, Dijkmans BA, van Lier RA. Quantitation of interferon gamma- and interleukin-4-producing T cells in synovial fluid and peripheral blood of arthritis patients. Rheumatology.(Oxford.) 1999;38:214-220

7. Funauchi M, Ikoma S, Enomoto H, Horiuchi A. Decreased Th1-like and increased Th2-like cells in systemic lupus erythematosus. Scand.J Rheumatol 1998;27:219-224

8. van der Linden S, Valkenburg HA, Cats A. Evaluation of diagnostic criteria for ankylosing spondylitis. A proposal for modification of the New York criteria. Arthritis Rheum. 1984;27:361-368

9. Jenkinson TR, Mallorie PA, Whitelock HC, Kennedy LG, Garrett SL, Calin A. Defining spinal mobility in ankylosing spondylitis (AS). The Bath AS Metrology Index. J Rheumatol 1994;21:1694-1698

10. Jones SD, Steiner A, Garrett SL, Calin A. The Bath Ankylosing Spondylitis Patient Global Score (BAS-G). Br.J Rheumatol 1996;35:66-71

11. Bucht A, Larsson P, Weisbrot L, et al. Expression of interferon-gamma (IFN-gamma), IL-10, IL-12 and transforming growth factor-beta (TGF-beta) mRNA in synovial fluid cells from patients in the early and late phases of rheumatoid arthritis (RA). Clin Exp Immunol 1996;103:357-367

12. Dolhain RJ, van der Heiden AN, ter Haar NT, Breedveld FC, Miltenburg AM. Shift toward T lymphocytes with a T helper 1 cytokine-secretion profile in the joints of patients with rheumatoid arthritis. Arthritis Rheum. 1996;39:1961-1969

13. Canete JD, Martinez SE, Farres J, et al. Differential Th1/Th2 cytokine patterns in chronic arthritis: interferon gamma is highly expressed in synovium of rheumatoid arthritis compared with seronegative spondyloarthropathies. Ann.Rheum.Dis.2000;59:263-268

14. Simon AK, Seipelt E, Sieper J. Divergent T-cell cytokine patterns in inflammatory arthritis. Proc.Natl.Acad.Sci.U.S.A. 1994;91:8562-8566

15. Kitagawa M, Mitsui H, Nakamura H, et al. Differential regulation of rheumatoid synovial cell interleukin-12 production by tumor necrosis factor alpha and CD40 signals. Arthritis Rheum. 1999;42:1917-1926

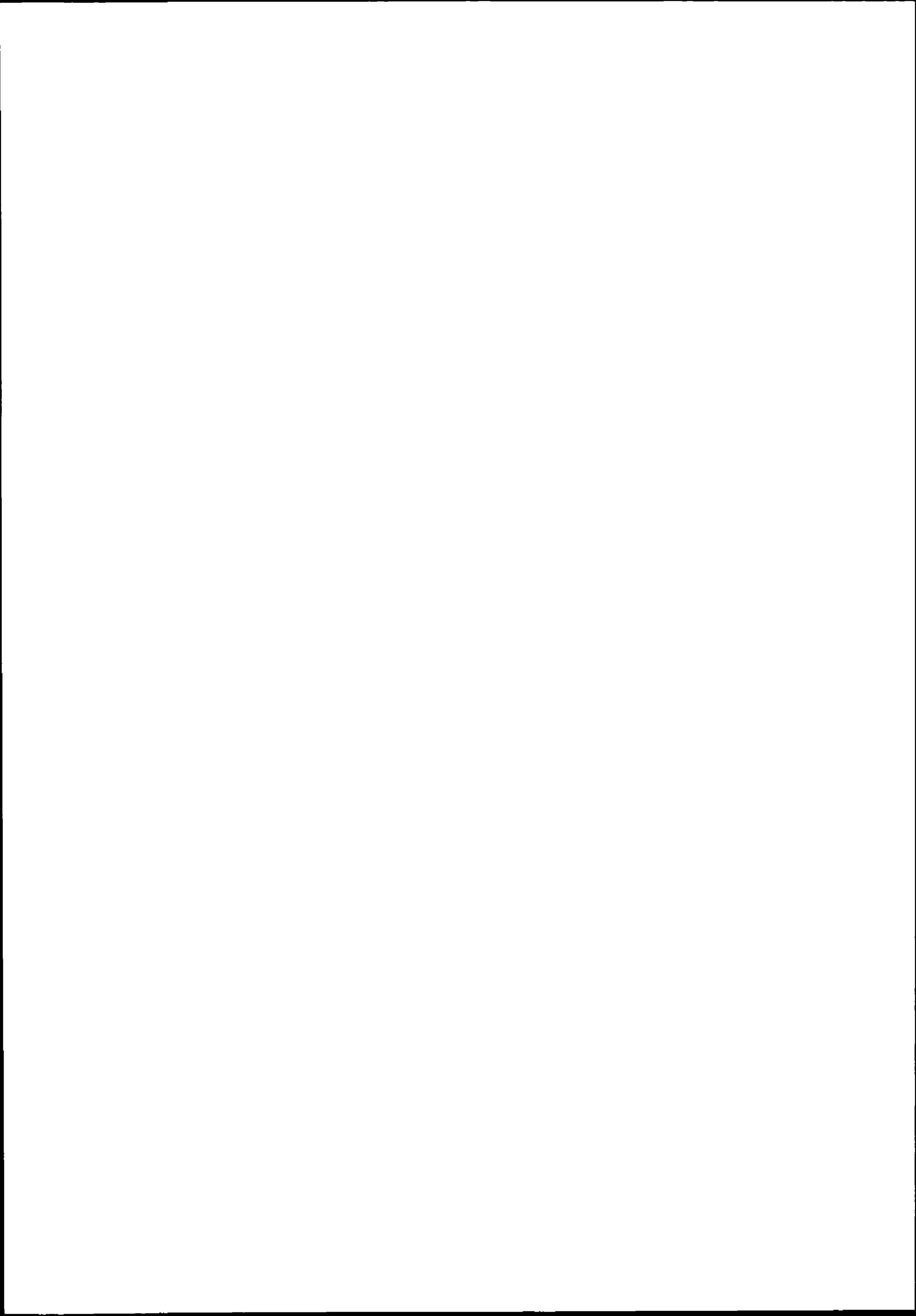
16. Wong WM, Howell WM, Coy SD, Cawley MI, Smith JL. Interleukin-2 is found in the synovium of psoriatic arthritis and spondyloarthritis, not in rheumatoid arthritis. Scand.J Rheumatol 1996;25:239-245

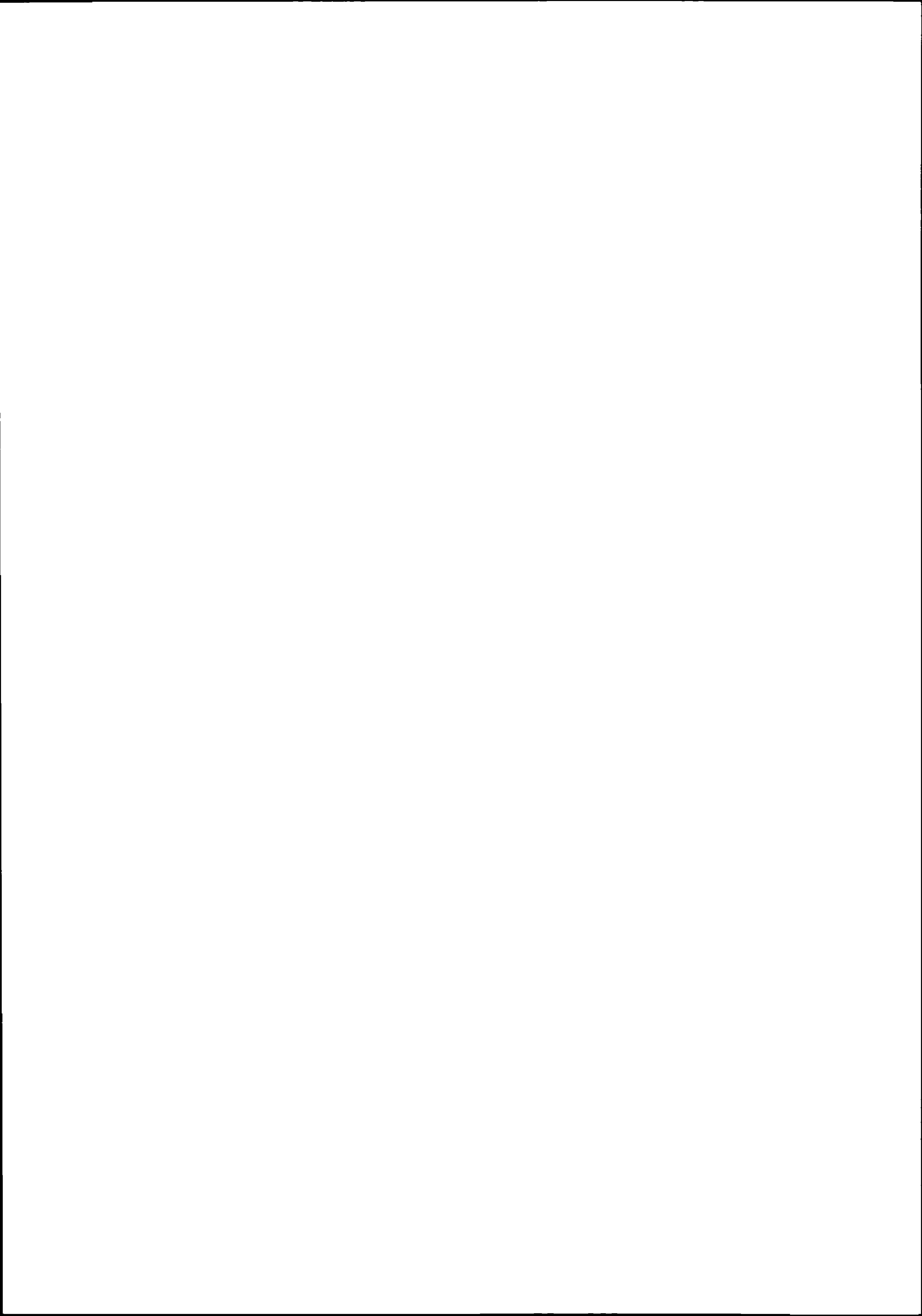
17. Howell WM, Warren CJ, Cook NJ, Cawley MI, Smith JL. Detection of IL-2 at mRNA and protein levels in synovial infiltrates from inflammatory arthropathies using biotinylated oligonucleotide probes in situ. Clin Exp Immunol 1991;86:393-398

18. De Jong R, Brouwer M, Hooibrink B, Van der Pouw-Kraan T, Miedema F, van Lier RA. The CD27- subset of peripheral blood memory CD4+ lymphocytes contains functionally differentiated T lymphocytes that develop by persistent antigenic stimulation in vivo. Eur.J Immunol 1992;22:993-999

19. van Lier RA, Pool MO, Kabel P, et al. Anti-CD27 monoclonal antibodies identify two functionally distinct subpopulations within the CD4+ T cell subset. Eur.J Immunol 1988;18:811-816

20. Appay V, Zaunders JJ, Papagno L, et al. Characterization of CD4(+) CTLs ex vivo. J Immunol 2002;168:5954-5958





Chapter 5

Treatment with monoclonal anti-tumor necrosis factor α antibody results in an accumulation of Th1 CD4^{+} T cells in the peripheral blood of patients with rheumatoid arthritis

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Arthritis & Rheumatism Vol. 42, 10:2166–2173

Chapter 5

TREATMENT WITH MONOCLONAL ANTI–TUMOR NECROSIS FACTOR α ANTIBODY RESULTS IN AN ACCUMULATION OF Th1 CD4+ T CELLS IN THE PERIPHERAL BLOOD OF PATIENTS WITH RHEUMATOID ARTHRITIS

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Objective. **In rheumatoid arthritis (RA), treatment with tumor necrosis factor α (TNFα) binding agents has proven to be highly effective. Down-regulation of the proinflammatory cytokine cascade and a reduced migration of leukocytes into the joints have been proposed as modes of action of TNFα blockade. We investigated whether alterations in the number of circulating pro- and antiinflammatory T cell subsets contribute to the therapeutic effect of monoclonal antibodies (mAb) against TNFα in RA patients.**

Methods. **Phenotypic analysis of peripheral blood T cell subsets was performed on blood from RA patients before and after treatment with an anti-TNFα mAb.**

Results. **An accumulation of primed CD45RA− T cells of both the CD4+ and the CD8+ T cell population was seen shortly after treatment. Most notably, within the CD4+,CD45RA− T cell subset, the number of interferon-γ–producing T cells was significantly increased after anti-TNFα mAb treatment, resulting in a significant rise in the Th1:Th2 ratio. In addition, an increase in the number of CD4+ T cells expressing the homing receptor CD49d in high density was observed after treatment, which correlated positively with the increase in the Th1:Th2 ratio.**

Conclusion. **We show that the Th1:Th2 ratio in the peripheral blood is raised by anti-TNFα mAb treatment.**

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Submitted for publication January 20, 1999; accepted in revised form June 8, 1999.

Rheumatoid arthritis (RA) is associated with a chronic inflammation of peripheral joints and, ultimately, the destruction of joint structures. The pathogenic events that lead to the development of RA have not yet been clarified, but an immunopathogenic component has been strongly implicated. Immunohistologic examinations of RA tissues revealed an infiltration of the joints with high numbers of mononuclear cells and a local, sustained overproduction of proinflammatory cytokines such as interleukin-1 (IL-1), IL-6, IL-8, granulocyte–macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor (TNF) (1). In the search for agents that interrupt the immunologic cascade in RA, the effects of administering monoclonal antibodies (mAb) against cell surface molecules and soluble mediators of inflammation have been studied extensively (2–17). Extremely promising results have been reported from randomized, placebo-controlled, clinical studies in which RA patients were treated with either a chimeric fusion protein of a murine anti-TNFα mAb and a human IgG1-Fc (cA2) (5) or with a recombinant human TNFα receptor (p75)–Fc fusion protein (18).

The rationale for blockade of TNFα was based upon several observations that point to a key role of TNFα in the pathogenesis of RA. High concentrations of TNFα were found in synovial fluid, synovial fluid cells, and synovial tissue specimens of patients with RA (19–21). Moreover, injection of TNFα in animals resulted in a transient synovitis with infiltration of lymphocytes, monocytes, and neutrophils in the joint cavity (hypothetically mediated by TNFα-induced up-regulation of adhesion molecules such as intercellular

adhesion molecule 1, vascular cell adhesion molecule 1 [VCAM-1], and endothelial leukocyte adhesion molecule 1, and by local induction of chemotactic factors such as IL-8) (for review, see ref. 22). Monoclonal antibodies against TNFα were observed to diminish the production of IL-1 and GM-CSF by synovial cells in vitro (23), and to ameliorate synovial hyperplasia and joint destruction in the collagen-induced arthritis model in DBA/1 mice, even after onset of arthritis (24).

The beneficial effect of anti-TNFα mAb administration in RA patients has been attributed to downregulation of cytokine activity and to the reduction of leukocyte trafficking to the joints, the latter being based on observations such as reduced expression of adhesion molecules on synovial endothelium, reduced cellularity of joints, and lymphocytosis in the peripheral blood of RA patients after treatment with anti-TNFα mAb (for review, see ref. 25).

We postulated that alterations in pro- and anti-inflammatory T cell subsets could contribute to the clinical effect of anti-TNFα mAb treatment. Depending upon the set of lymphokines that is secreted, T cells can be divided into discrete effector populations. Human CD4+ T cells that secrete interferon-γ (IFNγ) and TNFβ and are involved in cell-mediated immunity are called Th1 responders, and CD4+ T cells that secrete IL-4 and mediate humoral responses are called Th2 responders (26–28). These polarized sets of lymphokines exert mutual cross-regulatory or inhibitory effects (29). In organ-specific autoimmunity, the activation of proinflammatory Th1 cells and/or the insufficient counterbalance by Th2 cells is believed to be important in the development of disease and to correlate with tissue injury (30,31). In the present study, we analyzed the effects of anti-TNFα mAb on phenotypic and functional characteristics of peripheral blood T cells in RA patients.

PATIENTS AND METHODS

Patients and cells. Seventeen patients with severe RA were recruited from our outpatient clinic (4 men and 13 women, median age 56 years, range 41–74). Patients fulfilled the criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) for the diagnosis of RA (32), had a minimum disease duration of 6 months, a history of unsuccessful treatment with ≥1 disease-modifying antirheumatic drug, and radiographic evidence of erosive disease of hands and feet. Further inclusion and exclusion criteria have been described previously (5).

A human/murine chimeric mAb of IgG1κ isotype (cA2) (Centocor, Malvern, PA) is specific for human TNFα. The construction and characterization of cA2 has previously been described (33). The antibody was supplied as a sterile solution containing 5 mg/ml of cA2 in phosphate buffered saline (PBS) containing 0.01% polysorbate 80 (pH 7.2).

On the day of entry, patients were admitted to the hospital and randomly assigned to 1 of 3 treatment groups (6 patients per group). The first group received a single infusion of placebo (0.1% human serum albumin in the same buffer as described above). The other 2 groups each received 1 infusion of cA2, either 1 mg/kg (low dosage) or 10 mg/kg (high dosage).

At several time points during treatment, starting on day 0, heparinized blood was obtained. Peripheral blood mononuclear cell (PBMC) fractions were isolated from heparinized blood by Ficoll-Hypaque density gradient centrifugation and were cryopreserved immediately. To minimize interassay variability, samples from individual patients from all time points were analyzed in 1 experiment.

Membrane phenotyping. PBMC were washed twice with PBS supplemented with 0.5% bovine serum albumin (BSA) and sodium azide (5 μg/ml). Immunofluorescence staining was performed by incubation of PBMC with saturating amounts of combinations of the following mAb in PBS/BSA: CD4– or CD8–peridin chlorophyll protein (PerCP) (Becton Dickinson, San Jose, CA), CD27–fluorescein isothiocyanate (FITC) (CLB-27/3; Central Laboratory of the Red Cross Blood Transfusion Service [CLB], Amsterdam, The Netherlands), and CD45RA–phycoerythrin (PE) (2H4-RD1; Coulter, Miami, FL). Stained cells were washed twice and 10^4 viable lymphocytes were analyzed using a fluorescence-activated cell sorter (Becton Dickinson). Percentages of positive cells of each subset were calculated. Absolute cell numbers were found by determining the percentage of CD4+ and CD8+ cells within the lymphocyte gate (defined by forward and sideward scatter) and by counting of absolute numbers of lymphocytes.

Flow cytometric measurement of intracellular cytokine production. Measurement of cytokine-producing cells was performed as previously described (34,35). Briefly, 0.5×10^6 cells/ml were stimulated for 4 hours with phorbol myristate acetate (1 ng/ml) and ionomycin (1 μM) in the presence of the protein-secretion inhibitor monensin (1 μM). All subsequent steps were performed at 4°C. After cell surface staining with CD4–PE or CD8–PE combined with CD45RA–FITC (Becton Dickinson), cells were washed twice with PBS and fixated for 5 minutes with PBS/4% paraformaldehyde. Fixation was followed by permeabilization for 10 minutes with PBS/0.1% saponin (Sigma, Zwyndrecht, The Netherlands)/10% human pooled serum. PBS/0.1% saponin/0.5% BSA was used for all subsequent washing and incubation steps. Staining of the cytokines with 5 μg/ml biotinylated anti–IL-4 mAb (Hölzel Diagnostika, Cologne, Germany) or biotinylated anti-IFNγ mAb (MD1; gift from Dr. P. van der Meiden, Biomedical Primate Research Center, Rijswijk, The Netherlands) for 60 minutes was followed by incubation with streptavidin–RED670 (Gibco BRL, Breda, The Netherlands) for 60 minutes. Analysis was performed as described for the measurement of membrane markers.

Determination of adhesion molecules on Th1 and Th2 cells. CD4+ cells (>97% CD3+,CD4+) of 3 healthy donors were obtained by incubating PBMC with saturating amounts of CD8, CD19, CD16, and CD14 mAb (CLB), followed by positive depletion using goat anti-mouse Ig–coupled Dynalbeads (Dynal, Oslo, Norway) as previously described (36).

Table 1. Peripheral blood counts of lymphocytes, CD4+, and CD8+ T cell subsets in rheumatoid arthritis patients before and after treatment with monoclonal anti–tumor necrosis factor α (anti-TNFα) antibody*

	Treatment with anti-TNFα (n = 11)			Treatment with placebo (n = 6)		
	Median	SD	Range	Median	SD	Range
Lymphocytes						
Day 0	1,320	656	820–2,910	1,675	926	810–3,040
Day 3	2,540†	585	1,300–3,000	1,665	972	490–3,200
CD4+						
Day 0	549	315	227–1,425	631	450	188–1,441
Day 3	917†	364	501–1,647	668	315	155–952
CD4+,CD45RA+						
Day 0	176	129	88–499	254	207	70–548
Day 3	296†	229	114–567	234	127	58–357
CD4+,CD45RA−						
Day 0	319	228	96–926	420	263	118–894
Day 3	692†	242	290–1,016	433	192	98–609
CD4+,CD45RA−,CD27−						
Day 0	28	100	6–337	87	109	22–292
Day 3	49†	168	8–602	140	65	18–152
CD8+						
Day 0	243	400	93–1,379	387	453	166–1,377
Day 3	488†	356	137–1,165	310	323	106–1,014
CD8+,CD45RA+,CD27+						
Day 0	55	56	19–202	55	60	21–150
Day 3	85†	105	43–308	40	30	14–82
CD8+,CD45RA+,CD27−						
Day 0	37	179	7–585	46	64	7–585
Day 3	49	156	1–492	57	48	26–149
CD8+,CD45RA−,CD27+						
Day 0	88	62	25–198	110	56.3	44–190
Day 3	205†	94	50–319	104	71.5	30–226
CD8+,CD45RA−,CD27−						
Day 0	20	166	2–565	78	434	16–1,025
Day 3	39	111	3–349	52	299	19–717

* Values are the number of cells × 10^6/liter.
† $P < 0.05$ by Wilcoxon's signed rank test.

After stimulation as described above, cells were stained with antibodies against CD49d, CD29, CD11a, CD11b, CD11c, or CD2, or with negative control and FITC-coupled goat anti-mouse Ig (5 μg/ml) (all purchased from CLB). After blocking with normal mouse serum (1:10), cells were fixed, permeabilized, and stained with anti–IL-4–PE (Becton Dickinson) and biotin-coupled anti-IFNγ (MD-1) (Gibco BRL), and subsequently with streptavidin–RED670 (Gibco BRL).

Statistical analysis. Differences in the numbers of T cell subsets and in the levels of cytokine production before and after therapy were calculated using Wilcoxon's signed rank test. Correlations between increases in the Th1:Th2 ratio and the Disease Activity Score (DAS) or the number of high CD49d–expressing cells were analyzed by Spearman's rank correlation.

RESULTS

Anti-TNFα treatment induces an increase in the number of CD4+, CD45RA− T cells in the peripheral blood. In accordance with previous findings (37,38), treatment with anti-TNFα resulted in an increase in lymphocyte numbers in the peripheral blood of RA patients shortly after infusion (Table 1). Because previous data as well as our own findings showed that the increase in lymphocyte numbers was most pronounced shortly after infusion with anti-TNFα and correlated well with clinical benefit (37, 38), we decided to analyze the alterations in circulating T cell subsets occurring 3 days after infusion with anti-TNFα mAb in RA patients.

The analysis of CD45RA expression on CD4+ T cells (Table 1) revealed a significant increase in the absolute number of both CD45RA+ ($P = 0.018$) and CD45RA− ($P = 0.006$) cells at day 3 after infusion in the group of anti–TNFα-treated patients, but not in the placebo-treated group. The increase in CD45RA− cells was far more pronounced than the increase in CD45RA+ cells, leading to an increase in the percentage of CD45RA− memory cells within the CD4+ T cell population after anti-TNFα mAb treatment. CD45RA− memory CD4+ T cells can be further subdivided into CD27+ and CD27− T cells, of which the latter subset

represents highly differentiated memory T cells that have undergone prolonged antigenic stimulation (39). Despite a significant increase in number ($P = 0.033$) (Table 1), the percentage of CD27− T cells within the CD4+,CD45RA− population did not show significant changes after treatment.

The number of CD8+,CD45RA− T cells is increased after anti-TNFα treatment. Analogous to the behavior of CD4+ T cells, the number of CD45RA− T cells within the CD8+ T cell subset increased significantly after anti-TNFα therapy ($P = 0.003$) (Table 1). As a consequence of the simultaneous increase in both CD4+ and CD8+ memory T cells, the CD4:CD8 ratio was not affected by anti-TNFα therapy (not shown). CD8+ T cells can be subdivided into naive, memory, and effector subsets based upon their CD45RA and CD27 expression pattern (40). As shown in Table 1, the number of both the naive CD45RA+,CD27+ and the memory CD45RA−,CD27+ subset of the CD8+ population were significantly increased after treatment. However, the rise in the number of CD8+,CD45RA−,CD27+ T cells was greater than that of the CD8+,CD45RA+, D27+ T cells. Therefore, when calculated as a percentage of the total CD8+ population, only the CD45RA−,CD27+ memory T cell population was significantly increased ($P = 0.026$).

Increase of the Th1:Th2 ratio of CD4+,CD45RA− T cells in the peripheral blood after anti-TNFα treatment. We next investigated whether anti-TNFα mAb therapy alters the amount of differentiated Th1 and Th2 cells within the peripheral blood of RA patients. Since the secretion of Th1 and Th2 cytokines is largely confined to the CD45RA− subset of CD4+ T cells, the amount of IFNγ- and IL-4–producing T cells was analyzed within this subset. The numbers of both IL-4– and IFNγ-producing CD4+,CD45RA− T cells were significantly increased after anti-TNFα mAb treatment, but not after placebo treatment. However, the rise in IFNγ-producing T cells was more pronounced than the rise in IL-4–producing T cells, leading to a significant increase in the Th1:Th2 ratio in the peripheral blood ($P = 0.007$) (Figure 1).

For CD8+ T cells, the production of IFNγ is not confined to the CD45RA− subset. Therefore, the numbers of both IFNγ- and IL-4–positive cells were calculated for the total CD8+ T cell population. In contrast to the findings for CD4+ T cells, the numbers of both IFNγ- and IL-4–positive cells, as well as their ratio, were not significantly altered within the CD8+ subset after treatment with anti-TNFα mAb (Table 2).

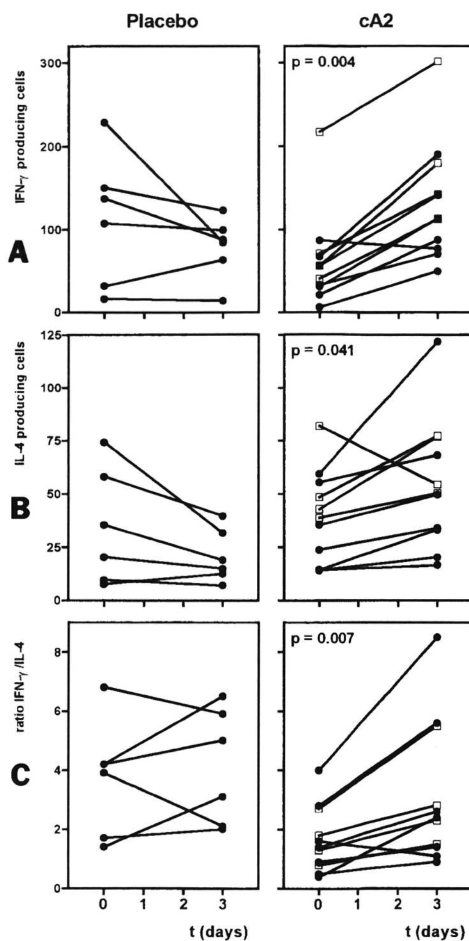


Figure 1. Effects of anti–tumor necrosis factor α (anti-TNFα) treatment on the number of interferon-γ (IFNγ)– and interleukin-4 (IL-4)–producing cells. CD4+ T cells (×10^6/liter) producing the Th1 cytokine IFN-γ were significantly increased in number on day 3 after anti-TNFα treatment (**A**). IL-4–producing CD4+ T cells increased in number more slowly (**B**), leading to a significantly increased IFN-γ/IL-4 ratio (**C**). Open squares represent patients who received 10 mg/kg of chimeric anti-TNFα monoclonal antibody (cA2). Closed circles represent patients who received 1 mg/kg of cA2.

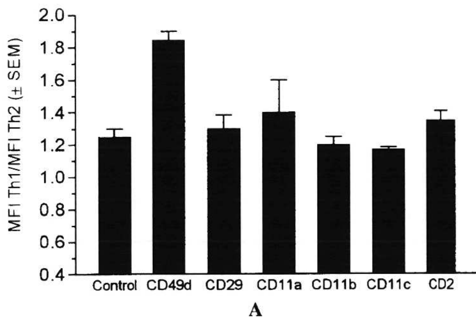
High CD49d–expressing CD4+,CD45RA− T cell numbers are increased after anti-TNFα mAb treatment. The selective accumulation of Th1-like cells in the

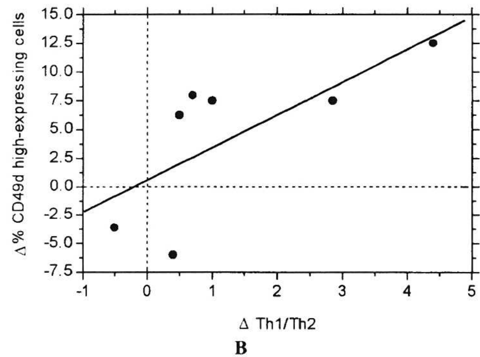
Table 2. Peripheral blood counts of interleukin-4 (IL-4)– and interferon-γ (IFNγ)–producing CD8+,CD45RA− T cells in rheumatoid arthritis patients before and after treatment with monoclonal anti–tumor necrosis factor α (anti-TNFα) antibody*

	Treatment with anti-TNFα (n = 11)			Treatment with placebo (n = 6)		
	Median	SD	Range	Median	SD	Range
IL-4+,CD8+						
Day 0	16	23	0–74	22	22	9–61
Day 3	12	37	0–106	22	16	0–38
IFNγ+,CD8+						
Day 0	83	309	11–1,003	119	336	39–916
Day 3	109	206	15–617	69	126	36–363

* Values are the number of cells × 10^6/liter.

peripheral blood could be explained by a therapy-induced inhibition of homing of those cells to the inflamed tissues. This assumption implies a different homing pattern of Th1 and Th2 cells, which could be reflected in a difference in the expression of adhesion molecules. An important pathway in the migration of T cells to inflamed peripheral tissues involves very late activation antigen 4 ($\alpha4\beta1$)/VCAM-1 (41), and VCAM-1 expression in the synovia of RA patients is reduced after anti-TNFα therapy (42).





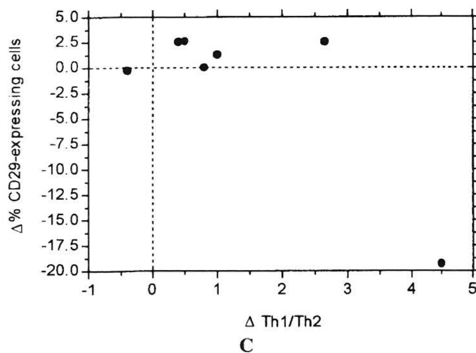


Figure 2. Comparison of the expression of adhesion molecules on IFNγ-producing Th1 and IL-4–producing Th2 CD4+ T cells from healthy individuals. The mean fluorescence intensity (MFI) for the different adhesion molecules was determined for IFNγ+,IL-4− (Th1) and IFNγ−,IL-4+ (Th2) cells by flow cytometry. The difference in antigen density between Th1 and Th2 cells is expressed as the MFI Th1/MFI Th2 ratio (mean and SEM of 3 donors) (**A**). After anti-TNFα treatment, the percentage of CD49d high-expressing CD4+ T cells in the peripheral blood of rheumatoid arthritis patients was increased and correlated significantly with the increase in the Th1/Th2 ratio (**B**). The alteration in the percentage of CD29-expressing CD4+ T cells did not correlate with the alteration in the Th1/Th2 ratio (**C**). See Figure 1 for other definitions.

To test whether CD49d (α4 integrin) is important in the homing of Th1 T cells to the synovium, we first analyzed the expression levels of CD49d and its associated β_1 chain, CD29, on the cell surface of Th1 and Th2 CD4+ T cells isolated from healthy individuals. In contrast to CD29 and the other adhesion markers that were analyzed, CD49d was expressed in markedly higher density on Th1 cells when compared with Th2 cells (Figure 2A). Next, we analyzed the expression levels of CD49d and CD29 on CD4+,CD45RA− T cells in 6 RA patients before and after treatment with anti-TNFα. In contrast to CD29 (Figure 2C), the percentage of high CD49d–expressing cells was increased after treatment and, moreover, correlated significantly with the alterations in the Th1:Th2 ratio ($P = 0.04$) (Figure 2B).

DISCUSSION

The present study analyzed the effects of anti-TNFα mAb treatment on the phenotypic and functional characteristics of T cell subsets in the peripheral blood of RA patients. A significant increase in the number of CD45RA− memory T cells of both the CD4+ and the CD8+ T cell population was observed shortly after anti-TNFα therapy, with a concomitant rise in the Th1:Th2 ratio of the CD4+,CD45RA− T cell population, but not of the CD8+ T cell population. These findings indicate that the increase in peripheral blood T cell numbers after anti-TNFα mAb therapy is mainly due to an increase in CD45RA− memory T cells, which, with respect to the CD4+ T cell subset, have a Th1-like phenotype.

The expression of adhesion molecules on the synovial endothelium and the density of synovial infiltration of inflammatory cells were both found to be reduced after anti-TNFα mAb treatment of RA patients (42). It has been proposed that the diminished activation of endothelial cells in the synovial microvasculature leads to a decreased migration of leukocytes to the joints. In accordance with this hypothesis, the rapid increase of lymphocyte counts in the peripheral blood of treated patients correlated with clinical benefit (37,38).

In the present study, we refined the model of the mode of action of anti-TNFα therapy by demonstrating that the increase in lymphocyte numbers is greater for Th1 cells than for Th2 cells. The data therefore are consistent with the assumption that CD4+,CD45RA− Th1-like T cells preferentially migrate to the inflamed tissue in the joints of RA patients. In accordance with this, T cells isolated from synovium or synovial fluid of RA patients were observed to predominantly express Th1 cytokines (43,44), and the selective homing of Th1 cells, and not Th2 cells, into inflamed joints of mice was recently demonstrated (45). Moreover, the present data confirm the finding of an inverse relationship between serum TNFα levels and the ratio of IFNγ:IL-4 production of peripheral blood T cells (46). The finding of diminished migration of Th1-like T cells into the inflamed tissues after anti-TNFα treatment is supported by the observed increase in the proportion of high CD49d–expressing CD4+,CD45RA− T cells. In accordance with the finding that Th1 cells express a markedly higher density of CD49d on the cell surface when compared with Th2 cells, the increase in the number of high CD49d–expressing CD4+ T cells in anti-TNFα–treated patients correlated with the increase in the Th1:Th2 ratio. The rapid down-regulation of VCAM-1 in the synovium (42) could therefore be an important factor for inhibiting Th1 cells from migrating into the synovium.

Within the CD4+,CD45RA− T cell population, cells that have lost the CD27 molecule from the cell surface represent highly differentiated memory T cells that can secrete considerable amounts of either IFNγ or IL-4 upon stimulation (39). No alteration in the percentage of CD27− T cells within the CD4+,CD45RA− population was observed. It could therefore very well be that both CD27+ and CD27− memory CD4+ T cells migrate equally well to the peripheral inflamed tissues. The described increase in CD27−,CD4+ T cells in the cellular infiltrates in the synovium (47,48) could therefore be explained by a postmigratory loss of the CD27 molecule from the cell surface, as previously proposed (47).

In a similar way of reasoning, one could hypothesize that the CD45RA−,CD27+ subset of the CD8+ T cell population, which is also increased in both number and percentage in the peripheral blood after anti-TNFα mAb therapy, is inhibited from migrating to the inflamed tissues. Accordingly, an enrichment of CD27+,CD8+ T cells in the RA synovium and synovial fluid has been demonstrated (47). The recent analysis of the phenotypic and functional properties of phenotypically separated CD8+ T cell subsets revealed that this CD8+,CD45RA−,CD27+ subset in healthy individuals consists of memory-type cells, which produce a wide range of cytokines and can provide helper activity for B cell differentiation (40). Thus, this CD8+ T cell subset, in addition to the previously proposed CD4+,CD45RA−,CD27+ T cell subset (47), might contribute to the B cell activation and subsequent immunoglobulin production as observed in the RA synovium. However, no increase in the IFNγ:IL-4 ratio was observed for CD8+ T cells, indicating that no joint-specific CD8+ T

cell subset could be identified on the basis of IL-4 and IFNγ production.

Selective inhibition of migration of CD45RA− T cells could contribute to the therapeutic efficacy of anti-TNFα. Patients with higher increases in the Th1: Th2 ratio after therapy tended to show higher increases in the number of circulating lymphocytes and stronger decreases in DAS (data not shown), but this did not reach statistical significance.

Previously, improved T cell mitogen– and recall antigen–induced proliferative responses of PBMC were reported to occur after anti-TNFα treatment of RA patients, which was suggested to be caused by a restoration of T cell function after removal of TNFα (49). In view of our findings, the increased responsiveness could be explained by an increase in the proportion of the memory T cell subset, which is likely to contain an increased frequency of recall-reactive T cells (50–53). Responses to recall antigens (tetanus toxoid, purified protein derivative, streptokinase–streptodornase) analyzed from anti-TNFα mAb–treated RA patients are known to be mainly mediated by Th1-like T cells (54–56), which are also found to be increased in number after anti-TNFα treatment.

In conclusion, the present findings show that after anti-TNFα treatment, the increase in lymphocyte numbers in the peripheral blood is greater for Th1 cells than for Th2 cells. Down-regulation of adhesion molecules on the synovial endothelium, selectively inhibiting the homing of Th1-like, memory CD4+ T cells to inflamed joints, might explain this observation.

ACKNOWLEDGMENT

We gratefully acknowledge Centocor, Malvern, PA, for cooperation.

REFERENCES

1. Feldmann M, Brennan FM, Chantry D, Haworth C, Turner M, Abney E, et al. Cytokine production in the rheumatoid joint: implications for treatment. Ann Rheum Dis 1990;49:480–6.
2. Van der Lubbe PA, Dijkmans BAC, Markusse HM, Nässander U, Breedveld FC. A randomized, double-blind, placebo-controlled study of CD4 monoclonal antibody therapy in early rheumatoid arthritis. Arthritis Rheum 1995;38:1097–106.
3. Moreland LW, Sewell KL, Trentham DE, Bucy RP, Sullivan WF, Schrohenloher RE, et al. Interleukin-2 diphtheria fusion protein (DAB_{486}IL-2) in refractory rheumatoid arthritis: a double-blind, placebo-controlled trial with open-label extension. Arthritis Rheum 1995;38:1177–86.
4. Kirkham BW, Thien F, Pelton BK, Pitzalis C, Amlot P, Denman AM, et al. Chimeric CD7 monoclonal antibody therapy in rheumatoid arthritis. J Rheumatol 1992;19:1348–52.
5. Elliott MJ, Maini RN, Feldmann M, Kalden JR, Antoni C, Smolen JS, et al. Randomized double-blind comparison of chimeric monoclonal antibody to tumour necrosis factor a (cA2) versus placebo in rheumatoid arthritis. Lancet 1994;344:1105–10.
6. Moreland LW, Bucy RP, Tilden A, Pratt PW, LoBuglio AF, Khazaeli M, et al. Use of a chimeric monoclonal anti-CD4 antibody in patients with refractory rheumatoid arthritis. Arthritis Rheum 1993;36:307–18.
7. Horneff G, Burmester GR, Emmerich F, Kalden JR. Treatment of rheumatoid arthritis with an anti-CD4 monoclonal antibody. Arthritis Rheum 1991;34:129–40.
8. Reiter C, Kakavand B, Rieber EP, Schattenkirchner M, Riethmüller G, Krüger K. Treatment of rheumatoid arthritis with monoclonal CD4 antibody M-T151: clinical results and immunopharmacologic effects in an open study, including repeated administration. Arthritis Rheum 1991;34:525–36.
9. Didry C, Portalès P, Andary M, Brochier J, Combe B, Clot J, et al. Treatment of rheumatoid arthritis with monoclonal anti-CD4 antibodies: clinical results [abstract]. Arthritis Rheum 1991;34 Suppl 9:S92.
10. Strand V, Lipsky PE, Cannon GW, Calabrese LH, Wiesenhutter C, Cohen SB, et al. Effects of administration of an anti-CD5 plus immunoconjugate in rheumatoid arthritis: results of two phase II studies. Arthritis Rheum 1993;36:620–30.
11. Kremer JM, Petrillo GF, Rigby WFC, Plehn SJ, Woodworth TG, Parker KC, et al. Phase I/II, open-label trial of DAB_{389}IL-2 administered to patients with active rheumatoid arthritis receiving treatment with methotrexate [abstract]. Arthritis Rheum 1994;37 Suppl 9:S341.
12. Isaacs JD, Watts RA, Hazleman BL, Hale G, Keogan MT, Cobbold SP, et al. Humanised monoclonal antibody therapy for rheumatoid arthritis. Lancet 1992;340:748–52.
13. Kavanaugh AF, Davis LS, Nichols LA, Norris SH, Rothlein R, Scharschmidt LA, et al. Treatment of refractory rheumatoid arthritis with a monoclonal antibody to intercellular adhesion molecule 1. Arthritis Rheum 1994;37:992–9.
14. Wendling D, Radacot E, Wijdenes J. Treatment of severe rheumatoid arthritis by anti-interleukin 6 monoclonal antibody. J Rheumatol 1993;20:259–62.
15. Elliott MJ, Maini RN, Feldmann M, Long-Fox A, Charles P, Katsikis P, et al. Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumor necrosis factor α. Arthritis Rheum 1993;36:1681–90.
16. Moreland LW, Margolies GR, Heck LW, Saway PA, Jacobs C, Beck C, et al. Soluble tumor necrosis factor receptor: results of a phase I dose-escalation study in patients with rheumatoid arthritis [abstract]. Arthritis Rheum 1994;37 Suppl 9:S295.
17. Drevlow B, Lovis R, Haag MA, Sinacore J, Jacobs C, Blosch C, et al. Phase I study of recombinant human interleukin-1 receptor administered subcutaneously in patients with active rheumatoid arthritis [abstract]. Arthritis Rheum 1994;37 Suppl 9:S339.
18. Moreland LW, Baumgartner SW, Schiff MH, Tindall EA, Fleischmann RM, Weaver AL, et al. Treatment of rheumatoid arthritis with a recombinant human tumor necrosis factor receptor (p75)-Fc fusion protein. N Engl J Med 1997;337:141–7.
19. Buchan G, Barrett K, Turner M, Chantry D, Maini RN, Feldmann M. Interleukin-1 and tumor necrosis factor mRNA expression in rheumatoid arthritis: prolonged production of IL-1α. Clin Exp Immunol 1988;73:449–55.
20. Firestein GS, Alvaro-Gracia JM, Maki R. Quantitative analysis of cytokine gene expression in rheumatoid arthritis. J Immunol 1990;144:3347–53.
21. Saxne T, Palladino MA Jr, Heinegård D, Talal N, Wollheim FA. Detection of tumor necrosis factor α but not tumor necrosis factor β in rheumatoid arthritis synovial fluid and serum. Arthritis Rheum 1988;31:1041–5.
22. Brennan FM, Maini RN, Feldmann M. Cytokine expression in chronic inflammatory disease. Br Med Bull 1995;51:368–84.

23. Brennan FM, Chantry D, Jackson A, Maini RN, Feldmann M. Inhibitory effect of TNF-α antibodies on synovial cell interleukin-1 production in rheumatoid arthritis. Lancet 1989;334:244–7.
24. Williams RO, Feldmann M, Maini RN. Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. Proc Natl Acad Sci U S A 1992;89:9784–8.
25. Feldmann M. What is the mechanism of action of anti-tumour necrosis factor-α antibody in rheumatoid arthritis? Int Arch Allergy Immunol 1996;111:362–5.
26. Bottomly K. A functional dichotomy in CD4+ T lymphocytes. Immunol Today 1988;9:268–74.
27. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989;7:145–73.
28. Romagnani S. Human TH1 and TH2 subsets: doubt no more. Immunol Today 1991;12:256–7.
29. Seder RA, Paul WE. Acquisition of lymphokine-producing phenotype by CD4+ T cells. Annu Rev Immunol 1994;12:635–73.
30. Charlton B, Lafferty KJ. The Th1/Th2 balance in autoimmunity. Curr Opin Immunol 1995;7:793–8.
31. Druet P, Sheela R, Pelletier L. Th1 and Th2 cells in autoimmunity. Clin Exp Immunol 1995;101 Suppl 1:9–12.
32. 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;31:315–24.
33. Knight DM, Trinh H, Le J. Construction and initial characterization of a mouse-human chimaeric anti-TNF antibody. Mol Immunol 1993;30:1443–53.
34. Jung T, Schauer U, Heusser C, Neuman C, Rieger C. Detection of intracellular cytokines by flow cytometry. J Immunol Methods 1993;159:197–207.
35. Hamann D, Baars PA, Hooibrink B, van Lier RAW. Heterogeneity of the human CD4+ T-cell population: two distinct CD4+ T-cell subsets characterized by coexpression of CD45RA and CD45RO isoforms. Blood 1996;9:3513–21.
36. De Jong R, Brouwer M, Miedema F, van Lier RAW. Human $CD8^+$ T lymphocytes can be divided into CD45RA+ and CD45RO+ cells with different requirements for activation and differentiation. J Immunol 1991;146:2088–94.
37. Paleolog EM, Hunt M, Elliott MJ, Feldmann M, Maini RN, Woody JN. Deactivation of vascular endothelium by monoclonal anti-tumor necrosis factor α antibody in rheumatoid arthritis. Arthritis Rheum 1996;39:1082–91.
38. Lorenz H-M, Antoni C, Valerius T, Repp R, Grünke M, Schwerdtner N, et al. In vivo blockade of TNF-α by intravenous infusion of a chimeric monoclonal TNF-α antibody in patients with rheumatoid arthritis: short term cellular and molecular effects. J Immunol 1996;156:1646–53.
39. De Jong R, Brouwer M, Hooibrink B, van der Pouw-Kraan T, Miedema F, van Lier RAW. The $CD27^-$ subset of peripheral blood memory CD4+ lymphocytes contains functionally differentiated T lymphocytes that develop by persistent antigenic stimulation in vivo. Eur J Immunol 1992;22:993–9.
40. Hamann D, Baars PA, Rep MHG, Hooibrink B, Kerkhof-Garde S, Klein MR, et al. Phenotypic and functional separation of memory and effector human $CD8^+$ T cells. J Exp Med 1997;186:1407–18.
41. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 1994;76:301–14.
42. Tak PP, Taylor PC, Breedveld FC, Smeets TJM, Daha MR, Kluin PM, et al. Decrease in cellularity and expression of adhesion molecules by anti-tumor necrosis factor α monoclonal antibody treatment in patients with rheumatoid arthritis. Arthritis Rheum 1996;39:1077–81.
43. Simon AK, Seipelt E, Sieper J. Divergent T-cell cytokine patterns in inflammatory arthritis. Proc Natl Acad Sci U S A 1994;91:8562–6.
44. Dolhain RJEM, van der Heiden AN, ter Haar NT, Breedveld FC, Miltenburg AMM. Shift toward T lymphocytes with a T helper 1 cytokine-secretion profile in the joints of patients with rheumatoid arthritis. Arthritis Rheum 1996;39:1961–9.
45. Austrup F, Vestweber D, Borges E, Löhning M, Bräuer R, Herz U, et al. P- and E-selectin mediate recruitment of T-helper-1 but not T-helper-2 cells into inflamed tissues. Nature 1997;385:81–3.
46. Van Roon JAG, Verhoef CM, van Roy JLAM, Gmelig-Meyling FHJ, Huber-Bruning O, Lafeber FPJG, et al. Decrease in peripheral type 1 over type 2 T cell cytokine production in patients with rheumatoid arthritis correlates with an increase in severity of disease. Ann Rheum Dis 1997;56:656–60.
47. Tak PP, Hintzen RQ, Teunissen JJ, Smeets TJ, Daha MR, van Lier RA, et al. Expression of the activation antigen CD27 in rheumatoid arthritis. Clin Immunol Immunopathol 1996;80:129–38.
48. Kohem CL, Brezinschek RI, Wisbey H, Tortorella C, Lipsky PE, Oppenheimer-Marks N. Enrichment of differentiated $CD45RB^{dim}$, CD27− memory T cells in the peripheral blood, synovial fluid, and synovial tissue of patients with rheumatoid arthritis. Arthritis Rheum 1996;39:844–54.
49. Cope AP, Londei M, Chu NR, Cohen SBA, Elliott MJ, Brennan FM, et al. Chronic exposure to tumor necrosis factor (TNF) in vitro impairs the activation of T cells through the T cell receptor/CD3 complex; reversal in vivo by anti-TNF antibodies in patients with rheumatoid arthritis. J Clin Invest 1994;94:749–60.
50. Sanders ME, Makgoba MW, Sharrow SO, Stephany D, Springer TA, Young HA, et al. Human memory T cells express increased levels of three adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL-1, CDw29, and Pgp-1) and have enhanced IFN-γ production. J Immunol 1988;140:1401–7.
51. Tedder TF, Clement LT, Cooper MD. Human lymphocyte differentiation antigens HB-10 and HB-11. II. Differential production of B-cell growth and differentiation factors by distinct helper T cell subpopulations. J Immunol 1985;134:2983–8.
52. Damle NK, Childs AL, Doyle LV. Immunoregulatory T lymphocytes in man. J Immunol 1987;139:1501–8.
53. Smith SH, Brown MH, Rowe D, Callard RE, Beverley PC. Functional subsets of human helper-inducer cells defined by a new monoclonal antibody. Immunology 1986;58:63–70.
54. McHugh SM, Deighton J, Stewart AG, Lachmann PJ, Ewan PW. Bee venom immunotherapy induces a shift in cytokine responses from a TH-2 to a TH-1 dominant pattern: comparison of rush and conventional immunotherapy. Clin Exp Allergy 1995;25:828–38.
55. Shirakawa T, Enomoto T, Shimazu S, Hopkin JM. The inverse association between tuberculin responses and atopic disorder. Science 1997;275:77–9.
56. Sabin EA, Araujo MI, Carvalho EM, Pearce EJ. Impairment of tetanus toxoid-specific Th1-like immune responses in humans infected with Schistosoma mansoni. J Infect Dis 1996;173:269–72.

Chapter 6

T cell modifying effects of disease modifying anti-rheumatic drugs in arthritis patients

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Abstract

OBJECTIVE: Disease modifying anti-rheumatic drugs (DMARDs) are widely used in the treatment of arthritis. Although DMARDs have been reported to suppress T cell function *in vitro* their mode of action *in vivo* is still unclear. Synergy between individual drugs does however suggest the existence of several mechanisms. To examine whether DMARDs have comparable effects on T cells *in vivo*, we analyzed the phenotype and function of peripheral blood T cells in patients with early arthritis treated with hydroxychloroquine (HCQ), sulfasalazine (SASP) or methotrexate (MTX).

METHODS: The following parameters were evaluated in peripheral blood T cells: 1. Differentiation state as determined by expression of CD45RA, CD27, Beta-1-, Beta-2 integrins, CCR5, CXCR-3 and CCR3. 2. Cytokine profile as determined by intracellular staining for IFN-γ and IL-4 with flowcytometric analyses and 3. Proliferative capacity was determined by thymidine incorporation after CD2 and CD28 stimulation in vitro. A comparison was made just before and 3 months after the start of a new DMARD.

RESULTS: After treatment for three months all groups improved clinically, reflected by a significant decrease in the disease activity score. In SASP treated patients the percentages of IFN-γ positive CD4+ and CD8+ cells decreased significantly in contrast to IL-4 positive cells that were only affected in CD4+ cells. No significant changes in T cell phenotype as determined by the expression of the differentiation markers CD45RA and CD27 were observed during this treatment. In addition a concomitant reduction in the adhesion molecules CD29, CD49d, CD49e and CD18 was observed on T cells in SASP treated patients. We could not find any cytokine reducing effect in HCQ or MTX treated patients nor was there any difference in expression of adhesion molecules on T cells. MTX treated patients showed a reduction of the chemokine receptor CCR5 while HCQ treated patients showed reduced T cell proliferative responses to the combination anti-CD2 and anti-CD28 antibodies.

CONCLUSION: These results indicate that in vivo DMARDs have distinct T cell modulating effects, which could be an explanation for their synergistic effect in the treatment of arthritis.

Introduction

The use of disease modifying anti-rheumatic drugs (DMARDs) is essential in the treatment of chronic arthritis. Despite DMARDs can diminish the amount of inflammation in the joints, their effect is only partial. In rheumatoid arthritis (RA) the combination of DMARDs is more effective than monotherapy[1-5]. Since there is still uncertainty about both the key mechanisms in the perpetuation of arthritis as well as the major mode of action of DMARDs in vivo, the background for synergy of DMARDs is unclear.
T lymphocytes are a major component of the synovial infiltrate in many inflammatory joint diseases[6]. The contribution of these cells to the inflammatory process can be assumed on the observation that they are highly enriched for the proinflammatory type-1 (IFN-γ

producing) cells and lack the anti-inflammatory type-2 (IL-4 producing) subset[7-14]. The anti- inflammatory effect of DMARDs could be ascribed in part by affecting T cells, since specific effects like inhibition of T cell proliferation and cytokine production by T cells have been documented in vitro[15-22]. Additional mechanisms to interfere with T cells could be modulating their expression of molecules used for extravasation and adherence to the synovium (homing). This is a multistep procedure in which selectins, integrins and chemokine receptors are involved. Little research has been performed on the effect of DMARDs on any of those participating molecules expressed by T cells.
Special attention has been made to the chemokine receptors CXCR3 and CCR5. They seem to play an important role in lymphocyte homing to inflamed tissue since the majority of the T cells in the synovial compartment express both receptors[23-25].
In contrast to the anti-inflammatory type-2 T cells, type-1 cells express high levels of integrins[26,27] and both CXCR3 and CCR5, which could contribute to the overrepresentation of this celltype in the synovial compartment. Whether DMARDs can affect the expression of any of those specific proinflammatory homing receptors is still not known.
The aim of this study is to investigate whether the various types of DMARDs can affect the phenotype and function of T cells in arthritis patients.

Methods

Patients studied:
Patients were recruited from the outpatient clinic for early arthritis of the Jan van Breemen Institute, Amsterdam. Diagnoses after one year of follow-up consisted of rheumatoid arthritis, undifferentiated arthritis and psoriatic arthritis. The diagnosis rheumatoid arthritis was made according to the ACR criteria[28]. All patients diagnosed with undifferentiated arthritis had oligo- or polyarthritis. To study the in-vivo effects of DMARDs blood was obtained at the start of treatment and after 3 months of oral monotherapy with hydroxychloroquine (200mg/day), sulphasalazine (2000mg/day) or methotrexate (7.5 mg/week).
Peripheral blood mononuclear cells (PBMC) were isolated with Ficoll-hypopaque and stored in liquid nitrogen until use.

Antibodies:
Monoclonal antibodies (mab's) directed against the following antigens were used in this study:
CLB, Amsterdam: CD27-FITC (3A12), CD18, CD11a (Tb-133), CD2 (2H4, Hik-27,) CD28 (15E8); *Becton Dickinson:* CD8-PerCP,IFN-γ-FITC, IL-4-PE; *Caltag:* CD4-APC; *Immunotech:* CCR5-PE; *R&D systems:* CXCR3

T cell proliferation
Whole blood of arthritis patients was diluted 1:10 with Iscove's modified Dulbecco's Medium Monoclonal antibodies directed against CD2 and CD28 were added for three days. Proliferation was measured by incorporation of tritium labeled thymidin and expressed as counts per minute (cpm).

T cell phenotype
Four color flowcytometry was performed on 0.2 million thawed PBMC stained with directly labeled monoclonal antibodies (mab's) against CD4 (APC) and CD8 (PerCP) simultaneously with directly labeled (FITC, Phycoerythrin) or indirectly (goat-anti-mouse phycoerythrin) mab's directed against differentiation markers, adhesion molecules and chemokine receptors.
Data were analyzed for CD4+ and CD8+ cells separately and expressed as percentages positive cells or mean fluorescence intensity (MFI).

Intracellular staining technique:
0.5 million PBMC were stimulated with 1 ng phorbol mystirate acetate (PMA) (Sigma chemicals) and 1μM Ionomycin (Sigma chemicals) during 4 hours in the presence of 1 μM Monensin (Sigma chemicals). After incubation cells were stained with PerCP-anti CD8 and APC-anti CD4 mAb and subsequently fixed with 4% paraformaldehyde. Permeabilisation of the cells was done with 0.1% saponin for 45 minutes. Intracellular staining was performed with either IFN-γ-FITC and IL-4-PE or IL-2 FITC and IL-13-PE. Stained cells were analyzed in a FacsCalibur (Becton Dickinson) and expressed as percentages cytokine positive CD4+ and CD8+ cells.

Results

Patients:
43 arthritis patients were included, clinical characteristics are presented in table 1. Diagnoses did not change during the 3 months of follow-up. Patients using hydroxychloroquine or sulphasalazine had never used DMARDs before while methotrexate was used by patients not responding to sulphasalazine or hydroxychloroquine. Only patients were chosen that did not use DMARDs for 3 months during the start of methotrexate treatment.

Table 1. Clinical characteristics arthritis patients studied

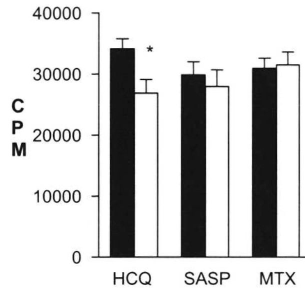
Treatment group	Hydroxy-chloroquine	Sulphasalazine	Methotrexate
Mean DAS* at start	5.3	5.2	5.2
Mean DAS at 3 months of therapy	4.8	4.6	4.7
Total number of patients	13	17	13
RA	8	12	11
Psoriatic arthritis	3	2	0
Undifferentiated arthritis	2	3	2

* DAS =Disease activity score

T cell proliferation

Results are shown in Figure 1. Three months of treatment with hydroxychloroquine resulted in significant lower proliferative responses to anti-CD2 and anti-CD28 antibodies in contrast to treatment with sulfasalazine and methotrexate. These results show that although each DMARD is reported to diminish proliferative T cell responses in vitro, this can only be shown for hydroxychloroquine in vivo.

Figure 1.
Proliferative response in whole blood of arthritis patients after stimulation with anti-CD2 and anti-CD28 antibodies. Shown are counts per minute (CPM) before (closed bars) and after 3 months of DMARD treatment (open bars). Given are means and SEM, *: $p<0.05$



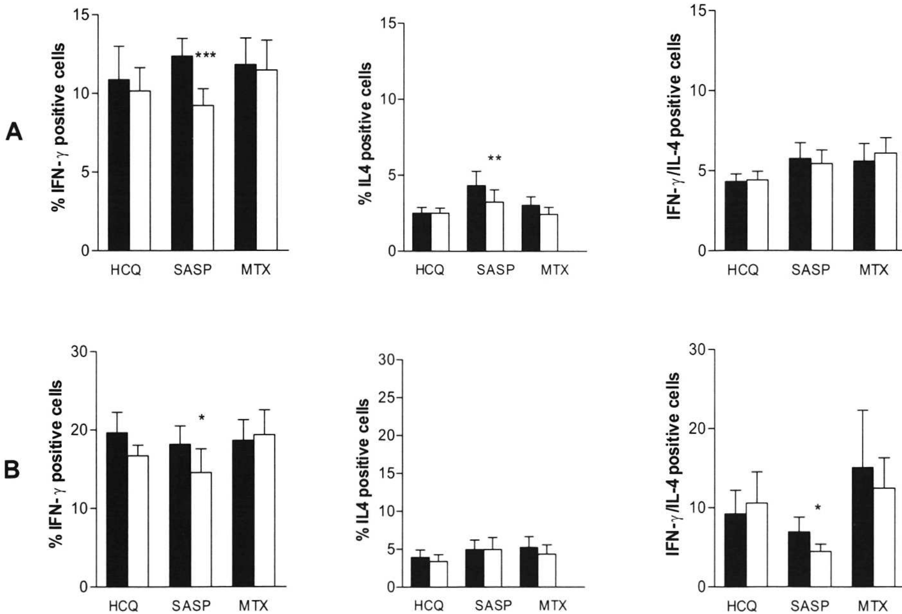


Figure 2.
Percentage of IFN-γ and IL-4 producing cells and their ratio within the CD4+ (A) and CD8+ (B) peripheral blood cells of arthritis patients, before (closed) and after 3 months of DMARD treatment (open). Given are means and SEM
*: $p<0.05$ **: $p<0.01$ ***: $p<0.001$

Cytokine producing cells

All three DMARDs investigated have been shown to influence the type-1/type-2 T cell balance by decreasing the production of IFN-γ in vitro[18,21,22]. To investigate whether this effect could be observed in vivo as well percentages of IFN-γ and IL-4 producing cells were determined in the peripheral blood of arthritis patients before and three months after DMARD treatment (figure 2). In patients treated with hydroxychloroquine or methotrexate no alterations were seen in percentages IFN-γ+ cells, IL-4+ cells or their ratio. However, a significant decrease in IFN-γ positive cells could be observed during sulfasalazine both in CD4+ and CD8+ T cells. The ratio between type-1 and type-2 cells was only significantly changed in CD8+ cells since within the CD4+ subset the percentage IL-4+ producing cells was decreased as well.

Expression of differentiation markers

Since changes in cytokine profiles during sulfasalazine could be a reflection of altered differentiation state of the T cells, flowcytometric analysis for the expression of differentiation markers CD45RA and CD27 was performed. As shown in Table 2, subsets defined by these markers were not changed during treatment suggesting a direct cytokine suppressive effect of sulfasalazine.

Table 2. Percentages of different subsets of CD4+ and CD8+ T cells before and after 3 months treatment with sulfasalazine

	Start		*3 months*		
	Mean	*SEM*	Mean	*SEM*	p*
CD4+					
CD45RA+CD27+	43.6	*4.4*	45.0	*5.0*	0.333
CD45RA-CD27+	42.6	*3.5*	42.2	*3.7*	0.969
CD45RA-CD27-	10.6	*2.4*	9.4	*2.0*	0.252
CD45RA+CD27-	3.3	*0.8*	3.3	*0.9*	0.359
CD8+					
CD45RA+CD27+	41.7	*4.8*	42.1	*4.9*	0.600
CD45RA-CD27+	20.0	*3.5*	18.8	*3.7*	0.330
CD45RA-CD27-	5.2	*0.8*	5.0	*1.0*	0.426
CD45RA+CD27-	33.1	*6.1*	34.1	*24.1*	0.804

* p= Wilcoxon signed rank test

Effect of DMARDs on the expression of adhesion molecules and chemokine receptors
The homing of leukocytes to the synovium is an essential step in the formation of the inflammatory infiltrate. This is mediated by the expression of integrins and chemokine receptors. Integrins are heterodimers of an α- and β chain and play a role in firm adhesion of the T cells to the synovial endothelium and extracellular matrix. Determination of the mean fluorescence intensity of two β chains during DMARD treatment revealed that only during sulfasalazine treatment the β_2 chain (CD18, figure 3) was downregulated on both CD4+ and CD8+ cells while the expression of the β_1 chain (CD29, figure 4) was not significantly changed by any of the DMARDs used. However of the tested α chains associated with β_1, CD49d was diminished on T cells of sulfasalazine treated patients (figure 4).
Methotrexate was the only DMARD that significantly influenced the expression of the chemokine receptor CCR5 on CD4+ and CD8+ cells, while CXCR-3 and CCR3 expression were not affected by any DMARD (figure 5).

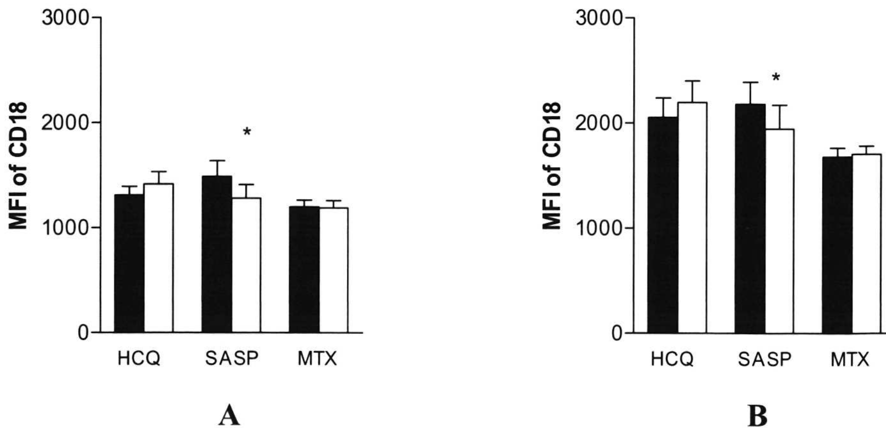
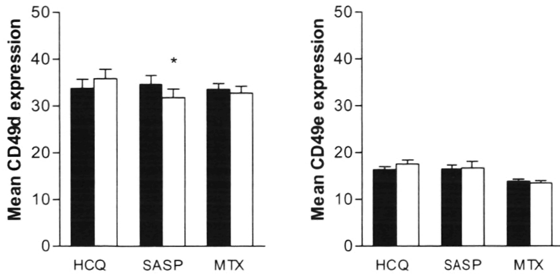


Figure 3.
Mean fluorescence intensity (MFI) of CD18 on CD4+ (A) and CD8+ (B) peripheral blood cells of arthritis patients, before (closed bars) and after 3 months of DMARD treatment (open bars). Given are means and SEM. *: $p<0.05$



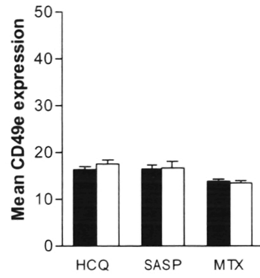
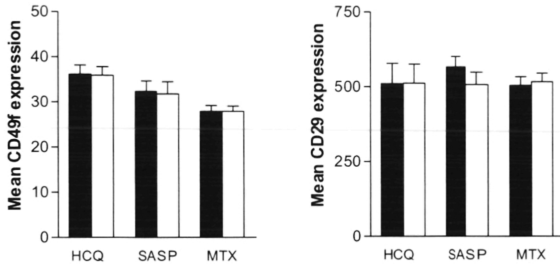


Fig 4A



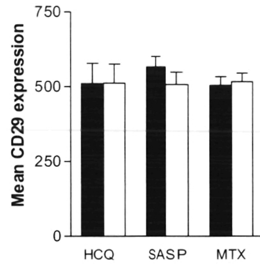
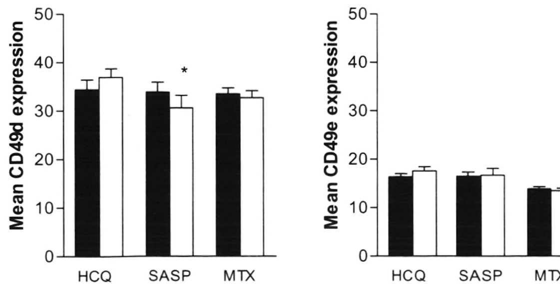


Figure 4.
Percentage of CD49 and CD29 positive cells within the CD4+ (A) and CD8+ (B) peripheral blood cells of arthritis patients, before (closed bars) and after 3 months of DMARD treatment (open bars). Given are means and SEM, *: $p<0.05$



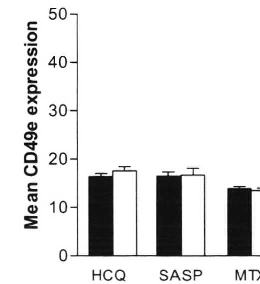
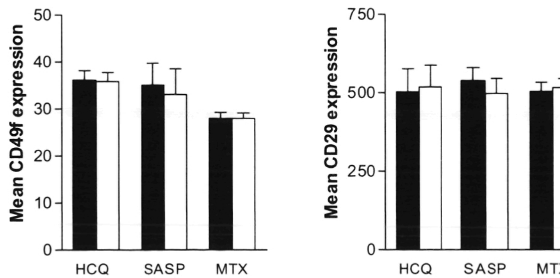
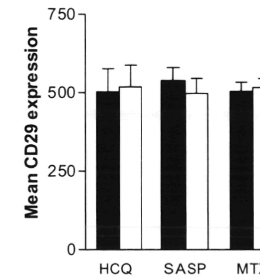


Fig 4B





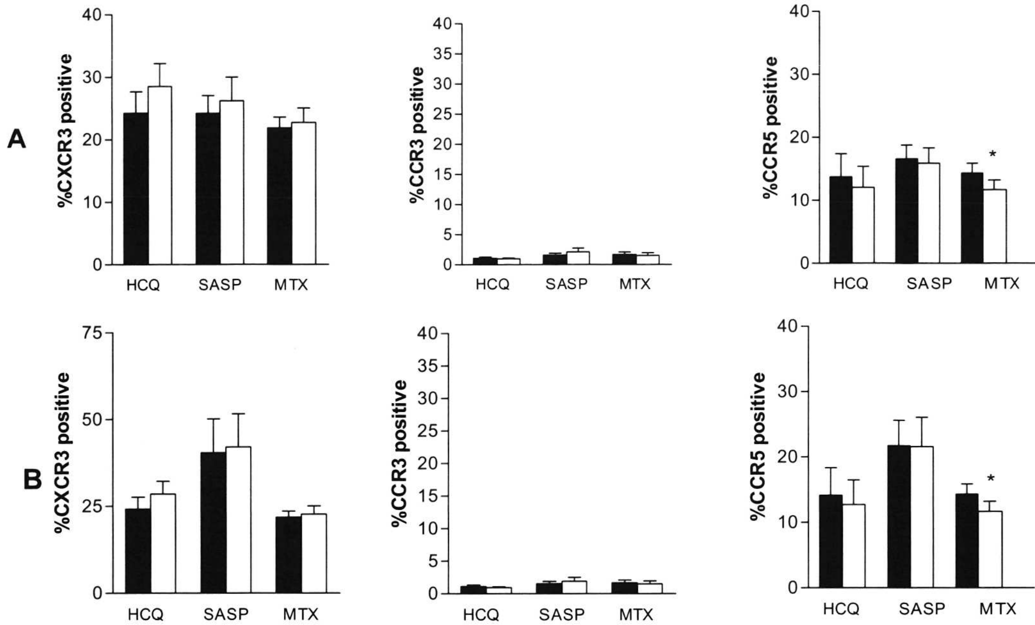


Figure 5.
Percentage of chemokine receptor positive cells within the CD4+ (A) and CD8+ (B) peripheral blood cells of arthritis patients, before (closed bars) and after 3 months of DMARD treatment (open bars). Given are means and SEM, *: $p<0.05$

Discussion:

In this study we investigated the effects of hydroxychloroquine, sulfasalazine and methotrexate on the phenotype and function of circulating T cells in arthritis patients. Although all three drugs are reported to decrease the proliferative responses of T cells in vitro[15-17,29,30], we only observed a significant inhibition of proliferation by hydroxychloroquine in vivo. Since the in vitro anti-proliferative effect of chloroquine is mediated by the inhibition of IL-2 production, we determined whether hydroxychloroquine treatment resulted in a decrease of IL-2 producing cells. However, we could not find a significant change in this cell type after 3 months of therapy.
Studies have shown that hydroxychloroquine can interfere with the process of antigen presentation[31] and could therefore interfere in T cell differentiation. Flowcytometric analysis of circulating T cells before and during hydroxychloroquine treatment in our study however did not indicate a shift towards a less differentiated, more naive phenotype as

determined by CD45RA and CD27 expression. This finding suggests that the action of hydroxychloroquine in the treatment of arthritis is not mediated via reduced antigen presentation.
The interference of DMARDs with cytokine production of T cells has been widely suggested as a mode of action in arthritis. Despite the reported decrease in the production of the proinflammatory cytokine IFN-γ by T cells in the presence of different DMARDs in vitro[18,19,21,32], we show that only sulfasalazine was able to induce a significant reduction in IFN-γ producing T cells in arthritis patients. A possible explanation could be that sulfasalazine can interfere with IL-12 production by antigen presenting cells as was demonstrated in a mouse model[21]. However we could demonstrate that in arthritis patients sulfasalazine reduced the IL-4 producing CD4+ cells as well making IL-12 not a likely target. Since IL-4 producers were not affected in the CD8+ subset it can be concluded that the balance between proinflammatory IFN-γ producing (type-1) and IL-4 producing (type-2) cells is shifted by sulfasalazine towards type-2.
Apart from the influence on proliferative and cytokine producing responses we investigated whether DMARD treatment could alter the expression of molecules on T lymphocytes involved in the homing to and retention in the synovium. Both β1 and β2 integrins are important in the adhesion to activated endothelium and the extracellular matrix in the synovium, while gradients of chemokines give direction to the T cells moving into the synovium. In this study we show that in sulfasalazine- in contrast to hydroxychloroquine and methotrexate treated patients, the expression of both the β2 integrins (CD18) and the β1 associated CD49d is diminished.
The finding of methotrexate being a moderator of CCR5 expression could point to a new mode of action for methotrexate in arthritis. CCR5 and CXCR-3 are the most abundantly expressed chemokine receptors in the rheumatoid synovium and they are also associated with interferon producing type-1 T cells. Therefore, although methotrexate does not influence the percentage of type-1 cells in the circulation of rheumatoid arthritis patients, it could influence the proportion of these cells in the synovial infiltrate.
In summary, in this study we show that although various overlapping effects of DMARDs on T cells are reported in vitro, distinct effects can be observed on the T cells in treated arthritis patients, which could account for synergistic anti-inflammatory action.

References

1. Dougados M, Combe B, Cantagrel A, et al. Combination therapy in early rheumatoid arthritis: a randomised, controlled, double blind 52 week clinical trial of sulphasalazine and methotrexate compared with the single components. Ann.Rheum.Dis. 1999;58:220-225

2. Haagsma CJ, van Riel PL, de Jong AJ, van de Putte LB. Combination of sulphasalazine and methotrexate versus the single components in early rheumatoid arthritis: a randomized, controlled, double-blind, 52 week clinical trial. Br.J Rheumatol 1997;36:1082-1088

3. Mottonen T, Hannonen P, Leirisalo-Repo M, et al. Comparison of combination therapy with single-drug therapy in early rheumatoid arthritis: a randomised trial. FIN-RACo trial group. Lancet 1999;353:1568-1573

4. O'Dell JR, Haire CE, Erikson N, et al. Treatment of rheumatoid arthritis with methotrexate alone, sulfasalazine and hydroxychloroquine, or a combination of all three medications. N.Engl.J Med 1996;334:1287-1291

5. Tugwell P, Pincus T, Yocum D, et al. Combination therapy with cyclosporine and methotrexate in severe rheumatoid arthritis. The Methotrexate-Cyclosporine Combination Study Group. N.Engl.J Med 1995;333:137-141

6. Miossec P. Are T cells in rheumatoid synovium aggressors or bystanders? Curr.Opin.Rheumatol 2000;12:181-185

7. Miltenburg AM, van Laar JM, de Kuiper R, Daha MR, Breedveld FC. T cells cloned from human rheumatoid synovial membrane functionally represent the Th1 subset. Scand.J Immunol 1992;35:603-610

8. Schlaak J, Hermann E, Ringhoffer M, et al. Predominance of Th1-type T cells in synovial fluid of patients with Yersinia-induced reactive arthritis. Eur.J Immunol 1992;22:2771-2776

9. Quayle AJ, Chomarat P, Miossec P, Kjeldsen-Kragh J, Forre O, Natvig JB. Rheumatoid inflammatory T-cell clones express mostly Th1 but also Th2 and mixed (Th0-like) cytokine patterns. Scand.J Immunol 1993;38:75-82

10. Simon AK, Seipelt E, Wu P, Wenzel B, Braun J, Sieper J. Analysis of cytokine profiles in synovial T cell clones from chlamydial reactive arthritis patients: predominance of the Th1 subset. Clin Exp Immunol 1993;94:122-126

11. Dolhain RJ, van der Heiden AN, ter Haar NT, Breedveld FC, Miltenburg AM. Shift toward T lymphocytes with a T helper 1 cytokine-secretion profile in the joints of patients with rheumatoid arthritis. Arthritis Rheum. 1996;39:1961-1969

12. Kotake S, Schumacher HRJ, Yarboro CH, et al. In vivo gene expression of type 1 and type 2 cytokines in synovial tissues from patients in early stages of rheumatoid, reactive, and undifferentiated arthritis. Proc.Assoc.Am.Physicians. 1997;109:286-301

13. Yin Z, Braun J, Neure L, et al . T cell cytokine pattern in the joints of patients with Lyme arthritis and its regulation by cytokines and anticytokines. Arthritis Rheum. 1997;40:69-79

14. van der Graaff WL, Prins AP, Niers TM, Dijkmans BA, van Lier RA. Quantitation of interferon gamma- and interleukin-4-producing T cells in synovial fluid and peripheral blood of arthritis patients. Rheumatology.(Oxford.) 1999;38:214-220

15. Landewe RB, Miltenburg AM, Verdonk MJ, et al. Chloroquine inhibits T cell proliferation by interfering with IL-2 production and responsiveness. Clin Exp Immunol 1995;102:144-151

16. Stevens C, Lipman M, Fabry S, et al. 5-Aminosalicylic acid abrogates T-cell proliferation by blocking interleukin-2 production in peripheral blood mononuclear cells. J Pharmacol.Exp Ther. 1995;272:399-406

17. Fujiwara M, Mitsui K, Yamamoto I. Inhibition of proliferative responses and interleukin 2 productions by salazosulfapyridine and its metabolites. Jpn.J Pharmacol. 1990;54:121-131

18. Constantin A, Loubet-Lescoulie P, Lambert N, et al. Antiinflammatory and immunoregulatory action of methotrexate in the treatment of rheumatoid arthritis: evidence of increased interleukin-4 and interleukin-10 gene expression demonstrated in vitro by competitive reverse transcriptase-polymerase chain reaction. Arthritis Rheum. 1998;41:48-57

19. Hildner K, Finotto S, Becker C, et al. Tumour necrosis factor (TNF) production by T cell receptor-primed T lymphocytes is a target for low dose methotrexate in rheumatoid arthritis. Clin Exp Immunol 1999;118:137-146

20. Neurath MF, Hildner K, Becker C, et al. Methotrexate specifically modulates cytokine production by T cells and macrophages in murine collagen-induced arthritis (CIA): a mechanism for methotrexate-mediated immunosuppression. Clin Exp Immunol 1999;115:42-55

21. Kang BY, Chung SW, Im SY, Choe YK, Kim TS. Sulfasalazine prevents T-helper 1 immune response by suppressing interleukin-12 production in macrophages. Immunology 1999;98:98-103

22. van den Borne BE, Dijkmans BA, de Rooij HH, le Cessie S, Verweij CL. Chloroquine and hydroxychloroquine equally affect tumor necrosis factor- alpha, interleukin 6, and interferon-gamma production by peripheral blood mononuclear cells. J Rheumatol 1997;24:55-60

23. Mack M, Bruhl H, Gruber R, et al. Predominance of mononuclear cells expressing the chemokine receptor CCR5 in synovial effusions of patients with different forms of arthritis. Arthritis Rheum. 1999;42:981-988

24. Qin S, Rottman JB, Myers P, et al. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. J Clin Invest. 1998;101:746-754

25. 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;11:553-559

26. Colantonio L, Iellem A, Clissi B, et al. Upregulation of integrin alpha6/beta1 and chemokine receptor CCR1 by interleukin-12 promotes the migration of human type 1 helper T cells. Blood 1999;94:2981-2989

27. Maurice MM, van der Graaff WL, Leow A, Breedveld FC, van Lier RA, Verweij CL. Treatment with monoclonal anti-tumor necrosis factor alpha antibody results in an accumulation of Th1 CD4+ T cells in the peripheral blood of patients with rheumatoid arthritis. Arthritis Rheum. 1999;42:2166-2173

28. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 1988;31:315-324

29. Fairbanks LD, Ruckemann K, Qiu Y, et al. Methotrexate inhibits the first committed step of purine biosynthesis in mitogen-stimulated human T-lymphocytes: a metabolic basis for efficacy in rheumatoid arthritis? Biochem J 1999;342:143-152

30. Nakajima A, Hakoda M, Yamanaka H, Kamatani N, Kashiwazaki S. Divergent effects of methotrexate on the clonal growth of T and B lymphocytes and synovial adherent cells from patients with rheumatoid arthritis. Ann.Rheum.Dis. 1996;55:237-242

31. Fox RI. Mechanism of action of hydroxychloroquine as an antirheumatic drug. Semin.Arthritis Rheum. 1993;23:82-91

32. Landewe RB, Miltenburg AM, Breedveld FC, Daha MR, Dijkmans BA. Cyclosporine and chloroquine synergistically inhibit the interferon- gamma production by CD4 positive and CD8 positive synovial T cell clones derived from a patient with rheumatoid arthritis. J Rheumatol 1992;19:1353-1357

Chapter 7

Prognostic value of Th1/Th2 ratio in rheumatoid arthritis

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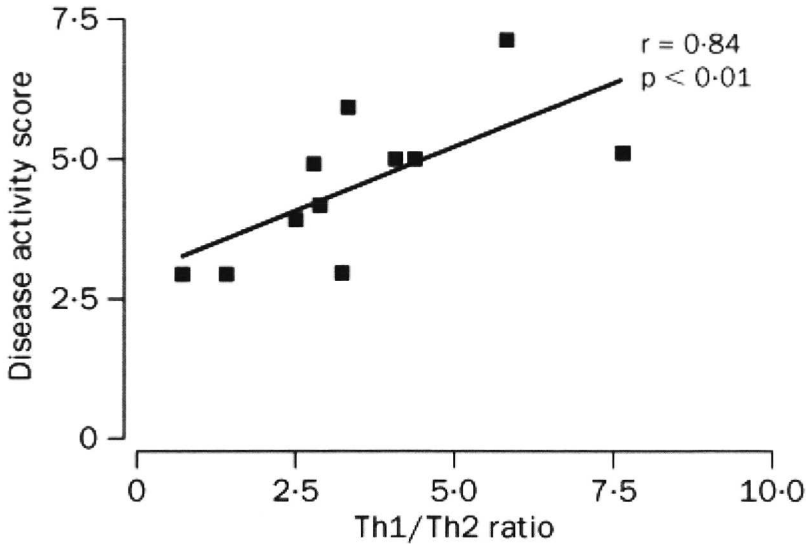
Lancet 351:1931,1998

Prognostic value of Th1/Th2 ratio in rheumatoid arthritis

Imbalance between proinflammatory T-helper-1 (Th1) and anti-inflammatory T-helper-2 (Th2) cells and, subsequently, their products interferon-γ and interleukin 4, respectively, is believed to be important in the development of autoimmune diseases. Dominance of Th1 over Th2 cells is thought to be important in the pathogenesis of rheumatoid arthritis.[2,3] We investigated whether the Th1/Th2 ratio in the peripheral blood of patients with rheumatoid arthritis is related to disease severity, measured by response to disease-modifying antirheumatic drugs (DMARDs).
We recruited patients with early rheumatoid arthritis (duration ≤1 year) who had not previously taken DMARDs. We determined disease activity by a modified disease activity score[4] based on scores for 28 joints. 11 patients with high disease activity (scores ≥6·0, median 6·4 [range 6·0-6·9]) were started on DMARDs; ten on sulphasalazine (2000 mg/day), one on hydroxychloroquine (200 mg/day). We measured Th1/Th2 ratios in peripheral blood by intracellular staining assay with flowcytometric analysis.[5] The percentages of Th1 and Th2 cells were determined by double staining for CD4 and either interferon-γ or interleukin-4. Data were analysed with Wilcoxon's signed rank test.
The median disease activity score decreased to 5·0 (range 3·3-6·9) after 3 months and 4·5 (range 2·9-7·1) after 9 months of therapy. Although activity scores were within a narrow range at baseline, we found large differences in Th1/Th2 ratios between patients. Th1/Th2 ratios did not correlate with disease activity scores or C-reactive-protein concentrations (data not shown). During the first 3 months of therapy, Th1/Th2 ratios increased significantly (median 3·2 vs 6·0, p=0·001) because of decreases in Th2 cells (median 5·0 vs 2·3%, p=0·002). After 9 months the initial Th1/Th2 ratio did not differ (median 3·2 vs 4·8; p=0·16.
This transient increase in the Th1/Th2 ratio during the first 3 months of treatment could have resulted from redistribution of Th1 and Th2 subsets, immunoregulatory effects of the medication, or from a combination of both. The effect, however, seemed to be of no importance to the clinical outcome since changes in the Th1/Th2 ratios and disease activity scores did not correlate after 3 months or 9 months. By contrast, the initial Th1/Th2 ratio correlated significantly with the disease activity score after 9 months (figure).
These data suggest that patients with low Th1/Th2 ratios in the peripheral blood during clinical active phases of rheumatoid arthritis are likely to respond well to the first course of DMARD treatment. The prognostic value of the Th1/Th2 ratio measured by intracellular staining assay in early rheumatoid arthritis needs to be assessed in larger groups of patients for longer times.
This study was supported by The Dutch League Against Rheumatism



Correlation between Th1/Th2 ratio before treatment and disease activity score after 9 months

References

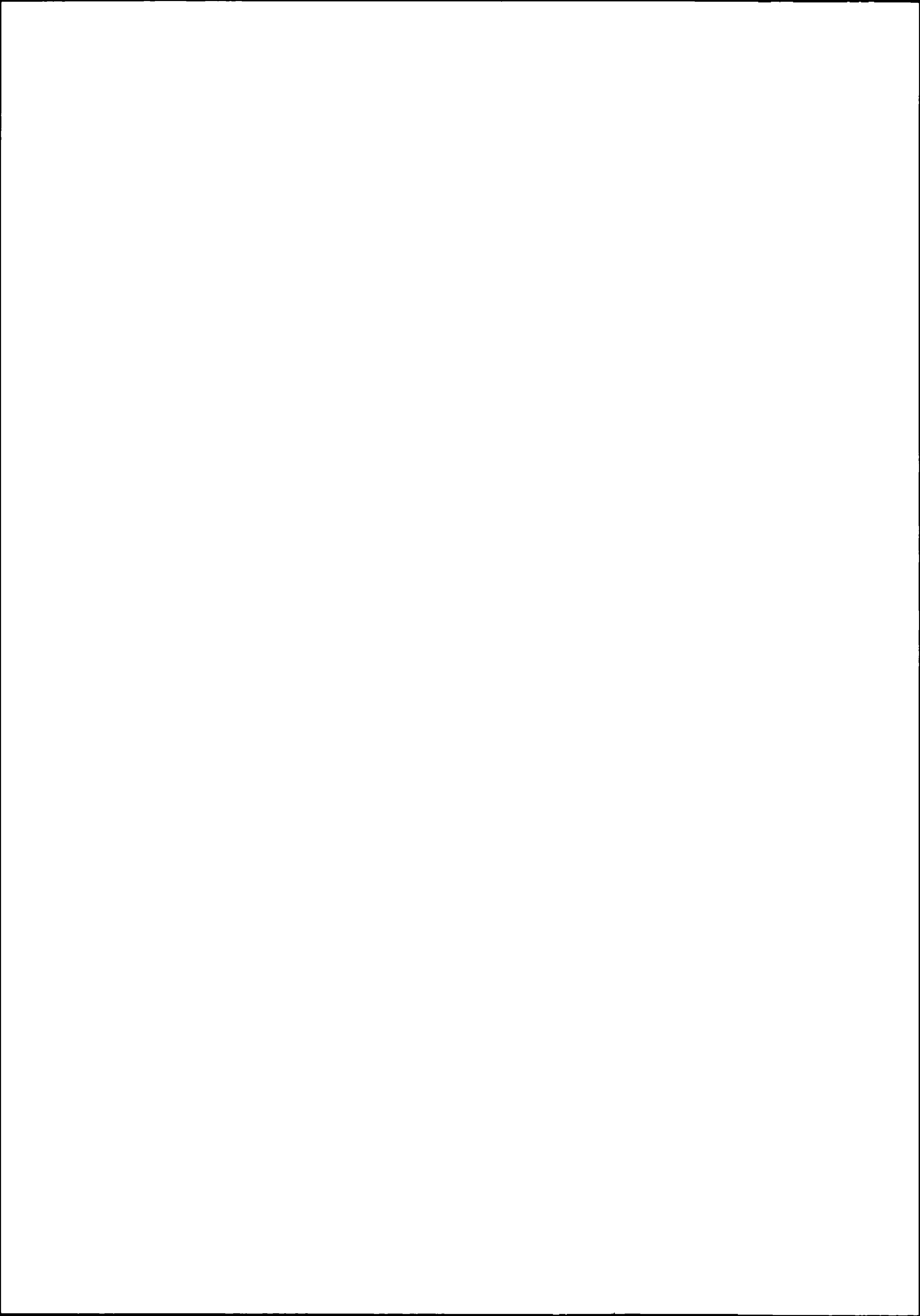
1 Romagnani S. Human TH1 and TH2 subsets: doubt no more. Immunol Today 1991; 12: 256-57.

2 Simon AK, Seipelt E, Sieper J. Divergent T-cell cytokine patterns in inflammatory arthritis. Proc Natl Acad Sci USA 1994; 91: 8562-66.

3 Dolhain RJ, van der Heiden AN, ter Haar NT, Breedveld FC, Miltenburg AM. Shift toward T lymphocytes with a T helper 1 cytokine-secretion profile in the joints of patients with rheumatoid arthritis. Arthritis Rheum 1996; 39: 1961-69.

4 Prevoo MLL, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LBA, van Riel PLCM. Modified disease activity scores that include twenty-eight-joint counts. Arthritis Rheum 1995; 38: 44-48.

5 Rep MH, van Oosten BW, Roos MT, Ader HJ, Polman CH, van Lier RA. Treatment with depleting CD4 monoclonal antibody results in a preferential loss of circulating naive T cells but does not affect IFN-gamma secreting TH1 cells in humans. J Clin Invest 1997; 99: 2225-31



Chapter 8

T cell subsets in early rheumatoid arthritis association with disease activity and prognosis

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Abstract

OBJECTIVE: To test the predictive value of T cell subset composition on the clinical outcome of early rheumatoid arthritis.

METHODS: A cohort study was initiated in which peripheral blood mononuclear cells (PBMC) of 65 patients who presented with rheumatoid arthritis of recent onset were obtained (mean disease activity score (DAS) 5.7, 54% rheumatoid factor positive). T cell differentiation markers (CD4, CD8, CD45RA, CD27) and T-cell cytokine production ability at presentation were related to DAS after one year DMARD treatment.

RESULTS: High percentages of differentiated helper T cells, characterized by the $CD4^{+}CD27^{-}$ phenotype, at the start of the therapy correlated with relatively low DAS after one year ($p=0.01$). In a subgroup of patients characterized by high initial disease activity (DAS>6), disease activity after one year was considerably lower in male (DAS 2.6) than in female patients (DAS 4.5) ($p<0.001$). Finally, in the group of female patients with high initial DAS, but not in the total cohort, a low $T_{HELPER(H)}1/T_{H}2$ ratio appeared to be related with better outcome scores ($p<0.05$).

CONCLUSION: T cell subset analyses revealed association between markers of T cell differentiation and disease activity after one year. T-cell subset and function measurements may prove useful in identifying patients that respond poorly to conservative DMARD therapy.

Introduction

Rheumatoid Arthritis (RA) is a disease of chronic inflammation of the joints with a presumed autoimmune origin. This usually leads to severe joint damage resulting in progressive disability. Tumor necrosis factor (TNF) α locally produced by monocytes plays a central role in the inflammatory cascade and neutralization of TNFα with either monoclonal antibodies or soluble receptor constructs ameliorates inflammation and has rapidly become an important treatment modality[1,2]. However, the trigger for TNFα production in the joint is still unknown.
The success of TNFα neutralization has formally demonstrated the involvement of inflammatory cascades in the pathogenesis of RA. Next to this, several indirect lines of evidence exist for contributions of the adaptive immune system to the etiology and/or pathophysiology of the disease. Perhaps most importantly, an association is found between certain HLA class II alleles, especially those containing the so-called shared-epitope (SE) sequence, and disease severity[3]. Because the function of class II molecules is to present antigenic peptides, it is supposed that SE-containing class II molecules contribute to an arthritogenic $CD4^{+}$ T cell repertoire[4-7]. Furthermore, autoantibodies directed to immunoglobuline (rheumatoid factor, RF) or citrullinated peptides are frequently found in RA patients. Although the role of these antibodies in the pathogenesis of the disease is unclear, they are related with more aggressive disease[8-11].

In contrast to indirect clues in humans, compelling evidence has been gathered in experimental animal models for a direct arthritogenic role of T cells. An important finding in these studies has been that the intrinsic ability of the T cell system to respond in qualitative different manners is an important variable for disease induction and outcome. Specifically, mice that are prone to respond to antigenic challenge in a $T_{HELPER(h)}1$ type fashion, which is characterized by an predominance of $CD4^+$ T cells secreting IFNγ, are highly sensitive to induced-arthritis[12]. In marked contrast, Th2 biased mice that generate relatively high numbers of IL-4 secreting T cells, do not develop disease[13]. It is suggested that these differential effects may be related to the opposing actions of Th1- and Th2-type helper cells on inflammatory cells, Th1 being pro-inflammatory in contrast to the anti-inflammatory Th2 cells. The Th2 biased Balb/c mouse is resistant to collagen-induced arthritis while it can develop a Th1 mediated arthritis when challenged with proteoglycan which is easily prevented by administration of IL-4[14]. We aimed to investigate whether also in humans the propensity of T cells to respond to antigenic challenges in a Th1- or Th2-biased fashion would be a contributing factor in the clinical course of RA. For this, a cohort study was initiated in which cellular immune functions were analyzed in patients who presented with early rheumatoid arthritis and results of these measurements were related to disease activity after one year. Two separate types of analysis were employed to evaluate $CD4^+$ T cell differentiation aspects. First, membrane expression of CD45RA and CD27 molecules were performed to enumerate naïve ($CD45RA^+$), memory-type ($CD45RA^-$) and differentiated memory-type helper T cells ($CD27^-$). Second, Th1 and Th2 were evaluated by intracellular measurement of IFN-γ and IL-4 in polyclonally stimulated cells.

Material and Methods

Patients

Patients with polyarthritis were recruited from an early arthritis clinic. The diagnosis rheumatoid arthritis was made according to the ACR guidelines[15] at one year of follow-up. Follow-up was at least a year with regular visits at the clinic every three months. Peripheral blood was obtained before start of treatment with disease modifying antirheumatic drugs. Initial treatment consisted of either hydrochloroquine or sulfasalazine as freely chosen by the treating physician, in the absence of a clinical response methotrexate was given as described in detail elsewhere[16]. Patients that had used DMARDs before presentation to the clinic were not included. Disease activity score[17]and CRP were determined before and after one year of the treatment. 43 out of 65 patients were analyzed for erosive scores of hands and feet[18], 32 out of 43 had erosive disease.

T cell phenotyping

Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll and stored in liquid nitrogen until use.

For membrane determination bound molecules, 0.2 million-thawed PBMC were incubated with directly labelled monoclonal antibodies for 30 minutes. After three washing steps cells were analysed on a four colour flowcytometer (FACScalibur). Results were expressed as percentage positive cells within CD4+ and CD8+ subsets.

For determination of cytokine production 0.4 million PBMC were shortly (four hours) stimulated with phorbol ester and Ionomycin as described previously, after fixation with paraformaldehyde, cells were permeabilized with saponin and incubated with directly labeled monoclonal antibodies against cytokines, CD4 and CD8. After analyses with four-color cytometry, cytokine positive cells were expressed as a percentage of CD4 and CD8 positive cells.
Antibodies: Directly labeled monoclonal antibodies (mab's) directed against the following antigens were used in this study: CLB, Amsterdam: CD27-FITC (3A12), Becton Dickinson: CD8-PerCP, IFN-γ-FITC, IL-4-PE, IL-2-FITC Caltag: CD4-APC.

Results

Description of the patient cohort.
Patients were selected from a group of 254 patients who presented with polyarthritis to the early arthritis clinic at our institute. During the first year of follow-up, 132 were diagnosed as having rheumatoid arthritis. Of these, 78 were selected because of the availability of clinical data and mononuclear cells from the start of the study and clinical data after one year of follow-up (11-13 months). Thirteen patients were excluded because of previous DMARD or steroid usage. Clinical parameters and DMARD usage of the 65 analyzed patients are summarized in table 1.

Table 1. Clinical data of 65 rheumatoid arthritis patients presenting at the Amsterdam early arthritis clinic

	Start		After one year of treatment	
	Mean	SD	Mean	SD
Disease activity score (DAS)	5.7	1.1	3.8	1.4
C-reactive protein	40	42	16	25
Age	56.7		57.7	
	Number			
Gender				
Male	20			
Female	45			
Rheumatoid factor (IgM) positive	35			
Erosive disease	32 (out of 43)			
DMARD usage				
Hydroxychloroquine	5		2	
Sulphasalazine	52		33	
None	7		6	

Correlation of T cell differentiation markers with measures of disease activity.
At the start DAS scores weakly negatively correlated with the percentage of circulating $CD45RA^-$, i.e. memory-type, helper T cells (table 2). This association might be related to the fact that memory-type cells have the ability to localize in inflamed tissue[19,20] and are therefore relatively depleted from the circulating pool. No correlation between any of the phenotypically defined T cells subsets and CRP levels were found (data not shown). Furthermore, at the start of the study no correlations were demonstrable between DAS and CD4 phenotype (table 2) or DAS and either IFN-γ (Th1) or IL-4 (Th2) producing $CD4^+$ T cells or the Th1/ Th2 ratio.

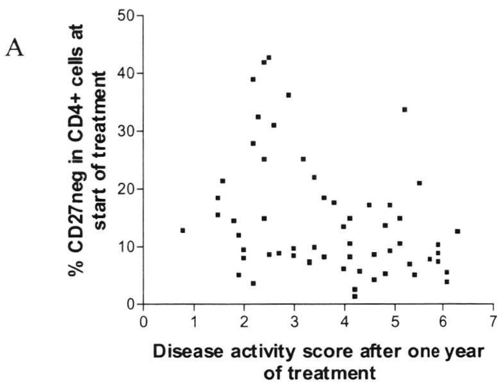
Table 2 Pearson correlation coefficient between CD4+ T cell phenotype in the peripheral blood and disease activity score (DAS) at inclusion

	Pearsons correlation coefficient	p	
All RA patients			
CD45RA positive	0.27	0.04	*
CD45RA negative	-0.27	0.04	*
CD27 negative	-0.01	0.93	ns
IL-4 positive	-0.26	0.19	ns
IFN-γ positive	0.00	0.99	ns
Th1/Th2	0.07	0.74	ns
IL-2 positive	-0.14	0.50	ns

*P<0.05, ns=not significant

Prognostic value of T cell differentiation markers.
Disease activity at one year was independent from the percentage of memory-type T cells at entry. The percentage of differentiated $CD4^+CD27^-$ showed a considerable variation in the cohort and inversely correlated with DAS at one year (Figure 1a and Table 3). No such correlations were found for Th1 and Th2 markers (Table 3).
RA is a heterogeneous disease, which may obscure possible relations between T-cell function parameters and prognosis. To analyze T cell differentiation markers in a more homogenous subgroup we separately analyzed patients presenting with high disease activity (DAS>6.0). Again, a relatively high percentage of $CD27^-$ T cells was associated with a rather good prognosis at one year, whereas neither $CD45RA^-$ percentages nor T_H1/ T_H2 measurements showed such correlation (Figure 1b and Table 3).

A



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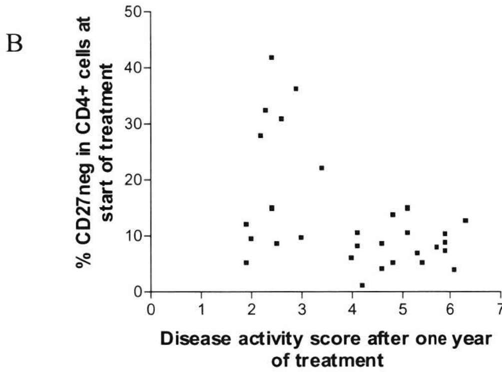

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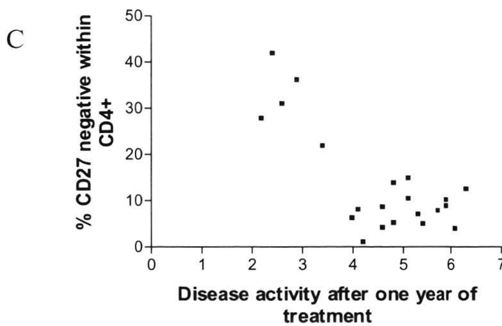


Figure 1.

Pearson correlation coefficient between the percentage of peripheral blood CD4 cells lacking CD27 at presentation and the Disease Activity Score (DAS) after one year of DMARD treatment in
A: all rheumatoid arthritis (RA) patients,
B: RA patients presenting with DAS >6,
C: female RA patients presenting with DAS >6.

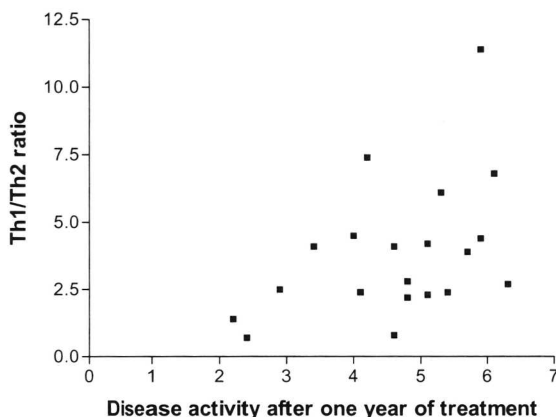
Table 3. Pearson correlation coefficients between CD4+ T cell phenotype in the peripheral blood at the start of treatment and disease activity score one year after treatment.

	Pearson correlation coefficient	p	
All RA patients			
CD45RA positive	0.04	0.8	ns
CD45RA negative	-0.04	0.8	ns
CD27 negative	-0.34	0.01	**
IL-4 positive	-0.1	0.49	ns
IFN-γ positive	-0.04	0.78	ns
Th1/Th2	-0.05	0.71	ns
IL-2 positive	-0.1	0.55	ns
Patients presenting with disease activity score >6			
CD45RA positive	-0.04	0.86	ns
CD45RA negative	-0.04	0.86	ns
CD27 negative	-0.5	0.01	**
IL-4 positive	-0.26	0.19	ns
IFN-γ positive	0	0.99	ns
Th1/Th2	0.07	0.74	ns
IL-2 positive	-0.14	0.5	ns

** $p<0.01$, ns=not significant

In a previous study, we found that a low T_H1/ T_H2 ratio was predictive of a relatively low DAS at one year(21). Since, this relation was not evident in the current high activity group (DAS>6.0) we reanalyzed the patients that were entered in this study. All patients tested in the previous study were females whereas in the present cohort contained 9 males (out of 30 patients presenting with DAS>6.0). Remarkably, in the present cohort of patients presenting with high disease activity scores, mean DAS scores at one year were 2.8 (standard deviation 1.3) for males versus 4.6 (standard deviation 1.2) for females ($p<0.001$, data not shown). Since this huge scatter in DAS might mask any potential influence of immunological parameters we analyzed these separately in the group of female patients with high disease activity at entry. Concerning $CD27^-$ differentiated T cells a similar relation was found as in the total cohort and the high DAS subgroup (Figure 1c). However, confirming our earlier findings, we corroborated that a high percentage of IL-4 producing cells and a low Th1/ Th2 ratio is associated with low DAS scores at one year in female patients (Figure 2).

Figure 2.
Correlation between the ratio between Th1 and Th2 cells at inclusion in the peripheral blood and disease activity score after one year of treatment in female patients presenting with high disease activity.



Discussion

The present cohort study was initiated to test whether markers of T-cell differentiation, such as naïve, memory and effector T cell numbers and cytokine production ability, can be used as predictive markers for disease course in patients with early RA. Three main conclusions can be drawn from the analyses. First, both in the total cohort and in the group of patients who presented with a high disease activity (DAS > 6), the presence of high numbers of differentiated memory $CD4^+$ T cells, defined by the absence of the TNF-R family member CD27, correlated with relatively low disease activity scores one year after entry in the study. Second, in the subgroup of patients with high disease activity male patients had a much more favorable prognosis than female patients. Third, a beneficial effect of a low Th1/Th2 ratio was observed in female patients with initial high disease activity, corroborating earlier findings by our group[21].

The inverse correlation of the percentage $CD4^+CD27^-$ with DAS at one year is unexpected and intriguing. We have previously shown that memory-type $CD4^+$ T cells are enriched in the rheumatoid synovium and strongly increase in the circulation after treatment with anti-TNFα mAb(22). Still, we consider it unlikely that the correlation between $CD27^-$ helper cells and prognosis might be explained by enhanced localization of these cells in the synovium in patients with severe disease. This because we did not find any correlation between disease activity at a given time point and the percentage of circulating $CD4^+CD27^-$ T cells.
$CD4^+CD27^-$ T cells appear to be specialized helper T cells that develop after persistent antigenic challenge[23,24]. Recently, Appay and colleagues[25] showed a relative increase in these cells in patients with persistent viral infection, i.e. cytomegalovirus and human immunodeficiency virus. In this respect, the presence of this subset in RA patients could

reflect an ongoing immune response to a persistent pathogen. In this scenario, patients that are able to form high numbers of these effector-type $CD4^+$ T cells do better because of their relatively good ability to cope with microbes. However, although viral and bacterial pathogens have been implicated in the etiology of RA proof for their role is lacking.
$CD4^+CD27^-$ T cells display a number of effector functions that are not found in $CD4^+CD27^+$ memory-type T cells. First, they exclusively secrete interleukin-5 and are enriched in interleukin-4 secreting T cells[24,26,27]. Moreover, they are able to synthesize the cytolytic mediators granzyme B and perforin and can execute cytolysis. The physiological role of cytotoxic $CD4^+$, class II-restricted T cells has been debated for many years by immunologists[28] but one appealing idea is that they may terminate immune reactions by eliminating peptide-bearing antigen presenting cells. If in the context of auto-immunity this would mean regulation of disease-maintaining auto-antigen cells, it could be envisaged that high numbers of these cells would correlate with favorable outcome.
Finally, in contrast to $CD4^+CD27^+$ memory-type T cells, $CD4^+CD27^-$ T cells are poor helpers for T-cell dependent immunoglobulin production[29]. Although the role of antibodies and B cells in RA remains heavily disputed, recent studies in showing that B-cell depleting CD20 antibodies ameliorate RA have renewed interest in this area. Low numbers of $CD4^+CD27^-$ T cells in RA could coincide with a relatively good helper activity of $CD4^+CD27^+$ T cells. In this respect it is of interest to note that Systemic Lupus Erythematosus, a disease in which the pathophysiological role of antibodies is hardly disputed, is characterized by low amounts of circulating $CD4^+CD27^-$ T cells (unpublished results).

Although it is well established that incidence of RA is three-fold higher in females[30], the finding that in the subgroup of patients with high disease activity at entry males had a far better prognosis at one year under standard DMARD treatments was striking. In line with this observation, Anderson et al.[19], reviewing cases of 1,435 patients treated in controlled trials with second-line anti-rheumatic drugs recently showed that female gender is associated with a relatively poor response to treatment. In addition *Kuiper et al* described an early arthritis cohort in which the DAS was higher for female patients than for male patients during follow-up while the DAS was the same at inclusion[31].

In a previous study we found that a low T_H1 and T_H2 ratio in a subgroup of patients with high disease activity at entry is associated with a relatively favorable prognosis after one year[32]. In addition, other studies have suggested similar relations between Th1 and Th2 ratio's and prognosis in RA[33-37]. In the total cohort no significant correlation between Th1 and Th2 ratio's and disease activity could be found. Also in the subgroup of patients with high disease activity at entry the relation was weak and non-significant. A significant relation was however found when females with high DAS scores at entry, that dominated the initial study group, were analyzed. These findings do not support a strong disease-modulating role for functionally differentiated T cells in RA. Yet, it should be realized that RA is a heterogeneous disease and that, in analogy to certain murine models for arthritis dominant effects of the Th1 and Th2 balance could be found if homogenous groups of patients can be identified and analyzed.
Recently, regulatory T cells (T_{REG}) have been identified in both rodents and humans[38-40]. It has been shown that these $CD4^+$ T cells can maintain self-tolerance in mice and it is

suggested that they have a similar function in humans. It will be interesting to test whether a disease-modifying role of these differentiated T cells can be found in RA.
In summary T cell subset analyses revealed association between markers of T cell differentiation and the progression of RA during a year. T-cell subset and function measurements may prove useful in identifying patients that respond poorly to conservative DMARD therapy.

References

1. Furst DE, Breedveld FC, Burmester GR, et al. Updated consensus statement on tumour necrosis factor blocking agents for the treatment of rheumatoid arthritis (May 2000). Ann.Rheum.Dis.2000;59 Suppl 1:i1-2.:i1-i2
2. Smolen JS, Breedveld FC, Burmester GR, et al. Consensus statement on the initiation and continuation of tumour necrosis factor blocking therapies in rheumatoid arthritis. Ann.Rheum.Dis.2000.Jul.;59.(7.):504.-5. 59:504-505
3. Gregersen PK, Silver J, Winchester RJ. The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum. 1987;30:1205-1213
4. Penzotti JE, Nepom GT, Lybrand TP. Use of T cell receptor/HLA-DRB1*04 molecular modeling to predict site-specific interactions for the DR shared epitope associated with rheumatoid arthritis. Arthritis Rheum. 1997;40:1316-1326
5. Gebe JA, Novak EJ, Kwok WW, Farr AG, Nepom GT, Buckner JH. T cell selection and differential activation on structurally related HLA-DR4 ligands. J Immunol 2001; 167:3250-3256
6. Gregersen PK, Silver J, Winchester RJ. The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum. 1987;30:1205-1213
7. Zanelli E, Krco CJ, Baisch JM, Cheng S, David CS. Immune response of HLA-DQ8 transgenic mice to peptides from the third hypervariable region of HLA-DRB1 correlates with predisposition to rheumatoid arthritis. Proc.Natl.Acad.Sci.U.S.A. 1996;93:1814-1819
8. Meyer O, Labarre C, Dougados M, et al. Anticitrullinated protein/peptide antibody assays in early rheumatoid arthritis for predicting five year radiographic damage. Ann.Rheum.Dis.2003; 62:120-126
9. Bas S, Perneger TV, Seitz M, Tiercy JM, Roux-Lombard P, Guerne PA. Diagnostic tests for rheumatoid arthritis: comparison of anti-cyclic citrullinated peptide antibodies, anti-keratin antibodies and IgM rheumatoid factors. Rheumatology.(Oxford.) 2002; 41:809-814
10. Schellekens GA, Visser H, de Jong BA, et al. The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. Arthritis Rheum.2000;43:155-163

11. van Jaarsveld CH, ter Borg EJ, Jacobs JW, et al. The prognostic value of the antiperinuclear factor, anti-citrullinated peptide antibodies and rheumatoid factor in early rheumatoid arthritis. Clin Exp Rheumatol 1999;17:689-697

12. Matyniak JE, Reiner SL. T helper phenotype and genetic susceptibility in experimental Lyme disease. J Exp Med 1995;181:1251-1254

13. Ortmann R, Smeltz R, Yap G, Sher A, Shevach EM. A heritable defect in IL-12 signaling in B10.Q/J mice. I. In vitro analysis. J Immunol 2001;166:5712-5719

14. Finnegan A, Mikecz K, Tao P, Glant TT. Proteoglycan (aggrecan)-induced arthritis in BALB/c mice is a Th1-type disease regulated by Th2 cytokines. J Immunol 1999;163:5383-5390

15. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 1988;31:315-324

16. Jansen LM, van Schaardenburg D, van DH-B, I, Bezemer PD, Dijkmans BA. Predictors of functional status in patients with early rheumatoid arthritis. Ann.Rheum.Dis.2000; 59:223-226

17. Prevoo ML, van 't H, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum. 1995;38:44-48

18. van der Heijde DM, van Leeuwen MA, van Riel PL, van de Putte LB. Radiographic progression on radiographs of hands and feet during the first 3 years of rheumatoid arthritis measured according to Sharp's method (van der Heijde modification). J Rheumatol 1995;22:1792-1796

19. Anderson JJ, Wells G, Verhoeven AC, Felson DT. Factors predicting response to treatment in rheumatoid arthritis: the importance of disease duration. Arthritis Rheum.2000; 43:22-29

20. Koch AE, Robinson PG, Radosevich JA, Pope RM. Distribution of CD45RA and CD45RO T-lymphocyte subsets in rheumatoid arthritis synovial tissue. J Clin Immunol 1990;10:192-199

21. van der Graaff WL, Prins AP, Dijkmans BA, van Lier RA. Prognostic value of Th1/Th2 ratio in rheumatoid arthritis. Lancet 1998;351:1931

22. Maurice MM, van der Graaff WL, Leow A, Breedveld FC, van Lier RA, Verweij CL. Treatment with monoclonal anti-tumor necrosis factor alpha antibody results in an accumulation of Th1 CD4+ T cells in the peripheral blood of patients with rheumatoid arthritis. Arthritis Rheum. 1999;42:2166-2173

23. Baars PA, Maurice MM, Rep M, Hooibrink B, van Lier RA. Heterogeneity of the circulating human CD4+ T cell population. Further evidence that the CD4+CD45RA-CD27- T cell subset contains specialized primed T cells. J Immunol 1995;154:17-25

24. De Jong R, Brouwer M, Hooibrink B, Van der Pouw-Kraan T, Miedema F, van Lier RA. The CD27- subset of peripheral blood memory CD4+ lymphocytes contains functionally differentiated T lymphocytes that develop by persistent antigenic stimulation in vivo. Eur.J Immunol 1992;22:993-999

25. Appay V, Zaunders JJ, Papagno L, et al. Characterization of CD4(+) CTLs ex vivo. J Immunol 2002; 168:5954-5958

26. Elson LH, Nutman TB, Metcalfe DD, Prussin C. Flow cytometric analysis for cytokine production identifies T helper 1, T helper 2, and T helper 0 cells within the human CD4+CD27- lymphocyte subpopulation. J Immunol 1995;154:4294-4301

27. Elson LH, Shaw S, van Lier RA, Nutman TB. T cell subpopulation phenotypes in filarial infections: CD27 negativity defines a population greatly enriched for Th2 cells. Int.Immunol 1994;6:1003-1009

28. Sun Q, Burton RL, Lucas KG. Cytokine production and cytolytic mechanism of CD4(+) cytotoxic T lymphocytes in ex vivo expanded therapeutic Epstein-Barr virus-specific T-cell cultures. Blood 2002; 99:3302-3309

29. van Lier RA, Pool MO, Kabel P, et al. Anti-CD27 monoclonal antibodies identify two functionally distinct subpopulations within the CD4+ T cell subset. Eur.J Immunol 1988;18:811-816

30. Spector TD. Rheumatoid arthritis. Rheum.Dis.Clin North Am. 1990;16:513-537

31. Kuiper S, van Gestel AM, Swinkels HL, de Boo TM, da Silva JA, van Riel PL. Influence of sex, age, and menopausal state on the course of early rheumatoid arthritis. J Rheumatol 2001; 28:1809-1816

32. van der Graaff WL, Prins AP, Dijkmans BA, van Lier RA. Prognostic value of Th1/Th2 ratio in rheumatoid arthritis. Lancet 1998;351:1931

33. Rudwaleit M, Yin Z, Siegert S, et al. Response to methotrexate in early rheumatoid arthritis is associated with a decrease of T cell derived tumour necrosis factor alpha, increase of interleukin 10, and predicted by the initial concentration of interleukin 4. Ann.Rheum.Dis.2000; 59:311-314

34. Verhoef CM, van Roon JA, Vianen ME, Bruijnzeel-Koomen CA, Lafeber FP, Bijlsma JW. Mutual antagonism of rheumatoid arthritis and hay fever; a role for type 1/type 2 T cell balance. Ann.Rheum.Dis. 1998;57:275-280

35. van Roon JA, Verhoef CM, van Roy JL, et al. Decrease in peripheral type 1 over type 2 T cell cytokine production in patients with rheumatoid arthritis correlates with an increase in severity of disease. Ann.Rheum.Dis. 1997;56:656-660

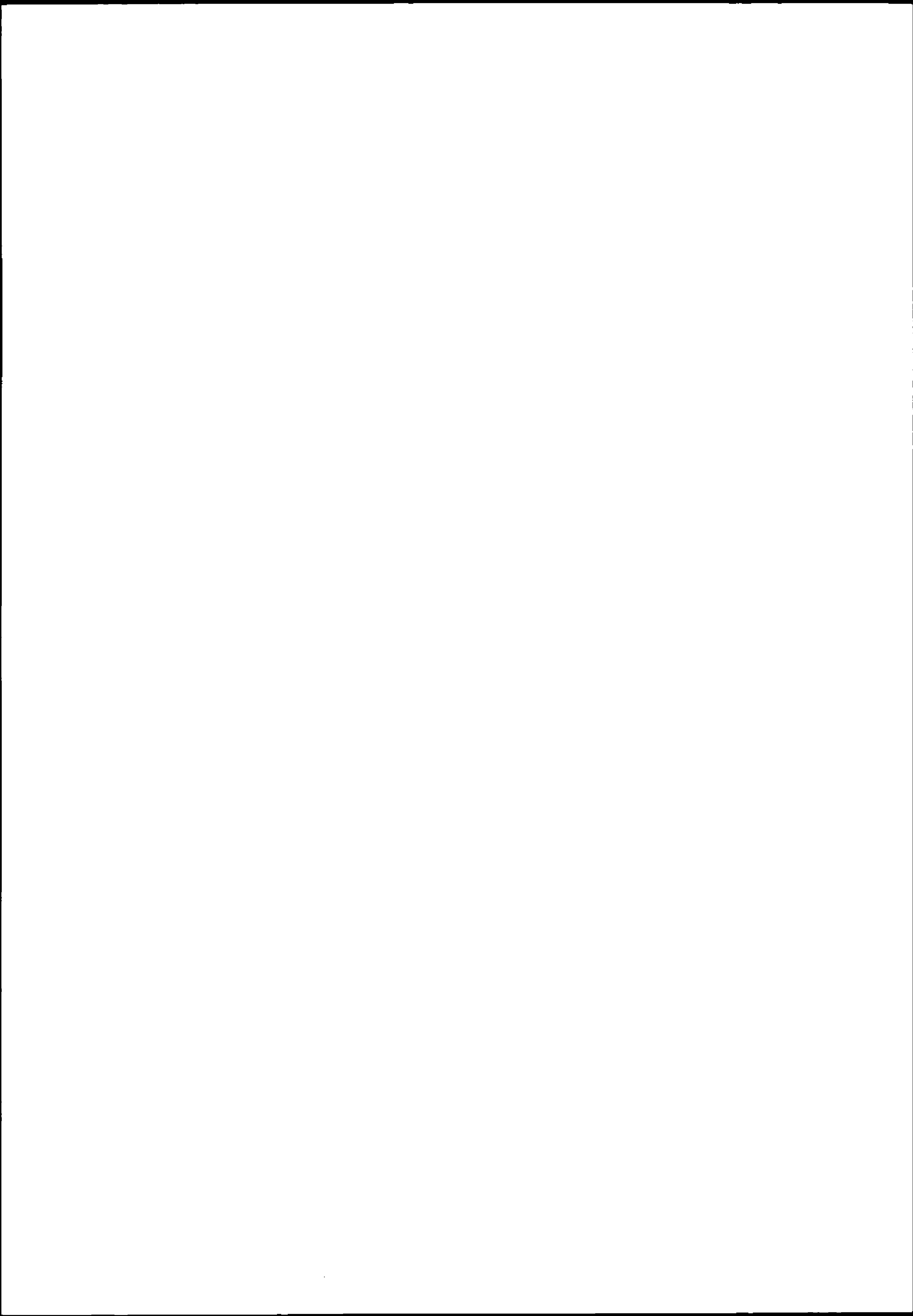
36. Hilliquin P, Allanore Y, Coste J, Renoux M, Kahan A, Menkes CJ. Reduced incidence and prevalence of atopy in rheumatoid arthritis. Results of a case-control study. Rheumatology.(Oxford.) 2000; 39:1020-1026

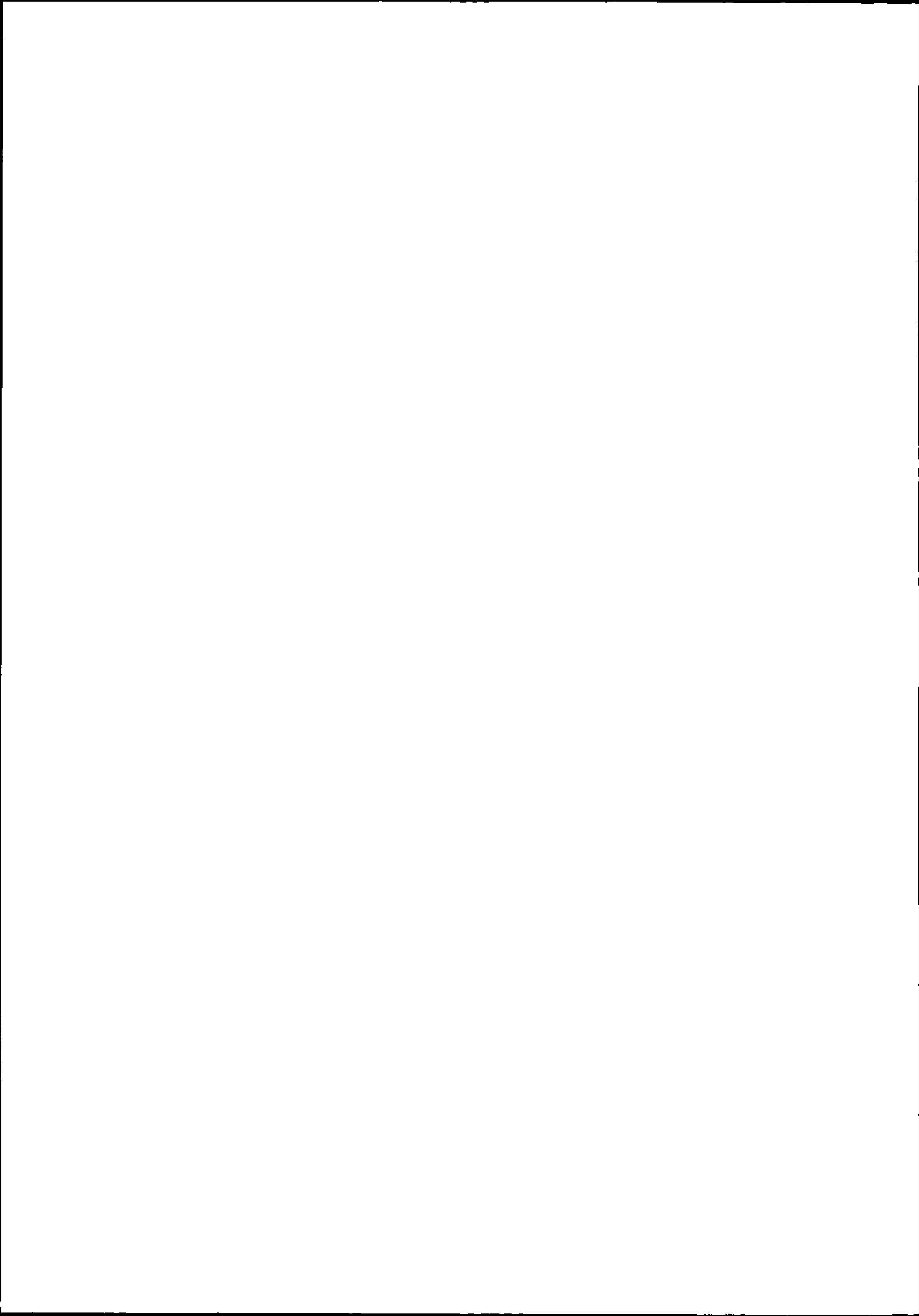
37. Allanore Y, Hilliquin P, Coste J, Renoux M, Menkes CJ. Decreased prevalence of atopy in rheumatoid arthritis. Lancet 1998;351:497

38. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995;155:1151-1164

39. Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med 1998;188:287-296

40. Suri-Payer E, Amar AZ, Thornton AM, Shevach EM. CD4+CD25+ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. J Immunol 1998;160:1212-1218





Chapter 9

Summary and general discussion

The balance between different T cell subsets is believed to play a role in development or prevention of autoimmune diseases. Especially an imbalance in the mutually antagonizing type-1 and type-2 T cells could result in damage to the host. Type-1 cells are pro-inflammatory and it has been postulated that an overshoot type-1 immune response could lead to organ-specific autoimmune disease such as arthritis, multiple sclerosis, thyreoiditis and diabetes mellitus. Type-2 cells on the other hand are effective inducers of antibody production by B cells and can antagonize the effects of type-1 T cells. Unopposed type-2 reactivity could lead to antibody-mediated disease such as allergy and systemic lupus erythematosus. In this thesis it has been tested if the balance between T helper 1 and T helper 2 cells plays a role in human rheumatic diseases. Both cell types were identified by flowcytometry with antibodies against IFN-γ, a prototypical T helper 1 cytokine and IL-4, a marker for type-2 T cells.

As shown previously, synovial fluid (SF) T cells of rheumatoid arthritis patients are primed cells as they express CD45R0[1,2] and CD95[3]. However while both type-1 and type-2 cells can be found in the peripheral blood of rheumatoid arthritis patients, SF T cells have predominantly a type-1 phenotype. This finding is not specific for rheumatoid arthritis but can also be observed in reactive arthritis, undifferentiated arthritis and psoriatic arthritis (chapter 2).
Whether this is a result of a mechanism where type-1 T cells are preferentially homing from the blood to the inflamed synovium or that after entry, type-1 cells can further differentiate in the synovium while type-2 cells are inhibited in their differentiation process is not entirely clear, but support for both mechanisms can be found.

The possibility of selective homing is supported by the observation that type-1 T cells express the adhesion molecule CD49d (alpha4) (chapter 5) and chemokine receptors like CCR5 and CXCR-3[4,5] of which the ligands are abundantly expressed on endothelial cells and in the extracellular matrix of the inflamed synovium (VCAM-1, Fibronectin, RANTES, IP-10 and MIG, respectively).
Chapter five gives *in vivo* support for the selective homing of type-1 T cells to the rheumatoid synovium. The humanized monoclonal antibody $_{C}$A2 blocks the activity of TNF-α and is shown to interfere with the inflammatory cascade in the joints. This results in the downmodulation of adhesion ligands like VCAM-1 and ICAM-1 on synovial endothelium[6-8] thereby obstructing the process of adherence of peripheral blood T cells to the inflamed endothelium and subsequently migration into the synovium. Indeed, the lymphocytosis that can be seen in the blood three days after administration of the antibody to rheumatoid arthritis patients is a likely the result of T cell redistribution. We found that this increase in lymphocytes is caused by the increase of CD45R0 positive T cells with a type-1 phenotype expressing CD49d suggesting that specifically these cells had lost the ability to adhere to the endothelium.

The second explanation for the enrichment of type-1 T cells in the inflamed synovium is local differentiation. Polarization into type-1 cells of synovium entering T cells can result from the presence of IL-12, IL-18 and IL-15[9].

The pro-inflammatory action of type-1 cells in the synovium is subsequently exerted by both IFN-gamma production and direct contact with macrophage type cells[10]. Since this induces increased local production of TNF-α this leads to amplification of the inflammatory response that in turn attracts more type-1 T cells to the synovium resulting in chronic inflammation. The assumption of this amplifying circle is supported by the observation that the ratio between type-1 and type-2 CD4 cells in the synovial fluid and the extent of inflammation as estimated by the erythrocyte sedimentation rate is correlated (chapter 2). However it is still unclear how the vicious circle is initiated and which factors in non-rheumatoid individuals are involved in switching it off.

In contrast to the synovium the ratio between type-1 and type-2 cells in the peripheral blood is not correlated to inflammation (chapter2, 8). While the ratio is significantly lower in the presumed type-2 disease SLE (chapter 3), in rheumatoid arthritis patients as well as in ankylosing spondylitis patients it is not different from healthy controls (chapter 2 and 4).
Even though treatment of arthritis patients during three months with DMARDs results in a reduction of disease activity, this is not accompanied by a comparable change in the type-1/type-2 ratio in the peripheral blood (chapter 6). This might mean that the ratio in peripheral blood is not only dependent on redistribution induced by inflammation, but that in longer time periods other mechanisms are involved. Since even in healthy controls substantial differences exist in this ratio in the peripheral blood, it can be argued that it is influenced by genetic factors as well as T cell responses to previously encountered pathogens.
In mice, it has been shown that the outcome of a T cell response to a particulate antigen is dependent on pathogen-derived signals as well as genetic factors of the host and that the possibility to develop a strong type-1 immune response can be essential in the resistance against certain pathogens[11]. It might therefore be that the tendency to develop type-1 response is an advantage in the defence against intracellular pathogens, while the prize could be a higher susceptibility to chronic inflammatory conditions like rheumatoid arthritis.
This theory is supported by the observation of a decreased occurrence of atopic disease which is type-2 mediated in rheumatoid arthritis patients[12-15] but contradicting data exist[16,17].

Rheumatoid arthritis is a multifactorial disorder and shows a wide spectrum of clinical phenotypes from mild disease to severe arthritis.
Epidemiological studies have revealed that multiple genetic factors are involved in the susceptibility as well as the severity of the disease[18,19]. In this thesis it is studied whether the type-1/type-2 ratio in the peripheral blood of rheumatoid arthritis patients, is related to disease severity. Again heterogeneity of rheumatoid arthritis is indicated in chapter 7 and 8. Although the measured type-1/type-2 ratio in the peripheral blood shows a relation with persistence of disease activity during one year (chapter 7), this seemed only to be true when a homogeneous group a patients was analysed (chapter 8). Nevertheless this suggests that a tendency to react with a type-1 immune response is unfavourable with respect to the course of rheumatoid arthritis. Because of the heterogeneity of rheumatoid arthritis the unpredictable clinical outcome and the increasing capability to prevent long term damage, there is a strong need for parameters to identify severe disease at an early stage. The data

obtained during our studies show that the T helper 1/T helper 2 ratio is not a strong enough predictor to be used in an unselected patient population. Still, it will be interesting to test its possible clinical relevance combined with other prognostic parameters or during longer follow-up periods.

References

1. Koch AE, Robinson PG, Radosevich JA, Pope RM. Distribution of CD45RA and CD45RO T-lymphocyte subsets in rheumatoid arthritis synovial tissue. J Clin Immunol 1990;10:192-199
2. Damle NK, Doyle LV. Ability of human T lymphocytes to adhere to vascular endothelial cells and to augment endothelial permeability to macromolecules is linked to their state of post-thymic maturation. J Immunol 1990;144:1233-1240
3. Hoa TT, Hasunuma T, Aono H, et al. Novel mechanisms of selective apoptosis in synovial T cells of patients with rheumatoid arthritis. J Rheumatol 1996;23:1332-1337
4. 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;187:875-883
5. Bonecchi R, Bianchi G, Bordignon PP, et al. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. J Exp Med 1998;187:129-134
6. Nakada MT, Tam SH, Woulfe DS, Casper KA, Swerlick RA, Ghrayeb J. Neutralization of TNF by the antibody cA2 reveals differential regulation of adhesion molecule expression on TNF-activated endothelial cells. Cell Adhes.Commun. 1998;5:491-503
7. Paleolog EM, Hunt M, Elliott MJ, Feldmann M, Maini RN, Woody JN. Deactivation of vascular endothelium by monoclonal anti-tumor necrosis factor alpha antibody in rheumatoid arthritis. Arthritis Rheum. 1996;39:1082-1091
8. Tak PP, Taylor PC, Breedveld FC, et al. Decrease in cellularity and expression of adhesion molecules by anti-tumor necrosis factor alpha monoclonal antibody treatment in patients with rheumatoid arthritis. Arthritis Rheum. 1996;39:1077-1081
9. Gracie JA, Forsey RJ, Chan WL, et al. A proinflammatory role for IL-18 in rheumatoid arthritis. J Clin Invest. 1999;104:1393-1401
10. McInnes IB, Leung BP, Sturrock RD, Field M, Liew FY. Interleukin-15 mediates T cell-dependent regulation of tumor necrosis factor-alpha production in rheumatoid arthritis. Nat.Med 1997;3:189-195
11. Locksley RM, Scott P. Helper T-cell subsets in mouse leishmaniasis: induction, expansion and effector function. Immunol Today 1991;12:A58-A61
12. Allanore Y, Hilliquin P, Coste J, Renoux M, Menkes CJ. Decreased prevalence of atopy in rheumatoid arthritis. Lancet 1998;351:497

13. Hilliquin P, Allanore Y, Coste J, Renoux M, Kahan A, Menkes CJ. Reduced incidence and prevalence of atopy in rheumatoid arthritis. Results of a case-control study. Rheumatology.(Oxford.) 2000; 39:1020-1026

14. Reckner OA, Skogh T, Wingren G . Comorbidity and lifestyle, reproductive factors, and environmental exposures associated with rheumatoid arthritis. Ann.Rheum.Dis.2001; 60:934-939

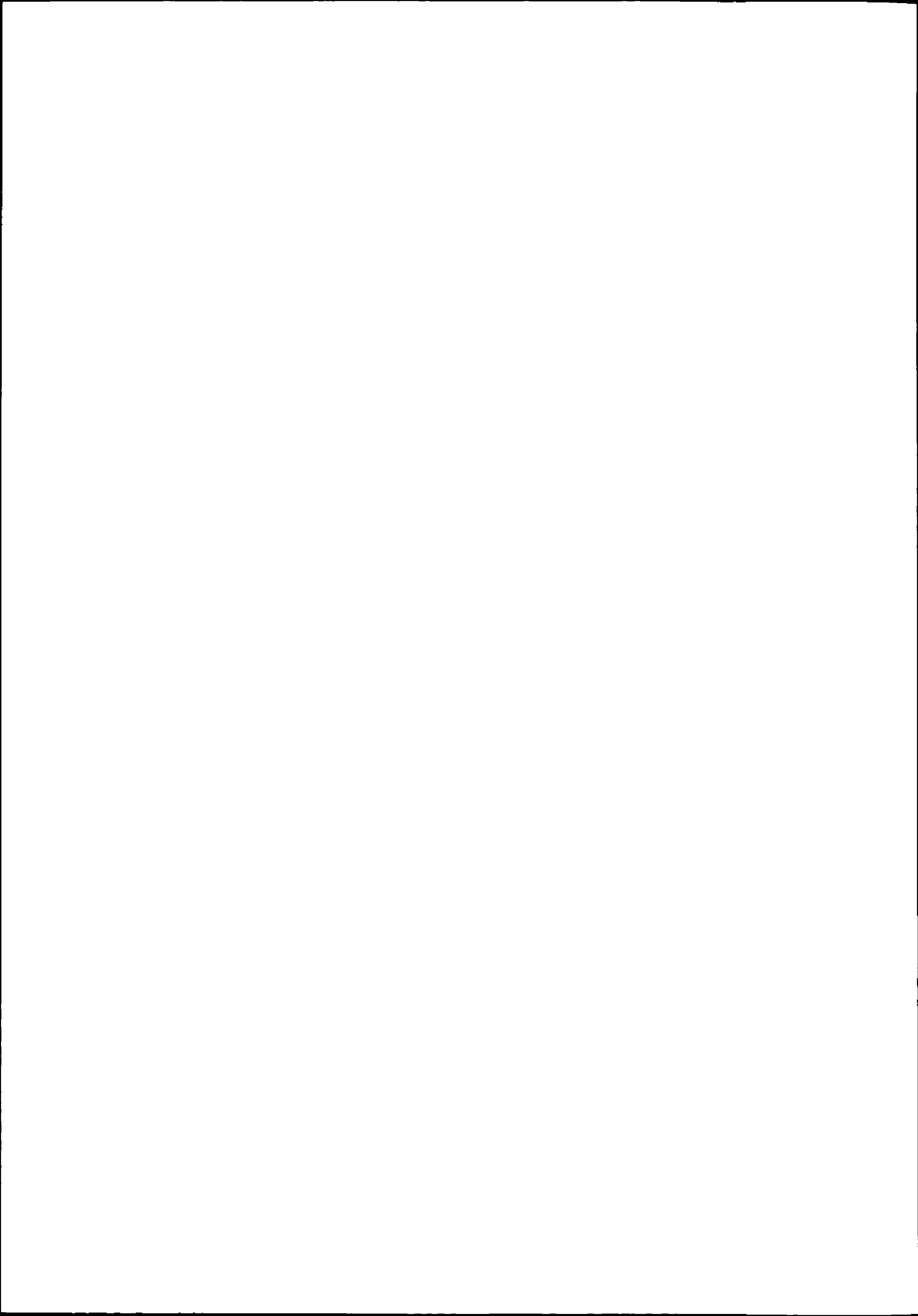
15. Verhoef CM, van Roon JA, Vianen ME, Bruijnzeel-Koomen CA, Lafeber FP, Bijlsma JW. Mutual antagonism of rheumatoid arthritis and hay fever; a role for type 1/type 2 T cell balance. Ann.Rheum.Dis. 1998;57:275-280

16. O'Driscoll BR, Milburn HJ, Kemeny DM, Cochrane GM, Panayi GS. Atopy and rheumatoid arthritis. Clin Allergy 1985;15:547-553

17. Simpson CR, Anderson WJ, Helms PJ, et al. Coincidence of immune-mediated diseases driven by Th1 and Th2 subsets suggests a common aetiology. A population-based study using computerized general practice data. Clin Exp Allergy 2002; 32:37-42

18. Barton A, Ollier W. Genetic approaches to the investigation of rheumatoid arthritis. Curr.Opin.Rheumatol 2002;14:260-269

19. Jawaheer D, Gregersen PK. Rheumatoid arthritis. The genetic components. Rheum.Dis.Clin North Am.2002; 28:1-15, v



Samenvatting

Samenvatting voor niet ingewijden

Het immuunsysteem is een complex van cellulaire en humorale facoren die als gezamelijk doel hebben lichaamsvreemde ziekteverwekkers (pathogenen) te herkennen en te elimineren. Als het een pathogeen gelukt is de buitenste grenzen van het lichaam (huid en slijmvliezen) te passeren wordt hier in eerste instantie op gereageerd door het aangeboren aspecifieke immunsysteem. Dit is in staat een aantal gemeenschappelijke onderdelen van ziekteverwekkers te herkennen en ter plaatse een ontstekingsreaktie te beginnen. De onstekingsreaktie heeft tot doel een omgeving te creeren waarin in zo kort mogelijke tijd met zo min mogelijk schade voor de gastheer het pathogeen geelimineerd kan worden.

Het aangeboren immuun systeem zal in eerste instantie zelf beginnen met het aanvallen van de indringer door het actief worden van oplosbare moleculen zoals het complement systeem. Daarnaast zijn er gespecialiseerde cellen die pathogenen direkt kunnen herkennen en opnemen (fagocytose) zoals macrofagen. In niet ontstoken weefsel zitten maar weinig macrofagen, echter door de ontstekingsreaktie onstaat er bloedvatverwijding en kunnen er meer macrofagen en fagocyterende neutrofiele granulocyten het weefsel binnengaan. Zij hechten zich aan het bloedvat door de ontstekings geinduceerde adhesiemolekulen, en worden de onstoken weefsels ingeleid door vrijgekomen chemokines.

Naast de direkte anti-pathogene werking speelt het aangeboren immuunsysteem een belangrijke rol in het activeren en mobiliseren van het verworven specifieke immuun systeem. Dit systeem is aanwezig in alle gewervelde dieren en bestaat uit T- en B-lymfocyten. B cellen produceren antistoffen die pathogenen direkt kunnen neutraliseren of door binding aan het oppervlak het pathogeen gevoeliger maken voor fagocytose.
T cellen kunnen onderverdeeld worden in een aantal subsets die verschillende functies hebben. Sommige kunnen geinfecteerde lichaamscellen opruimen (cytotoxie, met name door CD8 positieve T cellen) en andere spelen een belangrijke regulerende rol in zowel het specifieke als niet specifieke immunsysteem (voornamelijk CD4 positieve T cellen).
T cellen worden actief als er lichaamsvreemde eiwitten (antigenen) worden aangeboden door het niet specifieke immuun systeem met name door macrofagen en dendritische cellen (antigeen presenterende cellen of APC's). Tijdens een infectie migreren APC's vanuit het geinfecteerde gebied naar lymeklieren waar ze onderdelen van de binnengedrongen pathogenen (peptiden) aanbieden aan de aanwezige T cellen. Iedere T cel heeft een T cel receptor die een aantal peptiden kan herkennen. Als een specifieke T cel kan reageren met een aangeboden peptide ontstaat er T cel activatie waardoor de T cel gaat delen en differentieren. Er bestaan een aantal verschillende differentiatie paden die elk leiden naar afzonderlijke functies.

CD4 positieve T cellen kunnen klassiek differentiëren in type-1 of type-2 cellen. Type-1 cellen worden beschouwd als proinflammatoir. Ze migreren gemakkelijk naar ontstoken weefsels waar ze de onstekingsreaktie kunnen versterken en fagocyterende en cytotoxische cellen helpen bij het elimineren van pathogenen. Type-2 cellen zijn anti-inflammatoir in de zin dat ze effecten van type-1 cellen kunnen tegengaan. Daarnaast zijn ze beter in staat hulp te verlenen aan B cellen bij het maken van specifieke antistoffen.

Of er tijdens een immuunreaktie voornamelijk type-1 of type-2 cellen onstaan hangt af van verschillende factoren. APC's kunnen door de productie van cytokines sturen of T cellen in type-1 of type-2 differentieren. Vooral bij de aanwezigheid van intracellulaire pathogenen wordt er een sterk type-1 differentierend signaal gegeven terwijl de aanwezigheid van extracellulaire parasieten voornamelijk resulteert in de ontwikkeling van type-2 T cellen.
Het feit dat type-1 en type-2 T cellen elkaars ontwikkeling en uiteindelijke functie afremmen heeft tot de hypothese geleid dat een type-2 respons uiteindelijk weer uitgedoofd kan worden door type-1 cellen en vise versa, teneinde teveel schade aan de gastheer te voorkomen. Zo zou een te sterke of te landurige type-1 respons leiden tot chronische ontsteking en een te sterke type-2 reaktie tot allergieën.

Reumatoide artritis is een ziektebeeld dat gekenmerkt wordt door chronische ontsteking van de gewrichten. Ondanks intensieve research is het tot nu toe niet gelukt om lichaamsvreemd materiaal of pathogeen in de gewrichten aan te tonen. Wel zijn er aanwijzingen dat er immuunreakties optreden tegen lichaamseigen antigenen. Zowel geaktiveerde cellen van het aangeboren aspecifieke als van het verworven specifieke imuunsysteem zijn in de reumatoide gewrichten aanwezig. Geaktiveerde macrofagen maken grote hoeveelheden TNF-alfa hetgeen resulteert in rechtstreekse destructie van het kraakbeen en aan de andere kant rekrutering van steeds meer ontstekingscellen waaronder type-1 T cellen. Het is niet bekend of het primair de macrofagen zijn die ontregeld zijn of dat een dysbalans in de verhouding tussen pro- en anti-inflammatoire T cellen de drijvende kracht is voor deze TNF-alfa produktie.

In dit proefschrift worden de verhoudingen tussen verschillende T cel subsets, met name type-1 en type-2 T cellen, in reumatoide artritis patienten bepaald. Deze verhoudingen worden vergeleken met die in patienten met andere autoimuunziekten zoals systemische lupus erythematosus (SLE) en morbus Bechterew (ankyloserende spondylitis). Daarnaast is er bij reumatoide artritis patienten onderzocht of er een relatie bestaat tussen T cel subsets en de ernst van de ziekte.

Type-1 en type-2 T cellen zijn niet direkt identificeerbaar. Het belangrijkste onderscheid is de produktie van verschillende cytokinen. De produktie van interferon-γ (IFN–γ) is typisch voor type-1 cellen terwijl interleukine-4 (IL-4) door type-2 cellen wordt geproduceerd.
Met flowcytometrie kunnen verschillende T cel subsets worden geidentificeerd door het aankleuren van oppervlakte moleculen (differentiatie markers). Daarnaast kunnen na korte activatie van de T cellen in vitro, IFN-γ en IL-4 aangekleurd worden waardoor de verhouding tussen type-1 en type-2 gedifferentieerde T cellen benaderd kan worden.

In *hoofdstuk 2* wordt deze techniek gebruikt om aan te tonen dat het vooral type-1 T cellen zijn die in de synoviale vloeistof voorkomen zowel bij reumatoide artritis als ook bij reaktieve- en ongedifferentieerde artritis. Deze laatste twee hebben over het algemeen een minder chronisch beloop dan reumatoide artritis. Wel wordt er een relatie gevonden tussen de ontstekingsaktiviteit, gemeten als bezinkingssnelheid van de erythrocyten, en de ratio type-1/type-2 cel in de synoviale vloeistof. Dit kan betekenen of dat een hoge TNF-alfa produktie in de gewrichten resulteert in een verhoogde rekrutering van type-1 T cellen, of dat primair de opeenhoping van type-1 T cellen in het synovium resulteert in de produktie

van TNF-alfa en daardoor een hogere bezinking wordt gemeten. In *hoofdstuk 5* worden aanwijzingen gevonden dat de produktie van TNF-alfa leidt tot het uittreden van type-1 T cellen uit het bloed, mogelijk naar de ontstekingsgebieden als de gewrichten. Na selectieve blokkade van TNF-alfa met antistoffen bij reumatoide artritis patienten onstaat er na drie dagen een toename van T cellen in het bloed, die voor een groot deel uit type-1 cellen blijkt te bestaan.

Dat de verhouding tussen type-1 en type-2 cellen in het bloed niet volledig afhankelijk is van de mate van ontsteking in de gewrichten, kan worden afgeleid uit de resultaten van *hoofdstuk 6*. In dit hoofdstuk wordt bij patienten met artritis de type-1/type-2 ratio in het bloed bepaald met een interval van drie maanden. Ondanks dat er grote verschillen zijn in activiteit van de ziekte tussen deze twee tijdspunten zijn er geen correlaties met veranderingen in de type-1/type-2 ratio.

In *hoofdstuk 2* wordt er, net als bij ankyloserende spondylitis (*hoodstuk 4*), geen verschil waargenomen tussen de type-1 en type-2 ratio in het bloed bij patienten met reumatoide artritis en gezonde personen, terwijl deze ratio bij SLE patienten duidelijk lager is (*hoofdstuk 3*).
Het lijkt dus zo te zijn dat ondanks het feit dat er verschillen bestaan in de perifeer bloed type-1/type-2 ratio's tussen individuen, deze ratio niet sterk gerelateerd is aan het ontwikkelen van artritis. Bovendien blijkt dat bij het aanwezig zijn van artritis, de maat van onsteking niet gereflecteerd wordt in de type-1/type-2 ratio in het bloed maar eerder door die in het gewricht.

Dat de type-1/type-2 verhouding in het perifeer bloed op termijn toch invloed kan hebben op het verloop van reumatoide artritis wordt gezien in *hoofdstuk 7 en 8*. Hier blijkt dat in een cohort van patienten met "early arthritis" deze ratio wel gerelateerd is met het persisteren van ziekteactiviteit gedurende een jaar. Dit verband wordt echter alleen gezien bij vrouwen die met hoge ziekte activiteit binnen komen. Deze waarneming bevestigt vooral de multifactoraliteit van het ontstaan en het beloop van reumatoide artritis maar maakt tegelijk onderzoek naar de relatie tussen de type-1/type-2 ratio in een vroeg stadium tot progressie van de ziekte op langere termijn de moeite waard.

List of publications

1. van der Graaff WL, Borleffs JC. 1994. Eosinophilia in patients with HIV-1 infection. *Eur J Haematol. 52:246-7*

2. Benne CA, Harmsen M, van der Graaff W, Verheul AF, Snippe H, Kraaijeveld CA. 1997. Influenza virus neutralizing antibodies and IgG isotype profiles after immunization of mice with influenza A subunit vaccine using various adjuvants. *Vaccine.15:1039-44*

3. van der Graaff WL, Prins AP, Dijkmans BA, van Lier RA.1998. Prognostic value of Th1/Th2 ratio in rheumatoid arthritis. *Lancet. 351:1931.*

4. van der Graaff WL, Prins AP, Niers TM, Dijkmans BA, van Lier RA.1999. Quantitation of interferon gamma- and interleukin-4-producing T cells in synovial fluid and peripheral blood of arthritis patients. *Rheumatology (Oxford). 38:214-20.*

5. Maurice MM, van der Graaff WL, Leow A, Breedveld FC, van Lier RA, Verweij CL. 1999. Treatment with monoclonal anti-tumor necrosis factor alpha antibody results in an accumulation of Th1 CD4+ T cells in the peripheral blood of patients with rheumatoid arthritis. *Arthritis Rheum. 42:2166-73*

6. Killestein J, Den Drijver BF, Van der Graaff WL, Uitdehaag BM, Polman CH, Van Lier RA. 2001. Intracellular cytokine profile in T-cell subsets of multiple sclerosis patients: different features in primary progressive disease. *Mult Scler.7:145-50*

Hierbij wil ik iedereen bedanken die heeft bijgedragen aan het tot stand komen van dit proefschrift.