

Effects of biological response modifiers in psoriasis and psoriatic arthritis

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The studies presented in this thesis were conducted at the Department of Dermatology, the Department of Clinical Immunology and Rheumatology, and the Laboratory of Experimental Dermatology, Academic Medical Center, University of Amsterdam, the Netherlands.

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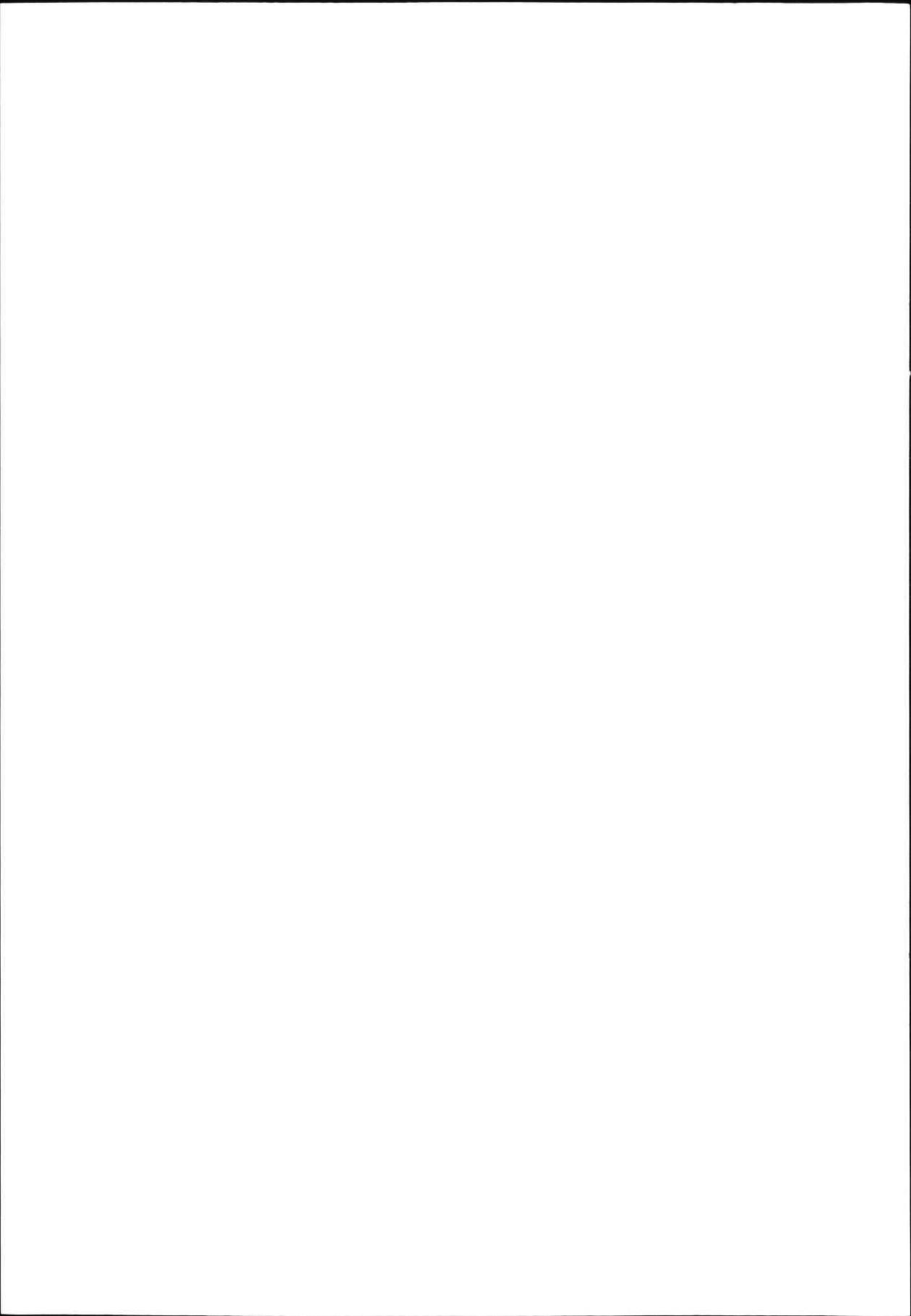
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Twenty years from now you will be more disappointed by the things that you didn't do than by the ones you did. So, throw off the bowlines. Sail away from the safe harbor. Catch the trade winds in your sails.

Explore. Dream. Discover.

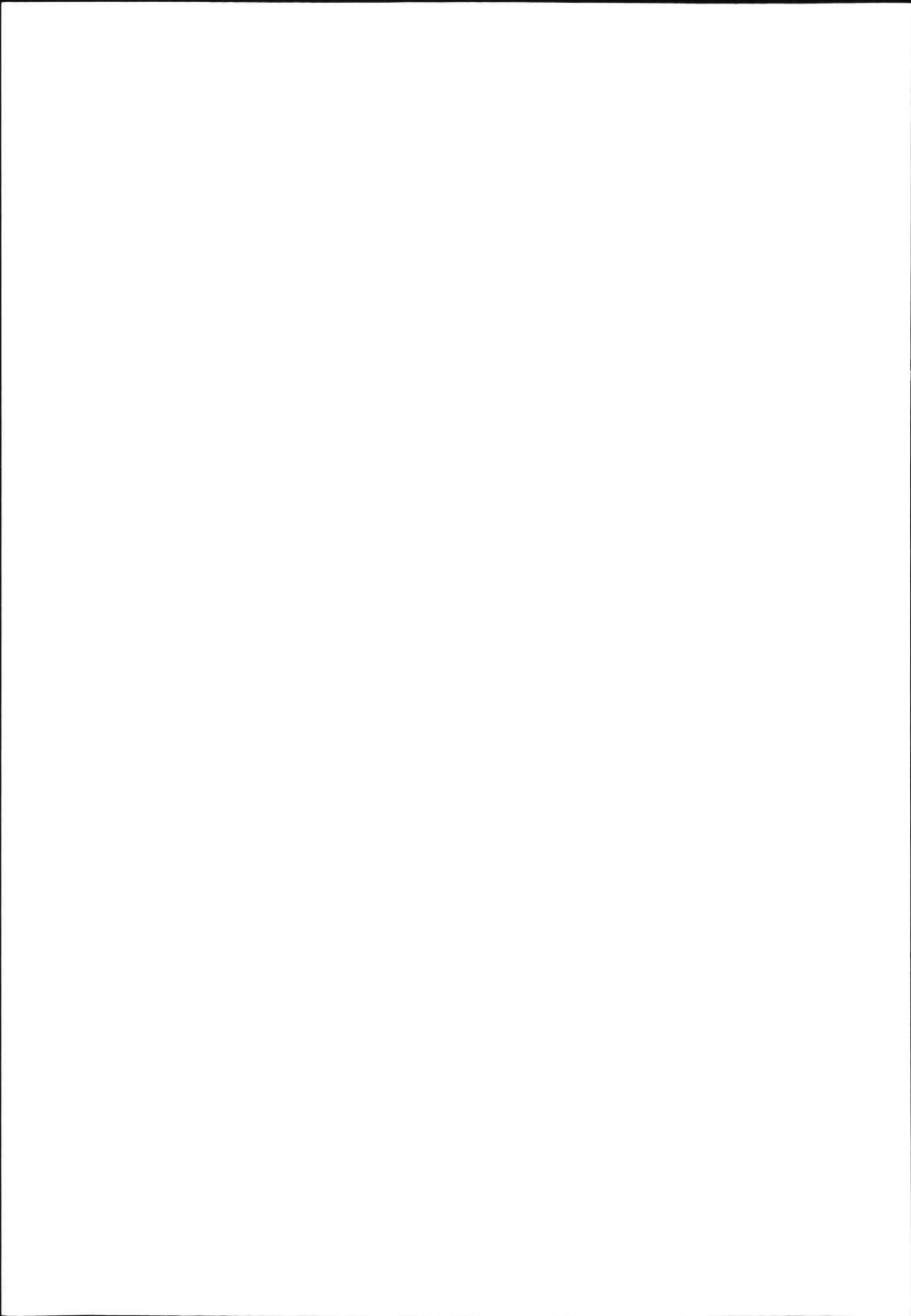
Mark Twain (1835 – 1910)

Aan mijn vader



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chapter



General introduction and aims of the thesis



Introduction to psoriasis and psoriatic arthritis

Psoriasis is a chronic inflammatory skin disease with an estimated prevalence of 2% worldwide. The term 'psoriasis' was first used by the Greek physician Galen, and is derived from the word 'psora' meaning itch. Patients suffering from psoriasis may experience physical discomfort as well as a great psychological burden. The clinical manifestations of psoriasis are abundant and diverse, and the intensity of disease activity varies in time. The most common clinical presentation is plaque-type psoriasis or psoriasis vulgaris, which is characterized by sharply demarcated erythematous plaques with silvery scaling presenting symmetrically on the extensor side of the elbows and knees, and the lumbosacral region. This type of psoriasis is often accompanied by involvement of nails and scalp. Other types of psoriasis are divided into those primarily describing the form (guttate, pustular, annular) or the distribution (flexural, palmoplantar, disseminated). Guttate psoriasis is characterized by numerous small coin-sized papules distributed over the whole body, and flexural psoriasis is characterized by erythematous plaques in body folds such as the groins, axillae, and the submammary region. Erythrodermic psoriasis and generalized pustular psoriasis are potentially life-threatening manifestations of psoriasis often requiring hospitalization. An arthritis associated with psoriasis was first recognized in the mid-nineteenth century and in 1860 Paul Bazin coined the term 'psoriasis arthropathique'^{1,2}. It was not until 1964 that the American Rheumatism Association recognised it as a separate entity. Approximately 25-34 percent of patients with skin psoriasis develop psoriatic arthritis (PsA)^{3,4}, a seronegative destructive joint disease with 5 different subtypes: asymmetrical oligo-articular arthritis (70%), symmetrical poly-arthritis (15%), distal interphalangeal arthritis (5%), spinal/axial arthritis (5%), and arthritis mutilans (5%)⁵. Without proper treatment PsA can lead to joint degeneration and loss of function. Usually the skin psoriasis precedes the arthritis, but in 19% of the cases arthritis is present before skin lesions develop³.

Immunopathology of psoriasis and psoriatic arthritis⁶

Introduction Although the exact origin of psoriasis and PsA has not been established yet, the immunopathogenetic pathways leading to the development of a psoriatic plaque have been subject to extensive studies in the last decades. In addition, the introduction of new biological therapies for psoriasis has proven to be a powerful tool in the investigation of the psoriatic immune responses. In general, the initiation and exacerbation of both psoriasis and PsA probably result from an interaction of genetic, environmental, and immunological factors. The morphology of lesional psoriatic skin is characterised by 3 major histological changes: 1) epidermal thickening, parakeratosis, and hyperkeratosis; 2) a pronounced dermal vascular plexus; and 3) the presence of inflammatory cells (e.g. T cells and neutrophilic granulocytes) in the superficial dermis and in the epidermis. Few studies have analyzed the immunohistochemical changes in synovial tissues in PsA. Histological features of synovial tissue include infiltration by macrophages, T cells, and other inflammatory cells⁷⁻⁹, hyperplasia of the synovial lining, and vascular changes, described as tortuosity and higher intensity of villous vascularization^{9,10}. In addition, high IL-8 levels were found in PsA synovial fluid, outlining the importance of this chemokine in the recruitment of inflammatory cells in patients with PsA⁸. In addition to cell-mediated immune responses, humoral immune responses are believed to play a role in the pathogenesis of PsA as well. For example, serum levels of IgA and IgG are higher in PsA patients¹¹, and synovial membranes from patients with PsA contain higher numbers of plasma cells positive for IgG or IgA than patients with meniscal tears¹².

T cells in psoriasis Increasing evidence suggests that T cells play a key role in the pathogenesis of psoriasis. In 1983, Bos et al. showed that the majority of the dermal inflammatory infiltrate consists of partially activated CD4⁺ and CD8⁺ T cells¹³. These cells express CD45RO on the surface, indicating their effector / memory status^{14,15}. Most of the infiltrating T cells also express markers such as the interleukin-2 receptor (CD25) and HLA-DR, indicating early and mid-to-late activation, respectively^{13,16-18}. The infiltration of T cells into the skin precedes epidermal proliferation and endothelial cell activation¹⁹. The key role for T

lymphocytes in the pathogenesis of psoriasis was supported by reported beneficial effects of specific T-cell targeted therapies, such as cyclosporine A and DAB389IL-2 toxin, and more recently alefacept²⁰⁻²³. Because of this, psoriasis is now suggested to be a T-cell mediated inflammatory disease.

How do T cells play a role in the development of a psoriatic plaque? To create a better understanding of this process, it can be broken down in three separate steps:

- 1 the activation of T cells
- 2 the migration of T cells into the lesional skin
- 3 the release of cytokines by activated T cells in the skin.

Ad 1) Initial T-cell activation requires stimulation of the T-cell receptor (TCR) by the major histocompatibility complex (MHC I or II) on the antigen presenting cell (APC). Although this is believed to be an antigen-specific process, the antigenic peptide responsible in psoriasis has as yet not been identified. The adhesion of the T cell with the APC is facilitated by the interaction of surface molecules, such as CD2 on the T cell with leukocyte function associated antigen (LFA)-3 on the APC, or LFA-1 (CD11a) on the T cells with intercellular adhesion molecule (ICAM)-1 on the APC²⁴. After the initial activation via the TCR, a second, non-antigen specific signal is needed to complete the activation. This 'costimulatory' signal results from interactions between molecules on the T cell and their ligands on APCs, for example CD2/LFA-3, very-late antigen 4 (VLA-4)/vascular cell adhesion molecule 1 (VCAM-1), LFA-1/ICAM-1, CD40/CD40L, and others²⁵⁻³⁰. Simultaneous delivery of both signals is essential for T cell activation, and if the costimulatory signal is inhibited, T cells can become unresponsive or 'anergic'.

A number of new biological therapies for psoriasis have been developed to inhibit T cell activation in one way or another, for example efalizumab, a humanized anti-CD11a monoclonal antibody, and alefacept, a LFA-3/IgG1 fusion protein. These and other biologicals will be discussed further on in this introduction

Ad 2) Once activated, T cells obtain certain cell surface proteins, which are necessary for migration from lymph nodes and blood vessels into extranodal

tissue, such as the skin. T-cell trafficking to the skin is a complicated process that requires intensive interaction between the activated T cell and the endothelium. First, T cells must be slowed down in the blood stream in order to be immobilized and to bind to the endothelium. This process, called 'tethering', is mediated by the glycoprotein cutaneous lymphocyte antigen (CLA), which is expressed on the surface of activated T cells in psoriasis³¹. CLA is an adhesion molecule that interacts with E-selectin and P-selectin, which are strongly upregulated on endothelium during cutaneous inflammation³². Both lesional and non-lesional skin of psoriasis patients shows upregulation of these adhesion molecules³³. Temporary binding of the selectins with the receptors on the T cell surface creates a rolling motion, which slows the T cell down^{34,35}. This allows the T cell to be exposed to chemokines that activate T-cell surface proteins, such as LFA-1 and very-late antigen (VLA)-4. These integrin receptors form high-affinity bonds with respectively ICAM-1 and VCAM-1 on endothelial cells, resulting in an arrest of the rolling process and subsequent flattening of the activated T cell, which facilitates the diapedesis of the T cell through the blood vessel wall³⁶. After extravasation through the blood vessel wall, skin-homing T cells migrate to the dermis and epidermis in response to chemotactic gradients. The chemokines that enhance T-cell trafficking are produced by endothelial cells, keratinocytes, monocytes, and Langerhans cells, and their release is stimulated by interferon (IFN)- γ and tumour necrosis factor (TNF)- α ³⁷.

Ad 3) Activated T cells produce a certain cytokine profile and based on this T cells are generally divided in two types. Type 1 T cells produce the pro-inflammatory cytokines interleukin (IL)-2, IFN- γ , and TNF- α , whereas type 2 T cells produce cytokines such as IL-5, IL-4, and IL-10. Activated T cells isolated from psoriasis lesions showed a predominant type 1 cytokine profile^{38,39}. Both CD4⁺ and CD8⁺ T cells can produce type 1 cytokines^{40,41}. There have been several reports describing a predominance of cytotoxic CD8⁺ T cells in psoriatic lesional epidermis, whereas CD4⁺ cells are the predominant type in lesional dermis^{13,14,42}. The secretion of cytokines by activated T cells influences neighbouring cells, such as keratinocytes and dendritic cells, which in turn release additional cytokines, creating a chronic inflammatory cascade⁴¹. IFN- γ has been shown to

induce epidermal hyperplasia when injected into non-lesional skin of psoriasis patients⁴³. Also, IFN- γ stimulates the expression of ICAM-1 by epidermal keratinocytes, facilitating the binding of T cells to keratinocytes. TNF- α is another important proinflammatory cytokine that plays a role in the pathogenesis and maintenance of psoriasis. The role of TNF- α and TNF- α inhibitors will be discussed further on in the introduction.

T cells in psoriatic arthritis In synovial tissue, activated memory CD4+ T cells predominate^{7,44}, which are focally distributed near small blood vessels and the intimal lining layer⁶, whereas in synovial PsA fluid activated CD8+ T cells predominate⁴⁵. The demonstration of oligoclonal expansions of T cells derived from synovial fluid of patients with PsA supports the hypothesis that T cells are involved in the pathogenesis of PsA⁴⁶. Findings of common T cell receptor β V expansions in psoriatic skin and synovium suggest an important role for cognate T-cell responses and suggest that the inciting antigens are identical or homologous between afflicted skin and synovium⁴⁷. Numerous chemokines and cytokines, such as TNF- α , IL-1 β , IL-2, IL-8, IL-10, IL-15, IL-18, and IFN- γ are believed to play a role in triggering cell proliferation and sustaining joint inflammation in PsA⁴⁸⁻⁵². The expression of the cellular adhesion molecules ICAM-1, VCAM-1, and E-selectin facilitate migration of activated T cells through the vascular endothelium and formation of an infiltrate in synovial PsA tissue⁵³. Further evidence that T cells play a role in the pathogenesis is provided by the observation that anti-T-cell targeted therapy has proven to be beneficial in PsA⁵⁴.

TNF- α in psoriasis TNF- α is a pro-inflammatory cytokine produced by activated T cells, keratinocytes, monocytes, and dendritic cells in human skin⁵⁵. It exists as a membrane-bound molecule on cells that produce it, as a soluble protein in the circulation, and bound to cell surface receptors on target cells, such as keratinocytes, dendritic cells, T cells, NK cells, and endothelial cells⁵⁶. TNF- α has numerous effects on the immune response, correlating with the clinical and histological pathology seen in psoriatic skin. TNF- α can induce the expression of adhesion molecules, such as ICAM-1, VCAM-1, and E-selectin^{57,58}, and vascular growth factors, such as vascular endothelial growth

factor (VEGF)⁵⁹ in the skin, promoting T cell trafficking. In addition, TNF- α has been demonstrated to increase the expression of other proinflammatory cytokines, such as IL-1, IL-5, IL-6, and transforming growth factor, and chemokines, such as IL-8, a member of the CXC chemokine family, thereby enhancing the infiltration of T cells into the epidermis^{60,61}. Nuclear factor κ B (NF κ B), a nuclear transcription factor that is crucial in inflammation, is also activated by TNF- α ⁶². Finally, TNF- α stimulates mature Langerhans cells to migrate from the skin to the lymph nodes, where antigen presentation and T-cell activation take place⁶³. Substantial evidence suggests that TNF plays a fundamental role in the pathogenesis of psoriasis. Increased levels of TNF- α compared to controls have been reported in psoriatic lesional skin^{58,64}. Levels of TNF- α in psoriatic lesional skin have been found to correlate with severity of psoriasis⁶⁵. In addition, levels of TNF in skin and serum of psoriasis patients have been demonstrated to decrease after successful therapy⁶⁶⁻⁶⁸. The most convincing evidence, linking TNF- α to psoriasis, is the ability of TNF- α inhibitors such as etanercept, infliximab, and adalimumab to ameliorate clinical symptoms of psoriasis. Clinical trials with infliximab and etanercept for psoriasis and PsA are discussed elsewhere in the introduction.

TNF- α in psoriatic arthritis In PsA, TNF- α activates NF- κ B, leading to synovial cell proliferation, leukocyte trafficking, further proinflammatory cytokine production, and up-regulation of RANKL-mediated osteoclastogenesis^{50,53,69}. A significantly higher concentration of TNF- α and its receptors have been reported in PsA synovial fluid compared with osteoarthritis^{48,49,53,70}. Examination of serial synovial biopsies in four PsA patients, who participated in an open study on the effects of the chimeric anti-TNF antibody infliximab, has shown that clinical improvement of peripheral arthritis activity is associated with decreased intimal lining layer hyperplasia, reduced vascularity, and reduced polymorphonuclear cell and macrophage infiltration⁷¹.

Angiogenesis in psoriasis Neovascularization appears to play an important role in the evolution of a psoriatic plaque. Epidermal proliferation is closely associated with vascular expansion in the superficial dermis early in the development of a psoriatic plaque. Studies demonstrated that abnormal blood

vessel growth could predict the area of skin to be involved in the inflammatory process⁷². Microvascular changes include exaggerated tortuosity, pronounced dilatation, increased permeability, and endothelial cell proliferation within the capillaries in the dermal papillae^{73,74}. Vascular proliferation is driven by the local expression of angiogenic molecules (mostly derived from keratinocytes), such as transforming growth factor- α , TNF- α , plasminogen activator inhibitor (PAI)-1, platelet-derived endothelial cell growth factor, endothelial cell stimulating angiogenesis factor (ESAF), and vascular endothelial growth factor (VEGF)^{75,76}. VEGF induces microvascular hyperpermeability and acts as an endothelial cell-specific mitogen, and is recognized as a central regulator of angiogenesis^{77,78}. Both ESAF and VEGF were found to be elevated in plaques of psoriasis as compared with uninvolved skin and normal skin⁷⁹. Tissue and serum levels of EASF, PAI-1, and VEGF correlated with the clinical severity of psoriasis, suggesting a pathogenetic role in psoriasis⁸⁰⁻⁸². Indeed, a randomized phase I/II trial with Neovastat, an inhibitor of angiogenesis, revealed a dose-dependent effect of this drug in the improvement in psoriasis⁸³.

Angiogenesis in psoriatic arthritis In previous studies of PsA the most significant histological findings were vascular changes, described as tortuosity and higher intensity of villous vascularization^{9,10,84,85}, supporting the theory that microvascular changes play an important role in the pathogenesis of PsA⁸⁶. In addition, synovial fluid metalloproteinases such as matrix metalloproteinase (MMP)-9 correlate with the pattern of neo-vascularization and synovial fluid VEGF levels⁸⁷. Elevated VEGF concentrations, produced by macrophages and fibroblast-like synoviocytes, have been reported in serum and synovial fluid of patients with PsA^{81,88,89}. Other growth factors that control angiogenesis are the angiopoietins (Ang). Ang1 induces stable maturation of blood vessels, whereas Ang2 plays a role in vessel remodelling and maturation. Ang2 and VEGF mRNA expression and protein levels were significantly higher in early PsA compared with rheumatoid arthritis synovium^{81,86}. Angiogenesis also plays a role in bone formation, since invasion of the cartilage by new blood vessels precedes osteoblastic transformation and ossification. However, data on vascular changes in PsA synovial tissue are sparse and sometimes conflicting, possible due to differences in patient's selection.

Genetics in psoriasis and psoriatic arthritis⁹⁰

Population and twin studies support the concept that psoriasis and PsA have a genetic basis^{91,92}. Genetic linkage studies have confirmed a genetic predisposition. It has been estimated that the HLA-associated allele PSORS1 on chromosome 6p accounts for 30 to 50% of the genetic contribution to psoriasis⁹³. This locus contains genes coding for HLA-C, corneodesmosin (Cdsn), and alpha-helix coiled-coil rod homolog (HCR) which were found to be expressed at higher levels in psoriatic lesional skin than in normal skin⁹⁴⁻⁹⁶.

18 Predisposing loci other than HLA include 17q24-q25 in a variety of Caucasian populations⁹⁷⁻⁹⁹, 4qter (PSORS3) in Irish families¹⁰⁰, 1q21 (PSORS4) in Italian and U.S. families^{101,102}, 3q21 (PSORS5) in Swedish families¹⁰³ and some other loci. Strong evidence of familial aggregation in PsA has been found as well, the risk of PsA was found to be 50 times greater in first-degree relatives of PsA patients than in a control population⁹¹. However, association of the HLA locus with PsA has been less clear-cut than with psoriasis¹⁰⁴. HLA B27 has traditionally been associated with spinal inflammation¹⁰⁵, and associations of PsA with other loci have been described as well^{106,107}. Finally, promoter polymorphisms of the genes encoding TNF- α and IL-1 β have been found to be associated with different subtypes of psoriasis characterized by early and late disease onset^{108,109}.

It is clear that the genetic predisposition for psoriasis and PsA is complex and cannot be ascribed to a single gene. For now, the cause of psoriasis and PsA can be considered to be multifactorial, resulting from an interaction of genetic, environmental, and immunological factors.

Biological response modifiers in psoriasis and psoriatic arthritis

Why do we need new therapies for psoriasis and PsA? Current therapies such as photo(chemo)therapy, cyclosporine A, and methotrexate are effective for psoriasis, but are limited in their use because of their potential side-effects. Cyclosporine may cause hypertension and renal failure, and methotrexate commonly causes malaise and hepatotoxicity. Photo(chemo)therapy, in particular PUVA, increases the risk of skin cancer, which limits its use in a chronic disease such as psoriasis. In addition, a National Psoriasis Foundation Survey showed that only 26% of patients with psoriasis are satisfied with their

current treatment¹¹⁰. Therapeutic options for patients suffering from PsA have been limited as well during the last decades. In contrast to rheumatoid arthritis, no disease-modifying anti-rheumatic therapy has been available for PsA except for methotrexate. Instead, symptom-modifying drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) are used to ameliorate pain. For these reasons there is a clear need for effective and long-lasting anti-psoriatic and anti-PsA treatments with limited side-effect profiles.

Improved understanding of the immunopathogenetic mechanisms in psoriasis and PsA has led to the rapid development of the so-called biological response modifiers ("biologicals"), a versatile group of engineered bioactive proteins. Biologicals can be divided into three main groups according their molecular structure: monoclonal antibodies, fusion proteins, and cytokines. The generic names of the biologicals comply to a strict nomenclature: chimeric monoclonals end with -ximab, humanized monoclonals end with -zumab, human monoclonals end with -umab, and receptor-antibody fusion proteins end with -cept⁶. Table 1 shows the biological response modifiers currently approved or under development for psoriasis and/or PsA.

Monoclonal antibodies

anti-adhesion *Efalizumab* Efalizumab is a recombinant, humanized monoclonal IgG1 antibody directed against the α subunit (CD11a) of leukocyte function-associated antigen (LFA)-1¹¹¹. In psoriasis, binding of LFA-1 on memory T-cells to ICAM-1 on keratinocytes and vascular endothelial cells leads to T-cell activation as well as trafficking of T cells from the circulation into the skin¹¹². This provides the rationale for blocking the LFA-1/ICAM-1 interaction with efalizumab in the treatment of psoriasis¹¹³. Data from in vitro studies demonstrated that efalizumab inhibits multiple key pathogenic steps in psoriasis: T-cell activation¹¹⁴, T-cell trafficking to the skin¹¹⁵, and T-cell adhesion to keratinocytes¹¹⁴. The biological effects of efalizumab and clinical activity were demonstrated in clinical trials^{116,117}. Gottlieb et al (2000) demonstrated in an open label study that intravenous administered efalizumab resulted in improvement in psoriasis area and severity index (PASI), decreased numbers of epidermal and dermal T cells, decreased expression of ICAM-1 on keratinocytes and blood vessels, and epidermal thinning. There was an inverse

relationship between sustained CD11a down-modulation and saturation and improvement of histological parameters¹¹⁸. Another open-label study by the same author confirmed these observations, and demonstrated a dose-response relationship both clinically and histological. The mean PASI decrease in the highest dosage group was 47 percent¹¹⁷. In the peripheral blood, administration of efalizumab to psoriasis patients resulted in an increase in circulating leukocytes, which was largely caused by an increase in T-cell numbers as opposed to other leukocytes. The largest increase was observed in memory CD8⁺ T cells, suggesting that efalizumab blocks cutaneous entry of memory CD8⁺ T cells¹¹⁹. Immunohistochemical changes induced by efalizumab were mirrored by reports on clinical improvement in psoriasis patients treated with efalizumab. In a randomized, double-blind, placebo-controlled multicentre phase II trial 144 psoriasis patients received either placebo or i.v. efalizumab 0.3 mg/kg for 8 weeks. The percentage of efalizumab-treated patients achieving more than 50% improvement in physician's global assessment at day 56 was 48%, compared to 15% of the placebo-treated patients. Epidermal thickness was reduced by 37% in efalizumab-treated patients and 19% in placebo-treated patients. Treatment was well tolerated; mild to moderate flu-like complaints were the most common adverse events. Depletion of circulating lymphocytes did not occur¹²⁰. To provide a more convenient mode of administration, a subcutaneous formulation of efalizumab was developed and used in phase III clinical trials. Five hundred ninety-seven patients with chronic plaque psoriasis were treated with either efalizumab (1 or 2 mg/kg s.c.) or placebo once weekly for 12 consecutive weeks in a phase III multicentre, randomized, placebo-controlled, double-blinded study. Depending on the response after 12 weeks, subjects received an additional 12 weeks of treatment with efalizumab or placebo. At week 12, PASI 75 (75% or more reduction in PASI) was achieved by 22% of the patients who had received 1 mg/kg of efalizumab and 28% of those who had received 2 mg/kg of efalizumab, as compared with 5% of the subjects in the placebo group. Efalizumab-treated subjects had greater improvement than those in the placebo group as early as week 4. After the discontinuation of efalizumab at week 24, an improvement of 50 percent or more in the PASI was maintained in approximately 30% percent of patients during the 12 weeks of follow-up.

Efalizumab was well tolerated, and adverse events were generally mild to moderate¹²¹. Finally, in a recently performed phase III, multicentre, randomized, double-blinded, placebo-controlled study 556 patients with moderate to severe psoriasis were treated with efalizumab 1 mg/kg s.c. or placebo weekly for 12 weeks. At week 12, 27% of efalizumab-treated patients achieved PASI 75, and 59% of efalizumab-treated patients achieved PASI 50 (50% or more reduction in PASI). After the first 12-week treatment period, all patients were treated with 1 mg/kg efalizumab weekly for another 12 weeks. After 24 weeks of continuous efalizumab therapy, 44% of patients achieved PASI 75 and 67% achieved PASI 50, suggesting that extending efalizumab treatment from 12 to 24 weeks leads to improved efficacy. There was a decline in adverse events during the study without evidence of cumulative toxic effects¹²². In phase III studies, efalizumab has been anecdotally reported to improve PsA. This finding is in agreement with the notion that the interaction between LFA-1 and ICAM-1 represents a major adhesion pathway in lymphocytic homing in PsA¹²³. Recently, efalizumab was tested in a phase II randomized trial in 107 patients with PsA. Preliminary results showed that after 12 weeks of treatment, 28 percent of the efalizumab-treated patients achieved a 20% or more reduction in American College of Rheumatology (ACR¹²⁴) response criteria, compared to 19 percent of the placebo-treated patients¹²⁵. Efalizumab is approved for the treatment of moderate to severe psoriasis in the U.S.A. and most countries in Europe.

anti-cytokine

Infliximab Infliximab is a monoclonal chimeric antibody composed of a murine anti-TNF Fab fragment joined to the constant region of human IgG1. Each molecule of infliximab has two antigen-binding sites for TNF- α , allowing for increased binding avidity to both membrane-bound and soluble TNF- α ⁵⁶. Infliximab can neutralize both transmembrane-bound TNF- α on the cells that synthesize it (e.g. T cells, keratinocytes, and dendritic cells), and soluble circulating TNF- α , thereby inhibiting the pro-inflammatory effects of TNF- α . In addition, *in vitro* studies have suggested that binding of infliximab to membrane-bound TNF- α could lead to lysis of TNF- α producing cells via activation of complement-dependent or antibody-dependent cell-mediated

toxicity¹²⁶. The clinical efficacy of infliximab in psoriasis was first demonstrated when a patient treated with infliximab for Crohn's disease showed a dramatic improvement of her psoriasis lesions as well¹²⁷. Next, the clinical efficacy of infliximab was confirmed in a small investigator-initiated, randomized, double-blinded, placebo-controlled trial of 33 patients with plaque type psoriasis, who were treated with either infliximab i.v. 5 mg/kg, infliximab i.v. 10 mg/kg or placebo at weeks 0, 2, and 6. At week 10, PASI 75 was achieved by 82 percent of the patients in the 5 mg/kg infliximab and 73 percent in the 10 mg/kg infliximab group, compared with 18 percent of patients in the placebo group. The infliximab-treated group showed a safety profile similar to that of the placebo group, with headache being the only adverse event recorded more frequently in the 10mg/kg group¹²⁸. At the end of this study, 'non-responding' patients in the placebo-group were randomized to receive either 5 mg/kg or 10 mg/kg infliximab at weeks 10, 12, and 16, and responding patients in the infliximab groups were evaluated for relapse (loss of at least half of the improvement in PASI at week 10) and retreated with open-label infliximab (5 or 10 mg/kg) as needed. In all, 29 patients received either 5 or 10 mg/kg of infliximab. At week 26, 55 percent of patients maintained a PASI 50 or better, and 48 percent of patients maintained at least PASI 75¹²⁹. Subsequently, the efficacy and safety of infliximab for the treatment of psoriasis were investigated in a phase II trial (SPIRIT trial) from which the results were recently published. In this study, 249 patients with severe plaque psoriasis were randomized to receive infliximab 5 mg/kg i.v., infliximab 3 mg/kg i.v., or placebo at weeks 0, 2, and 6. At week 10, 88 percent of the patients in the 5 mg/kg infliximab group and 72 percent of the patients in the 3 mg/kg infliximab group achieved PASI 75, compared to 6 percent in the placebo group. Furthermore, patients treated with infliximab showed rapid onset of improvement from baseline in psoriasis. Maximum response to infliximab therapy was observed 10 weeks after the first infusion. The duration of response was variable for individual patients, but in general patients started to lose response after 10 weeks (3 mg/kg group) and 14 weeks (5 mg/kg), respectively. Four patients were considered to have serious adverse events related to infliximab therapy, which included squamous cell carcinoma, cholecystitis, diverticulitis, and pyelonephritis with sepsis. Infusion reactions, such as chills, headache, nausea, and dyspnoea,

were reported in 18 and 22 percent of patients in the infliximab (3 and 5 mg/kg) groups, respectively, compared with 2 percent in the placebo group. There were no serious or life-threatening infusion reactions. Although the incidence of infusion reactions at week 26 was approximately 2 to 3-fold higher for patients with antibodies to infliximab relative to those who were negative for antibodies, the majority of patients with antibodies did not have any infusion reaction. The incidence of newly positive anti-nuclear antibodies (ANAs) observed in this study was 22-25 percent, however, no patients in this study developed symptoms of drug-induced lupus or lupus like syndrome. Other safety concerns such as the development of tuberculosis or malignant lymphoma were not observed during this study¹³⁰. In addition to the beneficial effect of infliximab on skin lesions in psoriasis, infliximab has been demonstrated to reduce clinical signs and symptoms of PsA as well. Ten patients with severe polyarticular PsA were treated with infliximab 5 mg/kg i.v. in combination with their current therapy at weeks 0, 2, and 6 in a small open-label study. At week 10, 8 of 10 patients achieved an ACR 70 response¹³¹. In another open-label study, 9 patients with active PsA were treated with infliximab 3 mg/kg i.v. at weeks 0, 2, 6, 14, and 22. At week 22, ACR 20, ACR 50, and ACR 70 were achieved by 8, 5, and 2 patients, respectively¹³². The observations in these studies led to a larger-scale, 16-week placebo-controlled trial followed by a 34-week open label extension to test the efficacy and safety of infliximab 5 mg/kg in 102 patients with PsA (IMPACT trial). At week 16, ACR 20, ACR 50, and ACR 70 were achieved by 69, 49, and 29 percent of patients in the infliximab group and 8, 0, and 0 percent of patients in the placebo group. The improvement observed in this study was not clearly related to the continued use of concomitant disease-modifying anti-rheumatic drugs¹³³. Recently, results were published from the IMPACT2 trial, a phase III study in which the efficacy and safety of infliximab were investigated in a larger population of patients with PsA. A total of 200 patients with active PsA were randomized to receive either placebo or infliximab 5 mg/kg i.v. at weeks 0, 2, 6, 14, and 22, in combination with stable doses of MTX. At week 14, ACR 20, ACR 50, and ACR 70 was achieved by 58, 36, and 15 percent of infliximab-treated patients, and 11, 3, and 1 percent of placebo-treated patients, respectively. The incidence of adverse events was comparable between the

infliximab group and the placebo group. No opportunistic infections, such as tuberculosis, or serious infusion reactions were observed. Five percent of infliximab-treated patients were positive for anti-infliximab antibodies at week 22. Newly positive ANAs were detected in 10 percent of infliximab-treated patients. None of the patients developed a lupus-like condition¹³⁴.

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Adalimumab Adalimumab is a fully human-derived recombinant monoclonal antibody that binds TNF- α and blocks its interaction with the p55 and p75 cell surface TNF receptors¹³⁵. Initially, adalimumab was developed to treat rheumatoid arthritis, but recently two patients with chronic severe recalcitrant psoriasis and PsA were reported who experienced significant improvement in both skin and joint disease after treatment with adalimumab 40 mg s.c. every other week¹³⁶. The results of a phase II, double-blinded, placebo-controlled, randomized trial were presented as a poster publication at the American Academy of Dermatology meeting in February 2004. Patients with moderate to severe plaque psoriasis (n=148) were treated with adalimumab 40 mg s.c. weekly, adalimumab 40 mg s.c. every other week (eow), or placebo. At week 12, PASI 50 was achieved by 88 percent of patients treated with adalimumab weekly, by 76 percent of patients treated with adalimumab eow, and by 17 percent of placebo-treated patients. PASI 75 was achieved by 80 percent of patients treated with adalimumab weekly, 53 percent of patients treated with adalimumab eow, and by 4 percent of placebo-treated patients. Adalimumab was well tolerated, and injection site reactions were the primary side effect¹³⁵.

The effectiveness of adalimumab in PsA was evaluated in a placebo-controlled, double-blind study, called Adalimumab Effectiveness in Psoriatic Arthritis Trial (ADEPT). Three-hundred and thirteen patients with active PsA received placebo or 40 mg of adalimumab s.c. every other week. Of the 69 patients with greater than three percent of body surface involvement who were treated with adalimumab, 42 percent achieved a PASI 90 response at 24 weeks. Sixty percent of patients achieved an ACR 20 response at week 12, and sustained response through week 24. One-fourth of these patients achieved an ACR 70 response at week 24. The rates of adverse events and serious adverse events in the study were comparable for adalimumab and placebo. Results from the ADEPT trial were reported at the American College of Rheumatology congress in San Antonio, Texas in October 2004.

Cases of tuberculosis have been reported in clinical trials with adalimumab and other TNF blockers in rheumatoid arthritis¹³⁷, and patients should be screened for latent tuberculosis prior to treatment. The incidence of lymphoma was also increased during adalimumab therapy in RA patients compared to the incidence in the general population. However it is known that patients with rheumatoid arthritis have an increased risk of lymphoma compared to the general population¹³⁸. The assumption that adalimumab is less immunogenic than other anti-TNF- α agents remains to be proven by long-term safety studies.

Fusion proteins

Alefacept Alefacept is a fully human fusion protein consisting of the first extracellular domain of LFA-3 fused to the hinge, CH₂, and CH₃ sequences of IgG₁. The LFA-3 domain of alefacept binds CD2 on T cells and blocks the co-stimulatory LFA-3/CD2 interaction¹³⁹⁻¹⁴¹, thereby inhibiting T-cell activation and proliferation. In addition, when alefacept binds CD2 on memory T cells and engages with FC γ RIII IgG receptors on natural killer cells, granzyme-mediated apoptosis (programmed cell death) of T cells is induced^{142,143}. Because CD2 expression is higher on memory-effector (CD45RO⁺) CD4⁺ and CD8⁺ T cells^{26,144} compared with naïve (CD45RA⁺) T cells, alefacept is thought to produce a selective reduction in memory T cells. In addition, a small population of circulating dendritic cells is also CD2⁺, suggesting that alefacept could have an additional effect on this cell population¹⁴⁵.

In a phase II, multicentre, randomized, placebo-controlled, double-blinded study alefacept was evaluated as a treatment for psoriasis. Two hundred twenty-nine patients with chronic plaque psoriasis received either alefacept i.v. or placebo weekly for 12 weeks. Twenty-four percent of patients who had received alefacept were clear or almost clear after 12 weeks of therapy, and the average duration of remission in those patients who were clear was 8 months. Alefacept reduced peripheral-blood memory effector T-cell (CD45RO⁺) counts, which was correlated with the improvement in psoriasis. Alefacept was well tolerated and no serious adverse events related to alefacept were observed²³. The reduction in levels of circulating memory T-cell subsets after alefacept therapy was confirmed in other studies¹⁴⁶.

A phase III, randomized, double-blinded, placebo-controlled study was conducted to evaluate efficacy of two courses of alefacept in patients with chronic plaque psoriasis. Five hundred fifty-three patients received two 12-week courses of once-weekly intravenous alefacept 7.5 mg or placebo. During treatment and follow-up, PASI 75 was achieved by 28% of alefacept-treated patients, and PASI 50 was achieved by 56% of alefacept-treated patients. After a single course of alefacept, patients achieving PASI 75 maintained PASI 50 for a median duration of 7 months. In addition, 40% of patients who received 26 2 courses of alefacept achieved PASI 75 and 71% of patients achieved PASI 50, indicating that a second course of alefacept increases efficacy¹⁴⁷.

A multicentre, randomized, double-blinded, placebo-controlled phase III trial investigated the efficacy of intramuscular alefacept. A total of 507 patients with chronic plaque psoriasis were treated with alefacept i.m. (10 mg or 15 mg) or placebo once weekly during 12 consecutive weeks. Mean reductions in PASI in the 15-mg alefacept, 10-mg alefacept, and placebo groups reached a maximum of 46%, 41%, and 25%, respectively, at 6 weeks post dosing. Twenty-one percent of the 15-mg dose group achieved PASI 75 at 2 weeks post dosing. Improvement was long-lasting, and 12 weeks after completion of treatment, mean PASI in both alefacept groups had not returned to baseline values. There were no opportunistic infections and no cases of disease rebound¹⁴⁸. Preliminary data suggest that the efficacy of alefacept therapy might be enhanced by combination with narrowband or broadband UVB. In addition to improvement of clinical parameters, alefacept therapy has been shown to be associated with improvement of quality of life of patients with psoriasis^{149,150}. Regarding the safety of alefacept therapy, no opportunistic infections or organ toxicity related to alefacept therapy have been reported as to this date. The only adverse events that had a $\geq 5\%$ higher incidence in the alefacept group than the placebo group were chills, pharyngitis, and accidental injuries¹⁴⁷. However, some concerns have risen due to the fact that an adequate secondary immune response to infectious agents or antigens depends on memory-effector (CD45RO⁺) T cells, which are affected by alefacept therapy. For this reason a study was performed to assess the effect of alefacept therapy on both primary and secondary responses to a newly encountered antigen and the acquired immune response to a recall antigen (tetanus toxicoid).

Results of this study showed that alefacept did not impair primary or secondary antibody responses to a neoantigen or memory responses to a recall antigen¹⁵¹. Data available from patients treated with up to nine cycles of alefacept indicate that there is no increase in toxicity over time. Other safety issues concern the possible development of lymphoproliferative disorders associated with immunosuppressive therapy. Indeed, three cases of lymphomas were reported during the trials. Future use of alefacept in the postmarketing period will elucidate this important issue. Alefacept is approved for the treatment of moderate to severe psoriasis in the U.S.A., and to date more than 9000 patients with psoriasis have been treated¹⁵².

Etanercept Etanercept is a recombinant molecule comprising the human TNF- α p75 receptor fused to the Fc portion of human IgG1 molecule. By blocking the binding of TNF- α to cell surface receptors, etanercept neutralizes the biologic activity of TNF- α . In a double-blinded, placebo-controlled study 60 patients with psoriasis and PsA were randomized to receive either placebo or etanercept 25 mg s.c. twice weekly for 12 weeks. After 12 weeks, the median PASI improvement was 46 percent in etanercept-treated patients versus 9 percent in placebo-treated patients. PASI 75 was achieved by 26 percent of etanercept-treated patients, compared to none of the placebo-treated patients. ACR 20 was achieved by 73 percent of etanercept-treated patients versus 13 percent of placebo-treated patients. No serious adverse events were reported¹⁵³. Another randomized, double-blinded, placebo-controlled study to investigate the efficacy and safety of etanercept in 112 psoriasis patients showed similar data. After 12 weeks of treatment, 30 percent of the etanercept-treated patients achieved PASI 75, compared to 2% of placebo-treated patients. The observed improvement was sustained in time, and by 24 weeks of treatment 56 percent of etanercept-treated patients versus 5 percent of placebo-treated patients achieved PASI 75. Adverse events were similar among etanercept and placebo groups, except for injection site reactions which occurred more frequently in patients treated with etanercept¹⁵⁴. This phase II proof-of-concept study demonstrated that etanercept in patients with psoriasis was well tolerated and significantly improved the signs and symptoms of disease¹⁵⁵. Based on these results, a larger trial followed. In a 24-

week, double-blinded, phase III study 652 psoriasis patients were treated with either etanercept s.c. at a low dose (25 mg once weekly), a medium dose (25 mg twice weekly), a high dose (50 mg twice weekly), or with placebo. After 12 weeks, patients in the placebo-group began treatment with etanercept 25 mg twice weekly. At week 12, PASI 75 was achieved by 14 percent of patients in the low-dose group, 34 percent of patients in the medium dose group, and 49 percent in the high dose group, compared to 4 percent of patients in the placebo-group. The clinical responses continued to improve with longer treatment. At week 24, PASI 75 was achieved by 25 percent of patients in the low-dose group, 44 percent of the patients in the medium-dose group, and 59 percent of patients in the high-dose group. No occurrence of opportunistic infections or tuberculosis was reported during the course of the study. Eight etanercept-treated patients had serum samples that tested positive for non-neutralizing anti-etanercept antibodies, but no differences in efficacy or adverse events were observed in these patients compared to patients without anti-etanercept antibodies¹⁵⁶. The efficacy of etanercept in PsA was confirmed in a placebo-controlled, double-blinded trial in which 205 patients with active PsA received either placebo or 25 mg etanercept s.c. twice weekly plus a stable dose of methotrexate for 24 weeks. Differences in clinical response between the groups were evident at week 4, and were maintained throughout the treatment period. At 12 weeks, ACR 20, ACR 50, and ACR 70 was achieved by 59, 38, and 11 percent of patients in the etanercept group and 15, 4, and 0 percent of patients in the placebo group, respectively. After 24 weeks of treatment, ACR 20, ACR 50, and ACR 75 was achieved by 50, 37, and 9 percent of etanercept-treated patients, whereas the corresponding placebo responses were 13, 4, and 1 percent, respectively. In general etanercept therapy was well tolerated, however one patient in the etanercept group developed multiple sclerosis at the end of the study¹⁵⁷. Regarding the safety profile of etanercept, some published reports have reported drug-induced systemic lupus erythematosus (SLE) in association with etanercept therapy^{158,159}. However, the lupus-like symptoms in all reported cases resolved following discontinuation of therapy. Etanercept is approved for treatment of psoriasis and PsA in the U.S.A and Europe.

Cytokines and chemokines

rhIL-4 Psoriasis is characterized by the presence of type-1 cytokine-producing T cells in lesional skin³⁹. In experimental animal models of type-1 mediated autoimmune diseases immune deviation of type-1 into anti-inflammatory type-2 responses generally improves the disease, without inducing general immunosuppression. Recombinant human IL-4 (rhIL-4), which induces a type-2 phenotype, has been developed for treatment of psoriasis. In a prospective dose-escalating study, rhIL-4 s.c. was administered to 20 patients with severe psoriasis 3 times daily, 5 days a week, for 6 weeks. PASI decreased in all patients treated with rhu-IL-4; psoriasis improved more than 50% (PASI 50) in 19 patients. On immunohistochemical evaluation of lesional skin, rhu-IL-4 induced a switch from type-1 to type-2 responses, with close correlation between clinical improvement and reversal of the IFN- γ /IL-4 ratio. Adverse events were mild and included fever, headache, and oedema¹⁶⁰.

rhIL-11 Recombinant human IL-11 (rhIL-11) has demonstrated anti-inflammatory effects in vitro and in vivo. In animal models, treatment with rhIL-11 reduced pro-inflammatory cytokine levels produced by T cells and macrophages, such as IFN- γ and TNF- α ¹⁶¹, and polarized the T-cell response toward a type-2 response with increased IL-4 production¹⁶². In an open-label, dose-escalating, phase I clinical trial rhIL-11 s.c. was administered to 12 patients with psoriasis every day for 8 weeks. Eleven of 12 patients experienced reduction in PASI, ranging from 20 to 80 percent. Amelioration of disease by rhIL-11, as shown by reduced keratinocyte proliferation and cutaneous inflammation, was associated with decreased expression of products of disease-related genes, including k16, iNOS, IFN- γ , IL-8, IL-12, TNF- α , IL-1 β , and CD8, and with increased expression of endogenous IL-11¹⁶³.

Other

Tadakinig-alpha (rh-IL-18 BP) Tadakinig-alpha is a recombinant unmodified form of the naturally occurring human IL-18 binding protein that is capable of neutralising the biological activity of IL-18. IL-18 was first identified as an IFN- γ -inducing factor^{164,165}, and it has costimulatory functions on other type-1 cytokines such as TNF- α and IL-1 as well. It is believed that by reducing the levels of these

pro-inflammatory cytokines by rh-IL-18 BP, immunological balance in psoriasis and PsA will be restored. Phase I studies of r-hIL-18 binding protein are now completed, and a phase II clinical study is on-going in psoriasis and PsA.

Aims of the studies

30 In the last decade a whole range of new 'biological' response modifiers have emerged for the treatment of psoriasis and PsA. Although clinical efficacy has been monitored in multicentre, randomized, placebo-controlled, double-blinded trials, less is known about the actual mechanism of action of these drugs in lesional skin and synovium of patients with psoriasis and PsA in situ. Because biological response modifiers act on very specific steps in the immunological cascade, investigation of changes in immunohistochemical markers in lesional skin and synovium might provide us with more insight into the immunopathogenesis in psoriasis and PsA. Traditionally, the evaluation of the cellular infiltrate and protein expression in skin tissue sections is done by manual quantification. However, for reliable evaluation of histology in the development of new anti-psoriatic treatments there is a need for a more time-efficient and reproducible method. To test the use of digital image analysis in this situation we compared the assessment of immunohistochemically stained skin sections with the more traditional manual quantification and semi-quantitative analysis (**chapter 2**). The digital image analysis was used in subsequent studies with biological response modifiers. In **chapter 3**, we investigated the immunohistochemical changes in lesional psoriatic skin after alefacept therapy, a LFA-3/IgG1 fusion protein that interferes with the activation and proliferation of T cells by binding to the CD2 receptor on their surfaces. We focussed on lesional memory-effector T cells in particular, since it is known that alefacept selectively reduces memory-effector T cells in peripheral blood²³. In a similar way, we investigated changes in the inflammatory infiltrate, in particular memory-effector T cells, in synovial tissue in patients with PsA after alefacept therapy. This was the first time alefacept was administered to patients with PsA, and the clinically efficacy of this drug in PsA was reported as well (**chapter 4**). Next to biological response modifiers interfering with T cell activation, such as alefacept, the use of TNF- α -inhibitory drugs has proven to induce rapid and profound

improvement in clinical signs and symptoms of psoriasis and PsA. The mechanism of action of infliximab, a chimeric anti-TNF- α antibody, has not been fully elucidated yet. In vitro studies suggest that besides neutralization of TNF- α , infliximab might be able to induce apoptosis of TNF- α producing cells via activation of complement-dependent or antibody-dependent cell-mediated toxicity. In **chapter 5**, we first investigated the early effects (after 48 hours) of infliximab on serial skin and synovial tissue biopsy samples of patients with PsA, focussing in particular on apoptosis of T cells. Next, we evaluated the influence of infliximab on T cell infiltration and expression of adhesion molecules after 4 weeks of infliximab therapy in the same group of PsA patients (**chapter 6**). Another TNF- α -inhibitory drug that is known to improve clinical signs and symptoms of psoriasis and PsA is etanercept, a fusion protein consisting of two identical chains of a recombinant human TNF receptor (p75) monomer fused to the Fc portion of human IgG1. By competitive inhibition of the interaction of circulating TNF- α with cell surface-bound TNF-receptors, etanercept is thought to prevent TNF-mediated cellular responses by rendering TNF biologically inactive. To compare the mechanism of action of etanercept with infliximab, we studied the effects of etanercept therapy on the T-cell infiltrate, expression of adhesion molecules, and expression of angiogenesis markers in lesional skin of patients with chronic plaque psoriasis in a double-blinded placebo-controlled study (**chapter 7**).

Table 1. List of biological response modifiers that are approved or under development for psoriasis and/or psoriatic arthritis.

Binding characteristics	Generic name	Brand name	Molecular structure	Mode of action	Indication / Status
<i>Monoclonal antibodies</i>					
Anti-CD11a	Efalizumab	Raptiva	Humanized Mab	Binds to α subunit of LFA-1; blocks LFA-1 / ICAM-1 interaction	Psoriasis, FDA and EMEA approved
Anti-TNF- α	Infliximab	Remicade	Chimeric Mab	Neutralizes TNF- α ; mediated lysis of TNF- α cells	Psoriasis and PsA, phase III
Anti-TNF- α	Adalimumab	Humira	Humanized Mab	Neutralizes TNF- α	Psoriasis and PsA, phase II and III
<i>Fusion proteins</i>					
Anti-CD2	Alefacept	Amevive	Fusion protein of extracellular domain of human LFA-3 and Fc part of human IgG1	Inhibits LFA-3 / CD2 interaction, induces NK- cell mediated T-cell apoptosis	Psoriasis, FDA approved
Anti-TNF- α	Etanercept	Enbrel	Fusion protein of the extracellular domain of the human TNF- α R and Fc part of human IgG1	Neutralizes TNF- α	Psoriasis and PsA, FDA and EMEA approved
<i>Recombinant cytokines and chemokines</i>					
IL-4	Rhu-IL-4	?	Recombinant human IL-4	Immune deviation from Th1 to Th2	Psoriasis, phase II
IL-11	Rh-IL-11	?	Recombinant IL-11	Immune deviation from Th1 to Th2; suppression of inflammation	Psoriasis, ?
<i>Other</i>					
IL-18BP	Tadekinig -alpha	?	recombinant human IL-18 binding protein	Neutralizes IL-18	Psoriasis and PsA, phase II

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chapter

2

Digital Image Analysis for the evaluation of the inflammatory infiltrate in psoriasis

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Abstract

Traditionally the evaluation of the cellular infiltrate and protein expression in skin tissue sections is done by manual quantification. However, for reliable evaluation of histology in the development of new anti-psoriatic treatments there is a need for a more time-efficient and reproducible method. To test the use of digital image analysis (DIA) in this situation we compared the assessment of immunohistochemically stained skin sections with the more traditional manual quantification (MQ) and semi-quantitative analysis (SQA).

42 The number of CD3⁺ T cells and the expression of E-selectin were evaluated in stained paired skin biopsies from 11 patients with chronic plaque psoriasis before and after initiation of anti-psoriasis therapy. We observed significant correlations between MQ and DIA for the number of T cells (epidermis: $r=0.88$, $p \leq 0.01$, dermis $r=0.87$, $p \leq 0.01$). Both DIA and MQ were equally effective in detecting reductions of T-cell numbers in active-treated patients. MQ took 20 hours, compared to 6 hours for DIA. We also observed significant correlations between SQA and DIA for the expression of E-selectin ($r=0.88$, $p \leq 0.01$), although DIA was more sensitive than SQA to detect (early) changes. SQA took 10 hours, compared to 4 hours for DIA.

In conclusion, the quantification of the inflammatory infiltrate in psoriatic lesional skin by DIA generated similar results as MQ and SQA in a reliable, reproducible and more time efficient fashion.

Introduction

Increasing knowledge of inflammatory pathways and components involved in chronic plaque psoriasis, together with technological advances in drug manufacturing, have resulted in a whole range of new targeted therapies, using biologicals and targeted small molecules [1-5].

Traditionally, treatment effects are assessed by clinical tools such as the Psoriasis Area and Severity Index (PASI) [6] and the Body Surface Area (BSA). Both have limitations, especially in the first phases of clinical development. Efficacy of the therapeutic intervention using the PASI is usually expressed as the percentage of patients reaching reductions of 90%, 75%, and 50%, respectively. The interpretation may be an uncertain factor. For example, inter-observer variation may be substantial, although intra-observer variability is usually limited. Similarly, the inter-observer variation for the BSA is quite large. In addition, there is a general overestimation of the BSA involved [7].

These limitations, together with the high specificity of new compounds and the need for proof of concept early in the development process, has nourished the interest to take paired skin biopsies before and after therapy in order to monitor intracutaneous alterations as surrogate markers for efficacy.

Tissue sections can be analyzed by routine staining techniques, such as haematoxylin eosin staining, as well as by immunohistochemical staining to detect markers of epidermal proliferation and differentiation, inflammation [8] and cell activation [9].

The current 'gold standard' for quantification of the cellular infiltrate is manual quantification (MQ) using a transmitted light microscope equipped with a calibrated ocular grid. Protein expression of cytokines and adhesion molecules detected by immunohistochemical techniques are usually quantified by semi-quantitative analysis (SQA). These are established and reliable techniques but MQ is laborious, whereas SQA is time efficient but less sensitive to change. For both techniques documentation, reproducibility and standardization are major challenges, especially for comparing results generated at different sites. Computer-assisted quantification might combine efficacy with a high sensitivity to change. Therefore, we developed a digital image analysis (DIA) system to quantify the cellular infiltrate and the expression of adhesion molecules in psoriatic lesional skin.

The aim of this study was to compare DIA, MQ and SQA for the analysis of immunohistochemical staining of lesional psoriatic skin in patients treated with anti-psoriatic therapy.

Materials and Methods

44 **Patients** Eleven patients with active mild to severe chronic plaque psoriasis were included in the study. Patients participated in a single randomized clinical trial comparing the early effects of infliximab on the local and systemic activity of their disease. Demographic and clinical data have been published previously [10, 11]. Skin biopsies from all patients were taken at baseline, two days after initiation of treatment (either infliximab (n=6) or placebo (n=5)), and 28 days after initiation of treatment. Patients treated with placebo at baseline received infliximab on day 2. All patients received infliximab at day 14. The protocol was reviewed and approved by the medical ethical committee and all patients gave their written informed consent before enrolment. The study was conducted according to the Declaration of Helsinki principles.

Biopsy procedure Four-mm punch biopsies were taken from the inside border of a target psoriatic plaque at baseline, after 2 days, and 4 weeks after initiation of treatment, preferentially from a non-sun exposed area. Serial biopsies from each individual patient were obtained from the same target lesion, separated by at least 1 cm. The biopsy samples were randomly coded, snap-frozen in Tissue- Tek OCT (Miles, Elkhart, IN), and stored at -70°C until further processing. Five-micrometer cryostat sections were cut in a cryostat and mounted on glass slides (Star Frost adhesive slides, Knittelgläser, Germany), and stored at -70°C until immunohistochemical staining was performed. All samples were analyzed in triplicate to minimize random variation.

Antibodies Monoclonal antibodies (mAb) used for immunohistochemistry were anti-CD3 (BD-Pharmingen, San Jose, CA) to detect T cells and anti-E-selectin (CD62E, 68-5H11, BD-Pharmingen).

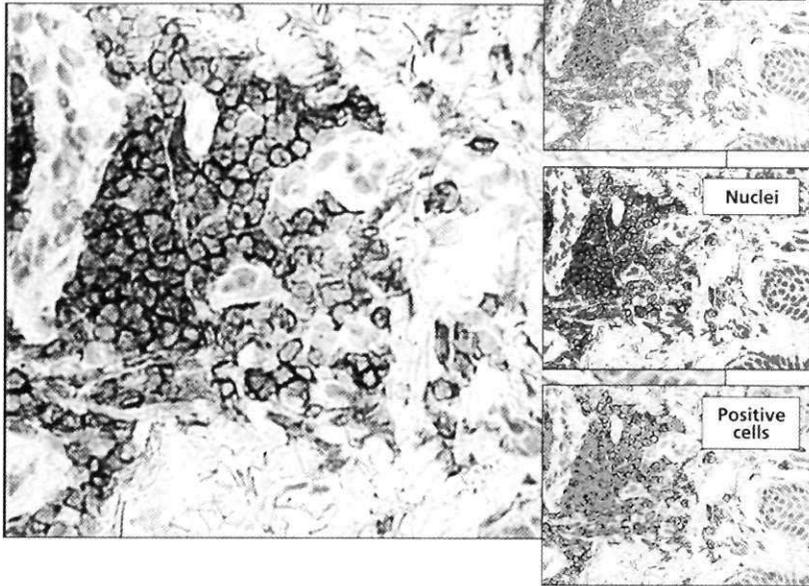
Immunohistochemical staining All sections were simultaneously stained under standardized conditions to minimize possible variation in incubation times and reagent volumes and concentrations. Sections were fixed in 100%

acetone for 10 min at 4°C, endogenous peroxidase activity was blocked (0.1% sodium azide and 0.3% hydrogen peroxide in Tris-buffered saline (TBS) for 20 min), and incubated for 15 min in 10% normal goat serum in TBS. Sections were incubated for 1 hour with the primary antibody in 1% bovine serum albumin (BSA; Sigma-Chemical Co, St. Louis, MO) in TBS, rinsed with TBS, incubated with biotin-conjugated goat-anti-mouse antibody (Dako, Glostrup, Denmark) for E-selectin, or with rabbit-anti-FITC (Dako) for CD3 in 10% normal human serum (NHS) in TBS for 30 minutes. Following a wash step with TBS, sections were incubated with horseradish peroxidase (HRP)-conjugated streptavidin (Dako) for E-selectin or with HRP-conjugated goat-anti-rabbit antibody (Dako) for CD3 in 1% BSA in TBS for 30 minutes. After a final rinse with TBS, HRP activity was visualized as a brown-red color by incubation with 3-amino-9-ethylcarbazole (AEC, Sigma-Aldrich). Sections were counterstained with Mayer's haematoxylin and mounted in glycerine-gelatine (Dako).

Manual quantification (MQ) of CD3⁺ T cells All sections were manually counted by two independent observers blinded for order, patient and clinical data. A standard binocular light microscope (Olympus, Tokyo, Japan) at a 200X magnification with a calibrated 0.5 x 0.5 mm ocular grid was used to count each separate CD3⁺ (red-stained) cell in the entire section. Only in case appropriate red staining was associated with a blue-stained nucleus, a cell was identified as CD3⁺. Positive cells were counted separately for the epidermis and dermis. The results were expressed as the number of cells per mm (epidermis) or per mm² (dermis). The mean MQ of the two independent observers was used. The time required to complete the MQ was formally recorded.

Semiquantitative analysis (SQA) of E-selectin expression Sections were analyzed by SQA by two independent observers blinded for order, patient and clinical data. With a standard binocular light microscope (Olympus) at 200X magnification the expression of E-selectin was scored on a 5-point scale (range 0-4) [12]. A score of 0 represented no expression, while a score of 4 represented abundant expression of E-selectin on dermal capillaries. Differences between observers were resolved by consensus. The time required to complete the SQA was formally recorded.

Digital image analysis (DIA) One observer (AYG) blinded for order, patient and clinical data performed both image acquisition and subsequent digital image analysis for all sections. The calibration procedure and differentiation between epidermis and dermis for the digital image analysis were repeated by an independent second observer. From each section a representative region of 1.45 x 1.45 mm (including epidermis and dermis) was identified at low power and separated in 20 consecutive high power fields (HPF). The HPF images were acquired with a 40X objective on a fully automated Leica DMRXA microscope (Leica, Wetzlar, Germany) with a Prior stage table, captured using a 3-chip CCD (Charged Coupled Device) video camera (Sony, Tokyo, Japan), and digitized using a Matrox 32bit color video digitizer card, using a highly standardized macro program written in the program language QUIPS for the Leica Qwin image analysis software (Leica, Cambridge, England) controlling stage table, condensor, diaphragms, light source and filters. The resultant color images were in a 740x570 pixel RGB format with a 24-bit resolution, enabling the use of 16,581,375 colors. For each acquisition session the microscope, camera, and computer were calibrated according to a standardized procedure, settings were recorded and stored and used for the entire session. The acquired images were stored using tagged image file (TIF) compression on a writable CD-ROM. Each acquisition was performed in one single session for each marker analyzed with fixed variables as derived from the calibration. The obtained TIFF-images were analyzed using a specialized algorithm written in the program language QUIPS operating a Qwin-based (Qwin Pro V2.4, Leica, Cambridge, U.K.) computer-assisted color video image analysis system [13]. To calibrate the thresholds for one marker at least 1 representative image from each section was used to select the pixels with color information compatible with a positive staining. The resulting composite threshold used for the final analysis was the consensus value of all calibration images used for the specific marker. Subsequently, this threshold was used and kept constant for all subsequent measurements with the same marker. For quality control during the analysis the area of interest (AEC red staining) and the total number of nuclei were determined, outlined in a pseudocolor (Fig 1) and displayed on a monitor screen. Subsequently, the software identified positive cells by combining the 2 masks where areas of a nucleus surrounded

CD3⁺

E-selectin

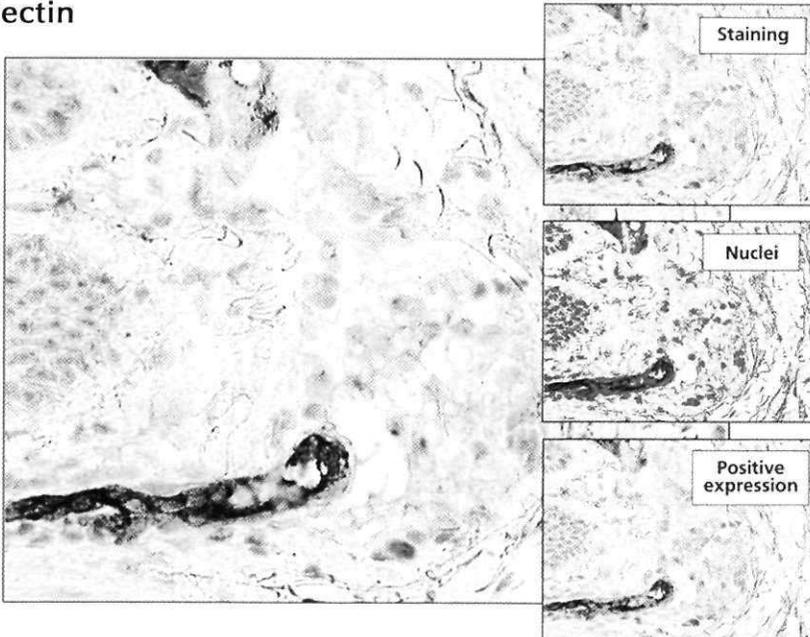


Figure 1. Example of Digital Image Analysis of CD3⁺ T cells (upper) and E-selectin expression (lower). Left side shows a high-power field image of a CD3 or E-selectin-stained tissue section (original magnification X400). Right side shows identification of red-brown staining with a green pseudo-color (upper corner), blue-stained nuclei with a purple pseudo-color (middle), and integration of blue-stained nuclei and red-brown staining, identifying CD3-positive stained cells or expression of E-selectin with a green pseudo-color (lower corner).

by a red-brown staining were identified as positive cells and isolated blue (nuclei without staining) or red were ignored.

In CD3-stained sections, the images were edited by marking the epidermis and dermis, enabling separate analysis of epidermis and dermis. Epidermal cell numbers were expressed as positive cells per mm; dermal cell numbers were expressed as positive cells per mm². For the assessment of the expression of E-selectin, the mean optical density, which is proportional to the cellular concentration of the protein, was multiplied by the area of positive staining, resulting in the integrated optical density (IOD) [14, 15].

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Statistical analysis SPSS 10.1.4 for Windows (SPSS, Chicago, IL, USA) was used for the statistical analysis. Correlations between the results obtained by the different methods to quantify the T-cell infiltrate and to quantify the expression of E-selectin, and correlations in outcome between different observers were calculated using the Spearman rank test. To calculate whether the difference between the two measurements was related to the magnitude of the measurement, the difference between the two methods was plotted against the standard (MQ), as suggested by Bland and Altman [16].

Results

Clinical improvement Improvement in PASI after four weeks of active treatment in each individual patient is shown in Table 1. Eight of 11 patients experienced a PASI reduction $\geq 50\%$, from whom 2 patients experienced a PASI reduction $\geq 75\%$ [10].

Table 1. PASI of each individual patient at baseline and after 4 weeks of active treatment.

	Baseline	Week 4	Reduction
1	29.8	12.6	58%
2	19.0	6.4	66%
3	14.1	6.0	57%
4	1.0	0.6	40%
5	14.9	5.4	64%
6	15.7	11.9	24%
7	9.2	7.6	17%
8	7.0	2.9	59%
9	8.3	2.3	72%
10	11.6	2.6	78%
11	5.0	0.6	88%

Changes in the number of CD3⁺ T cells; baseline versus 48 hours Already two days after treatment a significant difference in T-cell numbers in actively treated patients (n=6) versus placebo treated patients (n=5) was observed in the epidermis. Both MQ and DIA were able to detect the differences between the baseline and the 48 hours biopsies in a comparable fashion. Results of both analyses are shown in Figure 2.

Changes in the number of CD3⁺ T cells; baseline versus week 4 There was a significant reduction in the mean T cell number in the epidermis and dermis after 4 weeks of treatment (n=11). Both MQ and DIA were able to detect this reduction (Figure 2).

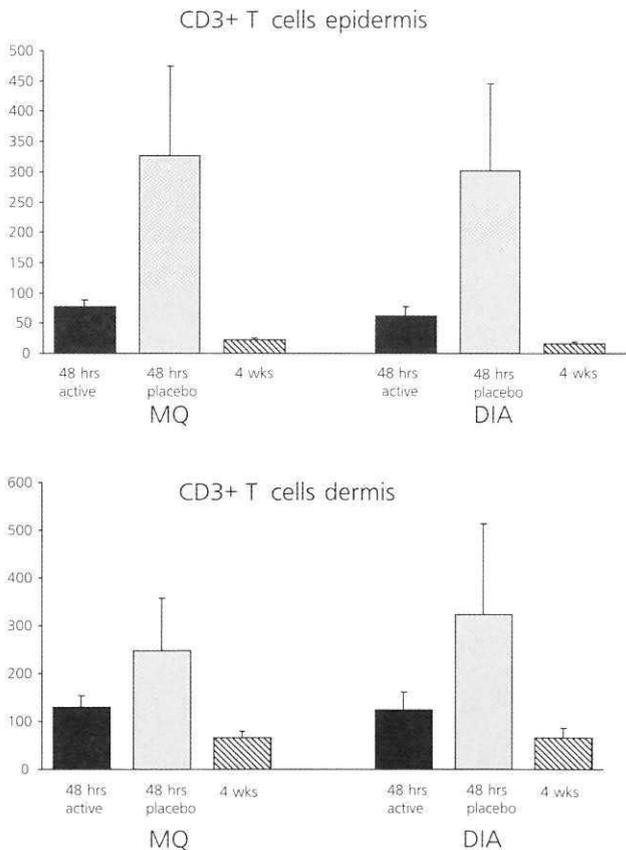


Figure 2. Mean reductions in T cell numbers. Epidermis and dermis were analyzed with both MQ and DIA. Baseline values are set as 100%, bars represent percentages from baseline. Results of MQ and DIA are shown for patients treated with active treatment (n=6, black bars) and placebo treatment (n=5, grey bars) after 48 hours of treatment, and after 4 weeks of active treatment (n=11, striped bars).

Correlations between MQ and DIA in CD3⁺ T cell infiltrate quantification

Three CD3-stained sections prepared from each biopsy from all patients (n=11) at baseline, after 48 hours and 4 weeks after initiation of treatment were analyzed by both MQ and DIA. In total 97 sections were analyzed by both methods, 2 sections were not suitable for analysis. Comparison between the mean and the s.e.m. of the MQ and the DIA revealed similar cell numbers (epidermis 45 cells/mm ± 4.3 (MQ) vs. 33 ± 4.5 (DIA), dermis 223 ± 15.8 (MQ) vs. 167 ± 18.8 (DIA)). We found strong Spearman rank correlations between MQ and DIA for both epidermal and dermal CD3⁺ T-cell numbers (rho 0.88, p ≤ 0.01 and rho 0.87, p ≤ 0.01 respectively). The scatter plots are shown in Figure

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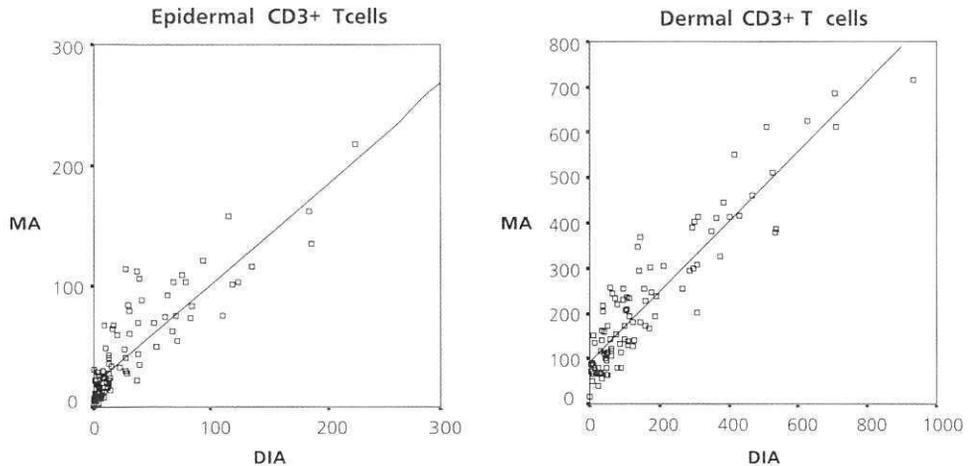


Figure 3. Scatter plot of T cell numbers, evaluated by MQ and DIA. Graph showing T-cell numbers of all skin tissue sections at baseline, 48 hours and 4 weeks (n=97), evaluated with MQ and DIA.

3. The Bland-Altman analysis showed no difference between MQ and DIA related to the magnitude of the cell infiltrate (epidermis rho -0.13, p=0.206, dermis rho 0.13, p=0.194) (Figure 4).

Changes in the expression of E-selectin; baseline versus 48 hours After 48 hours a significant reduction in the expression of E-selectin in the actively treated group was observed by DIA, which was not detected by SQA.

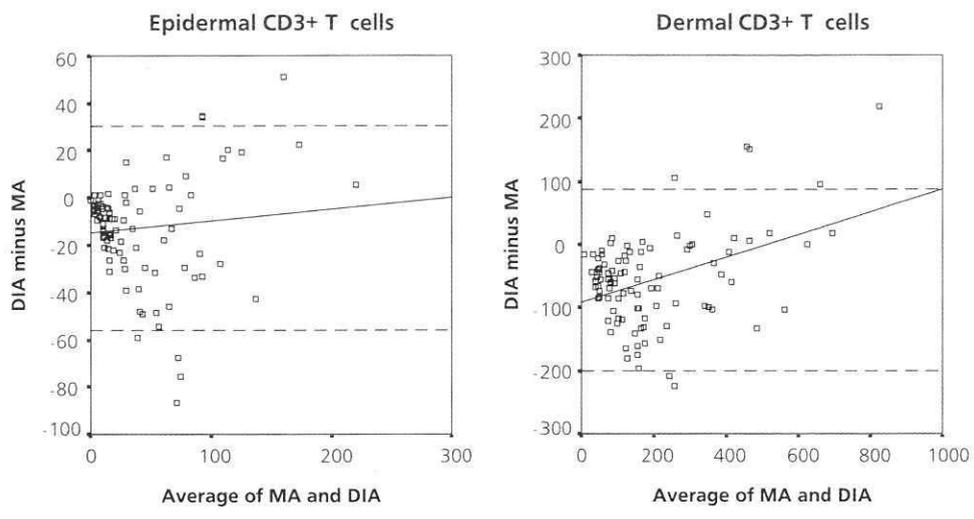


Figure 4. Scatter plot of T cell analysis according to Bland and Altman. Graph showing the difference between MQ and DIA against the average of MQ and DIA, with 95% limits of agreement (dashed lines) and regression line.

Changes in the expression of E-selectin; baseline versus week 4 There was a significant reduction in the expression of E-selectin after 4 weeks of treatment, assessed by DIA. SQA of the same section showed only a minor reduction in the expression of E-selectin after treatment.

Correlation between SQA and DIA in expression of E-selectin We compared SQA and DIA for their suitability to analyze the sections stained with anti-E-selectin-antibody (n=99). There was a strong correlation between

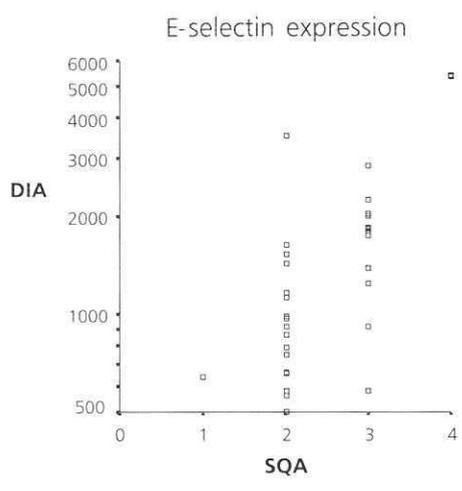


Figure 5. Scatter plot of expression of E-selectin. Graph showing the expression of E-selectin in all skin tissue sections at baseline, 48 hours and 4 weeks, evaluated with SQA and DIA.

SQA and DIA for detection of E-selectin expression (ρ 0.88, $p \leq 0.01$). A Bland-Altman analysis could not be performed because SQA and DIA are performed using different scoring systems. The scatter plot is shown in Figure 5.

Time required to complete the analysis We found a substantial difference in the time required to complete MQ, SQA and DIA.

52 MQ of T cell infiltration for all 97 sections (both epidermis and dermis) took approximately 20 hours to complete for each individual observer (approximately 10 minutes per section). The same sections were analyzed by DIA by a single observer in 6 hours, which includes 3 hours for acquisition of the images, and 3 hours for the quantification of T cells in both epidermis and dermis. An extra advantage of DIA is that cell numbers are automatically stored in an Excel-file and are available for further statistical analysis, in contrast to MQ.

SQA of E-selectin expression of 99 sections took approximately 10 hours (for each individual observer), whereas evaluation by DIA took 4 hours (3 hours for acquisition, 1 hour for quantification).

Inter-observer variability We did not find a significant inter-observer variability between the two independent observers for MQ in this study. Spearman rank correlation between two observers for MQ for T-cell infiltration showed ρ 0.92 and $p \leq 0.01$ for both epidermis and dermis. Correlation analysis between observers for SQA for E-selectin expression showed ρ 0.77 and $p \leq 0.01$.

Spearman rank correlation between two observers for DIA for T-cell infiltration and E-selectin expression showed ρ 0.83 (epidermis), ρ 0.93 (dermis) and ρ 0.86 (E-selectin) respectively, with $p \leq 0.01$ for all correlations (Figure 6).

Discussion

We compared the evaluation of immunohistochemically stained skin sections for CD3⁺ T cells by digital image analysis (DIA), and manual quantification (MQ). Despite the limited number of participating patients ($n=11$), and expected considerable inter-patient variability, T-cell numbers generated by both techniques before and after treatment showed strong correlations. The accuracy of DIA compared with MQ was not dependent on the magnitude of

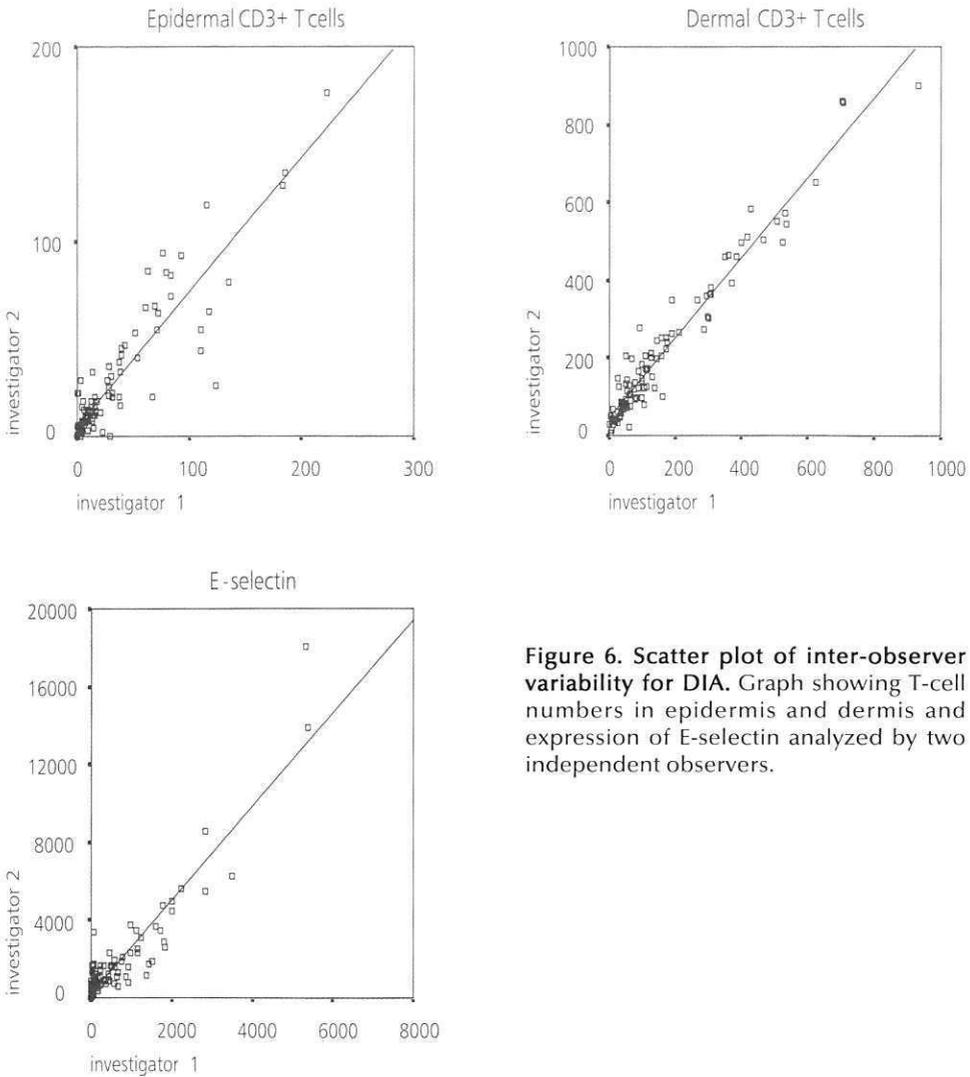


Figure 6. Scatter plot of inter-observer variability for DIA. Graph showing T-cell numbers in epidermis and dermis and expression of E-selectin analyzed by two independent observers.

the measurement, as shown by the Bland-Altman analysis [16]. Interestingly the absolute numbers obtained by DIA and MQ differed in range, apparently as result of the different algorithms used between the human brain and software. Both were equally effective in showing reductions in mean T cell numbers in the epidermis of actively treated patients compared with placebo treated patients after 48 hours, despite the wide minimum-maximum range observed in the measurements. There was also no difference in accuracy between these two methods when the epidermis and dermis were analyzed after 4 weeks of active treatment.

When we compared DIA with semi quantitative analysis (SQA) for the expression of E-selectin, we found DIA more competent to detect (early) changes in the expression of E-selectin after active treatment. This observation can be explained by the conservative nature of a 5-point scale of SQA compared to the numeric scale of DIA. However, the Spearman rank test showed good correlations between DIA and SQA for the detection of the expression of E-selectin. .

54 While CD3- and E-selectin staining are generally strong, crisp and easy to recognize, other antigen staining might be more difficult to analyze by DIA. For this reason we explored other antigen recognition such CD4, CD8, ICAM-1 and VCAM-1, which showed equal correlation between DIA and MQ/SQA (data not shown).

From the above-mentioned correlations, one can conclude that the use of DIA is a good substitute for MQ and SQA for a reliable, reproducible, and sensitive and time efficient analysis of multiple tissue sections. In the past 2 decades, several studies have been performed on the use of computer-assisted image systems [17, 18] for quantification of cells in immunohistochemically stained sections in different compartments of the human body, including synovial tissue [13-15, 19] and the central nervous system [20], as well as in malignant tumors [21-25]. There have also been reports on the use of computer-assisted video image analysis systems in dermatological diseases [26-28], especially in pigmented skin disorders [29, 30]. However, to our knowledge, in contrast to studies in synovial tissue [13, 31], there has been no report on the comparison between DIA systems versus the more traditional MQ and SQA for the quantification of the inflammatory infiltrate in skin tissue. Since synovial tissue and skin have different properties and reaction patterns, data from previous studies in synovial tissue cannot simply be extrapolated to psoriatic skin. However, as we have shown in the present study, DIA would be an adequate substitute for MQ and SQA in the immunohistochemical analysis of psoriatic lesional skin, and most likely also for other inflammatory disorders such as atopic dermatitis. The major advantage of DIA compared with MQ and SQA is the sensitivity of MQ combined with the reduction in time necessary to complete the analysis as in SQA. Another important advantage includes the standardization of the

analysis. A variety of scoring methods are currently used for MQ and especially SQA, since there is no clear consensus for these quantification techniques. For SQA, the intensity of staining has been quantified on 5-point scores or on visual analogue scales [9, 12]. Using DIA, the staining intensity is expressed as an absolute number in the IOD, providing an advantage over the subjective scales. High correlation coefficients were observed when the calibration analysis of the DIA was performed by two independent observers, suggesting that DIA is reliable and reproducible. The fact that one of the observers was untrained for DIA only strengthens this assumption. We did not find a significant inter-observer analysis with MQ and SQA in the present study either, probably due to extensively training of both observers for manual quantification prior to start of the study.

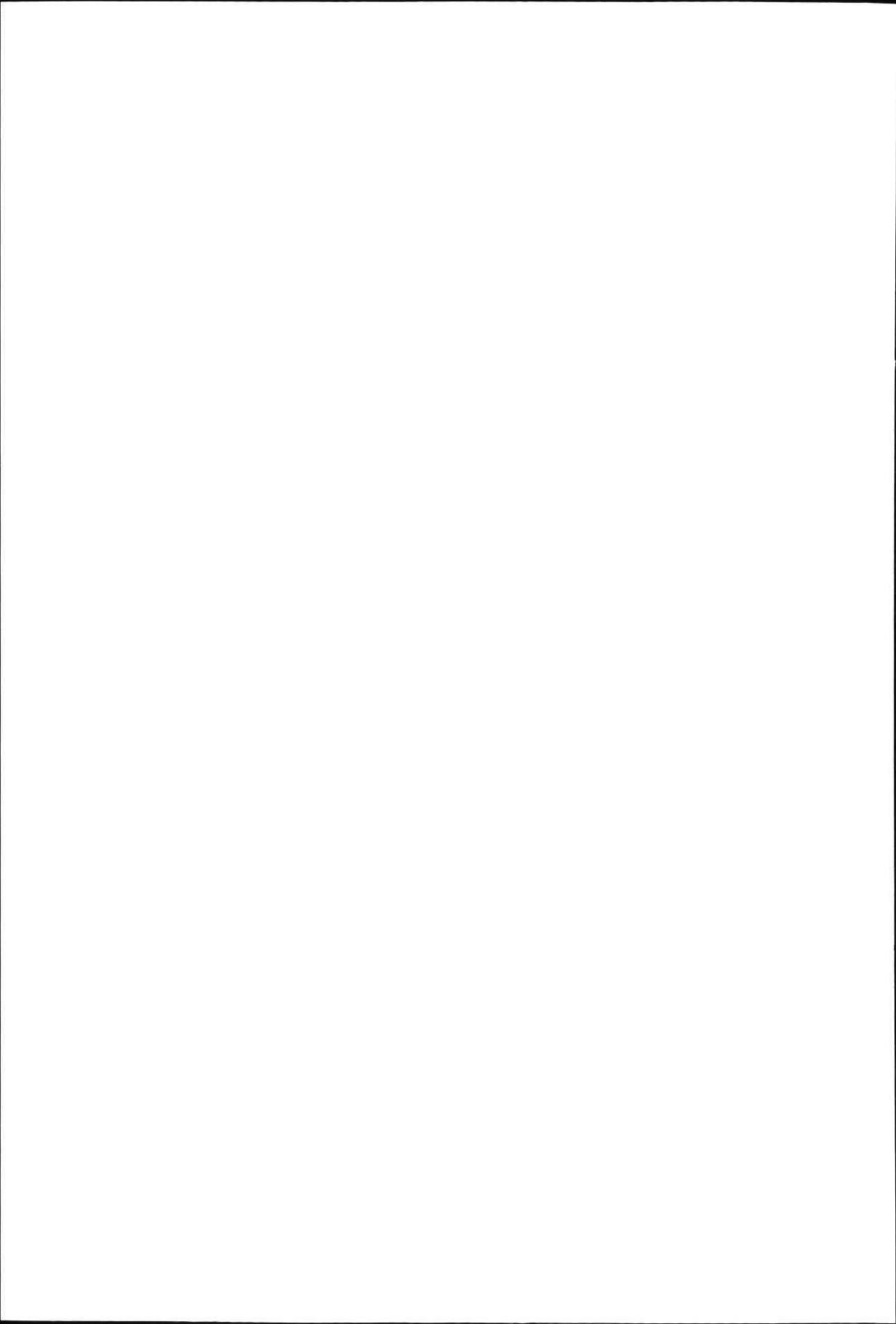
In contrast to the qualities mentioned above, DIA has some limitations and potential drawbacks as well. A computer is not able to interpret differences in staining intensity between different sections as the human eye and brain are. For example, aggregates of cells in thick-cut sections might be observed by a computer as a single object. For DIA it has proven to be necessary to follow standardized protocols for the cutting procedures and for the immunohistochemical staining to prevent irregularity in the number of superimposed cells. Secondly, observers have to be trained to be able to use the DIA system, which includes a learning curve. Without adequate training, DIA might be subject to some intra- and inter-observer variability as well [32]. Last but not least, the hardware and software of the DIA system are expensive compared to the normal light microscope used for MQ and SQA. Besides the initial costs of the DIA system itself, the systems usually require ongoing maintenance and upgrades. However, even when the above-mentioned disadvantages are taken into account, we believe DIA beholds promising options for future immunohistochemical studies in inflammatory skin diseases.

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chapter

3

Alefacept therapy reduces the effector T-cell population in lesional psoriatic epidermis

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Abstract

Alefacept, a LFA-3/IgG1 fusion protein, interferes with the activation and proliferation of T cells by binding to the CD2 receptor on their surfaces. The clinical efficacy of this drug has been demonstrated in chronic plaque psoriasis. We performed a single-centre, open-label study to investigate the immunohistochemical effects in psoriatic lesional skin. A group of 11 patients with plaque psoriasis all received 12 weekly doses of 7.5 mg alefacept intravenously. Skin biopsies were obtained at baseline and on days 8, 43 and 60 92, and were evaluated by digital image analysis after immunohistochemical staining. After completion of treatment, 8 out of the 11 patients experienced a PASI reduction of 50% or more compared to baseline. Immunohistochemical analysis displayed a gradual decrease in the number of cutaneous T cells during therapy, with a significant reduction in epidermal CD8⁺ cells and dermal CD4⁺ cells on day 92. Patients with a reduction in PASI of 50% or more after therapy had a clearance of effector / memory T cells from the epidermis, in contrast to patients with a reduction in PASI of less than 50%. These findings support the hypothesis that effector / memory T cells play a prominent role in the pathogenesis of psoriasis, and that alefacept is capable of reducing these cells in lesional psoriatic skin.

Introduction

Psoriasis is a common dermatological inflammatory disorder affecting about 2% of the Caucasian population. In the last two decades the cell infiltrate of psoriatic skin lesions has been investigated extensively (1-4). Lesions are characterised by epidermal hyperproliferation with abnormal differentiation of keratinocytes, dermal vascular proliferation, and accumulation of inflammatory cells in the epidermis and papillary dermis. The inflammatory infiltrate is primarily composed of CD4⁺ and CD8⁺ T lymphocytes, expressing CD45RO on the surface, indicating their effector/memory status (5,6). A substantial proportion of the infiltrating T cells also express markers such as the interleukin-2 receptor (CD25) and HLA-DR, indicating respectively early and mid-to-late activation. (3, 7-9). The beneficial effects of specific T-cell targeted therapies such as cyclosporine A and DAB389IL-2 toxin, support the key role for T lymphocytes in the pathogenesis of psoriasis (10-12).

Since the current therapeutic arsenal is not very specific, new immunomodulating drugs have been produced to treat patients with psoriasis. One of these new drugs is alefacept, a recombinant human LFA-3/IgG1 fusion protein designed to inhibit the interaction between CD2 and its ligand, leukocyte-function-associated antigen type 3 (LFA-3). LFA-3 is expressed on many cell types, including antigen-presenting cells. CD2 is weakly expressed on natural killer cells and on naive (CD45RO⁻) T cells, but is strongly upregulated on effector/memory (CD45RO⁺) T cells upon activation. (13,14). The LFA-3/CD2 interaction provides a costimulatory signal during the activation of T cells both by directly signalling T cells, and by enhancing the adhesive interaction of T cells and antigen-presenting cells. (15-18) The LFA-3 part of alefacept binds to the CD2 receptor on T cells and interferes with T-cell activation and proliferation (19,20). A recent study has also indicated an important role for the IgG domain of alefacept, which interacts with FcγR type III⁺ cells (e.g. natural killer cells and macrophages) to induce selective apoptosis of sensitive CD2⁺ cells (21). Since CD2 is upregulated on effector/memory (CD45RO⁺) T cells, alefacept is supposed to induce a selective reduction in these cells. This mechanism has recently been confirmed in *in vitro* studies (22).

Clinical data on the efficacy of alefacept therapy have been collected in phase 2 and 3 multicentre trials. In a recently reported placebo-controlled clinical

62 trial, alefacept was administered to patients with chronic plaque psoriasis once a week for 12 consecutive weeks. Alefacept proved to be an effective and well-tolerated treatment, and clinical improvement was sustained after the 12-week treatment period. A selective reduction was observed in the CD45RO⁺ T cell subpopulation in the peripheral blood during alefacept therapy (23). Still, only fragmentary information is available on the effects of alefacept therapy on the inflammatory infiltrate in lesional skin of patients with psoriasis. Kobayashi et al reported that clinical improvement of psoriasis by alefacept treatment correlates with a reduction in epidermal IFN- γ -producing T cells (24). Krueger et al. have reported a reversal of K16 expression in lesional epidermis in 8 of 13 patients with plaque psoriasis treated with alefacept for 12 weeks (25). This response was accompanied by a reduction in epidermal hyperplasia and a reduction in mean T cell counts, predominantly epidermal CD8⁺ and CD103⁺ (epithelial homing) T cells. These results indicate a therapeutic selectivity of alefacept on the major T cell subsets found in the epidermis. By investigating the changes in immunological cells and molecules in the dermal and epidermal compartment accompanying alefacept-induced resolution of psoriatic lesions, we aimed to elucidate the relative contribution of different cellular elements in the pathogenesis of psoriasis. In the present study we focussed on the changes in different T-lymphocyte subsets, activation markers and adhesion molecules in lesional skin of patients with plaque psoriasis treated with alefacept. We found a reduction in the number of epidermal effector/memory T cells, which correlated with clinical improvement. Reductions were present in both CD4⁺ and CD8⁺ epidermal T cells. We also observed a reduction in the presence of activation markers on T cells and a decreased expression of the adhesion molecule ICAM-1 after 12 weeks of alefacept treatment, indicating a downregulation of inflammation.

Materials and methods

Study design and patients This open-label, fixed-dose, single-centre study was conducted in adult patients with chronic plaque psoriasis, diagnosed at least 12 months prior to dosing. Participants had at least two representative psoriatic plaques. Patients with erythrodermic, guttate or generalized pustular psoriasis, or with a serious local or systemic infection within 3 months prior to dosing were excluded. Prior to the first dose, there was a wash-out phase

of 4 weeks for systemic retinoids, systemic fumarates, systemic steroids, methotrexate, cyclosporine A, azathioprine, thioguanine, investigational drugs, phototherapy (including artificial tanning beds) and high potency topical corticosteroids. In addition, there was a washout period of 2 weeks for moderate potency topical corticosteroids, vitamin D analogues or topical retinoids, keratolytics and coal tar (other than for scalp, palms, groin and/or soles of the feet). All patients were naive to alefacept. Patients who met the entry criteria received 7.5 mg of alefacept by i.v. bolus once a week for a total of 12 consecutive weeks and subsequently entered a follow-up period for 12 weeks. The protocol was reviewed and approved by the medical ethical committee and all patients gave their written informed consent before enrolment. The study was conducted according to the Declaration of Helsinki principles.

Clinical efficacy assessments The severity of psoriasis was assessed by the Psoriasis Area and Severity Index (PASI) and Total Body Area (TBA) at baseline, on days 15, 29, 43, 57 and 78 during the treatment period, and on days 92 (i.e. two weeks after the last dose), 106, 134 and 162 during the follow-up period.

Biopsies At baseline and on days 8, 43 and 92, 4-mm punch biopsies were taken from the inside border of a target psoriatic plaque under local lidocaine / adrenaline anaesthesia. All biopsies of each individual patient were taken from the same target lesion, separated at least by 1-2 cm. After being coded randomly, the biopsies were immediately embedded in TissueTek OCT compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands), snap-frozen by immersion in liquid nitrogen, and stored at -80°C until processing. Cryostat sections were cut at 5 µm, mounted on glass slides (Star Frost adhesive slides, Knittelgläser, Braunschweig, Germany), air-dried at room temperature, wrapped in aluminium foil, and stored at -80°C until staining. All sections were processed in a single staining session for each primary antibody.

Single staining procedures After fixation in 100% acetone for 10 min at 4°C, and blocking of endogenous peroxidase activity with 0.1% sodium azide and 0.3% hydrogen peroxide in Tris-buffered saline (TBS) for 20 min, the sections were incubated for 15 min in 10 % normal goat serum in TBS. Next, the sections

were incubated for 1 h with the primary antibody in 1% bovine serum albumin (BSA, Sigma-Chemical Co, St. Louis, MO) in TBS. The following mouse-anti-human monoclonal antibodies were used: FITC-conjugated anti-CD3 (clone SK 7, Becton Dickinson, San Jose, CA), anti-CD4 (SK3, Becton Dickinson), anti-CD8 (SK1, Becton Dickinson), anti-CD45RO (OPD4, Dako, Glostrup Denmark), anti-CD45RA (4KB5, Dako), anti-ICAM-1 (BBIG-L1, R&D Systems Inc., Minneapolis, MN), anti-E-selectin (68-5H11, Becton Dickinson), and anti-VCAM-1 (51-10C9, Becton Dickinson). After rinsing with TBS, sections were further incubated with biotin-conjugated goat-anti-mouse antibody (Dako) or, in case of CD3 staining, with rabbit-anti-FITC (Dako) in 10% normal human serum (NHS) in TBS for 30 minutes. Following a wash step with TBS, sections were subsequently incubated with horseradish peroxidase (HRP)-conjugated streptavidin (Dako) or, in case of CD3 staining, with HRP-conjugated goat-anti-rabbit antibody (Dako), in 1% BSA in TBS for 30 minutes. After a final rinse with TBS, the HRP activity was visualised as an orange-red colour by incubation with 3-amino-9-ethylcarbazole (AEC, Sigma-Aldrich). Sections were counterstained with Mayer's hematoxylin and mounted in glycerine-gelatine (Dako).

Double staining procedures In the double-staining experiments we used FITC-conjugated anti-CD3 (Becton Dickinson) together with either anti-HLA-DR (L243, Becton Dickinson) or anti-CD25 (ACT-1, Dako) antibodies. After inhibition of the endogenous peroxidase activity and blocking nonspecific binding sites with 10% normal goat serum in TBS, the sections were incubated with the first primary antibody (anti-HLA-DR or anti-CD25) for 1 h at room temperature.

For the CD3/HLA-DR staining, the sections were rinsed with TBS, and incubated with goat-anti-mouse peroxidase labeled antibody (Dako) in 10% NHS in TBS 30 min. After rinsing with TBS again, a second blocking for nonspecific binding sites was performed with 10% normal mouse serum in TBS. Next, sections were incubated with FITC-conjugated anti-CD3 in 1% BSA in TBS for 1 h, then washed with TBS and incubated with rabbit-anti-FITC in 10% NHS in TBS for 30 min. Subsequently they were incubated with goat-anti-rabbit alkaline phosphatase-labeled antibody (Dako). After a final rinse with TBS, the phosphatase activity was visualized using Fast Blue Salt (Sigma), resulting in a blue color. To visualize the peroxidase activity, AEC was used to give a red color.

For the CD3/ CD25 staining, the sections were rinsed with TBS, and successively incubated with goat-anti-mouse biotin-labeled antibody (Dako) in 10% NHS in TBS for 30 min; HRP-conjugated streptavidin (Dako) in 1% BSA in TBS for 30 min; 10% normal mouse serum in TBS to block nonspecific binding sites; FITC-conjugated anti-CD3 in 1% BSA in TBS for 1 h; rabbit-anti-FITC in 10% NHS in TBS for 30 min and finally goat-anti-rabbit alkaline-phosphatase-labeled antibody (Dako). After a final rinse with TBS, the phosphatase and peroxidase activity were visualized as above.

Digital image analysis All single-stained sections were randomly coded and analysed by digital image analysis by a blinded observer. From each section a representative region of 1.45 x 1.45 mm, including epidermis and dermis, was captured and digitised, and divided in 20 separate high power fields. Subsequently, sections were examined using a specialised algorithm written in the program language QUIPS operating a Qwin-based (Qwin Pro V2.4, Leica, Cambridge, U.K.) computer-assisted colour video image analysis system (26). Cellular-stained sections were separately counted for epidermal and dermal region. Epidermal counts were expressed as positive cells per mm; dermal counts were expressed as positive cells per mm². The presence of adhesion molecules was expressed as Integrated Optical Density (IOD) per mm². Each biopsy was analysed in triplicate to minimise random variation in infiltration.

Manual quantification Double-stained sections were manually counted by two independent blinded observers. Using a 0.5 x 0.5 mm ocular grid and at 200x magnification, single-red, single blue, and double-positive cells were counted in the entire section. The epidermal and dermal regions were separately counted. Results were expressed as the number of double-positive cells per millimeter (epidermis) or per millimeter squared (dermis).

Peripheral blood measurements The number of CD45RO⁺ T lymphocytes in peripheral blood was quantified by flow cytometric analyses at each study visit.

Statistical analysis SPSS 10.1.4 for Windows (SPSS, Chicago, IL) was used for statistical analysis. Values were expressed as median and range. To determine

significant differences between the baseline and subsequent values the Wilcoxon-signed rank test was used. All statistical tests were 2-sided; p-values less than 0.05 were considered to be significant. The Spearman rank correlation test was used to determine correlations between different variables.

Results

Alefacept treatment reduces clinical signs of disease activity during the treatment period Nine males and two females were included in the study.

66 Their median age was 45 years (range 35 – 70 years), their median PASI at baseline was 11.7 (range 1.6 – 39.2) and their median TBA was 15% (range 1% - 55%). All patients had a longstanding history of psoriasis; the median duration was 20 years (range 10 - 40 years). Ten out of the 11 patients had a history of systemic treatment, including methotrexate (ten patients), UVB (three patients) and cyclosporine A (one patient). All patients finished the 12-week treatment without the need for concomitant medication and without major side effects. During the subsequent 12-week follow-up period, after the day-92 biopsy, four patients started topical therapy and three patients started systemic therapy because of worsening of psoriatic lesions or arthritis. After 4 weeks of treatment a decrease in median PASI was noticeable that was sustained throughout the treatment period (Fig 1). On day 92, two weeks after the last dose, 7 out of 11 patients (64%) had improved in PASI, the mean

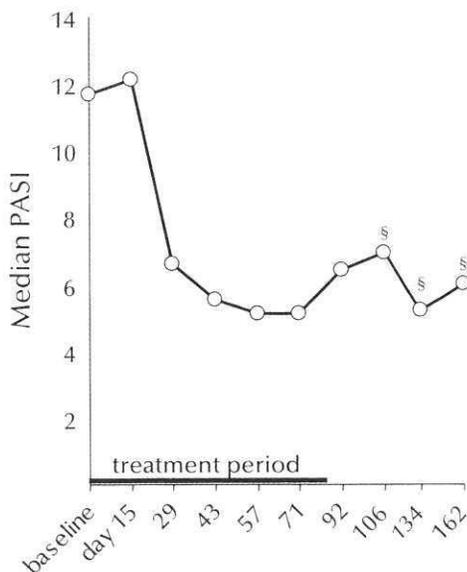


Fig. 1 Clinical efficacy of alefacept assessed by PASI. Numbers represent median PASI during and after alefacept therapy. The § symbols indicate the start of concomitant medication by one or more patients after the treatment period.

reduction in these 7 patients being 43%. Three patients (27%) experienced a PASI reduction of 50% or more. At any point within the study, 9 out of 11 patients (82%) showed an improvement in PASI, and 4 patients (36%) showed a reduction in PASI reduction of 50% or more. The median TBA was reduced from 15% (range 1 - 55) at baseline to 10% (range 1 - 70) on day 92.

Alefacept treatment reduces psoriasis lesional T-cell numbers Both epidermal and dermal T-cell numbers decreased during alefacept treatment (Table 1). The median epidermal T-cell number was decreased by 31% on day 92; the median dermal T cell number was decreased by 35% on day 92 ($p \leq 0.05$). There was a gradual reduction in epidermal thickness throughout the treatment period from 45 μm (range 29-70) at baseline to 36 μm (26-64) on day 92.

Table 1. Results of immunohistochemical single-staining of skin biopsies at baseline, and on days 8, 43 and 92. The data are median (range) numbers of positive cells per millimeter (epidermal cells) or per millimeter squared (dermal cells).

	Baseline	Day 8	Day 43	Day 92
CD3 ⁺ cells				
Epidermal	68 (1-709)	37 (3-405)	41 (1-234)	47 (0-155)
Dermal	200 (12-401)	129 (18-586)	186 (19-389)	130 (9-244) *
CD4 ⁺ cells				
Epidermal	30 (0-54)	14 (0-73)	17 (1-162)	13 (1-62)
Dermal	193 (20-550)	153 (5-512)	137 (12-615)	64 (11-313) *
CD8 ⁺ cells				
Epidermal	5 (1-37)	3 (1-40)	9 (1-39)	2 (0-18) *
Dermal	29 (0-146)	43 (6-112)	18 (1-141)	12 (1-42)

* $p \leq 0.05$ versus baseline

Alefacept treatment decreases the number of both CD4⁺ and CD8⁺ T cells in psoriatic lesional skin In the epidermis, the gradual reduction in the number of CD8⁺ cells reached statistical significance on day 92 (Table 1). There was also a decrease in the number of epidermal CD4⁺ cells during therapy, but this reduction did not reach statistical significance. In the dermis, there was a significant decrease in the number of CD4⁺ cells on day 92. The decrease in the number of dermal CD8⁺ cells did not reach statistical significance. Changes per patient in the number of CD4⁺ and CD8⁺ cells are shown in Fig 2. Reductions in the number of both epidermal CD4⁺ cells and CD8⁺ cells correlated with reduction in epidermal thickness ($\rho=0.64$, $p \leq 0.05$; $\rho=0.68$,

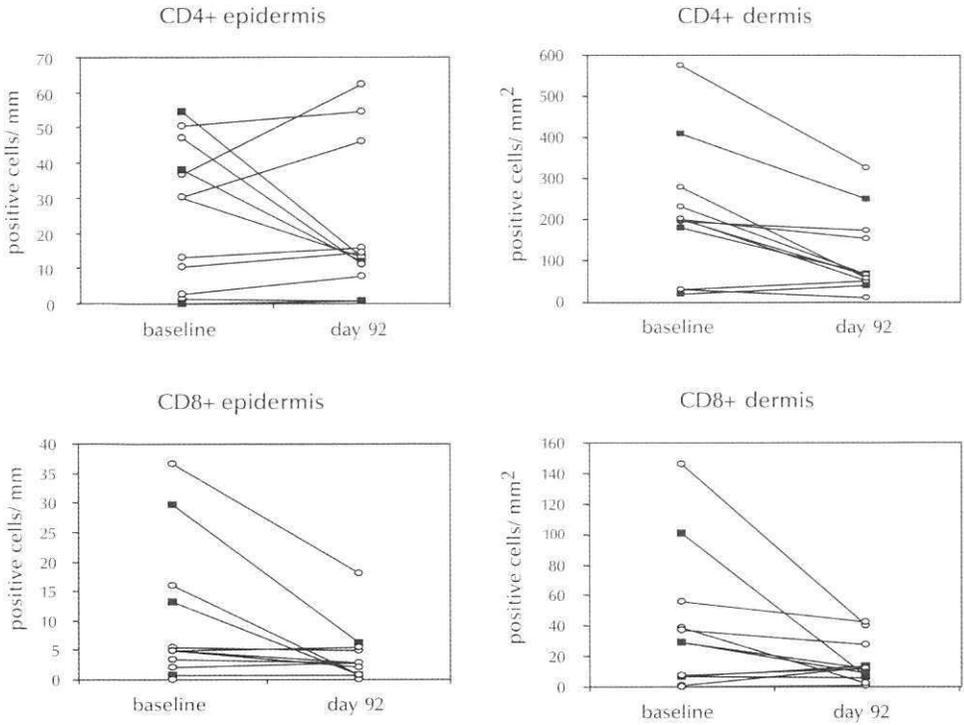


Fig. 2a-d. Individual effects of alefacept therapy on T cell subsets. Quantitative assessment of epidermal CD4⁺ (a), dermal CD4⁺ (b), epidermal CD8⁺ (c), and dermal CD8⁺ (d) cells per patient before and after therapy (day 92). Each data point corresponds to the average of triplicate determinations. The values indicate the number of positive cells per millimeter (epidermis) or positive cells per millimeter squared (dermis). O patients with a reduction in PASI of less than 50% on day 92 compared to baseline (n=8); □ patients with a reduction in PASI of 50% or more on day 92 compared to baseline. The bold line connects the median values at the two time points.

$p \leq 0.05$, respectively). The median epidermal CD4⁺/CD8⁺ ratio increased from 1.8 (range 0.1-73) at baseline to 2.9 (range 0.3-79) on day 92 ($p \leq 0.02$). The median dermal CD4⁺/CD8⁺ ratio (baseline ratio 5.8, range 2.9-273) did not change significantly during therapy. Although not statistically significant, we did observe a more profound reduction in the number of epidermal and dermal CD4⁺ and CD8⁺ cells in the high response group (PASI reduction at day 92 $\geq 50\%$ from baseline) compared to the low-response group (PASI reduction $< 50\%$).

Alefacept-induced reduction in the number of epidermal effector/memory T cells in psoriatic lesional skin is significantly higher in high-responding patients than in low-responding patients

A substantial proportion of the lesional T cells were effector/memory (CD45RO⁺) T cells. The number of naive T cells present in the lesions was insignificant; at baseline the median number of CD45RA⁺ cells in the epidermis was 0 (range 0-3) and the median number of dermal CD45RA⁺ cells was 1 (range 0-7).

As early as day 8, a decrease of 67% could be noticed in the median number of CD45RO⁺ cells in the epidermis, which was sustained throughout the treatment period (Fig 3). The number of dermal CD45RO⁺ cells was initially increased on day 8, but decreased by 35% at day 43 and by 73% on day 92. Representative immunohistochemical staining for CD3⁺ and CD45RO⁺ in lesional skin is shown in Fig 4.

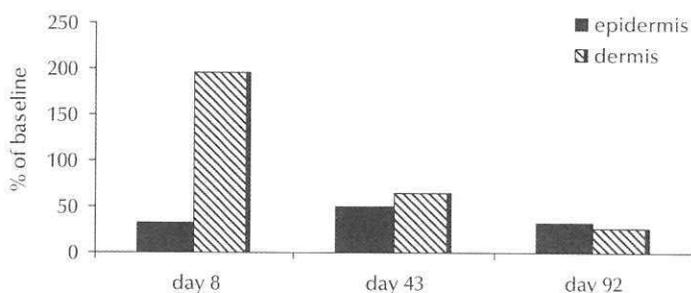


Fig. 3 Changes in CD45RO⁺ cells in epidermis and dermis during alefacept therapy. Numbers represent percentages of CD45RO⁺ cells in relation to the baseline median values (100%) on days 8, 43 and 92

There was a significant difference in response of memory/effector T cells between patients with a high clinical response at day 92 (PASI reduction 50% or more, n=3) and patients with a low or absent clinical response (PASI reduction less than 50%, n=8). In high-responding patients a clearing of epidermal memory/effector T cells had occurred by day 92. In patients with a lower or absent clinical response, the median memory/effector T cell number in the epidermis had increased ($p < 0.05$). In addition, the number of dermal CD45RO⁺ cells on day 92 showed a significantly greater decrease in the high-responding patients than in the low-responding patients (90% reduction vs. 41% reduction, $p < 0.02$). These different responses of the two groups of

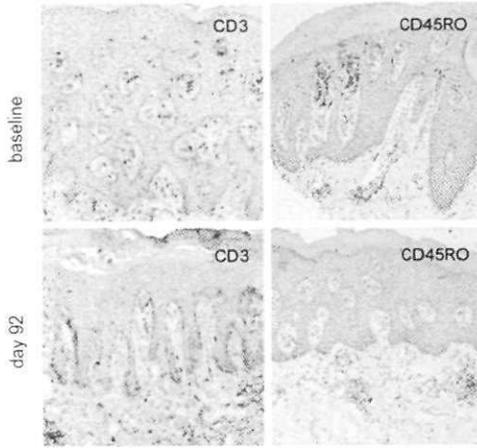


Fig. 4 Representative immunohistochemical staining of CD3⁺ cells and CD45RO⁺ cells at baseline and on day 92 (x100)

patients were also paralleled by a greater reduction in CD45RO⁺ T cells in the peripheral blood in high-responding patients than in low responding patients on day 92 ($p \leq 0.05$; Fig 5).

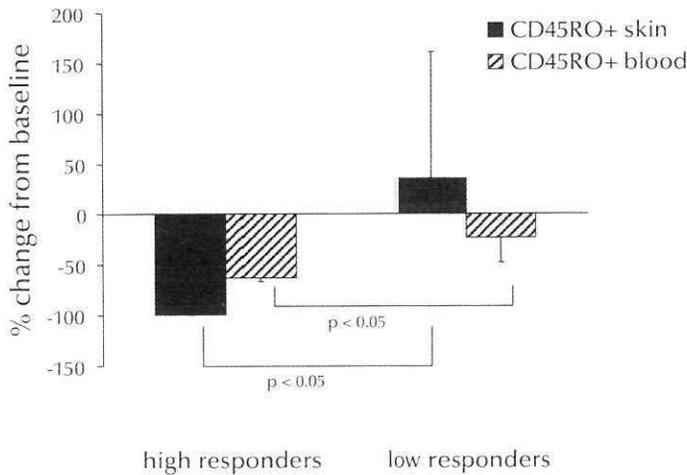


Fig. 5 Percentage change in CD45RO⁺ cells in epidermis and peripheral blood of high-responding patients and low-responding patients. High-responders are defined as patients with a reduction of 50% or more in baseline PASI on day 92 ($n=3$); low-responders are defined as patients with a reduction of less than 50% in baseline PASI on day 92 ($n=8$). Values are the means of the individual changes per patient, and the SD. Negative values indicate a mean reduction in cell numbers; positive values indicate a mean increase in cell numbers.

Alefacept treatment decreases both the number of activated T cells and non-activated T cells in lesional epidermis and dermis After the start of alefacept treatment, there was a gradual reduction in the number of epidermal CD25⁺ T cells (Table 2). On day 92, there was a 42% decrease compared to baseline ($p \leq 0.05$). A change in the number of dermal CD25⁺ T cells was not seen until day 92, when there was a 45% decrease from baseline ($p \leq 0.05$). The median number of epidermal HLA-DR⁺ T cells at baseline was 23 (3-56), decreasing by 48% on day 8 and by 61% on day 92 ($p \leq 0.02$). The number of dermal HLA-DR⁺ also decreased significantly after alefacept therapy (Table 2). Although not statistically significant, the reduction in the number of activated T cells in the epidermis and dermis was more profound in high-responding patients than it was in low-responding patients.

Table 2. Results of immunohistochemical double-staining of skin biopsies at baseline, and on days 8, 43 and 92. The data are as median (range) numbers of double-positive cells per millimeter (epidermal cells) or per millimeter squared (dermal cells)

	Baseline	Day 8	Day 43	Day 92
CD3+HLADR+ cells				
Epidermal	23 (3-56)	12 (4-94)	18 (3-94)	9 (4-34) *
Dermal	309 (187-836)	393 (114-786)	271 (118-500)	278 (48-426) *
CD3+CD25+ cells				
Epidermal	31 (17-27)	21 (7-57)	27 (4-96)	18 (1-67)
Dermal	276 (215-498)	275 (155-590)	249 (85-424)	152 (38-423) *

* $P \leq 0.05$ versus baseline

We also looked at the percentage of CD25⁺ T cells in relation to the total number of T cells. At baseline, 59% of the number of epidermal T cells were CD25⁺ and 57% of the T cells were HLA-DR⁺. After 12 weeks of treatment, these percentages had not changed significantly, due to a decrease in both non-activated T cells and activated T cells.

Alefacept treatment reduces the expression of the adhesion molecule ICAM-1, but not that of E-selectin and VCAM-1, in psoriatic plaques. To investigate the effect of alefacept therapy on T-cell migration, we evaluated the expression of three adhesion molecules in treated psoriatic lesional skin (Table 3). The expression of ICAM-1 (intercellular adhesion molecule-1) gradually reduced after starting therapy, reaching a 63% decrease on day 92. This decrease was, however, not statistically significant, presumably due to the small number of

Table 3. Expression of adhesion molecules during alefacept treatment. The data are median (range) Integrated Optical Density (IOD) per millimeter squared of lesional skin.

	Baseline	Day 8	Day 43	Day 92
E-selectin	778 (19-1963)	693 (16-1297)	353 (25-3340)	690 (12-2700)
ICAM-1	10275 (92-51157)	3952 (66-34367)	1480 (773-17951)	3829 (253-32642)
VCAM-1	13332 (485-300472)	21535 (1359-139808)	7898 (405-16500)	11463 (18-73851)

72 patients. There was a gradual reduction in expression of E-selectin from the start of the therapy until day 43. However, on day 92 the level of expression was equal to the baseline level. Apparently, the expression of E-selectin is not directly correlated with the number of T cells in lesional skin. The expression of VCAM-1 (vascular cell adhesion molecule-1) did not change significantly during therapy.

Discussion

The data presented here suggest that the T-cell population in lesional psoriatic skin is affected by intravenously administered alefacept therapy. We observed a reduction in the number of CD4⁺ and CD8⁺ T-cell subsets in the epidermis as early as 8 days after the first dose of alefacept, whereas reductions in both T-cell subsets in the dermis were not observed until day 43. Not all results were statistically significant, which might be explained by the limited number of patients participating in the study and the wide interpatient variability. Also, because of the open-label character of the study we were not able to compare the immunohistochemical data with results of placebo-treated patients. However, the consistent reductions in the immunological markers show a trend towards downregulation of inflammation that cannot be explained by expectation bias or regression to the mean, and are likely to be a result of alefacept therapy. The interpatient variability was bypassed by using patients as their own control.

Although the role of CD4⁺ T cells and CD8⁺ T cells in psoriasis has been investigated in numerous studies, the individual contribution of both subsets to the pathogenesis and clearance of psoriatic lesions has not yet been fully elucidated. There have been several reports describing a preponderance of cytotoxic CD8⁺ T cells in lesional epidermis (3,5,27,28), but this observation could not be confirmed in our study, as CD4⁺ cells were predominant over CD8⁺ cells in both epidermis and dermis. A previous study has demonstrated

that alefacept targets both CD4⁺ and CD8⁺ effector/memory T cells in peripheral blood (23). We demonstrated that alefacept targets both subsets in lesional skin as well, but nevertheless we found an increase in the CD4⁺/CD8⁺ ratio in the epidermis during therapy. This agrees with reports describing how reduction in disease severity is correlated with depletion of epidermal CD8⁺ T cells (10,29,30).

Alefacept is thought to affect predominantly the CD45RO⁺ (effector/memory) T cell population, because these cells have a high expression of CD2 on their surfaces. Cooper et al. have shown that alefacept selectively promotes NK cell-mediated deletion of CD45RO⁺ human T cells in vitro (22). In addition, Ellis and Krueger have shown that a reduction in memory T cells in peripheral blood induced by alefacept treatment is correlated with clinical improvement (23). Our own study not only confirmed these observations but, perhaps more importantly, also suggested that a correlation exists between a reduction in effector/memory T cells in lesional psoriatic epidermis and improvement in clinical symptoms. Even after one dose of alefacept a decrease in epidermal effector/memory T cells could be observed, which preceded clinical improvement. These findings support the concept that effector/memory T cells have a prominent role in the pathogenesis of psoriasis and demonstrate that alefacept is capable of reducing effector/memory T cells in lesional skin. In addition, an association between a reduction in the effector T cell population in peripheral blood and synovial tissue and improvement of clinical signs of psoriatic arthritis during alefacept therapy has been reported recently (31). However, our observations should be confirmed on a larger group of high responding patients before definite conclusions can be drawn. The two activation-associated molecules investigated in this study are known to be expressed during early activation (CD25) and late activation (HLA-DR) of T cells (9). The presence of both molecules at baseline on most epidermal and dermal T cells supports the hypothesis of persistent T cell activation in skin lesions of psoriasis patients. The reduction in both markers during alefacept therapy indicates a reduction in T cell activation in both epidermis and dermis. However, besides the reduction in activated T cells, alefacept treatment also reduced the number of non-activated (HLA-DR⁻/CD25⁻) T cells, suggesting that the effect of this drug on T cells is not completely related to

the state of activation of T cells. This could be either a direct result of alefacept, or due to the overall downregulation of proinflammatory cytokines, adhesion molecules and costimulatory molecules reducing the total number of T cells migrating to the inflamed area.

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It has been reported that alefacept has a long-lasting effect, with patients treated with alefacept having a median time to relapse (i.e. patients no longer clear or almost clear) of 306 days (23). This long-lasting remission cannot be explained just by a reduced capacity for T cells to be activated. We think it would be more likely that the long-lasting effect of alefacept is due to the selective reduction in memory-effector T cells in lesional skin, supposedly by an apoptotic mechanism (21). Also, the reduced expression of ICAM-1 observed in this study, although not statistically significant, might inhibit the migration of T cells into the inflamed area and the subsequent activation. ICAM-1 is expressed on a variety of cytokine-stimulated cells, including endothelial cells, antigen-presenting cells and keratinocytes, and binds to its ligand LFA-1 (leukocyte function-associated antigen 1) on T cells (32). ICAM-1 facilitates the trans-endothelial migration of T cells and the adhesion to T cells to keratinocytes, and is thought to provide costimulatory signals necessary for T-cell activation. Expression of ICAM-1 is related to progression of local immune responses (33,34). Possibly the downregulation of ICAM-1 after alefacept therapy is caused by a decreased release of cytokines such as TNF- α , IFN- γ and IL-1 that induce ICAM expression (35,36).

We observed substantial interindividual variations in the clinical and immunohistochemical response to alefacept, which could not be explained by disease severity or disease duration. At the present time it is not clear why some patients do not respond well to alefacept therapy. It is already known that different subgroups exist among psoriasis patients. Henseler and Christophers have reported a more severe disease course and a stronger influence of genetic factors in patients with early-onset psoriasis compared to late-onset psoriasis (37). Recently these observations have been confirmed by Reich et al, who reported that promoter polymorphisms of the genes encoding TNF- α and IL-1 β , are associated with different subtypes of psoriasis, characterized by early and late disease onset, respectively (38). Concerning the possible involvement of polymorphisms, the variability in response to

alefacept might be due to heterogeneity in CD2 or Fc γ R type III among patients. Perhaps future clinical trials, supported by further immunohistochemical analysis could identify patient subpopulations that are likely to respond to alefacept therapy.

In conclusion, the results presented in this study indicate that alefacept reduces signs of inflammation in psoriatic lesional skin. By the different observations made in this study, we assume that multiple mechanisms of action of alefacept are likely. First, clinical improvement in high-responding patients induced by alefacept treatment was associated with a clearance of effector/memory T cells from lesional psoriatic epidermis, possibly caused by an apoptotic mechanism. Second, the decrease in the expression of activation markers might indicate a diminution of T-cell activation in lesional skin, with resulting decreases in proinflammatory cytokines.

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chapter

4

Alefacept treatment in psoriatic arthritis. Reduction of the effector T cell population in peripheral blood and synovial tissue is associated with improvement of clinical signs of arthritis

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Abstract

Objective. To investigate whether alefacept (a fully human lymphocyte function-associated antigen 3 [LFA-3]/IgG1 fusion protein that blocks the LFA-3/CD2 interaction) is able to reduce the signs and symptoms of joint inflammation in patients with active psoriatic arthritis (PsA).

Methods. Eleven patients with active PsA were treated with alefacept for 12 weeks in an open-label and explorative study. Clinical joint assessment and laboratory assessments were performed at baseline and after 4, 9, 12, and 16 weeks of treatment. Serial synovial tissue (ST) biopsy from an inflamed index joint (knee, ankle, wrist, or metacarpophalangeal joint) were obtained by arthroscopy at baseline and after 4 and 12 weeks of treatment.

Results. At the completion of treatment, 6 of 11 patients (56%) fulfilled the Disease Activity Score (DAS) response criteria. Nine patients (82%) fulfilled the DAS response criteria at any point during the study. There was a statistically significant reduction in CD4⁺ lymphocytes ($P < 0.05$), CD8⁺ lymphocytes ($P = 0.05$), and CD68⁺ macrophages ($P < 0.02$) in the ST after 12 weeks of treatment compared with baseline. The ST and peripheral blood of those patients fulfilling the DAS response criteria contained more CD45RO⁺ cells at baseline and displayed a significant reduction in these cells compared with nonresponding patients.

Conclusion. The changes in ST, together with the improvement in clinical joint scores, after treatment with alefacept support the hypothesis that T-cell activation plays an important role in this chronic inflammatory disease. Furthermore, since alefacept, a T-cell-specific agent, led to decreased macrophage infiltration, the data indicate that T-cells are highly involved in synovial inflammation in PsA.

Introduction

Coexisting arthritis and psoriasis has been recognized as a clinical entity since the 19th century. Yet, the formal clinical distinction between psoriatic arthritis (PsA) and rheumatoid arthritis (RA) was made only in 1961, and was based on a composition of clinical features, including asymmetric pauciarticular arthritis involving the distal interphalangeal joints and dactylitis in the absence of rheumatoid factor (1). Currently, the diagnosis of PsA remains difficult due to the absence of specific markers and is therefore still mainly determined by clinical signs and symptoms (2). In the UK, the prevalence of PsA is estimated as 0.1% of the population, while RA has an estimated prevalence of 0.5–1.0% (3). Although PsA is perceived as a less aggressive disease compared with RA, it can cause severe disability in a significant proportion of patients.

Meanwhile, data on the treatment of PsA are limited. Only a few small, controlled clinical trials have been performed with interventions, such as sulfasalazine (4-6), methotrexate (7), and cyclosporine A (8, 9). The increased awareness of the success of early, aggressive, and novel treatment strategies in RA (10, 11) has encouraged an identical approach to treatment of patients who have disabling PsA, but using new modalities, such as etanercept (12). More detailed information about the effects of these regimens in PsA is needed before they can be developed further. Because serial synovial tissue (ST) sampling may help to screen for possible effects at the site of inflammation and could provide insight into the mechanism of action of PsA (13), results of this screening should be included as an end point in evaluations of these new biologic therapies (14).

T lymphocytes have been proposed as key players in the pathogenesis of psoriasis and are therefore a potential target for its treatment (15). This notion is based on the presence of T lymphocytes in early psoriasis lesions (16), the beneficial effects of T-lymphocyte-targeted therapies like cyclosporine A (8), and the altered relationship between psoriatic keratinocytes and interferon- γ (IFN- γ) compared with normal keratinocytes (17). Data on the role of T cells in PsA are limited, but it has been suggested that they play a central role in its pathogenesis as well (18-20).

Alefacept (human lymphocyte function-associated antigen 3/IgG₁ [LFA-3/IgG1] fusion protein; Biogen, Cambridge, MA) is a recombinant protein designed to inhibit the interaction between LFA-3 and CD2. The LFA-3 portion binds to

the CD2 receptor on T lymphocytes, blocking T-cell activation and proliferation *in vitro* and *in vivo*, while the IgG1 domain interacts with Fcγ receptor type III on accessory cells (e.g., macrophages and natural killer cells) to induce selective apoptosis of memory-effector T lymphocytes (21-25). In a recently completed phase II clinical trial, alefacept was documented to be safe and clinically efficacious compared with placebo in patients with active plaque psoriasis (26). In this same study, it was documented that alefacept administration resulted in a significant and selective reduction in effector T cells without major safety problems. This clinical efficacy in plaque psoriasis supports a potential role for alefacept in PsA, but its effects in these patients have as yet not been determined. Therefore, we performed an explorative, open-label clinical study with serial synovial biopsies in patients with active PsA.

Patients and methods

Patients and study protocol In a period of 6 months, 11 patients with plaque psoriasis (diagnosed at least 12 months before enrolment) who had active joint inflammation were recruited in a prospective, single-centre, open-label, clinical trial investigating the effects of alefacept treatment. Active disease was defined as ≥ 2 swollen and ≥ 2 tender joints. In all cases, both the physician and patient assessed disease activity as being moderate or poor. All patients had at least one clinically involved knee, wrist, metacarpophalangeal (MCP) joint, or ankle joint. Prednisone therapy was not allowed. Concomitant treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) at a stable dosage was allowed. None of the patients had previously been treated with alefacept. When applicable, all treatments other than NSAIDs were stopped and, after a washout phase of 28 days, patients received 7.5 mg of alefacept intravenously once a week for 12 weeks. The criteria for administering each dose of alefacept were a total lymphocyte count $\geq 67\%$ of the lower limit of the normal range within 24 hours before injection and an absolute CD4⁺ T lymphocyte count $\geq 300/\text{mm}^3$ in the previous week. All 11 patients in this study met these criteria during the entire study period.

Clinical assessments were performed at baseline and after 4, 12 (end of treatment phase), and 16 weeks, and included a 30-joint count (28-joint count (27) and both ankles) for joint swelling and tenderness, and physician and patient assessment of disease activity, morning stiffness, pain assessed by a visual analog

scale (VAS), and serum levels of serum C-reactive protein (CRP). The clinical effect after treatment was calculated using the Disease Activity Score (DAS) (28), with CRP as the acute-phase reactant and 28 joints counted. All patients gave informed consent, and the study protocol was approved by the Medical Ethics Committee of the Academic Medical Centre/University of Amsterdam.

Arthroscopy In all patients, 3 serial arthroscopically guided synovial biopsies of the same index joint, at baseline, after 4 weeks of treatment, and at the end of the treatment period at 12 weeks were performed under local anaesthesia (29). The joints subjected to the arthroscopic biopsy procedure were the knee joint (n=7), the wrist joint (n=2), ankle joint (n=1), and MCP joint (n=1) (30;31).

Knee arthroscopy The arthroscopy procedure was performed in the inflamed knee with a small-bore, 2.7-mm arthroscope (Storz, Tuttlingen, Germany) under local anaesthesia (lidocaine 1%) using an infrapatellar skin portal for macroscopic examination of the synovium and a second suprapatellar portal for the biopsy procedure. During each arthroscopy, synovial biopsy samples were obtained from the suprapatellar pouch, the synovium-cartilage junction, the patellar gutters, and the tibiofemoral junction, using a grasping forceps (Storz) (32).

Small joint arthroscopy Arthroscopy of the wrist, ankle, or MCP joint was performed using a small-bore 1.9-mm arthroscope (Storz) under local anaesthesia through 2 skin portals, as described previously (30). Both portals were used for macroscopic examination of the synovium and for the biopsy procedure. During each arthroscopy, synovial biopsies were obtained using a grasping forceps (Storz) (30).

If there was macroscopic variation of synovitis in knee or small joint, multiple samples were obtained from both macroscopically inflamed and macroscopically noninflamed regions. To reduce sampling error, a minimum of 6 tissue specimens were processed for immunohistochemistry and for formalin fixation, each as described previously (33-35).

Immunohistochemical analysis The specimens for immunohistochemistry were directly collected *en bloc* in a mold, embedded in Tissue-Tek OCT (Miles

Diagnostics, Elkhart, IN), and subsequently snap frozen by immersion in liquid nitrogen (-180°C) after being randomly coded. The frozen blocks were stored in liquid nitrogen until processed. Shortly before staining, 5- μ m sections were cut and mounted on glass slides (Star Frost adhesive slides, Knittelgläser, Germany); the slides were air dried at room temperature, carefully packed, sealed airtight, and stored at -80°C until immunohistochemical analysis could be performed in a single session.

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Serial sections were stained with the following mouse monoclonal antibodies (mAb): anti-CD68 (EBM11, Dako, Glostrup Denmark), anti-CD3 (SK7, Becton-Dickinson, San Jose, CA), anti-CD4 (SK3, Becton-Dickson), anti-CD8 (DK25, Dako), and anti-CD55 (Mab67, Serotec, Oxford, UK). Endogenous peroxidase activity was inhibited using 0.1% sodium azide and 0.3% hydrogen peroxide in phosphate buffered saline (PBS). Staining for cell markers and cytokines was performed as described previously (35). Following a primary step of incubation with mAb, bound antibody was detected according to a 3-step immunoperoxidase method. The primary antibodies were incubated for 60 min. Affinity-purified and horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (DAKO) was added for 30 min, followed by subsequent incubation with affinity-purified and HRP-conjugated swine anti-goat Ig (Biosource, Etten-Leur, the Netherlands) for 30 min. HRP activity was detected using hydrogen peroxide as substrate and aminoethylcarbazole (AEC, Sigma, St. Louis, MO) as dye. Slides were counterstained with Mayer's hematoxylin (Sigma) and mounted in Kaiser's glycerol gelatin (Merck, Rahway, NY).

Digital image analysis Subsequently, all sections were coded and analyzed by digital image analysis in random order as described previously (36), by an independent observer (MCK), who was blinded for the clinical data. Briefly, 3 separate representative regions, including the intimal lining layer and synovial sublining, were chosen for the evaluation of each section. Six consecutive high-power fields (HPFs) from each region were captured and digitized, resulting in a total of 18 HPFs (surface area \approx 2.1 mm²). Subsequently, sections were examined using a specialized algorithm written in the program language QUIPS operating a Qwin-based (Qwin Pro V2.4, Leica, Cambridge, U.K.) computer-assisted color video image analysis system. (36;37).

Double-labeling measurements for CD4⁺,CD45RO⁺ cells ST samples from 8 patients with evaluable baseline and 12-week biopsies were assessed for the co-expression of CD45RO and CD4 on serial sections of the block using double immunofluorescence methods, as described previously (38). First, the anti-CD45RO (UCHL-1, CLB, Amsterdam, The Netherlands) mAb was incubated on the sections, followed by incubation with tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse antibody (Nordic, Tilburg, The Netherlands). Ten percent normal mouse serum in PBS was applied as blocking serum. This was followed by incubation with fluorescein isothiocyanate (FITC)-conjugated mAb anti-CD4 (CLB-T4/2, 6D10, CLB). Subsequently, rabbit anti-FITC antibody (Dako) was incubated. Finally, FITC-conjugated swine anti-rabbit antibody (Dako) was added. The sections were embedded in Vectashield mounting medium (Vector, Burlingame, CA) and analyzed by 2 independent assessors (MCK, TJMS) who were unaware of the clinical data and the order of the biopsies (38). During the analysis, all CD4 and CD45RO double-positive cells in the entire section were counted and given as percentage of the absolute number of CD4⁺ cells in the biopsy specimens.

Peripheral blood measurements Flow cytometric analyses were performed at each study visit to quantify populations of CD4⁺, CD8⁺, CD45RA⁺, and CD45RO⁺ T lymphocytes. The cumulative reduction in the baseline counts over the 12-week treatment period was reported as the area under the curve.

Statistical analysis Access database and Excel spreadsheet software (Microsoft, Redmond, WA) were used for data collection and selection. SPSS for Windows, version 9.1 (SPSS, Chicago, IL) was used for statistical analysis. The Wilcoxon's signed-rank test was used to determine significant differences between the baseline and subsequent biopsies. Kendall's coefficient was calculated to see whether changes in one variable correlated with changes in others. All statistical tests were 2-sided; *P* values less than 0.05 were considered significant. The sponsor of the study collected the data; the authors performed the statistical analysis, interpreted the data, prepared their presentation, and wrote this report. Values are expressed as the mean ± SEM.

Results

Patients Eleven patients were included in the study and underwent all procedures without complications; 1 patient refused the third arthroscopy for personal reasons. The mean age of the patients was 46 years (range 35–70), 9 were male and 2 were female, mean disease duration was 18 months (12–124), and the mean previous number of disease-modifying arthritis drugs used was 1.3 (0–4). The study included all of the types of PsA manifestations (39); 1 patient had an isolated distal arthritis, 3 patients had asymmetric oligoarthritis, 5 patients had symmetric polyarthritis, 2 patients had arthritis mutulans, and 1 patient had spondyloarthropathy.

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Clinical efficacy There was a gradual decrease in the mean DAS during the dosing period, with a sustained response after cessation of the therapy (5.0 at baseline to 3.7 after 16 weeks) (Table 1). At the end of the 12-week dosing period, 6 of 11 treated patients (55%) fulfilled the DAS response criteria; 9 patients (82%) fulfilled the DAS response criteria at any point within the study. Analysis of the individual elements of the response criteria revealed a similar pattern (Table 1). Mean tender and swollen joint counts were significantly decreased as early as 4 weeks after treatment, and this was maintained for 4 weeks after therapy was completed (week 16). Mean CRP levels decreased over the study period, with the decrease achieving statistical significance at 16 weeks. VAS scores were significantly decreased at 12 and 16 weeks after treatment. The mean Psoriasis Area and Severity index (40) was reduced by

Table 1. Clinical data on the patients[#].

	Baseline	4 Weeks	12 Weeks	16 Weeks
DAS, mean (range)	5.0 (3.3-7.2)	4.3 (2.7-7.1) [†]	4.0 (1.5-6.0) [†]	3.7 (1.8-5.3) [†]
Tender joint count [§]	7.6 ± 2.0	5.6 ± 2.1 [†]	4.5 ± 1.6 [†]	4.8 ± 1.4 [†]
Swollen joint count [§]	7.1 ± 2.0	5.9 ± 1.9 [†]	5.4 ± 1.5 [†]	4.9 ± 1.3 [†]
Disease activity, 1-10 scale				
Investigator assessment	3.4 ± 0.2	3.4 ± 0.2	2.8 ± 0.2	2.8 ± 0.3
Patient assessment	3.6 ± 0.2	2.9 ± 0.2	2.9 ± 0.2	2.7 ± 0.3
Morning stiffness, minutes	319 ± 28	174 ± 21	37 ± 2	34 ± 3
CRP, mg/liter	38 ± 13	23 ± 6	22 ± 6	14 ± 2 [†]
VAS, mm	53 ± 8	40 ± 7	38 ± 8 [†]	28 ± 7 [†]

[#] Except for the Disease Activity Score (DAS), values are the mean ± SEM. CRP = C-reactive protein; VAS = visual analog scale (100 mm). [†] P ≤ 0.05 versus baseline. [‡] P < 0.01 versus baseline. [§] Of 30 joints counted

13% at 4 weeks (SEM 8%), 23% at 12 weeks (SEM 10%), and 28% at 12 weeks (SEM 11%).

Safety profile The aggregated safety data displayed a favourable safety profile. The most common side effects likely or definitely associated with the study drug were flu like syndrome (54%) and infection (18%). Two severe adverse events were documented; both were judged as non-study related.

PB measurements Total lymphocyte counts (mean \pm SEM) decreased slightly during the dosing phase (1667 ± 124 at baseline to 1313 ± 141 after 12 weeks), but recovered 4 weeks after cessation of the drug (1520 ± 82). CD4⁺ lymphocyte counts displayed an identical pattern (775 ± 95 at baseline, 557 ± 66 after 12 weeks, and 602 ± 44 after 16 weeks). CD8⁺ lymphocyte counts also decreased

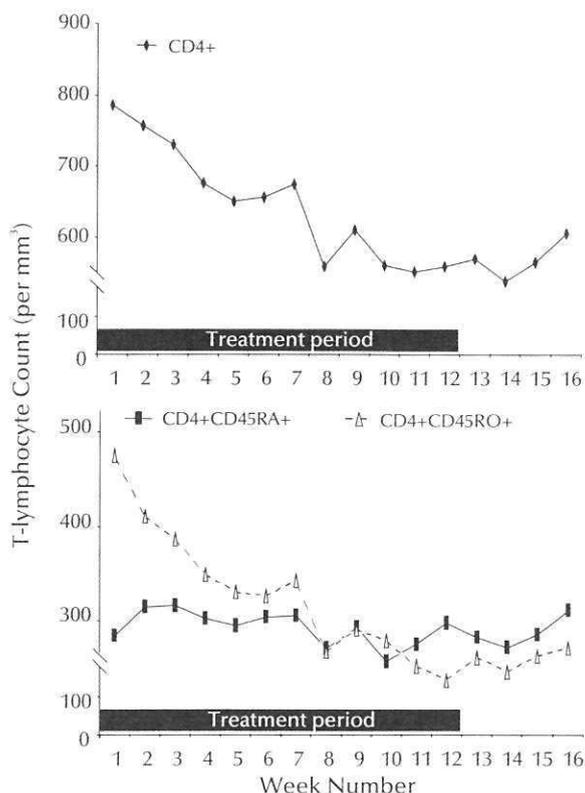


Figure 1. Changes in CD4⁺, CD4⁺CD45RA⁺, and CD4⁺CD45RO⁺ cell counts in the peripheral blood at baseline, during alefacept treatment, and after cessation of treatment. Numbers depict the absolute number of positive cells.

Table 2. Results of immunohistochemical staining of synovial biopsies at baseline, after 4 weeks of treatment, and at the end of the treatment phase (week 12)*.

	Baseline(n=9)	4 weeks(n=11)	12 weeks(n=11)
CD3 ⁺ T cells	431 ± 117	323 ± 90	219 ± 67
CD4 ⁺ T cells	384 ± 77	221 ± 70 [†]	158 ± 56 [†]
CD8 ⁺ T cells	191 ± 62	166 ± 45 [†]	77 ± 27 [†]
Intimal lining macrophages	528 ± 58	375 ± 43	313 ± 63
Sublining macrophages	1007 ± 171	1009 ± 191	574 ± 134 [†]
Fibroblast-like synoviocytes	1633 ± 1633	1616 ± 226	1599 ± 187

* Values are the mean ± SEM number of cells in 2.1 mm² of synovial tissue (± s.e.m.). Baseline synovial tissue biopsies of 2 patients were not assessable due to the absence of an identifiable intimal lining layer and were excluded from the immunohistochemical analysis. [†]P ≤ 0.05 versus baseline

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(282 ± 39 at baseline, 203 ± 29 after 12 weeks, and 248 ± 44 after 16 weeks). The number of naïve CD4⁺,CD45RA⁺ lymphocytes in the PB remained unchanged throughout the study (284 ± 43 at baseline, 297 ± 52 after 12 weeks, and 309 ± 39 after 16 weeks).

In contrast, the number of memory-effector CD4⁺,CD45RO⁺ lymphocytes was significantly reduced during the treatment period (mean ± SEM 461 ± 67 at baseline to 236 ± 30 after 12 weeks; P < 0.01) with sustained reductions after completion of dosing (270 ± 24 after 16 weeks) (Figure 1). Patients fulfilling the DAS response criteria at 12 weeks demonstrated a significant reduction in the percentage of CD45RO⁺ cells (mean 65% ± 5% at baseline to 49% ± 4% after 12 weeks; P < 0.05) compared with nonresponders, who did not have a

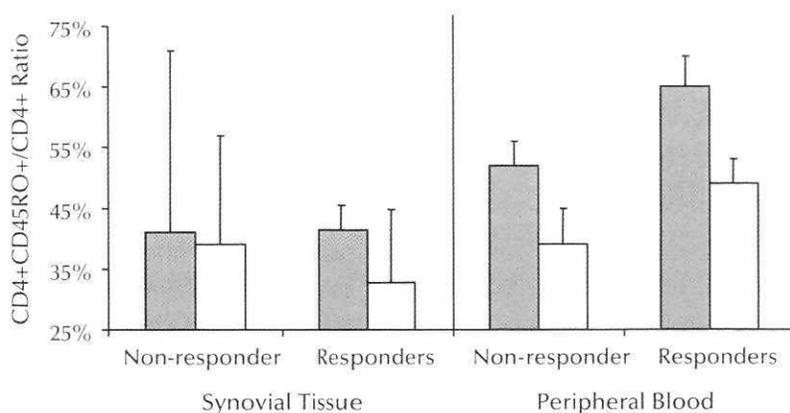


Figure 2. Percentage of CD4⁺, CD45RO⁺ cells in the peripheral blood and synovial tissue at baseline (shaded bars) and after alefacept treatment (open bars). Values are the mean and SEM and are for patients fulfilling the Disease Activity Score response criteria and for nonresponders.

significant reduction in the percentage of CD45RO⁺ cells (mean of 52% ± 4% at baseline to 39% ± 6% after 12 weeks) (Figure 2).

Arthroscopy In conjunction with the clinical improvement, there was a reduction in macroscopic signs of synovitis at arthroscopy after 4 weeks, and this was even more pronounced after 12 weeks. In the absence of a validated scoring system for macroscopic synovitis, examples are provided in Figure 3.

Immunohistochemical analysis The results of the immunohistochemical analysis at baseline and after 4 and 12 weeks are depicted in Table 2. Mean macrophage numbers were significantly reduced in the synovial sublining after 12 weeks, but not in the intimal lining layer. CD3⁺ T-cell numbers were also reduced, but this difference did not reach statistical significance. Both the CD4⁺ T cells and CD8⁺ T cells were significantly reduced after 4 weeks and 12 weeks. Representative examples of the immunohistochemical staining for CD3⁺, CD4⁺, CD8⁺, and CD68⁺ cells are shown in Figure 4. Fibroblast-like synoviocyte numbers did not change during the study.



Figure 3. Macroscopic appearance of the knee joint of the same patient at baseline, after 4 weeks and after 12 weeks of treatment with alefacept.

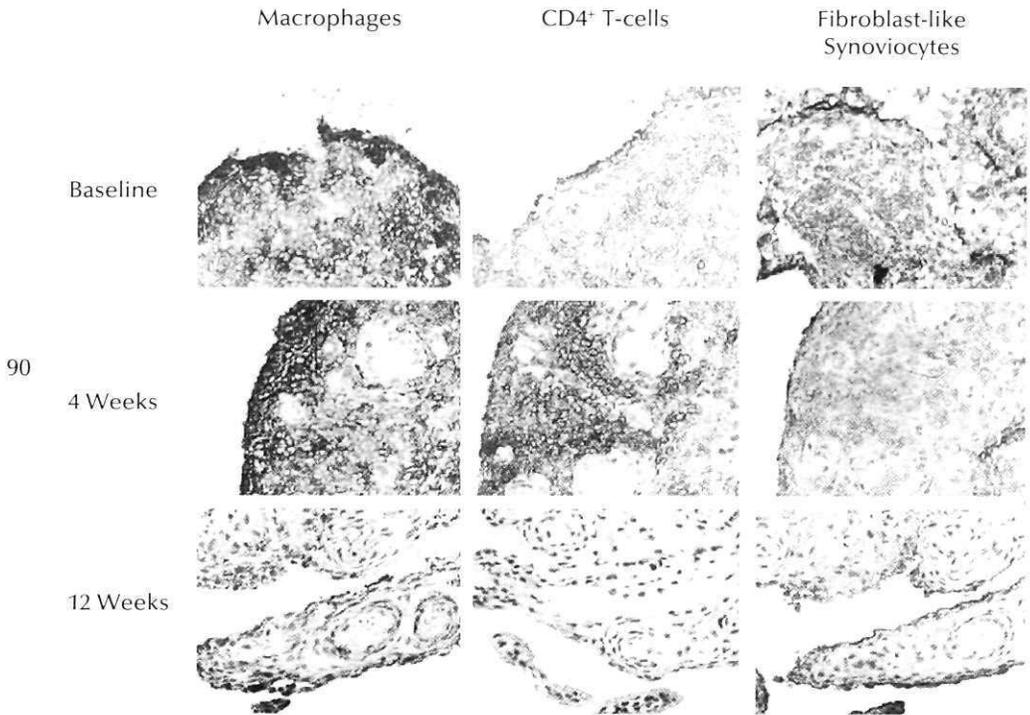


Figure 4. Immunohistochemical staining for macrophages, fibroblast-like synoviocytes, and CD4⁺ T-cells of one patient at baseline and after 4 and 12 weeks of alefacept treatment.

Double labelling CD4 and CD45RO The mean percentage of CD45RO⁺ lymphocytes (mean \pm SEM) in the ST was gradually reduced during the treatment phase (41% \pm 8% at baseline to 35% \pm 9% after 12 weeks), but this reduction did not reach statistical significance, presumably due to the relatively small number of patients. Patients fulfilling the DAS response criteria at 12 weeks exhibited a reduction in the percentage of CD45RO⁺ lymphocytes (42% \pm 4% at baseline to 33% \pm 12% after 12 weeks), this reduction was marked but also did not reach statistical significance because of the relatively low number of patients. In contrast, the percentage of CD45RO⁺ cells in the nonresponders was unaltered after treatment (41% \pm 30% at baseline to 39% \pm 18% after 12 weeks) (Figure 2).

Discussion

The data presented here show for the first time a beneficial effect of alefacept, an inhibitor of the LFA-3/CD2 interaction, in patients with PsA. There was a

reduction in arthritis activity and serum levels of acute-phase reactants. Clinical improvement was associated with a reduction in the number of macrophages and T effector cells in the synovium.

Recently increased awareness of the effects of chronic inflammatory joint diseases on the individual and the society, together with the expansion of treatment modalities, has increased the demand for new therapeutics. The number of treatment options available for patients with PsA is restricted to agents currently used in the treatment of RA, including sulfasalazine (4-6), methotrexate (7), and cyclosporine A (8;9). The recent development of novel agents such as etanercept (12) has shown the possibilities of targeted therapies. The importance of T cells in the pathogenesis of PsA has encouraged the development of compounds like alefacept, which interferes with T-cell activation and induces selective T-cell apoptosis (21-25). The data presented in this study support the notion that targeted therapies are effective in patients with PsA, as recently shown in plaque psoriasis (26).

To limit the placebo effects and expectation bias of an open-label design, which could suggest a more favourable response for the new treatment, we included serial measurement of biological markers that may be less susceptible to this bias (41). The relevance of ST analysis has been underscored by the observation that clinical arthritis activity is accompanied by persistent histological signs of synovitis after treatment with the mAb CAMPATH-1H, despite profound depletion of circulating lymphocytes (42). Previous work has shown that analysis of serial ST samples from RA patients who received either placebo or unsuccessful treatment with recombinant human interleukin-10 (IL-10) did not reveal any synovial changes (43). Similarly, there was no clear-cut change in serial biopsies after treatment with IL-1 receptor antagonist at 30 mg/day (44), which appears to have very limited effects on arthritis activity.

Thus, these studies support the view that changes in serial biopsy samples cannot be explained by placebo effects, regression to the mean, expectation bias, or by the arthroscopy procedure itself. Rather, they reflect biological effects of the treatment. In the present study, clinical improvement was associated with significant changes at the site of inflammation, which were consistent with the presumed mechanism of action of the compound.

Examination of serial synovial biopsy specimens may be more sensitive to change than clinical parameters (45;46), which allows its use as a screening method for novel therapies (13;45-47). However, a meaningful clinical effect obviously still needs to be shown in larger, well-controlled studies. The data presented here provide the rationale for such trials.

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The suggested mechanism of action of alefacept is by interaction with T-cell activation and induction of apoptosis, resulting in reduced numbers of memory-effector T cells. For that reason, we focused on the number of memory-effector T cells in serial samples of both the PB and synovial compartment. In the PB, we confirmed the reduction of circulating CD4⁺,CD45RO⁺ T cells during the course of treatment, as previously observed in patients with plaque psoriasis (26). Moreover, there was a similar reduction in the synovial compartment. Interestingly, the 6 patients fulfilling the DAS response criteria (after 12 weeks of treatment) displayed more profound reductions in both the numbers of circulating as well as synovial memory-effector T-cells compared to the 5 nonresponding patients. Since composite clinical response criteria, such as the DAS, were designed for measurement of clinically relevant disease activity, this observation supports the notion that effective blockade of the LFA-3/CD2 interaction leads to clinical improvement. It should be noted that the DAS has been validated in RA. We assume that the DAS may provide useful information in other forms of active arthritis as well, although its use in PsA remains to be validated. Using this composite index, it appears that patients with pre-existing high numbers of memory-effector T cells are more likely to respond to alefacept therapy than those with lower numbers. This could suggest that PsA is a heterogeneous disease and that activation of T-cells may be more important in the subset of patients who respond well to LFA-3/CD2 blockade.

The success of T-cell targeted therapy in PsA appears to contrast with the previous experience in RA (48). This could be explained by differences in pathogenesis, but also by differences in treatment. It has been shown previously that Th1-like cells are relatively spared treatment with anti-CD4-depleting antibody (49;50). In contrast, alefacept treatment specifically reduces the memory cell population. A potential drawback of this approach might be an increased risk of infection. However, we did not observe any increase in

infection rate, which is consistent with previous observations (26).

The favourable clinical response is also associated with a reduction in the number of macrophages during the treatment period. This is consistent with previous studies showing a strong correlation between macrophages and arthritis activity (35;43;51;52). It appears unlikely that alefacept had a direct effect on ST macrophages in light of its specificity. Therefore, we suggest that the effect of this novel biologic therapy on effector T cells is responsible for the observed reduction in macrophage numbers. The decrease in macrophage infiltration after alefacept treatment supports the view that activated T cells, presumably antigen driven (18), stimulate macrophage infiltration and activation in patients with PsA.

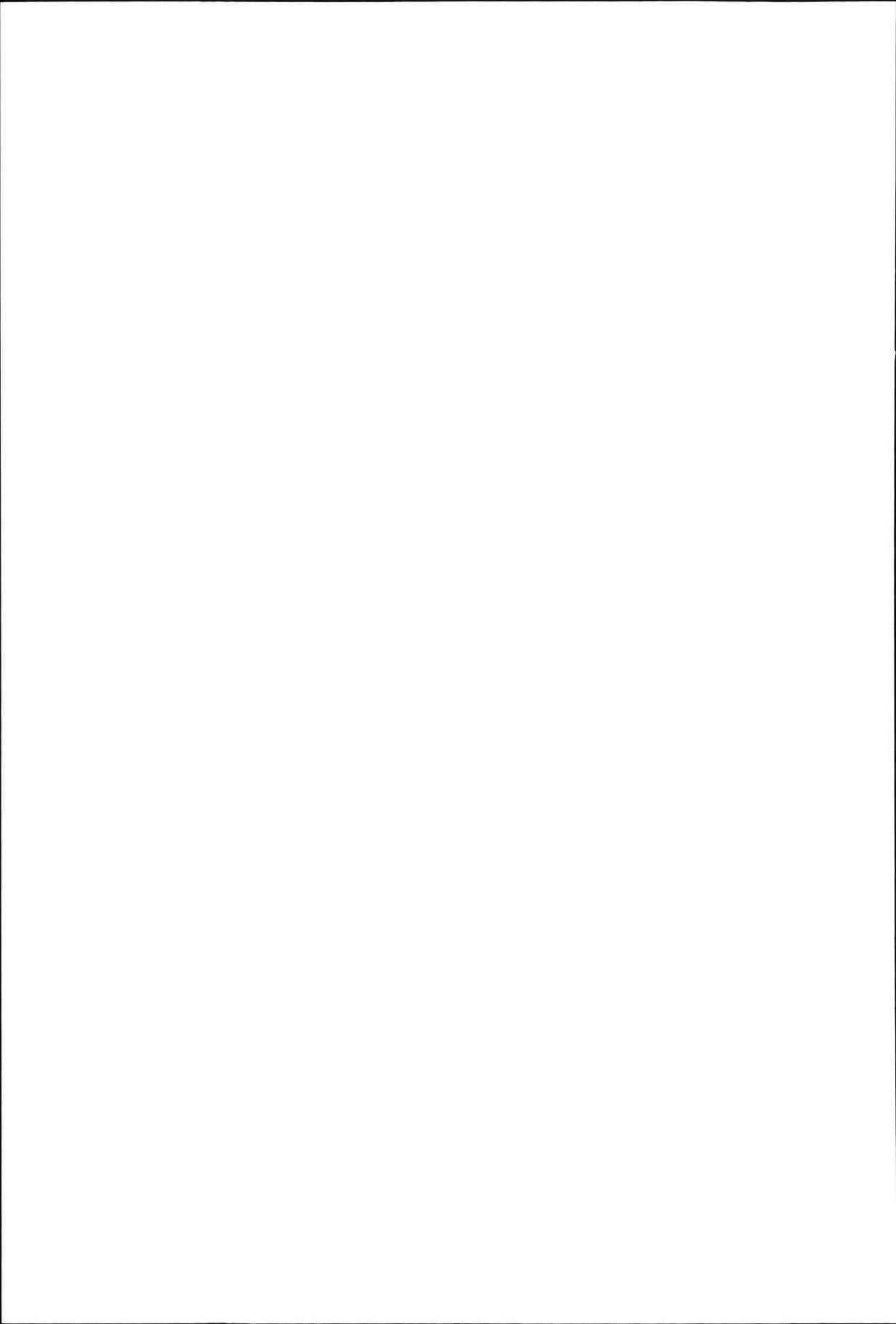
In conclusion, the clinical improvement is associated with a reduction in inflammation after treatment with alefacept. Furthermore, since alefacept, a T cell-specific agent, leads to decreased macrophage infiltration, the data indicate that T cells are highly involved in synovial inflammation in PsA.

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chapter

5

Early effects of tumour necrosis factor α blockade on skin and synovial tissue in patients with active psoriasis and psoriatic arthritis.

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Abstract

Background: Tumour necrosis factor α (TNF α) blockade using infliximab, a chimeric anti-TNF- α antibody, is an effective treatment for both psoriasis and psoriatic arthritis (PsA).

Objective: To analyze the early effects of infliximab treatment on serial skin and synovial tissue biopsy samples.

Methods: Twelve patients with both active psoriasis and PsA received a single infusion of either infliximab (3mg/kg) (n=6) or placebo (n=6) intravenously.

98 Synovial tissue biopsies and lesional skin biopsy specimens were obtained at baseline and 48 hours after treatment. Immunohistochemical analysis was performed to analyze the inflammatory infiltrate. In situ detection of apoptotic cells was performed by TUNEL assay and by immunohistochemical staining with anti-caspase-3 antibodies. Stained tissue sections were evaluated by digital image analysis.

Results: A significant reduction in mean (SEM) T-cell numbers was found in both lesional epidermis (baseline 37 (11) cells/mm, 48 hours 26 (11), $p = 0.028$) and synovial tissue (67 (56) cells/mm² v 32 (30), $p = 0.043$) after infliximab treatment, but not after placebo treatment (epidermis 18 (8) v 43 (20), NS; synovium 110 (62) v 46 (21), NS). Similarly, the number of macrophages in the synovial sublining was significantly reduced after anti-TNF α treatment (100 (73) v 10 (8), $p = 0.043$). The changes in cell numbers could not be explained by induction of apoptosis at the site of inflammation.

Conclusion: The effects of anti-TNF α therapy in psoriasis and psoriatic arthritis may be explained by decreased cell infiltration in lesional skin and inflamed synovial tissue early after initiation of treatment.

Introduction

Tumor necrosis factor α (TNF α) is a pivotal cytokine in various chronic inflammatory disorders, including rheumatoid arthritis (RA) and Crohn's disease. The central role of this cytokine has been emphasized by the therapeutic efficacy of infliximab, a chimeric TNF α neutralizing antibody (1-3). Psoriasis is a common dermatological disorder, affecting approximately 1.5% of the population, and is characterized by epidermal hyperproliferation, increased dermal angiogenesis and infiltration of mononuclear cells in dermis and epidermis. Psoriatic arthritis (PsA) affects 5-40% of the patients with psoriasis, and is diagnosed by clinical signs and symptoms, such as absence of rheumatoid factor, and a presentation of symmetric, oligoarticular, axial and/or distal interphalangeal joint involvement (4). Like RA, PsA can cause considerable joint damage, disability, and impairment of quality of life in a significant proportion of patients, with the additional handicap of skin involvement. The synovium of patients with PsA has not been studied as extensively as that of patients with RA. Recent studies suggest that the histology shows both differences and similarities between the two inflammatory joint diseases (5,6). The cell infiltrate in both joint diseases is composed predominantly of CD3⁺ T lymphocytes, located around the small blood vessels and near the hyperplastic intimal lining layer. Other cell types found in the synovial tissue of patients with PsA include macrophages and some neutrophils, located near the intimal lining layer and around the blood vessels (7).

Although the cause of psoriasis and PsA is still unknown, increasing evidence shows that the inflammatory response is primarily initiated by activated T cells in the epidermis and dermis of psoriatic lesions and in the synovium of affected joints (8-11). Proinflammatory cytokines, such as TNF α , have a key role in the inflammatory cascade in psoriasis and PsA as illustrated by the increased TNF α expression in psoriatic skin lesions (12, 13) and inflamed synovial tissue (14, 15). Consistent with this notion, infliximab has been reported to be clinically effective for both psoriasis and PsA (16-20), but the mechanism of action is not precisely known. To provide more insight into the effects of infliximab treatment in psoriasis and PsA, we performed a single centre, randomized, placebo controlled study to investigate the early changes at the site of inflammation.

Patients and methods

100 **Patients** Twelve patients with both active skin disease and active joint inflammation, diagnosed with PsA at least 12 months before inclusion, were evaluated in this prospective, single centre, double blind, randomized, placebo controlled study. Active arthritis was defined as at least three tender joints (28 joint count and both ankles (21)), and physician's and patient's joint assessment as moderate or worse, despite concurrent methotrexate (MTX) treatment at maximal tolerable dose (5-20 mg/week). Active psoriasis was defined as at least two psoriatic plaques. The dosage of MTX was kept stable at least 28 days before inclusion in the study. Stable doses of non-steroidal anti-inflammatory drugs were allowed, but prednisolone therapy was not. Only patients with a swollen knee or wrist joint were included. After randomization patients received a single infusion of infliximab 3 mg/kg or placebo. All patients gave informed consent before inclusion, and the study protocol was reviewed and approved by medical ethics committee of the Academic Medical Center/University of Amsterdam. The study was conducted according to the Declaration of Helsinki principles.

Synovial biopsies At baseline and 48 hours after infusion of either infliximab or placebo, small bore arthroscopy was performed under local anesthesia of the same knee (n= 8) or wrist (n= 4) joint. An average of at least 12 synovial tissue samples was obtained from the entire joint using a 2.5 mm grasping forceps (Storz, Tuttlingen, Germany) on each occasion, as described previously (22). Six samples were fixed in formaldehyde and embedded in paraffin, 6 samples were snap-frozen en bloc in Tissue Tek OCT (Miles, Elkhart, IN), and stored in liquid nitrogen until sectioning. Sections (5 µm) were cut in a cryostat and mounted on glass slides (Star Frost adhesive slides, Knittelgläser, Germany), which were stored at -70°C until immunohistochemical analysis could be performed.

Skin biopsies At baseline and 48 hours after infusion with either infliximab or placebo, 4 mm punch biopsies were taken from the inside border of a target psoriatic plaque, preferentially from an area not exposed to sun. Biopsy samples from each individual patient were obtained from the same target lesion, separated by at least 1 cm. The biopsies were randomly coded, snap-

frozen in Tissue Tek OCT (Sakura Finetek Europe, Zoeterwoude, The Netherlands), and stored at -70°C until further processing. Cryostat sections ($5\ \mu\text{m}$) were cut and mounted on glass slides (Star Frost adhesive slides), and stored at -70°C until immunohistochemical staining.

Immunohistochemistry The synovium and skin sections were stained with the monoclonal antibodies anti-CD3 (Becton Dickinson, San Jose, CA) to detect T lymphocytes, and anti-caspase-3 (Pharmingen, Becton Dickinson (skin), Cell Signaling Technology, Leusden, The Netherlands (synovium)) to detect apoptotic cells. In addition, the synovial tissue was stained with anti-CD68 (clone EBM11, Dako, Glostrup, Denmark) to detect macrophages. The staining procedure was performed as described previously (23). After a primary incubation step with monoclonal antibodies, bound antibody was detected according to a three step immunoperoxidase method. Horseradish peroxidase activity was detected using a hydrogen peroxide as substrate and aminoethylcarbazole as dye, producing a reddish color.

TUNEL assay A TUNEL assay was performed according to the manufacturer's instructions (Roche, Mannheim, Germany). In short, apoptotic cells in frozen synovial tissue en skin tissue were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling of apoptosis induced DNA strand breaks, using in situ cell death detection assay.

Digital image analysis All sections were randomly coded and analyzed by computer assisted image analysis, as previously described in detail (24). For the synovial tissue samples, three separate regions of six high power fields ($2.1\ \text{mm}^2$) were evaluated. Macrophage (CD68) expression was analyzed separately in the intimal lining layer and the synovial sublining. Caspase-3 expression was measured as integrated optical density, a product of staining area and intensity.

For the skin tissue samples, one single region of 20 high power fields ($2.1\ \text{mm}^2$) including both epidermis and dermis was analyzed. The images were acquired and analyzed using Syndia algorithm on a Qwin based analysis system (Leica, Cambridge, UK), as described previously.

Statistical analysis SPSS 10.1.4 for Windows (SPSS, Chigago, IL) was used for statistical analysis. The Wilcoxon signed rank test for matched pairs was used to compare data within each group. Results were expressed as mean (SEM).

Results

Patient characteristics Six men and six women were included in the study, and randomly allocated to receive either infliximab or placebo. Clinical baseline characteristics in both groups were comparable (table 1). From one of the patients, skin biopsy samples were not obtained for technical reasons. From another patient included in the study, the synovial tissue biopsy at baseline was not eligible for immunohistochemical analysis for quality reasons and, therefore, all samples from this patient were excluded from analysis.

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Table 1. Baseline characteristics

	Infliximab (n=6)	Placebo (n=6)
Age	53 (35-70)	45 (26-60)
Male: female	3:3	3:3
Duration of joint disease (years)	9 (5-13) *	9 (1-22)
Duration of skin disease (years)	24 (10-41)	17 (2-38) *
DAS	6.8 (5.0-7.9) *	5.7 (4.8-8.2)
30 Tender joint count	19 (2-26) *	9 (2-25)
30 Swollen joint count	15 (9-21) *	7 (2-26)
VAS	81 (65-90) *	68 (36-93)
CRP	23 (10-36) *	28 (7-65)
PASI		13.7 (1-29.8)
10.5 (5-19.0) *		
MTX dosage (mg/week)	10 (5-20)	10 (5-20)

* n=5. DAS, Disease Activity Score; VAS, visual analogue scale; CRP, C reactive protein; PASI, Psoriasis Area and Severity Index; MTX, methotrexate. Results are shown as mean (range).

Immunohistochemical analysis The severity of the inflammatory infiltrate in lesional epidermis and synovial tissue was comparable in both treatment groups at baseline. Forty eight hours after infusion, a significant reduction in the number of epidermal T cells was seen in patients treated with infliximab (baseline 37 (11) cells/mm, 48 hours 26 (11), $p = 0.028$), in contrast with patients treated with placebo (18 (8) v 43 (20), NS). This observation was mirrored by analysis of synovial tissue, showing a decrease in the total number of T cells 48 hours after treatment with infliximab (67 (56) cells/mm² v 32 (30), $p = 0.043$), but no significant change in the placebo group (110 (62) v 46 (21), NS). Analysis of

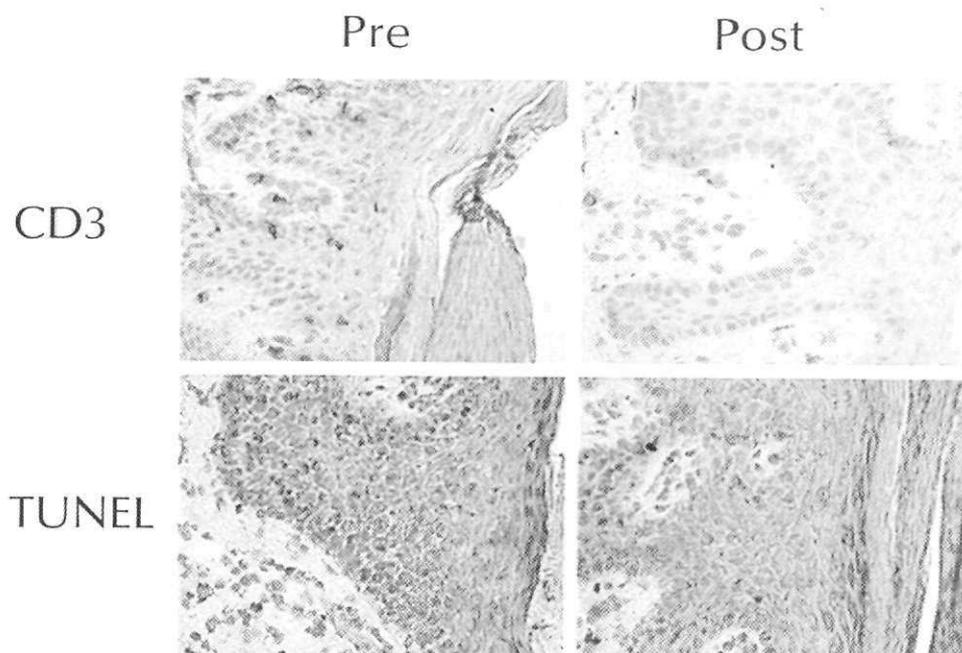


Figure 1 Representative images of CD3⁺ immunohistochemical staining and TUNEL assay in lesional psoriatic skin at baseline and 48 hours after initiation of infliximab treatment. Original magnification X400.

Table 2. Results of immunohistochemical staining and TUNEL assay of lesional skin and synovial biopsies.

	Infliximab 3 mg/kg		Placebo	
	Before	After	Before	After
CD3 ⁺ epidermis	37 (11)	26 (11)*	18 (8)	43 (20)
CD3 ⁺ dermis	159 (84)	136 (54)	101 (34)	167 (54)
CD3 ⁺ synovium	67 (56)	32 (30)*	110 (62)	46 (21)
CD68 ⁺ intimal lining layer	48 (41)	5 (3)	81 (25)	53 (36)
CD68 ⁺ synovial sublining	100 (73)	10 (8)*	111 (41)	67 (20)
TUNEL ⁺ epidermis	12 (7)	10 (6)	33 (13)	45 (26)
TUNEL ⁺ synovium	28 (10)	22 (10)	58 (20)	43 (14)

* significant reduction from baseline, $p < 0.05$. Epidermal counts are given as positive cells/mm. Dermal and synovial counts are given as positive cells/mm². Results are expressed as mean (SEM)

the synovial sublining also showed a significant reduction in the number of sublining macrophages in infliximab treated patients (100 (73) v 10 (8), $p = 0.043$), but not in the control group (111 (41) v 67 (20), NS). The decrease in macrophages in the intimal lining layer did not reach statistical significance (infliximab 48 (41) v 5 (3), NS; placebo 81 (25) v 53 (36), NS). Figure 1 and 2 and table 2 show the changes in the inflammatory infiltrate in both treatment groups.

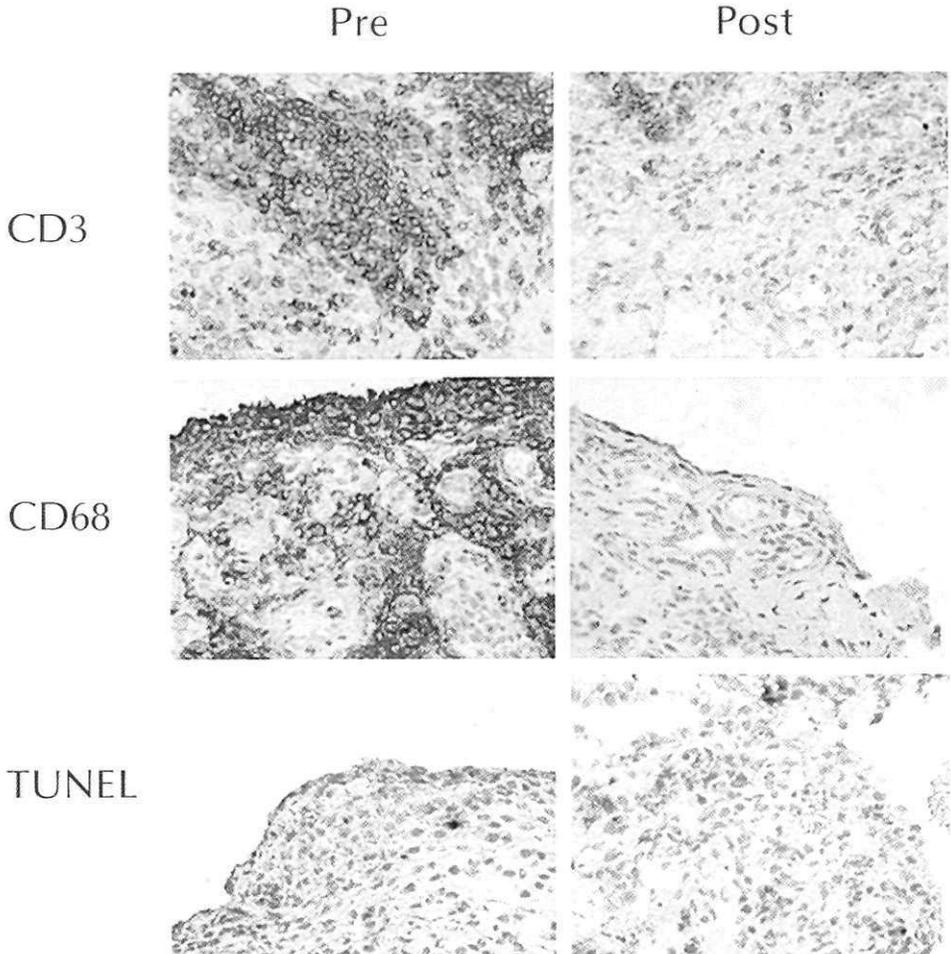


Figure 2 Representative images of CD3⁺ and CD68⁺ immunohistochemical staining and TUNEL assay in synovial tissue at baseline and 48 hours after initiation of infliximab treatment. Original magnification X400.

Apoptosis assays The presence of apoptotic cells was determined by TUNEL assay. Sections treated with DNase (Roche) to induce DNA fragmentation were included as positive controls.

Of interest, 48 hours after baseline, the number of apoptotic cells in both skin and synovium was unaltered in both the infliximab group (epidermis: baseline 12 (7) cells/mm², 48 hours 10 (6); synovium: baseline 28 (10) cells/mm², 48 hours 22 (10), NS) and the placebo group (epidermis: baseline 33 (13), 48 hours 45 (26); synovium: baseline 58 (20), 48 hours 43 (14), NS) (fig 2). To exclude

the possibility of a false negative result as a consequence of decreased cellularity, the analysis was repeated after correction for total cell counts. This confirmed that the number of TUNEL positive cells was not increased after treatment (data not shown).

Skin sections and synovial tissue were also analyzed for caspase-3 expression, as a marker of apoptosis. As positive controls, sections from UVB treated psoriatic skin were included, which clearly showed increased caspase-3 expression. Consistent with data obtained by TUNEL assay, the expression of caspase-3 did not significantly change after treatment in either group (data not shown).

Discussion

The data presented in this study show that a single infusion of a relatively low dose of infliximab (3 mg/kg) significantly decreases T cell and macrophage infiltration in synovial tissue of patients with PsA 48 hours after treatment. Similarly, we observed a reduction in T cell numbers in lesional epidermis. The reductions in the cell infiltrate could not be explained by an increase in the number of apoptotic cells at the site of inflammation.

Theoretically, the effects reported here could be influenced to some extent by the concurrent treatment with MTX. This drug may be capable of inhibiting synovial cell infiltration, in part by reducing the expression of adhesion molecules in synovial tissue (25). However, all patients had active disease at the time of inclusion despite MTX, and doses were kept stable at least 28 days before the start and during the study. Therefore, it appears unlikely that concomitant MTX treatment had a significant effect on the changes in synovial and skin biopsy samples. This notion is supported by the absence of significant changes in the patients who received placebo who also continued MTX treatment.

For analysis of synovial tissue we chose to select infiltration by T cells and macrophages because both cell types are considered crucial players in the pathogenesis of synovial inflammation in PsA. Moreover, previous work in RA patients has shown a reduction in numbers of T cells (26) and macrophages (27) 2-4 weeks after a single infusion of 10 mg/kg infliximab, suggesting that TNF α blockade might exert its effects, in part, by targeting these cells in the synovium, at least in RA. The a priori restriction of the number of immunohistological variables obviously decreases the chance of erroneously reporting statistically significant effects due to multiple comparisons.

The significant decrease in inflammatory cell infiltration in synovial tissue demonstrated in the present study is consistent with the reduction in synovial inflammation shown by gadolinium-DTPA uptake at week 10, which was previously described in patients with PsA who received infliximab treatment at 5 mg/kg at weeks 0, 2, and 6 (28). In addition, an open study in a heterogeneous group of eight patients with spondylarthropathy treated with infliximab at 5 mg/kg according to the same regimen showed a decrease in synovial macrophage infiltration 12 weeks after initiation of treatment (29). The infliximab dose used in the present study (3 mg/kg) is markedly lower than that used in previous studies evaluating the effects of infliximab in psoriasis and PsA. The results suggest that the lower dosage may also be effective. Obviously, clinically meaningful effects remain to be shown in larger, clinical studies.

In line with the changes in the synovium, we describe a decrease in T cell infiltration in paired skin biopsy samples after infliximab treatment. T cells are believed to have a central role in the pathogenesis of psoriasis, based on the presence of T cells in early psoriasis lesions (30), the beneficial effects of T cell-targeted therapies like cyclosporine A (31) and alefacept (32), and the altered relation between psoriatic keratinocytes and interferon- γ compared with normal keratinocytes (33). It is tempting to speculate that a decrease in antigen driven T cells at the site of inflammation might explain, in part, the beneficial effect of anti-TNF treatment in patients with psoriasis, similar to the effects of biologic therapies specifically targeting activated T cells in psoriasis and PsA (11, 32).

The changes in skin and synovial tissue were detected very early after initiation of treatment. Similar results were recently reported in patients with RA treated with infliximab (34). Consistent with the early immunohistological changes is the sometimes rapid onset of clinical improvement and changes in the acute phase response in patients with inflammatory disorders treated with infliximab. Clinical improvement and a reduction in C reactive protein levels may occur as early as 48 hours after initiation of therapy (20, 35, 36).

In line with recent observations in RA synovium (34), the decrease in cell infiltration could not be explained by induction of apoptosis at the site of inflammation, as shown by both TUNEL assay and caspase-3 staining. Thus, it appears that the mechanism of action of infliximab therapy might differ

between RA, PsA, and psoriasis on the one hand, and Crohn's disease (37, 38) on the other. In the last condition, an increase in the number of apoptotic cells in the lamina propria of the gut has been detected after infliximab therapy. A possible explanation for the discrepancy might be the difference in disease pathogenesis and tissue-specific properties. Neutralization of the effects of TNF α appears sufficient to induce clinical improvement in RA, PsA, and psoriasis even without induction of apoptosis at the site of inflammation. It should be noted that the available data suggest that treatment with both anti-TNF α antibodies and soluble TNF receptors are equally effective in RA, PsA, and psoriasis (39, 40) but not in Crohn's disease (41), where induction of apoptosis by anti-TNF antibody may be key to inducing clinical improvement. The decrease in cell infiltration seen in both skin and synovial tissue might be explained by reduced cell trafficking after TNF α blockade. Studies in patients with RA have shown that infliximab treatment decreases expression of adhesion molecules (26) and chemokines (27), molecules that are intimately involved in cell migration. Detailed studies addressing the effects of anti-TNF treatment on cell trafficking in PsA and psoriasis are, as yet, not available. In addition, we cannot exclude the possibility that infliximab may induce apoptosis in compartments other than skin and synovium, such as the bone marrow and peripheral blood, thereby affecting migration of inflammatory cells towards the synovial compartment and the skin. This remains to be shown in future studies.

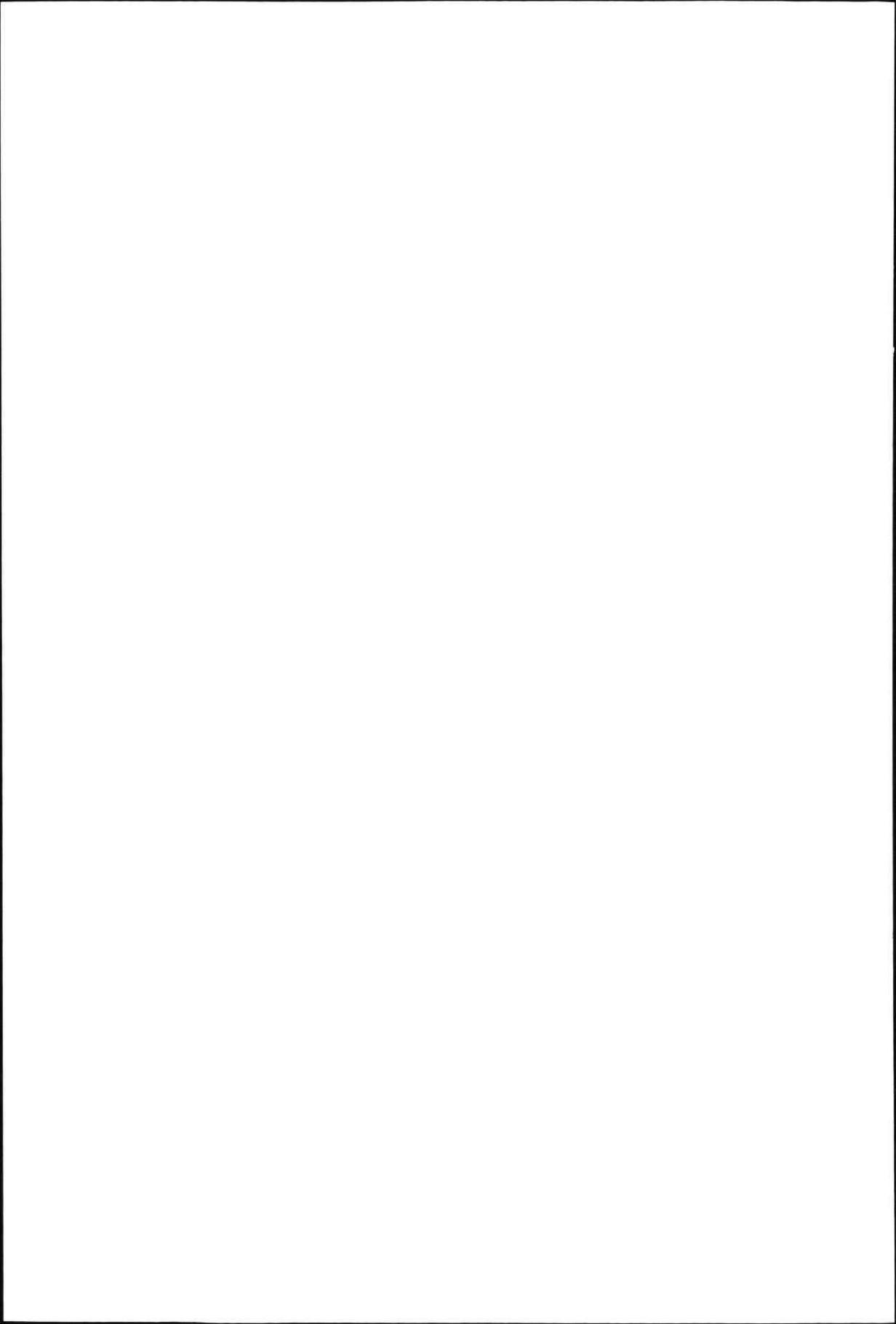
In conclusion, this study demonstrates a significant reduction in cell infiltration in both lesional epidermis and synovial tissue of patients with PsA by 48 hours after a single infusion of infliximab. The data support the view that TNF α is one of the key mediators in both psoriasis and PsA.

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chapter

6

Deactivation of endothelium and reduction in angiogenesis in psoriatic skin and synovium by low dose infliximab therapy in combination with stable methotrexate therapy:
a prospective single-centre study

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Abstract

Psoriasis and psoriatic arthritis are inflammatory diseases that respond well to anti-tumour necrosis factor- α therapy. To evaluate the effects of anti-tumour necrosis factor- α treatment on expression of adhesion molecules and angiogenesis in psoriatic lesional skin and synovial tissue, we performed a prospective single-centre study with infliximab therapy combined with stable methotrexate therapy.

112 Eleven patients with both active psoriasis and psoriatic arthritis received infusions of infliximab (3 mg/kg) at baseline, and at weeks 2, 6, 14 and 22 in an open-label study. In addition, patients continued to receive stable methotrexate therapy in dosages ranging from 5 to 20 mg/week. Clinical assessments, including Psoriasis Area and Severity Index (PASI) and Disease Activity Score (DAS), were performed at baseline and every 2 weeks afterward. In addition, skin biopsies from a target psoriatic plaque and synovial tissue biopsies from a target joint were taken before treatment and at week 4. Immunohistochemical analysis was performed to detect the number of blood vessels, the expression of adhesion molecules and the presence of vascular growth factors. Stained sections were evaluated by digital image analysis.

At week 16, the mean PASI was reduced from 12.3 ± 2.4 at baseline to 1.8 ± 0.4 ($P \leq 0.02$). The mean DAS was reduced from 6.0 ± 0.5 to 3.6 ± 0.6 ($P \leq 0.02$). We found some fluctuations in DAS response compared to the change in PASI, with the latter exhibiting a steady decrease over time. After 4 weeks the cell infiltrate was reduced in both skin and synovium. There was a significant reduction in the number of blood vessels in dermis and synovium at week 4. A significant reduction in the expression of $\alpha_v\beta_3$ integrin, a marker of revascularization, was also found in both skin and synovium at week 4. In addition, a significant reduction in the expression of adhesion molecules was observed in both skin and synovium at week 4. We also observed a trend toward reduced expression of vascular endothelial growth factor in both skin and synovium.

In conclusion, low-dose infliximab treatment leads to decreased neoangiogenesis and deactivation of the endothelium, resulting in decreased cell infiltration and clinical improvement in psoriasis and psoriatic arthritis.

Introduction

Tumour necrosis factor (TNF)- α has been recognized as a pivotal proinflammatory cytokine in several inflammatory diseases, including Crohn's disease and rheumatoid arthritis. Binding of TNF- α by infliximab, a chimeric IgG1 anti-TNF- α antibody has been shown to reduce clinical signs and symptoms of disease activity in several clinical trials (1-3). Psoriasis and psoriatic arthritis (PsA) are inflammatory diseases that also respond to anti-TNF- α therapy (4-10). Psoriasis is a common chronic skin disease characterized by hyperproliferation and abnormal differentiation of keratinocytes, as well as by infiltration of activated T cells in the epidermis and papillary dermis. PsA develops in 5-25% of patients with psoriasis. This destructive joint disease is characterized by symmetrical, oligoarticular, axial and/or distal interphalangeal joint involvement without the presence of rheumatoid factor (11). Histological features of PsA synovial tissue include infiltration by macrophages, T cells, and other inflammatory cells (12-14).

In addition to the inflammatory component described above, more recent studies on the histology of psoriasis and PsA revealed an important role for endothelial cells. In psoriasis, an abundance of blood vessels is present in the papillary dermis, showing microvascular changes such as pronounced dilatation and tortuosity (15). Expansion of the microvascular dermal plexus is believed to be mediated by angiogenesis, which is an active vasoproliferative process (16, 17). In PsA the synovium appears more vascular than in rheumatoid arthritis. Macroscopic observations of distinct changes in vascularity in PsA suggested possible pathogenetic differences between the two diseases. A typical morphology described as tortuosity and higher intensity of villous vascularization has been reported in PsA (12, 18).

Blood vessels in both psoriatic skin and synovial tissue express a variety of adhesion molecules, including intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin (13, 19). In addition, over-expression of vascular endothelial growth factor (VEGF), which is involved in neoangiogenesis, and of its endothelial cell receptors have been reported in psoriatic skin (20) and synovium (21). The prominent role played by neovascularization in the evolution of psoriatic plaques is underscored by the reported dose-dependent effect of neovastat, an inhibitor of angiogenesis, which resulted in improvement of psoriasis (22). Since TNF- α is

known to promote angiogenesis (23, 24), TNF- α blockade might be capable of inhibiting angiogenesis. Of interest, previous studies in patients with rheumatoid arthritis have shown that infliximab is able to deactivate the synovial endothelium (25, 26). There are only limited data for PsA, but examination of serial synovial biopsies in four patients suggested an inhibitory effect on synovial vascularity 12 weeks after initiation of therapy with 5 mg/kg infliximab (27). The aim of the present study was to evaluate the early effects of low-dose anti-TNF- α therapy on vascularity, in both psoriatic lesional skin and PsA synovial tissue, in relationship to the clinical effects. In short, we found that low-dose infliximab treatment in combination with methotrexate therapy leads to decreased neoangiogenesis and deactivation of the endothelium, resulting in decreased cell infiltration and clinical improvement in psoriasis and PsA.

Materials and methods

Study design The study was a 24-week, single-centre, prospective, open-label trial. Adult patients with a diagnosis of active PsA despite concomitant methotrexate therapy were recruited at the Academic Medical Centre/University of Amsterdam. Active psoriasis was defined as at least two psoriatic plaques, active arthritis was defined as at least three tender and swollen joints, and physician's joint assessment as moderate or worse.

A wash-out period of 28 days before study entry was applied in those patients who were receiving topical high-potency corticosteroids, phototherapy (including artificial tanning beds) and disease modifying anti-rheumatic drugs other than methotrexate. A wash-out period of 14 days was applied in those patients who were receiving low and moderate potency topical corticosteroids, topical vitamin D analogues, topical retinoids, keratolytics, or coal tar, other than on the scalp, palms, groins and/or soles of the feet. No topical treatment was allowed during the study except for emollients. The dosage of methotrexate was kept stable at least 28 days before inclusion. After inclusion, patients received infusions of 3 mg/kg infliximab at baseline, and at weeks 2, 6, 14 and 22.

The protocol was reviewed and approved by the medical ethical committee, and all patients gave their written informed consent before enrolment. The study was conducted according to the principles set out by the Declaration of Helsinki.

Assessments *Clinical evaluation* Clinical assessments were performed at baseline and at weeks 2, 4, 6, 8, 12, 14, 16, 20, 22, and 24. The clinical response of psoriatic skin lesions was measured using the Psoriasis Area and Severity Index (PASI), body surface area and the Physician's Global Assessment on a 7-point scale (ranging from 0 [clear] to 6 [very marked plaque elevation, scaling or erythema]). The percentage of patients achieving a 50%, 75% or 90% reduction in PASI from baseline (PASI 50, PASI 75, and PASI 90, respectively) was calculated. The clinical response of arthritis was measured by a modified Disease Activity Score (DAS; 28 joints + ankles [DAS30]) (28) and using the Health Assessment Questionnaire (29).

Skin biopsies. At baseline and 4 weeks after initiation of treatment, 4-mm punch biopsies were taken from the inside border of a target psoriatic plaque, preferentially from a non-sun-exposed area. Biopsies from each individual patient were obtained from the same target lesion, separated by at least 1 cm. The biopsy samples were randomly coded, snap-frozen in Tissue- Tek OCT (Miles, Elkhart, IN), and stored at -70°C until further processing. Cryostat sections (5 μm thick) were cut and mounted on glass slides (Star Frost Adhesive Slides, Knittelgläser, Germany), and stored at -70°C until immunohistochemical staining. All skin biopsies were analysed in triplicate to minimize random variation.

Synovial biopsies. At baseline and 4 weeks after initiation of treatment, a small-bore arthroscopy was performed under local anaesthesia of the same knee or wrist joint, which had been clinically active joint at the time the first biopsy was performed. An average of at least 12 synovial tissue samples was obtained from the entire joint using a 2.5-mm grasping forceps (Storz, Tuttlingen, Germany) on each occasion, as described previously (30). Six samples were fixed in formaldehyde and embedded in paraffin, and six samples were snap-frozen en bloc in Tissue-Tek OCT (Miles), and stored in liquid nitrogen until sectioning. Sections (5 μm thick) were cut in a cryostat and mounted on glass slides (Star Frost Adhesive Slides), which were stored at -70°C until immunohistochemical analysis could be performed.

Immunohistochemistry. Skin and synovial tissue sections were stained with anti-CD3 mAb (Becton Dickinson, San Jose, CA) to detect T cells. In addition, synovial tissue sections were stained with anti-CD68 mAb (clone EBM11, Dako, Glostrup, Denmark) to detect macrophages. Epidermal hyperproliferation was

evaluated by keratin-16 expression (Sigma, Saint Louis, MI). To analyze the expression of adhesion molecules, sections were stained with anti-VCAM-1 (CD106, 51-10C9, Becton Dickinson), anti-ICAM-1 (CD54, BBIG-L1, R&D Systems Inc., Minneapolis, MN), and anti-E-selectin (CD62E, 68-5H11, Becton Dickinson) mAbs. To study (factors involved in) vascularity, sections were stained with anti-VEGF (Santa Cruz Biotechnology, Inc., Santa Cruz, Ca), anti- $\alpha_v\beta_3$ integrin (CD51/CD61, Santa Cruz Biotechnology, Inc.), and anti-von Willebrand Factor (anti-vWF, Dako) mAbs. The staining procedure was performed as described previously (31). After a primary step of incubation with mAb, bound antibody was detected according to a three-step immunoperoxidase method. Horseradish peroxidase activity was detected using a hydrogen peroxide as substrate and amino-ethylcarbazole as dye, producing a reddish colour.

Digital image analysis. All sections were randomly coded and analyzed by computer-assisted image analysis as described previously (32). In short, images were acquired and analyzed using a Syndia algorithm on a Qwin based analysis system (Leica, Cambridge, U.K.). In skin biopsies, 20 high-power fields/section were analyzed. In synovial biopsies, 18 high-power fields from different parts of the section were analyzed. Positive staining of cellular markers was expressed as positive cells/mm² (dermis and synovium) or as positive cells/mm (epidermis). Positive staining of adhesion molecules, angiogenesis markers and growth factors was expressed as integrated optical density/mm². In skin sections, epidermal thickness was measured and expressed in millimetres.

Statistical analysis SPSS 10.1.4 for Windows (SPSS, Chigago, IL) was used for statistical analysis. The Wilcoxon signed rank test for matched pairs was used to compare baseline data with week 4 data. Results were expressed as mean \pm standard error of the mean.

Results

Clinical improvement of skin disease and arthritis activity after infliximab treatment Eleven patients with active PsA were included in the study and received infusions with low-dose infliximab (3 mg/kg). Baseline characteristics are summarized in Table 1. Patients had active disease despite methotrexate treatment. Two patients experienced adverse events during the study. One

patient suffered from a bursitis of the elbow and from a cold, another patient experienced headache, dry eyes, and restless feet. These adverse events were listed as mild events and were all of short duration. No serious adverse events were observed during the course of this study.

Table 1. Demographic and clinical data of study patients at baseline

Parameter	Data
Age (years)	49 (26-70)
Male: female ratio	6:5
Duration of joint disease (years)	9 (1-22)
Duration of skin disease (years)	21 (2-41)
Disease Activity Score	6.2 (4.8-8.2)
Tender joint count	14 (2-26)
Swollen joint count	11 (9-21)
Visual analogue scale for pain	69 (36-90)
C-reactive protein (mg/ml)	26 (7-36)
Psoriasis Area and Severity Index	12.2 (1.0-29.8)
Methotrexate dosage (mg/week)	10 (5-20)

Except for male: female ratio, data are expressed as mean (range) for the 11 patients evaluated. Visual analogue scale values were scored by the patient on a range 0-100 mm.

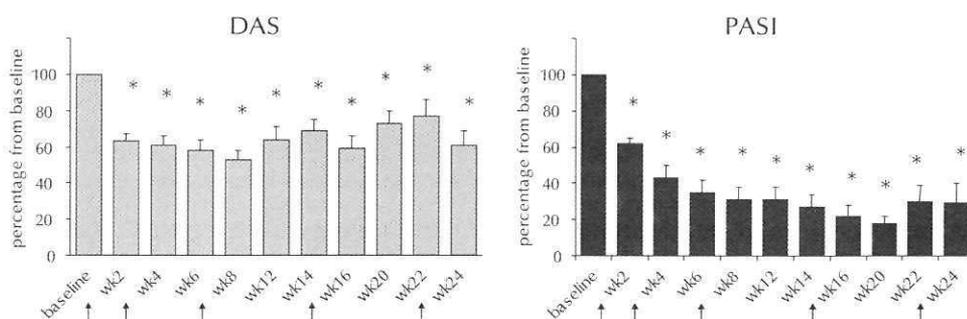


Figure 1. Clinical effects of low-dose infliximab (3 mg/kg). Shown are the Disease Activity Score (DAS 30, see Materials and Methods) results and Psoriasis Area and Severity Index (PASI) results. Results represent reductions from baseline, shown as mean \pm standard error of the mean. Arrows represent infliximab infusions. * $P \leq 0,02$ versus baseline.

After the first infusion of infliximab there was already a significant decrease in PASI, which was maintained throughout the study (Fig. 1). At week 16 the mean PASI was 1.8 ± 0.4 as compared with 12.3 ± 2.4 at baseline ($P \leq 0.02$). PASI 50 was achieved by 91% (10/11) of the patients at week 10. At the same time point, PASI 75 was achieved by 82% (9/11), and PASI 90 was achieved by 18% (2/11). The body surface area was reduced from $16.3 \pm 4\%$ at baseline to $4 \pm 1\%$ at week 16 ($P \leq 0.02$). Clinical pictures of a representative patient are shown in Fig. 2.

Baseline

Week 8

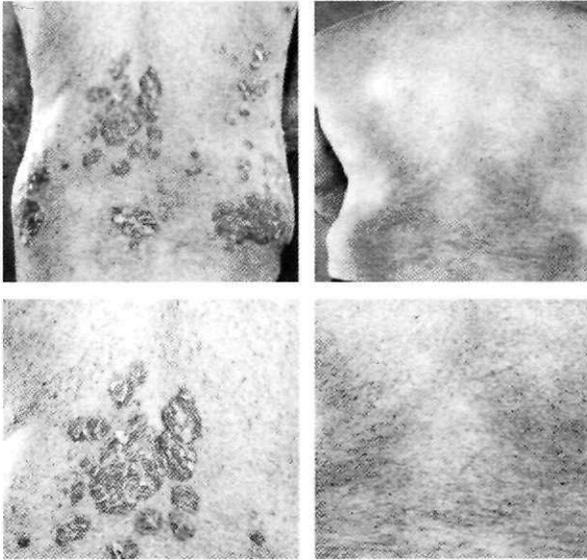


Figure 2. Pictures of a representative patient before (baseline) and 8 weeks after initiation of infliximab therapy.

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Amelioration of skin disease was associated with improvement of arthritis. Two weeks after the first infusion of infliximab a significant and clinically relevant decrease in DAS was observed. At week 16 the mean DAS was 3.6 ± 0.6 , as compared with 6.0 ± 0.5 at baseline ($P \leq 0.02$). Ten out of 11 patients (91%) exhibited a DAS response, defined as a decrease of at least 1.2 points. However, there was some fluctuation in the DAS response depending on the time points (Fig. 1). Approximately 6 weeks after the last infusion of the loading period (infusions at weeks 0, 2, and 6), DAS tended to be increased, and thereafter it decreased after each subsequent infusion. In contrast, for skin psoriasis we observed steady improvement in erythema and scaling of psoriatic plaques (Fig. 1). The mean Health Assessment Questionnaire score exhibited a rapid and sustained decrease from 3.2 ± 0.5 at baseline to 0.9 ± 0.3 at week 16 ($P \leq 0.02$).

Immunohistochemical changes in skin and synovium after infliximab treatment Skin biopsies from 11 patients were obtained at baseline and week 4. At the same time points, synovial biopsies were obtained from nine patients of the knee joint ($n=7$) or wrist ($n=2$). Baseline synovial biopsies from the other 2 patients were not suitable for immunohistochemical evaluation.

Table 2. Infiltration by T cells and macrophages in tissue samples before and 4 weeks after initiation of infliximab therapy

	Baseline	Week 4
CD3 epidermis	28 ± 7	3 ± 1 **
CD3 dermis	132 ± 47	58 ± 19 *
CD3 synovium	83 ± 46	14 ± 6
CD68 intimal lining layer	67 ± 27	47 ± 27
CD68 synovial sublining	112 ± 46	36 ± 18

Epidermal counts represent positive cells/mm. Dermal and synovial counts are shown as positive cells/mm². The data are expressed as mean ± standard error of the mean. ** P ≤ 0,02, * P ≤ 0,05, versus baseline

Decreased Cellularity. The cellular staining findings are shown in Table 2. At week 4 a significant decrease in the mean number of CD3⁺ T cells was observed in both lesional dermis and epidermis. Similarly, the number of CD3⁺ T cells and CD68⁺ macrophages in the synovium tended to be decreased, although the difference did not reach statistical significance, possibly due to the relatively small number of patients.

The mean epidermal thickness was reduced from 0.43 ± 0.04 mm to 0.16 ± 0.02 mm (P ≤ 0.02). Normalization of keratinocyte hyperproliferation, measured using epidermal keratin-16 expression, occurred in all biopsies obtained at week 4 (P ≤ 0.02).

Deactivation of endothelium. Results of the immunohistochemical analysis of the expression of all adhesion molecules are demonstrated in Table 3. A significant reduction in the expression of all adhesion molecules studied in lesional skin was observed 4 weeks after baseline. Mean E-selectin expression was reduced by 95% at week 4 compared with baseline (P ≤ 0.02). Mean ICAM-

Table 3. Expression of adhesion molecules

	Baseline	Week 4
ICAM-1 skin	2539 ± 425	532 ± 81 **
ICAM-1 synovial sublining	45382 ± 18097	10617 ± 3385 *
VCAM-1 skin	12242 ± 1334	6916 ± 1386 *
VCAM-1 synovium	4071 ± 1205	2419 ± 1052
E-selectin skin	625 ± 179	30 ± 8 **
E-selectin synovium	731 ± 224	494 ± 344

Expression of adhesion molecules in lesional skin and synovial biopsies (integrated optical density/mm²) before and 4 weeks after initiation of infliximab therapy. The data are expressed as mean ± standard error of the mean. ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule. ** P ≤ 0,02, * P ≤ 0,05, versus baseline.

Table 4. Vascularity

	Baseline	Week 4
vWF skin	4738 ± 1353	430 ± 158 **
vWF synovium	93121 ± 26511	32739 ± 7152 *
$\alpha_v\beta_3$ skin	9780 ± 1631	3580 ± 518 **
$\alpha_v\beta_3$ synovium	2003 ± 684	274 ± 97 *
VEGF skin	8230 ± 1651	5675 ± 1700
VEGF synovium	1784 ± 540	674 ± 236

120 Blood vessels positive for von Willebrand factor (vWF; all blood vessels) and $\alpha_v\beta_3$ (newly formed blood vessels) as well as expression of vascular endothelial growth factor (VEGF; integrated optical density/mm²) before and 4 weeks after initiation of infliximab therapy. The data are expressed as mean ± standard error of the mean. ** $P \leq 0,02$, * $P \leq 0,05$, versus baseline

1 expression was reduced by 79% ($P \leq 0.02$), and mean VCAM-1 expression was reduced by 44% ($P \leq 0.05$).

In synovial tissue, there was a significant reduction (81%) in the expression of ICAM-1 on synovial capillaries ($p \leq 0.05$). Decreased expression of both E-selectin and VCAM-1 was observed in the synovial tissue as well, although the change did not reach statistical significance.

Reduced vascularity. In both lesional dermis and synovial tissue, vascularity was significantly diminished after infliximab therapy, as shown by examination of haematoxylin stained sections. The mean number of blood vessels/mm² dermis was reduced from 27 ± 3 at baseline to 17 ± 2 at week 4 ($P \leq 0.02$). The number of blood vessels/mm² of synovial tissue was reduced from 18 ± 4 to 4 ± 1 ($P \leq 0.02$).

Consistent with these observations, there was a significant decrease in vWF-positive blood vessels and $\alpha_v\beta_3$ -positive newly formed blood vessels in the dermis ($P \leq 0.02$). A similar trend was seen for the expression of VEGF ($P = 0.37$) in lesional dermis. This growth factor is involved in blood vessel development. Evaluation of synovial tissue revealed the same pattern, with significant downregulation of both vWF and $\alpha_v\beta_3$ -positive vessels after infliximab treatment ($P \leq 0.05$), and a decrease in the expression of VEGF ($P = 0.07$; Table 4). Representative images of immunohistochemical staining are shown in Fig. 3.

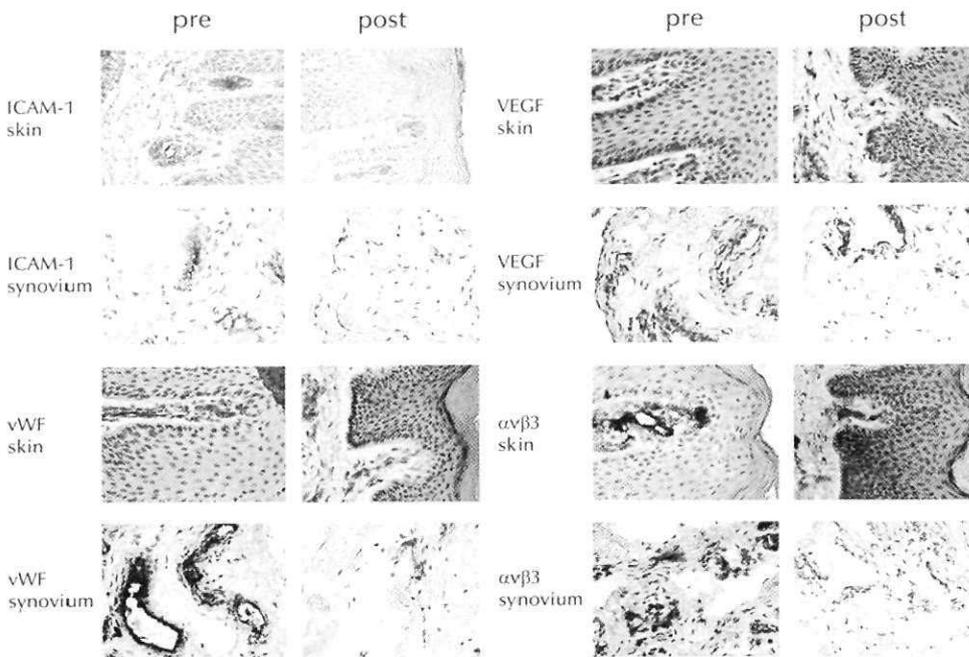


Figure 3. Representative pictures of immunohistochemical staining. Pictures represent the expression of von Willebrand Factor (vWF), $\alpha\text{v}\beta_3$ integrin, vascular endothelial growth factor (VEGF) and intercellular adhesion molecule (ICAM)-1 in skin and synovium before (pre) and 4 weeks after (post) initiation of infliximab therapy. Original magnification: 400x.

Discussion

The results of the present study confirm that anti-TNF- α treatment with infliximab is effective in reducing clinical signs and symptoms of both psoriasis and PsA. In comparison with previously performed clinical trials in PsA with 5 mg/kg infliximab (33), we demonstrated that a low-dose treatment regimen with 3 mg/kg in combination with methotrexate was also efficacious, exhibiting a rapid decrease in both PASI and DAS after the first dose of infliximab. The clinical effects confirm and extend the results of another recently reported trial (34). However, it should be noted that, although the decrease in PASI was sustained at a steady level throughout the study period, the DAS exhibited some fluctuation over time. After each administration of infliximab, a decrease in DAS was observed that was sustained for approximately 6 weeks, after which the score slowly increased to approximately 75 % of the baseline value until the next infusion. These data suggest that optimal infliximab therapy for the treatment of PsA might require a shorter dose interval period or higher

dosages. In contrast, low-dose infliximab treatment every 8 weeks appears to be sufficient to treat moderate-to-severe plaque psoriasis, at least in patients on stable concomitant methotrexate therapy.

122 The immunohistochemical evaluation performed in this study may provide insights into the immunomodulatory effects of infliximab on psoriatic skin and synovium in situ. We chose to conduct the immunohistochemical analysis at week 4 in order to ensure observation of the early effects of infliximab. It is known from clinical experience that after 2 weeks of infliximab therapy a beneficial clinical effect can be observed in both skin lesions and inflamed joints in PsA. In addition, we have recently shown in patients with rheumatoid arthritis that marked changes can be detected in the synovial tissue as soon as 48 h after the first infusion with infliximab (35). Apart from a reduction in clinical parameters of psoriasis and PsA, a decrease was observed in the number of inflammatory cells in lesional skin and synovial tissue biopsies at week 4. Although the reduction in CD3⁺ T cells and CD68⁺ macrophages in synovial tissue did not reach statistical significance, this might be accounted for by the relatively small number of patients from whom synovial biopsies could be obtained (n=9).

The mechanism by which the number of lesional inflammatory cells is decreased by low-dose infliximab in psoriasis and PsA is apparently not induction of apoptosis at the site of inflammation, as we recently demonstrated (36). Conceivably, infliximab treatment might reduce cell migration as well as retention of inflammatory cells in the skin and synovial tissue. A similar mechanism appears to be operative in rheumatoid arthritis (25, 35, 37).

In the present study, we found that infliximab is capable of reducing the expression of the adhesion molecules ICAM-1, VCAM-1 and E-selectin on endothelium in psoriatic dermis and synovial tissue. ICAM-1 is a member of the immunoglobulin superfamily and is widely distributed in psoriatic skin and synovial tissue (13, 19). Synthesis and expression of ICAM-1 on endothelial cells and keratinocytes can be induced by TNF- α (38, 39). The interaction between leukocyte function-associated antigen (LFA)-1 and ICAM-1 mediates adherence of leucocytes to endothelial cells, facilitating migration of inflammatory cells to inflamed areas (40). VCAM-1 is expressed on activated endothelial cells and stimulates transendothelial cell trafficking by binding to its ligand very late antigen (VLA)-4 on T cells and monocytes (41). E-selectin

mediates T-lymphocyte trafficking to psoriatic lesional skin by binding to cutaneous lymphocyte-associated antigen (CLA) (42, 43). The role of E-selectin-mediated cell trafficking in PsA synovium is less clear (44), but studies conducted in rheumatoid arthritis suggest a potential role in the pathogenesis of synovial inflammation (45). The observed decrease in adhesion molecule expression could be explained in part by the reduction in vascularity discussed below. It should be noted, however, that there was also clearly decreased expression of molecules per blood vessel (Fig. 3).

We found a significant and profound decrease in vascularity and neoangiogenesis in both skin and synovium after treatment. This might be particularly important in psoriasis and PsA because of the prominent role of hypervascularization, and the typical tortuous morphology of the capillaries, in these diseases (12, 15, 18). Previous work has shown that serum and tissue levels of VEGF are elevated in psoriasis and PsA compared with normal individuals (46-49). The effect of infliximab on vascularity, as shown in the present study, might be explained in part by reduced VEGF expression at the site of inflammation. Other factors could be involved as well. For instance, recent studies indicate a role for angiogenic peptides such as endothelial-cell stimulating angiogenesis factor (ESAF) and plasminogen activator inhibitor type-1 (PAI-1) in psoriasis (47, 50). The effects reported here could in theory be influenced to some extent by the concurrent treatment with methotrexate. This drug has been reported to inhibit neovascularization *in vitro* and *in vivo* (51). Therefore, it might be more difficult to detect an additional reduction in vascularity after adding infliximab to the therapeutic regimen. However, because the dosages of methotrexate were relatively low and were kept stable throughout the study, we do not consider it likely that concurrent methotrexate therapy influenced our results to a great extent.

Conclusion

TNF- α targeted therapy with low-dose infliximab in combination with stable methotrexate therapy confers improvement in clinical signs and symptoms of psoriasis and PsA. Decreased cell infiltration in both skin and synovial tissue associated with clinical improvement might be explained in part by deactivation of vascular endothelium and by inhibition of vascularity, resulting in decreased inflammatory cell migration.

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chapter

7

In situ effects of etanercept therapy in psoriatic lesional skin: downregulation of adhesion molecule expression leads to decreased influx of T cells

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Manuscript submitted for publication

Abstract

128 In the last decade, TNF- α targeted therapy has proven to be a successful treatment for plaque psoriasis. Of this class etanercept has recently been approved for the treatment of psoriasis. Etanercept is a fusion protein consisting of two identical chains of a recombinant human TNF receptor (p75) monomer fused to the Fc portion of human IgG1. By competitive inhibition of the interaction of circulating TNF- α with cell surface-bound TNF-receptors, etanercept is thought to prevent TNF-mediated cellular responses by rendering TNF biologically inactive. Since little is known about the actual mechanism of action of etanercept in psoriasis, we studied the effects of etanercept in lesional psoriatic skin in situ in a double-blind placebo-controlled study. Patients were treated with either 50 mg s.c. twice weekly (n=1), 25 mg s.c. twice weekly (n=2), or placebo (n=3). Skin biopsies were performed at baseline, after 4, and after 12 weeks of treatment. We observed a strong reduction in infiltrating T cells in lesional epidermis and dermis after 4 weeks in etanercept-treated patients, in contrast to placebo-treated patients. There was a reduction in the expression of the adhesion molecules E-selectin, VCAM-1, and ICAM-1 in the etanercept-treated patients. No changes in the expression of vWF, α v β 3, and VEGF were observed. In conclusion, etanercept induces a decreased influx of activated T cells in lesional skin of psoriasis patients in association with a downregulation of the expression of adhesion molecules on endothelial cells.

Introduction

Although the exact pathogenesis of psoriasis and psoriatic arthritis (PsA) is not yet identified, multiple clues point in the direction of TNF- α as a key cytokine in the inflammatory cascade that characterizes these diseases. For example, levels of TNF- α and TNF- α -receptors are increased in psoriatic lesional skin compared to uninvolved and normal skin (1, 2). Second, TNF- α is a versatile cytokine capable of inducing numerous pro-inflammatory cytokines and chemokines exerting specific effects in the pathogenesis of psoriasis. Infliximab and etanercept are two immunobiologicals targeted against TNF- α that have demonstrated significant improvement in clinical parameters of psoriasis and PsA. Etanercept is a fusion protein consisting of two identical chains of a recombinant human TNF receptor (p75) monomer fused to the Fc portion of human IgG1. By interfering with the binding of circulating TNF- α to cell surface TNF-receptors, etanercept is thought to prevent TNF-mediated cellular responses by rendering TNF biologically inactive. Infliximab has identical effects on circulating TNF- α but has additional effects on membrane and receptor bound TNF- α not observed with etanercept (3). Recently performed trials with etanercept s.c. 25 mg twice a week as monotherapy for psoriasis demonstrated that approximately 30% of patients achieved a reduction in psoriasis area and severity index (PASI) of 75% after 12 weeks of treatment, and 44-56% of patients reached this level after 24 weeks of treatment (4,5). A comparable study with infliximab for treatment of moderate to severe psoriasis demonstrated that 88% of patients achieved PASI 75 after 10 weeks of treatment, and 58% achieved PASI 90 by week 10 (6). Clinical efficacy of infliximab in psoriasis was supported by changes in immunohistochemical parameters in lesional psoriatic skin after infliximab treatment, as seen by reductions in T cell numbers after only 48 hours after initiation of therapy, as well as reduced expression of adhesion molecules after 4 weeks of treatment (7).

Although comparable immunohistochemical effects are expected after etanercept therapy (8), no evaluation of the changes in the inflammatory infiltrate in psoriasis lesions during etanercept therapy has been performed as yet. For this reason, we performed an explorative placebo-controlled, randomised, double-blinded, single-centre study to evaluate changes in the inflammatory infiltrate, adhesion molecule expression, and angiogenesis in psoriatic lesional skin of patients treated with etanercept.

Methods

Patients Six patients with moderate to severe plaque-type psoriasis (PASI \geq 10 at baseline) were included in the biopsy analysis. There was a 4-week washout period for PUVA therapy and systemic therapy, and a 2-week washout period for UVB therapy and topical therapy. After inclusion, patients were treated in a double-blinded regimen with either etanercept 50 mg s.c twice weekly (n=1), etanercept 25 mg s.c twice weekly (n=2), or placebo twice weekly (n=3) for 12 consecutive weeks. Clinical improvement was evaluated by PASI at baseline and after 4 and 12 weeks of therapy.

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Skin biopsies At baseline, 4 weeks, and 12 weeks after initiation of treatment, 4-mm punch biopsies were taken from the inside border of a target psoriatic plaque, preferentially from a non-sun exposed area. Biopsies from each individual patient were obtained from the same target lesion, separated by at least 1 cm. The biopsy samples were randomly coded, snap-frozen in Tissue-Tek OCT (Miles, Elkhart, IN), and stored at -70°C until further processing. Five-micrometer cryostat sections were cut and mounted on glass slides (Star Frost adhesive slides, Knittelgläser, Germany), and stored at -70°C until immunohistochemical staining was performed. For each staining three sections of each biopsy were analysed to minimize random variation.

Immunohistochemical analysis Skin sections were stained with anti-CD3 monoclonal antibody (Mab) (BD Pharmingen, San Jose, CA) to detect T cells. T-cell subpopulations were detected with anti-CD4 (BD Pharmingen) and anti-CD8 (BD Pharmingen) Mab. Epidermal hyperproliferation was evaluated by keratin-16 expression (Sigma, Saint Louis, MI). To analyze the expression of adhesion molecules, sections were stained with anti-vascular cell adhesion molecule (VCAM)-1 (CD106, BD Pharmingen), anti-intercellular adhesion molecule (ICAM)-1 (CD54, R&D Systems Inc., Minneapolis, MN), and anti-E-selectin (CD62E, BD Pharmingen) Mab. To study (factors involved in) vascularity, sections were stained with anti-vascular endothelial growth factor (VEGF, Santa Cruz Biotechnology, Inc., Santa Cruz, Ca), anti-alpha-v-beta-3-intergrin ($\alpha_v\beta_3$, CD51/CD61, Santa Cruz Biotechnology, Inc.), and anti-von Willebrand Factor (vWF, Dako, Glostrup, Denmark) Mab. The staining procedure was performed as described previously (9). Following a primary

step of incubation with Mab, bound antibody was detected according to a 3-step immunoperoxidase method. Horseradish peroxidase activity was detected using hydrogen peroxide as substrate and amino-ethylcarbazole (AEC) as chromogen, producing a reddish colour.

Digital image analysis Stained sections were randomly coded and analyzed by computer-assisted image analysis as described previously (9). In short, images were acquired and analyzed using Syndia algorithm on a Qwin based analysis system (Leica, Cambridge, U.K.). Twenty high power fields per section were analysed. Positive staining of cellular markers was expressed as positive cells/mm² (dermis) or as positive cells/mm (epidermis). Positive staining of adhesion molecules, angiogenesis markers, and growth factors was expressed as integrated optical density/mm² (IOD/mm²).

Semi-quantitative analysis For keratinocyte expression of K16 keratin, a semi-quantitative score was used ranging from 0 to 4+. Scores of 0 or 1+ were considered as normal expression, scores of 2+, 3+, or 4+ were considered as abnormal expression. Epidermal thickness was expressed in mm.

Results

Etanercept reduces clinical signs of psoriasis activity We observed a reduction in PASI in 2 of 3 patients treated with etanercept (17% and 41% reduction compared to baseline respectively) after 4 weeks of treatment, and a minor reduction in one of the placebo-treated patients at this time point (7% reduction compared to baseline). After 12 weeks of treatment, all 3 patients treated with etanercept showed a reduction in PASI (56%, 52%, and 78% reduction compared to baseline respectively). There was no difference in the clinical response between the patient receiving 50 mg etanercept twice weekly and the patients receiving 25 mg etanercept twice weekly. One patient treated with placebo responded with a 36% reduction in PASI compared to baseline, another patient showed a reduction in PASI of 8% reduction compared to baseline. The individual PASI values of the patients are shown in Table 1.

Table 1. Individual PASI values at baseline, and after 4 and 12 weeks of either etanercept treatment (25 mg twice weekly or 50 mg twice weekly) or placebo treatment. Numbers between brackets represent % reduction in PASI from baseline.

Patient	1 (50 mg)	2 (25 mg)	3 (25 mg)	4 (placebo)	5 (placebo)	6 (placebo)
Baseline	10.9	11.4	21.7	15.2	15.2	14.1
Week 4	9 (17%)	6.7 (41%)	28.8 (0%)	18.4 (0%)	19.8 (0%)	13.1 (7%)
Week 12	4.8 (56%)	5.5 (52%)	4.7 (78%)	9.8 (36%)	16.5 (0%)	13 (8%)

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Etanercept reduces the number of T cells and T-cell subpopulations in lesional epidermis and dermis A strong reduction in the number of CD3⁺ T cells in both epidermis and dermis was found in all three patients after 4 weeks of etanercept treatment, which was even more pronounced after 12 weeks of treatment. The mean reduction in epidermal T-cell numbers was 93% and the mean reduction in dermal T-cell numbers was 80%. There was no difference in response between the patient receiving 50 mg etanercept twice weekly and patients receiving 25 mg etanercept twice weekly. Of the placebo-treated patients, only one patient showed a transient reduction in epidermal and dermal T-cell numbers at week 4, which returned to the baseline observation

Table 2. Absolute numbers of CD3⁺ T cells and CD4⁺ and CD8⁺ T-cell subpopulations before and after treatment. Data are expressed as cells/mm (epidermis) or cells/mm² (dermis).

Patient		1 (50 mg)	2 (25 mg)	3 (25 mg)	4 (placebo)	5 (placebo)	6 (placebo)	
CD3	epidermis	Baseline	63	20	45	7	27	20
		Week 4	19	12	26	22	14	63
		Week 12	5	1	2	32	43	12
	dermis	Baseline	185	373	185	69	391	37
		Week 4	51	75	92	179	110	246
		Week 12	59	42	35	299	388	102
CD4	epidermis	Baseline	4	63	23	3	6	47
		Week 4	4	26	10	4	4	47
		Week 12	0	1	2	4	17	8
	dermis	Baseline	15	480	159	64	77	409
		Week 4	12	186	57	44	164	653
		Week 12	8	24	18	49	96	249
CD8	epidermis	Baseline	8	1	5	8	6	59
		Week 4	4	0	3	0	5	49
		Week 12	0	0	1	2	16	3
	dermis	Baseline	16	13	11	38	21	37
		Week 4	10	4	7	6	11	89
		Week 12	3	10	16	20	22	17

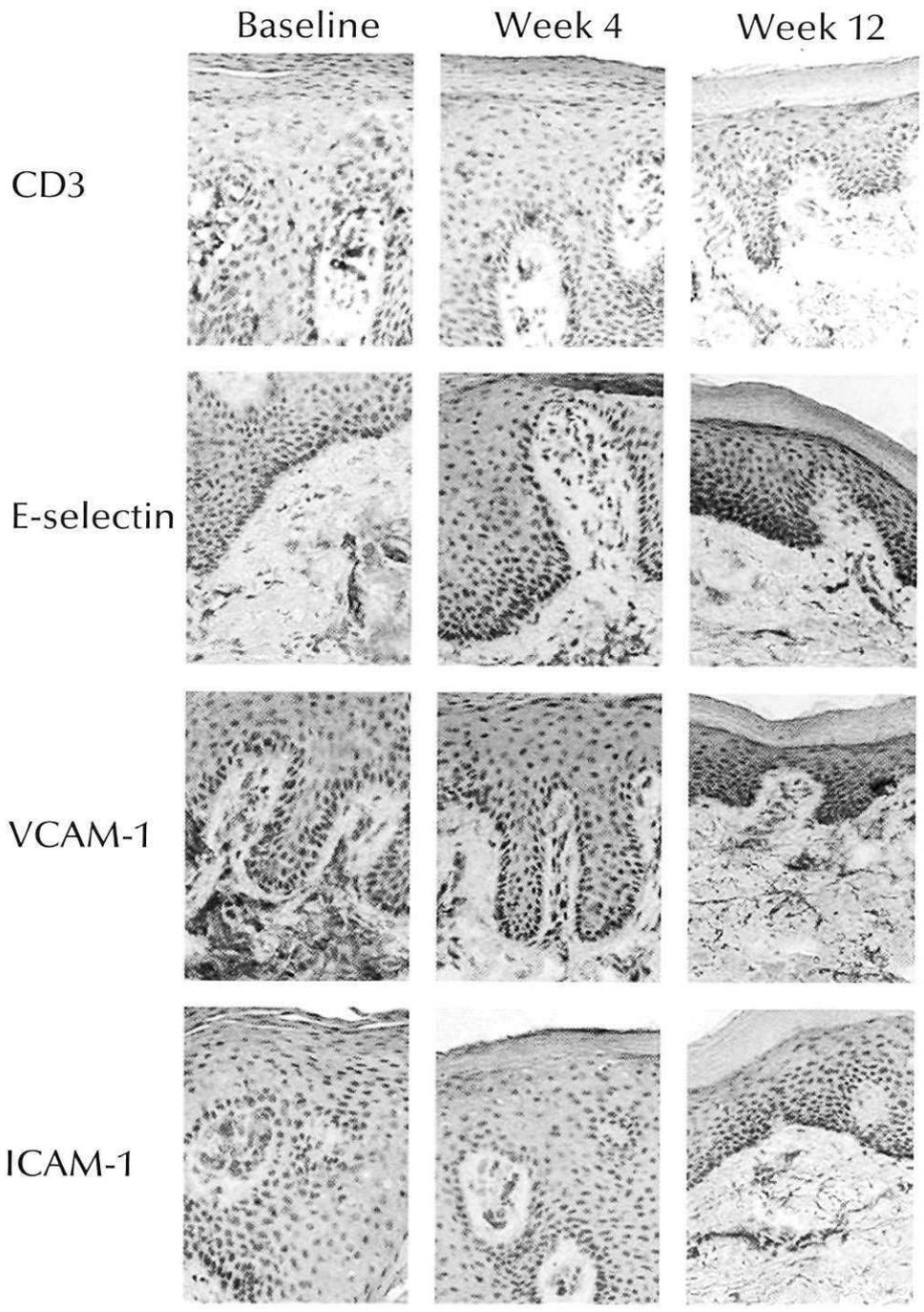


Figure 1. CD3, E-selectin, VCAM-1, and ICAM-1 immunohistochemical staining of lesional skin of patient 1 at baseline and after 4 and 12 weeks of etanercept treatment (50 mg s.c. twice weekly). Original magnification: 400x.

in the biopsy taken after 12 weeks. The epidermal thickness was reduced in all etanercept-treated patients at week 4 (mean reduction 34%) and week 12 (mean reduction 56%), in contrast to no changes in the placebo-treated patients. After 12 weeks of etanercept treatment keratin-16 expression was normalized in all patients, whereas at the same time point this expression was still abundantly present in placebo-treated patients.

Looking at T-cell subpopulations, both epidermal and dermal CD4⁺ T cell numbers showed a reduction at week 4 and week 12 in all patients treated with etanercept. We also observed a reduction in epidermal CD8⁺ T cell numbers in all three patients treated with etanercept. Dermal CD8⁺ T cell numbers of these patients showed also a reduction at week 4, which was less evident at week 12. In the patients treated with placebo the CD4 and CD8 expression was quite variable and did not show a clear decrease as was found in the etanercept-treated patients. Absolute T-cell numbers before and after therapy are shown in Table 2.

Etanercept reduces the expression of adhesion molecules A strong reduction in the expression of E-selectin was observed in all etanercept-treated patients at both timepoints, which was not observed in the placebo-treated patients. The expression of VCAM-1 was reduced at week 12 in all etanercept-treated patients and in none of the placebo-treated patients. The expression of ICAM-1 was reduced in the week 4 and 12 biopsies in 2 etanercept-treated patients and 1 placebo-treated patient (Table 3).

Table 3. Expression of E-selectin, VCAM-1, and ICAM-1 on endothelial cells at baseline and after 4 and 12 weeks of either etanercept treatment (25 mg s.c. twice weekly or 50 mg s.c. twice weekly) or placebo treatment. Data is expressed as IOD/mm².

Patient		1 (50 mg)	2 (25 mg)	3 (25 mg)	4 (placebo)	5 (placebo)	6 (placebo)
E-selectin	Baseline	439	1105	1102	980	1371	3056
	Week 4	205	125	738	394	427	6575
	Week 12	250	35	395	1741	1024	2502
VCAM-1	Baseline	7685	5221	5326	3816	5001	6683
	Week 4	3591	6220	1542	1734	2334	7875
	Week 12	2821	846	1670	5532	5472	9731
ICAM-1	Baseline	1707	258	396	609	4628	2081
	Week 4	943	318	382	987	3994	1802
	Week 12	75	554	180	1683	1906	1954

Etanercept does not induce a significant change in vascularity and angiogenesis The expression of vWF, $\alpha v\beta 3$, and VEGF in etanercept-treated patients after 4 weeks and 12 weeks of treatment was quite variable and no clear shift could be detected (Table 4).

Table 4. Expression of vWF, $\alpha v\beta 3$, and VEGF at baseline and after 4 and 12 weeks of either etanercept treatment (25 mg s.c. twice weekly or 50 mg s.c. twice weekly) or placebo treatment. Data is expressed as IOD/mm².

Patient		1 (50 mg)	2 (25 mg)	3 (25 mg)	4 (placebo)	5 (placebo)	6 (placebo)
vWF	Baseline	15032	5624	21967	4243	21165	28685
	Week 4	3083	3922	37203	23858	39862	36053
	Week 12	2733	11609	25840	15563	63350	23901
$\alpha v\beta 3$	Baseline	1554	482	1379	2145	3985	1522
	Week 4	2390	884	1071	1781	2274	669
	Week 12	1604	2608	475	1588	2240	797
VEGF	Baseline	1331	84	647	295	817	2017
	Week 4	313	412	175	297	893	704
	Week 12	1195	110	952	101	799	313

Discussion

Despite extensive research in different immunological areas, the exact pathogenic mechanism in psoriasis remains an unsolved puzzle. Besides excessive T-cell activation as a trigger for the development of a psoriatic plaque (10, 11), more recently TNF- α has been recognized to play a pivotal role in the inflammatory cascade. TNF- α has multiple effects; it induces production of other pro-inflammatory cytokines, such as IL-1, IL-6, and IFN- γ , and is capable of increasing the production of NF κ B (12, 13) resulting in protection against apoptosis. TNF- α is also involved in T-cell trafficking to lesional skin via the induction of chemokines, and it stimulates the upregulation of adhesion molecules such as E-selectin, vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 on endothelial cells (14-17). In connection with this, a positive correlation between the expression of E-selectin and VCAM-1 on endothelial cells and the number of infiltrating T cells in active psoriatic skin was identified (18). Finally, TNF- α has been shown

to induce the expression of vascular endothelial growth factor (VEGF) mRNA in keratinocytes (19), thus stimulating angiogenesis in lesional psoriatic skin. The two biological anti-TNF- α agents currently used for the treatment of psoriasis and PsA are infliximab and etanercept. Infliximab has been shown to induce complement-mediated cell lysis, antibody-dependent cytotoxicity, and caspase-dependent apoptosis of cells expressing membrane TNF- α *in vitro* (3, 20, 21), in contrast to etanercept (22). Also, the interaction between etanercept and membrane as well as soluble TNF- α is 'reversible' in that the inhibition of biological activity of TNF- α is less than that of infliximab and the binding is of lower affinity (3). Besides differences in efficacy, this might explain differences in adverse events, such as decreased host defence, between infliximab and etanercept.

The aim of this study was to investigate the immunohistochemical changes following etanercept treatment in patients with plaque psoriasis. Previous studies on lesional psoriatic skin after infliximab therapy demonstrated a significant reduction in T-cell infiltration already after 48 hours of therapy. This observation was accompanied by a significant reduction in the expression of adhesion molecules, e.g. E-selectin, VCAM-1, and ICAM-1 after 4 weeks of infliximab therapy (7), supporting that a reduction in leukocyte trafficking might be an important aspect of the mechanism of infliximab. In addition, a reduction in the expression of vWF and $\alpha v\beta 3$ on endothelial cells was observed, substantiating that infliximab is capable of regulating angiogenesis in psoriatic lesional skin, interfering with leukocyte recruitment as well. These assumed mechanisms of actions of infliximab are consistent with observations in synovial tissue in patients with rheumatoid arthritis (RA) treated with infliximab (23-25). The results of the present study suggest that etanercept is an effective treatment for moderate to severe plaque psoriasis. All patients treated with etanercept in our study achieved more than 50% reduction in PASI after 12 weeks of treatment, and one patient even more than 75% reduction in PASI after 12 weeks of treatment. This improvement was associated with a normalization of epidermal proliferation as seen by reduction of expression of keratin-16, as well as a decrease of more than 50% of epidermal thickness. Despite the small number of patients participating in the biopsy study, we

observed reductions in the number of infiltrating T cells in both lesional dermis and epidermis already after 4 weeks of treatment, and an even more pronounced reduction after 12 weeks of treatment. The observed reduction in T cells reflected a reduction in both epidermal and dermal CD4⁺ T cells, as well as epidermal CD8⁺ T cells. Interestingly, we did not detect consistent changes in the expression of IFN- γ and IL-4 in lesional skin of these patients (data not shown). Data on the effects of anti-TNF- α therapy, e.g. etanercept and infliximab on type 1 and 2 cytokine profiles in various inflammatory disorders are conflicting and not consistent. Both an increase in the expression and production of IFN- γ (26-28) and a reduction have been described (29-31) after anti-TNF- α therapy. Regardless of the conflicting data on the effects on cytokine profiles, infliximab and etanercept therapy have proven to be successful in RA, PsA and psoriasis, suggesting that neutralisation of soluble TNF- α without directly affecting T-cell function is sufficient to induce clinical remission of these diseases.

Focusing on adhesion molecules, we observed a trend towards downregulation of the expression of E-selectin and VCAM-1, and to a lesser extent ICAM-1, on endothelial cells of etanercept-treated patients. By downregulating adhesion molecules, etanercept presumably decreases cutaneous inflammation without causing depletion of T cells. These findings are consistent with previous reports on etanercept therapy in RA and uveitis (32, 33). As infliximab has a more direct effect on proteins involved in the angiogenesis (7), etanercept did not seem to have a profound effect on vascularity and angiogenesis in lesional psoriatic skin of patients treated in the present study. In general, we did not observe differences in clinical and immunohistochemical responses between the patient treated with 50 mg etanercept twice weekly and patients treated with 25 mg etanercept twice weekly.

In conclusion, we speculate that the mechanism of action of etanercept in psoriasis is a decreased influx of activated T cells in lesional skin caused by a downregulation of the expression of adhesion molecules on endothelial cells. The promising results of TNF- α -targeted therapy, e.g. infliximab and etanercept, in psoriasis, force us to deviate our thoughts about the pathogenesis of psoriasis from T-cell mediated origin toward the direction of an imbalance in TNF- α activity. One might speculate that due to a (genetic)

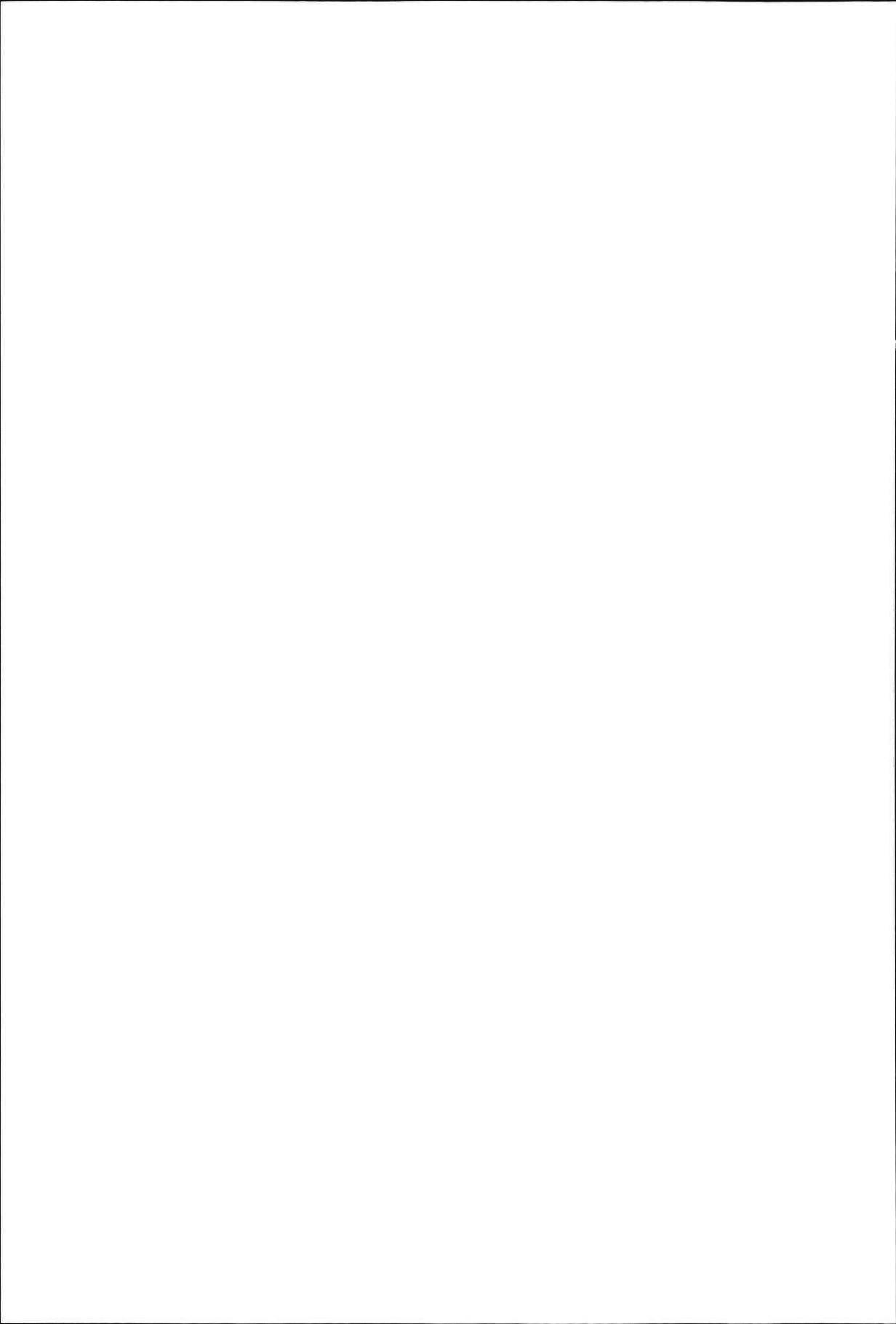
deficiency in downregulation of TNF- α activity, various stimuli might activate the innate immune response, leading to up regulation of adhesion molecules and recruitment of inflammatory cells, which in the end results in epidermal hyperproliferation. Future studies might help us to identify what specific triggers account for the initial production of TNF- α in psoriasis, possibly by defining the upstream cytokine network from TNF- α . Most likely both innate and adaptive immunity are found to be involved in the pathogenesis of psoriasis.

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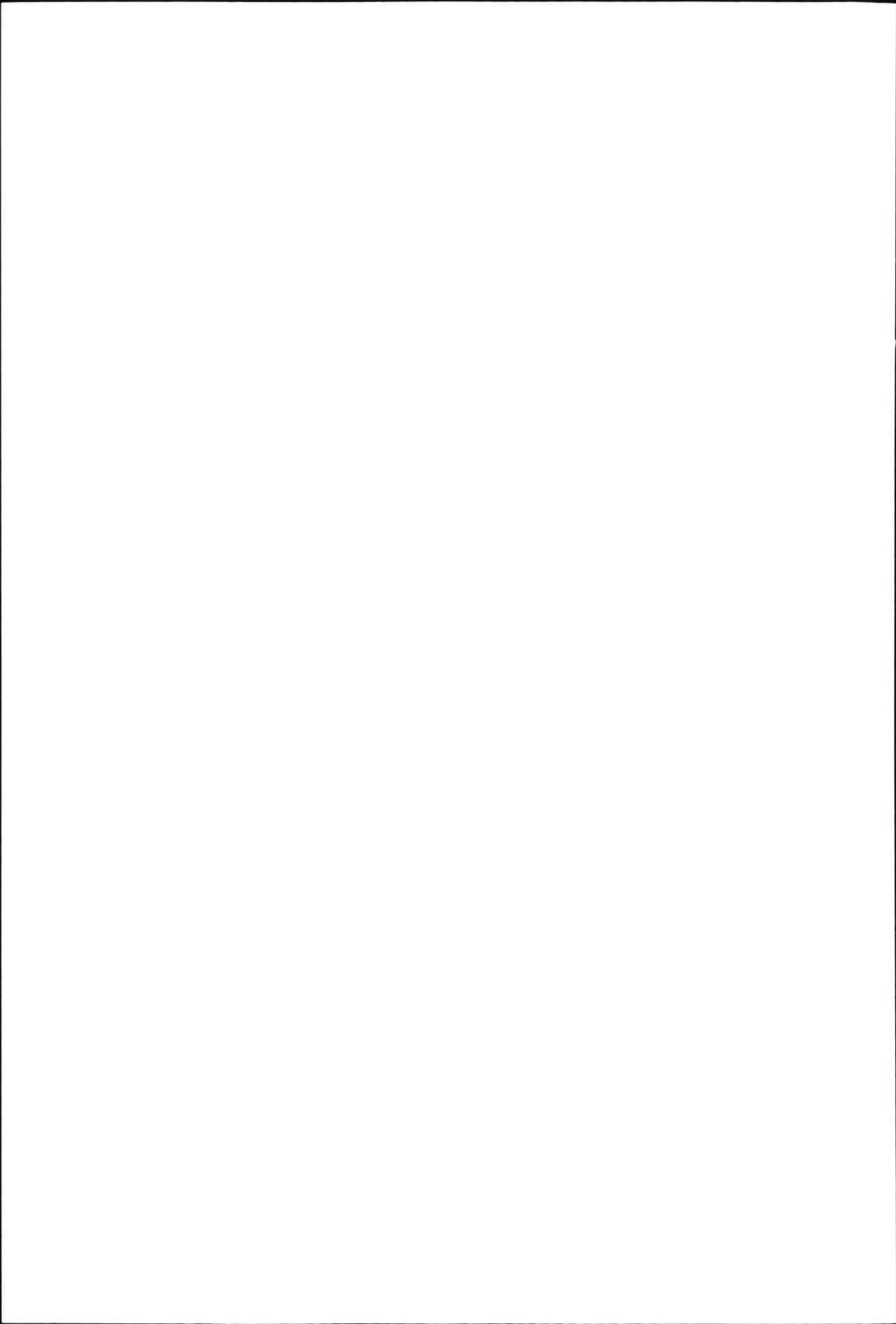
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chapter

8

Summary and conclusions



Summary

The aim of this thesis was to investigate the changes in lesional psoriatic skin and synovial tissue of patients with plaque psoriasis and psoriatic arthritis (PsA) after treatment with biological response modifiers. On the one hand this provides a tool to assess the efficacy of new drugs besides clinical assessment tools such as the psoriasis area and severity index (PASI) and American College of Rheumatology (ACR) response criteria. On the other hand this approach may help to understand the immunopathogenetic mechanisms of both psoriasis and PsA. Since psoriasis and PsA are considered to be T-cell mediated diseases, we were in particular interested in changes in T-cell subsets after therapy. In addition, because psoriasis and PsA are characterized by activated endothelium and increased vascularity in lesional skin and synovial tissue, we investigated changes in expression of adhesion molecules (involved in migration of T cells), angiogenesis markers, and growth factors (both involved in neovascularization) after therapy.

Traditionally, the evaluation of the cellular infiltrate and expression of proteins in skin tissue sections is done by manual quantification. However, for reliable evaluation of histology in the development of new anti-psoriatic treatments there is a need for a more time-efficient and reproducible method. To test the use of digital image analysis (DIA) in this situation we compared the assessment of immunohistochemically stained skin sections with manual quantification (MQ) and semi-quantitative analysis (SQA) in **chapter 2**. The number of CD3⁺ T cells and the expression of E-selectin were evaluated in stained paired skin biopsies from 11 patients with chronic plaque psoriasis before and after initiation of anti-psoriasis therapy. We observed significant correlations between MQ and DIA for the number of T cells. Both DIA and MQ were equally effective in detecting reductions of T-cell numbers in active-treated patients. MQ took considerable more time to complete than DIA. We also observed significant correlations between SQA and DIA for the expression of E-selectin although DIA was more sensitive than SQA to detect (early) changes. SQA took considerable more time to complete than DIA. From this study we concluded that the quantification of the inflammatory infiltrate in psoriatic lesional skin by DIA generated similar results as MQ and SQA in a reliable, reproducible and more time efficient fashion. We used DIA

to evaluate changes in the inflammatory infiltrate in lesional skin and synovium after **alefacept** therapy. Alefacept, a LFA-3/IgG1 fusion protein, interferes with the activation and proliferation of T cells by binding to the CD2 receptor on their surfaces. The clinical efficacy of this drug has been demonstrated in chronic plaque psoriasis. We performed a single-centre, open-label study to investigate the clinical efficacy and immunohistochemical effects on the T-cell population in psoriatic lesional skin and synovium (**chapters 3 and 4**). Eleven patients with plaque psoriasis and PsA all received twelve weekly doses of 7.5 mg alefacept intravenously. Skin biopsies were obtained at baseline and at day 8, 43 and 92, and synovial biopsies were obtained at baseline and at day 29 and 92. Skin and synovial tissue was evaluated by DIA after immunohistochemical staining. After completion of treatment, the majority of patients experienced a PASI reduction $\geq 50\%$ compared to baseline. Immunohistochemical analysis of lesional skin displayed a gradual decrease in the number of cutaneous T cells during therapy, in particular epidermal CD8⁺ cells and dermal CD4⁺ cells. Patients with a PASI reduction $\geq 50\%$ after therapy had a clearance of effector / memory (CD45RO⁺) T cells from the epidermis, in contrast to patients with $< 50\%$ PASI reduction. In addition to improvement of skin lesions, the majority of the patients fulfilled the Disease Activity Score (DAS) response criteria for arthritis. There was a significant reduction in CD4⁺T cells in the synovial tissue after therapy. In addition, patients who fulfilled the DAS response criteria after treatment displayed a significant reduction in effector / memory (CD45RO⁺) T cells compared with non-responding patients. These findings support the hypothesis that effector / memory T cells play a prominent role in the pathogenesis of psoriasis and PsA, and that alefacept is capable of reducing these cells in lesional psoriatic skin and synovium. This is also the first time alefacept therapy was demonstrated to improve symptoms of PsA.

In the following studies we focused on anti-TNF- α therapy, and the clinical and immunohistochemical effects of these drugs on lesional psoriatic skin and synovium. TNF- α plays a key role in the inflammatory cascade in psoriasis and PsA as illustrated by the increased TNF- α expression in psoriatic skin lesions and inflamed synovial tissue. Consistent with this notion, **infliximab**, an anti-TNF- α monoclonal antibody, has been reported to be clinically effective for both psoriasis and PsA, but the mechanism of action is not

precisely known. In vitro studies suggest that the binding of infliximab to membrane-bound TNF- α could lead to lysis of TNF- α producing cells via activation of complement-dependent or antibody-dependent cell-mediated toxicity. Indeed, an increase in the number of apoptotic cells in the lamina propria of the gut has been detected after infliximab therapy in Crohn's disease. To investigate whether apoptosis of T cells played a role in the mechanism of action of infliximab in psoriasis and PsA as well, we performed a single-centre, randomized, placebo-controlled study to investigate the early changes at the site of inflammation (**chapter 5**). Twelve patients with both active psoriasis and PsA were randomized to receive a single infusion of either infliximab (3mg/kg) or placebo intravenously. Synovial tissue biopsies and lesional skin biopsies were obtained at baseline and 48 hours after initiation of treatment, and evaluated by digital image analysis after immunohistochemical staining. A significant reduction in T cell numbers was found in both lesional epidermis and synovial tissue after infliximab treatment, but not after placebo treatment. However, the changes in cell numbers could not be explained by induction of apoptosis at the site of inflammation (investigated by both caspase-3-staining and TUNEL assay). Neutralization of the effects of TNF- α appears sufficient to induce clinical improvement in psoriasis and PsA even without induction of apoptosis at the site of inflammation, for example by reduced T cell trafficking to lesional skin and synovium. In line with this idea, studies in rheumatoid arthritis patients have shown that infliximab therapy results in decreased expression of adhesion molecules and chemokines, molecules that are essentially involved in cell migration. Blood vessels in both psoriatic skin and synovial tissue express a variety of adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), and E-selectin. In addition, overexpression of vascular endothelial growth factor (VEGF), which is involved in neoangiogenesis, and its endothelial cell receptors have been reported in psoriatic skin and synovium. To investigate the influence of infliximab on T-cell trafficking and vascularity, we treated 11 patients with psoriasis and PsA with infliximab (3 mg/kg) at baseline, and at weeks 2, 6, 14 and 22 in an open-label study (**chapter 6**). Clinical assessments, including PASI, and DAS, were performed at baseline and every 2 weeks afterwards. In

addition, skin biopsies from a target psoriatic plaque and synovial tissue biopsies from a target joint were taken before treatment and at week 4. The number of T cells, the number of blood vessels, the expression of adhesion molecules, and the presence of vascular growth factors were investigated by digital image analysis. At week 16, the mean PASI was reduced considerably from 12.3 at baseline to 1.8. The mean DAS was strongly reduced as well. Because the dosage of infliximab was lower (3mg/kg) than usual (5mg/kg), we found some fluctuations in DAS response compared to the change in PASI, the latter showing a steady decrease over time. After 4 weeks, the cell infiltrate was reduced in both skin and synovium, with a significant reduction in the number of blood vessels in dermis and synovium, and a significant reduction in the expression of alpha-v-beta-3 integrin ($\alpha_v\beta_3$), a marker of neovascularisation, in both skin and synovium. In addition, a significant reduction in the expression of adhesion molecules was observed in both skin and synovium at week 4. We also observed a trend towards reduced expression of vascular endothelial growth factor (VEGF) in both skin and synovium. The effects of infliximab on vascularity might be particularly important in psoriasis and PsA because of the prominent role of hypervascularization and the typical tortuous morphology of the capillaries in these diseases. In conclusion, low-dose infliximab treatment led to decreased neoangiogenesis and deactivation of the endothelium resulting in decreased cell infiltration and clinical improvement in psoriasis and PsA. To investigate whether these effects are restricted to infliximab therapy or apply to anti-TNF-alpha therapy in general, we performed a similar study with **etanercept** therapy in **chapter 7**. Etanercept is a fusion protein consisting of two identical chains of a recombinant human TNF receptor (p75) monomer fused to the Fc portion of human IgG1. By competitive inhibition of the interaction of circulating TNF- α with cell surface-bound TNF-receptors, etanercept is thought to prevent TNF-mediated cellular responses by rendering TNF biologically inactive. Compared to infliximab, the inhibition of biological activity of TNF- α by etanercept appears less effective. This might explain differences in efficacy between infliximab and etanercept. We studied the effects of etanercept in lesional psoriatic skin in situ in a double-blind placebo-controlled study. Six patients were treated with either 50 mg s.c. twice weekly, 25 mg s.c. twice

weekly, or placebo. Skin biopsies were performed at baseline, after 4, and after 12 weeks of treatment. The results suggest that etanercept is an effective treatment for moderate to severe plaque psoriasis. All patients treated with etanercept in our study achieved more than 50% reduction in PASI after 12 weeks of treatment, and one patient even more than 75% reduction in PASI after 12 weeks of treatment. We observed a strong reduction in infiltrating T cells in lesional epidermis and dermis after 4 weeks in etanercept-treated patients, in contrast to placebo-treated patients. There was a reduction in the expression of the adhesion molecules E-selectin, VCAM-1, and ICAM-1 in the etanercept-treated patients. However, in contrast to infliximab therapy, no changes in the expression of vWF, $\alpha_v\beta_3$, and VEGF were observed, suggesting that etanercept did not seem to have a profound effect on vascularity and angiogenesis in lesional psoriatic skin. In conclusion, etanercept induces a decreased influx of activated T cells in lesional skin of psoriasis patients in association with a downregulation of the expression of adhesion molecules on endothelial cells.

Concluding remarks

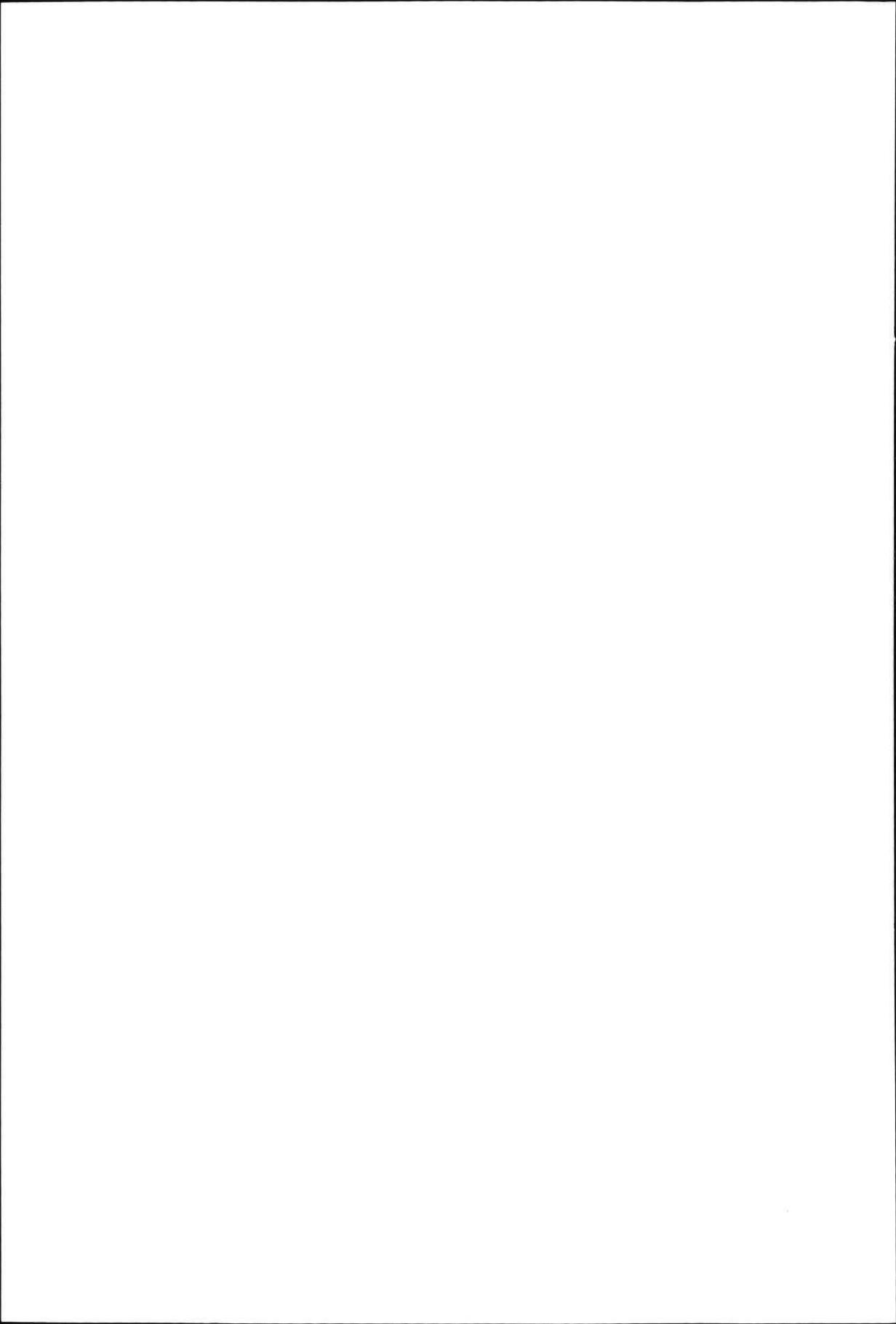
The aetiology of psoriasis and psoriatic arthritis (PsA) is still not known. Initially, psoriasis was thought to be a disorder of the keratinocytes, since hyperproliferation and abnormal differentiation of keratinocytes result in the silvery scales that are typical for psoriasis. The reason for disturbed keratinisation, however, could not be identified, nor could this hypothesis explain the joint disease associated with psoriasis. Approximately two decades ago the emphasis of psoriasis research shifted from keratinocytes to T cells because of two important observations. First, a remarkable improvement in clinical signs and symptoms of psoriasis was observed during cyclosporine treatment, a known T-cell inhibitor. The success of T-cell targeted therapy was recently confirmed when alefacept, an LFA-3/IgG1 fusion protein that inhibits T-cell activation, was found to ameliorate symptoms of both psoriasis and PsA (this thesis). Second, immunohistochemical staining of the inflammatory infiltrate of psoriasis revealed that activated effector / memory T cells were the first cells to arrive in early skin lesions and were present in lesional synovium as well. It is therefore suggested that psoriasis and PsA are

T-cell mediated diseases, in which activated T cells responding to an unknown antigen drive the initiation of the inflammatory cascade, including abnormal keratinocyte proliferation and differentiation. Many established and experimental anti-psoriatic treatments are based on this principle, such as cyclosporine A, UVB, methotrexate, and alefacept. Surprisingly, when anti-TNF- α therapy emerged in other inflammatory diseases, such as rheumatoid arthritis and Crohn's disease, it was discovered that both psoriasis and PsA responded remarkably well to these therapies, suggesting that TNF- α might play a more important role in the pathogenesis of psoriasis and PsA than previously anticipated. This suggestion is supported by the observations that the expression of TNF- α is increased in serum, skin, and synovium of patients with psoriasis and PsA. TNF- α is not only a regulator of the inflammatory response, but also plays an important role in bone metabolism by enhancing osteoclastogenesis. With TNF- α inhibition, there is a domino effect of decreased expression of other proinflammatory cytokines and decreased recruitment of activated cells, with consequent down-regulation of inflammation in both skin and joints. It should be noted, however, that severe infections in some of the patients treated with anti-TNF- α therapy underline the need for careful control. In addition, many of these treatments generally do not achieve complete remission in all patients, and it would be interesting to identify which patients are suitable for each specific anti-TNF- α therapy, perhaps by using pharmacogenomic techniques that will be tested in the near future.

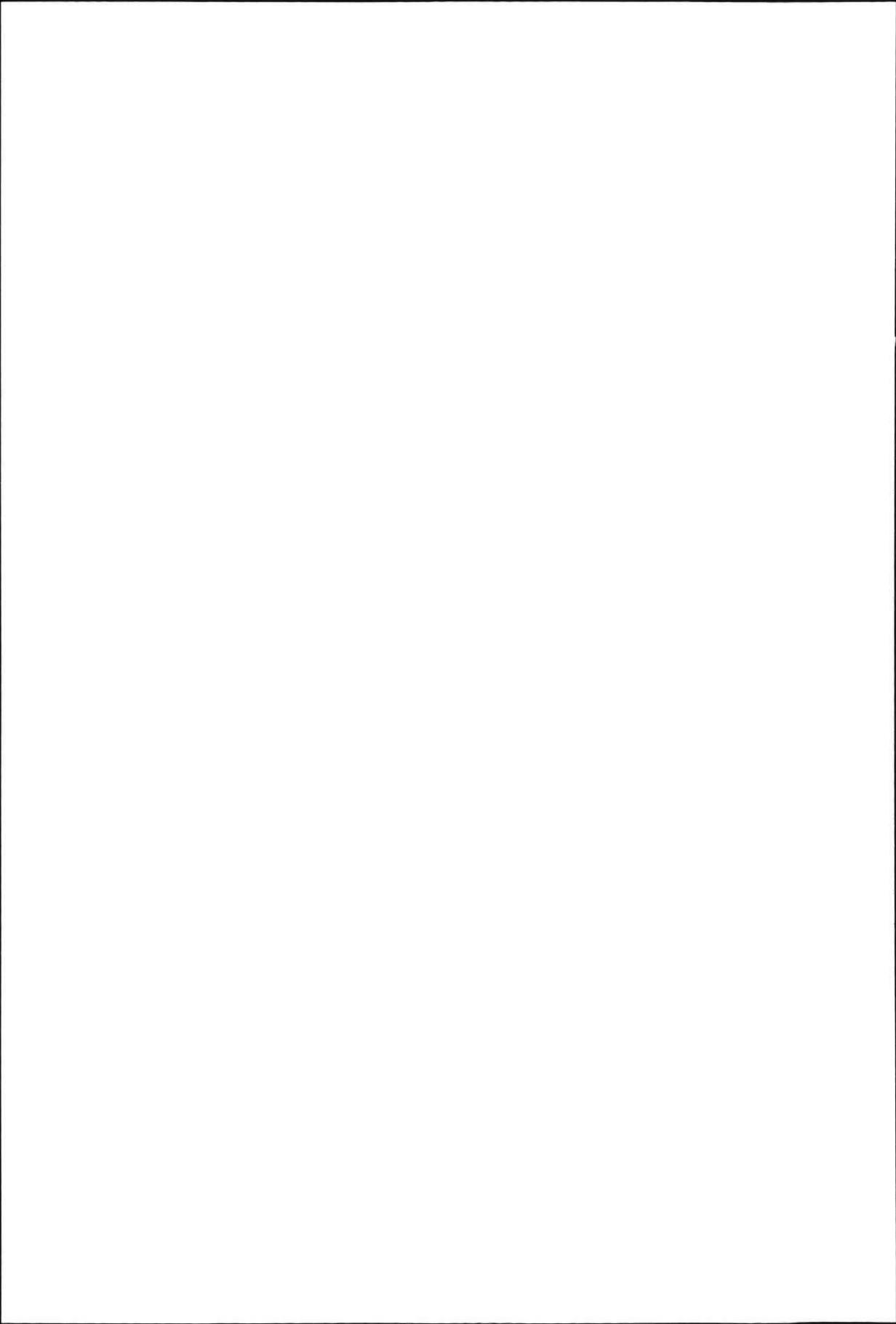
Recently, another cell type with pathogenic potential has been identified in psoriasis and PsA, the natural killer-like T (NK-T) cell. NK-T cells express both a conserved $\alpha\beta$ T cell receptor and a natural killer cell receptor, and are part of the innate immune system. NK-T cells can act without prior sensitization. It has been suggested that interaction of NK-T cell receptors with keratinocytes is the mechanism of keratinocytic hyperproliferation via the production of IFN- γ .

In conclusion, recent developments in psoriasis research, for instance the remarkable improvements of psoriasis and PsA after anti-TNF- α therapy, have shifted the focus from T-cells towards innate immunity. A possible hypothesis would be that in psoriasis there is a genetic deficiency in down-regulation of the innate immunity, in particular TNF- α , leading to increased angiogenesis and expression of adhesion molecules on endothelial cells which cause

increased trafficking of activated effector / memory T cells and NK-T cells towards lesional skin and synovium. These cells stimulate keratinocytes to hyperproliferation, and in synovial tissue increased levels of TNF- α induce osteoclastogenesis. The age of onset of psoriasis and PsA might be, in part, dependent on specific environmental or physical triggers, which are as yet unknown. NK and NK-T cells act as a first-line of defence against virally infected cells, which are lysed upon recognition by activated NK cells. It is possible that a viral antigen may be important in the pathogenesis and actual onset of psoriasis and PsA. Future research in psoriasis and PsA should focus more on the role of the innate immune system, in particular NK-T cells.



Nederlandse samenvatting



Nederlandse samenvatting

Psoriasis is een chronische huidziekte die voorkomt bij ongeveer 2 procent van de wereldbevolking. Psoriasis vulgaris, de meest voorkomende vorm van psoriasis, wordt gekenmerkt door scherp begrensde erythemateuze plekken met kaarsvetschilfering op de strekzijde van de ellebogen en knieën, en op de onderrug. Behalve de huid kunnen ook de gewrichten zijn aangedaan, in het kader van artritis psoriatica (PsA). Zonder adequate behandeling kan PsA leiden tot gewrichtsschade en functieverlies.

Hoewel de oorzaak van psoriasis vulgaris en PsA niet bekend is, wordt gedacht aan een interactie tussen genetische, immunologische en omgevingsfactoren. Bij histologisch onderzoek van lesionale huid en gewrichten wordt infiltratie van geactiveerde T cellen en een toename van bloedvaten ter plaatse gevonden. Verder is bekend dat de concentratie van TNF- α , een pro-inflammatoir cytokine, is verhoogd in lesionale huid en gewrichten.

De huidige therapieën voor psoriasis (en PsA), zoals methotrexaat, cyclosporine, en photo(chemo)therapie, worden in hun gebruik beperkt door de bijwerkingen. Recent zijn nieuwe therapieën, de zogenaamde biologicals, beschikbaar gekomen voor de behandeling van psoriasis en PsA. Doordat deze middelen selectief aangrijpen op bepaalde stappen in de immuncascade, wordt aangenomen dat het bijwerkingenprofiel gunstiger is dan de conventionele (immuunsuppressieve) middelen.

Het doel van deze thesis was om de immunohistochemische veranderingen te onderzoeken in lesionale huid en gewrichten van patiënten met psoriasis vulgaris en PsA tijdens de behandeling met deze nieuwe biologische therapieën. Aan de ene kant kunnen deze analyses worden gebruikt om de klinische effectiviteits parameters zoals de psoriasis area and severity index (PASI) en American College of Rheumatology (ACR) responsie criteria te ondersteunen bij het testen van nieuwe geneesmiddelen. Aan de andere kant hopen we door deze analyses meer inzicht te verkrijgen in de immunologische achtergrond van psoriasis en PsA.

De evaluatie van het infiltraat en de expressie van eiwitten in de huid wordt traditioneel gedaan door middel van handmatige tellingen. Echter, gezien de ontwikkelingen van nieuwe anti-psoriatische therapieën waarbij de evaluatie van de histologie van groot belang is, is er behoefte aan een meer repro-

duceerbare en tijdsefficiënte methode, zoals de digitale beeld analyse (DIA). Om dit nieuwe analyse systeem te testen vergeleken we de analyse van immunohistochemisch gekleurde huidcoupes met DIA, met handmatige tellingen (MQ), en met semi-kwantitatieve analyse (SQA) in **hoofdstuk 2**. Het aantal CD3⁺ T cellen en de expressie van het adhesiemolecuul E-selectine werden bepaald in huidbiopten van 11 patiënten met psoriasis vulgaris voor en na behandeling. We vonden significante correlaties tussen DIA en MQ wat betreft het aantal T cellen, en tussen DIA en SQA wat betreft de expressie van E-selectine. De handmatige tellingen (MQ en SQA) kosten significant meer tijd dan DIA. Concluderend kunnen we zeggen dat de analyse van het inflammatoire infiltraat in lesionale psoriasis huid door DIA dezelfde resultaten geeft als de handmatige tellingen, maar dat DIA betrouwbaarder is en minder tijd kost.

We gebruikten DIA om veranderingen in het inflammatoire infiltraat in lesionale huid en synovium te evalueren na alefacept behandeling. Alefacept, een LFA-3/IgG1 fusie eiwit, remt de activatie and proliferatie van T cellen door het binden aan de CD2 receptor op het oppervlak van deze cellen. In een open-label studie onderzochten we de klinische effectiviteit en de effecten op T cellen in lesionale huid en gewrichten van psoriasis patiënten (**hoofdstuk 3 en 4**). Elf patiënten met psoriasis vulgaris en PsA werden gedurende 12 weken wekelijks behandeld met 7.5 mg alefacept i.v.. Huidbiopten werden afgenomen op baseline en op dag 8, 43 en 92; synoviumbiopten werden afgenomen op baseline, dag 29 en 92. Na afloop van de behandeling had het merendeel van de patiënten meer dan 50% reductie in PASI vergeleken met baseline. Tevens zagen we een geleidelijke reductie van het aantal T cellen in de huid, vooral CD8⁺ cellen in de epidermis en CD4⁺ cellen in de dermis. Bij patiënten met meer dan 50% PASI reductie ('responders') vonden we tevens een complete afname van effector / memory (CD45RO⁺) T cellen uit de epidermis. Behalve de huid verbeterden ook de gewrichtsklachten na alefacept behandeling. We vonden een significante afname van het aantal CD4⁺ T cellen in het synovium na behandeling. Bovendien hadden patiënten die voldeden aan de DAS response criteria na behandeling een significante reductie in effector / memory (CD45RO⁺) T cellen in het synovium. Deze bevindingen ondersteunen de hypothese dat effector / memory T cellen een belangrijke rol spelen in de pathogenese van psoriasis en PsA, en dat alefacept in staat is deze cellen te

reduceren in lesionale huid en gewrichten van patiënten met psoriasis en PsA. In **hoofdstukken 5, 6, en 7** beschrijven we de effecten van anti-TNF- α therapie op lesionale huid en gewrichten van psoriasis patiënten. TNF- α is een belangrijk cytokine in de inflammatoire cascade in psoriasis and PsA. Infliximab, een anti-TNF- α monoklonaal antilichaam, is een effectieve behandeling van zowel psoriasis and PsA, echter het werkingsmechanisme van infliximab in deze ziekten is nog niet duidelijk. In vitro studies suggereren dat binding van infliximab aan membraangebonden TNF- α kan leiden tot lyses van TNF- α producerende cellen via activatie van complementafhankelijke of antilichaamafhankelijke celgedemedieerde toxiciteit. In een gerandomiseerde, placebogecontroleerde studie onderzochten we of apoptose een rol speelt in het werkingsmechanisme van infliximab in psoriasis en PsA (**hoofdstuk 5**). Twaalf patiënten met psoriasis and PsA werden eenmalig behandeld met infliximab (3mg/kg i.v.) of placebo. Op baseline en na 48 uur werden huid- en synoviumbiopsen afgenomen, waarin een significante afname van T cellen werd gevonden in de patiënten die behandeld waren met infliximab. Er werd echter geen toename van apoptotische cellen aangetroffen (caspase-3-kleuring en TUNEL assay). Kennelijk is neutralisatie van TNF- α voldoende om klinische verbetering te bewerkstelligen in psoriasis en PsA, zonder dat apoptose optreedt.

Bloedvaten in lesionale huid en synovium van psoriasis patiënten brengen een aantal adhesiemoleculen verhoogd tot expressie, waaronder ICAM-1, VCAM-1, en E-selectine. Tevens wordt er een overexpressie gevonden van endotheliale groeifactoren, zoals VEGF. Om de invloed van infliximab op endotheelcellen en angiogenese te testen werden 11 patiënten met psoriasis en PsA behandeld met infliximab (3 mg/kg i.v.) op baseline, en op week 2, 6, 14 en 22 (**hoofdstuk 6**). Huid- en synoviumbiopsen werden afgenomen op baseline en op week 4.

Klinische parameters, zoals de PASI en de DAS (een maat voor de activiteit van artritis) waren significant verlaagd op week 16. De reductie in DAS was minder stabiel dan de reductie in PASI, waarschijnlijk omdat de dosering infliximab lager was dan gebruikelijk (3mg/kg vs. 5mg/kg). In aansluiting op de klinische verbetering vonden we een significante afname in het aantal T cellen in zowel huid als synovium op week 4, evenals een significante afname van het aantal bloedvaten in dermis en synovium, een significante afname in

de expressie van $\alpha_v\beta_3$ integrin (een marker voor neovascularisatie) in huid en synovium, en een significante afname in de expressie van adhesiemoleculen in huid en synovium. Tevens vonden we een licht verlaagde expressie van VEGF in huid en synovium. Aangezien zowel psoriasis als PsA worden gekenmerkt door hypervascularisatie zijn dit relevante bevindingen. Concluderend vonden we dat infliximab behandeling leidt tot afname van de neoangiogenese en afname van expressie van adhesiemoleculen in de huid en in de gewrichten, wat resulteert in verminderde influx van ontstekingscellen en klinische verbetering.

156 Een ander soort anti-TNF- α behandeling is etanercept, een fusie-eiwit dat is opgebouwd uit twee identieke delen van een recombinante TNF receptor (p75) monomeer en het Fc gedeelte van humaan IgG1. Etanercept werkt door middel van competitieve inhibitie van de interactie van circulerend TNF- α met membraangebonden TNF-receptors. Om het werkingsmechanisme van etanercept te vergelijken met het werkingmechanisme van infliximab (**hoofdstukken 5 en 6**) behandelden we 6 patiënten met psoriasis met etanercept (50 mg of 25 mg s.c. twee keer per week) of placebo in een dubbelblinde studie (**hoofdstuk 7**). Huidbiopten werden afgenomen op baseline, en na 4 en 12 weken. Bij alle patiënten die werden behandeld met etanercept trad meer dan 50% PASI reductie op vergeleken met baseline, en bij 1 patiënt trad meer dan 75% PASI reductie op.

In de lesionale huid van patiënten die werden behandeld met etanercept zagen we een sterke afname van T cellen in epidermis en dermis na 4 weken, in tegenstelling tot patiënten die werden behandeld met placebo. Tevens zagen we een afname in de expressie van de adhesiemoleculen ICAM-1, VCAM-1, en E-selectine bij deze patiënten. In tegenstelling echter tot de patiënten die behandeld werden met infliximab zagen we geen veranderingen in de expressie van markers voor vasculariteit en neoangiogenese, zoals vWF, $\alpha_v\beta_3$, en VEGF.

Concluderend vonden we dat behandeling met etanercept in psoriasis leidt tot afname van influx van geactiveerde T cellen, waarschijnlijk ten gevolge van afname van expressie van adhesiemoleculen op endotheelcellen. Er was geen sprake van afname van angiogenese.

Conclusies

De oorzaak van psoriasis en artritis psoriatica (PsA) is tot op heden niet bekend. In het verleden werd aangenomen dat psoriasis ontstaat ten gevolge van een primaire stoornis in de keratinocyten, aangezien de abnormale differentiatie en proliferatie van de keratinocyten, zich uitend in kaarsvetschilfering, zo kenmerkend is voor psoriasis. Deze hypothese kon echter niet verklaren waarom sommige patiënten ook gewrichtsklachten ondervonden. Twee belangrijke observaties rond 1980 zorgden ervoor dat het psoriasisonderzoek zich meer ging richten op de rol van T cellen in de pathogenese van psoriasis. Allereerst werd een opmerkelijke verbetering van de klinische kenmerken van psoriasis waargenomen na behandeling met cyclosporine, een T-cel remmend geneesmiddel. Deze bevindingen werden onlangs bevestigd door de goede resultaten die werden bereikt met alefacept, een anti-T-cel therapie tegen psoriasis (deze thesis). Ten tweede wezen immunohistochemische kleuringen van lesionale psoriasis huid uit dat geactiveerde memory / effector T cellen de eerste cellen zijn die infiltreren in beginnende psoriasis plaques. Bovendien zijn T cellen aanwezig in ontstoken synovium van patiënten met PsA. Door deze bevindingen wordt tegenwoordig aangenomen dat psoriasis en PsA T-cel gemedieerde aandoeningen zijn, waarin (om onbekende reden) geactiveerde T cellen de inflammatoire cascade aansturen, eindigend in abnormale differentiatie en proliferatie van keratinocyten. Veel van de huidige anti-psoriasis behandelingen zijn gebaseerd op deze hypothese, zoals cyclosporine, UVB, methotrexaat, en alefacept.

Dat T cellen niet alleen verantwoordelijk zijn voor het ontstaan van psoriasis en PsA werd duidelijk toen bleek dat anti-TNF- α therapie, gegeven voor andere inflammatoire aandoeningen zoals reumatoïde artritis of de ziekte van Crohn, leidde tot opmerkelijke verbetering van psoriasis en PsA. Dit wekte de suggestie dat TNF- α een belangrijkere rol heeft in de pathogenese van psoriasis en PsA dan voorheen werd aangenomen. Dit vermoeden wordt versterkt door de observatie dat de concentratie van TNF- α is verhoogd in serum, huid, en synovium van patiënten met psoriasis en PsA. TNF- α heeft diverse functies in de inflammatoire cascade, en speelt bovendien een belangrijke rol in het botmetabolisme door bevordering van de osteoclastogenese. Door remming van TNF- α ontstaat er een domino effect dat leidt tot afname van de expressie

van andere pro-inflammatoire cytokines en afname van de influx van geactiveerde cellen. De keerzijde van anti-TNF- α therapie is de kans op het optreden van ernstige infecties, waardoor nauwkeurig monitoren tijdens therapie noodzakelijk is.

Onlangs werd een ander celtype ontdekt dat mogelijk een pathogene rol speelt in psoriasis en PsA, de natural killer-like T (NK-T) cel. NK-T cellen brengen zowel een $\alpha\beta$ T-cel receptor als een natural killer cel receptor tot expressie, en zijn onderdeel van het aangeboren (innate) immuun systeem.

158 Mogelijk speelt de interactie tussen NK-T cel receptoren en keratinocyten een rol bij de hyperproliferatie van keratinocyten.

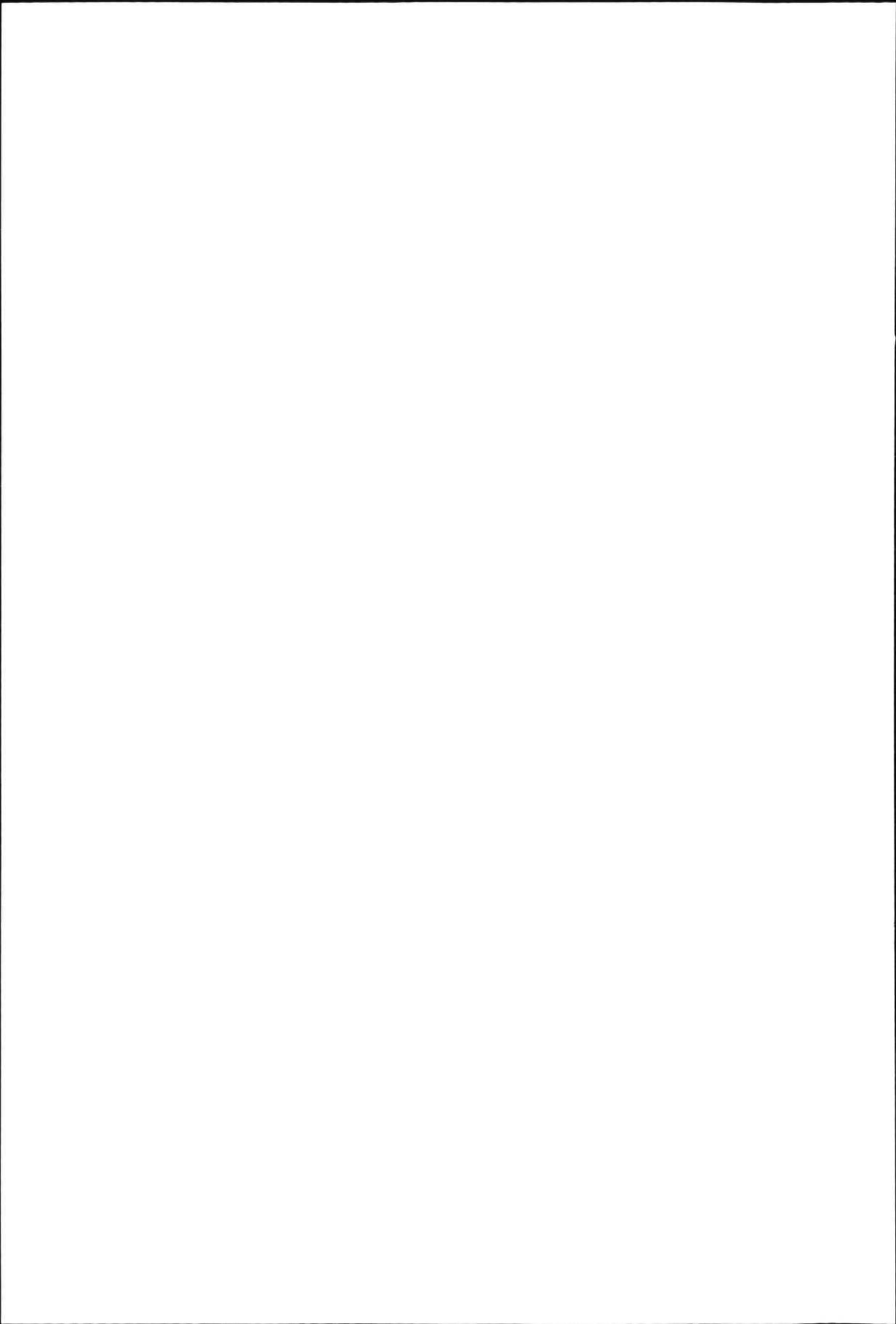
Concluderend kunnen we zeggen dat recent psoriasis onderzoek, zoals de invloed van anti-TNF- α therapie op psoriasis en PsA, de focus heeft verschoven van T cellen naar de aangeboren afweer. Een mogelijke hypothese voor de pathogenese van psoriasis is een genetisch bepaalde deficiëntie in de regulatie van de aangeboren afweer, in het bijzonder TNF- α , wat leidt tot toename van angiogenese en de expressie van adhesiemoleculen, resulterend in een verhoogde influx van geactiveerde T cellen en NK-T cellen in lesionale huid en gewrichten. Vervolgens stimuleren deze cellen de keratinocyten tot hyperproliferatie, en zorgt de verhoogde concentratie van TNF- α in de gewrichten voor osteoclastogenese. De leeftijd waarop psoriasis en PsA zich voor het eerst openbaren is mogelijk afhankelijk van specifieke (tot op heden onbekende) omgevingsfactoren. NK and NK-T cellen zorgen voor de directe afweer tegen viraal geïnfecteerde cellen door middel van cellyses. Mogelijk speelt een viraal antigeen een rol bij het ontstaan van psoriasis en PsA.

Toekomstig onderzoek naar psoriasis and PsA concentreert zich wellicht meer op de rol van het aangeboren immuun systeem, in het bijzonder NK-T cellen.

chapter

9

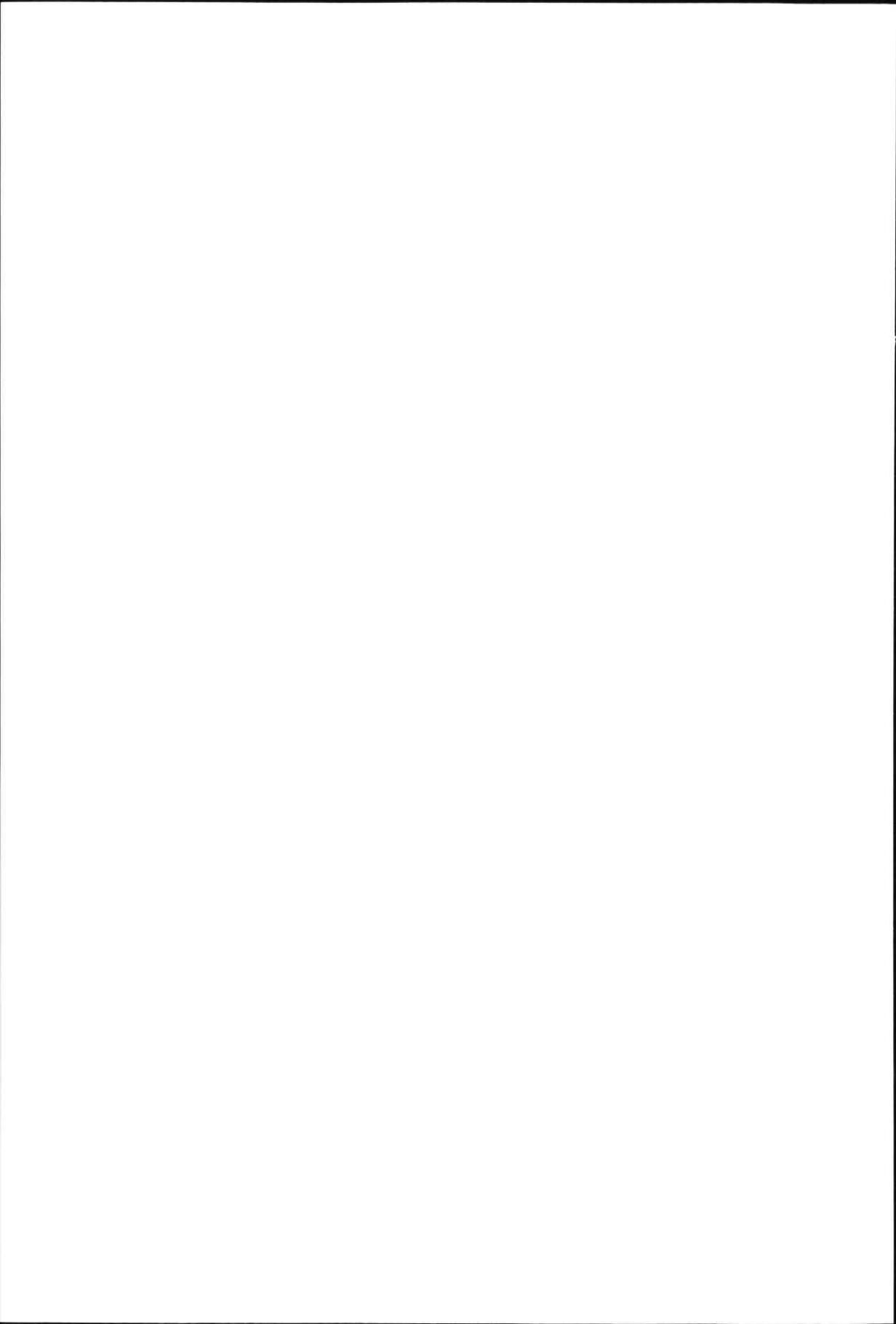
Curriculum vitae



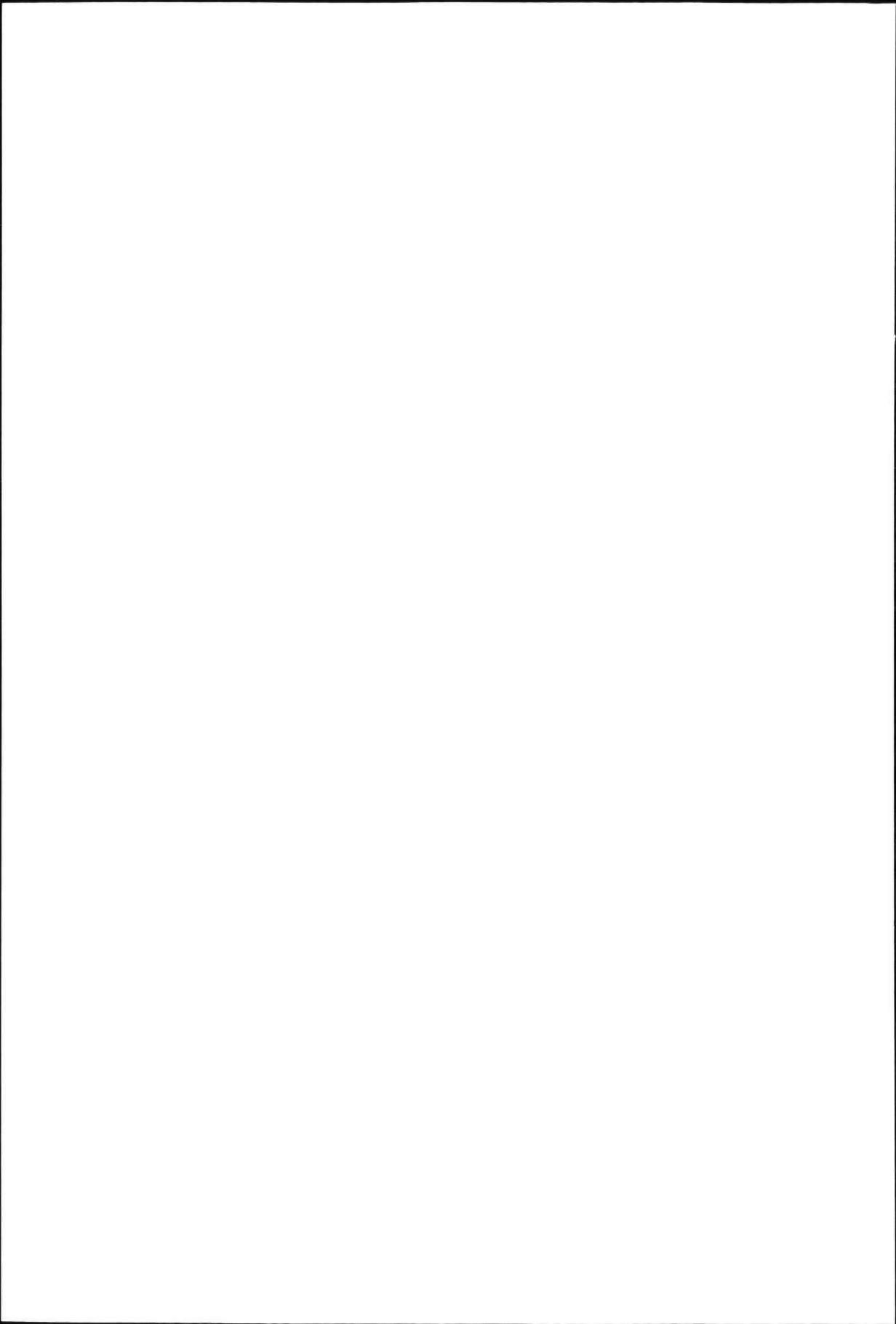
Curriculum vitae

Amber Goedkoop werd geboren op 22 augustus 1973 te Amsterdam als middelste kind in een gezin met 3 dochters. Ze groeide op in Uithoorn, en doorliep haar VWO opleiding aan het Snellius College te Amstelveen. Na het behalen van haar diploma volgde ze de opleiding medische biologie aan de VU te Amsterdam. Ze behaalde haar propedeuse een jaar later maar besloot toch zich in te schrijven voor de studie geneeskunde aan de VU, waar ze aan begon in september 1992. Tijdens haar co-schap periode volgde ze het keuze co-schap plastische chirurgie, waar ze haar eerste wetenschappelijke artikel publiceerde onder leiding van dr. J.J. Hage. Op 15 januari 1999 sloot ze haar opleiding geneeskunde af met het artsexamen. Ze begon daarna als arts-assistent plastische chirurgie in het Slotervaart Ziekenhuis te Amsterdam onder leiding van dr. R.B. Karim, waar ze nog enkele wetenschappelijke artikelen publiceerde. Na hier een jaar te hebben gewerkt begon ze in januari 2000 als arts-assistent plastische chirurgie in het Wilhelmina Kinderziekenhuis (UMCU) te Utrecht, onder leiding van prof. Kon. In april 2000 werd ze aangenomen als arts-onderzoeker op de afdeling Huidziekten van het AMC te Amsterdam (hoofd prof.dr. J.D. Bos), waar ze werkte aan studies naar de klinische en immunologische effectiviteit van 'biological therapies' als behandeling voor psoriasis en arthritis psoriatica. Deze onderzoeken vormden de basis van haar proefschrift 'Effects of biological response modifiers in psoriasis en psoriatic arthritis'. In oktober 2002 begon ze tevens aan haar opleiding tot dermatoloog in het AMC, deze verwacht ze begin 2007 af te ronden (opleider prof.dr. J.D. Bos).

Amber is getrouwd met Gert van Alphen en ze hebben een dochter van 10 maanden, Benthe Zomer.



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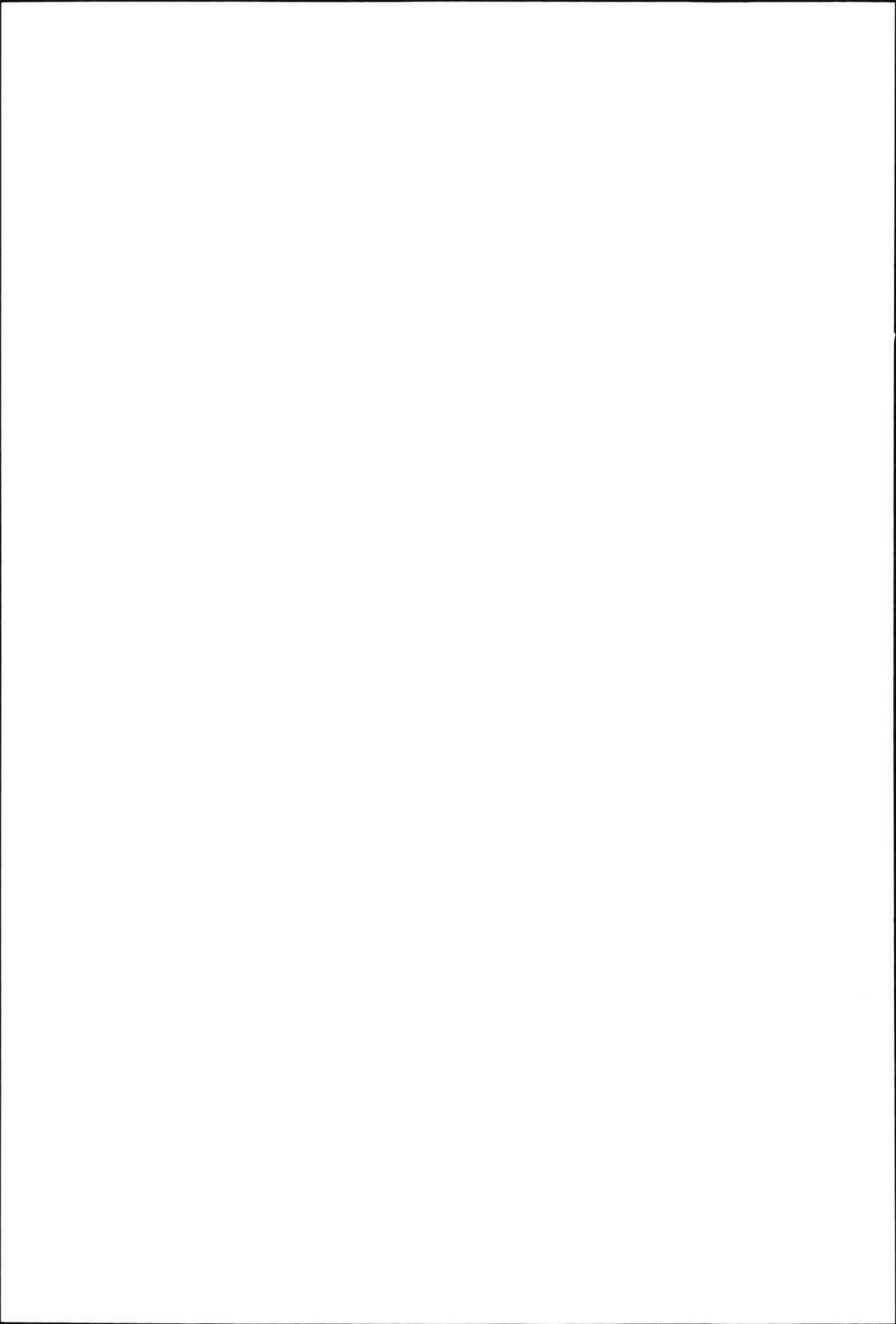


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Dankwoord



*- It's a sign of mediocrity when you demonstrate gratitude with moderation-
-Roberto Benigni-*

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Colour illustrations

Chapter 2

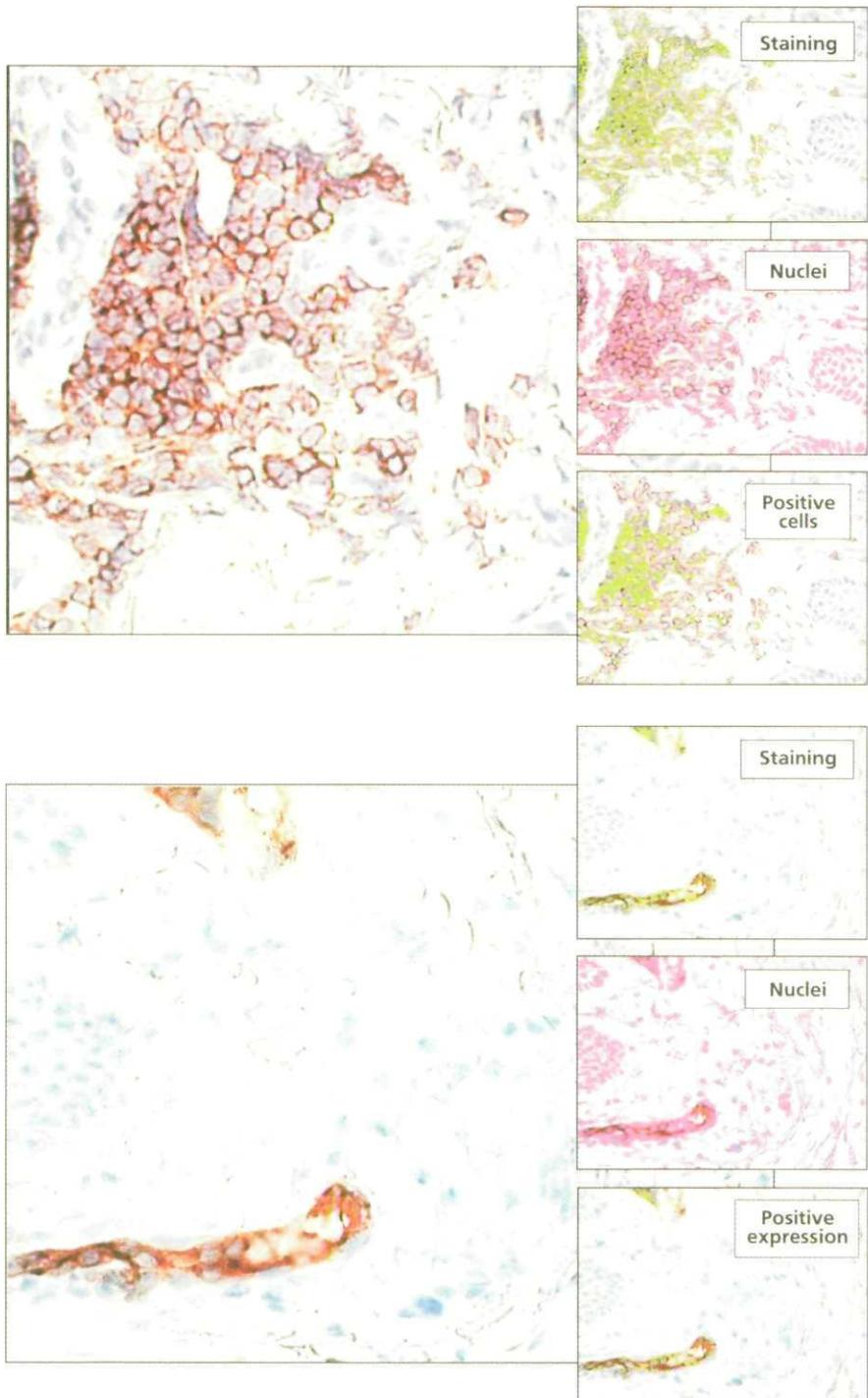


Figure 2-1

Chapter 3

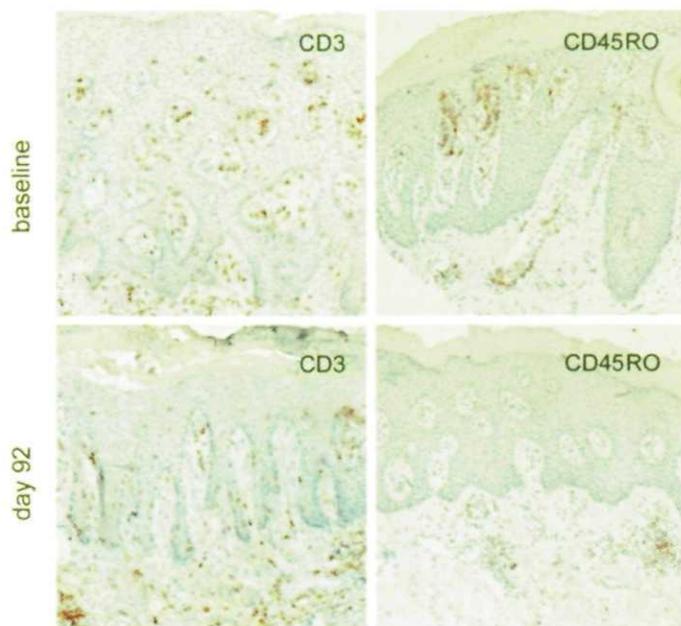


Figure 3-4

Chapter 4

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Figure 4-3

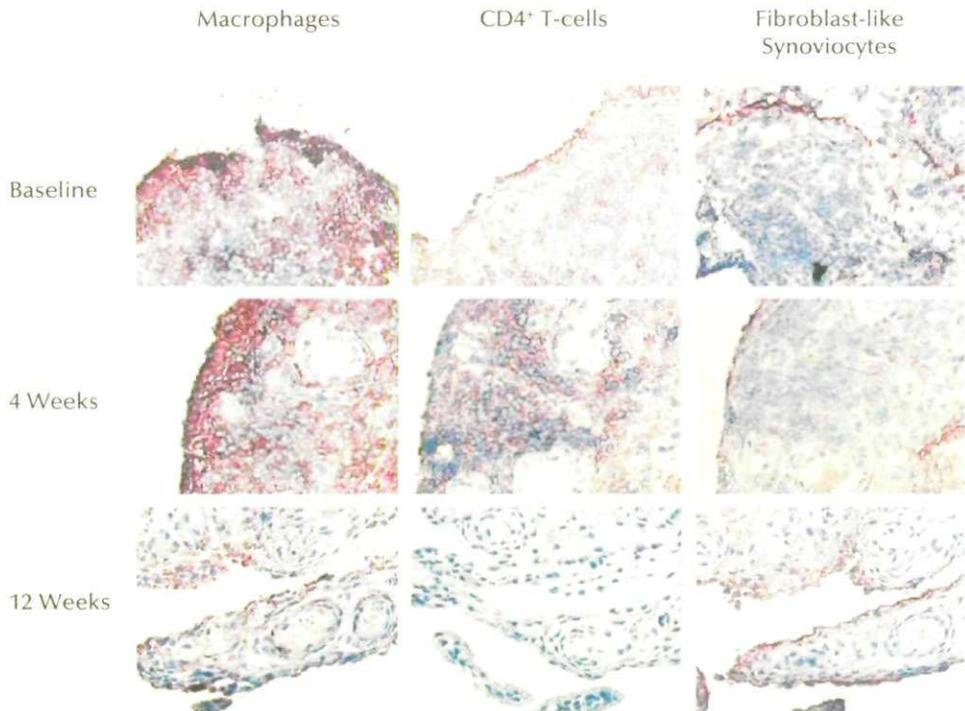


Figure 4-4

Chapter 5

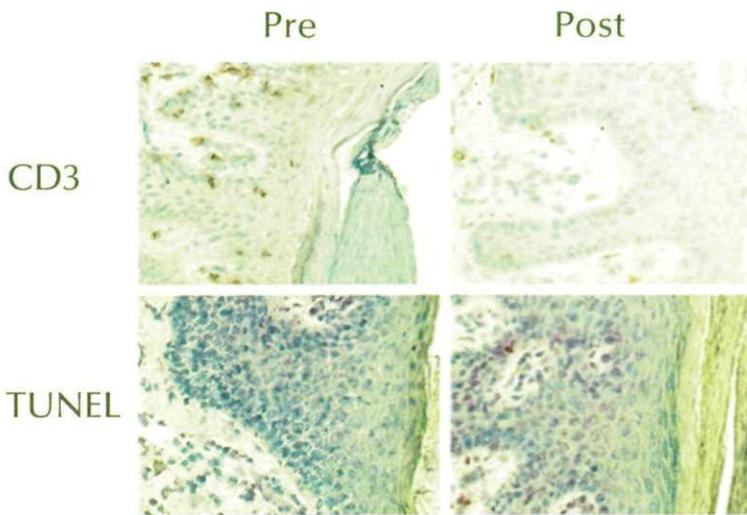


Figure 5-1

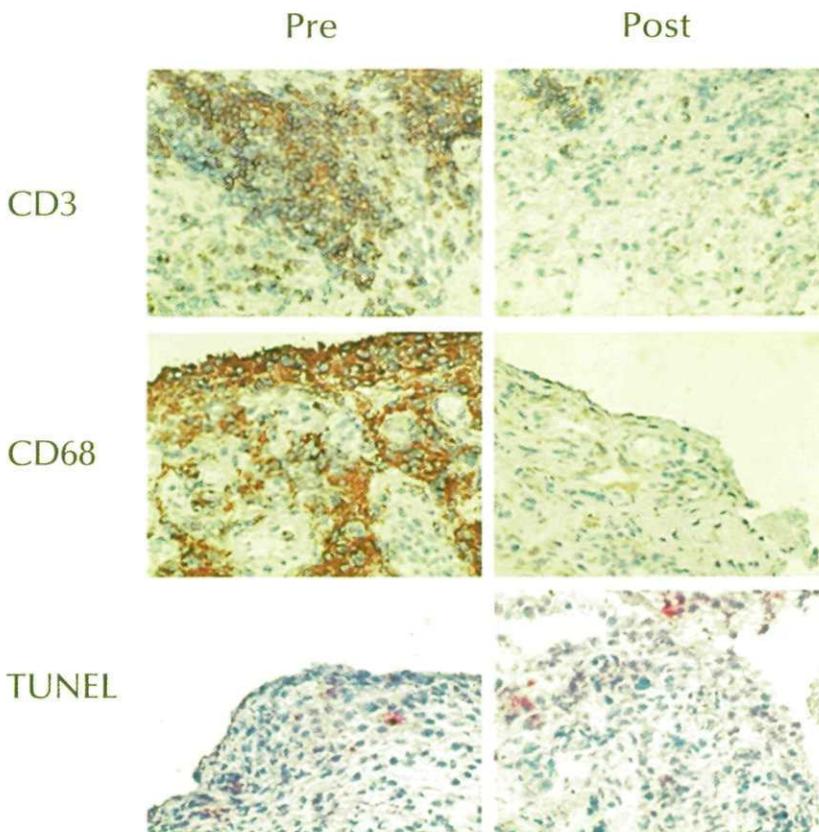


Figure 5-2

Chapter 6

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Figure 6-2

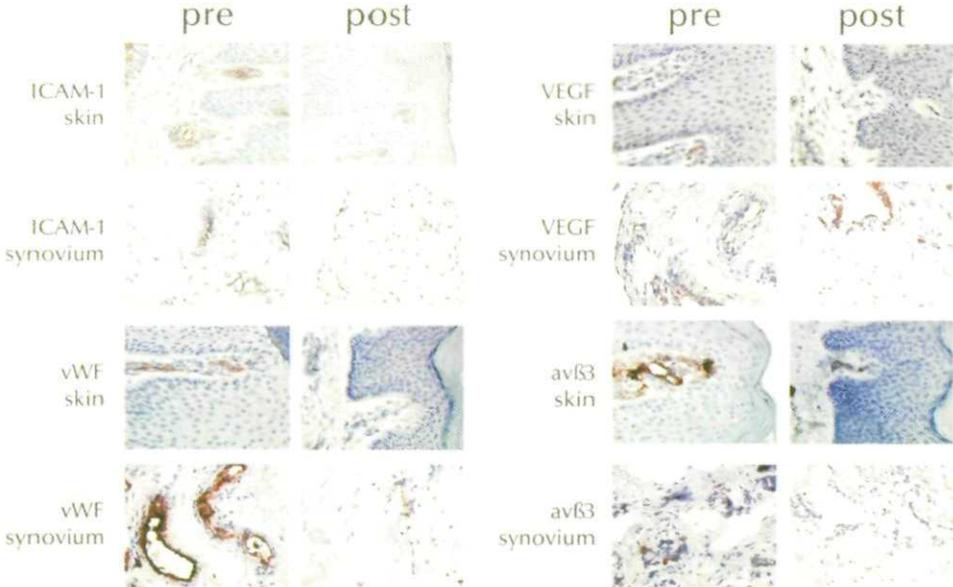


Figure 6-3

Chapter 7

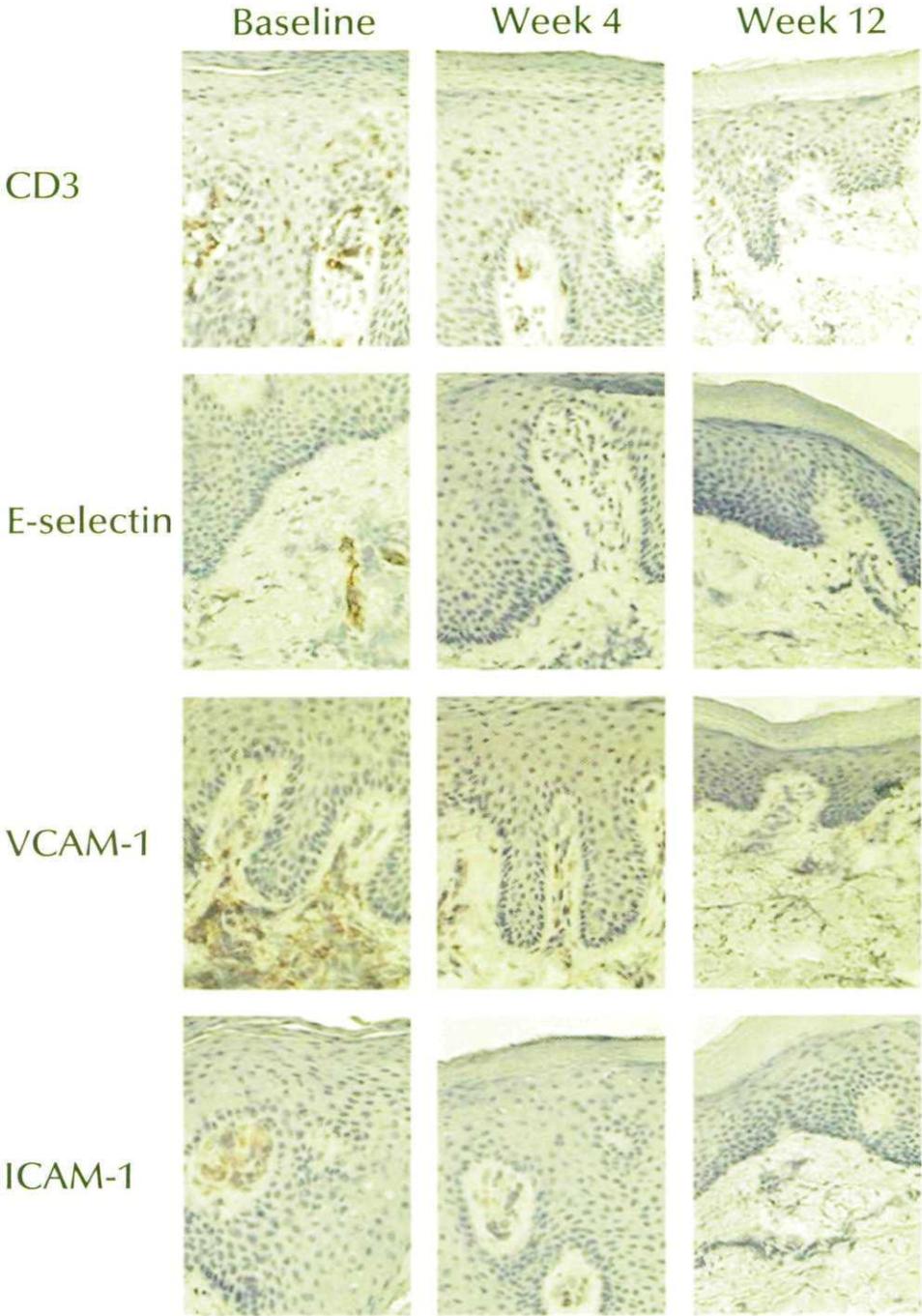


Figure 7-1

