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TARC and CTACK; Two Pivotal Chemokines in Atopic Dermatitis

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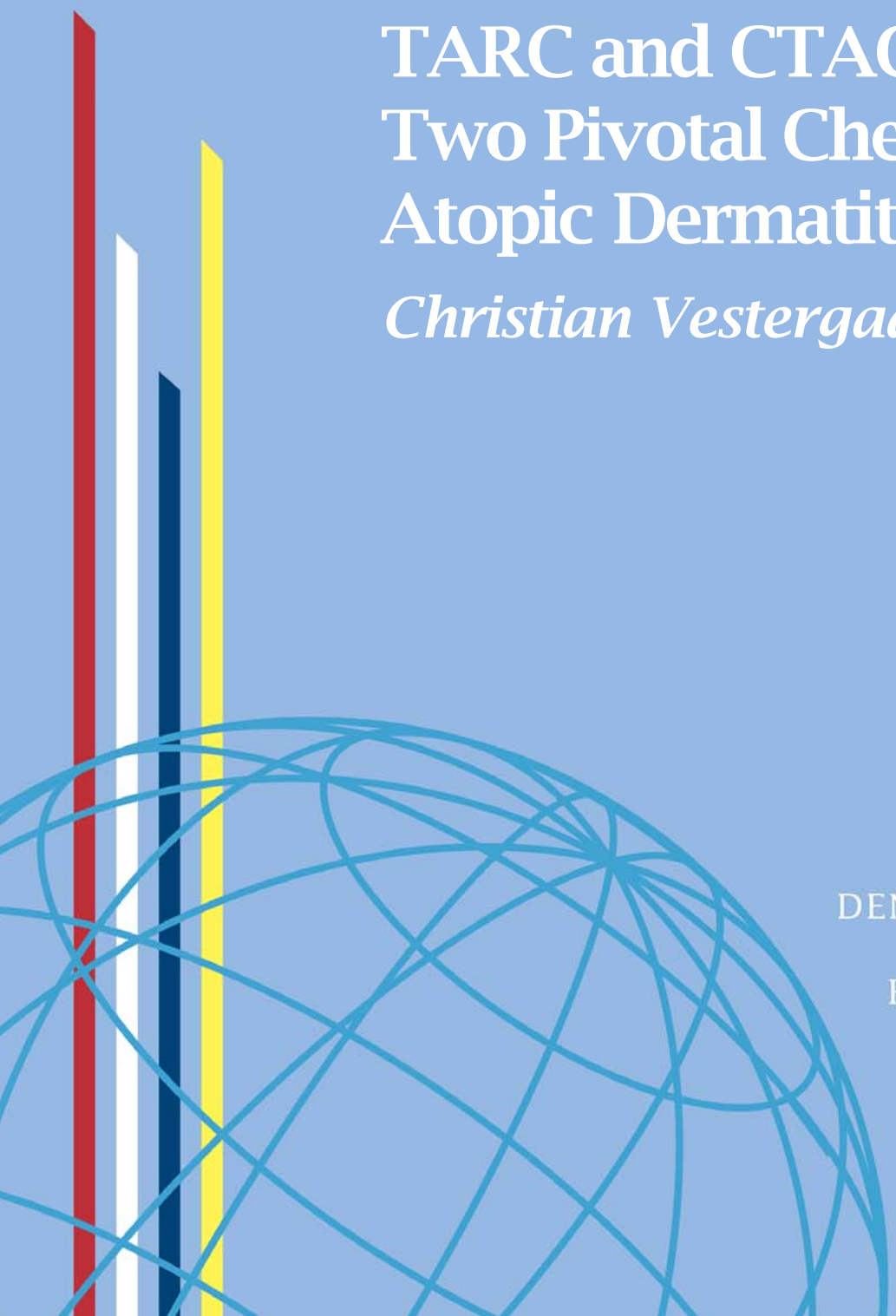

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TARC and CTACK; Two Pivotal Chemokines in Atopic Dermatitis

Christian Vestergaard

Department of Dermatology
Aarhus University Hospital
Denmark

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Denne afhandling er i forbindelse med nedenstående anførte tidligere publicerede artikler af Det Sundhedsvidenskabelige Fakultet ved Aarhus Universitet antaget til offentligt at forsvares for den medicinske doktorgrad.

This thesis is based on the following papers:

I. Vestergaard C, Yoneyama H, Murai M, Nakamura K, Tamaki K, Terashima Y, Imai T, Yoshie O, Irimura T, Mizutani H, Matsushima K. Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. J Clin Invest 1999; 104: 1097–1105.

II. Vestergaard C, Bang K, Gesser B, Yoneyama H, Matsushima K, Larsen CG. A Th2 chemokine, TARC, produced by keratinocytes may recruit CLA+CCR4+ lymphocytes into lesional atopic dermatitis skin. J Invest Dermatol. 2000; 115: 640–646.

III. Vestergaard C, Kirstejn N, Gesser B, Mortensen JT, Matsushima K, Larsen CG. IL-10 augments the IFN-gamma and TNF-alpha induced TARC production in HaCaT cells: a possible mechanism in the inflammatory reaction of atopic dermatitis. J Dermatol Sci 2001; 26: 46–54.

IV. Vestergaard C, Deleuran M, Gesser B, Larsen CG. Expression of the Th2 specific chemokine receptor CCR4 on CCR10 positive lymphocytes in atopic dermatitis but not psoriasis skin. Br J Dermatol 2003; 149(3): 457–463.

V. Vestergaard C, Deleuran M, Gesser B, Larsen CG. Thymus- and activation-regulated chemokine (TARC/CCL17) induces a Th2-dominated inflammatory reaction on intradermal injection in mice. Exp Dermatol 2004; 13(4): 265–271.

VI. Vestergaard C, Johansen C, Christensen U, Just H, Hohwy T, Deleuran M. TARC Augments TNF-alpha-induced CTACK production in keratinocytes. Exp Dermatol 2004; 13(9): 551–557.

VII. Vestergaard C, Just H, Baumgartner-Nielsen J, Thestrup-Pedersen K, Deleuran M. Expression of CCR2 on monocytes and macrophages in chronically inflamed skin in atopic dermatitis and psoriasis. Acta Derm Venereol 2004; 84(5): 353–358.

VIII. Vestergaard C, Johansen C, Otkjaer K, Deleuran M, Iversen L. CTACK/CCL27 (Cutaneous T-cell Attracting Chemokine) Production in Keratinocytes is Controlled by NF-kB. Cytokine 2005; 29: 49–55.

Arbejderne I-III har indgået som en del af PhD. afhandlingen ”Cross talk through chemokines between keratinocytes and lymphocytes in atopic dermatitis.” 2001.

De øvrige arbejder IV-VIII, eller deri indgående resultater, har ikke tidligere været indleveret med henblik på opnåelse af en akademisk grad.

FOREWORD

This thesis is a result of my work performed from 1998 to 2004 in the Department of Dermatology, Aarhus University Hospital, Denmark and the Department of Molecular Preventive Medicine, University of Tokyo, Japan. My studies would not have been possible without the help and support from all my co-authors and supervisors.

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To Halfdan, Sofie and Inger.

'Foragt ikke blindvejene. Det er dem, der leder os frem.'
Piet Hein.

INTRODUCTION

Atopic dermatitis is an eczematous disease in which the skin is invaded by lymphocytes macrophages, mastcells, and eosinophils. The cause of the disease remains unknown, although several theories spanning from bacterial infections in the skin to prenatal exposure and genetics have been presented, all of them probably representing part of the truth for the pathogenesis of this complex and multifactorial disease (Fig. 1).

The mechanism and the nature of the lymphocyte infiltration in the skin has also been studied intensively and some of the questions arising when studying atopic dermatitis are why is it only a certain subpopulation of cells that enters the skin? Why is the response initially dominated by Th2 cells? And why does it change as the disease progress? Is there any link between the lymphocytes and the keratinocytes in the skin or are there other cells contributing to the inflammatory reaction of the skin? And perhaps more importantly; can we manipulate the system which controls the inflammation and thereby help the patients suffering from atopic dermatitis?

To study the disease and to try to answer these questions we used a murine model for atopic dermatitis, the NC/Nga mouse. We investigated the expression of chemokines and correlated these to disease activity and found that especially two cytokines, TARC/CCL17 and MDC/CCL18 were highly expressed by keratinocytes and dermal dendritic cells respectively. These chemokines attracts Th2 cells and thus we hypothesized that they were pivotal to the pathogenesis of atopic dermatitis.

Nature is complex, and one answer, led to another question. Are there any differences in the expression of the receptors for these chemokines in healthy people and atopic dermatis patients? Are there other chemokines of importance for the inflammatory reaction? Can we block these chemokines and on what level? Can the overexpression of these chemokines be used as a diagnostic tool?

The work presented here has been performed over the last 6 years and during the course of our experiments and investigations other results from all over the world have been published and led to new and exciting questions, some of which we have also tried to address.

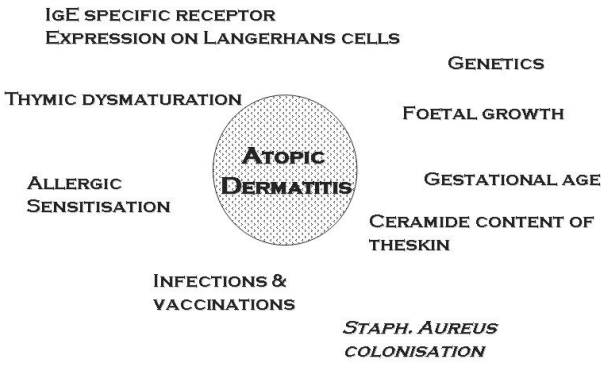

Fig. 1. Risk factors for the development of atopic dermatitis.

ATOPIC DERMATITIS – GENERAL CONSIDERATIONS

HISTORY

The diagnostic criterions for atopic dermatitis were primarily set up by Hanifin and Rajka in 1980, although several others exist. The term atopy is derived from the Greek word meaning 'out of place', and was first used by Coca and Cooke in 1923 to describe a type of hypersensitivity in humans. The main features of the disease described by Coca and Cooke were a) hereditary, b) limited to a small group, c) different from allergy and anafylaxis, d) a qualitatively abnormal response, e) clinical manifestations as asthma and hay fever, and f) associated with immediate-type skin reaction. Thus, Coca and Cooke's definition included all atopic diseases such as asthma, rhino-conjunctivitis and dermatitis. In 1933 Wise and Schultzberger described atopic dermatitis with the classical manifestations and distribution. Up until the 1960's population studies described the association between hay-fever, asthma, and atopic dermatitis. In the late 1960's the IgE molecule was described and the discovery of the elevated plasma IgE in atopic diseases made. Later studies, however, showed that not all atopic dermatitis patients have increased plasma IgE levels, which has lead to the concept of an intrinsic and an extrinsic type of atopic dermatitis (1).

EPIDEMIOLOGY

The incidence of atopic dermatitis has been rising in the western countries since World War II (2), a fact which has been ascribed to better living conditions and the western lifestyle (3, 4) although it seems to have levelled out during the 90's (5). Atopic dermatitis affects between 15% and 20% of all Danish children (6, 7) however; the number varies with a lifetime prevalence between 13% and 37% (8). In a Norwegian study 13% of the population under the age of 20 was affected by atopic dermatitis, whereas only 2% of the population over the age of 20 was affected (9).

In 60% of the cases the onset of the disease is in the first year of life and in 85% of the cases before the age 5 years (10).

Atopic dermatitis is considered as the first step in the 'atopic march' since patients suffering from atopic dermatitis have a higher risk for developing asthma and allergic rhinitis, a risk correlating with the severity of the disease (11).

The cause of atopic dermatitis is unknown. Patients suffering from atopic dermatitis often have a family history of atopic diseases. Monozygotic twins have a concordance rate of 77%, whereas in dizygotic twins it is 15%, suggesting a genetic background for the disease (12). However, atopic dermatitis is a complex disease and environmental factors certainly also play a role.

Foetal and early growth is linked to the development of diseases later in life (13). Increased gestational age and birth weight is associated with development of atopic dermatitis (6), whereas premature birth before gestational week 37 decreases the risk markedly (14). However, other studies shows no association between birth weight, gestational age and atopic dermatitis (15–17). Pregnant women have a Th2 skewed immunity (18), which is also found in neonatal immunological reactions (19). Newborn children with a Th2 skewed immunity and little IFN-γ response to allergens have a higher risk of developing atopic dermatitis (20). Furthermore an ectodermal dysmaturation of the thymic epithelium leading to a disturbed T-cell maturation has been suggested as a cause for atopic dermatitis (21), as have a decreased content of ceramide in the skin (22).

CLINICAL SYMPTOMS

Atopic dermatitis is a chronic and chronically relapsing inflammatory pruritic and eczematous disease often seen in conjunction with rhinitis and asthma (10, 23, 24).

The most prominent features of the disease are its pruritic nature and the distinct distribution of the lesions on the flexural areas of the body, especially in the childhood and adult phase (10). Atopic dermatitis is often complicated and aggravated by infections with *Staphylococcus Aureus* leading to impetigo (25). Severe eczema herpeticum is also a complication seen in children and adults with atopic dermatitis, as well as other viral infections such as warts and molluscum contagiosum (10).

The severity of atopic dermatitis can be measured with the SCORAD index (from The European Task Force on Atopic Dermatitis) based on subjective symptoms of the patients and objective descriptions of the lesions by a clinician (26, 27). An American scoring system, EASI, has also been suggested (28).

ELICITING FACTORS

Whether atopic dermatitis is an allergic disease or not is a long standing controversy. Between 35% and 40% of children with moderate to severe atopic dermatitis have food allergy and half of the children experienced marked improvement after elimination diet (29, 30). In addition T cells with specific reactivity to food allergens have been isolated from atopic dermatitis lesions (31). Yet, in a recent PhD thesis it was shown that only 12% of the children with atopic dermatitis had a positive atopy patch test for hens egg whereas 8% of healthy children had a positive test (32), although the diagnostic value of the atopy patch test in diagnosing food allergy is very low (33).

It has been shown that the number of positive skin prick tests increases the probability of having atopic dermatitis (32).

Skin lesions can erupt after intranasal or bronchial challenge with aero-allergens in sensitised patients (34), and epicutaneous application of aero-allergens elicits an eruption in 30–50% of the patients (23). Furthermore a reduction of house dust mites in the homes of the patients has shown significant reduction of their symptoms (35), but the number of atopic dermatitis patients with a positive patch test varies between 16% and 100% (36).

On the other hand association with a positive skin prick test seems to be restricted to atopic dermatitis patients with concomitant inhalant allergy, and only a positive skin prick test to *pityrosporum ovale* correlates to atopic dermatitis without inhalant allergy (37).

Thus the pathogenic significance allergies in atopic dermatitis is still a controversial subject.

Infections also play a role in the pathogenesis of atopic dermatitis. In a recent study it was shown that children who were subjected to the measles, mumbs and rubella vaccination or measles infection had a significantly higher risk of developing atopic dermatitis than children who were not vaccinated or had had measles (38).

S. Aureus, which colonizes the skin, can also exacerbate atopic dermatitis, probably through the production of superantigens. Some patients produces IgE antibodies against super antigens, and the disease activity is higher in patients sensitised to SEB (staphylococcal enterotoxin B) than in patients who are not sensitised (39). In the cutaneous lymphocyte associated antigen (CLA) positive subset of T cells from patients with atopic dermatitis, clonal expansion corresponding to the relevant Vβ chain in the T-cell receptor (which is able to bind the superantigens) is found (40). Furthermore, superantigens augments production of allergen specific IgE in the presence of IL-4 (41) and induces glucocorticoid resistance in peripheral blood mononuclear cells (PBMCs) of atopic dermatitis patients through induction of the glucocorticoid receptor β (42). Application of superantigens to the skin of atopic dermatitis patients induces an inflammatory reaction with the relevant clones of T cells (43). Finally, superantigens also induce CLA expression on T lymphocytes through induction of IL-12 and thereby contributes to the invasion of the skin by lymphocytes (44).

HISTOLOGY AND IMMUNOLOGICAL REACTIONS IN THE SKIN OF ATOPIC DERMATITIS

In atopic dermatitis the keratinocytes exhibit spongiosis (intracellular oedema), acanthosis (thickening of the stratum spinosum), parakeratosis (in the chronic lichenified lesions) (nuclei remaining in the stratum corneum), and hyperkeratosis (thickening of the stratum corneum) (24, 45). A severe cellular inflammatory reaction is seen in the dermis (46) consisting of lymphocytes (10, 24, 47), eosinophils (48), mast cells (49), monocytes (46) and dermal (50) as well as epidermal dendritic cells (51). The lymphocyte is the most predominant cell in the skin lesions of atopic dermatitis and the number of lymphocytes in the lesions have been calculated to 469.000/mm^3 compared to 124.000/mm^3 in uninvolved skin. This means that in an atopic dermatitis patient with 20% of the skin involved approximately 1.2×10^{10} lymphocytes will be in the skin compared to 1.25×10^{10} lymphocytes in the peripheral blood in the peripheral blood (52).

THE T CELLS IN ATOPIC DERMATITIS

The central role of the T cells in the pathogenesis of atopic dermatitis is demonstrated by the fact that increased serum IgE and eczematous reactions are cleared in primary T-cell immuno-deficiencies after bonemarrow transplantation (Wiskott-Aldrich syndrome) (53).

The CD4 positive T cells are divided into a Th1 subset, which is associated with the cellular immune response and a Th2 subset which is associated with the humoral immune response. The Th1 subset is typically described to produce IFN-γ and IL-2 whereas the Th2 subset is usually described to produce IL-4, IL-5 and IL-13 (54, 55) (Fig. 2). IL-4 induces IgE isotype switch and production in B cells (56, 57). Furthermore the Th1 subset and Th2 subset work antagonistic since IFN-γ inhibits Th2 maturation and IL-4 inhibits Th1 maturation (58).

In the peripheral blood of atopic dermatitis patients the T cells have an increased CD4/CD8 ratio (59). Moreover, they seem to be chronically activated, since their teleomer length is significantly reduced, and the teleomerase activity increased compared to healthy controls (60, 61).

Isolated T cells from peripheral blood of atopic dermatitis patients produce a large amount of IL-4 and less of IFN-γ, compared to T cells from healthy controls, when stimulated

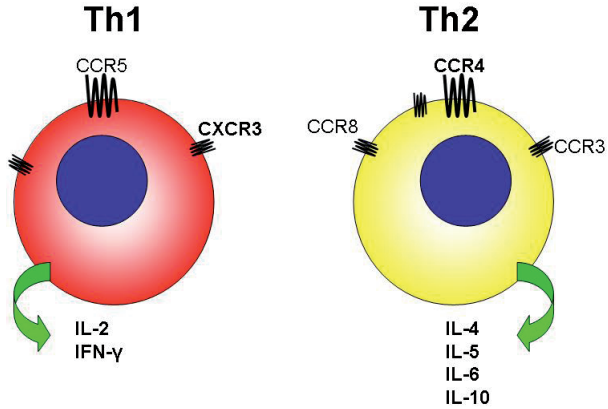

Fig. 2. Expression of chemokine receptors on Th1 and Th2 lymphocytes. Th1 lymphocytes express IL-2 and IFN-γ and the main chemokine receptor expressed on these cells is CXCR3. Th2 lymphocytes express IL-4, IL-5, IL-6, and IL-10, and the main chemokine receptor on these cells is CCR4.

with mitogen and allergen (62). This, in combination with the fact, that 80% of atopic dermatitis patients have increased serum-IgE, have led to the perception of atopic dermatitis as a Th2-dominated disease (10, 63).

A study of 920 children with insulin dependent diabetes mellitus (IDDM) and 9732 non-diabetic children showed that the children who developed IDDM had a lower risk of atopic dermatitis before the onset of IDDM than did the children who did not develop IDDM. However; after the debut of IDDM these children had the same risk for atopic dermatitis as did children who did not suffer from IDDM (64). This supports the concept of atopic dermatitis as a Th2 dominated disease since IDDM in animal models is associated with IFN-γ release and therefore with a Th1 dominated response (65, 66) which should then protect the children from atopic dermatitis until after the debut of IDDM.

The lymphocytes in the skin also have a high CD4/CD8 ratio, and are mainly CD3, CD4, and CD45RO positive T cells, which suggest that they belong to the memory subpopulation (47, 67). As already mentioned T cells from the peripheral blood of AD patients predominantly produce Th2 cytokines when stimulated. However, using RT-PCR analysis of mRNA isolated from the skin of atopic dermatitis patients, IFN-γ has been shown to be expressed concomitantly with IL-4 (68, 69). The majority of T-cell clones established from skin biopsies of atopic dermatitis are also able to express both IFN-γ and IL-4 (70, 71). Interestingly Higashi et al. (70) showed that stimulated T-cell lines isolated from atopic dermatitis skin primarily produced IL-4 and later IFN-γ. This suggests that the Th2 to Th1 shift may be a time dependent phenomenon.

When comparing the expression of cytokines in the acute and chronic lesions of atopic dermatitis the mRNA of the Th2 cytokines IL-4 and IL-5 are highly expressed in the acute atopic dermatitis lesions, whereas in the chronic lesions, the expression of IL-4 is low, and the expression of IL-5 and IFN-γ high (72). The change from a Th2 to a mixed Th1/Th2 has been further elucidated by studying the cytokine expression in atopy patch tests against house dust mite allergens. This showed an initial rise in Th2 cytokines followed by an increase in Th1 cytokines (73). The same pattern of cytokine expression has been observed during allergen late phase reactions in the skin (74). The mechanism for the shift from a Th2 to a Th1 dominated response may be mediated through IL-12 as suggested by Grewe et al (63). The hypothesis is based on the following observations: 1) chronic AD lesions have a higher IL-12 content than acute lesions (75), 2) in atopy patch test lesions IFN-γ expression was not observed after 24 hours of testing, only after 48 hours, 3) this was preceeded by IL-12 expression (76) and 4) eosinophils which are seen in the early phases of atopy patch test lesions (77) as well as monocytes (75), can be stimulated by IL-4 to produce IL-12 (78), and thereby contribute to the shift between a Th2 and a Th1 response.

TNF-α has also be described in the mast cells of atopic dermatitis, and it has been suggested that it may be responsible for the expression of adhesion molecules on the endothelium (79, 80).

T-CELLS IN PSORIASIS

Psoriasis is not the main subject in this thesis, but as skin biopsies and blood samples from this category of patients has been used in our study a very brief summary of psoriasis will be given. Psoriasis is also an inflammatory skin disease and affects approximately 2% of the population. The skin in psoriasis is characterized by marked epidermal hyperplasia and dermal infiltration by mononuclear cells (81). The lymphocytes invading the skin are mainly activated memory T lymphocytes, and are both CD4+ and CD8+ with the CD8+ cells forming the majority in psoriasis (47, 82, 83). Traditionally the T cells invading the skin in psoriasis are described as Th1 lymphocytes (84, 85), although some investigators have described both Th0 and Th2 lymphocytes in the skin of psoriasis (86). It is very likely that the T cells invading the skin of psoriasis have a mitogenic effect on the keratinocytes however their exact role still needs to be elucidated (81).

Thus, in a sense, psoriasis seems to be the reverse skin disease of atopic dermatitis in terms of T cells, and therefore we have used psoriasis as an 'opposite' control in our experiments included in this thesis (87, 88).

ANTIGEN PRESENTING CELLS

The major component of the cellular infiltrate in the skin lesions of atopic dermatitis is T lymphocytes, and since these cells are activated by antigen presenting cells, it has been hypothesized that the antigen presenting cells play a major role in the pathogenesis of the disease (89). The dendritic cells (DCs) are central in this process. They function as mobile sentinels who ingest and process antigens in the peripheral tissues and from there move to the lymphoid tissue and activate the T cells. The DCs capture antigens through the antigen capturing Fc-receptors, and present the antigens either on MHC II molecules to activate T-helper cells (CD4+) or on MHC I molecules to activate cytotoxic T cells (CD8+). Furthermore they are able to control the development of the T cells into Th1 or Th2 lymphocytes by means of there cytokine production, either IL-12 or IL-4 (90). DCs also function as activators of B-lymphocytes and play a vital role in the induction of tolerance. These subjects, however, are outside the scope of this thesis.

The first dendritic cell to be identified was the Langerhans' cell which was described by Paul Langerhans in 1868. The Langerhans' cell is derived from the bone marrow and circulating CD34+ hematopoietic cells, and is found in the epidermis of normal non-inflamed skin (91). It is characterized by its dendritic appearance, the content of Birbeck granules in the cytoplasm and expression of CD1a and MHC II (92). The Langerhans' cells form a network throughout the epidermis and acts as a first line antigen presenting network. Beside the above mentioned surface markers, the Langerhans' cells express numerous other markers some of which are disease specific (93).

The Langerhans' cells ingest antigens and process them in such a manner that they may be presented in the context of the MHC II molecule, from where interaction with the T-cell receptor and activation of the T-cell takes place (90).

Another population of antigen presenting cells are the inflammatory dendritic cells (IDECs) (94). The IDECs accumulate in the skin during inflammatory conditions, such as atopic dermatitis, and the number of IDECs found in the skin correlates to the duration and severity of the condition (92). Circumstantial evidence suggest that they may originate from monocytes (92).

IgE receptors on antigen presenting cells in atopic dermatitis

As described atopic individuals have a higher production of IgE, and in the mid 80's it was shown that Langerhans' cells, dermal dendritic cells, and monocytes from these individuals carry IgE molecules. Thus IgE was anticipated to play a role in an allergic eczematous reaction in the skin. Both Langerhans' cells and IDECs express the high affinity IgE-binding receptor FcεRI (94, 95), and the level of expression is increased in the skin lesions of atopic dermatitis patients, and correlates with the serum levels of IgE (93). FcεRI expression levels on monocytes also correlate with the severity of disease in atopic dermatitis patients (92). Antigen uptake through FcεRI/IgE and subsequent presentation has been suggested to be a key event in the pathogenesis of allergen induced atopic dermatitis, and a self-amplifying mechanism whereby antigen binding leads to activation of T cells, which in turn induces IgE production in B-lymphocytes and thus increases the number of IgE molecules found on the antigen presenting cells (92).

DCs in the dermis also produce lymphocyte attracting chemokines. We have shown that in the Nc/Nga mouse, dermal dendritic cells produce MDC/CCL22 (96), which is also the case in human patients (97). Thus; the dendritic cells may hold a central role in the pathogenesis of skin inflammation. They are involved in the activation of T lymphocytes and in allergy induced inflammation and here their role seems to be almost certain. Furthermore, they are able to produce chemoattractants for lymphocytes and other inflammatory cells.

However their role in the pathogenesis of atopic dermatis still remains to be elucidated.

ANIMAL MODELS FOR ATOPIC DERMATITIS

The use of animals to study atopic dermatitis presents certain problems. First and foremost the diagnosis of atopic dermatitis is, as mentioned above, based on the Lillehammer criterions, which are set up for humans. However; if some of the characteristics of atopic dermatitis is present in the animals, such as a Th2 dominated lymphocyte infiltrate in the skin, inflammation by mast cells, eosinophils, macrophages, and dendritic cells, hyperkeratosis resembling chronic lichenisation, high s-IgE, or pruritus, they may be used to study the different components of the disease.

Several animal models for atopic dermatitis exits, including dogs, horses, and cats (98). The murine models are the most attractive to work with due to the genetic homogeneity of the different species, to the fact that the murine immunesystem has been extensively studied, and to their physical properties. Below a few of the murine models for atopic dermatitis will be described.

A model for barrier disruption

A model using BALB/c mice was used to investigate the cutaneous reactions resulting from disruption of the skin barrier. The mice were shaved on the abdomen and adhesive tape was repeatedly applied to the skin to disrupt the skin barrier. This removed most of the stratum corneum and left the rest of the epidermis intact. Application of house dust mite allergens on the skin induced IL-4, but not IL-2 and IFN-γ mRNA in the skin draining lymph nodes (99), demonstrating the importance of the skin barrier. Moreover, epicutaneous sensitization resulted in skin lesions which microscopically resembled atopic dermatitis with an infiltration by CD3+CD4+ lymphocytes, mast cells, eosinophils and neutrophils (100). The method of epicutaneous sensitization was compared to conventional sensitization against OVA in BALB/c mice. This showed that epicutaneous sensitization elicited a significant higher IgE and IgG1 antibody response than intraperitoneal sensitization did.

A model using human PBMC's

An interesting animal model in which human PBMC's can be studied in mice has also been described (101). SCID mice, which are unable to elicit a response against foreign cells, were injected with PBMC's from human atopic dermatitis patients, and the effect of house dust mites and *S. Aureus* superantigens on dermal infiltration were studied. Application of both house dust mite antigen and SEB resulted in profound inflammation in both the dermis and epidermis. These experiments stresses the significance of the leucocytes and to certain extent the role of the above mentioned antigens in the pathogenesis of atopic dermatitis.

The IκBξ knock-out mouse

A recent described mouse in which the NF-κB inhibiting factor IκBξ has been removed shows normal growth until 4–8 weeks after birth. They then develop an eczematous inflammatory reaction in the skin in which expression of the Th2 attracting chemokine TARC (see below) is increased. Furthermore, increased IgE is seen in the serum of the mouse (102). Thus, this seems to be a promising model for atopic dermatitis.

All of these models have one or more specific eliciting factors of their condition, but atopic dermatitis is, as mentioned before, a multifactorial disease, without any well described specific eliciting factor. Several other models exist but one mouse model which resembles human atopic dermatitis, and in which no specific eliciting factor is known, is the NC/Nga mouse, which will be described below.

THE NC/NGA MOUSE

The NC/Nga mouse was established as an inbred strain in 1955 from the Japanese fancy mice (Nishiki-Nezumi) (103, 104). This strain of mice has a high susceptibility to radiation and anaphylactic shock induced by OVA. Japanese researchers noticed that the NC/Nga mouse spontaneously developed dermatitis just after weaning. H. Matsuda et al. were the first to describe the similarities of the skin lesions with atopic dermatitis (105). We described that the skin lesions appear on the back of the mice and have a nodular appearance at the age of 8 weeks after which the skin becomes dry and scaly and the mice start to itch and scratch themselves, leading to excoriations in the skin. Mice suffering from this condition becomes very aggressive, probably due to the constant itching (96) (Fig. 3A).

The lesions appear when the mice are kept in non-sterile conditions, but not when they are kept in sterile conditions (96, 105). The mice also develop increased s-IgE which reaches a maximum at 14 weeks of age (105). The increased s-IgE may be due to a constitutively and enhanced phosphorylation of the Janus Kinase 3 (106). We and others described that the histological appearance of the epidermis is characterized by hyperkeratosis, acanthosis, parakerathosis and spongiosis (Fig. 3B and C). The dermis is invaded by IL-4 producing CD-4+ lymphocytes (Fig. 3D and E), some CD8+ lymphocytes, mast cells, macrophages, eosinophils, and dermal dendritic cells, (96, 105). As in atopic dermatitis patients the cytokine balance is initially dominated by a Th2 response, but as the disease progress a more mixed expression of Th1 and Th2 cytokines can be observed, both on the mRNA level (96), and on the protein level (105).

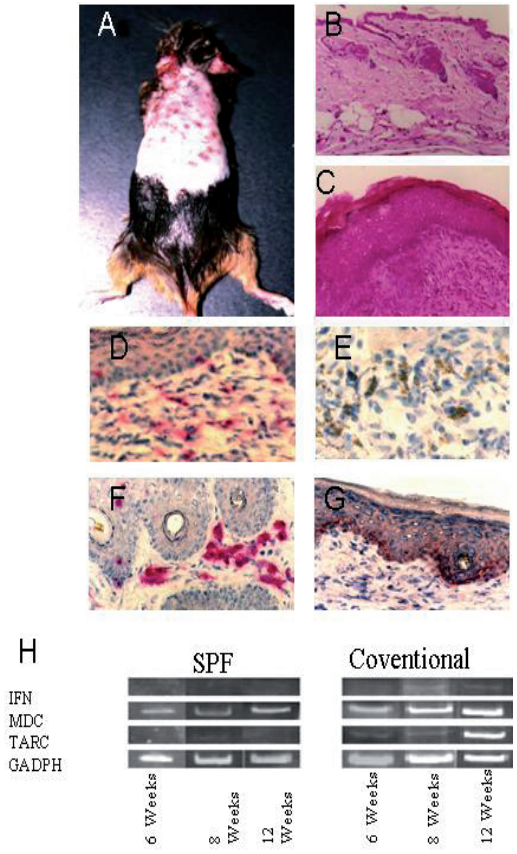

Fig. 3. A) The NC/Nga mouse at 14 weeks when kept under conventional conditions. B) HE staining of skin biopsy from a mouse kept under sterile conditions. C) HE staining from a mouse kept under conventional conditions. D) CD-4 staining of biopsy from a mouse kept under conventional conditions. Note that CD4+ lymphocytes are seen both in the dermis and epidermis. E)IL-4 staining of biopsy from a mouse kept under conventional conditions. The positive cells are, as judge by morphology, lymphocytes and mast cells. F) MDC staining of of skin biopsy from a mouse kept in conventional conditions. G) TARC staining of biopsy from a mouse kept under conventional conditions. H) RT-PCR performed on RNA isolated from the skin of mice kept either under sterile conditions or conventional conditions. IFN-g mRNA can be detected from week 8 in the mice kept under conventional conditions, whereas no IFN-g mRNA can be detected in the mice kept in sterile conditions. MDC can be detected in both mice kept in SPF conditions and mice kept under conventional conditions- TARC production is detected from week 6 in mice kept under conventional conditions and highly upregulated from week 12. No TARC production could be detected in the mice kept under sterile conditions.

The mechanisms for induction of the atopic dermatitis like condition in the NC/Nga mouse are still unknown. However, as mice kept in sterile conditions do not develop the disease environmental factors along with genetic factors specific for this strain must be responsible. Fur mites (*Myocoptes musculinus*) can induce the AD like condition along with increased s-IgE specific against the fur mite antigens (107). Application of a crude extract of house dust mite (*Dermatophagoides farinae*) also resulted in increased specific s-IgE and induction of the skin lesions, yet the major cell type in these lesions was the mast cell (108). The neuropeptide substance P, which is able to induce neurogen inflammation, is increased in both nerve endings and mast cells in the skin of the NC/Nga mouse which may make it more prone to develop the skin condition (109). Injection of staphylococcal enterotoxin B (SEB) and lipopolysaccharide (LPS) in the NC/Nga mice results in a low production of IFN-γ. The low response to SEB was attributed to the lack of Vβ8+ T cells and Vβ8+ NK cells, since Vβ8 is the main binding receptor of SEB in mice. The low response to LPS was attributed to lacking IL-18 production by monocytes. Treatment of the mice kept in conventional conditions with IFN-γ, IL-12 or IL-18 twice a week for 4 weeks ameliorated the disease (110), whereas injection of IL-18 neutralizing antibodies exacerbated the disease in the mice (111). If STAT6, a critical transcription factor for IL-4, is removed from the mice, they interestingly still develop the atopic dermatitis like lesions, but not the increased s-IgE and s-IL-4 (112). An observation, which is very interesting in the light of the ongoing discussion of intrinsic vs. extrinsic atopic dermatitis.

Furthermore, the NC/Nga mouse has an impaired skin barrier, and a decreased content of ceramide in the skin as described for atopic dermatitis patients (113). We showed that the condition of the mice can be successfully treated with topical steroids (96), whereas others have used tacrolimus hydrate (114), astragalin (a flavinoid from persimmon leaves) (115, 116). Clarithromycin (a macrolide antibiotic) delays the onset of the condition (117). This may be due to the anti-bacterial effect, but as another macrolide, roxithromycin, has a downmodulating effect on Th2 cytokines and the Th2 receptor CCR4 on lymphocytes (118) this could also explain the beneficial effects of the macrolides. Based on the histological appearances of the lesions, the marked Th2 response in the initial phase, followed by a more mixed Th2/Th1 response, the increased s-IgE, and the pruritic nature of the condition, we suggest that the NC/Nga mouse is an interesting and sufficient animal model for atopic dermatitis (119).

THE CHEMOKINES

The chemokines are a family of small proteins that mediate chemotaxis, activation, differentiation, and maturation of leucocytes, and are thus involved in lymphoid trafficking, wound healing, Th1/Th2 development, angiogenesis/angiostasis, metastasis, cell recruitment, lymphoid organ development, and inflammation. The term chemokine is derived from chemotactic cytokine and was suggested in 1992 (120). However, the names assigned to the different chemokines have more or less been associated with their function, or method of discovery, leading to Babylonian confusion when results were published. Therefore, a new nomenclature has been introduced using the same numbers as for the chemokine genes. Thus the CXC chemokines are named CXCL followed by the appropriate number; the CC chemokines are named CCL and so on (121). In this thesis, the chemokines studied will be introduced by their systematic and old names: yet, due to the readability they will be referred to by their old names.

The chemokines are divided into four families depending on two cysteine molecules in the N-terminal end of their amino acid structure. Thus there are the CXC-, the CC-, the CX3C, and the C-chemokines, in which C is the cysteine molecule in one letter code and X denotes any other amino acid interposed between the cysteine residues (Fig. 4).

The chemokines bind to the chemokine receptors which are 7 transmembranal G-protein coupled receptors. The receptors are also divided into 4 families depending on the chemokines they bind. The chemokine receptors may bind several different chemokines, but only from within one family, as well as the chemokines may bind to several different chemokine receptors from the same family (122–125).

Not all chemokines exhibit the same degree of promiscuity, since some chemokines bind only to one or very few chemokine receptors. The chromosomal location of these chemokines may explain this phenomenon. The majority of the CXC-chemokines are clustered at chromosome 4q12-13 whereas the majority of the CC-chemokines are clustered at chromosome 17q11.2, yet some CC- and CXC-chemokine genes are located outside of these clusters, and they tend to have a higher specificity than the clustered chemokines. This may reflect that the clustered chemokines, in evolutionary terms, are younger than the non-clustered chemokines, and it has been suggested that the clustered chemokines should be considered as single entities (121).

The chemokine families are a fast growing group of proteins, and to describe them all in detail is outside the scope of this thesis, thus some general considerations on the chemokines and their receptors will be made, and only the chemokines and the chemokine receptors studied in this thesis will be described in detail. (For up-to-date information on cytokines, chemokines and their receptors, the website ”www.copewithcytokines.de” is highly recommendable.)

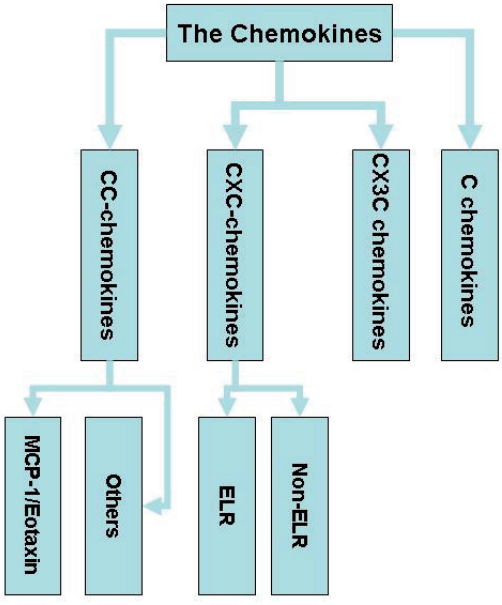

Fig. 4. The chemokine families.

THE CHEMOKINE FAMILIES

The CXC-chemokines are further subdivided into ELR and non-ELR CXC-chemokines based on the ELR (glutamate-leucine-arginine) motif in the N-terminal end of the amino acid sequence (Fig. 4). The ELR group of chemokines are highly chemotactic for neutrophils, but not for monocytes. The position of the ELR motif must be close to the N-terminus of the chemokine in order to have the neutrophil attracting activity (126, 127). This is demonstrated by two forms of IL-8: a 72 aminoacid long version which has high neutrophil attracting capability, whereas a 77 aminoacid long form, which has five extra amino acids in the N-terminal end embedding the ELR motif, is 10 times less potent in attracting neutrophils (128, 129). In addition, the ELR-group has an angiogenic capability which is seen in tumor growth, pannus formation in rheumatoid arthritis, psoriasis and idiopathic pulmonary fibrosis (125, 130). The non-ELR group does not have clear-cut common characteristics, except for angiostatic capability (130).

The CC-chemokines attract monocytes, eosinophils, basophils, neutrophils, and lymphocytes (123). Like the CXC-chemokines they can be divided into two groups; the monocyte-chemoattractant-protein (MCP)/eotaxin group in which the members have 65% or more homology, and the group containing all other CC-chemokines (131) (Fig. 4). The N-ter-

minal part of the CC-chemokines also seems to be crucial for their function although no clear motif like the ELR motif has been identified. This is demonstrated by the fact that addition of a single aminoacid in the N-terminal end of MCP-1 results in a 100–1000 fold reduced chemotactic potency (132, 133).

The CX3C- and C-chemokine families are not well examined (Fig. 4). Fractalkine, the only known CX3C-chemokine, exists in a soluble form and as membrane bound on top of a mucine like stalk (134). It mediates chemotaxis and cell adhesion (135). ATAC, lymphotactin and SCM-1α are the members of the C chemokine group. They induce chemokinesis in CD4+ and CD8+ cells (136, 137).

THE CHEMOKINE RECEPTORS

The chemokine receptors are divided into four families depending on the chemokines they bind, thus there are CXCR1-5, CCR1-11, CX3CR1 and CR1 chemokine receptors. The chemokine receptors are 7 transmembranal proteins bound to G-proteins (138), and as the name implies they have 7 intramembranal domains, connected through 3 intracellular domains and 3 extracellular domains, with the N-terminal end being extracellular and the C-terminal being intracellular (124, 139). As described for CCR2 the chemokine is bound with high affinity to the N-terminal end of the chemokine receptor, which leads to a conformational change in the molecule and brings the ligand into contact with a low affinity binding site on the second extra cellular loop (140, 141). Signalling is mediated through a highly conserved DRY (aspartic acid-arginine-tyrosine) sequence on the second intracellular loop (142). This is demonstrated through a mutation of DRY to GGA in the CCR5 which abolishes intracellular Ca^{2+} increase after stimulation (143). In contrast, changing D to V leads to constitutive stimulation with an accumulation of inositol phosphate (144). Furthermore, deletion of the C-terminal 44 aminoacids of CCR5 also abolishes the intra cellular increase of Ca^{2+} which is not the case if the last 32 aminoacids are deleted (143). Thus the intra cellular signalling seems to be dependent on at least two different sites; the DRY sequence in the second intra cellular loop and the C-terminal end between amino acids 32 and 44.

THE CHEMOKINE RECEPTORS ARE DIFFERENTIALLY EXPRESSED ON TH1 AND TH2 LYMPHOCYTES

Initially it was demonstrated that CCR3 is expressed on human Th2 cells (145). This was followed by a study which showed that T cells stimulated to either a Th1 response through stimulation with IL-12, anti-IL-4 and PMA, or a Th2 response through stimulation with IL-4, anti-IL-12, and PMA, expressed chemokine receptors in a differential manner, and as a consequence migrated in response to different chemokines. Through Northern blot it was documented that Th1 cells preferentially express CXCR3 and CCR5 whereas Th2 cells express CCR4 and to a lesser degree CCR3 (146). The expression of CCR5 by Th1 was described at the same time by Loetscher et al. (147), and the expression of CCR4 on Th2 cells was confirmed by Imai et al. (148) by analysing the IFN-γ and IL-4 expression in T cells responding to the ligands for CCR4, TARC and MDC (see below). CCR4 and CCR8 have also been shown to be upregulated on Th2 cells upon activation (149). Additionally, flowcytometric and Northern blot analysis of T-cell clones stimulated in Th1 or Th2 direction have shown that Th1 cell express CXCR3 whereas Th2 cells express CCR4 and CCR3 (150). However, very thorough flowcytometric analysis of chemokine receptor and interleukin expression in peripheral T cells have suggested that the T-cell subsets should be charaterized by more than one chemokine receptor. Thus 88% of the Th1 cells are CXCR3+CCR4- and 90% of the Th2 cells are CXCR3-CCR4+ (151) (Fig. 4). Some Th1 cells are expressing CCR5, but this does not seem to be as important a marker as suggested initially and CCR3 does not seem to be expressed on peripheral T cells. Furthermore non-differentiated Th0 cells seem to be expressing both CCR4 and CXCR3 (151, 152).

EXPRESSION OF CHEMOKINES IN ATOPIC DERMATITIS SKIN

As described above, atopic dermatitis changes from a Th2 dominated disease in the acute phase, to a mixed Th1/Th2 disease in the chronic phase. As also described, Th2 lymphocytes express CCR4, making its ligands, thymus and activation regulated chemokine (TARC) and monocyte derived chemokine (MDC), interesting in the context of atopic dermatitis. Another CC-chemokine, cutaneous T-cell attracting chemokine (CTACK), which is skin specific and attracts only skin-homing cells, expressing CCR10, is also interesting in the context of inflammatory skin diseases.

Thymus and activation regulated chemokine (TARC/CCL17) and monocyte derived chemokine (MDC/CCL22)

TARC is an 8 kDa CC-chemokine, which initially was shown to bind to T cells, but not to monocytes or granulocytes. Furthermore, pertussis toxin inhibited the chemotactic effect of TARC on T-cell lines (153) indicating that it is bound to a G-protein coupled receptor. The gene for TARC was assigned to chromosome 16q13 (154), unlike the clustered CC chemokines which are found on chromosome 17 (155). TARC is a highly specific ligand for CCR4 (156), and therefore chemoattractive for Th2 cells (148).

MDC is an 8 kDa CC chemokine also located to chromosome 16 (157). MDC also binds to CCR4 (158, 159) and thereby attracts Th2 lymphocytes. The production of MDC is induced by the typical Th2 cytokine IL-4, but not by the Th1 cytokine IFN-γ in human monocytes (160).

Expression of TARC by basal keratinocytes

The role of TARC and MDC in atopic dermatitis was first examined by us in the NC/Nga mouse (96). TARC is highly expressed in keratinocytes as the mice develop the full blown atopic dermatitis like condition (Fig. 3G). The expression is, however, preceded by a low expression of IFN-γ (Fig. 3H), which is in accordance with results obtained by stimulation of murine basal cell carcinoma (BCC) cells. These studies showed that IFN-γ and TNF-α as well as IL-1β were able to induce TARC production (96).

The expression of TARC by basal keratinocytes in atopic dermatitis lesions have been confirmed in several studies by both in situ hybridisation and immunohistochemistry (161–164). TARC is also expressed by keratinocytes in other inflammatory skin diseases such as bullous pemphigoid (165), mycosis fungoides (166), and in keratinocytes in atopic dermatitis in other species such as the dog (167) and the cat (168).

Others have not been able to show expression of TARC in the epidermis, but found it to be expressed in endothelial cells of the dermal vessels (169, 170), whereas others report expression in both the vessels and cells in the dermis (161, 171).

In our primary study of TARC expression in keratinocytes in atopic dermatitis patients (164), 4 out of 7 biopsies showed positive staining for TARC in the epidermis (see Fig. 8A). The others studies, whether they showed positive staining of the keratinocytes or not, also had a limited number of biopsies (ranging from 2 to 7 except in one study in which 17 patients were examined) (Table 1). In one study scales from bed linen of atopic dermatitis patients were collected and TARC levels were measured and compared with normal skin (from the sole of the foot) and showed that TARC was also significantly upregulated in these scales (172).

Table I. *The cellular source of thymus and activation regulated chemokine (TARC) in the skin lesions of atopic dermatitis (AD). The references describing TARC production in the skin are listed along with the number of biopsies examined and the method for detecting TARC in the study. The results are classified as positive or negative staining of the keratinocytes and positive or negative staining of dermal vessels or other dermal cells.*

Reference	No. of biopsies	Method	TARC expression in keratinocytes.	TARC expression in dermal vessel or dermal other cells
Campbell et al., 1999.	2	*In situ hybridisation*	Negative	Positive
Hijnen et al., 2004	5	IHC	Negative	Positive
Kakinuma et al., 2001.	3+3[1]	IHC	Positive	Positive
Horikawa et al., 2002.	3	IHC	Positive	Positive
Uchida et al., 2002.	17	IHC	Positive	Negative
Vestergaard et al., 2000.	7	IHC	Positive/Negative[2]	Negative
Zheng et al., 2003.	?	*In situ* RT-PCR	Positive	Positive

[1]The biopsies were from 3 patients with acute AD and 3 patients with chronic AD.
[2]Of 7 biopsies 4 stained positive for TARC in the keratinocytes wheras 3 were negative.
IHC: Immunohistochemistry.

To investigate the importance of TARC as an *in vivo* mediator of Th2 inflammation we injected TARC intradermally in BALB/c mice. We found a dose dependent infiltration in the skin after 48 hours of CD3+CD4+ Th2 lymphocytes as determined by mRNA analysis and immunohistochemistry (Fig. 5A and B), which yielded high expression of IL-4 and low expression of IFN-γ. The IL-4 and IFN-γ response was also dose dependent which may be a reflection of the dose dependent infiltration by the lymphocytes since they are the main source of these cytokines (173).

Interestingly injection of TARC also induced TARC production in the keratinocytes (173) (Fig. 5C). However, injection of TARC did not induce any eczematous reactions in our experiment. This may have two explanations; either the experiment was not carried out long enough (more than 8 days) or other factors need to be present in the skin. Given the complex nature of atopic dermatitis and eczema the likely explanation is that one or another factor is missing in this experimental setting.

Stressing the significance of TARC is the fact that TARC is elevated in the serum of atopic dermatitis patients and correlates with disease severity and might thus be an indicator for the severity of atopic dermatitis (169). In psoriasis patients s-TARC levels are the same or lower than in healthy controls (166, 171) which is in line with the perception of psoriasis as a Th1 dominated disease.

Individuals carrying a single nucleotide polymorphism (SNP) in the promoter region of the TARC gene (-431C>T) have a higher serum concentration of TARC than individuals who do not (174). One study has shown that the SNP (-431C>T) is associated with sensitization to *Derm. Pteronyssinus* and cat, as well as high succeptibility to blood eosinophilia and total serum IgE. Furthermore, a subgroup of children with asthma with the SNP had significantly higher TARC than children without the SNP (175). However, in children with atopic dermatitis, no association with the SNP can be found (176).

Regulation of TARC production

We, and others, have shown that TARC can be induced by IFN-γ and TNF-α in both keratinocytes and HaCaT cells (an immortalized keratinocytic cell line) (Fig. 6A) (164, 166, 171, 177). The effect of IFN-γ and TNF-α on TARC production in HaCaT cells has been shown by several others (178–181) but, surprisingly the typical Th2 cytokines IL-4 and IL-13 have an inhibitory effect (178, 179, 181). This might be interpreted as a counter regulatory mechanism as Th2 cytokines expressed by Th2 lymphocytes inhibit expression of a Th2 mediating chemokine from the keratinocytes.

IL-10, a Th2 cytokine which inhibits the production of Th1 cytokines (182, 183), is overexpressed in atopic dermatitis skin lesions by lymphocytes and keratinocytes (184). IL-10 is usually described as an anti inflammatory cytokine (185, 186), but surprisingly we found that IL-10 augments the TARC inducing effect of IFN-γ and TNF-α in HaCaT cells,

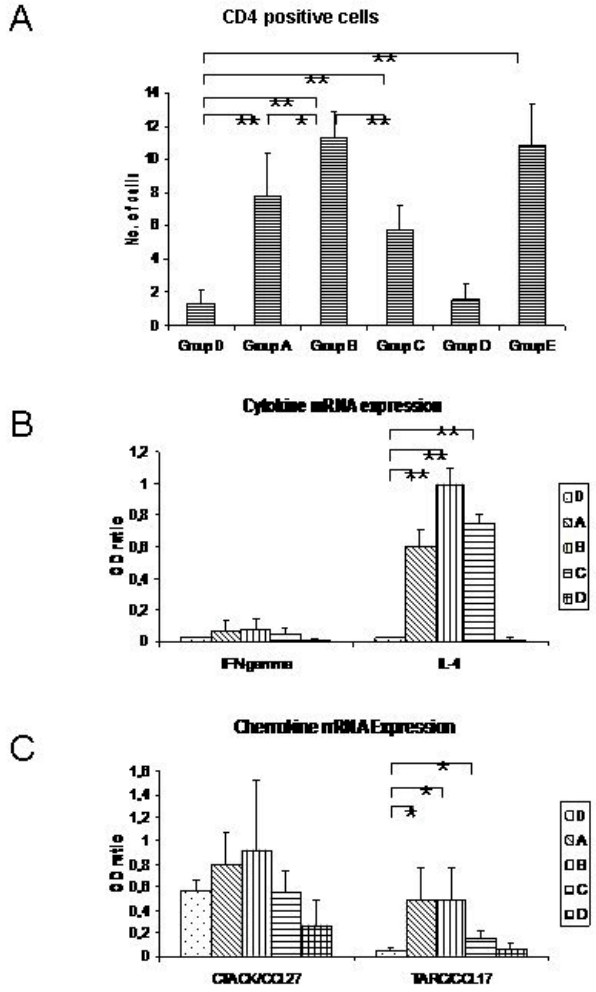

Fig. 5. A) BALB/c mice were injected with TARC (0=saline, A=0,1 ug/ml TARC, B=1 ug/ml, C=10 ug/ml, D= 1ug/ml TARC+50 ug/ml anti TARC, E=1 ug/ml TARC every second day for 8 days). The bars shows the number of CD4 positive cells in the dermis (counted in three consecutive fields at 400 times magnification) *n*=5 for each group. The results show a dose dependent infiltration of the skin by CD4 positive cells with a maximum at 1 ug/ml TARC. B) RT-PCR analysis of mRNA isolated from the skin of the mice in each group (*n*=5 in each group). The primers used were directed against IFN-γ and IL-4, and the result is shown as the OD (optical density) ratio between the cytokine examined and GAPDH (glutaraldehyde dehydrogenase), and shows a dose dependent increase in IL-4mRNA expression. This may be due to the dose dependent infiltration of the skin by the lymphocytes. C) Shows a dose dependent increase in TARC and CTACK mRNA, yet significant results were only obtained for TARC. (*=p<0,05, **p<0,01).

but does not have a TARC inducing effect on its own (177). The C-terminal 9 aminoacids, which have been called IT9302 (187), is the biological active part in this process as it has the same effect on TARC production as equimolar amounts of IL-10. Thus we concluded that IL-10 promotes the process

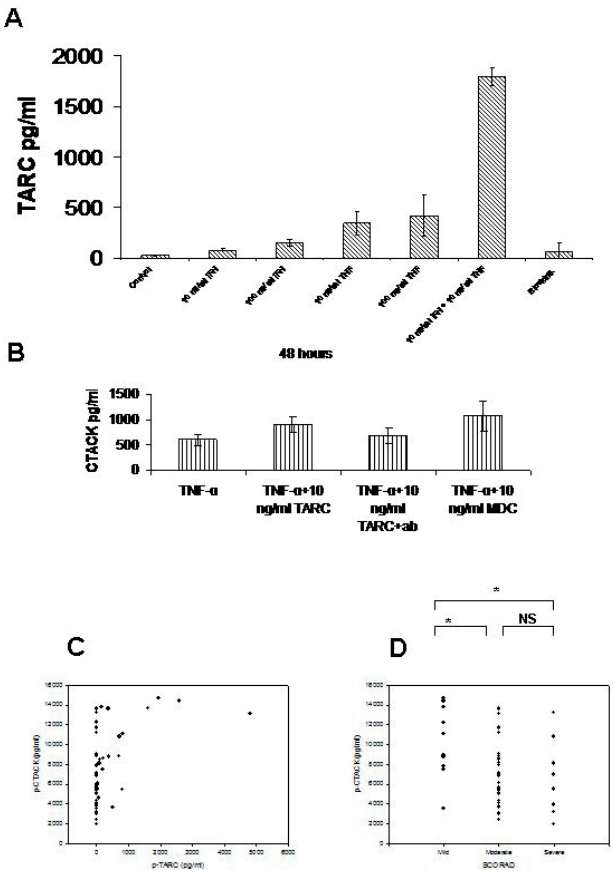

Fig. 6. A) TARC production i HaCaT cells after stimulation with TNF-α and IFN-γ. B) production of CTACK in keratinocytes after stimulation with TNF-α, TARC and MDC. C) P-concentrations of TARC and CTACK in 48 patients suffering from atopic dermatitis (AD). The correlation coefficient was found to be 0,478 ($p<0,05$) using Kendals T. D) P-concentrations of CTACK vs. SCORAD. Surprisingly this showed an inverse relationship between severity and p-CTACK. This might be due to a 'treatment effect'. Please see text for details. *$p<0,05$, NS=non significant.

leading to a Th2 dominated inflammatory process in the skin (177). This conclusion is supported by findings in a murine model for allergic dermatitis. IL-10 knock-out mice showed lack of IL-4 secretion as well as lack of IgE production when challenged with dermal OVA which was not the case in normal mice (188).

However, as with the cellular source of TARC *in vivo*, the induction of TARC in cultured human keratinocytes is still a matter of debate. In the above mentioned study (164) we cultured keratinocytes from patients undergoing plastic surgery until 2nd passage and stimulated with IFN-γ and TNF-α which resulted in TARC production. Others cultured neonatal human keratinocytes and found that TARC mRNA expression, but not TARC protein production, could be induced by IFN-γ (162, 181). Finally one group cultured keratinocytes until 4th passage in media containing, among other things, hydrocortisone, after which they were changed into regular media and stimulated with TNF-α and IFN-γ which did not result in any TARC induction (189).

Thus the regulation and the ability of keratinocytes to produce TARC *in vitro* is also a subject of debate, but the results obtained should be related to the experimental conditions of each study.

Dendritic cells and TARC production

Dendritic cells are also able to produce TARC when stimulated with LPS (190), the Th2 cytokine IL-4, and TNF-α (191) whereas the Th1 cytokine IFN-γ inhibited the production. An interesting observation is the induction of TARC and MDC production in the dermal CD11 positive dendritic cells by the cytokine thymic stromal lymphopoitin (TSLP) (192). TSLP is an IL-7 like protein first described almost 10 years ago (193) . TSLP stimulated dendritic cells activates naïve T cells to produce Th2 cytokines and TNF-α whereas the Th1 cytokines are downregulated. Interestingly TSLP is produced by epithelial cells, and especially by keratinocytes from atopic dermatitis (192).

MDC in atopic dermatitis

We showed that MDC is expressed continuously in the skin of the NC/Nga mouse (Fig. 3H). When the lesions start to develop in skin, MDC expression is upregulated on the mRNA level. The cellular source of MDC in the lesions of the NC/Nga mouse is dermal dendritic cells (Fig. 3F), as determined by immunohistochemistry with anti-NLDC- (non-lymphoid dendritic cell), anti-MHC-II-, and anti-MDC-antibodies (96). MDC is also produced by dermal dendritic cells in atopic dermatitis patients, and by skin infiltrating T cells (97, 192). However in atopic dermatitis patients MDC is produced by the keratinocytes as well, and is induced by IFN-γ (162). Thus, MDC seems to be a 'household' chemokine, which under normal conditions regulates the traffic of lymphocytes into the skin as part of the immunosurveillance, but also functions as an inflammatory chemokine. As with TARC, s-MDC concentrations correlate with disease activity (194).

Cutaneous T-cell attracting chemokine (CTACK/ CCL27)

CTACK is a 112 amino acid long CC-chemokine located outside the CC-chemokine gene cluster on chromosome 9p13. It is expressed first and foremost in the skin as determined by Northern blot analysis (195). CTACK binds specifically to the chemokine receptor CCR10 (196). CTACK attracts principally cutaneous lymphocyte-associated antigen (CLA+) skin homing T cells (see below) (195). CTACK is expressed by keratinocytes in inflammatory skin diseases such as atopic dermatitis, psoriasis and contact dermatitis (197, 198). One study using gene chip analysis has shown that CTACK mRNA expression is significantly lower in psoriasis than in

atopic dermatitis skin and healthy skin (199). Injection of CTACK in the skin of mice induced a lymphocyte dominated inflammation, whereas anti-CTACK antibodies reduced the inflammatory reaction seen in a contact dermatitis murine model, suggesting that CTACK holds a pivotal role as a mediator of cutaneous inflammation (197). S-concentrations of CTACK also correlates with the severity of atopic dermatitis (200). Furthermore, we and others have shown that CTACK is induced in keratinocytes by IL-1β and TNF-α (196, 197, 201). We also showed that injecting TARC intradermally into mice led to induction of CTACK mRNA production (173) (Fig. 5B), a finding which can be reproduced *in vitro* in keratinocytes prestimulated with TNF-α (201) (Fig. 6B). CTACK is also upregulated in the skin of piglets infected with *S. Hyicus* (202).

THE INTERDEPENDENCE OF TARC, MDC, AND CTACK

The different stimuli of TARC, MDC and CTACK are shown in Fig. 7. The significant role of these three chemokines is reflected by the fact that they all correlate with disease severity in atopic dermatitis (162, 198, 203), and the interdependence of these chemokines in atopic dermatitis is stressed by the fact that TARC correlates with MDC (162) and CTACK (201) (Fig. 6C), and CTACK with MDC (200). However, in our report (201) a negative correlation between CTACK and disease severity was observed (Fig. 6D). However our patients were all receiving treatment, and the more severe the disease, the more intense the treatment, and therefore the negative correlation could actually reflect the effect of the treatment. It has been shown that TARC decreases when the patients improve

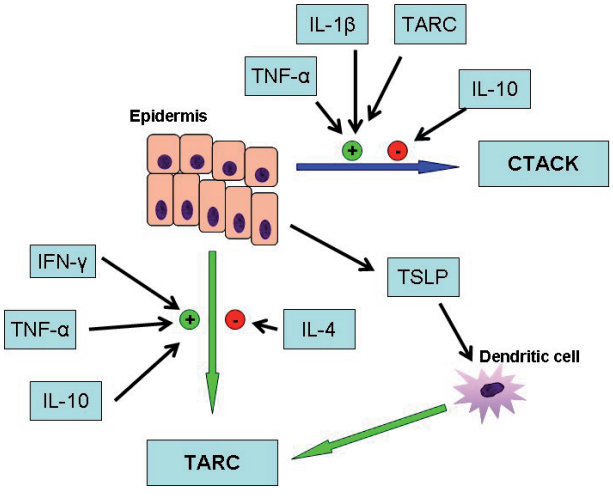

Fig. 7. Factors stimulating and inhibiting TARC and CTACK production in keratinocytes.

(204), which supports our findings. In other skin diseases the serum levels of TARC (mycosis fungoides and bullous pemphigoid) and CTACK (psoriasis) are also increased (165, 166, 198). In contrast, TARC is not increased in serum from psoriasis patients (166). As mentioned above TARC and MDC is able to induce CTACK production in keratinocytes (173, 201) and this could indicate that TARC drives Th2 dominated inflammation and also induces a mixed lymphocytic Th1/Th2 inflammatory reaction and thereby contributes to the shift in the Th1/Th2 balance in the skin.

CCR4 and CCR10, the receptors for TARC, MDC, and CTACK

As described above TARC, MDC and CTACK are overexpressed in atopic dermatitis skin. TARC and MDC attract Th2 lymphocytes and CTACK attracts skin homing lymphocytes through the CCR4 and CCR10 receptors respectively. As already described chemokine receptors mediate chemotaxis of cells which is migration of cells along a concentration gradient towards the higher concentrations. However, several more steps are involved in the process of cell migration in the inflammatory process.

Cell trafficking

The lymphocytes circulate between the different organs of the body, the lymphnodes , the lymphatic vessels and the blood, which ensures that the lymphocytes are able to encounter any foreign antigen that may enter the body. If an antigen enters the body the lymphocytes, along with other leucocytes, accumulate at the site of infection in the process known as inflammation. This process is controlled by intricate molecular signals which control cell movement, cell activation and the permeability of the vessels in the inflamed area. The process by which the leucocytes, including the lymphocytes, enter an organ can be described in a three step mode (205):

1) Tethering and rolling
2) Activation
3) Firm adhesion

This process of rolling is mediated by a family of proteins known as the selectines (L-, E-, and P selectines) (206, 207). Activation of the leucocytes can be mediated by a variety of molecules and among these the chemokines. The activation of the leucocytes leads to upregulation of the adhesion molecules belonging to the family of integrines which mediate the next step known as firm adhesion (206).

Cutaneous lymphocyte-associated antigen (CLA)

In the skin lesions of mycosis fungoides 85% of the invading lymphocytes expressed an isoform of P-selectin, CLA, which was also found to be expressed on the malignant cells, but not on malignant cells of non-cutaneous peripheral T-cell lymphomas (208), nor on lymphocytes in any other organ than the skin (209). CLA binds to a lectin, endothelial cell-leucocyte adhesion molecule 1, ELAM-1 (210), which is expressed by endothelial cells of the cutaneous venules

(211). The expression of CLA on lymphocytes is believed to be due to micro environmental factors in the skin draining lymphnodes or in the skin it self where the cells are activated (212). An example of this is the ability of IL-12 to induce CLA on T lymphocytes. IL-12 is produced by monocytes in the skin after stimulation with superantigens from *S. aureus* (44) and may thus contribute to the attraction of T cells in atopic dermatitis. Furthermore, when T cells are isolated from patients suffering from atopic dermatitis, only the CLA positive T lymphocytes proliferate in response to house dust mite antigen, towards which atopic dermatitis patients are often sensitized. However, if the patients also suffer from astma, both CLA positive and CLA negative T cells proliferate in response to house dust mite antigens (213). In addition CLA positive cells from patients with allergic contact dermatitis to nickel responded towards nickel which was not the case for CLA positive cells from atopic dermatitis patients (213). Lastly, CLA positive T cells often express the Vβ re-arrangement appropriate to staphyloccal superantigens produced by the bacteria often colonizing the skin (40). CLA is not the only molecule mediating binding to the vascular endothelium in the skin, however it only mediates binding to the endothelium in the skin.

CCR4 and CCR10 expression on lymphocytes in atopic dermatitis

CCR4 is expressed by CLA positive skin homing cells, but not by the α4β7 positive T cells bound for the intestinal mucosa. However, some T cells expressing neither CLA nor α4β7 also express CCR4 (170), and may be involved in pulmonary inflammation (214, 215). We showed that the level of CCR4 expression on CD4 positive T cells is upregulated in atopic dermatitis patients (164) (Fig. 8B). The level of CCR4 expression also reflects the severity of atopic dermatitis (216). Thus CCR4, considering the fact that its ligands TARC and MDC are overexpressed in the skin, seems to hold a central role in the attraction of Th2 positive cells into atopic dermatitis skin lesions. We, and others, have found CCR4 to be expressed by the T cells in the lesional skin of atopic dermatitis as shown by immunohistochemistry (88, 163, 164, 216) and by *in situ* RT-PCR (161). The same finding has also been made in canine atopic dermatitis (217). Interestingly, both TARC and MDC stimulation of lymphocytes lead to internalisation of CCR4 (218), which may represent a mechanism for retaining migrated lymphocytes at the site of inflammation. The staphylococcal superantigen SEB induces up regulation of CCR4 on lymphocytes from atopic dermatitis patients, but not on lymphocytes from healthy patients in which they upregulate CXCR3. Correspondingly Th2 cytokines are induced by SEB in lymphocytes from atopic dermatitis patients and Th1 cytokines in lymphocytes from healthy controls (218).

CTACK, which is expressed by the keratinocytes in atopic dermatitis lesions, attracts CLA positive memory (CD45-RO positive) CD4 positive and CD8 positive T cells but not CLA negative or naïve (CD45-RA positive) T cells (195). Using flowcytometry it was determined that not all CLA positive cells, but only a subset expressed CCR10 (197). Further characterization of these cells shows that a small subset of 'effector cells' CD4+CD45RA-CLA+CCR4+CD27-CCR7- all express CCR10, whereas only 30% of the CD4+CLA+ T cells express CCR10 (219).

CD27 is a receptor of the tumor necrosis factor receptor family and is expressed along with the chemokine receptor CCR7 on virtually all naïve T cells, whereas the cells lacking the expression of these markers contains the group of cells which have recently encountered antigens and respond more quickly to antigens than other T cells (220–222). It has been suggested that this particular group of CLA+ T lymphocytes may coordinate the response in the skin by the rest of the CLA+ T cells (219).

The close relation between CCR4 and CCR10 positive cells are further demonstrated in an animal experiment using CCR4 knockout mice. CCR4 deficient T cells were able to migrate into inflamed skin, a process which could be blocked by CTACK antibodies, whereas the migration of wildtype CCR4 expressing T cells were unaffected by CTACK antibodies (223). When examining lymphocytes in blister fluid from delayed type hypersensitivity reactions induced by *Candida* extracts the majority of the skin infiltrating lymphocytes are CLA+CCR4+ and only approximately 10% express CCR10 in accordance with the expression pattern in the peripheral blood (219). When we examined the CCR4 and CCR10 expression in lesional atopic dermatitis skin (Fig. 8C and F) 27% of the T cells expressing either CCR4 or/and CCR10 were double positive. Furthermore 70,6 % of the cells were only CCR4 positive and 2,2 % were only CCR10 positive (88). This supports the above mentioned results and indicates that at least three subsets (CCR4+CCR10+, CCR4+CCR10-, and CCR4-CCR10+) are involved in cutaneous inflammation. At the same time the CCR10 positive cells were found to express both IL-2 and IL-4 (Fig. 8D and E) indicating that they were both Th1 and Th2 cells (88) and suggesting that Th2 cells can be divided in at least two subpopulations, CCR10+ and CCR10- (as the majority of CCR4 positive cells are CCR10 negative).

When we examined psoriasis skin CCR10 positive cells did not express CCR4 nor did they express IL-4 which is in line with the understanding of psoriasis as a Th1 dominated disease (88). Other investigators have found very few CCR4+ cells in psoriasis (163), and some describe expression of CCR4 on perivascular CD3 positive T cells in psoriasis skin (224). Others again have described CCR4 expression on T cells isolated from the epidermis of psoriasis lesions (225), yet in immunohistochemical stainings of psoriasis no CCR4 positive cells (163), or only very few has been observed (224) in the epidermis. The differences in these observations may be due to the very low number of patients examined or one may speculate if the expression level of CCR4 is lower on lymphocytes in psoriasis skin making the detection more difficult. TARC concentrations in the serum of psoriasis patients

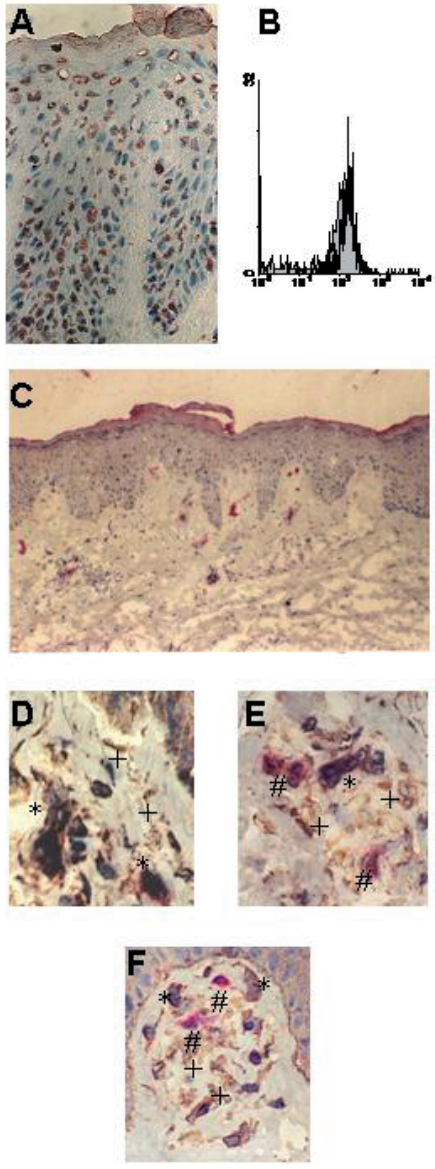

Fig. 8. A) An atopic dermatitis lesion stained for TARC (brown). The TARC positive keratinocytes are mainly seen in the basal layer of the epidermis. B) Flowcytometric analysis of lymphocytes from atopic dermatitis patients and healthy controls. The staining shown here is CCR4 on lymphocytes gated positive for CLA. The number of CCR4 positive CLA positive cells is higher in atopic dermatitis patients (no filling) than in healthy controls (gray filling). C) An atopic dermatitis lesion stained for CCR10 (red). The CCR10 positive cells are seen located throughout the dermis. D) An atopic dermatitis lesion stained against IL-2 (brown) and CCR10 (red) (+= single positive for IL-2, *= double positive for IL-2 and CCR10). E) An atopic dermatitis lesion stained against CCR10 (red) and IL-4 (brown). (#= single positive for CCR10, *=double positive for CCR10 and IL-4, and += single positive for IL-4). F) An atopic dermatitis lesion stained for CCR10 (red) and CCR4 (brown). (#= single positive for CCR10, *= double positive for CCR10 and CCR4, and += single positive for CCR4).

are not higher than in healthy controls (166, 171), and taken together with the observations described above this indicates that TARC and CCR4 plays a minute, if any role part in the pathogenesis of psoriasis.

MONOCYTES, MONOCYTE CHEMOTACTIC PROTEIN 1 (MCP-1/CCL2) AND, CCR2 IN ATOPIC DERMATITIS SKIN LESIONS

Monocytes make up a large proportion of the cells invading the skin in atopic dermatitis lesions, where they are able to differentiate into macrophages and act as antigen presenting cells (23). The circulating monocytes express the CC-chemokine receptor CCR2 (226) which binds MCP-1 (227). CCR2 is pivotal for the attraction of monocytes into inflamed tissue (228-230) and lack of CCR2 expression on the monocytes impairs their ability to adhere to endothelium and to clear certain intra cellular infections (*L. Monocytogenes*). Furthermore lack of CCR2 leads to a decreased Th1 response due to a decreased ability of the Langerhans' cells to relocate to the draining lymph nodes, decreased ability of the monocytes/macrophages to act as antigen presenting cells, and decreased invasion by monocytes into allogenic transplants (231). CCR2 has also been described as pivotal for the migration of monocytes/macrophages into the atherosclerotic lesions (232), and CCR2 expression is found on monocytes in inflamed synovium.

MCP-1(CCL2) (monocyte chemotactic protein 1) is a 15 kDa CC-chemokine also known as MCAF (monocyte chemoattracting factor) and was first described in 1989 (233, 234) as a chemokine which attracted and activated monocytes to tumoricidal activity. MCP-1 is expressed by keratinocytes in both atopic dermatitis and psoriasis (235–237), and we and others found that IFN-γ and TNF-α is able to induce the production in a dose dependent manner in vitro (87, 238, 239). Further indicating a role for MCP-1 in atopic dermatitis is the fact that serum concentrations of MCP-1 are increased in atopic dermatitis (240).

We, and others, found that CCR2 is expressed by virtually all circulating monocytes (CD14 positive cells) (89,9%). We also found that monocytes in atopic dermatitis and psoriasis patients had a slight but significant overexpression of CCR2 (87, 241) (Fig. 9A and B).

Macrophages (CD163 positive cells) located close to the dermal vessels in the skin lesions of atopic dermatitis and psoriasis also express CCR2, but the expression decreases as the monocytes move farther away from the vessels (87). This may be due to the effect of IFN-γ which downregulates the expression of CCR2 on monocytes/macrophages and thus have a retaining effect on the cells once they have reached their target organs (242). A similar result has been observed in rheumatoid arthritis in which 84% of the circulating monocytes express CCR2 and only 24% of the monocytes in synovial fluid express CCR2 (241).

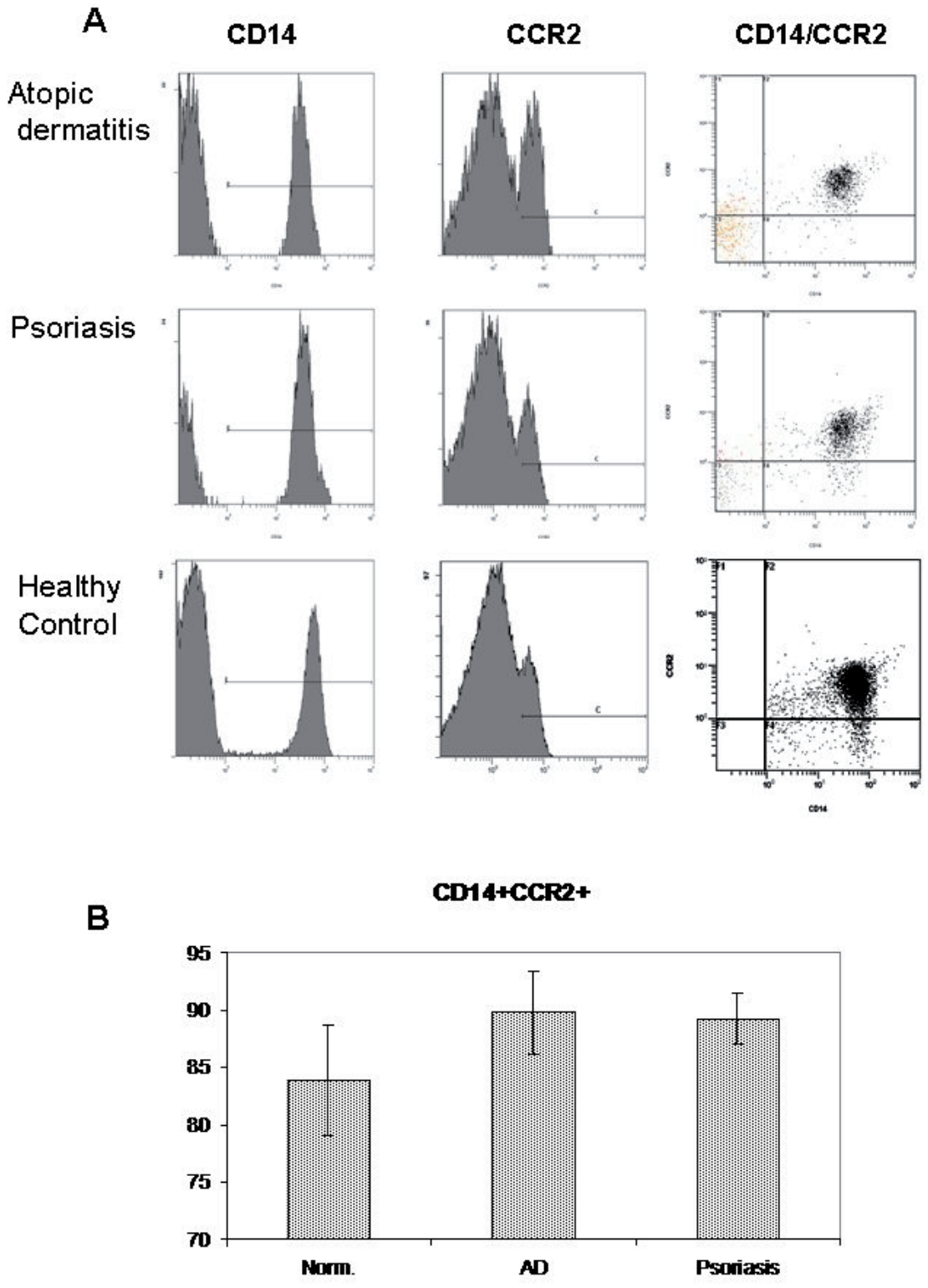

Fig. 9. shows flow cytometric analysis of monocytes. The monocytes identified on their expression of CD 14 (first column). These were positively gated and their expression of CCR2 was measured (column 2). Column three shows a double histogram with both CD14 and CCR2. B) shows the average percent CCR2 positive CD14 positive cells in healthy controls (norm), atopic dermatitis patients (AD) and psoriasis patients (Psoriasis). The result is an average of n=5 (healthy controls), n=6 (atopic dermatitis) and n=7 (psoriasis).

Thus MCP-1 and CCR2 seem to be pivotal in attracting monocytes to the skin, and other organs, and in the sense that CCR2 is down regulated once they reach their target organs, also in retaining the monocytes in their target organs.

CHEMOKINES AND KERATINOCYTES

Chemokine production

As already described the keratinocytes produces the chemokines TARC, MDC, CTACK, and TSLP, and thereby contributes to the inflammatory reaction in the skin. The fact that keratinocytes mediates inflammation is not new. In a review from 1991 (243) the production of IL-8, IL-1, IL-6, MCP-1 and ICAM-1 by keratinocytes is described. Later, chemokines such as RANTES, MIP-1α, MIP-1β and eotaxin are also described in keratinocytes from patients with atopic dermatitis (244). An interesting fact is that the keratinocytes from patients with psoriasis and from patients with atopic dermatitis show a distinct chemokine production profile in response to various T-cell stimuli. Thus, keratinocytes from atopic dermatitis patients showed a significant and higher RANTES production in response to TNF-α than keratinocytes from psoriasis patients. In contrast keratinocytes from psoriasis patients had significantly higher production of IL-8, MCP-1 and IP-10 in response to TNF-α and IFN-γ (235), indicating that the keratinocytes, through interaction with the inflammatory cells, play a major role in determining the nature of the inflammatory reaction in the skin. On the other hand keratinocytes from different skin diseases have, as described above, different potential to produces cytokines. Whether this is due to 'priming' during the course of the disease or to genetic difference remains unknown.

The keratinocytes in atopic dermatitis also undergo apoptosis leading to the characterstic epidermal spongiosis (245). This reaction is mediated through interaction between the Fas ligand secreted by the T cells and the Fas receptor expressed by keratinocytes. Fas receptor expression is induced by IFN-γ, and once the number of receptors expressed reaches a critical size (app. 40.000) the keratinocytes become succeptible to apoptosis (246). During apoptosis cleavage of E-cadhedrines, which are responsible for epidermal continuity of keratinocytes, leads to the characteristic spongioform appearance of the atopic dermatitis lesions (247).

Chemokine receptors on keratinocytes

Keratinocytes do not only produce chemokines, they also express chemokine receptors and are thus able to respond to stimuli mediated through chemokines. The main research of chemokine receptor expression by keratinocytes has been concentrated on the receptors for the CXC-chemokine IL-8, CXCR1 and CXCR2, which is known to regulate growth and differentiation of the keratinocytes. CXCR2 is expressed on the suprabasal keratinocytes in psoriasis lesions whereas CXCR1 is not (248). UVB irradiation of cultured keratinocytes results in significant decrease in the levels of CXCR2 expression, whereas CXCR1 is expressed continuously, and if healthy skin is subjected to 2 minimal erythema doses of UVB light, the same effect can be seen. In this study a faint staining for CXCR1 could be detected (249). CXCR2 also plays a role in wound healing. Experiments with CXCR2 knockout mice have shown that not only the recruitment of leukocytes to the wound, but also epithelial resurfacing of keratinocytes is dependent on CXCR2 (250). The CC-chemokine receptor CCR3, which binds the chemokines RANTES and eotaxin, is also expressed in keratinocytes. *In vitro* experiments shows that CCR3 is upregulated by RANTES but not IL-4 and IFN-γ and immunohistochemical stainings of skin biopsies have shown high expression in keratinocytes in atopic dermatitis lesions (251, 252). Expression of CCR-1 on HaCaT cells correlates inversely with the density of the cell culture (253) indicating that chemokine receptor expression on keratinocytes depends, at least in cell cultures, on intercellular mechanisms. As already described we found that TARC is able to augment TNF-α induced CTACK production in keratinocytes in a dose dependent manner. Furthermore MDC was able to induce the same effect suggesting that CCR4 is expressed on keratinocytes. Using immunohistochemistry, RT-PCR and western blot CCR4 was found to be expressed on both TNF-α stimulated and non stimulated keratinocytes *in vitro*, although TNF-α induced CCR4 mRNA production, thus explaining why TARC is able to stimulate keratinocytes, but not why TNF-α is needed, in respect to CTACK production (201). This may be due to some intra cellular activation of the CCR4 receptor signalling pathway, but the exact mechanism still remains to be elucidated. We also found that CCR4 was expressed by keratinocytes in some, but not all, skin biopsies from atopic dermatitis lesions. This was not demonstrated in any of the earlier publications, but that may be due to new, and perhaps, better monoclonal antibodies. However, if the work of Wakugawa et al. (216) is examined closely, keratinocytes in the basal layer of atopic dermatitis lesions in CCR4 stainings actually stain positive, yet this is not commented by the authors.

REGULATION OF CTACK TRANSCRIPTION IN THE SKIN

As described CTACK is overexpressed by keratinocytes in inflamed skin. A well known regulator of chemokines, as well as proinflammatory cytokines, adhesion molecules, MHC proteins, and apoptosis is the transcription factor NF-κB which is expressed ubiquitously in all tissues. NF-κB binds to specific κB sequences in the promoter regions of several genes and mediates transcription (254–256). NF-κB was first described by D. Baltimore in 1986 as a regulator of the transcription of the kappa-light chain of the immunoglobulins (257). However later studies have shown that NF-κB regulates other functions as mentioned above.

Nuclear Factor-κB (NF-κB)

NF-κB is a dimeric transcription factor formed by homo- or hetero dimerization of the five Rel-family proteins RelA (p65), RelB, cRel, p52 and, p50 (258). The Rel proteins contain a highly conserved N-terminal 300 aminoacid sequence, the Rel homology domain (RHD) (259). The Rel family is divided into two classes based on their aminoacid sequence C-terminal to the RHD. RelA(p65), c-Rel, and RelB belong to the first class and are characterized by an activation domain C-terminal to the RHD. The second class, p52 and p50, is characterized by ankyrin repeats similar to those of the IκB's (259). The first cloning of p52 and p50 revealed mRNA coding for proteins which were approximately 105 kDa and 100 kDa respectively. However, it was later demonstrated that these proteins were cleaved post-translationally resulting in p52 and p50 (260). NF-κB binds selectively to a consensus sequence known as the κB sequences in the promoter regions of genes under its control. The κB sequence is G/(T)GGRNNYYC/(T)C (T=thymine, G=guanine, C=Cytosine, R=purine, Y=pyrimidine and N=any base) (261).

NF-κB is situated in the cytoplasm of resting cells where it is retained through binding to one of the members of the NF-κB inhibitor protein families which are: IκBα, IκBβ, IκBγ, IκBε, Bcl-3 and the precursors of the Rel proteins p100 and p105 (259). This is mediated through interaction between the RHD and the ankyrin repeats, which characterizes the IκB family (262). When an appropriate stimuli reaches the cell, e.g. TNF-α binds to its receptor, the IκB kinases (IKK) are activated (263) and phosphorylates the IκB proteins, leading to degradation of the IκB protein and release and relocation to the nucleus of NF-κB, which binds to the κB sequence in the promoter regions of the appropriate genes and induces transcription (263, 264). The IKK's consists of two catalytic subunits, IKKα and IKKβ as well as a regulatory subunit IKKγ (263, 264). Interestingly proinflammatory stimuli leading to phosphorylation of IκB are solely dependent on IKKβ (265) (Fig. 10).

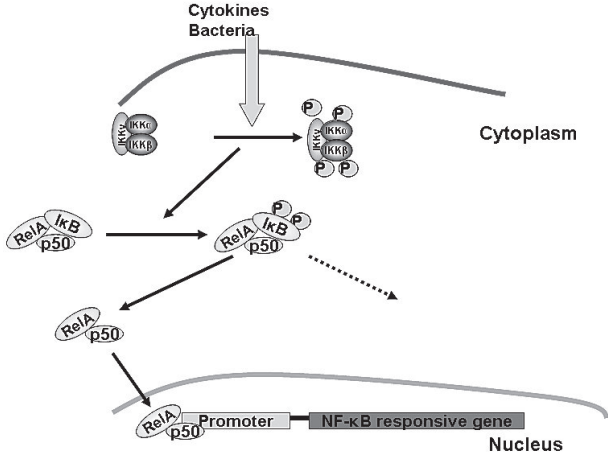

Fig. 10. A simplified and schematic representation of the activation of NF-κB. Once a stimuli arrives at the membrane it activates the IκB kinases thorugh phosphorylation. This in turn leads to phosphorylation of IκB which is bound to NF-κB and keeps it inactive and in the cytoplasma. Once phosphorylated IκB is sequestered and NF-κB is relocated to the nucleus. It binds to the promoter region of an appropriate gene, and activates the transcription of this gene.

Activation of gene transcription through NF-κB is thus a matter of minutes since no new synthesis of NF-κB upon stimulation of the cells is needed. Interestingly, transcription of IκBα is under the control of NF-κB thereby providing a negative feedback loop.

CTACK and NF-κB

TNF-α induces activity of NF-κB and production of CTACK in keratinocytes which led us to the hypothesis that production of CTACK is under the control of NF-κB. Sodiumsalicylate (SSC) (266), Phenylarsine oxide (PAO) (267) and, 3,4-dichloroisocoumarine (DCIC) (268) are non-specific inhibitors of NF-κB, as they act on different levels of the NF-κB activation pathway. When we incubated keratinocytes with either of these compounds it resulted in a down regulation of CTACK expression by keratinocytes stimulated with TNF-α. When we used antisense oligonucleotides directed against the p65 and p50 subunits of NF-κB in keratinocytes (Fig. 11A, B, C, and D), TNF-α induced CTACK production was blocked almost completely (75%–80%) (269). These results indicate that the NF-κB heterodimer p50/p65 is the main transcription factor for CTACK, and further substantiating this, is the fact that we found three regions containing the κB-site in the promoter region of CTACK.

Since the drugs used to treat inflammatory skin diseases, such as glucocorticoids (270), cyclosporine A (271), and perhaps anti-TNF-α-antibodies (272) inhibits NF-κB, one possible mechanism for their ameliorating effect could be inhibition of CTACK production. Yet other mechanisms and inhibition of other cytokines and chemokines are probably also responsible.

NF-κB and atopic dermatitis

A study has shown that treatment of the NC/Nga mouse with a NF-κB decoy, which inhibits the activity of NF-κB, ameliorates the condition of the mouse (273), indicating that NF-κB may be a potential therapeutic target in atopic dermatitis. Mice lacking IκBξ, an IL-1 nuclear protein with high homology to Bcl3 and other IκB family members (274), show lack of activation of genes in the Toll-like-receptor (TLR)/IL-1 signalling pathway (275) and severe impairment of IL-6 production. Furthermore mice that lack IκBξ (INAP, MAIL and, IκBζ) develops a condition similar to atopic dermatitis (102). And interestingly this condition is characterised by a pruritic condition in the skin, which shows alopecia, acanthosis, and hyperkeratosis as well as a dermal and epidermal infiltrate by CD4+- and CD8+-lymphocytes together eosinophils and Langerhans' cells. Furthermore the mice develop increased

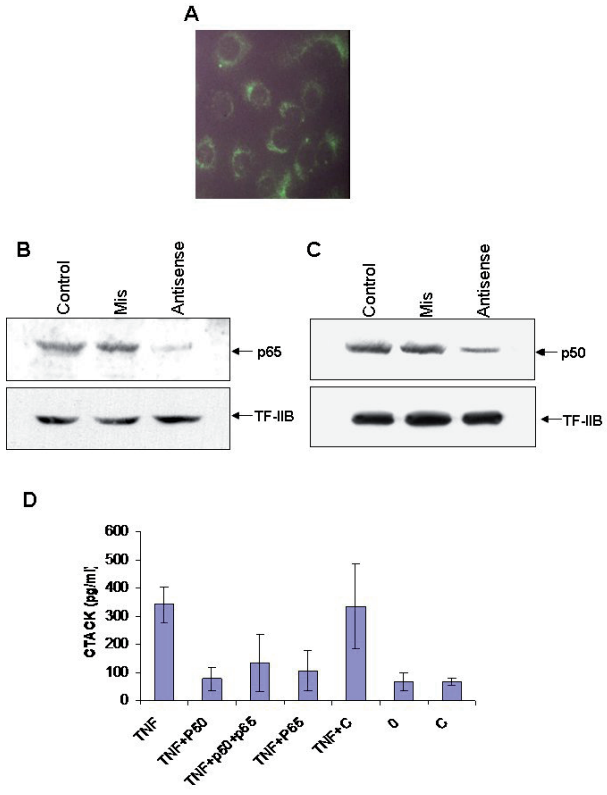

Fig. 11. Experiments with keratinocytes and anti-sense RNA directed against p65 and p50. A) shows keratinocytes incubated with a FITC- labeled oligonucleotide after 48 hours of incubation. The oligo nucleotides are clearly seen located perinuclearly corresponding to the rough endoplasmatic reticulum. This indicated that the method using anti-sense oligo nucleotides worked. B) and C) western blot of protein extracts from keratinocytes cultured either without oligo nucleotides (control), with a missense oligonucleotide, with an oligo nucleotide directed against p65, or an oligonucleotide directed against p50. TF-IIB is used as an internal standard. It is clearly seen that the oligo nucletides are able to down regulate the production p65 and p50 in keratinocytes. D) Shows the resulting production of CTACK after TNF-α stimulation either alone or with oligonucleotides directed against p50, p65 or a control oligonucleotide (c). It is clearly seen that inhibition of either p50 or p65 production inhibit TNF-α induced CTACK production.

s-IgE, and perhaps most interestingly express a high amount of TARC in the skin.

A mouse with epidermis specific deletion of IKK2, which activates IκB, and thereby lacking NF-κB activity in the epidermis also develops a severe inflammatory skin disease. The skin is characterized by a dermal inflammation by CD3+- and CD4+ -lymphocytes as well as granulocytes and macrophages. These mice however die between postnatal day 7 and 9. When these mice were crossed with TNF-α receptor I deficient mice they developed normally (276). These experiments stress the importance of both NF-κB and TNF-α in the development of inflammatory skin diseases.

Mice lacking RelB (RelB-/-) also develop an atopic dermatitis like condition with hyperkeratosis, acanthosis, parakeratosis and a dermal infiltration (277). The lymphocytes in the dermis are CD4+ lymphocytes and CD8+ lymphocytes with an overweight of CD4+ lymphocytes. The cytokines expressed in the skin are mainly of Th2 type, in line with other experiments in which the Rel/NF-κB signalling pathway in T cells was inhibited (278), stressing the similarity to atopic dermatitis. These mice also developed increased IgE and bronchial inflammation, and interestingly when crossed with mice lacking peripheral T cells the condition did not develop.

DISCUSSION

As described, the cellular source of TARC in atopic dermatitis is still a matter of debate. Some investigators have found that TARC is only produced in endothelial cells of the dermal vessels and inflammatory cells in the dermis (169, 170), whereas others have found that TARC is only produced in the keratinocytes of the epidermis (163, 164), and others again that TARC is produced in both keratinocytes and the endothelial cells (161, 162, 171). An important aspect is the fact that the number of biopsies examined from both atopic dermatitis patients and psoriasis patients is very limited. Thus, the papers on TARC expression in atopic dermatitis cited in this thesis have examined a total of 40 patients.

Furthermore the ability of human keratinocytes to produce TARC in vitro is also a matter of debate. Some investigators claim that keratinocytes are unable to produce TARC (189), others that keratinocytes produce mRNA, but not the protein (162), and others again that keratinocytes are able to produce TARC in vitro (164). In an immortalised keratinocytic cell line, HaCaT cells however, there is broad agreement of their ability to produce TARC (177–180).

At first glance these results do not make sense, but if the complex nature of atopic dermatitis is taken into consideration these divergent results might not be that strange. As already described atopic dermatitis patients may suffer from allergic rhinitis or asthma along with their skin disease (23), they may have a superimposed infection of *S. Aureus*, which is known to exacerbate the disease (23), or they may have food allergy or other allergies (29, 30, 34). Thus the group of patients suffering from atopic dermatitis is very heterogeneous. Keratinocytes from different skin diseases have different capabilities to produce certain chemokines and cytokines (235) and if the above mentioned differences actually represent different diseases, all with a common phenotypic endpoint namely atopic dermatitis, the keratinocytes may differ in their ability to produce TARC. Furthermore, the cytokine micro milieu in the skin changes as the skin disease progress (68–72) which could also explain the various expression pattern of TARC as a result of the biopsies being taken at different stages of the disease. The SNP (-431C>T) in the TARC gene, which is responsible for an over production of TARC, is present in both patients with atopic dermatitis and healthy controls, may influence these results. Thus, experiments testing for the ability to produce TARC in response to different stimuli, with keratinocytes heterozygous, or homozygous for the SNP (-431C>T) and keratinocytes without the SNP should be carried out.

In animal models of atopic dermatitis TARC is consistently expressed by the keratinocytes (96, 119, 167, 168). Yet these animal models have been selected on the basis of a Th2 dominated inflammatory reaction in the skin, and therefore this result is not surprising.

It is evident that TARC alone does not induce atopic dermatitis. When we injected BALB/c mice with TARC intradermally a lymphocytic inflammation was seen in the dermis, but no mast cells, macrophages or other cells were observed, and no clinical evident eczematous reactions was observed. One could speculate if this may be due to the short duration of the experiment; however as described in this thesis the development of atopic dermatitis is due to a multitude of factors, and in this experiment we only tested one of these. It is clear though that TARC attracts CCR4 positive T cells, which are mainly Th2 positive cells, and TARC is therefore still a strong candidate as one of the key Th2 attracting chemokines in the skin (148, 173).

As described, MDC is also a ligand for CCR4 and attracts Th2 lymphocytes (158, 159). MDC is expressed in high amounts in atopic dermatitis skin by dendritic cells and keratinocytes and is therefore also likely to play a role in the pathogenesis of atopic dermatitis (162, 192). In the NC/Nga mouse MDC was expressed continuously in the healthy skin and over expressed in the affected skin, which to us indicated that in healthy skin MDC may play a role as a household chemokine regulating the traffic of lymphocytes in the normal immuno surveillance of the skin (96).

CTACK on the other hand attracts both Th1 and Th2 lymphocytes to the skin as shown through the CCR10+ cells ability to produce both IL-2 and IL-4 as well as being either CCR4+ or CCR4- (88). CTACK is produced consistently in inflammatory skin diseases including both atopic dermatitis and psoriasis (197). One study of the chemokine mRNA expression in the skin shows significantly lower expression of CTACK in psoriasis skin as compared to atopic dermatitis and healthy skin. However the patients in this study are not described very well (199). Thus CTACK seems to be a central mediator of lymphocytic inflammation in the skin, yet not as a necessity as other chemokines may take over (223). CTACK may exert its main effect through a specialised subset of CD4+CLA+CD45RA-CCR4+CCR7-CD29-CCR10+ effector T lymphocytes that may act as a central controller of the immune response in the skin in atopic dermatitis and other inflammatory skin conditions (219).

Since TARC, MDC and CTACK all are over expressed in the skin in atopic dermatitis it is not surprising that the serum concentrations of these chemokines are elevated and correlates with disease severity (162, 198, 203). Are the serum concentrations of these chemokines diagnostic for atopic dermatitis? The correlations between the s-concentrations of the chemokines have been made to the severity of the disease and not to whether there is a disease or not, and since the atopic dermatitis diagnosis is based on clinical observations, family history and so on (279), using serum concentrations as a diagnostic tool is not meaningful. However; serum concentrations

of the chemokines may be valuable as a monitoring tool for treatment of atopic dermatitis since it decreases as treatment is instituted (201, 204), although further experiments are needed to describe the exact dynamics of these cytokines. As described, several stimuli induce both TARC, CTACK and, MDC, and they do have an effect on each others production (201) and it is therefore not surprising that the serum concentrations of these chemokines correlate with each other.

TARC and CTACK belong to the non clustered chemokines and have a high specificity to their receptor and it is remarkable that two of these cytokines are so intimately related to skin inflammation (197). CTACK seems to be closely related to the attraction of lymphocytes to the skin whereas TARC seems to important in determining the Th1/Th2 balance of the cells invading the skin. TARC also seems to be important in general for allergic diseases as a mediator of the Th2 response (280–283).This indicates that they both could be potential therapeutical targets.

The nuclear transcription factor κB (NF-κB) is an ubiquitously expressed transcription factor. It is known to be pivotal in the regulation of several inflammatory cytokines/chemokines (256). We have shown that blocking NF-κB in keratinocytes results in a decreased TNF-α induced production of CTACK (269). Furthermore several studies in mice have shown that disturbance of the NF-κB signalling pathway can result in inflammatory skin diseases. Interestingly, and in concordance with our results, inhibition of IκBξ, which under normal circumstances causes inactivation of NF-κB, in mice result in a condition very similar to the NC/Nga mouse, and in an increased production of TARC in the dermis (102). Furthermore, several reports point to NF-κB as an important regulator of the Th2 response in T lymphocytes (277, 278, 284). Thus, NF-κB seems to be central in the regulation of the inflammatory response in the skin, and in atopic dermatitis also in the regulation of the T-lymphocyte response. This is not surprising given the effects of NF-κB, yet it does indicate that NF-κB is a very attractive target for therapy, not only in atopic dermatitis, but in all inflammatory skin diseases.

MCP-1 has been shown to be expressed in both atopic dermatitis and psoriasis (235–237). It binds to the CCR2 receptor which is expressed on circulating monocytes (226, 227). MCP-1 and CCR2 are thought to play a vital role in the recruitment of monocytes to inflamed tissues, and also seems to play a central role in the recruitment of monocyte derived Langerhans' cells into the skin (226, 285–287). The MCP-1/CCR2 interaction may be a general inflammatory mechanism for the recruitment of the first line defence of the immune system.

We show that MCP-1 is produced by keratinocytes after stimulation with TNF-α and IFN-γ, and that in atopic dermatitis and psoriasis the level of CCR2 expression is upregulated on circulating CD14+ leucocytes compared to healthy controls (87). Furthermore we show that CD-163 positive cells in the skin are CCR2 positive close to the dermal vessels but as they migrate into the skin they do not express CCR2. IFN-γ is known to down regulate CCR2 on monocytes (242), and since this cytokine is expressed in the skin of atopic dermatitis and psoriasis it may also have the role of retaining the monocytes in the skin.

CONCLUSIONS

In this thesis we have studied the expression and regulation of two major mediators of lymphocytic infiltration in the skin, TARC and CTACK, in both a murine model of atopic dermatitis and in atopic dermatitis patients. We have also studied the expression of their receptors on lymphocytes in the peripheral blood and in the skin. We have extended these studies to the expression of a monocyte attracting chemokine MCP-1 and its receptor CCR2. Our studies have led us to the following conclusions:

1. The lesions of the NC/Nga mouse offer great similarity to atopic dermatitis, both histologically and immunologically. The mouse is therefore a sufficient and good model for atopic dermatitis (I).
2. TARC, which binds to CCR4 and attracts Th2 lymphocytes, is expressed in the skin lesions of the NC/Nga mouse as well as in humans. Controversies over the origin of this chemokine still exist, however if the literature on this subject is studied only very few patients have been examined, and one might suspect that TARC expression is dependent on the type of atopic dermatitis and the phase in which the patient is examined (I+II).
3. TARC production can be induced in keratinocytes through inflammatory cytokines such as IFN-γ and TNF-α, which are both found in atopic dermatitis lesions. IL-10, which is usually described as an anti inflammatory cytokine, is able to potentiate the effect of IFN-γ and TNF-α (II+III).
4. We have confirmed that TARC attracts CCR4 positive lymphocytes, which are Th2 lymphocytes, not only in vitro, but also *in vivo*. However, these experiments also show that TARC alone is not able to induce the eczema. TARC therefore is not 'the atopic dermatitis chemokine' (IV).
5. TARC augments TNF-α induced CTACK production in keratinocytes, both *in vivo* and *in vitro*. This is dependent on the expression of CCR4 on the keratinocytes, and MDC which also binds to CCR4 demonstrates the same ability. Thus, TARC, which mediates Th2 inflammation, may contribute to a general Th1/Th2 inflammation in the chronic phase of atopic dermatitis (V).
6. The serum concentrations of TARC and CTACK correlate with each other, probably as a reflection of the fact that the inducing stimuli are the same and that TARC is able to induce CTACK. One finding, however, is quite surprising namely that s-CTACK correlates inversely with disease severity in atopic dermatitis. This contradicts all other findings, but this may be due to the treatment received by the patients (VI).
7. We show that the receptors for TARC and CTACK both are expressed on lymphocytes in atopic dermatitis skin lesions. At least three subpopulations of lymphocytes are present in the skin, CCR4+CCR10-, CCR4+CCR10+, and CCR4-CCR10+, indicating that at least two Th2 subpopulations exits; CCR10+ and CCR10- Th2 cells. CCR10 positive cells also express both IL-2 and IL-4 indicating that both Th1 and Th2 cells are CCR10 positive. We did not find any CCR4 positive cells in psoriasis skin; however others have demonstrated various degrees of this receptor in psoriasis skin. Again the number of patients examined is very limited and therefore the different results may reflect natural variations (IV).
8. The TNF-α induced CTACK production is controlled through NF-κB in keratinocytes. This again demonstrates the pivotal role of NF-κB in inflammation and confirms that it is an attractive target for therapy in inflammatory skin diseases (VIII).
9. MCP-1 can be induced in keratinocytes through TNF-α and IFN-γ. They work as agonists in a dose dependent manner. The receptor for MCP-1, CCR2, is expressed on virtually all circulating CD14+ monocytes, with a slight but significant up regulation on CD14 positive cells in atopic dermatitis and psoriasis patients (VII).
10. CCR2 is also expressed on a small population of CD163+ monocytes/macrophages in the skin lesions of atopic dermatitis and psoriasis. These cells are located in the vicinity of the dermal vessels whereas CD163 single positive monocytes/macrophages are further away. Thus CCR2 may be down regulated on these cells as a retaining mechanism, or as a consequence of the cells differentiating into DCs (VII).

Future studies

One question regarding TARC still remains unanswered; why is there such a discrepancy in reports on the ability of keratinocytes to produce TARC? This could be due to the single nucleotide polymorphism, and studies with keratinocytes with or without this polymorphism and their respective ability to produce TARC in response to different stimuli should be carried out. Furthermore deletion of IκB-ξ in mice has shown increased TARC production, and experiments blocking this inhibitor of NF-κB would also be very interesting in this context. Naturally it would also be very interesting to examine the function of IκB-ξ in the skin of atopic dermatitis patients, and determine if atopic dermatitis patients carries a mutation in this gene, as well as to determine if IκB-ξ is functional in the NC/Nga mouse.

In the case of both TARC and CTACK, and their receptors, there is some doubts about their expression in both atopic dermatitis and psoriasis skin, and studies showing differential expression in the lesions of different types of atopic dermatitis and psoriasis would certainly also be interesting as this would

indicate differential invasion of the skin by the lymphocytes. Studies of keratinocytes cultured from skin biopsies from each patient category should also be performed. If differential ability between subpopulations of patients with either atopic dermatitis or psoriasis could be identified this would certainly help us in the understanding of these diseases.

Furthermore studies of TSLP expression in the skin and its effect on CTACK and TARC expression, as well as chemokine receptor expression would be very interesting since this chemokine seems to hold a central role in the regulation of chemokine expression in the skin.

Studies, so far, have demonstrated that CTACK is specifically expressed in keratinocytes. This high organ specificity is very unique given that CTACK belongs to the non clustered cytokines and studies of CTACK expression in other inflammatory diseases in humans should be performed.

DANSK RESUMÉ

Atopisk dermatitis er en inflammatorisk kløende hudsygdom der rammer mellem 15 og 20% af danske børn. Mikroskopisk ses der et inflammatorisk infiltrat i dermis og epidermis, der hovedsagligt består af lymfocytter, makrofager, eosinofile, dermale dendritiske celler og mast celler. Lymfocytterne er domineret af CD4 positive celler og i den akutte fase primært af Th2 lymfocytter, dvs. de producerer IL-4, IL-5, IL-6 og IL-10, medens de i den kroniske fase er mere blandede Th1/Th2 lymfocytter, dvs. de producerer også cytokinerne IL-2 og IFN-γ.

Kemokinerne er en undergruppe af cytokinerne (kemotaktiske cytokiner), der aktiverer og tiltrækker leukocytter. Kemokinerne binder sig til kemokinreceptorerne der er 7 transmembranale proteiner. Kemokin receptorerne udtrykkes differentieret på hhv. Th1 og Th2 lymfocytter. Således udtrykker Th1 cellerne fortrinsvist CXCR3 kemokin receptoren medens Th2 cellerne fortrinsvist udtrykker CCR4 kemokin receptoren. Derudover udtrykkes kemokin receptoren CCR10 udelukkende på hudspecifikke lymfocytter. Udfra dette er det nærliggende at spørge hvorvidt liganderne for CCR4, thymus and activation regulated chemokine (TARC/CCL17) og monocyte derived chemokine (MDC/CCL18), og for CCR10, cutaneous T-cell attracting chemokine (CTACK/CCL27) udtrykkes i atopisk dermatitis læsioner og hvordan de har en effekt på atopisk dermatitis' patogenese.

NC/Nga musen er beskrevet som en model for atopisk dermatitis idet den udvikler en eksematøs tilstand når den holdes under 'snavsede forhold' medens den forbliver rask når den holdes under 'rene forhold'. Vi beskriver at cytokinerne, der udtrykkes i huden på NC/Nga musen, er af Th2 typen i den akutte fase, medens de er af Th1/Th2 typen i den kroniske fase. Derudover beskriver vi at kemokinerne TARC og MDC udtrykkes i huden på musen, i henholdsvis keratinocytter og dermale dendritiske celler, når den har eksem. I overensstemmelse med dette fandt vi ligeledes CCR4 udtrykt i huden. TARC ekspressionen genfindes i hudbiopsier fra patienter med atopisk dermatitis, ligesom der ses en overekspression af CCR4 på cutaneous lymphocyte associated antigen (CLA) positive lymfocytter i perifert blod fra atopisk dermatitis patienter. Andre grupper har senere vist at graden af CCR4 ekspression på lymfocytter fra perifert blod, ligesom plasma koncentrationen af TARC og MDC korrelerer med sværhedsgraden af atopisk dermatitis.

TARC produktion kan induceres i keratinocytter af TNF-α og IFN-γ og denne proces kan forstærkes af IL-10. TNF-α og IL-10 findes i atopisk dermatitis hud i den akutte fase, og IFN-γ i den kroniske fase. For yderligere at undersøge TARC's betydning for udviklingen af Th2 responset i huden injicerede vi TARC i dermis på BALB/c mus. Dette medførte en dosis afhængig infiltration i huden på musene med Th2 dominerede lymfocytter, samt ekspression af TARC og CTACK.

Den tætte sammenhæng mellem CTACK og TARC genfindes i atopisk dermatitis patienter hvor vi finder korrelation mellem de to cytokiner, samt at TARC er i stand til at forstærke CTACK produktionen i keratinocytter. I modsætning til andre grupper finder vi en omvendt sammenhæng mellem s-CTACK og sværhedsgraden af atopisk dermatitis. Dette kan forklares ved at patienterne som vi har undersøgt alle var i behandling, og jo sværere sygdommen var desto kraftigere var behandlingen. Således kan den omvendte sammenhæng ses som et resultat af en effektiv behandling.

Lymfocytterne i huden ved atopisk dermatitis udtrykker CCR4 og CCR10. Langt den største gruppe udtrykker udelukkende CCR4 medens en mindre gruppe udtrykker CCR4 og CCR10, og den mindste gruppe udtrykker udelukkende CCR10. Dette er i overensstemmelse med hvad andre har beskrevet for lymfocytterne i perifert blod fra patienter med inflammatoriske hudlidelser. De CCR10 positive celler i huden udtrykker både IFN-γ og IL-4 og er derfor både Th1 og Th2 celler. Givet at de CCR4+ celler er Th2 lymfocytter, og eftersom der findes både CCR10+CCR4+ og CCR4+CCR10- celler, tyder dette på at Th2-undergruppen er inddelt i flere kompartments. Psoriasis betragtes som en Th1 domineret inflammatorisk hudlidelse og dermed i visse sammenhænge modsætningen til atopisk dermatitis. Vi har undersøgt psoriasis hud og fandt at lymfocytterne i psoriasis hud udtrykker CCR10 men ikke CCR4, og at disse lymfocytter udtrykker IFN-γ, men ikke IL-4.

Nuclear transcription factor κB (NF-κB) er en transskriptionsfaktor der udtrykkes i mange væv og har stor betydning for ekspressionen af cytokiner, adhesionsmolekyler, vævstype antigerne, samt apoptose. NF-κB er en hetero eller homodimer bygget op af proteiner fra Rel familien af proteiner, dvs. RelA (p65), RelB, cRel, p52 and, p50. Vi viser at blokering af produktionen af p65 og p52 i keratinocytter fører til en nedsat TNF- induceret CTACK produktion. Det er af andre vist at en NF-κB 'dummy' der blokerer NF-κB aktiviteten kan hæmme udviklingen af eksem i NC/Nga musen. Således synes NF-κB at være et attraktivt terapeutisk mål ved inflammatoriske hudlidelser.

Et andet kemokin MCP-1 (monocyte chemotactitic cytokine-1), der produceres af keratinocytter i både psoriasis og atopisk dermatitis, er rettet mod monocytterne. MCP-1 binder til CCR2 som udtrykkes på monocytter i det perifere blod. CCR2 er essentiel for monocytters evne til at trænge ud i vævene samt for Langerhans' cellers evne til at re-allokere til lymfeknuder og dermed aktivere Th1 responset. Vi viser at monocytter i perifert blod hos atopisk dermatitis patienter og psoriasis patienter udtrykker CCR2 i højere grad end monocytter fra raske kontroller. Dertil kommer at monocytter/makrofager i huden nedregulerer ekspressionen af CCR2, og at denne regulering bliver mere udtalt jo længere fra karrene cellerne kommer. Dette kunne være en mekanisme hvorved monocytter bliver tilbageholdt i inflammeret væv idet IFN-γ er i stand til at nedregulere CCR2 ekspressionen på monocytter/macrofager. Vi demonstrerer desuden at de inflammatoriske cytokiner TNF-α og IFN-γ virker agonistiske på MCP-1 produktionen i keratinocytter. MCP-1 og CCR2 synes på den måde at stå centralt i reguleringen af monocytter/makrofagers invasion i huden.

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