Studies on antigen presenting cells and T-cell activation in contact dermatitis

Involvement of antigen presenting cells and the pro-inflammatory cytokine Tumor Necrosis Factor-α

Steen Lisby

Medical science is like performing detective work, finding information in critical minor bits to finally reveal significant results.

PREFACE

The present thesis is based on investigations that were conducted during my employments at departments of Dermatology, Bispebjerg Hospital and Gentofte Hospital, Copenhagen, Denmark. Part of the studies was also conducted at the Department of Dermatology, Hôpital Cantonal Universitaire, Geneva, Switzerland. The initial studies were performed at Department of Dermatology, Bispebjerg Hospital under supervision of Dr. Gunhild Skovgaard whose dedication was an inspiration for me. In this phase, Dr. Kristian Thomsen spent many hours introducing me to the dermatological patients and thereby gave me a firm attachment to the field of Dermatology. Furthermore, I am grateful for the help by the technicians Merete Tjalve and Inge Aakilde, who assisted me during the often very time consuming *in vitro* analyses and Dr. Erik Obitz for a constructive collaboration and many hours of give full discussions. During the period at Bispebjerg Hospital and later at Department of Dermatology, Gentofte Hospital, Dr. Ole Baadsgaard functioned as my direct supervisor and mentor. His enthusiasm and knowledge within the field of immunodermatology was an inspiration for me and was an important aid in the development of new ideas. In 1994 to 1996, I worked at the Department of Dermatology, Hôpital Cantonal Universitaire in Geneva together with Dr. Conrad Hauser, whose enthusiasm and huge work efforts yielded a work environment, where the performance of many techniques within the field of molecular biology were made possible. During my research, I have been supported by grants from Danish National Research Council, Yde's Foundation and Aage Bangs Foundation. These grants were essential for the research conducted.

I want to thank my wife, Maj-Britt Mosegaard Hansen for her tolerance and acceptance of my many hours firmly attached to the computer. Her love and strength has inspired my finishing this thesis.

Finally, I dedicate this thesis to my child Victor, who has been a treasure to me. My love to him has been and still is a source of energy and inspiration in my daily life.

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III. Nickel-induced activation of T cells in individuals with negative patch test to nickel sulphate. *S Lisby*, LH Hansen, L Skov, T Menné, O Baadsgaard. Arch Derm Res 291: 247–252, 1999.

IV. Nickel induced proliferation of both memory and naive T cells in patch test negative individuals. *S Lisby*, LH Hansen, T Menné, O Baadsgaard. Clin Exp Immunol 117: 217–222, 1999.

V. Decreased number and function of antigen-presenting cells in the skin following application of irritant agents: Relevance for skin cancer. *S Lisby*, O Baadsgaard, KD Cooper, GL Vejlsgaard. J Invest Dermatol 92: 842–847, 1989.

VI. Nickel and skin irritants up-regulate tumour necrosis factor-α in keratinocytes by different but potentially synergistic mechanisms. *S Lisby*, KM Müller, VC Jongeneel, J-H Saurat, C Hauser. International Immunol 7: 343–352, 1995.

VII. Transcriptional regulation of TNFα in keratinocytes mediated by Il-1β and TNFα. *S Lisby,* C Hauser. Exp Dermatol 11:592–8, 2002

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LIST OF ABBREVIATIONS:

ACD	allergic contact dermatitis
AMLR	autologous mixed lymphocyte reaction
AP	activator protein
APC	antigen presenting cell
CAT	chloramphenicol acetyl transferase
CCR	chemokine receptor (CC)
CD	cluster of differentiation
CLA	cutaneous lymphocyte-associated antigen
CSF	colony stimulating factor
DMSO	dimethylsulphoxide
DNFB	dinitroflourobenzene
DTH	delayed type hypersensitivity
EGF	epidermal growth factor
ELR	epidermal cell lymphocyte reaction
ETAF	epidermal-derived thymocyte activating factor
FACS	fluorescent-activated cell sorting
FCS	fetal calf serum
GM-CSF	granolocyte-macrophage colony stimulating factor
ICAM-1	intercellular adhesion molecule-1
ICD	irritant contact dermatitis
ICE	interleukin-1 converting enzyme (Caspase-1)
IFN-γ	interferon-gamma
IL	interleukin
LC	Langerhans cell
LFA	lymphocyte function-associated antigen
LPT	lymphocyte proliferation test
MHC	major histocompatibility complex
MIP-1α	macrophage inflammatory protein-1 alpha
MMP	matrix metalloproteinase
MoAb	monoclonal antibody
mRNA	messenger RNA
PBMC	peripheral blood mononuclear cells
PK	protein kinase
PMA	phorbol 12-myristate 13-acetate
r	recombinant
SALT	skin-associated lymphoid tissue
SLS	sodium lauryl sulphate
Tc1	T-cytotoxic type 1
TCR	T-cell receptor
TGF-β	Transforming growth factor-beta
Th1	T-helper type 1
TNCB	trinitrochlorobenzene
TNFα	tumor necrosis factor-alpha
UV	ultraviolet
VCAM-1	vascular cell adhesion molecule-1
VLA	very late antigen

CONTENTS

1. INTRODUCTION

Contact dermatitis represents a clinical reaction, initiated either through sensitisation by allergens or haptens or through irritation induced by contact with skin irritants of external origin. The latter include a vide spectrum of skin afflictions ranging from mild to moderate irritants where perturbation of the skin barrier is a hallmark to strong irritants, where skin contact results in necrosis and cell death. In both allergic and irritant reaction types, immunological signals are initiated locally within the skin leading to skin infiltration of T lymphocytes and the clinical development of eczema. The immunological events underlying these reactions are the focus of this review.

Modern research has demonstrated a complex role of the skin in both immunological and non-immunological reactions. It is recognised that intact skin not only functions as a barrier inhibiting the penetration of external stimuli but also is involved in regulating the body temperature, preventing loss of body fluid[1], and the skin contains receptors belonging to the nervous system. Furthermore, the skin represents the outermost part of the immune system. In concert with the latter, the skin associated immune system has been profoundly investigated and the concept of skin-associated lymphoid tissue (SALT) has been developed[2-4]. Thus, today the skin is regarded as an integrated part of the immune system and research within the field of immunodermatology has expanded through the last decade.

The skin is composed of different cellular compartments. The epidermis constitutes the outermost part of the skin and harbours both constitive present cell populations i.e. the keratinocyte, the Langerhans' cell (LC), the melanocyte, as well as transient cell populations; e.g. T lymphocytes and non-LC antigen presenting cells (APCs), all capable of synthesising/releasing inflammatory or pro-inflammatory signals. Thus, the epidermis is involved both in the initiation and in the maintenance of inflammatory processes underlying many skin diseases.

One of the most common inflammatory skin diseases is contact dermatitis. Contact dermatitis can be divided into allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD). Contact dermatitis is characterised by epidermal spongiosis and skin infiltration of lymphocytes in particular T lymphocytes and macrophages and if induced by contact with skin allergens, this disease represent an example of an inflammatory skin disease where antigen presentation and/or T-cell activation is thought essential. In severe cases, in particular following skin contact with strong irritants, acantholysis can be observed and the cells become karyopyknotic. If the reaction continues, complete necrosis of the epidermal cells can occur, resulting in the formation of intra- or subepidermal vesicles. The pathogenesis differs between the two types of contact dermatitis, however, clinically mild to moderate allergic and irritant reactions are often impossible to distinguish.

ACD is elicitated by skin contact with exogenous agents. These agents are taken up by the epidermal LC, the only intact APC resident within non-inflamed human skin. Following antigen up-take, the LC subsequently migrates to the local lymphnodes, where presentation of antigens, or antigenic peptide-fragments to T lymphocytes occurs. The latter, in concert with locally released cytokines, chemokines and induction/upregulation of cell-adhesion molecules, finally leads to the induction and maintenance of inflammatory infiltrates within the skin.

The pivotal role of immunological mediators in the induction and elicitation of ACD is today generally accepted. One important study, performed by Piguet et al in 1991 demonstrated that injection of antibodies to tumor necrosis factor-alpha (TNFα) *in vivo*, inhibited the elicitation of ACD in mice[5]. Another study by Enk et al. has revealed that injection of recombinant cytokines *in vivo* resulted in phenotypical and histological changes in the skin comparable to findings in ACD[6]. Thus, local induction of immunological mediators in affected skin areas seems crucial for the development of clinical ACD reactions. Skin irritants are also capable of inducing the synthesis and release of keratinocyte-derived cytokines and chemokines as will be discussed in this review. The specificity of the latter is not fully described.

The end result of both types of contact dermatitis is inflammation with T-lymphocytes migrating to the skin. The importance of such T-lymphocyte activation is stressed by the observations that treatments blocking T-lymphocyte function; e.g. cyclosporin A, are very successful in the management of several dermatological diseases – like contact dermatitis, atopic dermatitis and psoriasis vulgaris. Thus, antigen presentation, cytokine release and T-lymphocyte recruitment and activation are crucial events occurring during the development of contact dermatitis.

2. CUTANEOUS IMMUNITY IN NORMAL EPIDERMIS

The epidermis constitutes the outermost part of the skin. The skin is continuously confronted with a variety of pathogenic microorganisms and environmental chemicals. It is therefore mandatory that the skin harbours an immunological network that can interact with these potential harmful molecules or microorganisms. In skin, both the innate and adaptive immunity is present. The innate immunity includes the presence of defensins, cathelicidin and other antimicrobial peptides and the complement system[7-9]. The adaptive immunity includes the generation of antigen-specific lymphocytes that becomes activated by antigens of external origin. In concert with the latter, the concept of SALT has been known for almost 20 years. The presence of SALT was first proposed by Streilein et al. in 1983[2], and later substantiated[3,10,11]. SALT is comprised of a unique set of APC and responding lymphoid cells. These includes the epidermal LC, that following antigenic/chemical uptake migrates to the local lymph nodes, subsets of T lymphocytes migrating through the paracortical regions of the lymph nodes and following specific activation, these T cells migrate towards the skin, and finally signalling molecules/chemokines released *in situ* by resident cutaneous cells, e.g. the keratinocyte.

2.1 EPIDERMAL KERATINOCYTES

Since 1981, the keratinocyte has been implicated in regulation of the skin immune system[12,13]. These studies described a keratinocyte-derived cytokine that was termed epidermal-derived thymocyte activating factor (ETAF). Later studies revealed that ETAF activity was identical to the interleukin (IL)-1[14,15]. Following these observations, the role of the keratinocyte in immunological reactions has been thoroughly investigated. Several studies have demonstrated that the keratinocyte is capable of producing and releasing a variety of immunologically active cytokines/chemotactic factors following stimulation both *in vivo* and *in vitro* (Table I).

To emphasise the complex role of the keratinocyte in the skin immune system, this cell type can also synthesise T-lymphocyte inhibitory factors, such as IL-1 receptor antagonist, contra IL-1, transforming growth factor-beta (TGF-β) and eicosanoids.

Beside cytokine expression, keratinocytes can express major histocompatibility complex (MHC) class II molecules both *in vitro* and *in vivo*. MHC class II molecules belong to a group of molecules crucial for antigen presentation (se chapter 4.2). Keratinocytes in normal, non-inflamed epidermis do not express detectable levels of MHC class II. However, in diseased skin, keratinocyte MHC class II expression is apparent. Both in ACD and ICD, *in vivo* keratinocyte MHC class II expression is detectable. Keratinocyte MHC class II expression is associated with the presence of lymphocytic infiltrates in the skin as reported in both murine and human systems [16-18]. *In vitro* studies have revealed that cytokines, in particular interferon-gamma (IFN-g), are responsible for the observed MHC class II expression on keratinocytes[19,20]. IFN-g is released from activated T lymphocytes and upregulation of keratinocyte MHC class II expression is thus secondary to the skin infiltration of T lymphocytes. In addition to IFN-γ also TNFα, released from both keratinocytes and epidermal LC, can induce or upregulate MHC class II molecules on keratinocytes *in vitro*. The role of MHC class II$^+$ keratinocytes in the immuneresponse is not fully clarified. Studies indicate that in contrast to "professional APC" (e.g. the epidermal LC) the keratinocyte does not seem to be capable of functioning as a complete APC. This has been emphasised in studies demonstrating that antigen presentation using MHC class II$^+$ keratinocytes as APC has resulted in the development of T-cell non-responsiveness or even tolerance[21–23]. These results suggest that the keratinocytes lack some co-stimulatory signals necessary for the proper T-cell activation.

Following stimulation, the keratinocyte can also express cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), now known as cluster of differentiation (CD)-54. Induction of such keratinocyte CD54 expression has been demonstrated *in vitro* and mediated by the cytokines IFN-γ and TNFα. Increased keratinocyte CD54 expression has also been observed *in situ* in a large number of inflammatory skin diseases and has been found to correlate with disease severity[24]. Moreover, cytokines released from epidermal keratinocytes are capable of upregulating e.g. E-selectin (CD62E) and vascular cell adhesion molecule-1 (VCAM-1, CD106) on dermal endothelial cells thereby contributing to extravasation of immunocompetent cells from the blood to the skin.

In conclusion, the keratinocyte seems to play a central role in the skin immunesystem and keratinocytes are involved in both the initiation and maintenance of an inflammatory infiltrate and in delivering signals, such as TNFα, important for the migration of the epidermal LC.

Table I. Examples on keratinocyte-derived mediators, including cytokines and chemokines (numbers indicates the reference)

Main function	Cytokines	human	murine
Pro-inflammatory Cytokines:	**IL-1α**	15	13,182
	IL-1β	15	***not synthesized***
	TNFα	183	182, Paper VI
Chemokines	**GRO-α /**		
	MIP-2	184,185	186
	MCP-1	187,188	189,190
	IL-8	191,192	
	IP-10	193	186,194
Cytokine regulation	**IL-10**	195	196
	IL-1RA	197,198	199
	TGF-b1		200
	IL-12	201,202	
	IL-18	203,204	205
T cell growth	**IL-7**	206	206–208
	IL-15	41	
CSF-activity	**GM-CSF**	209	210
	IL-3	211	212
	IL-6	213,214	215
	M-CSF	216	216
Other	**TGF-α**	166,217,218	200
	IL-20	219	220

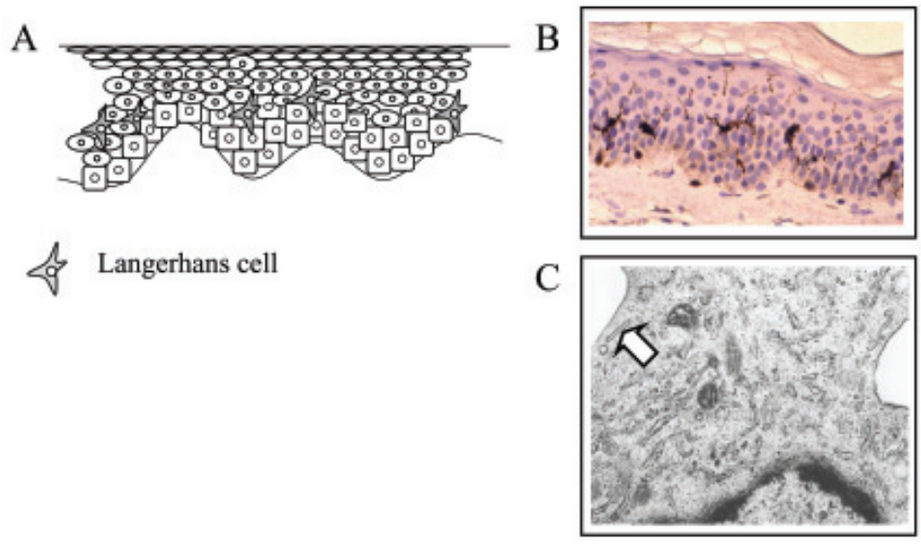

Fig. 1.
a) Langerhans' cell arrangement in epidermis.
b) immunophenotypic demonstration of epidermal LC using anti-S100. Basal cells represent melanocytes.
c) Electron microscopy of an epidermal LC, arrow indicates Birbeck's granules.

2.2 EPIDERMAL LANGERHANS' CELLS

The principal epidermal APC is the LC[25,26]. Langerhans first described this cell type in 1868[27], and the LC is today believed to be the major APC in normal non-inflamed skin. The LC is a complete APC and exhibits the capacity to present antigens or antigen-fragments to both naive and memory T cells. The LC contributes both to the initiation and the maintenance of inflammatory processes in the skin. The LC comprises 2% to 5% of the total epidermal cell population. It is constitutively present in the skin and is localised in the supra-basal part of the epidermis and form a strategely-arranged network to encounter external stimuli (Fig.1a,b). Thus, one can claim that the LC functions as the outermost frontier of the immune system. The LC originates from bone marrow-derived precursors[28,29], but mechanisms involved in the migration of the LC to skin and maturation of this cell type is still not fully understood. In addition to recruitment from bone marrow, some mitotic activities of LC has been described *in situ*[30,31].

The LC is a dendritic cell characterised by a surface expression of CD1a antigen, MHC class I, MHC class II (HLA-DR, -DP, -DQ), S-100, HLe-1, E-cadherin, as well as co-stimulatory molecules such as B7-1/B7-2 (CD80/CD86), CD54 and lymphocyte function-associated antigen (LFA)-3 (CD58)[32-37]. Furthermore, at the ultrastructually level, the LC demonstrates a unique phenotype in that it contains characteristic intracytoplasmic Birbeck granules (Fig.1c)[38]. The LC can synthesise and release a large number of inflammatory or pro-inflammatory cytokines upon stimulation. These mediators include macrophage inflammatory protein-1 alpha (MIP-1α), IL-1β, IL-6, IL-12, IL-15 and TNFα[39–45]. Thus, it is generally accepted that the LC is required for induction of primary immune responses in normal skin. *In situ*, the main task for the LC is to take up and process antigens/ antigenic peptides[46]. These "fresh" LC are not efficient APC as compared to dermal dendritic cells[47]. However, surface expression of MHC class II and the capacity to present antigens are greatly enhanced following short term culture *in vitro* [47,48] or *in vivo* following migration to the regional lymph node. In concert with this, the LC is the principal cell involved in the *in vitro* epidermal cell lymphocyte reaction (ELR, see chapter 7 for detailed description) where it is capable of stimulating allogeneic as well as antigen specific T cells[49–51]. The involvement of the epidermal LC in the ACD reaction is discussed in chapter 4.

3. NON-LC EPIDERMAL ANTIGEN PRESENTING CELLS

In 1985, Cooper et al. described the appearance of epidermal OKT6$^-$ (CD1a$^-$) non-LC APC following UV-induced inflammation of the skin. These cells were not detected in normal skin[52]. Later the non-LC APC were further characterised by the monoclonal antibody OKM5[53]. OKM5 (anti-CD36) binds to a transmembrane glycoprotein with a molecular weight of about 88 kDa [54]. It was initially characterised on the surface of platelets[55]. On immunocompetent cells, CD36 was first demonstrated on peripheral blood monocytes and together with the monoclonal antibody OKM1 (binding to CD11b), characterised a major subpopulation of monocytes in the peripheral blood[56–58]. Later, CD36 has been identified as the receptor for the adhesive glycoprotein thrombospondin-1[54] and collagen[59], involved in the cytoadherance of P. falciparum infected erythrocytes to endothelial cells[60], involved in phagocytosis of apoptotic cells [61–63] and to function as a class B scavenger receptor[64].

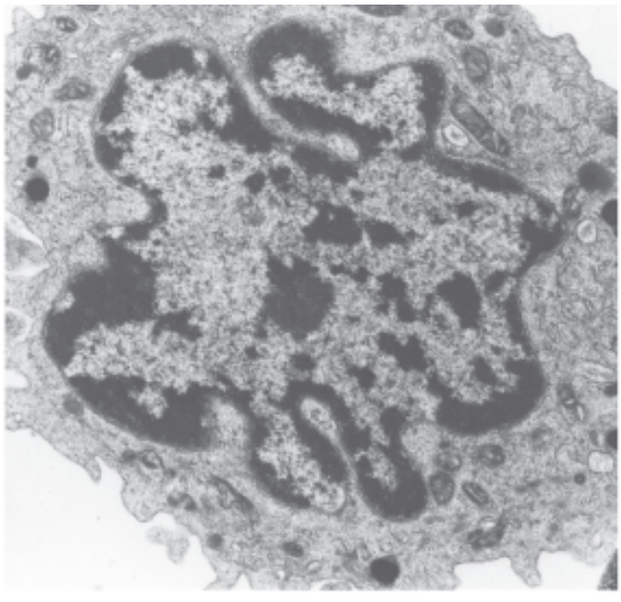

Fig. 2. Electron microscopy of a CD36$^+$ macrophage from involved skin in a patient with cutaneous T-cell lymphoma. Modified from ref. 70

Following UV-irradiation, CD36$^+$ APC was identified in the skin and phenotypically this cell population was identical to a minor subpopulation of peripheral blood monocytes, expressing a CD36$^+$CD11b$^-$ phenotype. This subpopulation exhibited an unique capacity to trigger an autologous mixed lymphocyte reaction (AMLR) as well as presenting soluble antigens[56,57]. Later studies in our group demonstrated a differential recruitment of cells expressing this CD36$^+$CD11b$^-$ phenotype type to skin using different wavelengths of *in vivo* UV-irradiation[65]. Furthermore, functional studies of these cells indicated, that they were functionally active and most possible involved in down-regulation of immune responses[66,67]. This modulation of the immune system was speculated to be involved in the immune-surveillance, and also possible be involved in termination of inflammatory responses. In support of this, studies have now shown that the UV-induced CD36$^+$ non-LC APC preferentially synthesise the inhibitory cytokine IL-10[68] and not IL-12, which is found released from normal epidermal LC.

Prompted by these observations, our group investigated the occurrence of CD36 in other skin diseases. I was the first to identify such bone marrow-derived CD36$^+$ non-LC in the skin of patients with mycosis fungoides[69]. This disease is distinguished from other lymphomas by marked T-cell affinity for the skin, and at the histopathological level is characterised by a clear epidermotrophic T lymphocyte infiltrate. Further analysis of these CD36$^+$ cells indicated that they were neither T- /B-cells, they did not belong to the major CD11b$^+$ blood monocyte population, but partly co-expressed the CD1a surface molecule. Despite the observed expression of CD1a, electron microscopy of the positively selected bone marrow-derived CD36$^+$ cells demonstrated that the cells lacked Birbeck granules and had an ultrastructual appearance of indeterminate cells/ macrophages (Fig. 2)[70]. To describe the functionally capacity of these bone marrow-derived cells, *in vitro* co-culture using epidermal cell subpopulations and T lymphocytes were performed (assay described in chapter 7) and it was demonstrated, that the CD36$^+$ cells indeed could activate T cells in the absence of added antigens/ mitogens. In addition, the CD36$^+$ APC were more potent, on a cell per cell basis than epidermal LC obtained from uninvolved skin from the same patients[69,71]. Thus, in diseased skin several types of professional APC are present.

4. ANTIGEN PRESENTATION AND T-CELL ACTIVATION IN THE ALLERGIC CONTACT DERMATITIS REACTION

Allergic contact dermatitis (ACD) is the result of interactions of the adaptive immune system with environmental molecules or chemicals. Most allergens are haptens i.e. small molecules with a molecular weight less than 400 Da. Following the penetration of the skin, the haptens require conjugation to epidermal/ dermal proteins, that can either be cell bound or soluble. Following this, the haptens can be recognised by the skin immune system. ACD differ from most other immunological reactions by its dose dependence during the elicitation phase. The capacity of an allergen to evoke ACD in sensitised individuals is only operative within a relative narrow dose range and it is well known that almost all skin allergens also can elicit irritative skin reactions, if applied in sufficient concentrations. These observations prompted Grabbe et al to investigate the need for irritative stimuli during elicitation of an ACD reaction. Using a set-up in which animals (mice) were sensitised to either oxazolone or trinitrochlorobenzene (TNCB), they demonstrated that application of the irrelevant hapten significantly affected the outcome of challenge with suboptimal concentrations of the specific hapten[72]. This study thus indicated the presence of non-antigen specific effects of epicutaneously applied haptens and demonstrated that allergens also have an irritant component and that this irritant component of an allergen is critical for the induction of clinical ACD. This hypothesis opens up for the introduction of a "danger" model, in which a primary, most probably antigen un-specific, reaction is mounted. This reaction could include the induction of cell adhesion molecules, chemoattractants and epidermal cytokines, of which IL-1β and TNFα are of particular interest (se chapters 4.1, 11 and 12). Following this, antigen specific reactions occur. The antigen specific reaction in ACD can be divided into two separate phases. In the afferent phase, antigens are taken up and presented to the immune system. In normal, non-inflamed skin the APC population is identical to the epidermal LC. In inflamed skin, however, other APCs are present (see chapter 3).

Following binding of haptens/ allergens, the LC migrates via the afferent lymphatic vessels to the paracortical areas of the regional lymph nodes where LC-T-cell interaction takes place. In this phase, the responding T-cell belongs to the $CD45RA^+$ (naïve) T-cell subset. These T-cells are then activated, clonal expansion occurs and immunological memory develops (see chapter 4.1).

In the second phase of ACD, also called the efferent phase, re-exposure to the antigen can lead to clinical ACD. This is the result of antigen presented to memory T cells either in the affected skin or in the local lymph nodes. In this phase, clear dose-responses are observed, as described earlier in this chapter.

4.1. LANGERHANS CELL MIGRATION IN CONTACT DERMATITIS REACTION

In order to act as an initiator of immunological responses, the LC has to migrate out of the epidermis to interact with naïve or memory T cells. Thus, when the LC encounters e.g. chemicals or microorganisms, they respond by migrating to the regional lymph nodes where they subsequently present the antigenic peptides to the T lymphocytes. The migration of LC from skin to the regional lymph node is orchestrated via a complex interaction of cytokines, chemokines and cell adhesion molecules and it is suggested that maturation of the LC occurs during this transfer. This maturation results in a shift from a cell which major function is antigen capture and processing to a cell that acts as a highly effective APC[47]. The pivotal role of LC migration in the ACD reaction has been underscored by observations that agents that inhibit LC migration[73] or application of contact allergens on skin areas depleted of epidermal LC inhibit subsequent contact sensibilisation[74].

Several cytokines have been implicated in LC migration, but particular interest has been addressed to the cytokines IL-1β and TNFα. The importance of these cytokines has been demonstrated mainly in the murine system where *in vivo* injection of recombinant IL-1β or TNFα are both capable of inducing LC migration[75-77]. Furthermore, blocking of IL-1β or TNFα with monoclonal antibodies inhibits LC migration from skin[73]. IL-1β and TNFα are found upregulated in ACD and ICD reactions, as reported in murine as well as in human systems (see chapter 10). The effect of TNFα on LC migration seems mediated via binding to the species-specific membrane TNF receptor type II (TNF-R2)[76,77]. These observations are supported by findings in mice lacking the TNF-R2, where a marked reduction in allergen-induced LC migration has been observed[78].

Another crucial event accompanying LC migration from the epidermis is the modulation of adhesion molecules on the cell surface of the LC. Following application of e.g. contact allergens, a decrease in LC E-cadherin expression has been reported[79]. E-cadherin is a calcium-dependent homophilic adhesion molecule that mediates cell-cell adhesion and in particular mediates adhesion of LC to keratinocytes[80]. Thus, E-cadherin has been suggested to be a key player in the retainment of LC within the epidermis under normal non-inflammatory conditions. Down-regulation of E-cadherin is today regarded as an early event facilitating the detachment of LC from the nabouring keratinocytes.

Following the detachment from the surrounding keratinocytes, the LC migrate towards the epidermal-dermal junction. At this point, the migrating cells have to cross the basal-membrane in order to enter the dermis. Interaction with the basal-membrane is facilitated by expression of very late antigen (VLA)-6, a surface laminin-binding molecule. Interestingly, VLA-6 has been found up-regulated on LC in ACD.

Furthermore, the LC have been shown to express the gelatinase-type matrix metalloproteinase (MMP)-9. This enzyme is capable of degrading type IV collagen[81,82], found in the basal-membrane and thereby MMP-9 expression possibly contributes to the migration of LC across the basal-membrane. The importance of MMP-9 in LC migration is demonstrated by the use of anti-MMP-9 antibodies, that inhibit the hapten-induced decrease in epidermal LC numbers and accumulation of dendritic cells in the regional lymph nodes[83]. Furthermore, expression of MMP-9 has been found upregulated on LC in ACD[84], but also on some invasive tumor cells and the molecule is thought important for tumor cell invasion[85,86].

Following departure from the epidermis, the LC migrates through the dermis and via the lymphatic system towards the regional lymph nodes. This trafficking is, at least in part, con-

trolled by expression of chemokines/ chemokine-receptors[87]. In studies using animals deficient in the c-c type chemokine receptor (CCR) type 7, hapten-induced LC depletion from the skin was comparable to wildtype animals. In contrast, LC accumulation in the regional lymph nodes following skin application of contact sensitisers was significantly decreased compared to normal wild type animals[88]. Similar results have been obtained with mice deficient in the CCR2 receptor[89]. Thus, surface expression of chemokine-receptors and possible receptor-ligand interactions seem to be important for directing the migrating LC to the regional lymph node.

Once LC reaches the lymph node, they accumulate in the paracortical areas where the LC-T lymphocyte interaction takes place.

4.2. MOLECULES INVOLVED IN APC – T-CELL INTERACTIONS.

The crucial interaction between antigens/antigenic peptides presented by MHC class I or II molecules and the T-cell receptor (TCR)/CD3 complex is well established. Following this, the signal is transduced into the cell and the T-cell is activated.

The antigens can bind to MHC molecules/ peptides associated with MHC molecules by different pathways. Some haptens directly bind to and subsequently alter the peptides bound in the MHC class I or II groove. Other haptens are internalised and processed before presented in the context of MHC molecules. Different pathways for antigen processing excist[90]. Haptens can penetrate the cell membrane of the APC and conjugate to cytoplasmic proteins. Following this, the complex is transported to the endoplasmic reticulum where it combines with MHC class I molecules[91]. This complex is then transported to the cell surface and antigens processed by this „endogenous" pathway are presented to $CD8^+$ T lymphocytes[92]. This route has been described for endogeneous as well as exogeneous lipophilic haptens[93]. Another pathway involves phagocytosis or endocytosis of the hapten, followed by degradation in the endosome/lysosome compartment. Following degradation, the peptide fragments are associated with MHC class II molecules and expressed on the cell surface. This pathway leads to the activation of T lymphocytes belonging to the $CD4^+$ subset. The complexity of these pathways is underscored by observations that both pathways can process the same antigens[93].

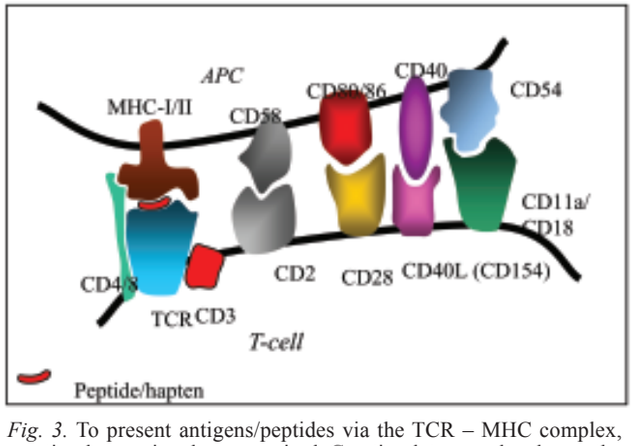

Fig. 3. To present antigens/peptides via the TCR – MHC complex, co-stimulatory signals are required. Co-stimulatory molecules on the APC include CD40, and binding to this molecule results in upregulation of other cell adhesion molecules, including B7/BB1 (CD80/86), LFA-3 (CD58), and ICAM-1 (CD54).

The MHC-TCR/CD3 complex inter-action, however, is not sufficient to maintain a sustained APC-T cell interaction and co-stimulative signals are required for proper T-cell activation to occur. Several studies have demonstrated that e.g. presentation of antigen by MHC class II^+ keratinocytes or by purified MHC class II molecules inserted in lipid membranes lead to tolerance and not sensitisation[22,94]. These secondary signals, obligatory for the activation of T lymphocytes include cytokine release (e.g. IL-1 and TNFα), and the interaction between receptor-ligand molecules on the surface of the APC and T-lymphocyte (Fig. 3). These co-stimulatory proteins include the CD40ligand (CD154) that interacts with CD40 on the APC. This binding results in an increased expression of cell adhesion molecules on the APC, including B7/BB1 (CD80/86) that interacts with CD28/ CD152 on the T-cell[95-97]. The end result is a stabilisation of the T-cell-APC interaction necessary for signal transduction. Other cell adhesion molecules on the APC include CD58 that binds to CD2 on T-cells[98-100] and CD54 that interact with LFA-1 (CD11a/CD18) on infiltrating leukocytes. On the T-cells the CD4 or CD8 molecules, that together with CD3 form an essential part of the T-cell receptor complex, are involved in recognising the type of MHC. Antigenic fragments, presented via MHC class II molecules interact with T-cells expressing CD4. In contrast, if the antigen is presented in association with MHC class I molecules, only $CD8^+$ T-cells respond. Thus, the type of MHC molecules associated with the antigen/antigenic peptide, dictates the subsequent T-cell response.

In conclusion, costimulatory receptor-ligand interactions are necessary for sufficient APC-T-cell interaction to take place. However, the minimal requirement for such T cell activation is still debatable.

4.3. T-CELL HOMING TO SKIN

Following the LC-mediated priming of the immune system, the activated T-cells tend to home to the skin. Thus, if one at a later time-point encounters the same antigen, the possibility of T-cells expressing the relevant T-cell receptor being present in the regional lymph node or even locally in the skin is greatly enhanced. Recently, it has been demonstrated that T lymphocytes entering the skin often are characterised by an increased expression of a surface molecule – cutaneous lymphocyte-associated antigen (CLA)[101-103]. The ligand for CLA is CD62E[104,105], which has been found upregulated in various skin diseases, including contact dermatitis[106].

The importance of CLA has been demonstrated *in vitro,* where blocking antibodies towards CLA resulted in an inhibition of transendothelial T-lymphocyte migration[107,108]. Furthermore, studies on T-lymphocytes from nickel contact allergic individuals have revealed that CLA^+ cells are preferentially activated following an *in vitro* activation using a specific antigen[109]. Finally, CLA expression in transitional T-cells (T-cells undergoing the CD45RA to CD45RO transition) in skin-associated peripheral lymph nodes has been demonstrated to be more than fivefold higher than in the corresponding cells within the mucosal micro-environment[103]. Thus, CLA^+ T-cells are selectively recruited to skin. This is further substantiated by the observation of a marked increase in CLA expression on suction blister-derived T-cells obtained from skin overlying delayed type hypersensitivity reactions (i.e. epidermal T-cells) compared to

peripheral blood T cells in the same persons[103]. Thus, the importance of CLA as a selective skin homing receptor for T-lymphocytes has been established and this molecule seems to play an important role in the recruitment of T-lymphocytes to the local inflammatory reaction site in the skin. Despite these observations, the role of CLA expression in irritant contact dermatitis is still not clarified.

CONTEXT OF CURRENT INVESTIGATIONS

5. AIM OF THE STUDY

Until recently, the only antigen-presenting cell in the epidermis was thought to be the LC. Studies of our group and those by Cooper et al. in the late 80ties revealed the presence of functional active non-Langerhans APC in skin following *in vivo* UV-irradiation. These cells were bone marrow-derived and characterised by surface expression of CD36. Preliminary studies suggested that these cells could be present in other inflammatory skin diseases.

The first goal of this study was therefore to describe the presence of such CD36 positive cells in different skin diseases. Furthermore, I wished to describe a functional role of these APCs with particular emphasis on allergic and irritant contact dermatitis, the former representing a disease in which presentation of antigen is thought essential. The goal was also to elaborate the knowledge of T-cell activation in contact dermatitis, with emphasis on nickel – the most common contact sensitiser in the Scandinavian countries. Furthermore, since a decrease both in number and function of APC in *in vivo* irritant skin reactions was observed, I also wanted to deepen the understanding of the intracellular signalling-mechanisms underlying irritant induced upregulation of the pro-inflammatory cytokine TNFα, a cytokine critically involved in LC migration and shown to be essential for elicitation of ACD as well as for the development of ICD.

Finally, I wanted to explore whether pro-inflammatory cytokines could contribute to the observed increased expression of TNFα in inflammatory skin diseases. Sections 6–9 and 11–12 will review the results of these investigations.

6. EXPRESSION OF SURFACE MARKER CD36 IN INFLAMMATORY SKIN DISEASES (PAPER I)

Today CD36 is known as a receptor (or a receptor family) with a broad binding capacity. In skin, CD36 expression was identified following UV-irradiation and in cutaneous T-cell lymphoma, a disease characterised by a marked epidermotrophic T-lymphocyte infiltrate (se chapter 3). To elaborate these observations, the expression of CD36 in both benign and malignant skin diseases was determined. Using a three-stage immunoperoxidase method on paraffin-embedded skin sections, keratinocyte expression of CD36 in both benign and malignant skin diseases was observed **(**paper I**)**. In contrast, no staining in samples from normal skin was seen. The specificity of the staining was shown by omitting the primary antibody or the application of an isotype control antibody which both yielded negative results. The observation of $CD36^+$ keratinocytes in several malignant skin diseases confirmed my earlier finding of $CD36^+$ keratinocytes in mycosis fungoides[69], but additionally $CD36^+$ keratinocytes were identified in skin samples from both basal cell carcinoma and squamous cell carcinoma lesions. Furthermore, in benign diseases keratinocytes were found to express CD36. The staining pattern was mainly restricted to the stratum granulosum and stratum spinosum, but in diseases like psoriasis, more abundant staining was observed. Thus, the CD36 expression on keratinocytes was not restricted to a particular layer of the epidermis. In skin punch biopsies from positive patch test reaction sites, a time response was indicated with no CD36 expression on keratinocytes in biopsies obtained 4 hours following application of patch test increasing to strong expression on keratinocytes obtained 72 hours following allergen application. Other studies have reported keratinocyte CD36 expression[110-113], however the function of such keratinocyte CD36 expression is currently unknown.

Of particularly interest, I was the first to demonstrate the presence of $CD36^+$ dendritic cells not only in cutaneous T-cell lymphoma but also in the ACD reaction **(**paper I**)** – a reaction characterised by T-lymphocyte infiltration in skin. The $CD36^+$ epidermal leukocytes were localised in the basal and suprabasal part of the epidermis and thus exhibited another staining pattern than observed on the keratinocytes. Due to the sparse occurrence of the $CD36^+$ leukocytic cells, analysis on histological stainings obtained from skin punch biopsies was not the most suitable method to describe these cells. Subsequent investigations were therefore performed on single cell populations obtained from suction blister-derived epidermal cells. The function of these $CD36^+$ dendritic cells could not be attributed in paper I because the immunohistopathology investigations were performed on paraffin embedded sections. However, the functional capacity of these $CD36^+$ epidermal cells was addressed by the use of an *in vitro* assay, epidermal cell lymphocyte reaction (ELR), in a later study[70]and in paper II.

Finally, up-regulation/induction of MHC class II expression on keratinocytes in biopsies from inflammatory skin diseases was found **(**paper I**)**. The staining pattern of HLA-DR differed from that observed using the OKM5 antibody and in concert with other groups[16-18], the keratinocyte MHC class II staining was found to be confined to cells located in the basal and suprabasal layers of the epidermis.

In conclusion, both keratinocyte and leukocyte expression of CD36 was present in skin. Since the keratinocyte CD36 expression was not directly correlated with the occurrence of lymphocytic infiltrates, my focus was directed towards the new $CD36^+$ leukocytic cells. These cells could potentially represent novel epidermal APC population and thus could be of importance for the development of skin inflammation.

7. REGULATION OF EPIDERMAL CD1A$^+$ AND CD36$^+$ ANTIGEN PRESENTING CELLS IN ALLERGIC CONTACT DERMATITIS REACTION (PAPER II)

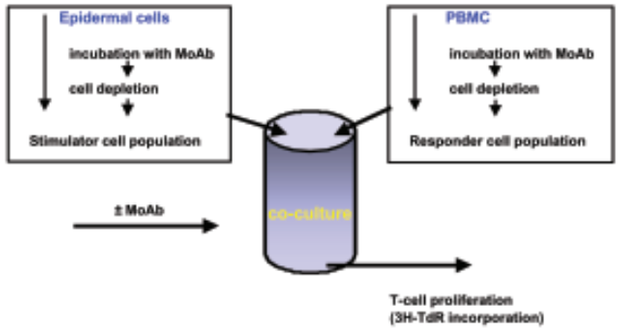

Fig. 4. Schematic description of an ELR. Both stimulator and responder populations can be modified by depletion of cell subpopulations using monoclonal antibodies and macnetic beads or complement. Furthermore, the use of blocking antibodies during culture yields information on function of different cell surface receptors. The readout of the ELR is T-cell activation monitored by ^{3}H-TdR incorporation in the T-cells.

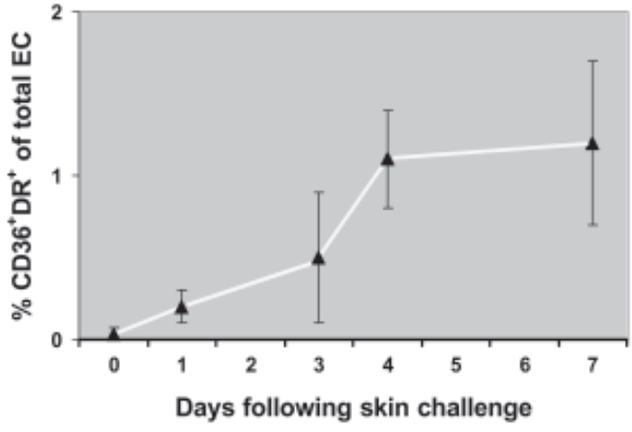

Fig. 5. Epidermal cells were stained with anti-CD36 and anti-HLA-DR. Percentage of double positive cells of total cells is depicted from vehichle areas (C) and ACD reaction sites, at indicated time points. The CD36$^+$ cells co-expressed CD45 (anti-HLe-1) demonstrating a bone marrow origin. Modified from paper II.

Intrigued by the above-mentioned observations, I set out to describe the presence of potentially non-LC APC within the epidermis of allergic contact dermatitis reactions. The study (paper II) was based on immunphenotypi of epidermal single cell populations and functional *in vitro* assays. The latter was achieved by application of ELR. The ELR is an efficient tool to explore the functional capacity of cell populations *in vitro*. The assay system is based on co-culture of epidermal cells and T-lymphocytes (Fig. 4). The co-culture of epidermal cells and allogeneic T lymphocytes yield information on functional antigen-presenting capacity, since this reaction is not affected by the presence of nominal antigen. In con-trast, co-culture of epider-mal cells and autologous T lymphocytes yield information on the capacity of APC to present nominal antigen in a MHC-restricted manner. Furthermore, the assay open up for depletion of cell subpopulations and thus can contribute to the description of individual cell populations. In paper II, patients with patch test-verified contact allergic reactions were recruited. These patients were tested with the relevant allergen and a vehicle control. The latter was based on the observation, that simple occlusion alters the number of phenotypically identifiable LC in skin (**paper II**).

When analysing suction blister-derived epidermal cells from such ACD reactions, a novel dendritic cell type characterised by cell surface expression of CD36 and HLA-DR was observed. Using the suction blister technique, epidermal sheets at approximately 2.5 cm^2 were obtained. Following the induction of single cell suspensions, between 10^6 and $2*10^6$ epidermal cells were obtained per sample and this technique was therefore superior to skin punch biopsies for the evaluation of changes in cell populations occurring in low numbers. Furthermore, the suction blister technique, which is based on the application of negative pressure on skin, results in the induction of pure epidermal sheets. The dermo-epidermal cleavage obtained by this technique has been demonstrated to be localised within the lamina lucida of the basement membrane[114] thus avoiding any dermal contamination. The CD36$^+$ cells were uniformly present within the epidermal compartments of challenged skin areas but not in vehicle control areas. A time-maximum of induction was observed between day 4 and day 7 following skin challenge using different irritants (Fig. 5). In addition to CD36$^+$HLA-DR$^+$ dendritic epidermal cells, an increase in CD1a$^+$ epidermal LC was observed. Interestingly, no co-expression of CD36 and CD1a was observed, as is also the case in UV-irradiation mediated inflammation[65]. The CD36$^+$ leukocytes expressed Hle1 but only a minor fraction expressed the monocytic marker CD11b (identified by the monoclonal antibody OKM1). Thus, a heterogeneous bone marrow-derived CD36$^+$ cell population was identified; the majority of these cells did not belong to the major CD11b$^+$ blood monocyte population.

To explore the functional capacity of the CD36$^+$ leukocytes, *in vitro* ELR were performed. One advantage of this method is the possibility to explore the capacity of APC to activate both autologous and allogeneic T-cells (see above). First, unfractionated, suction blister-derived epidermal cells were used as stimulator cells in an ELR. Increased allogeneic T-cell activation was observed when using cells obtained from positive patch test reaction sites as compared to cells obtained from control areas. This observation parallels findings by Grønhøj Larsen et al.[115]. Using the same set-up, I next investigated potential autoreactivity and performed cell depletion studies to evaluate the functional capacity of the different APC subpopulations present in the involved skin. In paper II, I observed an increased capacity of involved epidermal cells to stimulate autologous T cells as compared to unchallenged control epidermal cells.

When analysing the functional capacity of the different APC obtained from epidermal sheets overlying positive patch test skin reactions, removing $CD1a^+$ ($Leu6^+$) epidermal LC resulted in a partial inhibition of the ELR. Thus, the remaining $CD1^-$ cells were capable of activating allogeneic- as well as autologous-T lymphocytes. In contrast, removing $CD1a^+$ epidermal cells from untreated control areas completely blocked all APC activity in skin.

Upon removal of all $HLA\text{-}DR^+$ cells, i.e. $CD1a^+$ LC and $CD36^+$ non-LC APC from the cell suspensions, all APC activity within both allergen-challenged and normal control skin was abolished. Thus, a novel $CD36^+$ APC, located within the epidermis from areas with ACD, was described and it was shown that a substantial fraction of the capacity of involved epidermal cells to stimulate T lymphocytes was mediated by these $CD36^+$ non-LC APC.

In contrast to paper 1, no $CD36^+$ or $HLA\text{-}DR^+$ keratinocytes were observed in this study. Subsequent double stainings and analyses by immunhistochemistry revealed that the $CD36^+$ and $HLA\text{-}DR^+$ cells co-expressed the surface marker HLe1, identifying a bone-marrow origin. Both CD36 and HLA-DR are probably upregulated on keratinocytes by IFN-γ and other lymphokines, which are released from locally activated T-cells. In **(**Paper II**)** only weak ACD reactions (+ /++) were elicited and consequently only minimal T-cell infiltration and lymphokine release occurred. Furthermore, CD36 is susceptible to proteolytic degradation[58] and was probably affected during cell preparation with the proteolytic enzyme trypsin. Thus, minor keratinocyte CD36 expression was probably non-detectable due to technical procedures.

In the same study, an increase in total CD1a positive dendritic cells at day 3, 4, and 7 following skin challenge was observed. This indicates that although a fraction of epidermal LC migrates out of the skin following skin challenge, other cells are recruited simultaneously. This, together with the recruitment of non-LC APC to the involved skin areas indicates the presence of chemoattractants facilitating the migration of APC from the blood/ or dermal compartment to the epidermis. Later studies have indeed demonstrated the presence chemoattractants in the ACD reaction, such as IL-6 and IL-8[116-118].

In conclusion of paper II, a minor increase in epidermal LC and a concordant recruitment of $CD36^+$ leukocytes to the epidermis was observed. These non-LC APC exhibited a substantial APC capacity and contributed to an overall increase in stimulation of both allogeneic and autologous T-lymphocytes when involved epidermal cells were used as stimulators in our *in vitro* assay. I had thus identified and characterised a novel APC in human skin following *in vivo* application of contact allergens.

8. STUDIES ON NICKEL AND T CELL ACTIVATION (PAPERS III AND IV)

The pathogenesis of allergic contact dermatitis is thought to relay on the identification of specific antigen/hapten by APC, which subsequently present modified peptide fragments to T-lymphocytes expressing specific T-cell receptors. However, studies also indicate that all potent allergens exhibit some irritative effects (described in chapter 4). The combinatory effect seems necessary and the end result manifests in an activation of the immunesystem where antigen presentation leads to specific immuneresponses. As previously described, both intrinsic and extrinsic pathways can be involved in presenting antigen in context with MHC molecules on the cell surface on the APC. However, when using nickel as an antigen (hapten), the major hypothesis today is that nickel binds to a certain group of peptides present in the MHC groove, thereby modifying these peptides and make them antigenic[119]. Nickel-sulphate or -cloride often dissociate and nickel thus present as a divalent cation (Ni^{++}). Cations are capable of forming complexes with electron-rich groups in proteins and in particular ionised cysteine and histidine binds Ni^{++}[120-122]. In the following, I focus on nickel as a model of a very common allergen, which when exposed to non-sensitised individuals or animals also can act as a skin irritant.

In literature, many studies have focused on the capacity of nickel to stimulate T-lymphocytes, the key player in ACD. The lymphocyte transformation test also called the lymphocyte proliferation test (LPT) was in many years used as an *in vitro* assay to determine the allergy against nickel. However, this assay has never been standardised. Therefore, a large number of combinations, including different culture periods and different choices of serum added to the *in vitro* cell cultures, make direct comparison of the studies almost impossible. This is particularly important in studies using foetal calf serum (FCS), which by itself contains many mitogens. Therefore, the results of the LPT analyses varied and even in the control groups, i.e. persons without clinical ACD and with a negative patch test to nickel sulphate, variable T lymphocyte proliferation was observed. These findings were, however, only interpreted as "noise"[123–126].

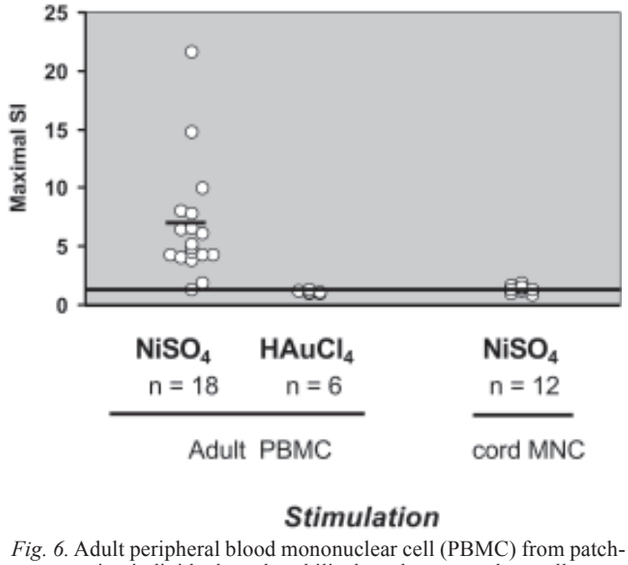

Fig. 6. Adult peripheral blood mononuclear cell (PBMC) from patch-test negative individuals and umbilical cord mononuclear cells were stimulated using graded concentrations of $NiSO_4$ and controls. The fig. show maximal stimulation index (SI), calculated as CPM stimulated cells/CPM unstimulated cells. Modified from paper III.

It is note-worthy, that low exposure to skin of immunogenic modalities has been demonstrated to exhibit profound effect on T lymphocytes, even in the absence of clinical manifestation. In support of this, our group has previously shown that UV-B, even when applied at ¼ minimal erythema dose to intact skin, induced cellular epidermal changes and increased T-lymphocyte autoreactivity[65]. In addition, a study by McLelland and co-workers has demonstrated that simultaneous topical application of two separate allergens, which by themselves do not suffice to evoke a clinical reaction, elicits clinical eczema in sensitised individuals[127]. Thus, even in the absence of clinically manifest reactions, antigen challenge can induce immunological alterations and the observed "noise" in the nickel-induced LPT could reflect an antigen-specific reaction due to low level of sensibilisation towards nickel.

Therefore, further elaboration on the observed nickel-inducible T-lymphocyte proliferation was conducted by analysing the clinically non-allergic individuals, i.e. those without eczema and with a negative patch-test to nickel sulphate. Using an *in vitro* system, in which autologous serum was added to the medium, a nickel-inducible proliferation of peripheral blood mononuclear cells (PBMC) was demonstrated in 16 of 18 individuals that subsequently demonstrated a negative skin-reaction to patch-testing using 5% $NiSO_4$ (Fig. 6, **Paper III**). In this assay set-up, autologous serum was chosen as growth medium to exclude the potentially stimulatory effect of FCS-mediated exogenous mitogens. The specificity of the finding was demonstrated by three different approaches. First, depletion of MHC class II positive APC from the *in vitro* cell cultures abrogated the nickel-induced T-lymphocyte response. Second, human cord blood cells, which are known to respond to mitogen – but not to antigen stimulation, did not respond in our *in vitro* assay system (Fig. 6, marked "cord MNC") and finally, when using mitogen-driven T-cell clones as responder cells, no proliferation in response to nickel was observed (**Paper III**). Thus, the concentrations of nickel used in these studies seemed not to induce antigen-unspecific/mitogenic activation of T lymphocytes.

To characterise the nickel-inducible T-lymphocytes at a functional level, cytokine measurements were performed following *in vitro* stimulation with nickel. Data were compared to findings from nickel-sensitised individuals. Interestingly, it was observed that PBMC obtained from both nickel-allergic and non-allergic individuals produced IFN-γ but no measurable IL-4 following stimulation (**Paper III**). These cytokine measurements were performed on crude PBMC populations, thus whether the responding cells belonged to T-helper type 1 (Th1) or T-cytotoxic type 1 (Tc1) T lymphocytes could not be distinguished.

To further characterise the nickel-responsive T-lymphocytes in the patch-test negative individuals, the technique of fluorescent-activated cell sorting (FACS) was used to separate the T-lymphocytes into $CD4^+$ and $CD8^+$ T-cell populations. Follow-

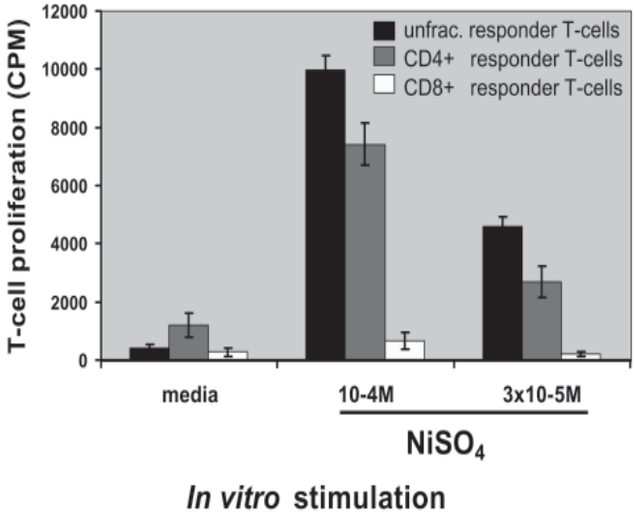

Fig. 7. $CD4^+$, $CD8^+$ or unfractionated T-cells were incubated with $NiSO_4$ and γ-irradiated PBMC for 7 days. Resulting T-cell proliferation was measured using ^{3}H-Thymidin incorporation during the last 18 h of culture. Modified from paper IV.

ing separation, the dif-ferent T-cell subsets were stimulated with gamma-irradia-ted PBMC and specific antigen (in our case; nickelsulphate). Using this set-up, the nickel-responsive T-lymphocytes were mainly confined to the $CD4^+$ subset (**Paper IV**). However some, albeit lesser proliferation in the $CD8^+$ T lymphocyte subset was also present (Fig. 7, open bars). The nickel-induced T-lymphocyte activation observed in our *in vitro* assay system resembles classical antigen presentation in that blocking MHC class II molecules abrogated the reaction (Fig. 8). In contrast, the usage of blocking antibodies towards MHC class I molecules did not result in significant lower T-cell activation than control cultures. The latter add to the observation, that in this assay system, predominantly $CD4^+$ T-cells were activated following *in vitro* stimulation with nickel.

It is generally accepted, that elicitation of ACD is the result of antigen specific activation of memory T-lymphocytes. This memory T-lymphocyte subset is characterised by surface expression of CD45RO, and no or only minor expression of CD45RA. To describe the nickel responsive $CD4^+$ T-cell subsets, $CD3^+CD4^+$ T-lymphocytes were separated into memory and naïve subsets by FACS sorting followed by *in vitro* stimulation using nickel as stimulant. Interestingly, both $CD45RO^+$ and $CD45RA^+$ $CD4^+$ T-lymphocytes proliferated in response to nickel *in vitro* (**Paper IV**). This finding is in contrast to findings using the classical recall-antigen tetanus toxoid, which stimulates memory cells only.

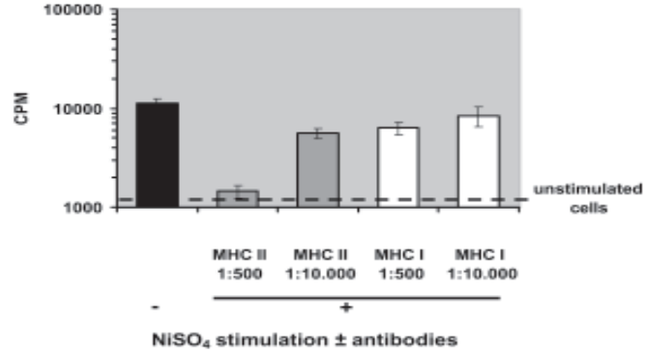

Fig. 8. Unfractionated PBMC were stimulated with NiSO4 for 7 days in the absence or presence of antibodies towards MHC class I or II. Resulting T-cell proliferation was measured using ^{3}H-Thymidin incorporation during the last 18 h of culture. Modified from paper IV.

Following our first publication of this controversial finding in 1995 as an abstract[128], the results were substantiated by Cavani et al. in 1998[129]. This study demonstrated an activation of nickel specific $CD4^+$ T cells in both allergic and non-allergic individuals. The frequency of nickel-specific T cells in the peripheral blood was determined using limiting dilution and a comparable frequency of nickel specific $CD4^+$ T lymphocytes was found in both groups. Of particular interest was the finding of nickel specific activation of $CD8^+$ T cell subset only in the nickel allergic individuals. The frequency of such nickel-specific $CD8^+$ T cells were detected in range of 1: 2600 to 1:31000[129]. These findings correlate well with own observations showing that predominantly $CD4^+$ T cells are activated upon *in vitro* nickel-stimulation in individuals without cutaneous allergy towards nickel.

The frequencies of nickel-specific T lymphocytes in peripheral blood is comparable to findings by Werfel et al., who investigated the frequency of T lymphocytes specific to the allergen dermatophagoides pteronyssinus in atopic dermatitis patients with known allergy towards dermatophagoides pteronyssinus [130] and Kalish et al. who demonstrated urushiol specific peripheral blood T cells in allergic individuals[131]. In conclusion, these studies support the concept that nickel activation of T lymphocytes represents a specific process and not a polyclonal, unspecific or mitogenic activation of the T lymphocytes. The specific mechanism of nickel-inducible T lymphocyte activation, however, is still under investigation. Furthermore, the functional role of nickel-specific $CD4^+$ T cells in non-allergic individuals awaits further clarification. Parenthetically, the presence of antigen specific T lymphocytes in patients without clinical disease is not restricted to nickel, since antigen specific T cell clones exhibiting specificity towards the major birch pollen allergen (Bet1) and dermatophagoides pteronyssinus have been identified in non-allergic individuals[132,133].

Although MHC class II restriction has been observed following *in vitro* nickel stimulation of T lymphocytes, no specific MHC restriction has been demonstrated in relation to the *in vivo* development of allergy towards nickel[134]. Despite this, an increased risk of developing ACD to nickel has been shown in 1st degree relatives of patients with nickel ACD[135] emphasising that genetic elements most possible are important in the development of nickel ACD. Furthermore, several studies have investigated the involvement of potentially specific or restricted TCR repertoire in T-lymphocyte responses to nickel. Some reports of skewed TCR Vb repertoire have been published[136-138], but data from different studies are not yielding comparable results and the TCR repertoire used in nickel-reactive T-lymphocytes seem restricted at the individual level. Due to observations that selective TCR Vβ are upregulated following stimulation with nickel, a superantigen-like effect could be suggested. However, a study by Vollmer et al. has excluded such superantigen-like recognition of nickel *in vitro*[139].

At this point, it is imperative to stress, that the precise cellular and molecular/ genetic mechanisms underlying nickel-induced ACD still awaits clarification and even the involvement of different T-cell subsets in the initiation and development of the ACD reaction is also controversial.

In many years, the prevailing opinion was that delayed type

hypersensitivity (DTH) reaction or contact hypersensitivity responses were mediated by antigen presentation to $CD4^+$ T-lymphocytes. Often no distinction was made whether the reaction was elicited by epicutaneous application of allergens, as in the contact hypersensitivity reaction, or by intradermal injection of the antigen as in the case of the DTH reaction. In the murine system, DTH has been demonstrated to be mediated by $CD4^+$ T lymphocytes. This is supported by observations by Gautam et al. who showed that contact hypersensitivity in mice could be transferred to naïve mice by intradermal injection of $L3T4^+$ ($CD4^+$) T-cells from mice painted with TNCB[140]. Furthermore, *in vivo* injection of monoclonal antibodies towards L3T4 has been demonstrated to diminish DTH reactions in mice[141]. In human systems, the involvement of $CD4^+$ T cells in ACD reaction has come from studies demonstrating the presence of nickel-specific $CD4^+$ T-cell clones in nickel-allergic individuals[142-145].

However, despite the above-mentioned findings, the growing evidence supports the concept that the major effector T-lymphocyte subset in contact hypersensitivity (the murine equivalent of allergic contact dermatitis in man) is the $CD8^+$ cytotoxic T-lymphocyte whereas the $CD4^+$ T-lymphocytes mediate regulatory functions. This hypothesis is supported by an observation using *in vivo* cell depletion, where it was shown that the murine DTH reaction was mediated by $CD4^+$ T-cells, whereas the murine contact hypersensitivity reaction was critically dependent upon $CD8^+$ T-cells although $CD4^+$ T cells also exhibited some effector functions[146]. In concert with the above mentioned study, it has been shown that MHC class I-deficient mice were unable to mount a contact hypersensitivity response to dinitroflourobenzene (DNFB) whereas in MHC class II-deficient mice, an exaggerated response were observed[147]. Furthermore, in MHC class II-deficient mice, the induction of contact hypersensitivity reactions could be inhibited by *in vivo* injection of anti-CD8 antibodies[148]. In the human system, nickel specific $CD8^+$ T-cell clones have been generated from nickel allergic persons[129,149,150]. In addition, contact hypersensitivity to urushiol has been demonstrated to be mediated by $CD8^+$ T-cells in mice[151] and urushiol specific T-cell clones has been established in the human system[131,152,153]. Thus, today $CD8^+$ T-lymphocytes are regarded as crucial mediators of the ACD reaction.

Taken all data into account, it seems likely that most haptens evoke specific T-lymphocyte responses and both $CD4^+$ and $CD8^+$ effector T-lymphocytes seems to contribute for full development of the contact allergic reaction, and that different allergens could require a different set of immunoreactive T-cells for optimal response to occur. In support of this hypothesis studies have shown that e.g. urushiol can generate activation of both $CD4^+$ and $CD8^+$ T-cell clones, depending upon the processing pathway used[93], and further supported by Wang et al who demonstrated the requirement of both $CD4^+$ Th1 and $CD8^+$ Tc1 T-lymphocytes in the full development of contact hypersensitivity reaction towards DNFB and oxazolone[154].

9. ANTIGEN PRESENTING CELLS AND T CELL ACTIVATION IN THE IRRITANT CONTACT DERMATITIS REACTION (PAPER V)

When the importance of non-LC APC in the ACD reaction (Paper II) was established, the involvement of similar cell populations were investigated in the irritant skin reaction. *In vivo* ICD reactions were therefore elicited on individuals with no history of allergic skin reactions or other skin diseases **(**Paper V**).** Irritants were applied to human volar forearm skin and time-response analyses were performed. Skin samples were obtained at day 1,3,4 and 7 following application of the skin irritants. This window was chosen due to findings both in ACD (Paper II) and following *in vivo* ultraviolet irradiation of human skin[65]. Again, a suction blister technique was used to separate the epidermis from the surrounding tissue, followed by trypsinisation of the epidermal sheets to obtain epidermal single cell populations. Immunophenotypic analyses were performed to describe changes in the epidermal LC population as well as to detect any induction of $CD36^+$ leukocytes following skin application of irritants and *in vitro* ELRs were performed (see fig. 4) to identify the functional capacity of the epidermal cell populations.

Using this setup, a time-dependent decrease in the number of $CD1^+HLA\text{-}DR^+$ epidermal LC was observed as determined by fluorescent microscopy (Fig. 9, **Paper V**). Furthermore, only very few $CD36^+$ epidermal leukocytes were detected, the majority of which co-expressed CD11b and thus belonged to the major $CD11b^+CD36^+$ blood monocyte population. The observation of a decrease in number of epidermal LC following irritant skin challenge was in concert with findings by Ferguson et al.[155], who analysed the number of LC in skin punch biopsies. The latter technique, though, is not a suitable technique to detect and quantify numbers of cells occurring in such low numbers as the epidermal LC. Whether the observation of a decrease in the numbers of epidermal LC following irritant skin challenge reflected a loss of surface markers on epidermal LC or migration of LC out of the epidermis could not be detected by the analyses used in the study. However, the concept of LC migration in ICD was subsequently supported by several studies. Electron microscopic analyses of epidermal and dermal cell populations underlying the irritant skin reaction have indicated migration of LC from the epidermis to the dermal compartment[156]. Furthermore, Brand et al. has described the occurrence of LC in the cutaneous sentinel lymph nodes following skin challenge with irritants[157]. Thus, evidence is now available that, as reported in the ACD reaction, the epidermal LC migrates out of the epidermis during the elicitation of an irritant skin reaction.

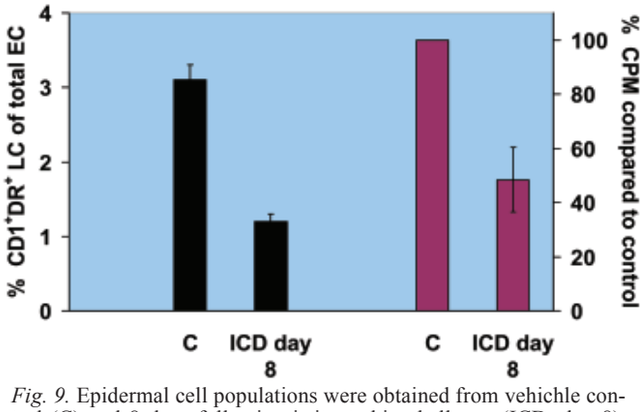

Fig. 9. Epidermal cell populations were obtained from vehichle control (C) and 8 days following irritant skin challenge (ICD, day 8). Immuno-phenotypic staining was performed using anti-CD1 and anti-HLA-DR, depicted in the black bars. In addition, allogeneic-ELRs were performed. Resulting T-cell proliferation was measured by ^{3}H-Thymidine incorporation during the last 18 h of culture, grey bars. Modified from paper V.

When the results of the immunophenotypings from irritant skin reactions were available, the next question was whether the observed decrease in number of identifiable epidermal LC was paralleled by a decrease in the functional capacity of the epidermal cells. Thus, epidermal cells were obtained at different time points following *in vivo* irritant skin patch testing and used as stimulators of allogeneic T lymphocytes. This assay would reflect the antigen presenting capacity of the residual epidermal cells. In parallel with the immunophenotypic findings, a time-dependant decrease in epidermal cell-induced T-cell activation was observed (Fig. 9). This decrease was paralleled by a phenotypic depletion of all $HLA\text{-}DR^+$ epidermal leukocytes (**paper V**). Thus, in contrast to the findings in the ACD reactions (**paper II**), a decrease both in number and function of epidermal LC was observed following irritant skin challenge. In two volunteers, the time-response study was prolonged to four weeks, and a decreased number of epidermal LC was still found at day 14, returning to normal values at four weeks (**Paper V**). In concert with this finding, a study by Hindsén et al. has demonstrated a decrease in sensibilisation if the skin had been exposed to an irritant (sodium lauryl sulphate, SLS) before the application of the sensitiser[158]. Despite the above-mentioned findings, irritant skin challenge does not always diminish subsequent elicitation of allergic skin reactions. This has been demonstrated by Seidenari et al. who demonstrated that skin application of SLS 24 hours prior to skin application of nickel sulphate, results in increased response to $NiSO_4$ and by findings by McLelland et al. who demonstrated that simultaneous application of a skin irritant an a skin allergen resulted in a response greater that to either alone[159,160]. Thus, the time between applications of different chemicals to the skin seems critical and possible determine the overall outcome of the exposure.

In conclusion, although LC migration occurs in ICD, the importance of this phenomenon is currently debatable. Furthermore, the low number of epidermal LC and lack in recruitment of significant numbers of other non-LC APC strongly indicates that other signalling mechanisms are the major determinants in the development and maintenance of the ICD reaction in skin.

10. PRO-INFLAMMATORY CYTOKINES IN CONTACT DERMATITIS REACTIONS

Taken in account the observations of a decreased number and functional capacity of epidermal LC during an irritant skin reaction, the LC seems not to be the essential for the development and maintenance of skin inflammation observed in ICD. Furthermore, studies by Piguet et al and Enk et al. have clearly demonstrated that blocking pro-inflammatory cytokines *in vivo* results in a decreased or even abrogated clinical reaction to allergens as well as skin irritants[5,6]. Thus, the importance of locally synthesised inflammatory mediators is well established. Much focus has been directed to the two pro-inflammatory cytokines, IL-1β and TNFα. In the ACD reaction, Enk et al. demonstrated that IL1β was the first cytokine to be found upregulated[161], and that injection of IL-1β *in vivo* resulted in clinical reactions resembling ACD reactions[6]. It has been suggested that the induction of IL-1β could differ the allergic versus irritant skin reaction, but IL-1β is not only found upregulated in ACD. A study by Hunziker et al. has also demonstrated increased levels of IL-1β protein in the sentinel lymph nodes underlying irritant skin reactions[162]. In both the elicitation phase of ACD and in the ICD reaction, Piguet et al. has demonstrated the importance of TNFα. In accordance with others they found an upregulation of TNFα in the irritant reaction. In this study, Piguet and co-workers used the strong skin irritant – TNCB, which following skin painting resulted in the development of a clinical irritant skin reaction as well as allergic skin reaction in previously sensitised animals. However, if they injected neutralizing antibodies to TNF before skin challenge, no skin reactions would subsequently develop[5]. Thus, anti-TNF antibodies were capable of inhibiting both elicitation of an ACD reaction as well as inhibiting an ICD reaction in mice. Furthermore, as discussed in chapter 4.1, both IL-1β and TNFα contribute to the LC migration observed in both ACD and ICD reactions. Besides inducing LC migration, these pro-inflammatory cytokines have been described to induce several other cytokines, including IL-1α, IL-6, and IL-8[163], which contribute to the pool of upregulated cytokines found both in ACD and ICD skin reactions. One important function of cytokines is to induce cell adhesion molecules as well as chemotactic polypeptides by different cell populations in the skin. These factors are critically involved in the induction and the maintenance of lymphocyte infiltrates in skin. As shown in table 1, the epidermal keratinocyte is capable of synthesising most of the identified cytokines thereby implicating the keratinocyte as a crucial player in the development of inflammatory skin reactions.

In conclusion, following epidermal challenge (both allergic and irritant), a cascade of molecular events including cytokine release from local LC and keratinocytes occur. Special emphasis has been attributed to IL-1β and TNFα, both of which are critically involved in the early phases of contact dermatitis. This common primary signal could represent a kind of "danger" signal. The upregulation of cytokines subsequently results among other things in the initiation of epidermal cell and endothelial cell adhesion molecule expression. The latter includes upregulation of CD62E and CD106, thereby facilitating the efflux of blood-derived cells into the dermal compartment. Furthermore, the synthesis of keratinocyte-derived chemotactic factors further attracts cells into the epidermal compartment. The keratinocyte CD54 expression is then involved in maintenance of the infiltrating cells in the epidermis.

Thus, the induction of the pro-inflammatory cytokines, especially IL-1β and TNFα seems to be a common primary event in both elicitation of an ACD as well as in the ICD reaction**.**

11. MECHANISMS OF IRRITANT INDUCTION OF TNFα (PAPER VI)

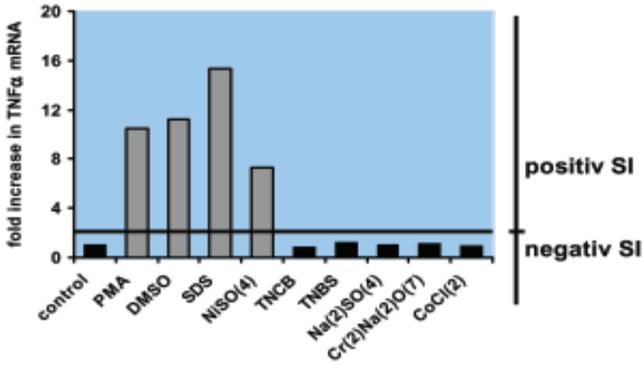

Fig. 10. Murine keratinocytes were stimulated *in vitro* with different irritants. Resulting TNFα mRNA induction, is depicted as fold increase compared to unstimulated cultures. All values are normalised to the housekeeping gene G_3PDH. Modified from paper VI.

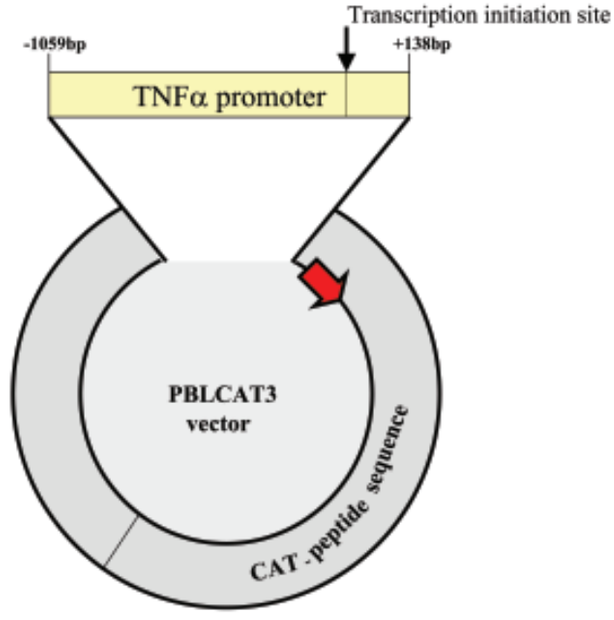

Fig. 11. The full length TNFα promoter, containing the – 1059bp to +138bp region was cloned into a PBL3 vector containing the gene coding for CAT[164], an enzyme not found in mamalian cells. Following transfection into keratinocytes, the cells were stimulated. Any stimulation, that increases transcription of the TNFα gene, results in an increase in TNFα promoter activity and in parallel an increase in activity of the CAT-gene. Thus, increased CAT-activity reflects an increased transcription of the TNFα promoter.

The capacity of skin irritants to induce keratinocyte TNFα has previously been described but other studies have not could confirm this observation[117]. However, since skin irritants potentially interact with cells by different signalling pathways, I set out to demonstrate the capacity of different irritants to induce the synthesis of TNFα in different keratinocyte lines as well as in freshly isolated murine keratinocytes. Furthermore, if irritants directly could upregulate TNFα in keratinocytes, the intracellular signal mechanisms underlying this irritant-induced upregulation of TNFα would be addressed **(Paper VI).**

In order to obtain this objective, the induction of TNFα messenger RNA (mRNA) was evaluated following *in vitro* irritant challenge. First, the strong irritant phorbol 12-myristate 13-acetate (PMA) was applied and upregulation of TNFα mRNA in freshly isolated murine keratinocytes was induced. Following this, the analyses were expanded to include several keratinocyte cell lines, including the C_3H-derived HD_{II} and HEL-30, C57BL/6-derived BDVIIa and BALB/c-derived PAM212 cell line. Next, several irritants were included in the study. It was observed that in addition to PMA, previously known to upregulate TNFα in keratinocytes, the irritants SDS, dimethylsulfoxide (DMSO) and the weak irritant nickel sulphate, applied in cell cultures obtained from non-sensitised animals, directly could upregulate TNFα mRNA in keratinocytes (Fig. 10). In contrast, TNCB was incapable of inducing TNFα mRNA in pure keratinocyte cell lines, a finding that questions the mechanism underlying the *in vivo* inducible increase in keratinocyte TNFa expression following skin application of TNCB. Thus many, but not all, skin irritants used could upregulate TNFa in keratinocytes. Next it was determined, whether a common signalling pathway, used by different irritants to upregulate TNFα expression in keratinocytes, could be detected.

Following application of non-toxic concentrations of skin irritants to cells, the irritant binds to the cell surface and a signal transduction cascade is initiated. The activation of keratinocytes by irritants may involve a number of signal transduction pathways, including several phospholipases, protein kinase (PK) C, PKA or cyclic nucleotides. Thus, the involvement of different kinases in the irritant-mediated upregulation of TNFa was investigated. In order to obtain this objective, different blocking agents were applied to the *in vitro* assay system. Using staurosporine and H7, a selective inhibitor of PKC, the irritant-mediated induction of TNF-α mRNA was inhibited in a dose-dependent fashion. However, in addition to inhibiting PKC, H7 is also an inhibitor of cAMP/cGMP dependent protein kinases. Therefore, the inhibitor HA1004 was included in the study. HA1004 is another isoquinoline-sulfonamide derivative that has a similar affinity for cyclic nucleotide-dependent protein kinases, but reduced affinity for PKC. Using this set-up it was observed that some agents, including SLS, DMSO and PMA all used PKC-dependent signalling pathways. In contrast, when stimulating the keratinocytes with $NiSO_4$, neither blocking PKC nor cGMP/cAMP-dependent kinases could inhibit the induction of TNFα mRNA in the stimulated keratinocytes. Thus, the different irritants used different intracellular signalling mechanisms to upregulate TNFα.

To determine whether the observed increase in TNFα mRNA in keratinocytes following irritant stimulation was the result of increased transcription of the TNFα gene, a chloramphenicol acetyl transferase (CAT) assay was applied. This assay is based on the introduction of CAT, an enzyme not present in eukaryote cells, into cells. We used a PBL3 vector with the full length TNFa promoter region inserted in front of the CAT gene[164] (Fig. 11). Following transient transfection of the keratinocytes, the cells were stimulated with different irritants. If a transcriptional

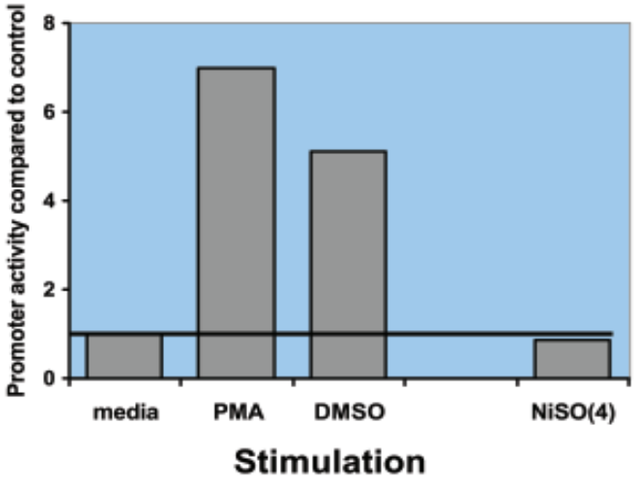

Fig. 12. Stimulation with irritants such as phorbol 12-myristate 13 acetate (PMA) and dimetylsulphoxide (DMSO) result in increased TNFα promoter activity compared to control cultures. In contrast, stimulation with $NiSO_4$ does not result in increased promoter activity. Modified from paper VI.

activation of the TNFα gene was induced in these cells, the result would be an increase in transcription of the CAT enzyme. Following stimulation of the cells, the cells were lysed and intracellular CAT-activity then determined in a separate *in vitro* assay based on the measurement of acety-lation of radioactive-labelled chloramphenicol.

Using this setup, it was found that different irritants used different intracellular signalling pathways. Skin irritants like PMA and DMSO upregulated TNF gene expression via increase in gene transcription (Fig. 12). In contrast to findings using PMA and DMSO as stimulants, nickel-stimulation did not result in an increased CAT-activity although total mRNA was increased revealed by Northern blot.

The technique applied in this study to investigate gene activation is a reproducible and stable technique. However, the actual readout is activation of a promoter region and not transcription of an entire gene. One can argue that in addition to the 5'promoter region, some regulatory sites can be present in the 3'end of the gene. This possibility cannot be ruled out by this assay. Another technique, that more directly indicates gene transcription, is the nuclear run-on assay. This assay is based on isolating the nuclei of activated cells and subsequently finishing the transcriptional process in the presence of radioactive nucleotides. This method, however, requires huge amounts of radioactive materials (in the amounts of mCi). Therefore, the CAT-assay was chosen as reporter assay to detect changes in gene transcription.

Thus taken the above-mentioned arguments in account, paper VI demonstrates that several skin-irritants directly induce gene transcription in keratinocytes. However, the nickel-induced regulation seemed mediated at a posttranscriptional level.

In order to investigate this phenomenon, TNFα mRNA stability analyses were performed. Again the keratinocytes were stimulated with different irritant agents. Following stimulation, actinomycin-D was added to cell cultures, thereby blocking further mRNA synthesis. Using this approach, cell samples were subsequently obtained at different time-points and total mRNA extracted. The remaining amounts of specific mRNA (in this case TNFα mRNA) can be used to calculate the $T_{½}$ of the transcribed material. An increase in $T_{½}$ equals an increase in the stability of the mRNA. To our surprise, a pronounced increase in TNFα mRNA stability was induced by stimulation using $NiSO_4$, but not the other irritants tested.

Thus, it was demonstrated that irritants are capable of upregulating the important pro-inflammatory cytokine TNFα in keratinocytes in the absence of other cell populations. In addition, it was demonstrated that different signalling pathways were used in the observed increase in TNF in response to skin irritants involving both transcriptional and post-transcriptional regulatory mechanisms. However, the observation that TNCB was incapable of inducing TNFα in keratinocyte cultures indicated that other, potential indirect, mechanisms were involved in the observations by Piguet et colleagues, who observed increased TNFα mRNA in the epidermis following irritant skin painting.

12. PRO-INFLAMMATORY CYTOKINES INDUCE TNFα IN KERATINOCYTES (PAPER VII)

The capacity of keratinocytes to synthesise and release TNFα in response to irritant stimulation is well established (se above). However, some divergence has been described between *in vivo* and *in vitro* experiments. One example is the TNCB-mediated upregulation of TNFα in keratinocytes as reported using *in vivo* skin application of the agent on non-sensitised animals[5]. In later *in vitro* studies, however, TNCB failed to up regulate TNFα mRNA in murine keratinocytes[6,(Paper VI)]. Thus, the TNCB-mediated regulation of TNFα appeared to be indirect and possibly secondary to the induction of other cytokines. One interesting candidate, possibly involved in this induction of TNFα, is IL-1β. IL-1β has been involved in regulation of TNFα in human astroglioma cells and ovarian cancer cell lines and reported to be upregulated in skin from both ACD and ICD reactions (see chapter 10). In the elisititation phase of allergic contact dermatitis, LC have been identified as the earliest cellular source of IL-1β and IL-1β mRNA has been demonstrated as early as 15 min. following stimulation[161]. Following the induction of IL-1β, this cytokine could subsequently induce epidermal TNFα expression[6].

In order to demonstrate that IL-1β was capable to induce TNFα in keratinocytes, stimulations using varying doses of IL-1β were performed on keratinocyte cell lines (**Paper VII**). Following stimulation, total RNA was extracted and specific mRNA for TNFα was measured by Northern blot. In addition, intracellular TNF protein was measured using a bioassay. Using this set-up, it was shown that recombinant (r) murine IL-1β was capable of increasing the expression of TNF-α mRNA and biologically active protein in murine keratinocyte cell lines. This upregulation was dose-dependant and the specificity of the reaction was verified by stimulating the keratinocytes with IL-1β in the presence of a surplus of IL-1 receptor antagonist that binds to the IL-1 receptor without signal transduction is initiated. Furthermore, soluble IL-1 receptor, that binds to IL-1 and thus inhibits IL-1-mediated signalling, was also included. Taken the above-mentioned controls in account, it was found that the IL-1-mediated induction of TNFα was specific and mediated via binding to the IL-1 receptor. However, due to the set-up used in paper VII, it could not fully be excluded that the induction of TNFα mRNA was secondary and mediated via other IL-1β-inducible cytokines. This is, though, less likely considering that increased TNFα mRNA was observed already 15 min. following stimulation. An observation of IL-1β inducible TNFα expression in murine epidermal cells[165], parralels my finding, however this study yielded no information on the number of contaminating I-A positive epidermal LC and a high concentration (up to 1000 U/ml) of IL-1β was used. In paper VII, the use of murine keratinocyte cell lines excludes the possibility of potentially contaminating LC or dermal mast cells in the system and thereby the keratinocyte was identified as an unequivocal source of TNF.

Since no information regarding signalling mechanisms involved in the IL-1β-mediated upregulation in keratinocytes was present, this phenomenon was further investigated. Keratinocyte cell lines were therefore stimulated with IL-1b in the presence of different protein kinase inhibitors to evaluate signalling pathways. Using this setup, it was shown that PKC-mediated signalling was required (se Fig. 13). Furthermore, the keratinocytes were transfected with the full length –1059 to +138 bp TNFα-CAT construct before stimulation using rIL-1β. Following *in vitro* stimulation, total RNA was extracted. parallel to these studies, intracellular protein content was measured using a TNF bioassay. It was found, that IL-1β stimulation of keratinocytes resulted in increased transcription of the TNFα gene and the upregulation was paralleled by an increase in intracellular TNF protein. These observations could then explain the discrepancy observed between *in vivo* and *in vitro* studies using TNCB, suggesting that the observed increase in keratinocyte-derived TNFa seen *in vivo* following skin painting using TNCB could reflect an indirect upregulation mediated by irritant-induced, LC-derived IL-1β.

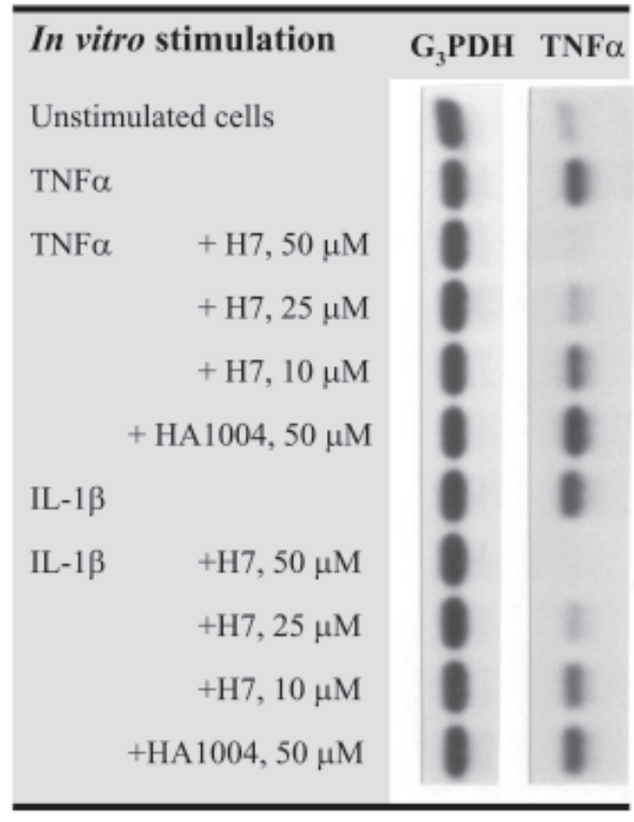

Fig. 13. Murine keratinocytes were stimulated with rTNFα or IL-1β in the absence or presence of inhibitors of PKC-dependent signalling (H7) or PKA (HA1004). Following culture, mRNA was extracted and hybridised to a probe for house keeping gene (G_3PDH) and TNFα. Modified from paper VII.

These observations further implicated the keratinocyte in the induction of an altered cytokine environment observed in ICD. Earlier studies have actually demonstrated that the keratinocyte could function as an amplifier with regard to release of IL-1β[166]. If this phenomenon could be widened to include other cytokines, this would implicate the keratinocyte as an important cell type in the induction and possible maintenance of inflam-matory skin diseases. It was therefore investigated whether TNFα contributed to the increased induction of keratinocyte-derived TNFα, and thus reflected an autocrine regu-

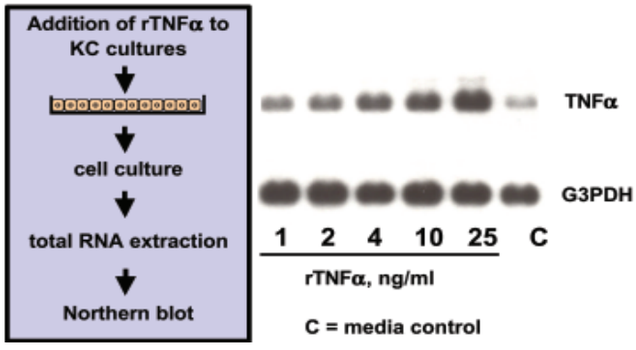

Fig. 14. Keratinocyte cultures were stimulated with rTNFα and mRNA extracted. Northern blots were performed and expression of TNFα mRNA corrected using the housekeeping gene (G_3PDH). Data obtained from paper VII.

latory path-way in skin. Keratinocytes were therefore stimulated using murine rTNFα and subsequently the induction of TNFα mRNA expression was determined by Northern blot. It was found that rTNFα indeed could upregulate its own expression in keratinocytes revealed by an increase in TNFα mRNA (Fig. 14). Furthermore, this upregulation seemed to be specific, involved increased gene-transcription and was mediated by PKC-dependent signalling pathways (see Fig. 13).

It is well known that TNFα can induce expression of IL-1, both IL-1α and IL-1β, in cells, the latter shown to regulate TNFα (se above). To exclude such indirect upregulation, stimulation of the keratinocytes using rTNFα was conducted in the presence of both soluble IL-1 receptor and IL-1 receptor antagonist. Neither of these agents could block the TNFα mediated upregulation of TNFα and subsequently the involvement of IL-1 was excluded.

Thus, the demonstration of an autocrine regulation of TNFα in keratinocytes, as earlier reported for IL-1α, suggests that even a minor induction of pro-inflammatory cytokines could have profound effects on the resulting cytokine milieu found in the skin. Furthermore, these results critically implicate the keratinocyte as an important player in inflammatory skin diseases and suggest that the keratinocyte can function as an amplifier of the pro-inflammatory epidermal cytokine secretion.

13. CONCLUSIONS AND PERSPECTIVES

The following 5 main conclusions may be drawn on the basis of the results presented in this thesis.

1. In diseased skin, CD36 expression is confined not only to keratinocytes but also contribute to identify bone marrow-derived non-LC APC in both allergic and to minor degree irritant contact dermatitis reactions.
2. The $CD36^+$ APCs obtained from ACD reaction sites exhibit a functional capacity to activate both allogenic as well as autologous T lymphocytes and as such contribute to the increased APC-activity found in skin underlying ACD.
3. In parallel to findings in ACD, T-cell response towards nickel is measurable in persons with negative epicutan test towards $NiSO_4$. This activation results in preferential $CD4^+$ T-cell proliferation and the release of IFN-γ. Both memory and naïve T-cell subsets were found to proliferate in response to nickel.
4. Skin irritants and the pro-inflammatory cytokine IL-1β directly induce TNFα in keratinocytes via transcriptional and post-transcriptional signalling pathways.
5. Autocrine regulation of TNFα expression is present in epidermal keratinocytes.

Today it is well recognised that both allergic and irritant contact dermatitis reactions represent complex immunological reactions. The presence of different APC and the induction of a local milieu of cytokines and chemokines all contribute to the observed inflammatory skin reaction. Our demonstration of non-LC APC in inflamed skin adds to the complexity by which the immunesystem is activated locally in skin. The regulatory function of such $CD36^+$ non-LC APC in contact dermatitis reactions is unknown. However, they may contribute to down regulate skin inflammation. This hypothesis is supported by a study using ultraviolet irradiation of skin[68]. Furthermore it has been shown that the binding of P.falciparum-infected erythrocytes to CD36 on dendritic cells or even the usage of anti-CD36 antibodies, block the normal maturation of the dendritic cells and subsequent the dendritic cells fail to activate T cells and preferentially secrete IL-10 and not IL-12[167,168]. Further studies are needed to evaluate whether the $CD36^+$ epidermal APC found in ACD reactions in skin exhibit the same cytokine-inhibitory profile, and thus acts as down regulators of ongoing inflammatory processes in skin.

In many years, much effort has been addressed to the question whether qualitative differences exist between the contact allergic and irritant reaction. Today quantitative rather than qualitative differences are described. Of particular interest is the findings of increased expression of IL-1β in the early phase of the contact allergic reaction, but as earlier stated IL-1β is also present in the irritant skin reaction.

The observation of an autocrine regulation of TNFα in keratinocytes, stresses the importance of even minor induction of cytokines by e.g. skin irritants. Thus, induction of an "inflammatory milieu" where pro-inflammatory mediators are upregulated may contribute to the maintenance or even exacerbation of a locally induced eczematous reaction in skin via a positive feedback. This can also add to the understanding why e.g. the chronic irritant skin reaction persists for month or even years after abrogation of the irritant challenge.

Taken the available data in account, it is intriguing to apply a model in which a common initial activation of the immune system occurs. When a signal, e.g. an allergen, irritant or a microorganism is recognised, an activation of the immune system occurs. This is probably not antigen-specific and involves induction or upregulation of pro-inflammatory cytokines, chemokines and cell adhesion molecules. The keratinocyte is supposed to play an important role in this phase. Later, a more specific immune reaction evolves. Thus, one should expect a common scenario in the initial phases of both ACD and ICD reactions. This is indeed reflected by histological evaluations, where ACD and mild to moderate ICD reactions are very difficult to differ from one another. A common primary immunological reaction is also supported by observations that in the ACD reaction, only a few percent of skin inflammatory T-cells are actually antigen specific, the rest are preferently $CD4^+$ regulatory T cells.

Then, why do all individuals not react equally upon comparable stimulations? Today no complete answer is present, but the term "susceptibility" gains increased focus. At the individual level, comparable stimulation may result in different activation of genes involved in inflammation. Differential activation of a gene can in part be attributed to the presence of functional allelic-polymorphisms associated with changes in e.g. cytokine production. One known example of this is the TNFα promoter. In humans, a polymorphism is present at –308 relative to the transcription initiation site of the TNFα gene. This polymorphism consists of a G to A transition, with the G form of the polymorphism being the most common[169]. If an individual have the G-form (TNF1 allele) an "activator protein-2" (AP-2) binding site is present in the TNFα promoter region. Binding to this AP-2 site is suggested to suppress the activity of the TNFα promoter, as demonstrated in a Jurkat T cell line *in vitro*[170]. Presence of the TNF2 allele (A allele) results in disruption of the AP-2 site, and is associated with high secretion of TNFα following stimulation[171,172]. In the clinic, individuals homozygous for TNF2 allele have been described to have an increased risk of death from cerebral malaria[173], a disease where TNFα plays a major role. Of particular interest, in collaboration with our group, Allan MH et al.[174] has reported that this polymorphism is also associated with susceptibility to irritant contact dermatitis. This was shown by investigating the presence of the –308 polymorphism in the human TNFα gene and correlation of these data to susceptibility towards *in vivo* irritant stimulation. It was shown that in individuals without skin diseases, a lower threshold towards SLS-mediated skin irritation was observed in individuals expressing the TNF2 allele. Thus, the –308 TNFα polymorphism can be used as a marker for irritant susceptibility.

In addition to polymorphisms in the TNFα gene, other gene polymorphisms are described. Some examples on biological important polymorphisms are reported within the IL-1β-, IL-2-, IL-6-, IL-10-, IL-13- and epidermal growth factor (EGF)-genes[175-180]. Thus, today clinical relevant gene-polymorphisms are described and studies are implicating these polymorphisms in the development of several diseases, including irritant contact dermatitis.

Furthermore, the capacity of different allergens and/or irritants to functionally interact needs more focus. In a normal