MODULATION OF PRO-INFLAMMATORY CYTOKINES IN NORMAL AND INFLAMED SKIN

Modulatie van pro-inflammatoire cytokinen in normale en ontstoken huid

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知不知，尚矣；不知知，病也。
圣人不病，以其病病。夫唯病病，是以不病。

Who recognizes his limitations is healthy;
Who ignores his limitations is sick.
The sage recognizes this sickness as a limitation.
And so becomes immune.

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CONTENTS

Chapter 1

GENERAL INTRODUCTION

Outline of this introduction

This thesis describes the expression and modulation of pro-inflammatory cytokines in normal and inflamed skin. During the last few decades it has become clear that the skin comprises a complex network of interacting cells including keratinocytes (KC), dendritic cells (such as Langerhans cells; LC), macrophages, endothelial cells, fibroblasts and T cells. During an infectional or trauma incident, resident and invading cells in skin initiate a complex cascade of events resulting in an inflammatory reaction and ultimately in the elimination of the infectious agent or recovery of the wound. Pro-inflammatory cytokines like members of the interleukin-1 (IL-1) family of proteins, tumor necrosis factor-α (TNF-α) and IL-6 play a prominent role in the induction and maintenance of this inflammatory response. When the expression of cytokines in skin is altered, it can result in an inflammatory disease like psoriasis. Thus the regulation of cytokine expression must be tightly orchestrated. The regulation of pro-inflammatory cytokines in normal and inflamed skin is still incompletely understood. During the study presented in this thesis the modulation and effects of pro-inflammatory cytokines in skin were investigated. This introduction is focussed on the expression of these pro-inflammatory cytokines. The following paragraphs deal with the architecture of the skin, the interaction and cross talk of the cells residing in it and the expression and regulation of the IL-1 family of proteins.

The skin architecture

The skin consists of two compartments: the epidermis and the dermis (Fig. 1A) which are separated by the basement membrane, a thin layer consisting of type IV collagen, laminin and glycosaminoglycan, a heparin sulfate. This membrane serves as an anchor for the KC which are attached to this layer by hemidesmosomes. The first single cell layer of KC forms the basal layer containing the proliferating cells. As the cells divide, they are pushed towards the outer layers and start to differentiate. First the cells enter the suprabasal layer and subsequently the granular layer. During this migration, KC start to shift their keratin profile. Keratin is a protein complex consisting of cytokeratin and keratohyalin. When KC progress towards the more outer layers of the epidermis, the lysosomes start to rupture and the cells go into apoptosis. Although the cells loose their nucleus, the contact between them persists because of the desmosomal junctions, which are still intact. At the same time an environment is created in which the keratin polymerizes thereby forming the outer layer of the epidermis: the stratum corneum. The pH in this layer is rather low (5-6) due to its specific fatty acid components, thus protecting the body against invading potentially pathogenic microorganisms.

Like KC in the epidermis, fibroblasts determine the structure of the dermis which is mainly composed of type I collagen and elastin. Fibroblasts are responsible for the repair of tissue damage by producing growth factors including keratinocyte growth factor and dermal matrix. Venules, together with the capillaries are the port of entry of for example T cells, monocytes and granulocytes which can leave the skin via lymphatic vessels. Additionally, also nerve fibers course through the dermis. Thus the dermis is a feeding layer of the epidermis providing it with growth factors, and is also the site of entrance of leukocytes during nor-

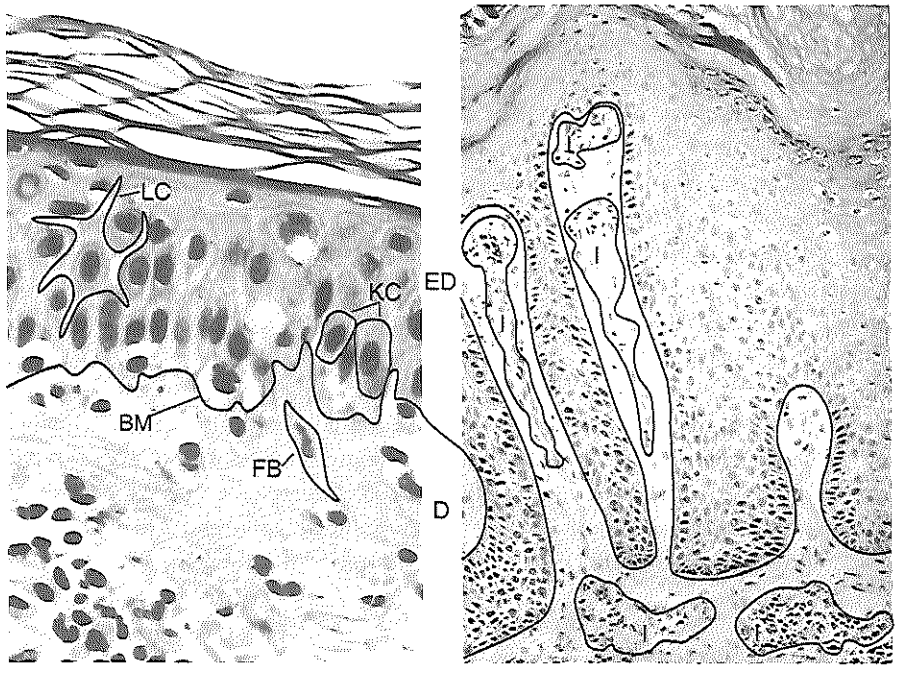

Fig. 1. Normal and psoriatic lesional skin architecture. A: Normal skin can be divided into an epidermal and dermal part separated by the basal membrane. Several cell types reside in the skin (see text for details) including keratinocytes and Langerhans cells in the epidermis, and fibroblasts, endothelial cells and dermal dendritic cells in the dermis. T cells (not shown) reside mostly in the dermis but are also present in the epidermis. (magnification: ×350) **B**: Psoriatic lesional skin is characterised by the thickening of the epidermis, elongation of the rete ridges and infiltrating leukocytes (I). (magnification: ×140) ED: epidermis; D: dermis; BM: basal membrane; KC: keratinocytes; LC: Langerhans cell; FB: fibroblast.

mal and disease state.

The function of the immunologically most important cells residing in the dermis and epidermis is discussed in more detail in the following paragraphs

The epidermis, first line of defense

Keratinocytes: the main building blocks, not only bystander cells

About 90% of the total number of cells in the epidermis are KC. Since long it has been thought that KC were bystander cells just covering the body and protecting it against the outside world. We now know that KC take active part in the regulation of the skin immune system. KC express many of the known anti- and pro-inflammatory cytokines and their receptors (Table 1) and are therefore able to recruit for example T cells, neutrophils and monocytes by producing chemokines like IL-8 (attracting neutrophils and T cells) and RANTES (attracting monocytes and T cells) (Fukuoka *et al.*, 1998b). Paracrine and autocrine regulation of the expression of adhesion molecules like ICAM-1 and E-selectin can be induced by

Table 1. Pro- and anti-inflammatory cytokines expressed by resident cells of the skin. Resident cells of the skin can produce several pro- and anti-inflammatory cytokines in different amounts (see text for details). Cytokines known to be produced by the different resident cells in skin are listed in this table. The references depicted in this table represent literature in which the expression of the involved cytokine is reported. Question marks (?) indicate that no literature data on that aspect is available. References are indicated between brackets and are listed below.

Cytokine	Resident cells in the dermis and epidermis		
	Keratinocytes	Skin dendritic cells (LC and DDC)	Fibroblasts
IL-1α/β	(1-3)	(4-6)	(7-8)
IL-18	(9-11)	(12-13)	(14)
IL-12	(15-17)	(18, 19)	?
TNF-α	(20, 21)	(22)	(21)
IL-6	(23, 24)	?	(25, 26)
IL-8	(26, 27)	?	(26, 28)
IL-10	(29)	?	?
IL-1ra	(30, 31)	?	(32, 33)

References: 1-3: Kupper *et al.*, 1986; Phillips *et al.*, 1995; Zepter *et al.*, 1997; **4-6**: Enk *et al.*, 1993; Matsue *et al.*, 1992; Mohamadzadeh *et al.*, 1997; **7-8**: Kawaguchi, 1994; Lambert *et al.*, 1998; **9-11**: Companjen *et al.*, 2000; Mee *et al.*, 2000; Naik *et al.*, 1999; **12-13**: Nakagawa *et al.*, 1999; Stoll *et al.*, 1998; **14**: Lu *et al.*, 2000; **15-17**: Aragane *et al.*, 1994; Kondo & Jimbow, 1998; Yawalkar *et al.*, 1996; **18-19**: Kang *et al.*, 1996; Yawalkar *et al.*, 1996a; **20-21**: Avalos-Diaz *et al.*, 1999; Malaque *et al.*, 1999; **22**: von Stebut *et al.*, 2000; **23-24**: Kupper *et al.*, 1989; Li *et al.*, 1996; **25-26**: Maruyama *et al.*, 1995; Yellin *et al.*, 1995; **27**: Kondo *et al.*, 1993; **28**: Fukuoka *et al.*, 1998; **29**: Becherel *et al.*, 1997; **30-31**: Bigler *et al.*, 1992; Phillips *et al.*, 1995; **32-33**: Higgins *et al.*, 1999; Krzesicki *et al.*, 1993.

KC through expression and release of IL-1 (Wyble *et al.*, 1997) and this can also be antagonized by expression of interleukin-1 receptor antagonist (IL-1ra). Expression of adhesion molecules enables KC to modulate the migration of neutrophils, LC and T cells through the epidermis. IFN-γ induces the expression of the major histocompatibility complex class II (MHC class II) on KC *in vitro* (Kemeny *et al.*, 1995; Kerr *et al.*, 1990; Nilsson *et al.*, 1989; Wikner *et al.*, 1986). In inflamed skin the MHC class II expression is elevated as well (Aubock *et al.*, 1986; Gottlieb *et al.*, 1986; Terui *et al.*, 1987; Weller *et al.*, 1995), probably due to T cell derived IFN-γ, as at present there is no evidence that KC can produce IFN-γ. MHC class II expression suggests that KC are able to present antigen and may even costimulate T cells. However, although it has been shown that KC can express at least CD80 message (Wakem *et al.*, 2000), there is no evidence that keratinocytes can costimulate T cells *in vivo* by interacting with CD28. Conversely KC transfected with CD86 can costimulate T cells (Nickoloff *et al.*, 1995) and *Candida albicans* infected transgenic mice specifically expressing CD86 in skin, show an exaggerated cutaneous delayed-type hypersensitivity (DTH) response (Gaspari *et al.*, 1998). CD40, another crucial costimulatory molecule of antigen presenting cells (APC) has also been shown to be expressed on KC and is upregulated by IFN-γ (Denfeld *et al.*, 1996). It has been shown that stimulation of CD40 on human KC results in the induction of several chemokines (e.g. IL-8, RANTES, MCP-1) (Denfeld *et al.*, 1996; Peguet-Navarro *et al.*, 1997; Pash, manuscript in preparation; this thesis, chapter 6). CD40 ligation on KC also induces the expression of pro-inflammatory cytokines like IL-6 and TNF-α (Gaspari *et al.*, 1996; Peguet-Navarro *et al.*, 1997; this thesis, chapter 6) and adhesion mol-

ecules like ICAM-1 (CD54) (Denfeld *et al.*, 1996). Whether CD40 expressed on KC can provide a mitogenic signal to T cells, is still a subject for debate (Gaspari *et al.*, 1996; Grousson *et al.*, 1998). Moreover, CD40-ligand (CD40L) interaction on human KC seems to inhibit their proliferation, but induces their differentiation uncovering a novel function for CD40 (Peguet-Navarro *et al.*, 1997). Finally it has been shown that human KC express CD1d, which could stimulate the expression of IFN-γ by NK-T cells present in psoriatic lesional skin (Bonish *et al.*, 2000) (see also below).

In conclusion, it is now clear that KC indeed play an active and important role in the regulation of inflammation in skin.

Langerhans cells; detectors of invading danger

LC are professional APC located in the epidermis and are characterized by CD1a expression and the expression of special organelles, called Birbeck granules. LC belong to the lineage of dendritic cells (DC), a cell type specialized in taking up and processing antigen (Ag). Subsequently, APC can present the Ag to naïve T cells in the context of MHC resulting in T cell activation. About 1-2% of the total epidermal cells are LC. These cells are mainly localized in the suprabasal layer of the epidermis. Apart from their dendritic morphology, LC are characterized by expression of MHC class II and CD1a (Meunier *et al.*, 1996). Human LC express E-cadherin (Blauvelt *et al.*, 1995) which mediates the adhesion to the surrounding KC (Tang *et al.*, 1993). E-cadherin ligation also interferes with LC maturation. When E-cadherin ligation is abrogated, expression of CD86 and CD83 are upregulated while CD1a expression diminishes: the LC achieves a more mature phenotype upon migration to the lymph node (Riedl *et al.*, 2000). Mature LC express CD86 and CD80 and consequently they can costimulate T cells (Yokozeki *et al.*, 1996). CD40 is functionally expressed by LC (Peguet-Navarro *et al.*, 1995) and ligation results in upregulation of CD54 and CD86 expression. Recent literature implies that the human hair follicle is a reservoir of $CD40^+$ LC (Gilliam *et al.*, 1998). These LC do not express CD80 or CD86 in culture and repopulate the skin after UVB exposure. This might implicate that these hair follicle derived LC are immature and may serve as a backup in case of for example UV damage.

LC are known to express several cytokines including pro-inflammatory cytokines like IL-1β (Enk *et al.*, 1993), IL-6 (Schreiber *et al.*, 1992) and IL-18 (Nakagawa *et al.*, 1999) (see also Table 1) and accordingly influence the cytokine environment in the epidermis. By influencing the cytokine microenvironment, LC can affect T helper cell differentiation.

Recently a new model of Th skewing by DC has been proposed (Kapsenberg *et al.*, 1999; Rissoan *et al.*, 1999). In this model DC producing high levels of IL-12 have a major effect on skewing Th0 cells towards the Th1 pole and are called DC1. Conversely, DC producing prostaglandin E_2 (PGE_2) have a Th2 skewing effect and are hence called DC2. Recent data from individuals challenged intradermally with lipopeptides from *Treponema pallidum* show that skin derived DC1, but not DC2 cells were more mature as defined by their CD1a, CD83, CD80 and CD86 expression than their peripheral blood counterparts (Sellati *et al.*, 2001). This implicates that DC1 might play an important role during inflammation in human

skin and may affect Th development.

When an unknown Ag is encountered, the LC starts to produce metalloproteinases like matrix metalloproteinase-9 (MMP-9) (Kobayashi *et al.*, 1999) to break down its connections with the surrounding tissue. Subsequently the LC migrates to the lymph node and becomes an interdigitating cell (IDC) in the paracortical T cell areas. There the Ag is presented to naïve T cells in the context of the MHC complex. Subsequently the activated T cells home to the site of infection (see below). After activation of T cells in the lymph node, the former LC goes into apoptosis.

Thus LC are professional APC located in the epidermis, capable of activating naïve T cells and are therefore potent inducers of an immune response in the skin.

Melanocytes as mediators of inflammation

Melanocytes are located in the basal layer of the epidermis. By generating the radical-scavenging pigment melanin, melanocytes protect the cells resident in skin against ultraviolet radiation. Additionally to this well-known protective role, melanocytes play an active part in the skin immune system. Melanocytes are capable of phagocytosis (Le Poole *et al.*, 1993) and express low levels of MHC-II and ICAM-1 which are upregulated upon stimulation with IFN-γ (Hedley *et al.*, 1998). Pro-inflammatory cytokines like IL-1 and TNF-α are also expressed by melanocytes (Barral *et al.*, 2000) indicating that they can induce an inflammatory response by secreting these molecules. Finally the role of melanocytes in immunity is illustrated by the observation that the absence of melanocytes in lesional vitiligo leads to impaired migration of Langerhans cells (Das *et al.*, 2001).

These observations suggest that melanocytes contribute to a protective immune barrier at the dermo-epidermal interface.

The dermis: the place of immune responses and entry and exit for leukocytes

T-cells: executioners of immune responses in skin

Most of the T cells in normal human skin are located in the dermis (Bos & Kapsenberg, 1993). The α/β T cell receptor (TCR) phenotype exceeds the γ/δ TCR phenotype in normal human skin (Foster *et al.*, 1990). This is in contrast to murine skin where the majority of the skin T cells are γ/δ T cells (Sugaya *et al.*, 1999). The majority of the T cells in human skin are of the memory cell phenotype ($CD45RO^+$). A large number of the T cells residing in the skin are $CD8^+$, indicating that they are cytotoxic T cells (Foster *et al.*, 1990). The VαVβ and VγVδ gene usage of skin T cells display a skewed phenotype (Dunn *et al.*, 1993; Holtmeier *et al.*, 2001; Uyemura *et al.*, 1991) indicating that T cells in the skin respond to a limited number of antigenic epitopes. Possibly these epitopes are pathogen derived. If so, T cells in skin are capable of reacting quickly to an invasion of such pathogens. Fifty percent of the skin T cells bear a specific homing receptor (cutaneous lymphocyte antigen (CLA)) (Bos *et al.*, 1993) for which E-selectin, expressed by endothelial cells and KC, serves as a ligand (Berg *et al.*, 1991; Rossiter *et al.*, 1994). The cytokine profile of skin T cells is dominated by IFN-γ expression. T cells in skin differ from peripheral blood T cells with respect to

IFN-γ expression. For example, upon stimulation skin T cells produce IFN-γ 17 times faster than peripheral T cells. Conversely, synthesis of IFN-γ persists for several days in peripheral T cells whereas it lasts for only 90 minutes in skin T cells (Hassan-Zahraee *et al.*, 1998). This is explained by the finding that skin T cells express IFN-γ mRNA constitutively in contrast to peripheral T cells. The already available IFN-γ mRNA is immediately translated upon stimulation while the mRNA expression is switched off.

During skin inflammation, E-selectin expression is elevated (Harari *et al.*, 1999; Henseleit *et al.*, 1996; Jones *et al.*, 1997) and CLA positive T cells are attracted. That T cells in human skin have a T helper 1 (Th1) like phenotype, might be explained by the observation that E-selectin mediates the recruitment of Th1 but not Th2 cells (Austrup *et al.*, 1997). The Th1 phenotype predominates in several inflammatory skin diseases (Austin *et al.*, 1999; Llorente *et al.*, 1997; Schlaak *et al.*, 1994). However, in some skin diseases, like atopic dermatitis (AD) predomination of Th2 cells has been reported, indicating that also Th2 cells can be attracted (Neumann *et al.*, 1996; Vestergaard *et al.*, 2000; Vowels *et al.*, 1994).

In conclusion, T cells in human skin exhibit mainly an α/β TCR phenotype with a skewed epitope specificity. These T cells are capable of producing IFN-γ very quickly. This suggests: (1) these T cells can induce an environment favourable for the elimination of common pathogens; (2) because of their TCR specificity they are able to mount an immune response against common epitopes present on putative pathogens resulting in the fast elimination of these pathogens; and (3) T cells in skin might also have a defensive role in that they are toxic for transformed cells and consequently protect against skin cancer.

Fibroblasts: not only producers of collagen

Fibroblasts are an important source of mediators of inflammation. Like KC fibroblasts can express pro-inflammatory cytokines like IL-1, IL-6 and TNF-α (Avalos-Diaz *et al.*, 1999; Higgins *et al.*, 1999; Maruyama *et al.*, 1995) (Table 1). Furthermore, fibroblasts express chemokines like RANTES and IL-8 upon stimulation with TNF-α or IL-1α (Fukuoka *et al.*, 1998a; Kristensen *et al.*, 1991) indicating that they play a role in the attraction of neutrophils, T cells and monocytes. KC growth is also regulated by fibroblasts. Using a model in which skin equivalents can be investigated, Maas-Szabowski showed that fibroblasts stimulate KC growth by producing keratinocyte growth factor (KGF) upon stimulation with KC derived IL-1 (Maas-Szabowski *et al.*, 2000). Apart from producing cytokines, dermal fibroblasts also interfere with the regulation of the immune response on other levels. For example, murine fibroblasts are able to express MHC class II and CD80 (Pechhold *et al.*, 1997) which suggests that murine fibroblasts may play a role in the costimulation of T cells. CD40 is functionally expressed by fibroblasts as well (Banchereau *et al.*, 1995; Fries *et al.*, 1995). CD40 expression on dermal fibroblasts is upregulated by IFN-γ, and ligation of CD40 on fibroblasts induces the expression of IL-6 and adhesion molecules like CD54 and CD106 (Fries *et al.*, 1995; Yellin *et al.*, 1995). In contrast to KC, CD40 stimulation of fibroblasts facilitates their proliferation rather than differentiation.

Thus human fibroblasts influence the immune response by expressing chemokines,

pro-inflammatory cytokines and adhesion molecules.

Endothelial cells: port of entry for leukocytes

Apart from covering the walls of a blood vessel, an important task of endothelial cells is the attraction and adhesion of leukocytes. Stimulation of endothelial cells with IL-1β, TNF-α or bacterial lipopolysaccharide (LPS) results in the upregulation of E-selectin, CD54 and VCAM-1 (Haraldsen *et al.*, 1996) and facilitates adhesion of T cells, neutrophils and monocytes. IL-8 and IL-6 are also expressed by endothelial cells (Kristensen *et al.*, 1991; Soderquist *et al.*, 1998), and their expression levels are modulated by stimulation with pro- and anti-inflammatory cytokines (Chen & Manning, 1996). Endothelial cells are even capable to pinocytose KC and fibroblast derived IL-8 and to translocate it across their own cytoplasm after which it is presented to leukocytes in a membrane bound form (Middleton *et al.*, 1997).

CD40 is also implicated in the expression of adhesion molecules and cytokines by endothelial cells. Endothelial cells express functional CD40 on their cell membrane, which, when ligated by CD154 (CD40L) on activated $CD45^+$ T cells, enhances the expression of E-selectin and CD54 and augments the TNF-α induced expression of VCAM-1 (Hollenbaugh *et al.*, 1995; Karmann *et al.*, 1995) facilitating adhesion and migration. Stimulation of CD40 on human endothelial cells also induces activation of caspase-1 (also known as ICE) and consequently the expression of mature IL-1β (Schonbeck *et al.*, 1997). Recently it has been shown that CD40L expressed on platelets can induce inflammatory reactions by endothelial cells (Henn *et al.*, 1998), which suggests an important role for CD40 signaling on endothelial cells during injury.

Thus upon stimulation, skin endothelial cells express chemokines like IL-8 and adhesion molecules like E-selectin and CD54 and as a consequence facilitate the attraction and entrance of leukocytes into the skin.

Dermal dendritic cells: cells traveling to and from the epidermis?

Morphologically, dermal dendritic cells (DDC) are difficult to distinguish from LC. Whether DDC are a distinct subset or related to LC is still unclear (Lenz *et al.*, 1993). Although some DDC express CD1a and possess Birbeck granules in low amount, the majority is CD1a negative and lack Birbeck granules (Nestle & Nickoloff, 1995). DDC are discriminated from LC by the expression of factor XIIIa (Cerio *et al.*, 1989), a transglutaminase which plays a role in the formation of fibrin clots (Dallabrida *et al.*, 2000). DDC play an important role in the regulation of the skin immune system. They express a high level of MHC class II and are potent APC (Nestle *et al.*, 1998; Nestle & Nickoloff, 1995). DDC express CD80, CD86 and CD40 and are capable of T cell costimulation. It is known from studies in psoriasis that DDC mediate a T cell response with high levels of IL-2 and IFN-γ (Nestle *et al.*, 1994). Therefore DDC might be considered to belong to the DC1 subset.

In conclusion, the majority of DDC lack LC markers, but are highly potent in antigen presentation and T cell stimulation. It is still unclear whether DDC are precursors of LC, LC

derived, or even a distinct DC subset in human skin.

The IL-1 system

Discovery

In 1948 a heat-labile protein was detected in acute granulocytic exudate fluid, capable of inducing fever when injected into rabbits (Beeson, 1948). This protein was identified in 1972 as a macrophage derived factor, acting as an activator of lymphocytes (lymphocyte-activating factor, LAF) (Gery *et al.*, 1972). Mizel *et al.* (Mizel *et al.*, 1978) characterized LAF and its purification was reported several years later (Lachman *et al.*, 1980). Since its discovery, many factors have been isolated similar if not identical to LAF (e.g.: thymocyte activating factor (TAF), tumor inhibitory factor-2 or fibroblast activating factor (FAF)), and after cloning of the gene a new name for this factor was proposed: interleukin-1 (IL-1). The decades of research on the action and function of IL-1 has revealed the existence of a vast family comprising different IL-1 isoforms and the corresponding receptors (Dinarello, 1996).

The IL-1 system: members, receptor binding and signal transduction

The first two IL-1 isoforms isolated and characterized were IL-1α and IL-1β (Auron *et al.*, 1984; Lomedico *et al.*, 1984). IL-1α is being produced as a 31 kD active polypeptide and IL-1β as a partially inactive 31 kD precursor protein, which has to be processed by caspase-1 (formerly known as interleukin-1 converting enzyme (ICE)) to yield a 17 kD active protein. IL-1α as well as IL-1β lacks a signal peptide. Both IL-1α and IL-1β bind to the IL-1-receptor type I (IL-1RI) and signal transduction is triggered after recruitment of the IL-1R accessory protein (IL-1RAcP). IL-1 signaling is inhibited by two IL-1 antagonists: IL-1 receptor antagonist (IL-1ra) and IL-1R type II (IL-1RII). The phenomenon of naturally occurring receptor antagonists is rather unique in cytokine biology whereas soluble receptors are more common (Arend *et al.*, 1991; Mantovani *et al.*, 2001). IL-1ra occurs in three forms: a secreted form (sIL-1ra) and two forms who remain intracellular (icIL-1ra), all encoded by the same gene (IL-1RN) (Arend *et al.*, 1998). IL-1RII occurs both as a secreted form (sIL-1RII) and as a membrane bound form. IL-1ra also binds to the IL-1RI chain, but lacks a binding site for IL-1RAcP, resulting in a blockade of signal transduction through the IL-1R complex (Fig. 2). IL-1RII is identical to the IL-1RI, but lacks the cytoplasmatic signaling domain and is therefore unable to transduce a signal upon IL-1 binding (Fig. 2). IL-1RI can be secreted as well, thus both IL-1RI and IL-1RII can function as a decoy receptor inhibiting IL-1α and IL-1β activity (Arend *et al.*, 1994). IL-1 activity is generated through the NF-κB signal transduction route (Fig. 2). Several signal transduction proteins are activated when the IL-1RI is stimulated by its ligand, resulting in the phosphorylation of IκB and subsequent activation of NF-κB.

New members of the IL-1 and IL-1R family of proteins

The discovery of new IL-1 isoforms and their receptor chains is ongoing (Barton *et al.*, 2000; Busfield *et al.*, 2000; Kumar *et al.*, 2000; Mulero *et al.*, 1999; Smith *et al.*, 2000a)

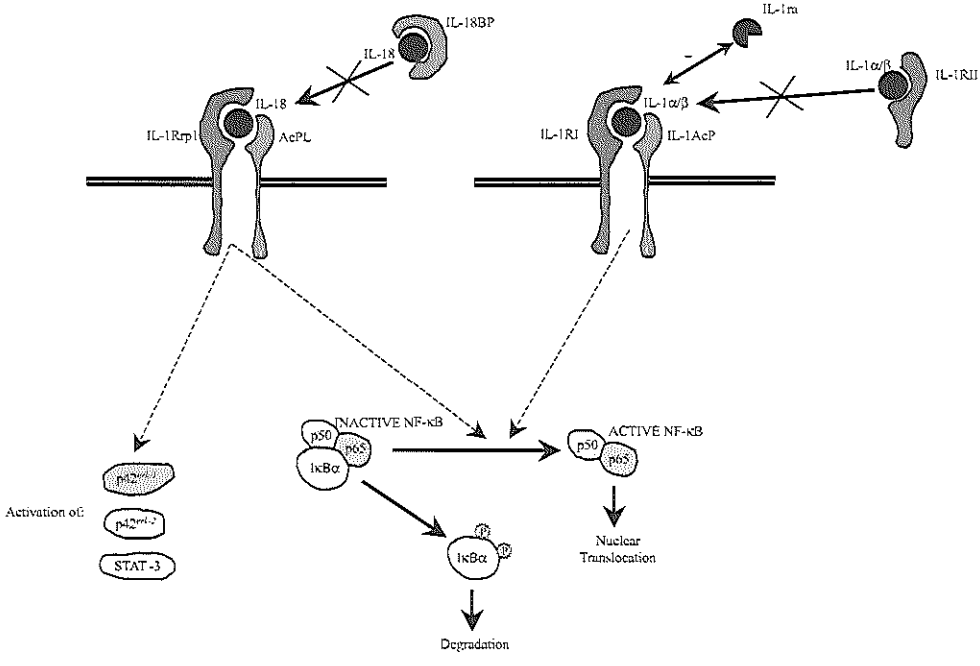

Fig. 2. Similarities in IL-1 and IL-18 receptor binding and signaling. Ligation of the IL-1RI by IL-1α/β or IL-1Rrp1 by IL-18 is followed by the recruitment of IL-1AcP (IL-1α/β) or AcPL (IL-18) which results in the interaction of different signal transduction proteins (see text for details). As a result NF-κB is activated. Inactivation of IL-1α/β or IL-18 occurs by binding to the IL-1RII or IL-18BP, respectively. Additionally the IL-1 signal can be blocked by IL-1ra. A functional IL-1ra homologue for IL-18 has not been described yet. Note that IL-18 can also activate other factors.

(Table 2). The data collected so far suggest that the IL-1 system probably originates from an ancient molecule which evolved through gene duplication and mutation resulting in the present complex system of agonists and antagonists (Beutler & Poltorak, 2001). IL-1 like molecules known as Toll or Toll like proteins are even found among invertebrates and plants (O'Neill, 2000), suggesting that IL-1 like proteins play an important role in the innate immune response.

In addition to the expansion of newly isolated IL-1 isoforms, also new IL-1R like proteins are being identified (Bonnert *et al.*, 1997; Carrie *et al.*, 1999; Chaudhary *et al.*, 1998; Li *et al.*, 2000; Nolan *et al.*, 1998; Parnet *et al.*, 1996; Sana *et al.*, 2000; Thomassen *et al.*, 1999). Because of the expansion of the IL-1R family, a numbering system for the IL-1R like genes has been suggested. Using this new system, IL-1RI, IL-1RII, IL-1RAcP, ST2, IL-1Rrp1 (IL-18Rα), IL-1Rrp2, AcPL (IL-18Rβ), SIGIRR and IL-1RAPL are now called IL-1R chain 1 (IL-1R1) to 9 respectively (Rock *et al.*, 1998; Sana *et al.*, 2000; Debets *et al. 2001*) (Table 3).

Most of the genes encoding the IL-1 family members are located on chromosome 2.

However, there are a few exceptions: IL-18 and SIGIRR (single Ig IL-1R-related molecule), are located on chromosome 11 (Nolan *et al.*, 1998; Thomassen *et al.*, 1999), IL-1RAPL and IL-1R9 are located on chromosome X (Carrie *et al.*, 1999; Sana *et al.*, 2000) and MyD88 and IL-1RAcP are located on chromosome 3 (Bonnert *et al.*, 1997; Dale & Nicklin, 1999).

Table 2. Novel IL-1 isoforms: their alternative names, chromosome location and predicted molecular weight. The discovery of novel IL-1 isoforms still continues. Different researchers describe similar isoforms. This tabel lists the different human IL-1 isoforms, their alternative names, chromosome location and predicted molecular weight. References (Ref.) are indicated between brackets. Mw: molecular weight; up/p: unprocessed/processed. References: 1. Mulero *et al.*, 1999; 2. Smith *et al.*, 2000; 3. Kumar *et al.*, 2000; 4. Okamura *et al.*, 1995; 5. Busfield *et al.*, 2000; 6. Barton *et al.*, 2000; 7. Pan *et al.*, 2001; 8. Bazan *et al.*, 1996; 9. Debets *et al.*, 2001.

IL-1 Isoforms	Ref.	alternative names	chromosomal localization	predicted Mw kD (up/p)
IL-1L1	(6)	FIL-1δ (2); IL-1RP3 (5); IL-1HY1 (1); IL-1δ (9)	2	~17
FIL-1ε	(2)	-	2	?
FIL-1η	(2)	-	2	?
IL-1H1	(3)	IL-1RP2 (5); IL-1ε (9)	2	20
IL-1H	(7)	IL-1H4 (3); IL-1RP1 (5); FIL-1ξ (2)	2	34/27
IL-1H2	(3)	-	?	?
IL-18	(4)	IL-1γ (8)	11	24/18

Table 3. IL-1R system based on the numbering of the different receptor chains. A system based on the numbering of the different receptor chains of the IL-1R family has been proposed by Rock *et al.*. This numbering system simplifies the nomenclature of the IL-1R system. This tabel lists the different human IL-1R chains, their numbering, chomosome location and predicted molecular weight. Mw: molecular weight.

IL-1R chain nr.	published names	chromosomal location	predicted Mw (kD)
1	IL-1RI	2	~80
2	IL-1RII	2	~60
3	IL-1RacP	3	~66
4	T1/ST2	2	~61
5	IL-1Rrp1 (IL-18Rα)	2	~64-100
6	IL-1Rrp2	2	~55-60
7	AcPL (IL-18Rβ)	2	~62
8	SIGIRR	11	~50-80
9	IL-IRAPL	X	~76
10	IL-1R10	X	~78

IL-1 expression and biology

Most cells are capable of producing IL-1. IL-1β for example, can be induced in leukocytic cells, like monocytes and dendritic cells, but can also be expressed by non-leukocytic cells including fibroblasts and keratinocytes. In humans IL-1α is expressed intracellularly and is released during disease and upon tissue injury. IL-1α is abundantly expressed in keratinocytes which are also the most important source of IL-1ra. This implies that the IL-1 system plays an important role in skin function during normal circumstances, injury and during state of disease. Indeed, many researchers reported the involvement of IL-1 in inflammatory skin diseases and repair after injury (Barone *et al.*, 1998; Cooper *et al.*, 1990; Debets *et al.*,

1997; Haas *et al.*, 1998; Hubner *et al.*, 1996; Kondo & Ohshima, 1996; Sato & Ohshima, 2000; Sauder *et al.*, 1990). Interestingly, IL-1 seems to play a role in stimulation of keratinocyte proliferation (Maas-Szabowski *et al.*, 2000) and differentiation (Eller *et al.*, 1995). Other IL-1 isoforms are also expressed in skin such as IL-18 (Companjen *et al.*, 2000a; 2000b; and chapters 3, 4 and 7), IL-1RP1 and IL-1L1 (Barton *et al.*, 2000; Busfield *et al.*, 2000). The function of the last two isoforms is currently unknown.

The IL-1 system exerts both local and systemic effects in the body. For example, a systemic effect like the induction of fever is mediated through IL-1β. A more local effect of IL-1β is the induction of ICAM-1, IL-8 and IL-6 expression in various tissues. Other IL-1 like molecules, like IL-18, are involved in the regulation of the Th-cell balance (Lebel-Binay *et al.*, 2000). Furthermore, it has been reported that IL-1APL (IL-1R8) and IL-1R9 play a role in the induction of mental retardation (Carrie *et al.*, 1999; Sana *et al.*, 2000).

IL-1 expression in inflammatory diseases

The role of IL-1 in inflammation was proposed in 1982 by Oppenheim *et al.* (Oppenheim *et al.*, 1982) and has been investigated using different murine IL-1 knock out models (Horai *et al.*, 1998; Labow *et al.*, 1997; Yamada *et al.*, 2000). Many inflammatory diseases are associated with an altered expression of IL-1. IL-1 activity has been implicated in rheumatoid arthritis. For example, in different animal models IL-1ra and IL-1RII attenuate the severity of the inflammatory response in the joint (Bessis *et al.*, 2000; Gabay, 2000) whereas administration of recombinant IL-1 induces a flare-up of the inflammation (van de Loo *et al.*, 1992). Furthermore, mononuclear cells from the lamina propria of patients suffering from Crohn's disease produce elevated levels of IL-1. The same holds true for PBMC of patients with multiple sclerosis (MS) and alveolar macrophages of patients with sarcoidosis (Matsuda *et al.*, 1991; Reinecker *et al.*, 1993; Steffen *et al.*, 1993). In asthma, IL-1ra levels in serum were elevated (Yoshida *et al.*, 1996) and IL-1β was detected in bronchialalveolar lavage (BAL) fluid (Borish *et al.*, 1992). The levels of IL-1β expression were reduced by treatment with corticosteroids.

IL-1 expression in skin

The regulation of the IL-1 system in skin has been investigated thoroughly since the discovery that human keratinocytes produce IL-1 (Kupper *et al.*, 1986). It has become clear that the balance of the different IL-1 agonists and antagonists in skin is of great importance. Disruption of the IL-1 balance can result in a state of chronic skin inflammation such as is the case in psoriasis (Debets *et al.*, 1997). Alterations of the IL-1 system balance have been extensively investigated in this disease (Bonifati *et al.*, 1997; Debets *et al.*, 1995; Groves *et al.*, 1994; Prens *et al.*, 1990; Rasmussen & Celis, 1993; Wei *et al.*, 1999). In psoriatic lesional skin a decrease of IL-1α and an increase of IL-1β levels were detected (Bonifati *et al.*, 1997). Also the expression of IL-1β and the balance of the IL-1/IL-1ra ratio in psoriatic versus normal KC during various differentiation stages is different (Hammerberg *et al.*, 1998). Finally the involvement of IL-1 in inflammatory skin diseases was investigated using a mouse

model. Skin specific IL-1α single and IL-1RI/IL-1α double transgenic mice spontaneously develop skin disease characterized by hair loss, scaling, inflammatory skin lesions and the induction of secondary cytokines in the epidermis (Groves *et al.*, 1995; 1996).

As mentioned before, IL-18, another IL-1 isoform, is also abundantly expressed in human skin. The regulation of this cytokine will be discussed in the next paragraph.

The IL-18 system

Discovery

IL-18 was first discovered as an interferon-γ inducing factor (IGIF) in sera of mice treated with *Mycobacterium bovis* BCG and challenged with LPS (Nakamura *et al.*, 1989). Cloning of the gene for the murine form of IL-18 (Okamura *et al.*, 1995) revealed that the gene encodes a precursor protein of 192 amino acids and a mature protein of 157 amino acids. Subsequently the human IL-18 gene was cloned and comparative analysis showed that it harbored a 65% homology with the murine form (Ushio *et al.*, 1996). The amino acid sequence of IL-18 includes a IL-1 like signature and the comparable three-dimensional structure of IL-18 suggested that IL-18 is a member of the IL-1 family of proteins (Bazan *et al.*, 1996). Subsequent investigations revealed that features of IL-18 regulation are indeed analogous to all members of the IL-1 system. Akita *et al.* and Ghayur *et al.* (Akita *et al.*, 1997; Ghayur *et al.*, 1997) independently found that IL-18, like IL-1β, has to be processed by caspase-1 to generate an active protein. Additionally, IL-18 can be degraded by caspase-3 which might imply a downregulating function of this protease (Akita *et al.*, 1997).

IL-18 signal transduction

Signal transduction proteins involved in IL-1α/β signaling are shared by IL-18 as well. IL-18 activates NF-κB in mouse Th1 cells (Matsumoto *et al.*, 1997) and IRAK and TRAF-6 are recruited when mouse EL-4 cells are exposed to IL-18 (Kojima *et al.*, 1998). Finally, Adachi (Adachi *et al.*, 1998) showed that mice lacking MyD88, an adapter molecule which interacts with IRAK and the IL-1RI, are defective in IL-18 signaling. Taken together, these studies imply that IL-18 can indeed be considered as a member of the IL-1 family of proteins. However, stimulation of NK cells with IL-18 results in the activation of signal transducer and activator of transcription factor-3 (STAT-3) and mitogen-activated protein kinases (MAPK) $p42^{erk-2}$ and $p44^{erk-1}$ (Kalina *et al.*, 2000b) demonstrating that IL-18 is not restricted to the IL-1 signal transduction pathway to exert its actions (Fig. 2). Stimulation of MAPK by IL-18 in murine Th1 cells was also demonstrated (Tsuji-Takayama *et al.*, 1999).

Organization and regulation of the IL-18 gene

The genomic location and organization of IL-18 have been described for both mouse and man. The murine IL-18 gene is located on chromosome 9 and consists of 7 exons, distributed over 26 kb (Tone *et al.*, 1997). Two promoter regions are located in the IL-18 gene, one of which acts constitutively, while the other is activated upon stimulation with, for example, LPS. The human IL-18 gene is located on chromosome 11 (Nolan *et al.*, 1998), and is

somewhat differently regulated than the mouse equivalent. The human IL-18 gene is composed of 6 exons, spanning 19.5 kb. So far one promoter region of the IL-18 gene has been isolated and studied. DNA protein binding experiments revealed that binding of STAT-5 results in increased induction of the IL-18 promoter (Kalina *et al.*, 2000a).

Members of the IL-18 system: Receptor chains and binding proteins

In 1997 Torigoe *et al.* (Torigoe *et al.*, 1997) isolated one of the IL-18 receptor chains which appeared to be identical to the IL-1Rrp1 (IL-1R chain 5, see previous paragraph) which till then did not have a known ligand. Because the binding activity of IL-18 to the IL-18 receptor (IL-18R) was rather low, the existence of yet another IL-18R chain, as is the case for the IL-1 receptor complex, was predicted. Indeed the second IL-18R chain, AcPL (IL-1R chain 7, see previous paragraph), was discovered soon after the discovery of IL-1Rrp1 being part of the IL-18R (Born *et al.*, 1998). Thus, similar to the IL-1R complex, the IL-18R complex consists of two receptor chains, IL-1Rrp1 and AcPL, which are now called IL-18Rα and IL-18Rβ, respectively.

Similar to IL-1 and the decoy receptor IL-1RII, an inhibitor of IL-18 activity was found: IL-18 binding protein (IL-18BP) (Novick *et al.*, 1999). This IL-18 inhibitor shows a remarkable resemblance with IL-1RII in that it binds IL-18 and consequently blocks IL-18 activity (Fig. 2). IL-18BP is secreted but, in contrast to the IL-1RII, membrane expression has not been reported yet. Based on differences in mRNA splicing, four variants of human IL-18BP have been isolated (IL-18BPa-d) two of which (IL-18BPa and c) can inhibit IL-18 activity by more than 95% and are therefore biologically active (Kim *et al.*, 2000). Compared to IL-18BPc, IL-18BPa is the most potent inhibitor of IL-18 activity. This is reflected in the dissociation constants of IL-18BPa and c which are 0.399 ± 0.034 nM and 2.94 ± 0.86 nM, respectively. IL-18BPb and c are unable to inhibit IL-18 activity. Regulation of expression levels of the biologically active and inactive IL-18BP variants might also modulate the IL-18 activity. Interestingly, proteins structurally related to IL-18BP have been isolated from pox viruses and these IL-18BP like proteins indeed block IL-18 activity (Calderara *et al.*, 2001; Smith *et al.*, 2000b).

An IL-18 receptor antagonist has not been identified yet, but recently a new IL-1 isoform has been cloned (IL-1H, see Table 2) which binds to the IL-18R but not to the IL-1R (Pan *et al.*, 2001). The protein sequence of IL-1H bears a similarity of 36% with IL-1ra and might be an IL-18ra candidate. Thus similar to the IL-1 system, the IL-18 system seems to consist of agonists and antagonists.

IL-18 expression and biology

IL-18 is widely expressed by both leukocytic and non-leukocytic cells including macrophages, dendritic cells, astrocytes, Kupffer cells, chondrocytes and epithelial cells like gut epithelial cells and keratinocytes.

Stimulation of IFN-γ production during inflammation is the most well known action of IL-18. In combination with IL-12p35/p40, IL-18 has a synergistic effect on IFN-γ induc-

tion by Th1 cells (Kohno *et al.*, 1997; Micallef *et al.*, 1996; Yoshimoto *et al.*, 1998). Also IFN-γ production by NK cells as well as the activation of NK cells are influenced by IL-18 (Hunter *et al.*, 1997; Lauwerys *et al.*, 1999; Micallef *et al.*, 1997; Okamura *et al.*, 1998; Walker *et al.*, 1999). This effect of IL-18 on Th1 and NK function was also demonstrated in studies using IL-18 deficient mice (Takeda *et al.*, 1998).

Other biological properties of IL-18 are for example its effect on Fas/FasL expression (Cho *et al.*, 2000; Dao *et al.*, 1996; Ohtsuki *et al.*, 1997; Tsutsui *et al.*, 1996; 1997) which at least in part accounts for the anti tumor effects of IL-18 (Hashimoto *et al.*, 1999). Furthermore, IL-18 stimulates GM-CSF production by osteoblasts and T cells and as such inhibits the formation of osteoclasts (Horwood *et al.*, 1998; Udagawa *et al.*, 1997).

Finally, IL-18 has a direct effect on TNF-α production by Th and NK cells (Puren *et al.*, 1998). Early literature data indicates that IL-18 is a cytokine that typically favours Th1 skewing. However, recent reports show that in the absence of IL-12 and the presence of IL-3 and cell types like basophils and mast cells, IL-18 can also induce a Th2 response (Hoshino *et al.*, 1999; 2000; Leite-De-Moraes *et al.*, 2001; Shi *et al.*, 2000b; Wild *et al.*, 2000). Hence the microenvironment is important in orchestrating IL-18 determined responses. Taken together, it is now clear that IL-18 is a typical pleiotropic cytokine involved in many inflammatory reactions.

IL-18 expression in inflammatory diseases

Various reports illustrate the involvement of IL-18 activity in different inflammatory diseases. IL-18 appears to play an important role in the pathology of sarcoidosis, Crohn's disease and rheumatic arthritis (Gracie *et al.*, 1999; Greene *et al.*, 2000; Joosten *et al.*, 2000; Kanai *et al.*, 2000; Monteleone *et al.*, 1999; Pizarro *et al.*, 1999; Shigehara *et al.*, 2000; Wei *et al.*, 2001). Also the onset of experimental autoimmune encephalomyelitis (EAE) seems to be mediated by IL-18 (Shi *et al.*, 2000a).

IL-18 expression in skin

Expression of IL-18 in skin has first been described by Stoll *et al.* (Stoll *et al.*, 1997) who showed that murine keratinocytes produce functional IL-18. Its involvement in skin inflammation was shown using skin-specific caspase-1 transgenic mice (Yamanaka *et al.*, 2000). Studies on the expression of IL-18 in human skin revealed that IL-18 is expressed in human keratinocytes predominantly in its unprocessed biologically inactive form (Companjen *et al.*, 2000a; 2000b; Mee *et al.*, 2000; Naik *et al.*, 1999). Furthermore it has been shown that keratinocytes also express the IL-18R (Mee *et al.*, 2000) and are capable of producing IL-18BP upon IFN-γ stimulation (Muhl *et al.*, 2000). Finally a study on the modulation of keratinocyte derived IL-18 expression during wound healing in skin showed that both IL-18 mRNA expression and protein secretion were altered upon stimulation with TNF-α, TGF-β and epidermal growth factor (EGF) (Kampfer *et al.*, 2000).

Psoriasis as a model for an inflammatory skin disease

Many studies on cutaneous inflammation use psoriatic skin as a model. The clinical characteristics of this chronic inflammatory skin disease is well known: erythema and extensive scaling of the skin. In psoriasis, KC show an unrestrained proliferation, their differentiation is altered and apoptosis is not induced when KC enter the stratum granulosum. This results in thickening of the epidermis, elongated rete ridges and absence of the granular layer (Fig. 1B). Since long it has been thought that psoriasis was primarily caused by an altered KC function. However, the observation of a dense infiltrate in psoriatic lesional skin, composed of different leukocytes in the dermis and to a lesser extent also in the epidermis, depending on the state of the disease, shows that inflammatory reactions play an important role in the pathogenesis of psoriasis. Early psoriatic skin lesions are characterized by the influx of $CD8^+$ and $CD4^+$ T cells (Onuma, 1994). Among the infiltrating cells in the epidermis, $CD4^+$ T cells are dominant in early lesions whereas $CD8^+$ T cells are dominant in chronic lesions. In later stages neutrophils invade the lesional skin causing the characteristic munro abscesses (Terui *et al.*, 2000).

Genetic factors determining the onset and progress of psoriasis

The acquisition of psoriasis is probably also dependent on genetic factors. Which genes determine the onset and progress of psoriasis in the western population is still indistinct, but a clear association with HLA-Cw6 has been found (Enerback *et al.*, 1997; Tiilikainen *et al.*, 1980). Several loci, which might contain susceptibility genes for psoriasis, have been assigned to different chromosomes, but a locus on chromosome 6 shows the highest association (Balendran *et al.*, 1999). Interestingly this locus also contains the MHC class I genes. Several candidates for the psoriasis susceptibility gene have been described but the function of the proteins encoded by these genes in the pathology of psoriasis is still unclear (Asumalahti *et al.*, 2000; Burden, 2000; Tazi Ahnini *et al.*, 1999; Tomfohrde *et al.*, 1994).

Which cells determine the onset of psoriasis?

Which factors initiate the onset of psoriasis is still subject of discussion, but that leukocytes play an important role is well accepted. For example, bone marrow transplantation (BMT) from psoriasis patients to non-psoriatic acceptors can result in the onset of psoriasis in the recipients (Gardembas-Pain *et al.*, 1990) and vice versa BMT from healthy donors to psoriatic acceptors can resolve the disease (Eedy *et al.*, 1990; Jowitt & Yin, 1990). Using the SCID-hu xenogenic transplantation model, in which normal appearing psoriatic skin (PN) is transplanted onto SCID mice, Boehncke (Boehncke *et al.*, 1997) and Wrone-Smith independently demonstrated that PN becomes psoriatic lesional skin (PP) when challenged with superantigen-stimulated autologous peripheral blood mononuclear cells (PBMC) (Wronesmith & Nickoloff, 1996).

Most investigators claim that psoriasis is a T cell mediated disease. This concept is supported by the following observations: (1) T cells are present in early lesions and precede the characteristic changes in psoriatic lesional skin; (2) treatment of psoriasis patients with

IL-2/diphteria toxin fusion proteins resolves the disease (Gottlieb *et al.*, 1995); (3) antibodies against CD4 have a therapeutic effect (Gottlieb *et al.*, 2000; Morel *et al.*, 1992) as well as T cell specific drugs like tacrolimus (FK506) (Jegasothy *et al.*, 1992) and cyclosporinA (Van Joost *et al.*, 1986); and (4) PN skin transplanted onto SCID mice is transformed into PP skin after injection of autologous T cells. During these studies it became clear that in this model both $CD4^+$ T cells and $CD8^+$ T cells play an important role in the onset of the disease (Nickoloff & Wrone-Smith, 1999).

It has to be stressed that the concept of induction of psoriasis by T cells is not totally explicative. Some researchers point out that also neutrophils, products from nerve cells or the production of nitric oxide play an important role in the onset of psoriasis (Nickoloff *et al.*, 2000b). It is therefore tempting to speculate that the onset of psoriasis may have more than one trigger which all feed into a final common pathway leading to the induction of psoriatic lesional skin.

Using the SCID-hu xenogenic skin transplantation model, Nickoloff *et al.* discovered a subset of T cells that play an important role in the pathogenesis of psoriasis (Nickoloff & Wrone-Smith, 1999). These T cells bear receptors originally characterized on NK cells like CD161, CD158 and CD94 (Mingari *et al.*, 1998) and hence are called NK-T cells. NK-T cells are present in psoriatic lesional skin and functional studies point out that they can induce a psoriatic lesional skin phenotype (Nickoloff *et al.*, 1999; 2000a).

NK-T cells are potent producers of IFN-γ and do so when triggered by CD1d expressed by psoriatic KC (Bonish *et al.*, 2000). Recent literature indicates that CD1 presents hydrophobic Ag, like lipids and glycolipids (reviewed by Porcelli & Modlin, 1999). Therefore the observation that the CD1d specific NK-T cells play an important part in the onset of the psoriatic phenotype, might suggest a role for a hydrophobic Ag in the pathology of psoriasis.

Expression of cytokines

Not surprisingly the regulation of pro- and anti-inflammatory cytokines like IL-1, IL-6, TNF-α, IFN-γ, IL-10 and IL-20, a newly discovered IL-10 homologue, plays a pivotal role in psoriasis (Asadullah *et al.*, 1998; Blumberg *et al.*, 2001; Castells-Rodellas *et al.*, 1992; Debets *et al.*, 1997; Grossman *et al.*, 1989; Wei *et al.*, 1999). The balance of IL-1 agonists and antagonists is severely dysregulated in psoriatic lesional skin (see below) and IL-6 and TNF-α expression are elevated compared to normal skin (Ettehadi *et al.*, 1994; Neuner *et al.*, 1991). Several studies demonstrated that the infiltrating $CD4^+$ T cells in lesional skin are mainly of the Th1 type (Austin *et al.*, 1999; Schlaak *et al.*, 1994; Szabo *et al.*, 1998; Uyemura *et al.*, 1993). Indeed elevated production of IFN-γ is a hallmark of psoriatic lesional skin. The importance of this cytokine in the pathogenesis of psoriasis has also been demonstrated by the observation that the skin of IFN-γ transgenic mice displays a psoriatic-like phenotype (Carroll *et al.*, 1997). This IFN-γ expression in skin might be induced by IL-12 and IL-18 which are produced by DC and KC (Companjen *et al.*, 2000a; 2000b; Mee *et al.*, 2000; Naik *et al.*, 1999; Yawalkar *et al.*, 1998; Yawalkar *et al.*, 1996). Finally, compared to normal skin

the expression levels of interferon regulating factors (IRF) 1 and 2 in psoriatic lesional skin is altered as well (van der Fits *et al.*, submitted). These factors are at least partly responsible for the upregulation of caspase-1 and IL-18 binding protein (IL-18BP) and consequently might affect the activity of IL-1β and IL-18 in psoriatic lesional skin.

During the pathogenesis of the disease, different chemokines like RANTES, IP-10 and IL-8 as well as the IL-8 receptor are dramatically increased in psoriatic lesional skin (Fukuoka *et al.*, 1998; Gillitzer *et al.*, 1991; Gottlieb *et al.*, 1988; Schulz *et al.*, 1993; Companjen *et al.*, submitted) and expression of several adhesion molecules like CD54 and E-selectin is elevated as well (Das *et al.*, 1994; de Boer *et al.*, 1994; Lee *et al.*, 1994; Paukkonen *et al.*, 1995).

A disease model for psoriasis

Summarizing these different observations leads to the following two-phase model for the onset and maintenance of psoriasis (Fig. 3). During the first phase, PN skin is triggered by elements which can initiate the onset of lesional skin (these elements might be pathogens or trauma). Subsequently IL-1β and IL-1α are released resulting in the upregulation of for instance IL-6, IL-8, RANTES, CD54 and E-selectin. LC presenting an as yet unknown psoriatic specific Ag, travel to the lymph node and activate naive T cells by stimulation and costimulation. That this co-stimulation plays an important role in the pathogenesis of psoriasis has been shown in a study in which the disease was cleared by downregulating co-stimulation through treatment with CTLA-4-Ig fusion proteins (Abrams *et al.*, 1999; 2000). The activated (NK-)T cells travel to the site of inflammation during the second phase and produce IFN-γ under the influence of IL-12 and IL-18 which leads to the induction of the psoriatic phenotype. CD40 expression is elevated via IFN-γ stimulation and triggering of CD40 on KC leads to the production of more IL-8 and RANTES resulting in the attraction of more T cells and neutrophils. Additionally CD40 stimulation also leads to the production of Bcl-x and consequently protects the KC against apoptosis. IFN-γ stimulation via induction of IL-1 results in the upregulation of regenerative maturation markers like transglutuminase kinase (TGk), keratin 16 and keratin 17 which are also upregulated during wound healing. Hence the psoriatic phenotype is established. As a reaction on the IFN-γ stimulation the IFN-γ receptor is downregulated and to counteract the IL-1 stimulus IL-1RII and IL-1ra are induced and secreted. In the case of normal inflammation the inflammatory response is reversed by anti-inflammatory cytokines like IL-10 and TGF-β. Why the inflamed skin by psoriasis patients is not normalized after inflammation is still unclear.

Introduction to the chapters

The human skin is prepared for inflammation. This is illustrated by the observation that an inflammatory response is common in simple skin trauma, bacterial and fungal infection and many skin diseases. It has been shown that normal human skin contains high levels of the pro-inflammatory cytokine IL-1α. Under normal circumstances, skin inflammation is prevented because IL-1 is safely stored intracellularly. However, upon stimulation by trauma

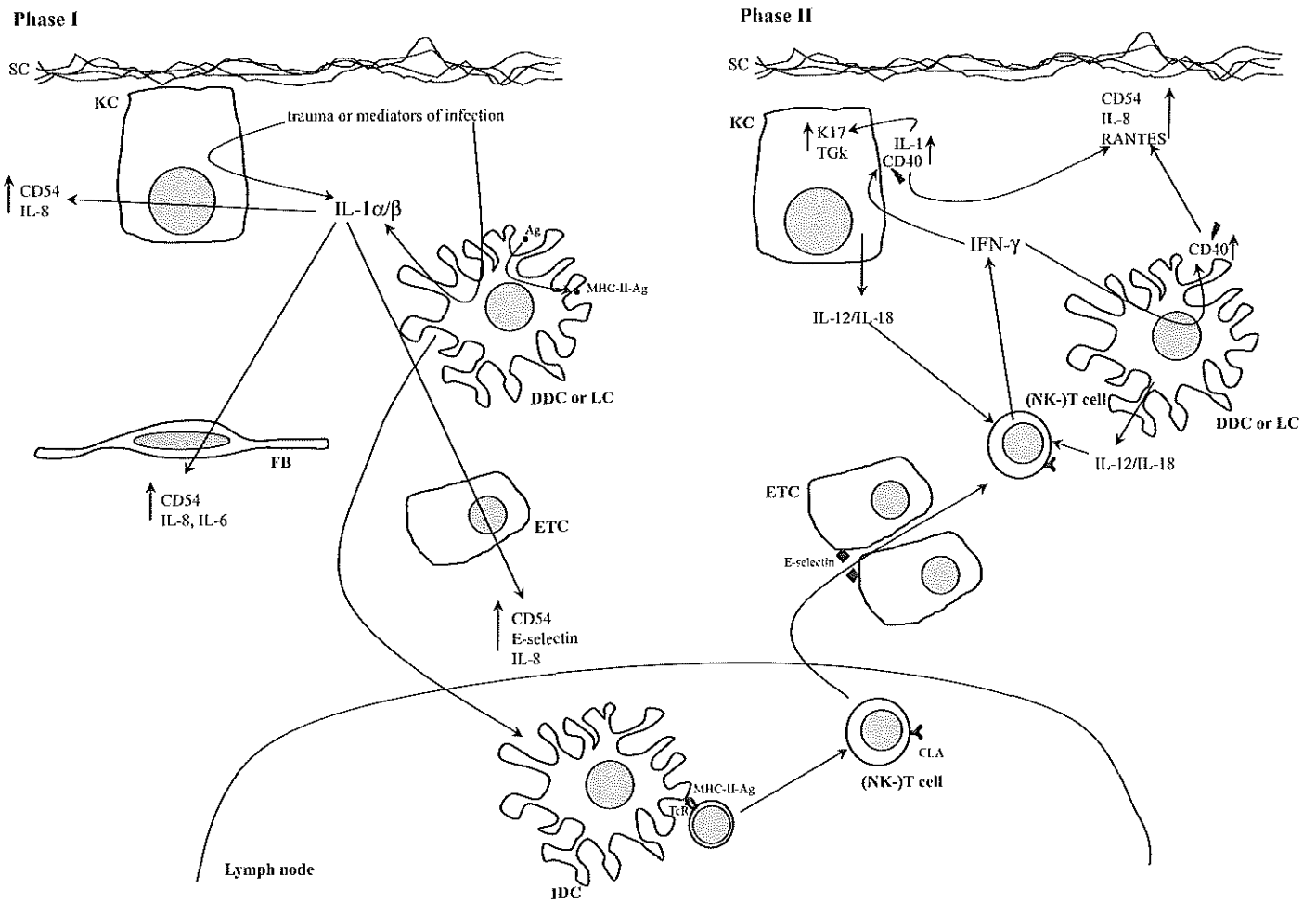
Phase I
SC
KC
trauma or mediators of infection
CD54
IL-8
IL-1α/β
Ag
MHC-II-Ag
DDC or LC
FB
CD54
IL-8, IL-6
ETC
CD54
E-selectin
IL-8
Lymph node
IDC
MHC-II-Ag
TCR
CLA
(NK-)T cell
Phase II
SC
KC
K17
TGk
IL-1
CD40
CD54
IL-8
RANTES
IFN-γ
CD40
IL-12/IL-18
(NK-)T cell
DDC or LC
IL-12/IL-18
ETC
E-selectin

Fig. 3. A two-phase model for the onset and maintenance of psoriasis. The pathology of psoriasis is depicted here as a two-phase model. During the initiation phase (phase I), resident cells in the skin express pro-inflammatory cytokines like IL-1 upon challenge by for example trauma, infection, bacterial residues or an as yet unknown Ag. This results in the expression of chemokines and adhesion molecules by keratinocytes, fibroblasts or endothelial cells. During phase II (NK-)T cells, neutrophils and monocytes (not shown) enter the inflamed skin. Subsequently these cells express different mediators of inflammation. IFN-γ is produced by (NK-)T cells upon stimulation with IL-12 and IL-18 derived from Langerhans cells and/or keratinocytes, resulting in the induction of the phenotype typical for psoriatic lesional skin. This includes the upregulation of CD40, CD54 (ICAM-1), maturation markers like keratin 17 (K17), transglutaminase kinase (TGk) and chemokines to attract more leukocytes to the site of inflammation. KC: keratinocyte; FB: fibroblast; LC: Langerhans cell; DDC: dermal dendritic cell; ETC: endothelial cell. (see left page).

or infection IL-1 is released, initiating an inflammatory reaction resulting in the induction and expression of a cascade of pro-inflammatory cytokines. To understand the regulation of inflammatory pathways in skin it is neccessary to investigate how pro- and anti-inflammatory cytokines are expressed and regulated in normal and inflamed skin. Therefore it is important to know if these cytokines are expressed, and if so how they are activated. Finally we have to determine their function in inflammatory responses. The emphasis in this thesis is on the expression and function of some of the members of the IL-1/IL-18 family of proteins. The postulated hypotheses tested in this thesis are:

1. *Epidermal cell derived IL-1β can be activated by proteases other than caspase-1.*
   Activation of IL-1β is thought to be dependent on processing by caspase-1. In chapter 2 we show that in normal and psoriatic lesional skin, IL-1β activation is not dependent on caspase-1 but can also be processed and activated by other proteases into a biologically active molecule.
2. *Human keratinocytes express high levels of IL-18 and predominantly produce the unprocessed form*
   Because IL-18 is a pro-inflammatory cytokine and a member of the IL-1 family of proteins, it is conceivable that the expression and regulation of IL-18 in normal human skin, like IL-1α, IL-1ra and IL-1β, is crucial for maintenance of the pro-inflammatory environment in skin. The expression level of IL-18 was investigated in normal skin (chapter 3) and in psoriasis as a model of skin inflammation (chapter 4). We found that normal human keratinocytes are major producers of IL-18 when compared to other cells like monocytes and that this IL-18 is expressed predominantly in the unprocessed form (chapter 3).
3. *IL-18 expression in psoriatic lesional skin is elevated compared to normal skin.*
   The balance of IL-1 agonists and antagonists is disturbed in psoriasis. Furthermore, IFN-γ expression is elevated in psoriatic lesional skin. Because IL-18 is involved in the production of IFN-γ, we studied its expression in psoriatic lesional skin. Although we found that IFN-γ levels were indeed elevated, IL-18 expression was not (chapter 4).
4. *Induction of IL-6 and IL-8 expression through CD40 stimulation is mediated via IL-1.*
   To investigate the regulation of inflammatory processes in skin it is important that the

microenvironment in culture remains intact. Therefore we developed an *ex vivo* skin organ culture system, to study the effects of mediators of inflammation on different inflammatory responses in normal human skin. In chapter 5 we show, using this model, that the modulation of expression of different markers by IL-1β and LPS can be investigated. The effects of IL-1β and LPS could be antagonized by both specific (IL-1ra) and general (dexamethasone) inhibitors. During the development of the skin organ culture system we observed that IL-1β is a potent inducer of IL-6 and IL-8 in intact human skin. Recent reports show that CD40 stimulation of human keratinocytes *in vitro* also leads to the induction of IL-6 and IL-8 expression. Using the skin organ culture system we show that CD40 stimulation is also relevant in intact skin and that the CD40/IFN-γ mediated upregulation of IL-6 and IL-8 in human skin is partly mediated via the endogenous production of IL-1 (chapter 6).

Chapter 2

BIOLOGICAL ACTIVITY OF HUMAN EPIDERMAL INTERLEUKIN-1β: COMPARISON WITH RECOMBINANT HUMAN INTERLEUKIN-1β

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ABSTRACT

We have recently presented evidence that human plantar stratum corneum and psoriatic scales contain biologically active interleukin–1β (IL–1β) which has been activated in a process not involving interleukin-1β converting enzyme. The aims of the present study was to compare this form of native IL–1β with recombinant mature human IL–1β as regards activity and the effects of inhibitors.

In an assay based on the ability of IL-1 to induce the expression of E-selectin in cultured endothelial cells, the maximal activity of IL–1β partially purified from plantar stratum corneum and recombinant IL–1β was approximately the same. The specific activity was slightly higher for recombinant IL–1β, although this difference was within one order of magnitude. An antibody to IL–1β caused total inhibition of both forms of IL–1β with no significant differences in dose-response curves of the antibody. Immunochemical analyses and experiments with neutralising antibodies specific for IL–1α and tumor necrosis factor α (TNFα) verified that the observed activity in the partially purified preparation was due to IL–1β, and not to IL–1α or TNFα. There were no significant differences between the two forms of IL–1β as regards the inhibitory effects of recombinant IL-1 receptor antagonist.

Partially purified IL–1β from plantar stratum corneum and from psoriatic scales were both highly active in the D10 proliferation assay. This activity could be totally inhibited with an IL–1β specific antibody.

This work thus confirms the presence in plantar stratum corneum and psoriatic scales of biologically active IL–1β. Alternatively activated IL–1β in the epidermis should be considered in future studies on skin biology and pathophysiology.

INTRODUCTION

The multipotent proinflammatory cytokine interleukin-1 (IL-1) occurs in at least two forms, IL–1α and IL–1β. Both forms are produced as 31 kD precursors, which are proteolytically processed to 17 kD mature forms. Whereas IL–1α is biologically active in precursor as well as mature form, only the mature form of IL–1β is active (Dinarello, 1984; Dinarello & Savage, 1989). In IL–1β producing cells, especially cells of the monocyte/macrophage lineage, IL–1β activation is mediated by a highly specific enzyme, interleukin-1 converting enzyme (ICE), which catalyses cleavage of the precursor C-terminal of Asp-116 (Black *et al.*, 1989; Cerretti *et al.*, 1992; Kronheim *et al.*, 1992; Sleath *et al.*, 1990; Thornberry, 1994).

IL-1 may play an important role in skin pathophysiology. Since it is produced by keratinocytes also under normal conditions (Barker *et al.*, 1991; Bell *et al.*, 1987; Kupper *et al.*, 1986; McKenzie & Sauder, 1990), its release by microbial, chemical, and physical insults may be a very early event in the induction of immune and inflammatory reactions (Barker *et*

al., 1991; Dinarello & Savage, 1989; Luger & Schwarz, 1990). Although IL–1α and IL–1β have very similar activities in most systems (Hirsch *et al.*, 1996; Saklatvala, 1995), there are situations in which IL–1β may play a key role. In delayed type hypersensitivity the presence of active IL–1β may be mandatory (Enk *et al.*, 1993; Enk & Katz, 1995; Shornick *et al.*, 1996). Its effect is associated with the activation and function of Langerhans cells (Kitajima *et al.*, 1995), and can not be substituted for by other proinflammatory cytokines such as IL–1α and TNFα. Many of the early events in the development of the psoriasis lesion could be attributed to IL-1. It has been shown that in the psoriasis lesion there is an upregulation of IL–1β production, whereas IL–1α is downregulated (Cooper *et al.*, 1990). It has also been shown that the autologous mixed epidermal cell - T-lymphocyte reaction in psoriasis can be inhibited by neutralising antibodies to IL–1β (Prens *et al.*, 1996).

There are at least two sources of epidermal IL–1β; keratinocytes (Barker *et al.*, 1991; Bell *et al.*, 1987; Kupper *et al.*, 1986; McKenzie & Sauder, 1990) and Langerhans cells (Enk *et al.*, 1993; Kitajima *et al.*, 1995). Of these two cell types, however, only Langerhans cells produce active ICE (Groves *et al.*, 1996a; Kitajima *et al.*, 1995). It has therefore been assumed that only IL–1β derived from these cells can take part in early epidermal inflammatory and immune reactions (Enk *et al.*, 1993; Jonuleit *et al.*, 1996; Kitajima *et al.*, 1995). Due to the lack of ICE-activity in keratinocytes it has been difficult to assign a role for keratinocyte-derived IL–1β. It has been suggested that it may not take part in early phases of a disease process, but serve to augment an inflammatory reaction after having become activated by proteases released from invading inflammatory cells (Kupper, 1990).

This picture may have to be modified due to results from studies on gene deficient mice. In mice with no functioning ICE-gene the production of mature IL–1β is drastically decreased, but there may not be a total absence of active IL–1β (Li *et al.*, 1995). Interestingly, these mice were still able to develop a cutaneous contact hypersensitivity reaction. This was in marked contrast to mice with a deletion of the IL–1β gene, in which contact sensitisation was drastically impaired (Shornick *et al.*, 1996). These results suggest that there may be a physiologically relevant alternative activation mechanism not involving ICE for IL–1β in the skin.

We have recently presented evidence that plantar stratum corneum as well as psoriatic scales contain biologically active IL–1β which has been activated by enzymes other than ICE (Brattsand & Egelrud, 1998; Egelrud & Jonsson, 1997; Lundqvist & Egelrud, 1997; Nylander-Lundqvist *et al.*, 1996). We have also shown that an epidermis-specific protease, stratum corneum chymotryptic enzyme, can catalyse the conversion of inactive IL-1β precursor to active IL–1β in vitro (Nylander-Lundqvist & Egelrud, 1997). Our results were contradictory to previous results by others, who were unable to detect active IL–1β in plantar stratum corneum (Camp *et al.*, 1990), or in extracts of dermatome specimens of psoriatic skin (Cooper *et al.*, 1990). We therefore found it important in the present work to extend our studies to a more thorough comparison of our preparations of epidermal IL–1β with recombinant mature human IL–1β as regards activity in different assay systems and the effects of inhibitors.

MATERIALS AND METHODS

Monoclonal neutralising antibodies to human IL–1β and TNFα were purchased from R&D Systems Europe (Abingdon, UK), and to human IL–1α from Genzyme (Cambridge, MA). Recombinant human IL-1 receptor antagonist (IL-1ra) was purchased from R&D. ELISA-kits for IL–1α, IL–1β, IL-1ra, and TNFα were obtained from R&D, and used according to the instructions provided by the manufacturer. All dilutions were carried out in Medium 199 with Hank's salts (Life Technologies, Paisley, UK). Recombinant human IL–1β was purchased from R&D. It had a sequence corresponding to that of mature, ICE-processed IL–1β except for an N-terminal methionine (N-terminal sequence Met-Ala-117-IL-1β). The provided stock solution was diluted in Medium 199 to get an IL–1β concentration of $20x10^3$ pg/ml, which was aliquoted and stored at -70°C until used in the experiments.

Extracts of plantar stratum corneum were prepared as previously described (Nylander-Lundqvist *et al.*, 1996), and subjected to cationic and anionic ion exchange chromatography as previously described for extracts of psoriatic scales (Lundqvist & Egelrud, 1997). The preparation obtained was checked by SDS-polyacryalmide gel electrophoresis followed by immunoblotting with an IL–1β specific antibody (Nylander-Lundqvist *et al.*, 1996), and analysed by ELISA for IL–1β content. A stock solution containing 20 ng/ml of stratum corneum derived IL–1β was prepared in Medium 199. This solution was analysed by ELISA also for IL–1α, IL-1ra, and TNFα (Table 1), aliquoted, and stored at -70 °C. IL–1β from psoriatic scales was partially purified as previously described (Lundqvist & Egelrud, 1997). A stock solution containing 100 ng/ml in Medium 199 was used for analysis of IL-1 activity in the D10 assay (see below).

Table 1. Results of analyses by ELISA of the stock solution of IL–1β partially purified from plantar stratum corneum used in the experiments presented in this paper. n. d. = not detectable.

Substance	Concentration
IL-1 β	20 ng/ml
IL-1 α	n. d. (< 7 pg / ml)
TNF α	n. d. (< 16 pg / ml)
IL-1 ra	n. d. (< 31 pg / ml)

Biological IL-1 activity was analysed as the ability to induce expression of E-selectin in cultured human umbilical vein endothelial cells (HUVEC) (Nylander-Lundqvist *et al.*, 1996). Preparations to be analysed, appropriately diluted in Medium 199, were incubated with HUVEC in 96-well titer plates for 4 h at 37°C. The cells were then fixed with 4% paraformaldehyde and the amount of induced cell-bound E-selectin quantified by a two-step ELISA using monoclonal antibodies to E-selectin followed by secondary alkaline-phosphatase-conjugated antibodies (Nylander-Lundqvist *et al.*, 1996). The absorbance at 405 nm was read after incubation with the substrate *p*-phenyl-phosphate. The results were expressed

as change in absorbance per hour (ΔA_{405}/h). When experiments with neutralising antibodies were performed all solutions to be analysed were pre-incubated at 37°C for 1 h prior to addition to HUVEC. The detection limit for recombinant human IL-1β in this assay is approximately 100 pg/ml.

IL-1 activity was also measured using a sub line (D10(N4)M) of the murine T cell line D10.G4 (Hopkins & Humphreys, 1989; Orencole & Dinarello, 1989). For neutralisation of IL-1β in this assay a polyclonal sheep antibody (Glaxo, Mol.Biol.Lab., Geneva) was used. Recombinant IL-1β obtained from Glaxo was used as standard in this assay. Measurements were carried out in triplicate. One unit of IL-1 activity in this assay is defined as the amount of IL-1 which gives half maximal stimulation.

RESULTS

Results of the analyses of immunoreactive IL-1β, IL-1α, TNFα, and IL-1ra in the preparation of partially purified IL-1β from plantar stratum corneum are shown in Table 1. Whereas the preparation contained 20 ng/ml IL-1β, the amounts of IL-1α, TNFα, and IL-1ra were below detection limits.

Fig. 1 shows dose-response curves for IL-1 from plantar stratum corneum and recombinant human mature IL-1β in the E-selectin assay. Both types of IL-1β induced expression

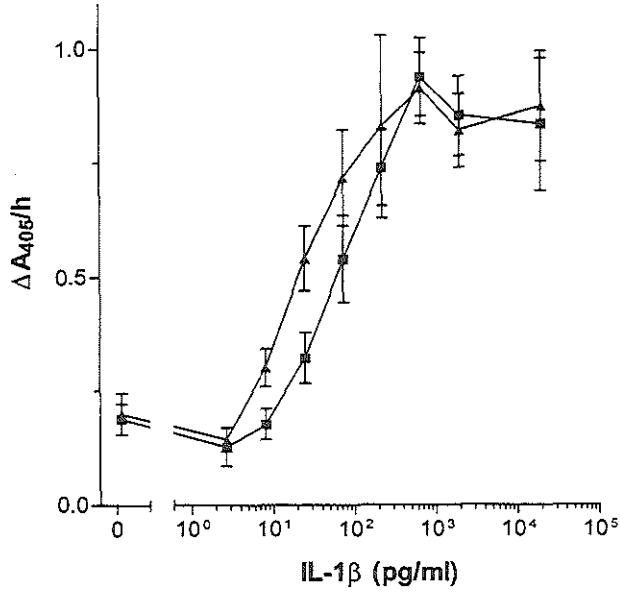

Fig. 1 Dose-response curves for the stimulation of E-selectin expression in HUVEC by IL-1β partially purified from human plantar stratum corneum (*filled squares*) and recombinant human IL-1β (*filled triangles*). Mean and SD, n = 8.

of E-Selectin by HUVEC in a dose dependent manner. The ascending parts of the dose-response curves of the two IL-1β preparations were parallel. Maximal induction with the keratinocyte IL–1β and rIL–1β did not differ significantly. The shift to the right of the dose response curve for epidermal IL–1β as compared to recombinant IL–1β suggested a lower specific activity of the former. This difference was within one order of magnitude.

The effect of pre-incubation of the IL–1β preparations with increasing concentrations of a neutralising IL–1β-specific monoclonal antibody on the E-selectin inducing activity in HUVEC is shown in Fig. 2. For both preparations the antibody caused a dose dependent inhibition of the E-selectin inducing activity, which was essentially total at approximately 100 ng of antibody per ml. No significant differences between the two IL–1β preparations were found as regards the dose-dependency of the antibody effect. No inhibition of the E-selectin inducing activity of the epidermal IL–1β preparation could be found with antibodies to IL–1α or TNFα (results not shown).

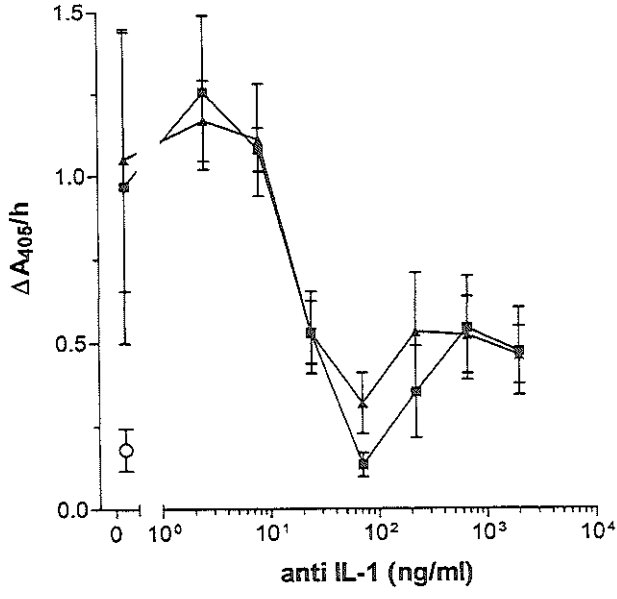

Fig. 2 Inhibition of the induction of E-selectin in HUVEC by IL–1β partially purified from human plantar stratum corneum (*filled squares*) and recombinant human IL–1β (*filled triangles*) by an IL–1β specific monoclonal mouse antibody. The antibody at concentrations shown were pre-incubated with 2 ng/ml of IL–1β for 1 h at 37 °C prior to addition to HUVEC. *Open circle* = HUVEC incubated with medium without additions. Mean and SD, n = 8.

Fig. 3 shows the effect on the E-selectin-inducing activity in HUVEC when IL–1β from plantar stratum corneum and recombinant IL–1β at fixed concentrations were mixed with increasing concentrations of IL-1ra. IL-1ra caused a dose dependent inhibition of both preparations. There was no significant difference in this respect for epidermal IL–1β and recombinant IL–1β. In the experiment shown the ratio of IL-1ra to IL–1β was in the range

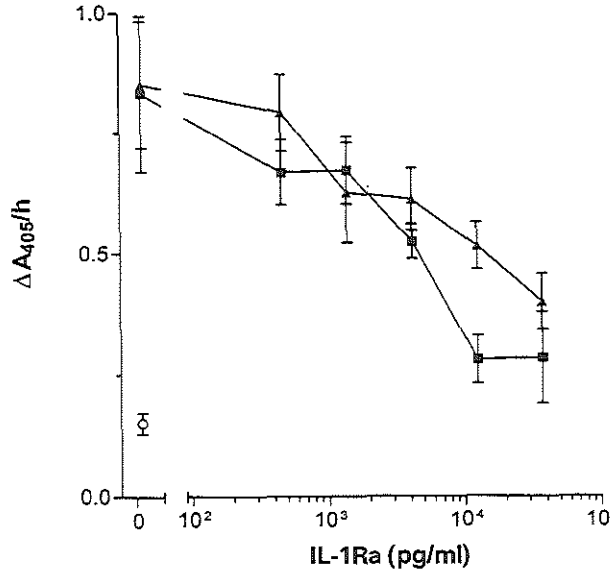

Fig. 3 Inhibition of the induction of E-selectin in HUVEC by IL–1β partially purified from plantar stratum corneum (*filled squares*) and recombinant human IL–1β (*filled triangles*) by IL-1ra. IL-1ra at concentrations shown were mixed with IL–1β at 1 ng/ml in medium immediately prior to addition to HUVEC. *Open circle* = HUVEC incubated with medium without additions. Mean and SD, n = 8.

0/1 - 37/1. The inhibition at the highest concentration of IL-1ra was not total. In other experiments (not shown), an essentially total inhibition of both IL–1β preparations was obtained at an IL-1ra/ Il–1β ratio of 100/1 and above.

In the D10 proliferation assay the specific activity of IL–1β partially purified from plantar stratum corneum and from psoriatic scales were found to be of the same order of magnitude as the specific activity of recombinant IL-1β. For both preparations the IL-1 activity could be totally inhibited with IL–1β– specific antibodies (Table 2).

Table 2. Summary of results from analyses of IL-1 activity in preparations of partially purified IL-1b from plantar stratum corneum and psoriatic scales in the D 10 proliferation assay. Mean and SD; n = 3. N.S. = not significantly different from blank incubations.

Source	IL-1 activity (U/mg)	
	No antibody	With anti-IL-1β
Recombinant IL-1β	4.8×10^8	-
Plantar stratum corneum	$1.6 \pm 0.6 \times 10^8$	N.S.
Psoriatic scales	$2.8 \pm 1.8 \times 10^8$	N.S.

DISCUSSION

In two recent reports we showed that hypertrophic but otherwise normal plantar stratum corneum as well as psoriatic scales contained IL–1β with a higher molecular mass and a more acidic isoelectric point as compared to recombinant mature human IL–1β. We also presented evidence that the IL–1β from these two epidermal sources could induce E-selectin expression in HUVEC (Lundqvist & Egelrud, 1997; Nylander-Lundqvist *et al.*, 1996). The major IL-1β species in plantar stratum corneum with isoelectric point 6.1 has been purified. It was found to have an N-terminal histidine and an ensuing amino acid sequence suggesting that it had been formed by cleavage of the IL-1β precursor C-terminal of valine 114. Comparison of isoelectric points of the IL-1β species prepared from epidermal extracts with isoelectric points of recombinant variants of IL-1β was compatible with the presence in plantar stratum corneum and psoriatic scales of His-115-IL-1β, isoelectric at pH 6.1, as well as of Val-114-IL-1β, isoelectric at pH 6.3 (Brattsand & Egelrud, 1998; Egelrud & Jonsson, 1997). Since processing of the IL-1β precursor by ICE produces Ala-117-IL-1β, isoelectric at pH 6.9, these results strongly suggested that there is an alternative activation mechanism for IL-1β in the epidermis not involving ICE, but one or several proteases which catalyse cleavage of the IL–1β precursor at amino acid residues N-terminal of the ICE cleavage site.

Our findings of active IL–1β in plantar stratum corneum and psoriatic scales seemed to be in variance with previously reported results (Camp *et al.*, 1990; Cooper *et al.*, 1990; Groves *et al.*, 1996a; Mizutani *et al.*, 1991). In this work we wanted to obtain further evidence that the activity we have observed in our epidermal preparations indeed is due to IL–1β. We also wanted to examine whether there are any differences between recombinant mature IL–1β and epidermal IL–1β which could possibly explain the differences between our results and the results of others.

An IL–1β preparation from plantar stratum corneum was used for comparative studies. This preparation was shown not to contain detectable amounts of IL–1α or TNFα. It could induce the expression of E-selectin in HUVEC with a maximal activity which did not significantly differ from recombinant IL–1β, and with a specific activity (expressed as E-selectin inducing activity per mg IL–1β) which was within one order of magnitude of the specific activity of recombinant IL–1β. This activity could be inhibited by antibodies to IL–1β and by IL-1ra, but not by antibodies to IL–1α or TNFα. These results, taken together with the facts that the only cytokines which in addition to IL–1β are known to induce the expression of E-selectin in HUVEC are IL–1α and TNFα (Klein *et al.*, 1995), that IL-1ra binds to the same receptors as IL–1α and IL–1β (McMahan *et al.*, 1991), and that human endothelial cells have been shown to express only the type I IL-1 receptor (Colotta *et al.*, 1993), strongly suggest that plantar stratum corneum contains biologically active IL–1β.

A possible explanation to our divergent findings could be the fact that we have used a bioassay for IL-1 activity based on endothelial cells, whereas in most other studies lymphoid cells have been used. This was ruled out, however, by the results obtained in the D10 assay. For two preparations, one prepared from plantar stratum corneum and one from psoriatic

scales, we found IL-1 activity which could be inhibited by antibodies to IL-1β. Also in this assay the specific activity of epidermal IL-1β was within one order of magnitude of the specific activity of recombinant IL-1β.

No significant differences between epidermal IL-1β and recombinant IL-1β could be found as regards maximal activity and the inhibitory effects of IL-1β antibodies and IL-1ra. In agreement with a previous report (Nylander-Lundqvist *et al.*, 1996), the results with the E-selectin assay as well as the D10 assay suggested that epidermal IL-1β may have a somewhat lower specific activity than recombinant IL-1β. This is supported by a report that IL-1β activated by digestion of the IL-1β precursor with chymotrypsin had about one fifth of the specific activity of mature IL-1β (Hazuda *et al.*, 1991). We can not rule out, however, that the observed differences may be due to as yet unidentified inhibitors in the partially purified preparations.

A lower specific activity of epidermal IL-1β may reflect lower affinity for the signal transducing IL-1 receptor type I. Since the endothelial cells express only this type of IL-1 receptor, a major difference in receptor affinity should have been possible to detect in the experiments with IL-1ra. We could not, however, detect any significant differences between epidermal IL-1β and mature IL-1β in these experiments. A clarification on this point will have to await binding studies with pure variants of IL-1β. In any case it seems unlikely that the observed difference in specific activity between the two types of IL-1β should be of any biological significance.

In conclusion we have presented further evidence for the presence of biologically active IL-1β in plantar stratum corneum and in psoriatic scales. No major differences were found between this type of IL-1β and mature IL-1β. These findings, together with the previously presented evidence of an alternative activation mechanism of epidermal IL-1β, should be considered and further evaluated in future studies on skin biology and pathophysiology.

Acknowledgedments

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Chapter 3

HUMAN KERATINOCYTES ARE MAJOR PRODUCERS OF IL-18: PREDOMINANT EXPRESSION OF THE UNPROCESSED FORM

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ABSTRACT

The cytokine network in the skin is a tightly regulated system in which IL-1 isoforms as well as their receptors and antagonists have a central role. The recently discovered IL-1 isoform IL-18 (also known as interferon gamma inducing factor (IGIF) or IL-1γ), promotes IFN-γ expression by T cells in concert with IL-12. Because IFN-γ plays an important role in many inflammatory skin diseases by facilitating the development of Th1 cells, it is important to elucidate the role of mediators which regulate the production of this cytokine. We demonstrate that human keratinocytes constitutively express IL-18 at the mRNA as well as at the protein level. The protein was mainly expressed intracellularly in the 24 kD unprocessed proform, but was also secreted. Histochemistry revealed a diffuse staining of IL-18 in the epidermis of normal skin, which is in line with our *in vitro* data. Furthermore, we show that the level of IL-18 expressed in freshly isolated normal human epidermal cells, whether or not containing HLA-DR$^+$ cells, significantly exceeded the expression levels of other cell types like monocytes and bronchial epithelial cells. Finally, our results show that stimulation of the keratinocyte cell line HaCaT with PMA LPS or IL-1β does not significantly affect intracellular or released (pro)-IL-18 levels. These experiments show for the first time that human keratinocytes relative to monocytes, PBMC or leukocytes produce a considerable larger amount of pro-IL-18, which is also readily released. High constitutive levels of IL-18 may contribute to the skewing towards a Th1-like environment, which is apparent in many human inflammatory skin diseases.

INTRODUCTION

IL-18 (interferon-γ inducing factor, IGIF) was initially discovered in studies of IFN-γ production in a *Propionibacterium acnes*-induced model of toxic shock (Nakamura *et al.*, 1989). The amino acid sequence of IL-18 is distinct from other cytokine sequences, but structural analysis and fold recognition studies suggest that IL-18 is a member of the IL-1 family (Bazan *et al.*, 1996). This is supported by the observation that IL-18, like IL-1β, is processed from an inactive precursor molecule into its bioactive form by caspase-1 (interleukin-1 converting enzyme, ICE) (Akita *et al.*, 1997; Fantuzzi & Dinarello, 1999; Ghayur *et al.*, 1997). Furthermore it has been shown that IL-18 is bound by members of the IL-1R family, namely IL-1Rrp1 and AcPL (Akita *et al.*, 1997 and Debets in preparation; Born *et al.*, 1998) and that IL-18 activates classical IL-1 signaling components, such as myD88, IRAK-1, TRAF-6 and NF-κB (Adachi *et al.*, 1998; Kojima *et al.*, 1998; Robinson *et al.*, 1997; Thomassen *et al.*, 1998). Despite the structural similarity, human IL-18 shows only 15-18% sequence homology with the IL-1 family of cytokines. To date the most well documented biological effects of IL-18 are induction and enhancement of IFN-γ production by Th1 cells (in combination with IL-12) (Ahn *et al.*, 1997; Robinson *et al.*, 1997; Yoshimoto *et al.*, 1998), enhancement of Th1

proliferation, and stimulation of cytolytic activity of NK-cells (Dao *et al.*, 1998; Kanakaraj *et al.*, 1999; Kohno *et al.*, 1997). Taken together, these data show that IL-18 plays an important role in inflammation.

In skin, interaction between keratinocytes and leukocytes is of vital importance for maintaining homeostasis, especially during defense (Barker *et al.*, 1991; Williams & Kupper, 1996). The balance between cytokines such as TNF-α, IFN-γ, IL-12, IL-4, IL-10 and members of the IL-1 family plays a pivotal role in maintaining the immune milieu of the skin (Horrocks *et al.*, 1997; Prinz *et al.*, 1994; Schlaak *et al.*, 1994). Expression and activity of these cytokines, expressed by different cell types (including keratinocytes) is disturbed in some skin disorders. Psoriatic lesions, for instance, are characterized by a disturbed balance between agonists and antagonists of the IL-1 system (Debets *et al.*, 1997) and by a disturbed responsiveness to (increased levels) of IFN-γ (Schmid *et al.*, 1994; Uyemura *et al.*, 1993). The main cytokines responsible for the induction and expression of IFN-γ are IL-12 and IL-18. Upregulation of IL-12 expression has been reported in psoriasis (Taha *et al.*, 1998; Yawalkar *et al.*, 1998). In contrast, little is known about the regulation of IL-18 expression and processing in human skin and keratinocytes. Given the obvious involvement of the IL-1 system in inflammatory skin diseases and the properties of IL-18, a role for IL-18 in the skin can be expected. Murine keratinocytes constitutively produce IL-18 mRNA and its expression can be upregulated *in vivo* using contact allergens, but not by irritants. Keratinocytes turned out to be a major source of active protein (Stoll *et al.*, 1997; Xu *et al.*, 1998). One study in human skin on the accessory cell function of epidermal cells (EC) in the mixed epidermal cell leukocyte reaction (MECLR), showed that endogenously produced IL-18 was of minor importance in this system (Suemoto *et al.*, 1998). Because regulation of IL-18 expression might be essential in inflammatory reactions in the skin, i.e. by maintaining the delayed type hypersensitivity (DTH) like environment and Th1 polarization, we aimed to analyze whether human keratinocytes are able to produce IL-18 and how its production is regulated. In this report we show that IL-18 mRNA and protein are constitutively expressed by human epidermal cells *in vitro* as well as *in vivo* and by the human keratinocyte cell line HaCaT. Intracellular IL-18 in epidermal cells and HaCaT cells is mainly in the unprocessed 24 kD pro-form. We observed considerably higher levels of pro-IL-18 in normal epidermal cells and HaCaT cells than in peripheral blood leukocytes and the bronchial epithelial cell line BEAS2B. Finally, strong stimulation of HaCaT cells, BEAS2B cells and PBMC by PMA, LPS or IL-1β did not affect intracellular or released levels of total IL-18 protein. These results show that human keratinocytes are major producers of IL-18, which is predominantly expressed in the unprocessed form.

METHODS

Epidermal cells

Dermatome specimens were obtained after informed consent from normal skin of

patients undergoing breast or abdominal plastic surgery in the University Hospital Rotterdam. The epidermis was detached from the dermis by trypsinisation. Epidermal cell suspensions (ECS) were prepared from epidermal sheets by incubating them at 37°C for 45 min in trypsinisation buffer (0.025% trypsin and 0.1% EDTA in PBS), to which 0.25% DNAse was added for the last 15 min of the incubation (Boehringer Mannheim, Mannheim, Germany). The cell suspension was filtered through a 30 µm mesh gauze and suspended in PBS containing trypsin inhibitors (Boehringer Mannheim), and adjusted to a concentration of 4×10^6 epidermal cells (EC) per ml.

HLA-DR$^+$ cells were depleted from total ECS, using para-magnetic "Dynabeads" coated with anti-HLA-DR mAb (Dynal, Oslo, Norway). The mixture of EC and dynabeads was centrifuged gently for 10 min (200×g at room temperature (RT)) and incubated for 30 min on ice. The HLA-DR$^+$ cells were removed from the total ECS using the Dynal magnetic particle concentrator (MPC-1®). ECS depleted of Langerhans and other HLA-DR$^+$ cells (ECS$^-$) were resuspended in 1 ml of Hanks' Balanced Salt Solution (HBSS) (Gibco BRL, Paisley, Scotland), supplemented with a broad mixture of protease inhibitors (Complete™, Boehringer Mannheim). Intracellular proteins were extracted by 4 freeze-thaw cycles and stored at -80°C.

Leukocytes, PBMC and monocytes

Heparanized blood was drawn by venipuncture of healthy volunteers after informed consent. Leukocytes were isolated using a standard procedure. In brief, 2 ml of whole blood was diluted in 50 ml lysis buffer (NH_4Cl, pH 7.4), incubated on ice for 20 min and centrifuged for 5 min (450xg at RT) followed by washing with PBS. Leukocytes were counted using a hemacyto-counter (Coulter ZM, Beckman Coulter, Fullerton, CA) and the cells were resuspended in HBSS, supplemented with a broad mixture of protease inhibitors (Boehringer Mannheim) and adjusted to a concentration of 75×10^6 cells/ml. PBMC were isolated using standard Ficoll gradient centrifugation. Normal human monocytes were kindly provided by Prof. Drexhage (dept. of Immunology, Erasmus University Rotterdam, The Netherlands) and were isolated by layering PBMC on a percoll solution followed by centrifugation for 40 min (300xg at RT). Cells at the interface were isolated and counted. Monocyte isolation resulted in a average purity of 88% (range: 83-93%). The intracellular proteins from the cells were isolated by 4 freeze-thaw cycles.

Cell lines and stimulation experiments

HaCaT cells (Boukamp *et al.*, 1988) were cultured in RPMI 1640 (GibcoBRL) supplemented with 5% Fetal Calf Serum (FCS) (BioWhittaker, Walkersville, MD) at 37°C and 5% CO_2. The cells were passaged every 5 days. Prior to an experiment, HaCaT cells were detached by using trypsinisation buffer, rinsed in PBS, taken up in IMDM (GibcoBRL) containing 1% human serum (HS) (Sigma, St. Louis, MO) and plated at 5×10^5 cells per well in 24-well plates (Nunc, Roskilde, Denmark). The cells were allowed to adhere to the plates for 18 hours, after which they were rinsed with PBS. IMDM containing 1% HS with or without

stimuli was then added to the cells which were subsequently cultured for different periods of time (0-72 hrs). HaCaT cells were stimulated with 10 µg/ml lipopolysaccharide (LPS) (Brunschwig, Amsterdam, The Netherlands), 10 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) or 250 U/ml IL-1β (Glaxo, Research Triangle Park, NC). Supernatants were collected after centrifugation for 1 min (17000×g at 4°C) and stored at -80°C until further analysis. Cells were detached as described above, washed in PBS and resuspended in 400 µl HBSS, supplemented with a broad mixture of protease inhibitors (Boehringer Mannheim). To obtain intracellular proteins, the cells were subjected to four cycles of freeze thawing, and the extracts were stored at -80°C until analysis. The bronchial epithelial cell line BEAS2B was cultured as described previously (van der Velden *et al.*, 1998) and PBMC were isolated as described above. Both cell types served as an control and were cultured and stimulated identical to the HaCaT cells.

Immunohistochemistry

Biopsies were obtained from 5 patients undergoing breast or abdominal plastic surgery. The biopsies were snap frozen in liquid nitrogen and cryosections were cut using a cryostat (Jung Frigocut 2800 E, Leica, Rijswijk, The Netherlands). Sections were fixed in acetone for 10 min and blocked for 10 min with PBS containing 0.05% Tween 20 (Merck, Whitehouse Station, NJ) and 1% HS at RT. Subsequently the fixed tissue was incubated for 18 hr at 4°C with a mouse anti-human IL-18 specific primary mAb (MAB318, R&D Systems, Minneapolis, MN), followed by an incubation for 30 min with a phosphatase-linked secondary rabbit anti mouse polyclonal antibody (pAb) (DAKO, Carpinteria, CA). Isotype controls were stained with an irrelevant antibody of the same isotype as the IL-18 specific antibody (anti-KLH IgG_{2A} mAb, MAB003, R&D Systems). 3-Amino-9-Ethylcarbazole (Sigma) was used as the chromogen.

Western blotting and immunodetection of IL-18

Proteins were separated using 15% SDS-PAGE gels according to Laemmli (Laemmli, 1970). The proteins were blotted onto Hybond-C membranes (Amersham, Little Chalfont, UK) using an electroblot system (BioRad, Hercules, CA). The membranes were blocked with Tris buffered saline (TBS) containing 5% low fat milk and 0.05% Tween20 for 1 hr at room temperature (RT). Blots were stained with a primary antibody against IL-18 (MAB318, R&D systems), followed by a secondary biotin-labelled anti-mouse polyclonal antibody (Amersham) and streptavidin poly-horseradish peroxidase (HRP) (CLB, Amsterdam, The Netherlands). Isotype controls were stained with an irrelevant antibody of the same isotype as the IL-18 specific antibody (anti-KLH IgG_{2A} mAb, MAB003, R&D Systems). IL-18 specific staining was detected using a chemoluminescence substrate (Pierce, Rockford, IL).

Cytokine specific ELISAs

Maxisorb ELISA plates (Nunc, Roskilde, Denmark) were coated for 18 hr at 4°C with 100 µl of either 2 µg/ml anti-human-IL-18 mAb (MAB318, R&D systems) or 0.5 µg/ml anti-

human-IL-6 mAb (Biosource, Camarillo, CA) followed by blocking with 1% BSA (Sigma). Hundred µl of IL-18 standard or sample and 50 µl of either 0.2 µg/ml biotin linked anti-human-IL-18 pAb (BAF318, R&D systems) or 0.2 µg/ml biotin linked anti-human-IL-6 pAb (Bioscource) detection antibody was simultaneously added to each well. The standards were diluted in sample buffer (HBSS or IMDM + 1% HS). Samples, standards and detection antibodies were incubated for 2 hours at RT. Cytokines were detected by using Streptavidin linked HRP (CLB) and TMB peroxidase substrate (Kirkegaard & Perry, Gaithersburg, MD). The OD was measured at 450 nm.

RNA isolation and RT-PCR

RNA was isolated from 1×10^6 cells using the guanidine thiocyanate extraction procedure (Chomczynski & Sacchi, 1987). RNA was reversed transcribed into cDNA and PCR reactions were performed as previously described (Debets *et al.*, 1997; van der Velden *et al.*, 1998). In each sample hypoxantine phosphatidyl ribosyltransferase (HPRT) cDNA was measured as a control.

The sequences of the primers were as follows: IL-18 (forward): 5'-GTC TTC GTT TTG AAC AGT GAA-3'; IL-18 (reverse): 5'-TAC TTT GGC AAG CTT GAA TCT-3'; HPRT (forward): 5'-GTG ATG ATG AAC CAG GTT ATG ACC TT-3'; HPRT (reverse): 5'-CTT GCG ACC TTG ACC ATC TTT GGA- 3'. The predicted sizes of the PCR products were 470 bp for IL-18, and 454 bp for HPRT. The products were separated on a 1 % agarose gel containing ethidium bromide, visualised by UV light and the gels were photographed.

RESULTS

IL-18 is constitutively produced by keratinocytes in normal skin

Biopsies from normal skin were taken to investigate expression of IL-18 in normal epidermis *in vivo* using immunohistochemistry. IL-18 expression was observed in both the dermis and epidermis (Fig. 1A). In the dermis strong positive staining in the cytoplasm of cells with dendritic morphology was seen (Fig. 1B). The epidermis showed diffuse staining,

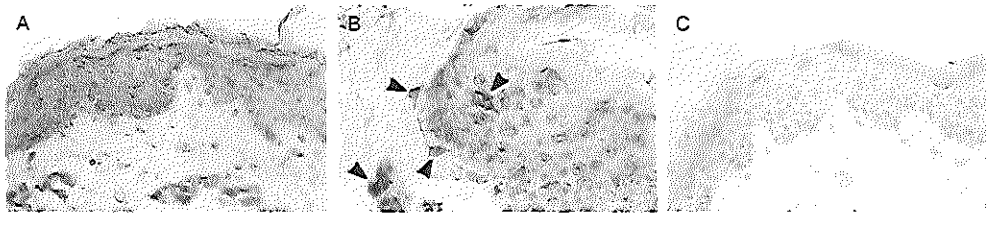

Figure 1. IL-18 expression in normal skin. Acetone-fixed cryostat sections were stained with an IL-18 specific monoclonal antibody (MAB318) as described in the material and methods section. A: IL-18 specific staining; B: IL-18 specific staining at an higher magnification showing cells with dendritic morphology in dermis and epidermis (arrow heads) (magnification: ×630); C: Isotype control antibody staining (magnification ×400).

but also local staining of some strong positive cells with dendritic morphology, probably Langerhans cells (Fig. 1B). Keratinocytes were more diffusely stained. Incubation of normal skin sections with an isotype control antibody did not show any reaction (Fig. 1C). These results indicate that keratinocytes express IL-18 *in vivo*.

IL-18 mRNA and protein are constitutively expressed by epidermal cells and HaCaT cells in vitro

IL-18 mRNA expression by normal epidermal cells (EC), HaCaT cells, PBMC and cells from the bronchus epithelial cell line BEAS2B, was analyzed using RT-PCR. IL-18 mRNA was expressed by all cell types, and no marked visual differences in mRNA levels were detected (Fig. 2). Detection of IL-18 protein in cellular lysates of HaCaT cells (by use of ELISA) revealed that HaCaT cells constitutively expressed intracellular IL-18 protein, i.e. extracts of 1×10^5 freshly harvested HaCaT cells contained 816 ± 140 pg IL-18. As a comparison, extracts of 1×10^5 EC contained about 100 pg IL-18, and EC suspensions depleted of HLA-DR$^+$ cells contained similar levels of IL-18 (see below). The intracellular IL-18 concentration in HaCaT cells did not change significantly during culture in medium (Fig. 3A). To assess whether human keratinocytes also secrete IL-18 protein, supernatants of HaCaT cells cultures were analyzed. After 3 hours of culture (without stimulation), an average of 115 ± 5 pg/ml IL-18 was measured. After subsequent culture for 24 hours, the IL-18 concentration had dropped to 86 ± 4 pg/ml and decreased further to 39 ± 1 pg/ml after 48 hours (Fig. 3B). This decreased detection of IL-18 secretion could be due to epitope blocking because of the binding of IL-18 to IL-18 binding protein (IL-18BP) or the IL-18 receptor. These data show that normal keratinocytes and HaCaT cells constitutively express IL-18 mRNA and protein.

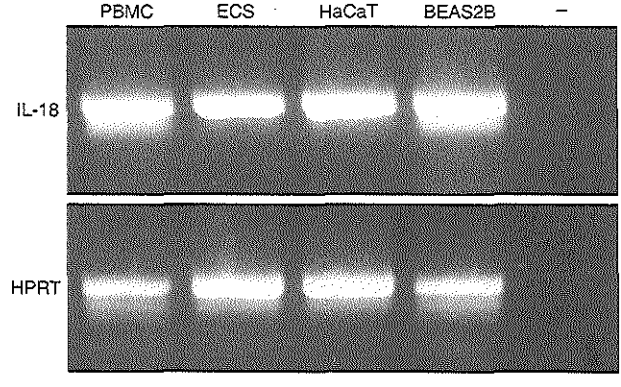

Figure 2. Production of IL-18 mRNA transcripts by different cell types. Detection by RT-PCR. HPRT mRNA expression was included as a positive control. PBMC: peripheral blood mononuclear cells; ECS: epidermal cell suspension; HaCaT: human keratinocyte cell line; BEAS2B: bronchial epithelial cell line and -: negative control (H_2O).

Intracellular produced IL-18 in epithelial cells is mainly in the unprocessed form

To determine whether the IL-18 produced by EC whether or not depleted of HLA-DR$^+$ cells, HaCaT and BEAS2B cells, was in the processed or the unprocessed form, we analyzed extracts and supernatants of these cells using Western blotting and a mAb specific for both the processed and unprocessed form of IL-18 (MAB318). All extracts contained the 24 kD unprocessed IL-18 and no processed IL-18 could be detected (Fig. 4). As illustrated in figure 4, HaCaT cells contained the most pronounced amount of pro-IL-18, followed by EC and BEAS2B cells. ELISA (data not shown) confirmed these data. The levels of secreted IL-18 were below the detection limits of the Western blotting assay used. These data show that normal keratinocytes, HaCaT and BEAS2B cells constitutively express unprocessed IL-18.

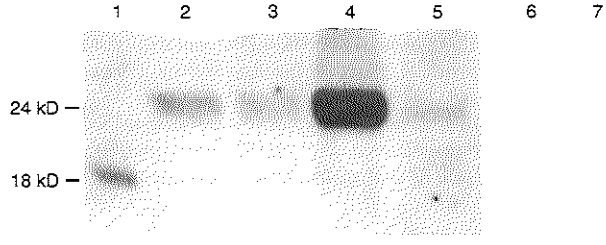

Figure 4. Intracellular IL-18 expression in different cell types. IL-18 was detected on Western blot using a monoclonal antibody against IL-18 (MAB318). Extracts from $5x10^4$ cells were loaded in each lane. Lane 1: processed recombinant human IL-18; lane 2: Normal human epidermal cells; lane 3: Normal human epidermal cells depleted of HLA-DR$^+$ cells; lane 4: HaCaT cells; lane 5: BEAS2B cells; lane 6: processed recombinant human IL-18, omission primary antibody and lane 7: normal human epidermal cells, omission primary antibody. Pro-IL-18: 24 kD, processed IL-18: 18 kD.

Normal human epidermal cells produce more (pro)-IL-18 than leukocytes, PBMC, monocytes and bronchial epithelial cells.

Next, we asked whether cells other than EC also produced (pro)-IL-18, and how this production relates to the amounts produced by epidermal cells. Cellular extracts from $3x10^5$ leukocytes, PBMC, monocytes, total EC and HLA-DR$^+$ cell-depleted EC were analyzed by Western blot for their IL-18 content. Amounts of (pro)-IL-18, detected in extracts of total leukocytes, PBMC or monocytes, were relatively low as compared to EC, depleted of HLA-DR$^+$ cells or not, these extracts contained high amounts of unprocessed IL-18 (Fig. 5). ELISA results show that the extracts of $1x10^5$ total EC and HLA-DR$^+$ cell-depleted EC contained 99.89 ± 23.48 pg (range: 83.25 - 116.49 pg) and 81.66 ± 12.43 pg (range: 68.71 - 95.85 pg) of IL-18 respectively. The extracts of $1x10^5$ leukocytes, PBMC and monocytes, however, contained 0.81 ± 0.61 pg (range: 0.99 - 1.39 pg), 3.36 ± 5.13 pg (range: 0.39 - 9.29 pg) and 1.22 ± 0.40 (range: 0.88-1.79 pg) of IL-18, respectively. During culture in normal medium, intracellular IL-18 expression in PBMC (Table 1) and BEAS2B cells (data not shown) was about 150 fold and 10 fold lower on average respectively when compared to IL-18

expression in HaCaT cells. IL-18 secretion by BEAS2B cells and PBMC was 6 fold lower on average than the production by HaCaT cells.

These data show that normal keratinocytes and HaCaT cells produce a considerable larger amount of total IL-18 protein than peripheral blood leukocytes and BEAS2B cells.

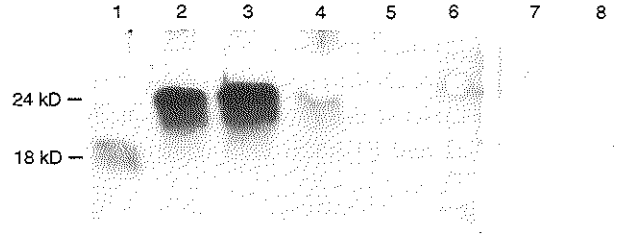

Figure 5. Intracellular expression of IL-18 in total and HLA-DR$^+$ cell depleted normal epidermal cells compared to leukocytes, PBMC and monocytes. IL-18 was detected on Western blot using a monoclonal antibody specific for IL-18 (MAB318). Extracts from $3x10^5$ cells were loaded in each lane.
Lane 1: processed recombinant human IL-18; lane 2: total epidermal cell suspension; lane 3: epidermal cell suspension depleted of HLA-DR$^+$ cells; lane 4: monocytes; lane 5: PBMC; lane 6: leukocytes; lane 7: processed recombinant human IL-18, isotype control and lane 8: total epidermal cell suspension, isotype control. Pro-IL-18: 24 kD, processed IL-18: 18 kD.

Stimulation of keratinocytes with LPS, PMA and IL-1β

To investigate whether IL-18 protein expression by keratinocytes could be altered by robust stimuli like LPS, PMA or IL-1β, HaCaT cells were cultured for different periods of time in the presence of these compounds. BEAS2B cells, representing bronchial epithelial cells, and PBMC were cultured similar to the HaCaT cells for comparison. Stimulation of HaCaT cells with LPS, PMA or IL-1β (Fig. 3) and PBMC (Table 1) or BEAS2B (data not shown) with LPS or PMA did not alter intracellular expression of total IL-18 protein, nor did it alter secreted levels of IL-18 protein. To confirm that the cells were able to respond to the given stimuli, IL-6 levels were measured in the supernatants. As expected, significant levels of IL-6 were detected after LPS or PMA stimulation, ranging from 9 to 10 times the secretion of non-stimulated cells (data not shown) confirming the activation of the cells.

DISCUSSION

This is the first study which provides *in vitro* and *in vivo* evidence that human keratinocytes are a potent source of pro-IL-18 when compared to other epithelial cells, leukocytes, PBMC or monocytes. Our studies on the regulation of mRNA and protein expression as well as the processing of pro-IL-18 into biologically active IL-18 revealed that keratinocytes preferably store high amounts of pro-IL-18 intracellularly, and potent stimuli like LPS, PMA and IL-1β have no effect on total IL-18 protein expression. We could not show a

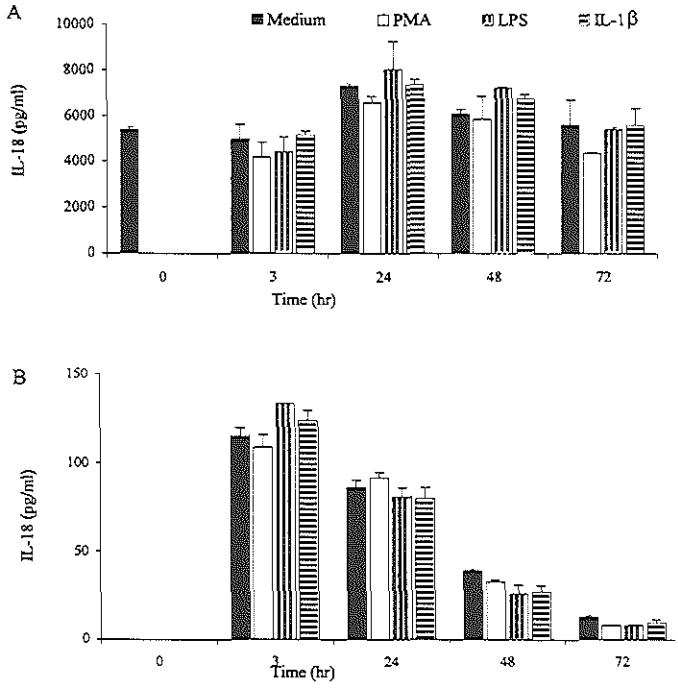

Figure 3. IL-18 protein expression by HaCaT cells during culture and after stimulation by LPS, PMA or IL-1β. A: total intracellular IL-18 expression; B: total IL-18 secretion. IL-18 levels were measured by ELISA. Total intracellular levels represent the IL-18 expression in $5x10^5$ cells. Data of one representative experiment out of three are shown. Readings were done in duplicate.

clear processing of IL-18 after stimulation in the cell extracts or culture supernatants by the methods used here.

Stoll *et al.* (Stoll *et al.*, 1997) showed that in murine skin, keratinocytes are the major source of IL-18. The data presented here show that human keratinocytes express and release IL-18 as well. Because bioactive IL-18 may promote IFN-γ synthesis in normal skin, we investigated whether IL-18 was expressed in the unprocessed (24 kD) or processed (18 kD) form. Our results show that IL-18 expressed by HaCaT and normal keratinocytes was essentially in the non-active, unprocessed 24 kD form. This is in accordance with the expression of IL-1β, which in normal skin is also synthesized in the unprocessed 32 kD form (Mizutani *et al.*, 1991). Expression of bioactive IL-1β in keratinocytes is dependent on caspase-1 activity (Zepter *et al.*, 1997). Whether keratinocytes express caspase-1 has been a matter of controversy, until Zepter *et al.* (Zepter *et al.*, 1997) showed that IL-1β in human keratinocytes is processed by caspase-1 which in turn is upregulated upon stimulation by urushiol and irritant chemicals. Pro-IL-18 may be converted into its active form in the same way. If so, keratinocytes are a reservoir of non-active IL-18, which can be processed by caspase-1 directly after stimulation. However pro-IL-18 may also be processed by other proteases as is the case

Table 1. IL-18 expression in PBMC during culture in normal medium, and after stimulation with PMA or LPS. IL-18 concentrations were measured by ELISA. Intracellular and extracellular IL-18 levels are in pg/ml (mean ± sd). Intracellular levels represent the total IL-18 production in $5x10^5$ cells. Data represent the mean of 3 separate experiments.
IL-6 secretion was measured as a control for activation of the cells. The IL-6 secreted after LPS or PMA stimulation, ranged from 9 to 10 times the secretion of non-stimulated cells.
T: Stimulation time (hr); ND: Not detected.

Stimulus	T (hr)	intracellular	extracellular
T = 0	0	12 ± 12	ND
Medium	3	20 ± 24	20 ± 15
	24	38 ± 25	39 ± 18
	48	34 ± 24	25 ± 26
	72	4 ± 1	18 ± 11
LPS	3	24 ± 15	19 ± 5
	24	13 ± 23	39 ± 13
	48	10 ± 17	22 ± 11
	72	ND	32 ± 30
PMA	3	45 ± 19	11 ± 5
	24	6 ± 10	51 ± 17
	48	ND	53 ± 36
	72	23 ± 39	33 ± 11

for IL-1β in the epidermis (Brattsand & Egelrud, 1998; Lundqvist *et al.*, 1998). An important difference between IL-18 and IL-1β in keratinocytes is that intracellular concentrations of pro-IL-18 are much higher than those of pro-IL-β. Pro-IL-18 levels correspond better with the intracellular concentrations of IL-1ra and IL-1α in skin, which are also high when compared to IL-1β (Phillips *et al.*, 1995). Therefore like IL-1α, IL-18 may have other functions in addition to its role in inflammatory response (Maier *et al.*, 1990).

That human keratinocytes can produce bioactive IL-18 after stimulation with LPS or PMA has recently been demonstrated by Naik *et al.* (Naik *et al.*, 1999). They also showed that IL-18 protein concentration is not altered after stimulation with PMA and LPS, which is confirmed by our studies. In addition we show that keratinocytes express significantly higher levels of IL-18 than bronchial epithelial cells, normal human leukocytes, PBMC and monocytes.

Constitutive expression of pro-IL-18 has also been reported to occur in other cell types. Pizarro *et al.* (Pizarro *et al.*, 1999) showed that like keratinocytes, gut epithelial cells solely expresses the unprocessed form of IL-18. They also found that in Crohn's disease, in which the Th cell balance is skewed towards the Th1 pole, pro-IL-18 is processed into the 18 kD form. A predominant Th1 environment is also apparent in psoriasis, a human inflammatory skin disease. In analogy to Crohn's disease, one would expect processing of IL-18 in psoriatic lesional skin. However, Western blot data did not reveal the 18 kD mature form of IL-18 in extracts of psoriatic lesional skin (unpublished data). Constitutive pro-IL-18 expres-

sion was also detected in human chondrocytes (Olee *et al.*, 1999) and stimulation of these chondrocytes with IL-1β generated the processed form of IL-18. Finally, Puren *et al.* (Puren *et al.*, 1999) showed constitutive pro-IL-18 expression in human PBMC. In our experiments the IL-18 concentration in the PBMC extracts and culture supernatants could be detected by ELISA but was below the detection limit of the Western blot method used. Therefore it was not possible to determine whether the IL-18 present was in the processed or unprocessed form.

The considerably lower expression of (pro)-IL-18 in monocytes compared to keratinocytes, might be explained by the severe and undesirable systemic side effects that may occur if high amounts of pro-IL-18 would be processed and released upon stimulation by monocytes. In skin, IL-18 could have a more local effect, in analogy to the IL-1 cytokine family. Excess production of IL-18 could then be overcome by the IL-18-BP, which has recently been described by Novick *et al.* (Novick *et al.*, 1999). IL-18BP is also expressed by human keratinocytes, at least on the RNA level (R. Groves, personal communication).

During skin inflammation, the locally intracellular stored pro-IL-18 may rapidly be processed, followed by the release of bioactive IL-18 by the keratinocytes. The released IL-18 may then skew T cells towards a Th1 phenotype, characterized by IFN-γ secretion. Therefore, IL-18, together with IL-12, may be a key cytokine for maintaining the Th1 environment in the skin after proinflammatory stimuli.

In conclusion, our data provide additional evidence that IL-18 might play a key role in facilitating the maintenance of a local Th1-like environment in skin during inflammation.

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Chapter 4

INCREASED IFN-γ EXPRESSION IN STABLE PLAQUE TYPE PSORIATIC LESIONS IS NOT ACCOMPANIED BY ELEVATION OF IL-18

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Re-submitted

ABSTRACT

Psoriasis is a T cell mediated inflammatory skin disease characterized by an elevated IFN-γ and IL-12p70 expression in lesional skin. Interleukin-18 (IL-18) is, together with IL-12, critical in inducing IFN-γ expression in Th1 cells. Since keratinocytes in normal skin produce and store high amounts of pro-IL-18, we hypothesized that expression of mature IL-18 in psoriatic lesional skin is elevated compared to normal skin. Therefore we investigated whether in psoriatic lesional skin the expression and processing of IL-18 are increased. As expected elevated IFN-γ expression was detected in extracts of psoriatic skin. IL-18 mRNA levels, assessed by quantitative real time PCR, and total protein concentration did not differ between normal and stable plaque type psoriatic lesions. In addition, processing of IL-18 in psoriatic lesional epidermis relative to normal epidermis was not increased. This study shows that IL-18 mRNA, protein synthesis and processing are not elevated in stable plaque type psoriatic lesions. Therefore in contrast to other inflammatory diseases like rheumatoid arthritis, Crohn's disease and sarcoidosis, elevated IFN-γ expression in psoriasis might be maintained by mediators other than IL-18.

INTRODUCTION

Psoriasis is an inflammatory skin disorder affecting approximately 2% of the western population. An important feature of this disease is the infiltration of T cells in the dermis and epidermis (Baker *et al.*, 1984; Onuma, 1994), which are thought to play a significant role in the pathogenesis of psoriasis (Barker, 1998; Nickoloff & Wrone-Smith, 1999; Schon *et al.*, 1997). Evidence has accumulated that the T cell infiltrate in psoriatic lesional skin comprises $CD4^+$ as well as $CD8^+$ T cells with a skewing towards T helper 1 (Th1) cells (Austin *et al.*, 1999; Schlaak *et al.*, 1994; Uyemura *et al.*, 1993). One of the hallmarks of Th1 cell activation is expression of interferon-γ (IFN-γ). Indeed an increased IFN-γ expression in psoriatic lesional skin has been reported (Bjerke *et al.*, 1983; Bonifati *et al.*, 1994; Livden *et al.*, 1989). IFN-γ induces the expression of the psoriatic phenotypic markers ICAM-1, HLA-DR and CD40, as well as other markers of the regenerative epidermal phenotype (Denfeld *et al.*, 1996; Gottlieb *et al.*, 1986; Paukkonen *et al.*, 1995; Wei *et al.*, 1999). In addition, IFN-γ transgenic mice exhibit a psoriatic like skin phenotype (Carroll *et al.*, 1997). Hence an important role for IFN-γ in psoriasis is apparent. Synthesis of IFN-γ is induced by IL-12 and IL-18 (Debets *et al.*, 2000) that act as Th1 skewing cytokines. Studies on the expression of IL-12 in keratinocytes, revealed that both IL-12p35 and IL-12p40 mRNA are constitutively expressed. Additionally, low levels of IL-12p70, the active form of IL-12, were detected in the supernatant of cultured normal keratinocytes (Yawalkar *et al.*, 1996). In psoriasis, an increase of IL-12p40 mRNA and IL-12p70 protein was reported (Yawalkar *et al.*, 1998), which suggests a role for IL-12 in the induction of IFN-γ in this disease.

IL-18 is expressed in many cell types, including keratinocytes. Stoll *et al.* (Stoll *et al.*, 1997) showed that murine keratinocytes produce IL-18 mRNA and functional protein after stimulation with contact allergens. Recently, it was found that human keratinocytes constitutively produce pro-IL-18 protein, which was processed upon activation with pro-inflammatory mediators and dinitrochlorobenzene (Mee *et al.*, 2000; Naik *et al.*, 1999). We confirmed and extended these data by showing that the production of IL-18 protein in human keratinocytes is on average a factor 100 higher than in other cell types such as leukocytes, PBMC, or monocytes and that this IL-18 is predominantly produced in the inactive unprocessed 24 kD form (Companjen *et al.*, 2000b). These data indicate that IL-18 might play an important role in the cytokine network in skin. However, the role of IL-18 in psoriasis remains undefined. Because IL-18 is a member of the IL-1 family, and the regulation of the IL-1 system in psoriasis is altered (Debets *et al.*, 1997), the regulation of this cytokine might be altered as well. The elevated IFN-γ expression in psoriatic lesional skin might be an indication for this imbalance. Therefore, IL-18 mRNA, protein expression and processing in psoriatic lesional skin was investigated. The data show that IL-18 mRNA, protein expression and processing in stable plaque type psoriatic lesions (sptPP) are not increased and suggest that IFN-γ expression in psoriasis might be maintained by mediators other than IL-18.

MATERIALS AND METHODS

Skin dermatome and biopsy specimens

Dermatome specimens for epidermal cell isolation or protein extraction were obtained using a portable dermatome (Padgett Instruments Inc., Kansas City, MO) after informed consent from 14 healthy donors and from 14 patients with stable plaque type psoriasis. Skin biopsies were obtained after informed consent from 8 healthy donors and 8 patients with psoriasis vulgaris. Biopsies with an average length of 3 mm were taken with a 3 mm diameter biopsy punch (Stiefel, Leuven, Belgium) and were snap frozen in Tissue Tek (Bayer, München, Germany). Biopsies were stored at -80°C until use. Dermatome specimens and biopsies from the borders of active and progressive psoriatic lesions (apPP) were taken from 3 patients. Dermatome specimens and biopsies from normal skin (NN) were obtained from healthy controls undergoing breast or abdominal plastic surgery in the department of Plastic Surgery of the University hospital Dijkzigt or the Sint Franciscus gasthuis Rotterdam. Dermatome specimens and biopsies from psoriatic lesional skin were obtained from psoriasis patients in the Dermatology department of the Hospital Walcheren, Vlissingen. Isolation of dermatome specimens and biopsies was approved by the Medical Ethical Committee of our hospital. Patients did not receive medical treatment for at least three weeks before isolation of dermatome specimens or biopsies.

Epidermal cell suspensions

Dermatome specimens were obtained as described above. The epidermis was detached

from the dermis by trypsinisation. Epidermal cell suspensions (ECS) were prepared from epidermal sheets by incubating them at 37°C for 45 min in trypsinisation buffer (0.025% (w/v) trypsin and 0.1% (w/v) EDTA in PBS), to which 0.25% DNAse was added for the last 15 min of the incubation (Roche, Basel, Switzerland). The cell suspension was filtered through a 30 μm mesh gauze and suspended in PBS containing trypsin inhibitors (5 mg/ml, Roche).

Epidermal protein extracts

Dermatome specimens were obtained as described above. The epidermis was detached from the dermis after incubation in Hanks' Balanced Salt Solution (HBSS, Gibco BRL, Paisley, Scotland), containing 1 U/ml dispase (Roche) at 4°C for 18 hr. The epidermal sheets were homogenized with a scalpel and freeze dried. The freeze dried samples were homogenized in 1 ml of HBSS, supplemented with a broad mixture of protease inhibitors (Complete™, Roche, concentration added according to the manufacturer's instructions). Proteins were extracted by ten freeze-thaw cycles in liquid nitrogen. To remove the cell debris, extracts were centrifuged (14000xg, 4°C) and the supernatant was isolated. The total protein concentration was measured using the BCA-200 protein assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Epidermal protein extracts were stored at -80°C until use.

Quantitation of mRNA expression

RNA was isolated from 1 to $2*10^6$ normal or psoriatic lesional epidermal cells of 7 normal donors and 9 psoriasis patients using RNAzol-B (Tell-Test Inc., Friendswood, TX) and reverse transcribed into cDNA as previously described (van der Velden *et al.*, 1998). cDNA was analyzed using a Taqman sequence detector (7700 Sequence detector, Applied Biosystems, Foster City, CA). The sequences of the primers and probes were as follows: IL-18 (forward, Sigma Genosys, Cambridge, UK): 5'-GCT TTA CTT TAT AGC TGA AGA TGA TGA A-3'; IL-18 (reverse, Sigma Genosys): 5'-CTC TAC AGT CAG AAT CAG TCA TAT CTT CAA ATA-3'; IL-18 (FAM labeled probe, Bioscource): 5'-TTC TCT TCA TTG ACC AAG GAA ATC GGC CT-3'. In each sample cDNA of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was measured as a control. A commercially available kit (Applied Biosystems) was used for GAPDH mRNA detection according to the manufacturer's instructions. mRNA isolated from the HaCaT keratinocyte cell line (Boukamp *et al.*, 1988) was used as a reference for IL-18 mRNA detection and mRNA isolated from the Th0 cell line B21 was used as a reference for GAPDH mRNA detection. The PCR signal detected for NN, stpPP and apPP epidermal cell samples were correlated to the signals obtained for the HaCaT (IL-18) or B21 (GAPDH) control mRNA. Subsequently the IL-18-GAPDH mRNA ratio was calculated to normalize for the variability in mRNA quality of the different samples.

Immunohistochemistry

Skin biopsies were immersed in TissueTek (Bayer, München, Germany) and snap

frozen in liquid nitrogen. Six µm cryosections were cut using a cryostat (Jung Frigocut 2800 E, Leica, Rijswijk, The Netherlands) and stored in a sealed box containing silica gel at -80°C prior to use. Sections were fixed in acetone for 10 min at room temperature (RT) and pre incubated for 10 min with PBS containing 0.05% Tween 20 (Merck, Whitehouse Station, NJ) at RT. Subsequently sections were incubated for 18 hr at 4°C with either anti-human-IL-18 (αhIL-18, mAb MAB318, R&D Systems, Minneapolis, MN, 2.5 µg/ml), αhIL-12p40/70 (detecting both the p40 monomer and the active p70 heterodimer of IL-12) (mAb C8.6, Pharmingen, San Diego, CA, dilution 1:200), αhCaspase-1p20 (pAb RPI-ICEP20Cabg, Research Diagnostics, Flanders, NJ, dilution 1:20) or αhIFN-γ (mAb MD-2, U-Cytech, Utrecht, The Netherlands, dilution 1:150). This incubation was followed by incubation with biotin-linked secondary rabbit-anti-mouse polyclonal antibody (pAb) (DAKO, Carpinteria, CA, dilution 1:400) and peroxidase-linked avidin (DAKO, dilution 1:200). Sections incubated with an antibody of the same isotype as the specific antibody but of irrelevant specificity served as a negative control. 3-amino-9-ethylcarbazole (Sigma, St. Louis, MO) was used as the chromogen. Staining intensity and numbers of positive cells were reproducibly ranked by two independent observers blinded to treatment using a semiquantitative scoring scale.

Cytokine ELISA

Maxisorb ELISA plates (Nunc, Roskilde, Denmark) were coated for 18 hr at 4°C with 100 µl of 0.5 µg/ml anti-human-IFN-γ (αhIFN-γ), αIL-12p40, αIL-1α, αIL-8 (Biosource, Camarillo, CA) or 2 µg/ml αhIL-18 mAb (MAB318, R&D Systems) followed by blocking with 0.5% BSA (Sigma) for two hours at RT. Hundred µl of the recombinant IFN-γ, IL-12p40, IL-1α, IL-8 (Biosource) or IL-18 (DNAX, Palo Alto, CA) standard or sample and 50 µl of 0.2 µg/ml biotin linked αhIFN-γ, αhIL-12p40, αIL-1α, αIL-8 (Bioscource) or αhIL-18 (BAF318, R&D systems) pAb detection antibody were simultaneously added to each well. The standards were diluted in PBS containing 0.5% BSA (Sigma) and 0.1% (v/v)Tween 20 (Merck). Samples, standards and detection antibodies were incubated for 2 hours at RT. Cytokines were detected using streptavidin linked peroxidase (CLB, Amsterdam, The Netherlands) and TMB peroxidase substrate (Kirkegaard & Perry, Gaithersburg, MD). The substrate was incubated for a maximum of 20 min, and subsequently the OD was measured at 450 nm. For caspase-1 detection the R&D caspase-1 ELISA kit was used (Human quantikine ELISA kit, R&D Systems). This assay was performed according to the manufacturer's instructions.

Western blotting and immunodetection

Proteins were separated using 15% SDS-PAGE gels according to Laemmli (Laemmli, 1970). The proteins were blotted onto Hybond-C membranes (Amersham, Little Chalfont, UK) using an electroblot system (BioRad, Hercules, CA). The membranes were blocked with Tris buffered saline (TBS) containing 5% low fat milk and 0.05% Tween20 for 1 hr at room temperature (RT). Blots were stained with a primary antibody against IL-18 (MAB318, R&D systems), followed by a secondary biotin-linked anti-mouse pAb (DAKO) and streptavidin

linked peroxidase (CLB, Amsterdam, The Netherlands). Incubation with an antibody of the same isotype as the specific antibody but of irrelevant specificity served as a negative control. IL-18 specific staining was detected using a chemoluminescence substrate (Pierce, Rockford, IL).

Statistical analysis

The Mann-Whitney test was used to determine the significance of differences between patients and normal controls and $p<0.01$ was considered to be significant.

RESULTS

Comparable expression of IL-18 mRNA in normal and psoriatic lesional epidermis

We compared IL-18 mRNA levels in normal (NN), psoriatic stable plaque type lesional (sptPP) and psoriatic active and progressive lesional (apPP) epidermal cells to determine whether the IL-18 mRNA expression in psoriatic lesional skin differs from the expression in normal epidermal cells. The IL-18 and GAPDH mRNA levels in samples of 7 NN, 7 sptPP and 2 apPP cell suspensions were assessed using real time quantitative PCR. No significant difference was observed between the IL-18/GAPDH mRNA ratio of NN and spt PP epidermal cells (Fig 1). Epidermal cells from active and progressive lesions displayed

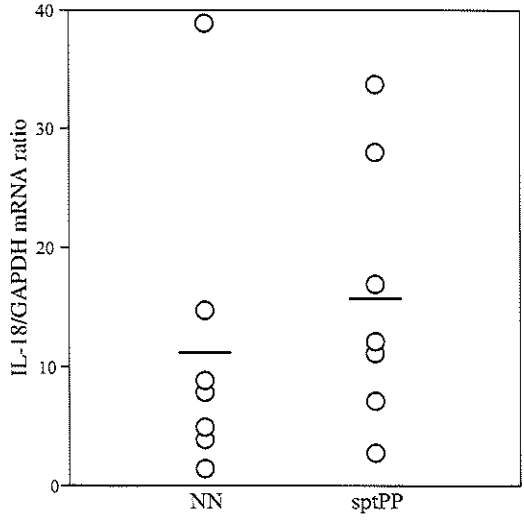

Figure 1. Similar IL-18 mRNA expression in normal versus psoriatic lesional skin. IL-18 mRNA expression in 7 normal (NN) and 7 psoriasis stable plaque type lesional (sptPP) epidermal cell suspensions was quantitated using a TaqMan sequence analyzer as described in the method section. The IL-18/GAPDH mRNA concentration ratio was calculated to normalize for the variability in mRNA quality in the samples. The horizontal bar represent the mean and the open circles represent the individual IL-18/GAPDH ratios of the different samples.

IL-18/GAPDH ratios similar to those found in normal and stable plaque type psoriatic lesions (data not shown). These data show that both normal epidermal cells and psoriatic lesional epidermal cells express IL-18 mRNA and that the expression levels are similar.

IL-18 protein expression in stable plaque type psoriatic epidermis is not elevated

Cryosections of NN, sptPP and apPP skin were incubated with an IL-18 specific antibody. The epidermis of NN and sptPP skin specimens displayed a diffuse granular staining pattern and no difference in IL-18 expression between NN and sptPP epidermis was observed (Fig 2a-c). In addition, only apPP epidermis displayed an increased expression of IL-18 especially in the basal layer (data not shown). The staining pattern was comparable with NN and sptPP skin, but additionally a more pronounced membrane staining was observed. Immunohistochemistry also revealed that there was no difference in IL-18 expression in psoriatic non-lesional versus normal skin (data not shown). As reported earlier (Companjen *et al.*, 2000b), keratinocytes and cells with dendritic morphology, probably Langerhans cells (LC), expressed IL-18. Cellular infiltrates in the dermal papillae of psoriatic lesional skin were clearly IL-18 positive and included cells with dendritic, macrophage and lymphocyte morphology.

IL-12 and IFN-γ expression was assessed by immunohistochemistry as well. IL-12p40/p70 expression was observed in NN, sptPP and apPP skin skin. Normal skin displayed a diffuse staining with more intensely stained individual cells scattered throughout the epidermis. In apPP and sptPP skin, keratinocytes in the super basal layer were highly

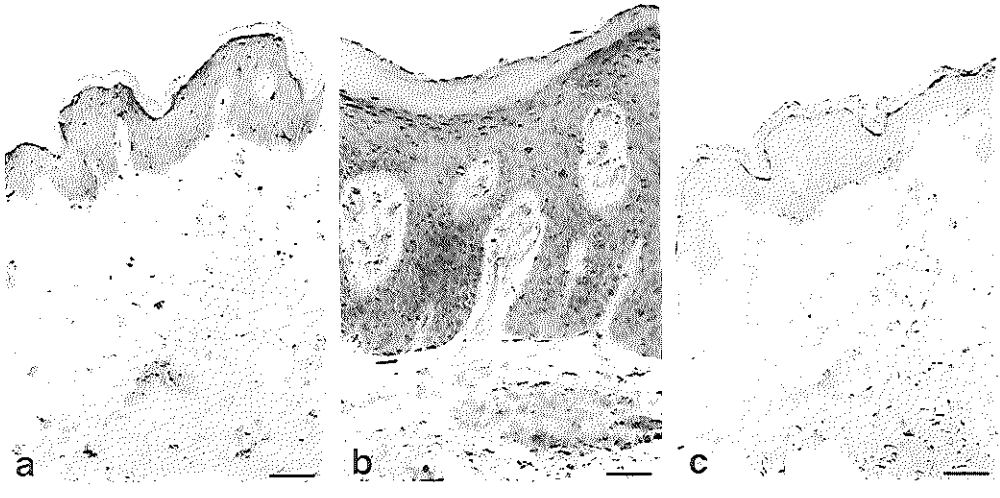

Figure 2. Similar IL-18 protein expression in normal and psoriatic lesional skin. Acetone-fixed cryostat sections were stained with an IL-18 specific MAb as described in the methods section. (a) normal skin. (b) stable plaque type psoriatic lesions. (c) isotype control antibody staining on normal skin. Representative specimens out of 16 are shown. Scale bar: 50 μm.

IL-12p40/p70 positive whereas basal keratinocytes showed a weaker staining (data not shown). No clear difference in IFN-γ expression between NN and sptPP skin could be detected by immunohistochemistry (data not shown).

The total IL-18 concentration (processed and unprocessed) in the epidermal protein extracts was measured by ELISA. In accordance with the data obtained by immunohistochemistry, no significant difference in total IL-18 protein expression between NN and sptPP epidermis was observed (Table I). For comparison, expression levels of cytokines known to be expressed differentially in psoriasis were also determined. In accordance with previous reports, IFN-γ, IL-12p40 and IL-8 levels were elevated whereas the IL-1α level was reduced in sptPP epidermis ($p<0.01$, Table I). Additionally IL-18 and IL-12p40 levels in extracts of apPP epidermis were determined. Compared with NN and sptPP epidermis, IL-18 expression in apPP epidermis was increased by a factor 4 to 3 respectively (concentration: 27186 ± 4098 pg/ml (mean ± SEM, n=3)). IL-12p40 expression in apPP epidermis was elevated by a factor 3 relative to sptPP epidermis but with considerable inter individual variation (concentration: 593 ± 250 pg/ml (mean ± SEM, n=3)).

Table I. Total IL-18 protein expression is not elevated in psoriatic lesional epidermis. Cytokine and enzyme concentrations were determined in normal (NN) and psoriatic stable plaque type lesional (sptPP) epidermal extracts by ELISA and were normalized for the total protein concentration. Data represent the mean cytokine or enzyme concentration (pg/mg total protein)(mean ± SEM) of 7 NN and 7 sptPP epidermal extracts.

Cytokine/enzyme	NN	sptPP
IL-18	6838 ± 1470	10083 ± 1691
IL-12p40	2.45 ± 1.73	205.03 ± 31.28*
IFN-γ	0.29 ± 0.09	1.40 ± 0.39*
IL-1α	2416 ± 343	145 ± 26*
IL-8	1.60 ± 0.47	747.83 ± 116.73*
Caspase-1	4205 ± 494	4037 ± 545

*: $p<0.01$

IL-18 in psoriatic lesions is predominantly expressed in the unprocessed form

IL-18 processing was assessed by Western blot. In SDS-PAGE, unprocessed IL-18 migrates at the 24 kD level whereas the active processed form migrates at the 18 kD level. Both pro-IL-18 and processed IL-18 can therefore conveniently be discriminated. Western blotting and subsequent immunostaining revealed that IL-18 present in extracts of sptPP epidermis is predominantly in the unprocessed form like in extracts of NN epidermis (Fig 3). IL-18 processing was also determined in extracts of apPP epidermis. Compared with NN and sptPP, no enhanced IL-18 processing was observed in apPP epidermis (data not shown). Pro-IL-18 is processed by caspase-1 to yield the 18 kD active form (Ghayur *et al.*, 1997). Because an increase of this processed form of IL-18 was absent in extracts of psoriatic lesional epidermis, caspase-1 levels were determined as well. Caspase-1 expression was evaluated by ELISA and immunohistochemistry. An antibody directed against both the inactive pro-form and the p20 subunit of the active complex of caspase-1 was used. No difference between the expression of total caspase-1 in extracts of NN and sptPP epidermis was found (Table I).

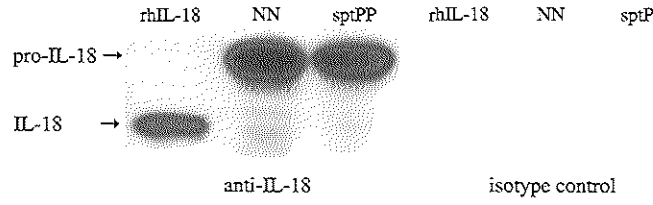

Figure 3. IL-18 in extracts of psoriatic lesional epidermis is predominantly in the unprocessed form. Western blots of extracts from normal and psoriatic lesional epidermis were probed with an IL-18 specific mAb as described in the methods section. Recombinant human IL-18 was used as a positive control and an isotype matched antibody as a negative control. Data of one representative extract out of 7 are shown. rhIL-18: processed recombinant human IL-18; NN: normal epidermis; sptPP: psoriasis stable plaque type lesional epidermis. Pro-IL-18, 24 kD; processed IL-18, 18 kD.

Additionally, immunohistochemistry revealed no clear difference in staining intensity in NN skin versus sptPP skin and infiltrates in both NN and sptPP skin were clearly positive (Fig 4a-d). The caspase-1 expression pattern in sptPP skin shows a clear membrane staining (Fig 4c). This was also observed in psoriatic non-lesional, apPP and NN skin (data not shown).

DISCUSSION

A role of IL-18 in the induction of IFN-γ expression has been implicated in some autoimmune and inflammatory diseases (Fassbender *et al.*, 1999; Gracie *et al.*, 1999; Monteleone *et al.*, 1999; Rothe *et al.*, 1997; Shi *et al.*, 2000a). Recently we showed that pro-IL-18 is abundantly present in normal human keratinocytes (Companjen *et al.*, 2000b), which suggests a likely role for IL-18 in skin inflammation and psoriasis. Our results showed no differences in IL-18 mRNA and total IL-18 protein expression between normal and stable plaque type psoriatic lesional epidermis. In contrast to IL-18, expression of the p40 subunit of IL-12, another IFN-γ inducing cytokine, was dramatically increased in psoriatic lesional epidermis, and ELISA readings revealed an elevation of IFN-γ as well. Elevation of IL-12p40 and IFN-γ in psoriatic lesional skin has also been described by others (Bonifati *et al.*, 1994; Yawalkar *et al.*, 1998). Immunohistochemistry confirmed the IL-18 and IL-12 results obtained by ELISA. Using immunohistochemistry, Naik *et al.* (Naik *et al.*, 1999) observed a difference in IL-18 protein expression between psoriatic lesional skin and normal skin. In this study normal and psoriatic lesional skin sections were fixed with formaldehyde. To exclude the possibility that the differences in observations by Naik *et al.* and our group was due to a different fixation method, we tested the effect of different fixation methods (acetone, formaldehyde, pararosaniline (Schrijver *et al.*, 2000) and zamboni) on IL-18 staining. However, no difference in IL-18 staining pattern was observed (data not shown). The discrepancy between the data obtained by Naik et al. and the data presented here might there-

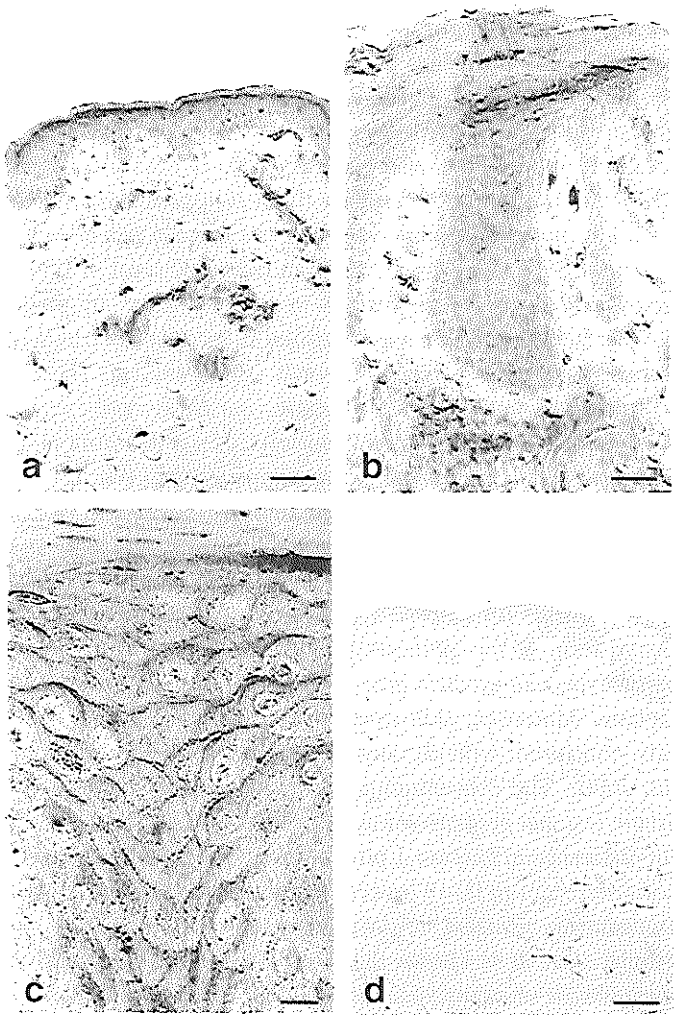

Figure 4. Caspase-1 expression in normal and psoriatic lesional skin. Acetone-fixed cryostat sections were stained with a caspase-1 specific goat polyclonal antibody as described in the methods section. (a) normal skin. (b) stable plaque type psoriatic lesion. (c) detail showing the membrane staining of caspase-1 in stable plaque type psoriatic lesions. Scale bar: 10 μm. (d) normal skin probed with purified normal goat IgG. Representative specimens out of 16 are shown. Scale bars: (a,b and d) 50 μm; (c) 10 μm.

fore be explained by the difference in the antibody used.

In Crohn's disease, increased levels of processed, active IL-18 were observed in extracts of lesional gut epithelial cells (Monteleone *et al.*, 1999; Pizarro *et al.*, 1999). We asked whether this also occurs in psoriatic lesional skin. However, compared to normal epidermis no elevation of the 18 kD active form of IL-18 in stable plaque type psoriatic lesion-

al epidermis could be detected on Western blot. Processed IL-18 might be bound to the IL-18 receptor or IL-18 binding protein (IL-18BP) potentially present in the psoriatic lesional epidermal extracts resulting in a difference in migration in SDS-PAGE. This could result in the observation of a lower amount of processed IL-18 than actually present in the extracts. However, electrophoresis of the epidermal extracts was done under reducing conditions, ruling out differences in the migration level of processed IL-18 because of binding to the IL-18 receptor or IL-18BP. The cause for the absence of an increased level of processed IL-18 in stable plaque type psoriatic lesional epidermis might be that the processed IL-18 is degraded at the time of isolation of the skin sample while an increased level of IFN-γ is still present. Therefore we also analyzed IL-18 expression in active borders of progressive psoriatic lesions. Total IL-18 protein expression was increased in these lesions, although there was no evidence of elevated IL-18 mRNA expression or IL-18 processing. These preliminary data suggest that IL-18 might be involved in IFN-γ induction during early stages of the disease. Future investigation of IL-18 involvement in psoriasis should therefore include early, active and progressive lesions in addition to stable plaque type lesional skin.

Activation of IL-18 depends on processing by caspase-1 (formerly known as Interleukin-1 Converting Enzyme (ICE)). The role of caspase-1 activity in human skin has been debated. Mizutani (Mizutani *et al.*, 1991) claimed that human keratinocytes are unable to process IL-1β, a substrate for caspase-1, suggesting that they are therefore incapable of producing the active form of this protease. In contrast, Zepter et al. (Zepter *et al.*, 1997) showed that keratinocytes can produce processed IL-1β after exposure to irritant chemicals and reactive haptens. Moreover, skin-specific caspase-1-transgenic mice spontaneously develop dermatitis, concurrent with elevated expression of mature IL-18 and IL-1β in skin (Yamanaka *et al.*, 2000). The concentration of caspase-1 in extracts of normal and stable plaque type psoriatic lesional epidermis was assessed but did not show a significant difference. This lack of difference in caspase-1 production might explain our findings demonstrating the absence of differences in IL-18 processing in normal versus psoriatic lesional epidermis. Immunohistochemistry revealed a distinct membrane staining in the epidermis of normal and psoriatic lesional skin. It is known that the p20 subunit of caspase-1 is exclusively expressed on the membrane of monocytes (Singer *et al.*, 1995). The membrane staining found in this study might therefore suggest that caspase-1 is constitutively active in normal and psoriatic skin. In accordance with this, occasionally both normal and psoriatic epidermal extracts contained limited amounts of processed IL-18 (data not shown), indicating that epidermal cell derived IL-18 can indeed be processed. However, this processing could also be due to other proteases potentially present in human skin (Lundqvist et al, 1998). Another protease which can activate IL-18 has recently been discovered[1]. The lack of elevated IL-18 processing in psoriatic lesional skin might also be due to inhibition of caspase-1 activity. A novel candidate for caspase-1 inhibition is ICEBERG (Humke *et al.*, 2000). Whether ICEBERG is expressed in human skin and elevated psoriatic lesional skin is presently unknown.

[1]Fantuzzi G, Karasek JA, Reznikov LL, Puren AJ, Cheronis J, Dinarello CA: A role for neutrophil proteinase-3 (PR-3) in the generation of active IL-18. (Abstr.) *European Cytokine Netw* 9:378, 1998

To get more insight into the function of IL-18 in psoriatic skin, IL-18 activation in epidermal extracts and medium of stimulated lesional skin biopsies should be evaluated.

The present data show that the expression or processing of IL-18 in stable plaque type psoriatic lesions are not increased in vivo. This is in contrast with IL-18 expression in other Th1 mediated inflammatory diseases and suggests that maintenance of IFN-γ expression in psoriatic lesional skin might be mediated by factors other than IL-18.

Acknowledgements

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Chapter 5

A MODIFIED *EX VIVO* SKIN ORGAN CULTURE SYSTEM FOR FUNCTIONAL STUDIES

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ABSTRACT

To investigate the immunological function of cells in skin during normal and disease states, under conditions approximating the *in vivo* situation, it is required to maintain the structural integrity of the tissue. To achieve this situation, freshly isolated skin has to be cultured *ex vivo*, or an *in vitro* constructed complete skin equivalent may be used. Different skin organ culture systems have been described in the past. Basically two systems prevail: submerged or air-exposed skin organ cultures. The former model has been used for measuring cytokine secretion by skin cells in the medium, the latter to study the expression of cell membrane proteins in situ and the kinetics of epidermal Langerhans cells. Here we present a modified *ex vivo* skin organ culture system which approaches the *in vivo* situation by maintaining the normal skin architecture without spontaneous induction of the regenerative maturation markers. This method allows measuring the expression of cell membrane proteins in situ, and quantitate the cytokine secretion by skin cells in the culture medium in the same experiment. In this system, both general and specific stimuli like LPS and IL-1β upregulated the expression of skin-derived cytokines like IL-8 and IL-6 in the medium and different markers, like ICAM-1, CD40 and CD86 on cells in the tissue in a 24 hour culture format. Elevation of both cytokine and cell marker expression could be blocked by dexamethasone and by IL-1ra which acts specifically on IL-1β mediated responses. The system presented here is both quick and simple and can be used as a model to study the behavior of skin cells in their natural microenvironment.

INTRODUCTION

After the initiation of inflammation in the skin, a complex network of immune reactions comes into effect. Cells interact with each other directly through cell-cell contact or indirectly through cytokine signalling. Interaction of the cells with the extracellular matrix also plays a crucial role during immune processes. Thus, the microenvironment of the skin dictates the outcome of these interactions. To investigate the interaction of cells in situ, an intact microenvironment is essential. One way to study the behavior of skin cells in their microenvironment, is by making use of the SCID-hu xenogenic transplantation model (reviewed by Boehncke (Boehncke, 1999)). In this system human skin is transplanted onto SCID mice in order to investigate different parameters of the immune system in intact skin. Boehncke and Wrone-Smith (Boehncke *et al.*, 1997; Wronesmith & Nickoloff, 1996) showed that this model is a valid tool for investigating cellular immunity in skin. In addition, it was recently shown that this model allows for the screening of anti-psoriatic drugs (Boehncke *et al.*, 1999). However, the SCID-hu xenogenic transplantation model also has some disadvantages. It is time-consuming, requires specific expertise and facilities, and furthermore, although the SCID mouse does not have functional T and B cells, it possesses other immuno-

cytes that might be of influence. An alternative to this model is the culturing of total skin. Skin organ culture models have been used for a long time (reviewed by Tammi *et al.* (Tammi & Maibach, 1987)). Different methods have been used, varying from culture of skin biopsies in immersed medium (Ameglio *et al.*, 1997; Tavakkol *et al.*, 1999) to methods in which skin is cultured on metal grids covered by filter paper (Rambukkana *et al.*, 1996; Tammi *et al.*, 1991). The latter method was developed in 1959 by Trowell (Trowell, 1959) and was later modified by Jensen *et al.* (Jensen *et al.*, 1964). Several studies have been performed using an *ex vivo* culture system. These studies include analysis of the behavior of epidermal Langerhans cells (Rambukkana *et al.*, 1995), differences in cytokine expression in normal and lesional psoriatic skin (Yoshinaga *et al.*, 1995), and the function of epidermal growth factor (EGF) in psoriatic skin (Varani *et al.*, 1998). These models were either used to investigate the modulation of certain cell markers in the tissue or secretion of cytokines in the medium. Moreover, in most studies the skin was cultured under normal conditions (5% CO_2 and 37°C) by which diseased skin like psoriatic lesional skin deteriorates. This makes monitoring of modulatory effects on diseased skin in these systems difficult.

The present study describes a modified skin organ culture model based on the method presented by Trowell (Trowell, 1959), which allows monitoring skin cell activation and differentiation in situ and cytokine release into the culture medium. Human skin biopsies were cultured in Iscove's Modified Dulbecco's Medium (IMDM) with 1% heath-inactivated human serum for 24 hours in a culture system at 32°C in sealed bags containing 95% O_2 and 5% CO_2. The skin biopsy is inserted through a hole in a transwell filter with the epidermis placed upwards at the liquid-air interface and the dermis suspended in the culture medium. The effects of two immunostimulators and suppressors on the expression of different immunologically relevant parameters in both culture medium and skin tissue were monitored simultaneously using this system. Lipopolysaccharide (LPS) stimulates inflammatory reactions through different receptors (Fenton & Golenbock, 1998; Kawai *et al.*, 1999), whereas IL-1β acts in a more specific manner and signals specifically through the type I IL-1 receptor which is present on most cells in the skin, including keratinocytes (Cumberbatch *et al.*, 1998; Grewe *et al.*, 1996). Using these stimuli we could stimulate secretion of IL-8 and IL-6 in the culture medium, as well as the expression of CD40, CD86 and ICAM-1 on cells in the tissue sample. In addition, we were able to block the elevation of these markers by pharmacological relevant agents like dexamethasone, a general immunosuppressive agent and by IL-1receptor antagonist (IL-1ra), a specific antagonist of IL-1. Additionally we show that IFN-γ secretion by psoriatic lesional skin biopsies is induced after stimulation of T cells through CD3.

This *ex vivo* culture system allows monitoring of different immunological parameters after stimulation or inhibition by different agents.

MATERIAL AND METHODS

Skin biopsies

Normal skin biopsies were obtained from 7 healthy volunteers undergoing a breast reduction in the department of Plastic Surgery of the Dijkzigt hospital or Sint Franciscus gasthuis Rotterdam. Psoriatic lesional skin biopsies were obtained from 7 patients with plaque type skin lesions. After informed consent biopsies with an average length of 3 mm were taken with a 3 mm diameter biopsy punch (Stiefel, Leuven, Belgium) and were either snap frozen in Tissue Tek (Bayer, München, Germany), or cultured (see below). After culture the biopsies were immersed in Tissue Tek (Bayer) and snap frozen in liquid nitrogen. Biopsies were stored at -80°C until use.

Organ culture conditions

The culture method presented here is based on the method presented by Trowell (Trowell, 1959). Biopsies were cultured as follows: a 2 mm hole was punched in a Transwell filter (pore size: 0.75 µm; Corning Costar, Corning, NY). The biopsy was inserted into the hole, and the filter containing the biopsy was placed in a 12 well culture plate (Corning Costar) containing 1 ml medium with or without stimulus. Culture plates containing the biopsies were placed in Tedlar culture bags (Pacwill Environmental, Fredericton, Canada). The bag was filled with 5% CO_2 and 95% O_2 through a valve, sealed with a removable clamp, and placed in an incubator as depicted in Fig 1. Skin biopsies were cultured in IMDM (GibcoBRL, Paisley, Scotland) containing 1% heat inactivated human serum (HS) (Sigma, St. Louis, MO), 100 U/ml penicillin and 100 µg/ml streptomycin (BioWhittaker, Verviers, Belgium) under normal conditions (5% CO_2, 37°C) or special conditions (95% O_2, 5% CO_2 and 32°C). All experiments were done at least three times, with triplicate readings. A schematic drawing of the culture procedure is shown in Figure 1.

Some biopsies were stimulated for 24 hours with either 10 µg/ml LPS (Brunschwig, Amsterdam, The Netherlands) or 250 U/ml IL-1β (Glaxo, Research Triangle Park, NC). Normal skin biopsies were also cultured in the presence of dexamethasone (Sigma, concentrations: 10^{-5} to 10^{-9} M) or 1 µg/ml IL-1ra (Synergen Inc., Denver, CO) with or without stimulation with 250 U/ml IL-1β or 2 µg/ml LPS. Psoriatic lesional skin biopsies were stimulated with CD3 stimulating antibodies (CLB, Amsterdam, The Netherlands; 1500 ng/ml).

Immunohistochemistry

Skin biopsies were snap frozen in liquid nitrogen and cryosections were cut using a cryostat (Jung Frigocut 2800 E, Leica, Rijswijk, The Netherlands) and stored in a sealed box containing silica gel at -80°C prior to use. Sections were fixed in acetone for 10 min at room temperature (RT) and pre incubated for 10 min with PBS (pH: 7.4) containing 0.05% Tween 20 (Merck, Whitehouse Station, NJ) at RT. Subsequently sections were incubated for 18 hours at 4°C with either anti-human-CD86 (1G10, Tanox Pharma BV, The Netherlands, dilution: 1:750), anti-human-CD40 (5D12, Tanox Pharma BV, dilution: 1:300), biotinylated anti-HLA-

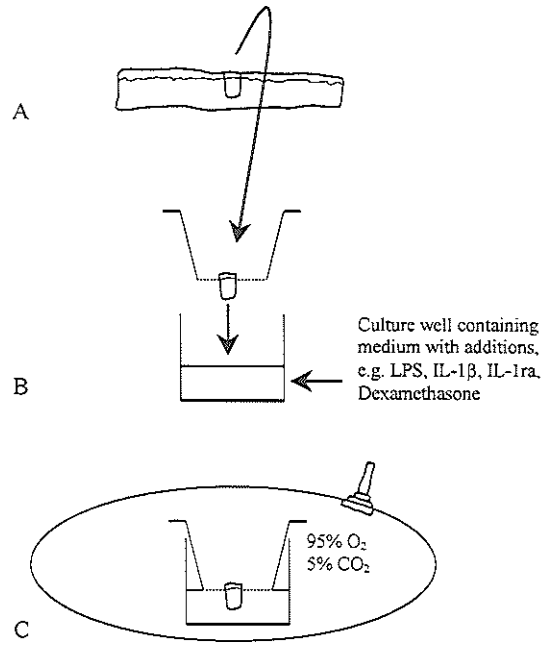

Fig. 1: Schematic overview of the skin organ culture system. **A:** A skin biopsy is isolated by use of a 3 mm diameter biopsy punch; **B:** The biopsy is inserted through a 2 mm opening in the transwell filter and placed in culture medium to which specific stimuli are added; **C:** Biopsies are placed at 32°C in a sealed bag containing 95% O_2 and 5% CO_2

DR (L243, Beckton Dickinson, dilution: 1:3000) or for 1 hour at room temperature with anti-human-ICAM-1 (BBA4, Boehringer Mannheim, Mannheim, Germany, dilution: 1:250), anti-human-keratin-17 (K17) (E3, DAKO, Carpinteria, CA, dilution: 1:100) or anti-human-Transglutaminase kinase (TGk) (BT-621, Biomedical Technologies Inc., Stoughton, MA, dilution: 1:200), followed by an incubation for 30 min with a peroxidase-linked secondary rabbit-anti-mouse polyclonal antibody (pAb) (DAKO, dilution: 1:400). Biotinylated anti-HLA-DR was directly detected with streptavidin linked peroxidase (DAKO). Sections incubated with an antibody of the same isotype as the specific antibody but of irrelevant specificity served as a control. 3-amino-9-ethylcarbazole (Sigma) was used as the chromogen. Staining intensity and numbers of positive cells were reproducibly ranked by two independent observers blinded to treatment using a semiquantitative scoring scale.

Cytokine ELISA

Maxisorb ELISA plates (Nunc, Roskilde, Denmark) were coated for 18 hr at 4°C with 100 µl of 0.5 µg/ml anti-human-IL-8, anti-human-IL-6 or anti-human-IFN-γ mAb (Biosource, Camarillo, CA) followed by blocking with 0.5% bovine serum albumin (BSA, Sigma) for two hours at RT. 100 µl of the recombinant cytokine standard or sample and 50 µl

of 0.2 μg/ml biotin linked anti-human-IL-8, anti-human-IL-6 or anti-human-IFN-γ pAb (Bioscource) detection antibody were simultaneously added to each well. The standards were diluted in PBS (pH: 7.4) containing 0.5% BSA (Sigma) and 0.1% Tween 20 (Merck). Samples, standards and detection antibodies were incubated for 2 hours at RT. Cytokines were detected using streptavidin linked peroxidase (CLB) and TMB peroxidase substrate (Kirkegaard & Perry, Gaithersburg, MD). The optical density (OD) was measured at 450 nm.

Statistical analysis

The Wilcoxon signed ranks test was used to determine the significance of differences between treatment and non-treatment and $p<0.05$ was considered to be significant.

RESULTS

Culture of skin biopsies under different conditions

We observed that the morphology of psoriatic lesional skin was negatively affected by incubation under standard culture conditions (5% CO_2 and 37°C) (Fig 2A). Therefore we

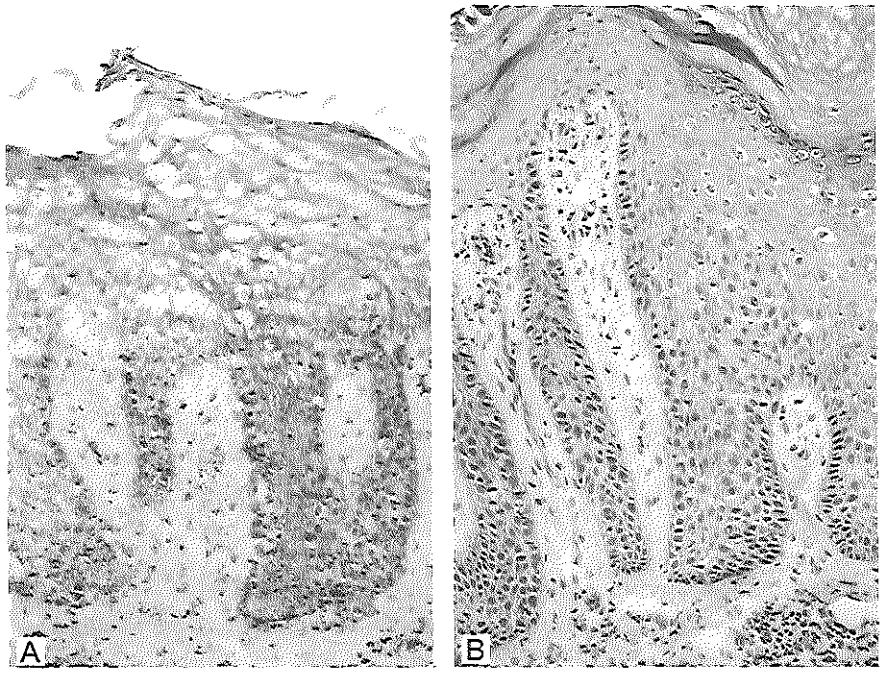

Fig. 2: Standard atmospheric culture conditions affect the morphology of psoriatic skin. Haematoxylin and eosin staining of a psoriatic lesional skin biopsy specimen cultured at **A:** 37° C and 5% CO_2 or at **B:** 32°C, 95% O_2 and 5% CO_2 for 24 hours (magnification: ×160).

asked whether culture under different atmospheric conditions could reverse the deterioration of the morphology. As illustrated in Figure 2B, we found that in this system the degenerative effect on the morphology of lesional skin biopsies was largely abolished during culture at 32°C in an atmosphere containing 95% oxygen. Deterioration of the morphology of normal skin did not occur at 5% CO_2 and 37°C, but to allow adequate comparison with lesional psoriatic skin, normal skin was cultured under identical conditions.

A main drawback of an organ culture system is the occurrence of spontaneous activation, a woundhealing reaction in the explanted skin sample, characterised by the expression of regenerative maturation markers. Because this effect might be induced by a high serum concentration in the medium, we assessed the effect of human serum on the induction of regenerative markers in our *ex vivo* model. Culturing in IMDM without human serum was compared with culturing in medium supplemented with 1% human serum. To measure the spontaneous activation of certain markers in normal skin after culture in medium, the secretion of IL-8 and IL-6 into the culture medium was compared. Differences in IL-8 or IL-6 secretion during culture in medium without human serum and medium supplemented with 1% human serum were not observed (data not shown). However, there was a slight upregulatory effect of human serum on the expression of keratin 17 and TGk.

Inter-sample variation of cytokine secretion

To investigate the variability in cytokine expression between different biopsies, IL-8 secretion in the medium of 4 biopsies from the same donor, cultured under the same conditions was compared.

Background secretion of IL-8 showed some variation, e.g., 487 to 1225 pg/ml, measured in the media of the 4 different biopsies from the same individual. The level of IL-8 secretion was always consistently increased upon LPS stimulation, and variation in secretion of this cytokine was observed as well (range 4153 to 11143 pg/ml). IL-6 secretion showed a similar trend. Background IL-6 secretion ranged from 577 to 990 pg/ml. Upon stimulation with LPS this secretion was elevated, and ranged from 7301 to 16090 pg/ml.

Variation in background expression of IL-8 was also observed among biopsies from different individuals (range 428 ± 96 pg/ml to 2076 ± 831 pg/ml, n = 7, readings in triplicate). Elevation of IL-8 secretion by biopsies of different individuals after LPS stimulation was consistent and ranged from 4- to 13-fold increase compared to background secretion. Variation of IL-6 expression similar to the variation in IL-8 expression was also observed (data not shown).

Increased cytokine secretion upon stimulation with LPS or IL-1β

IL-8 levels in the culture medium were significantly increased after culturing skin for 24 hours with LPS or IL-1β ($p<0.05$; Table 1). Similar results were observed for IL-6 secretion ($p<0.05$; Table I). Compared to background levels of expression, LPS stimulation resulted in an 7 ± 2 fold increase of IL-8 secretion and in an 12 ± 5 fold increase of IL-6 secretion. Upon IL-1β stimulation, IL-8 secretion was elevated by 7 ± 3 fold and IL-6 by 7 ± 2 fold

increase on average.

LPS and IL-1β induced cytokine secretion is suppressed after treatment by immunosuppressive mediators

Normal skin biopsies were pre-treated with dexamethasone or IL-1ra for 1 hour followed by stimulation with IL-1β or LPS. IL-8 secretion induced by IL-1β was partially suppressed by dexamethasone and was completely abolished to below the background level by IL-1ra (Table 1). This indicates that both induced and non-induced IL-1 signalling were blocked by this IL-1 antagonist. This also holds true for IL-6 secretion (Table 1). Similar results were obtained for LPS induced IL-8 and IL-6 expression after dexamethasone treatment (data not shown). The effect of dexamethasone on IL-1β induced cytokine expression was dose-dependently diminished at very low concentrations. For example, IL-1β induced IL-6 secretion was suppressed by 62% after treatment with 10^{-5} M dexamethasone and 18% after treatment with 10^{-9} M dexamethasone (data not shown).

Dexamethasone alone suppressed spontaneous IL-8 secretion below background level, indicating that dexamethasone suppresses medium-induced stimulation in this *ex vivo* system ($p<0.05$; Table 1).

Table 1 Modulation of IL-6 and IL-8 secretion after stimulation and suppression. Secretion of IL-6 and IL-8 by normal skin biopsies stimulated for 24 hours with 250 U/ml IL-1β or 10 μg/ml LPS alone and in combination with 10^{-5} M dexamethasone (dex) or IL-1ra (1 μg/ml). IL-6 and IL-8 levels were measured in the culture supernatant by ELISA. Data represent the mean ± SEM of at least four experiments. Readings were done in triplicate.

Stimulus	IL-6 (pg/ml)	IL-8 (pg/ml)
Medium	992 ± 177	1273 ± 252
IL-1β	6268 ± 1224*	7424 ± 1467*
IL-1β + IL-1ra	667 ± 74	754 ± 77
IL-1β + dex	4327 ± 424	3280 ± 528
IL-1ra	347 ± 108	389 ± 175
LPS	10134 ± 1299*	7131 ± 2049*
Dex	451 ± 53*	226 ± 45*

Modulation of the expression of CD86, CD40, ICAM-1 and HLA-DR

In freshly isolated skin CD40 was expressed on keratinocytes, especially in the basal layer, and on cells with dendritic morphology in both epidermis and dermis. CD86 and HLA-DR were present only on cells with dendritic morphology in epidermis and dermis. ICAM-1 expression was observed mainly in the dermis, and sporadically on keratinocytes in the basal layer.

Spontaneous upregulation of the tested markers was observed after culture in medium alone (data not shown). CD86 expression on cells with dendritic morphology in both dermis and the epidermis was further enhanced upon stimulation with LPS (Fig. 3a versus Fig. 3b and Table 2). LPS stimulation also resulted in elevation of CD40 expression on basal keratinocytes and cells with dendritic morphology in dermis and epidermis, and resulted in increased expression of ICAM-1 in the dermis and the basal epidermal layer (Table 2). The

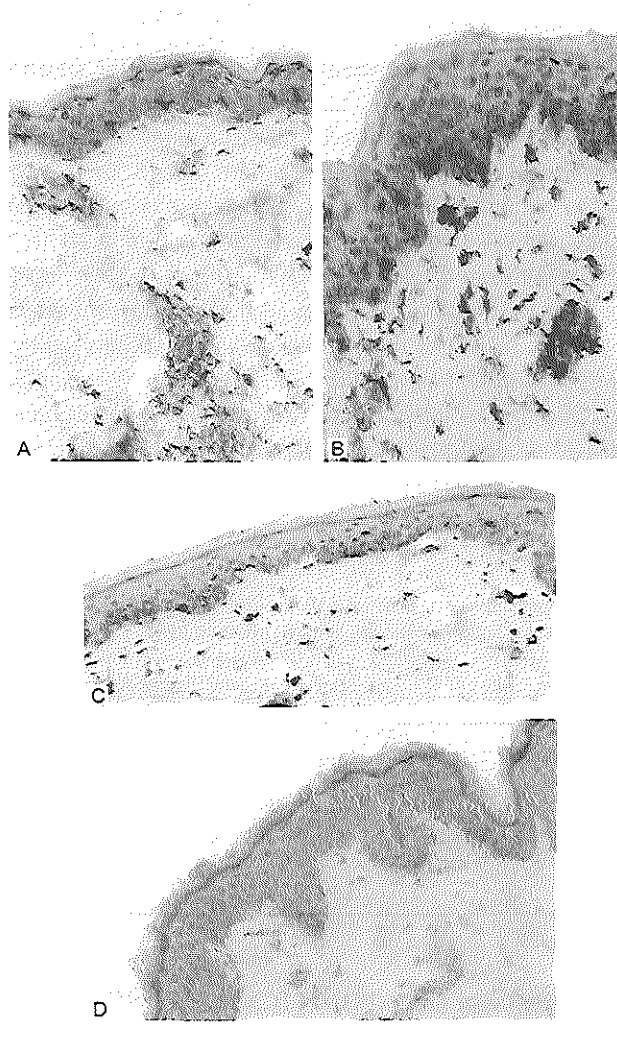

Fig. 3: CD86 expression is elevated after stimulation with LPS and downregulated after treatment with dexamethasone. **A:** Medium control; **B:** CD86 expression after LPS stimulation; **C:** CD86 expression after culture in medium in the presence of 10^{-5} M dexamethasone and **D:** isotype control antibody staining (magnifications: ×400). Data of one representative experiment out of four are shown.

expression of HLA-DR showed no further increase after the applied stimuli (Table 2).

Culturing of normal skin biopsies in medium containing 10^{-5} or 10^{-6} M dexamethasone, inhibited the expression of CD86, CD40 and ICAM-1, to levels below medium-induced expression. The effects on the expression of CD86 are shown in Fig. 3C. The effect of dexamethasone was dose-dependently diminished at low concentrations (e.g.: 10^{-7} M dexamethasone). After IL-1β stimulation an upregulation of CD40, CD86 and ICAM-1 was

observed similar to that seen after LPS stimulation (Table 2). IL-1β did not induce a further increase of HLA-DR expression. IL-1ra partially blocked the IL-1β induced effects (data not shown).

Table 2 Modulation of CD86, CD40, ICAM-1 and HLA-DR expression after stimulation and suppression. Expression of CD86, CD40, ICAM-1 and HLA-DR in normal skin biopsies stimulated for 24 hours with 250 U/ml IL-1β, 10 μg/ml LPS or 10^{-5} M dexamethasone (dex). Staining intensity and numbers of positive cells were reproducibly ranked by two independent observers blinded to treatment using a semiquantitative scoring scale (range: 0 (no staining) - 3 (high staining intensity)). Data represent the mean ± SEM of at least four experiments. Readings were done in triplicate.

Marker	no stimulus	LPS	IL-1β	dex
CD86	2.0 ± 0.0	3.0 ± 0.0	2.5 ± 0.3	1.4 ± 0.7
CD40	2.2 ± 0.2	3.0 ± 0.0	2.5 ± 0.3	1.4 ± 0.4
ICAM-1	1.4 ± 0.7	3.0 ± 0.0	2.8 ± 0.3	0.6 ± 0.5
HLA-DR	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0

IFN-γ induction by CD3 stimulation in psoriatic lesional skin biopsies

Secretion of IFN-γ was detected in supernatants of psoriatic lesional skin biopsies stimulated with CD3 stimulatory antibodies (237 ± 97 pg/ml; mean ± SEM). No IFN-γ was detected in supernatants of unstimulated biopsies. Biopsies were stimulated for three time points (24, 48 and 72 hours) but no significant differences in amount of IFN-γ secretion was detected in the medium (48 hours: 305 ± 122 pg/ml and 72 hours: 346 ± 107 pg/ml).

DISCUSSION

An *ex vivo* skin organ culture model is presented which allows close monitoring of the events following activation and suppression of the skin immune system in healthy human skin. In the present study this is illustrated on the basis of the stimulatory effects of LPS and IL-1β on the levels of IL-6 and IL-8 released in the culture medium and on the expression of cell signalling and adhesion molecules in situ. These stimulatory effects of LPS and IL-1 β could be inhibited by dexamethasone and IL-1ra. The system used here is based on the method developed by Trowell (Trowell, 1959), but modified in such a way, by using punctured transwell filters, that the dermal part of the skin biopsy is immersed in the culture medium and the epidermis remains exposed to air. This system allows to collect data simultaneously on the amount of cytokine released in the culture medium and the expression of cell activation markers on the cells in the cultured skin samples.

We showed that strong stimuli efficiently upregulate different markers in this system. However, strong stimulation could result in a decay of the skin architecture which could interfere with monitoring the modulation of specific inflammation markers using immuno histochemistry. Kondo and others showed that the morphology of psoriatic lesional skin remained preserved when it was cultured under special atmospheric conditions (5% CO_2/95% O_2 and 32°C) (Kondo, 1986; Tammi & Jansen, 1980; Yasuno *et al.*, 1981). These conditions might

mimic the natural environment of human skin. In particular inflamed skin needs a high oxygen supply to meet the demands of an enhanced tissue metabolism. We showed that in this system the morphology of psoriatic lesional skin remained intact when cultured under these conditions. Additionally we show that culturing under these specific conditions allows monitoring of upregulation and downregulation of certain markers of inflammation in the medium and skin.

Previous experiments in our lab showed that regenerative maturation markers, like keratin 17 and TGk were strongly induced during culture in medium containing a high serum concentration (i.e. 5% human serum). This hampered monitoring the assessment of the stimulation effect in the cultured skin biopsies (Wei, unpublished data). The background induction of the regenerative maturation markers was limited in this system, mainly due to the lower concentration of human serum and probably the elimination of tight adhesion of the biopsy sample to the culture plate. Earlier studies showed similar effects (Wei *et al.*, 1999).

Secretion of human skin cell derived cytokines into the medium after culture has been reported previously, (Ameglio *et al.*, 1997) using an *ex vivo* skin explant system in which submerged skin biopsies were cultured. Some fluctuation in the levels of IL-6 and IL-8 secretion between several normal skin donors were observed. Variation in cytokine levels after culturing of biopsies from different donors occurred in this system as well and we also observed variation in cytokine levels within a single individual. However, the level of cytokine induction after LPS or IL-1β stimulation and the degree of inhibition after treatment with immunosuppressive agents was significant.

Culturing with bacterial LPS and IL-1β resulted in a clear increase of IL-6 and IL-8 levels in the medium and of CD40, CD86 and ICAM-1 expression on skin cells. These effects could be suppressed by dexamethasone in a dose dependent fashion and IL-1ra specifically antagonised the effects of IL-1β. The suppressive effect of dexamethasone in skin was also observed by Furue *et al.* (Furue & Katz, 1989). Furthermore we were able to induce INF-γ secretion in psoriatic lesional skin biopsies, showing that cytokines associated with the pathology of psoriasis can be assessed as well.

Additionally, the release of soluble IL-1 receptor type 2 (sIL-1RII) and sICAM-1 in the culture medium by skin biopsies cultured under standard conditions was enhanced by IL-1β and IFN-γ stimulation respectively ((Kooy *et al.*, 1998) and Wei *et al.*, manuscript submitted for publication). This indicates that also secretion of soluble cell membrane proteins can be assessed in this system.

The present study describes a modified *ex vivo* skin organ culture model in which the different read out systems are combined. In addition, we culture under special conditions which preserves the morphology of diseased skin so that this model can be used to investigate modulation of different markers in disease models such as psoriasis (Wei *et al.*, manuscript submitted for publication). In this model the modulation of cytokine secretion and membrane protein expression in response to immunostimulators or suppressors can be measured in the same experiment.

This system provides a fast and simple method for functional studies using readily

available human skin and also allows quick screening of drugs for treatment of a variety of skin diseases.

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Chapter 6

CD40 LIGATION-INDUCED CYTOKINE PRODUCTION IN HUMAN SKIN EXPLANTS IS PARTLY MEDIATED VIA IL-1

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Submitted

ABSTRACT

CD40 ligation by $CD40L^+$ $CD4^+$ T-cells has been claimed to be involved in inflammatory responses in human skin. However, these data are derived from *in vitro* cell culture systems and immunohistochemistry, and the mechanisms involved have not been fully elucidated. We previously observed that cells in intact normal human skin secrete high levels of IL-6 and IL-8 upon stimulation with IL-1β. *In vitro* studies have shown that CD40 ligation on human keratinocytes results in the production of IL-6 and IL-8 as well. We used a novel tissue culture system with intact normal human skin and show that ligation of CD40 using an agonistic anti-CD40 monoclonal antibody (mAb) results in the induction of several pro- and anti-inflammatory cytokines. IL-6, IL-8, TNF-α, IL-12 and IL-1β were induced in the presence of the agonist CD40 mAb and IFN-γ, while IL-10 could be induced by the agonist mAb alone and was reduced again by the addition of IFN-γ. Since CD40 ligation on monocytes and dendritic cells *in vitro* results in the secretion of IL-1, which is pre-stored in high concentrations in normal human keratinocytes, we subsequently investigated whether CD40 induced IL-6 and IL-8 production in skin is mediated via IL-1. Indeed IL-1 receptor antagonist (IL-1ra) inhibited the CD40 ligation-induced IL-6 and IL-8 production. CD40 ligation-induced TNF-α and IL-10 production were not affected by IL-1ra, while IL-1β production was even further enhanced by the addition of IL-1ra. These data show that CD40 ligation-induced secretion of IL-6 and IL-8, but not TNF-α, IL-10 and IL-1β, is partially mediated via IL-1 and that IL-1 plays a prominent role in the inflammatory response initiated by CD40 ligation in intact human skin.

INTRODUCTION

CD40 is a member of the TNF-receptor family and was first discovered as a 50 kD surface antigen on B lymphocytes (Paulie *et al.*, 1985). The ligand of CD40 (CD40L or CD154) is a 39 kD glycoprotein and is functionally expressed on activated $CD4^+$ T cells (Armitage *et al.*, 1992). Research was initially focussed on the role of CD40 in the humoral immune response. The importance of CD40 in isotype switching is emphasized by the observation that mutations in the CD40L gene lead to X-linked hyper-IgM syndrome (Aruffo *et al.*, 1993; DiSanto *et al.*, 1993). CD40 is also expressed by non-B cells and appeared to be a key player in other immune responses as well. Stimulation of CD40 on dendritic cells results in the upregulation of co-stimulatory molecules like CD80 and CD86 (Caux *et al.*, 1994). Besides enhancing co-stimulation, CD40 ligation induces the secretion of inflammatory cytokines such as IL-1, TNF-α, IL-6 and IL-8 from monocytes (Kiener *et al.*, 1995). The IL-12-mediated activation of Th1 cells is promoted via CD40 stimulation on macrophages and dendritic cells which results in IL-12 production by these cells (Kato *et al.*, 1996; Schulz *et al.*, 2000). Furthermore, CD40 ligation-induced responses are also involved in tumoricidal activity and

nitric oxide production (Angulo *et al.*, 2000; Müerköster *et al.*, 2000).

CD40 expression is not restricted to leukocytes. Expression of CD40 on resting human keratinocytes was first observed by Denfeld *et al* (Denfeld *et al.*, 1996), and was shown to be upregulated by IFN-γ. Activation of CD40 on keratinocytes by CD40L results in elevated expression of ICAM-1, IL-8, IL-6 and TNF-α (Denfeld *et al.*, 1996; Gaspari *et al.*, 1996; Peguet-Navarro *et al.*, 1997). Ligation of CD40 on human fibroblasts *in vitro* leads to expression of ICAM-1, VCAM-1 and IL-6 production (Yellin *et al.*, 1995). The proliferation of skin cells is influenced by CD40 as well. CD40 triggering on keratinocytes inhibits proliferation and promotes differentiation (Grousson *et al.*, 2000; Peguet-Navarro *et al.*, 1997). Conversely, CD40 ligation on fibroblasts *in vitro* stimulates their proliferation (Fries *et al.*, 1995; Yellin *et al.*, 1995). CD40 is also functionally expressed on human epidermal Langerhans cells and its ligation results in enhancement of viability and upregulation of ICAM-1 and CD86 expression (Peguet-Navarro *et al.*, 1995).

The role of CD40-CD40L interactions during inflammation in human skin was studied in *Leishmania* and *Mycobacterium leprae* infections (Marovich *et al.*, 2000; Yamauchi *et al.*, 2000). Both studies clearly indicate that cognate interactions of $CD40L^+$ $CD4^+$ cutaneous T cells with CD40-expressing APC from patients suffering from *Leishmania major* or *Mycobacterium leprae* infection result in the induction of bioactive IL-12 (Marovich *et al.*, 2000; Yamauchi *et al.*, 2000). Furthermore, CD40 function may also play a role in the disease process of the inflammatory skin disease psoriasis, since upregulation of CD40 expression in psoriatic lesions has been reported (Denfeld *et al.*, 1996). Finally, it has been shown that CD40 ligation plays a role in skin allograft rejection (Larsen *et al.*, 1996) and migration of Langerhans cells from skin to the draining lymph nodes (Moodycliffe *et al.*, 2000). It is therefore plausible that CD40 has a function in skin inflammation.

The data on CD40 ligation-induced cytokine expression in human skin presented during recent years were derived from *in vitro* or immunohistochemical staining studies. However, knowledge of the mechanisms underlying CD40 ligation-induced cytokine expression by skin cells in their natural environment is lacking.

Therefore we used a novel culture system (Companjen *et al.*, 2001) to study the role of CD40 ligation in intact human skin. We previously showed that IL-6 and IL-8 production in normal human skin is increased after stimulation with IL-1β (Companjen *et al.*, 2001). It is also known that CD40 ligation on monocytes *in vitro* results in the induction of IL-1 (Wagner *et al.*, 1994) and that high concentrations of IL-1 are pre-stored in normal human keratinocytes (Arend *et al.*, 1998; Kupper & Groves, 1995). Therefore we asked whether the CD40 induced IL-6 and IL-8 production in skin is mediated via IL-1.

The data presented here show that CD40 stimulation in intact normal human skin results in the induction of several pro- and anti-inflammatory cytokines and that CD40 ligation-induced secretion of IL-6 and IL-8, but not TNF-α, IL-10 and IL-1β, is largely mediated via IL-1.

MATERIAL AND METHODS

Skin biopsies

Normal skin biopsies were obtained from 6 healthy volunteers undergoing a breast reduction in the department of Plastic Surgery of the Sint Franciscus Gasthuis Rotterdam, The Netherlands. After informed consent biopsies with an average length of 3 mm were taken with a 3 mm diameter biopsy punch (Stiefel, Leuven, Belgium) and were either snap frozen in Tissue Tek (Bayer, München, Germany), or cultured (see below). After culture the biopsies were immersed in Tissue Tek (Bayer) and snap frozen in liquid nitrogen. Biopsies were stored at -80°C until use.

Skin organ culture

Biopsies were cultured as described elsewhere (Companjen *et al.*, 2001). In brief, a 2 mm hole was punched in a Netwell filter (pore size: 0.75 µm; Corning Costar, Corning, NY). The biopsy was inserted into the hole (3 biopsies per filter), and the filter containing the biopsies was placed in a 12-well plate containing 1 ml medium. Skin biopsies were cultured in IMDM (GibcoBRL, Paisley, Scotland) containing 1% heat inactivated human serum (HS) (Sigma, St. Louis, MO), 100 U/ml penicillin and 100 µg/ml streptomycin (BioWhittaker, Verviers, Belgium) under special conditions (95% O_2 and 5% CO_2 at 32°C) in a culture bag. The biopsies were cultured under the following conditions: Biopsies were pre-treated for 24 hrs in medium containing 1000 U/ml IFN-γ (Boehringer Ingelheim, Alkmaar, The Netherlands) or in medium devoid of IFN-γ. After pre-treatment the filters containing the biopsies were washed in PBS and transferred to a new 12-well plate containing 1 ml medium with or without 1000 U/ml IFN-γ and stimulus. Biopsies were stimulated with an agonistic anti-CD40 mAb (clone 64 mouse-anti-human CD40; Tanox Pharma BV, Amsterdam, The Netherlands; isotype: mouse anti human IgG1; concentration: 20 µg/ml) alone or antibody in the presence of IL-1ra (Synergen, Denver, CO; concentration: 1 µg/ml). Biopsies cultured in the presence of an antibody of the same isotype as the anti-CD40 antibody, but of irrelevant specificity served as a control. After 3 days of stimulation the supernatants and biopsies were isolated, and stored at -80°C until use.

Cytokine ELISA

Maxisorb ELISA plates (Nunc, Roskilde, Denmark) were coated for 18 hr at 4°C with 100 µl of 0.5 µg/ml αhIL-6, αhIL-8, αhTNF-α, αhIL-1β or αIL-10 mAb (Biosource, Camarillo, CA) followed by blocking with 0.5% BSA (Sigma) for two hrs at RT. Hundred µl of the recombinant IL-6, IL-8, TNF-α, IL-1β or IL-10 (Biosource) standard or sample and 50 µl of 0.2 µg/ml biotin linked αhIL-6, αhIL-8, αhTNF-α, αhIL-1β or αhIL-10 (Bioscource) polyclonal detection antibody were simultaneously added to each well. The standards were diluted in PBS containing 0.5% BSA (Sigma) and 0.1% Tween 20 (Merck). Samples, standards and detection antibodies were incubated for 2 hrs at RT. Cytokines were detected using streptavidin linked peroxidase (CLB, Amsterdam, The Netherlands) and TMB peroxidase

substrate (Kirkegaard & Perry, Gaithersburg, MD). The OD was measured at 450 nm. ELISA readings were done in duplicate.

Immunohistochemistry

Skin biopsies were snap frozen in liquid nitrogen and cryosections were cut using a cryostat (Jung Frigocut 2800 E, Leica, Rijswijk, The Netherlands) and stored in a sealed box containing silica gel at -80°C prior to use. Sections were fixed in acetone for 10 min at room temperature (RT) and pre incubated for 10 min with PBS containing 0.05% Tween 20 (Merck, Whitehouse Station, NJ) at RT. Subsequently sections were incubated for 18 hrs at 4°C with biotin linked antibodies specific for hIL-12p40/p70 (C8.6, Pharmingen; dilution 1:200) followed by incubation with peroxidase linked avidin (DAKO). Sections by which the first antibody was omitted during the first incubation served as a control. 3-amino-9-ethylcarbazole (Sigma) was used as the chromogen. Staining intensity and numbers of positive cells were reproducibly ranked by two independent observers blinded to treatment using a semiquantitative scoring scale. Staining intensity scale: -: no staining to +++: high staining intensity. Scoring scale for the number of positive cells: -: no positive cells to +++: many positive cells (>25 per image field, magnification: 100×).

Statistical analysis

The Wilcoxon signed ranks test was used to determine the significance of differences between treatment and non-treatment. $p<0.05$ was considered to be significant.

RESULTS

CD40 ligation in normal human skin induces both pro- and anti-inflammatory cytokines

To assess whether CD40 stimulation in human skin results in the elevation of cytokine expression, normal human skin biopsies were cultured in the presence of an agonistic anti-CD40 mAb. Secretion of IL-1β by normal skin biopsies was significantly elevated upon stimulation with the agonistic anti-CD40 mAb. IL-1β was not detectable in supernatants of non-stimulated biopsies or biopsies cultured in the presence of IFN-γ. Secretion of IL-1β was not further enhanced by the addition of IFN-γ to the agonistic anti-CD40 mAb compared to stimulation with the mAb alone (Table 1).

Culturing of biopsies in medium alone resulted in spontaneous IL-6 and IL-8 secretion. This IL-6 and IL-8 release was significantly inhibited by the addition of IFN-γ (70 to 80%, Table 1, $p<0.05$). The agonistic anti-CD40 mAb alone stimulated IL-6 and IL-8 production. However, in the presence of IFN-γ, the agonistic anti-CD40 mAb was able to prevent the downregulation of the cytokines induced by IFN-γ alone (Table 1). IL-6 secretion by biopsies cultured in the presence of the agonistic anti-CD40 mAb and IFN-γ showed a 10-fold increase compared to biopsies cultured in the presence of IFN-γ alone (Fig. 1 and Tables 1 and 2). Culturing in the presence of an isotype-matched control antibody and IFN-γ had no

Table 1. IFN-γ differentially regulates CD40 ligation-induced cytokine secretion. Normal skin biopsies were cultured in the presence and absence of IFN-γ and either or not stimulated with agonistic anti-CD40 mAb. Later IL-6, IL-8, TNF-α, IL-1β and IL-10 concentrations were measured in the supernatant by ELISA. Data represent the mean ± SEM (pg/ml or ng/ml) of 6 donors in independent experiments of identical design. αCD40: agonistic anti-CD40 mAb. ▲: αCD40 compared to not stimulated: $p<0.05$; *: IFN-γ compared to no IFN-γ: $p<0.05$.

Cytokine		Additions			
		without IFN-γ		with IFN-γ	
		None	αCD40	None	αCD40
IL-6	(ng/ml)	4.37 ± 1.08	7.32 ± 1.48▲	0.63 ± 0.10*	6.41 ± 1.23▲
IL-8	(ng/ml)	14.26 ± 7.16	22.89 ± 9.87▲	1.71 ± 0.52*	13.92 ± 5.24▲
TNF-α	(pg/ml)	18.46 ± 5.40	84.14 ± 22.66▲	13.89 ± 2.85	120.68 ± 23.85▲
IL-1β	(pg/ml)	< 2.00	12.65 ± 3.62▲	< 2.00	10.83 ± 3.30▲
IL-10	(pg/ml)	6.03 ± 3.13	34.61 ± 13.27▲	< 1.00	3.59 ± 1.01▲

effect (unstimulated: 0.63 ± 0.10 pg/ml and isotype-matched control antibody: 1.16 ± 0.34 pg/ml; mean ± SEM, $p = 0.27$).

Similar effects were observed for IL-8 secretion, which showed a 9-fold increase upon stimulation of CD40 in the presence of IFN-γ (Table 1). Comparable levels of TNF-α were produced by biopsies cultured in medium alone or in the presence of IFN-γ. Addition of the

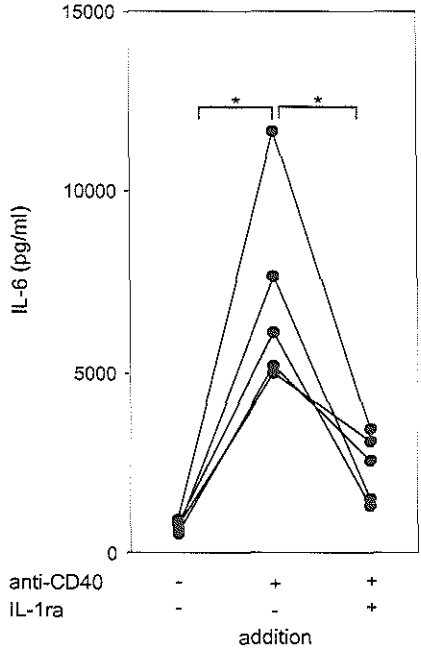

Fig. 1. CD40 ligation in normal human skin biopsies in the presence of IFN-γ stimulates IL-6 production. Normal skin biopsies were cultured for a total of 4 days in the presence of IFN-γ to upregulate CD40 expression and stimulated with an agonistic anti-CD40 mAb as described in the methods section. IL-6 concentrations were measured in supernatants of biopsies from 5 donors. Data of 5 independent experiments of identical design are presented. *: $p<0.05$.

agonistic CD40 mAb alone stimulated TNF-α production 5–fold, while agonist mAb combined with IFN-γ induced a 10-fold higher TNF-α production compared to medium or IFN-γ alone. Additionally, immunohistochemistry revealed that IL-12p40 and IL-12p70 expression in the tissue was elevated upon stimulation of CD40 as well (Fig. 2). Biopsies cultured in the presence of agonistic CD40 mAb and IFN-γ, displayed a diffuse staining in the epidermis with occasional stronger positive cells with dendritic morphology (probably Langerhans cells). In the dermis positively stained infiltrates were observed including cells with macrophage or dendritic morphology. All positively stained cells showed a cytoplasmatic staining pattern. Cells of biopsies cultured in the presence of IFN-γ alone also included IL-12p40/p70 positive cells, but numbers of positive cells were markedly lower compared to biopsies in which CD40 was stimulated. Biopsies in which CD40 was stimulated in he absence of IFN-γ displayed staining patterns similar to biopsies cultured in the presence of IFN-γ and agonistic CD40 mAb.

CD40 ligation also affected IL-10 secretion. In medium devoid of IFN-γ, IL-10 secretion was spontaneously induced in some skin biopsies (Table 1). Upon CD40 stimulation and in the absence of IFN-γ, IL-10 was significantly increased compared to the amount of IL-10 secreted by unstimulated biopsies (Table 1). Similarly to IL-6 and IL-8, IFN-γ inhibited IL-10 secretion by normal human skin biopsies. For IL-10, however, CD40 stimulation was not able to overcome the inhibitory effect of IFN-γ.

These data show that CD40 ligation in normal human skin explants results in the induction of both pro-inflammatory (IL-1β, IL-6, IL-8, TNF-α and IL-12) and anti-inflammatory (IL-10) cytokines.

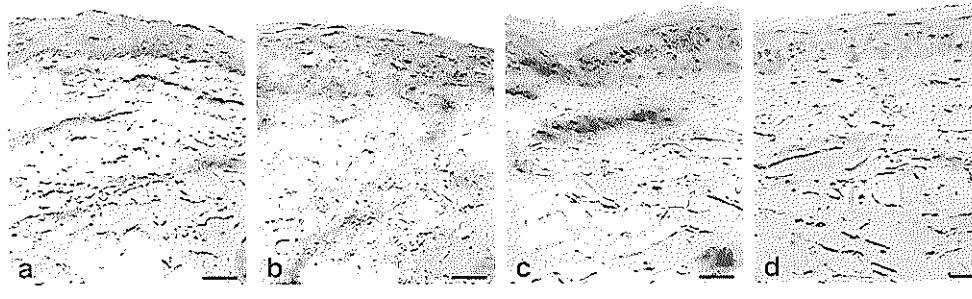

Fig. 2: IL-12p40/p70 expression is elevated upon CD40 stimulation. Acetone-fixed cryostat sections were stained with an IL-12p40/p70 specific mAb as described in the methods section. Culture conditions: (a) no stimulation. (b) 1000 U/ml IFN-γ. (c) 1000 U/ml IFN-γ and anti-CD40 mAb. a-c: stained with a mAb specific for IL-12p40/p70. (d) 1000 U/ml IFN-γ and anti-CD40 mAb, omission primary antibody. Scale bar: 50 μm.

CD40 ligation-induced secretion of IL-6 and IL-8 but not TNF-α and IL-10 is mediated via IL-1

To investigate whether IL-1 is involved in CD40 ligation-induced IL-6, IL-8, TNF-α

and IL-10 secretion, normal human skin biopsies were stimulated with the CD40 agonistic mAb and IFN-γ in the presence of IL-1ra. Culturing of normal skin biopsies in the presence of IL-1ra and IFN-γ significantly inhibited IL-6 and IL-8 secretion compared with culturing in the presence of IFN-γ alone (Table 2). No effect of IL-1ra on TNF-α and IL-10 secretion in cultures with IFN-γ was observed (Table 2). In the presence of IFN-γ, IL-1ra significantly inhibited CD40 ligation-induced IL-6 and IL-8 secretion by normal human skin biopsies by approximately 60% ($p<0.05$, Table 2 and Fig. 1). Conversely, in IFN-γ containing cultures CD40 ligation-induced TNF-α secretion was not affected by IL-1ra which was also observed for IL-10 (Table 1). CD40 ligation-induced IL-1β levels were also influenced by IL-1ra. Culturing of biopsies in the presence of CD40 agonistic mAb in combination with IL-1ra and IFN-γ resulted in an increase of IL-1β secretion (10.83 ± 3.30 vs. 26.09 ± 7.34 pg/ml, mean ± SEM, n = 5).

Comparable results were obtained when normal skin biopsies were stimulated with CD40 agonistic mAb in the presence of IL-1ra in cultures devoid of IFN-γ (data not shown).

These data show that CD40 ligation-induced secretion of IL-6 and IL-8, but not TNF-α or IL-10, is mediated to a major extent via IL-1.

Table 2. CD40 ligation-induced secretion of IL-6 and IL-8 but not TNF-α and IL-10 is mediated via IL-1. Normal skin biopsies were cultured in the presence of IFN-γ and stimulated with agonistic anti-CD40 mAb alone or antibody in the presence of IL-1ra. Later IL-6, IL-8 and TNF-α concentrations were measured in the supernatant by ELISA. ELISA readings were done in duplicate. Data represent the mean ± SEM (pg/ml or ng/ml) of 5 donors in independent experiments of identical design. αCD40: anti-CD40 mAb. ♠: αCD40, IL-1ra and αCD40 + IL-1ra compared to not stimulated: $p<0.05$; *: αCD40 compared to αCD40 + IL-1ra: $p<0.05$.

Cytokine		Additions			
		None	αCD40 mAb	IL-1ra	αCD40 mAb + IL-1ra
IL-6	(ng/ml)	0.72 ± 0.08	7.11 ± 1.23♠	0.19 ± 0.03♠	2.38 ± 0.42♠, *
IL-8	(ng/ml)	1.72 ± 0.63	15.20 ± 6.23♠	0.65 ± 0.40♠	5.56 ± 2.19♠, *
TNF-α	(pg/ml)	16.38 ± 1.70	138.04 ± 20.04♠	16.94 ± 3.66	129.06 ± 31.36♠
IL-10	(pg/ml)	< 1.00	3.65 ± 1.11♠	< 1.00	3.96 ± 1.75♠

DISCUSSION

Recent studies suggest that CD40 ligation plays a key role in inflammatory responses in skin. However, these studies used *in vitro*, *in vivo* or *in situ* analysis. Here we present functional data on the effects of CD40 ligation on cytokine expression in intact human skin using a recently developed *ex vivo* culture system (Companjen *et al.*, 2001). Our data indicate that CD40 stimulation in intact normal skin results in the induction of pro- and anti-inflammatory cytokines. Furthermore, we demonstrate that CD40 ligation-induced secretion of IL-6 and IL-8, but not TNF-α and IL-10, is partly mediated via IL-1.

First we showed that stimulation of CD40 in intact normal human skin results in the induction of IL-1β secretion. IL-1β induction upon CD40 stimulation has also been demonstrated in monocytes (Wagner *et al.*, 1994), dendritic cells (la Sala *et al.*, 2001) and

Langerhans cells (Nakagawa *et al.*, 1999). It is therefore possible that monocyte-derived dendritic cells or macrophages in skin are the primary source of the IL-1β measured in our system. Although it is known that human keratinocytes can produce IL-1β, Denfeld *et al* (Denfeld *et al.*, 1996) demonstrated that ligation of CD40 on keratinocytes *in vitro* did not induce IL-1β production. The difference in observation reported by Denfeld *et al* and our group can be explained by the difference in system used. We studied the effects of CD40 ligation on all cells present in normal skin whereas Denfeld *et al* investigated CD40 ligation on keratinocytes only. Furthermore, the IL-1β concentration in supernatant of CD40 stimulated keratinocytes might be too low to detect. Finally the difference in observation might suggest that CD40 ligation does not result in upregulation of IL-1β in keratinocytes and that the IL-1β detected in our system after CD40 stimulation is not keratinocyte derived or that factors induced upon CD40 ligation in cells other than keratinocytes induce IL-1β in skin resident cells.

Several reports show that CD40 ligation on skin cells *in vitro* induces IL-6, IL-8 and TNF-α expression. CD40 ligation-induced IL-6, IL-8 as well as TNF-α production by human keratinocytes in suspension has been reported (Denfeld *et al.*, 1996; Gaspari *et al.*, 1996; Peguet-Navarro *et al.*, 1997). Fibroblasts are also known to produce IL-6 upon CD40 triggering (Yellin *et al.*, 1995). CD40 stimulation on endothelial cells results in the upregulation of IL-6, IL-8 and TNF-α as well (Mach *et al.*, 1997). Our data confirm and extend the data presented by these groups by showing that induction of pro-inflammatory cytokines like IL-6, IL-8, TNF-α and IL-12 upon CD40 stimulation also occurs in intact human skin.

Induction of IL-10 by CD40 stimulation in skin has not been reported yet. Denfeld *et al* (Denfeld *et al.*, 1996) reported that CD40 ligation on keratinocytes did not result in the induction of IL-10. However, CD40 ligation-induced IL-10 expression has been reported in other *in vitro* systems. For example, IL-10 expression in PBMC from patients with Graves disease was upregulated upon stimulation of CD40 in combination with IL-4 (Itoh *et al.*, 2000) and CD40 mediated IL-10 induction was also observed by dexamethasone pre-treated dendritic cells (Rea *et al.*, 2000). This suggests that the probable IL-10 source in our system may be monocytes derived cells like dermal dendritic cells, Langerhans cells or macrophages which are present in normal human skin.

Next we investigated whether IL-1 plays a role in the CD40 ligation-induced cytokine expression. We observed that IL-1ra could inhibit CD40 induced IL-6 and IL-8, but not the TNF-α or IL-10 expression. However, IL-6 and IL-8 levels observed after culturing in medium containing CD40 agonistic antibodies and IL-1ra did not equal the levels observed after culturing with IL-1ra alone. This indicates that CD40 ligation-induced IL-6 and IL-8 secretion is also influenced by factors other than IL-1. TNF-α might be a candidate because it has been shown that this cytokine can induce IL-6 and IL-8 secretion by synovial fibroblasts (Yoshida *et al.*, 1999) and TNF-α is also elevated upon CD40 stimulation in our system. In contrast to the decreased IL-6, IL-8 and unchanged TNF-α secretion, IL-1β secretion was elevated after stimulation of CD40 in combination with IL-1ra. The explanation for this may be that IL-1β binding to the IL-1 receptor is blocked by IL-1ra in cultures containing both

IL-1ra and anti-CD40. Consequently this IL-1 receptor blockade results in the secretion of IL-1β normally bound to its receptor in cultures lacking IL-1ra.

During our studies we observed that the spontaneous IL-6 and IL-8 secretion in the culture medium was downregulated upon treatment with IFN-γ. Downregulation of IL-6 expression by IFN-γ represents a novel observation whereas inhibition of IL-8 expression by IFN-γ in thymic epithelial cells has been reported (Galy & Spits, 1991). An explanation for the downregulation of IL-6 and IL-8 secretion could be that IFN-γ induces expression of IL-6 and IL-8 receptors. Subsequent binding of IL-6 and IL-8 to these receptors could result in a decreased secretion into the medium. Previous studies reported that IL-6 receptor expression by monocytes and intestinal epithelial cells is upregulated by IFN-γ (Panja *et al.*, 1998; Sanceau *et al.*, 1991). Additionally we showed that the agonistic CD40 mAb was able to prevent the IFN-γ mediated downregulation of IL-6 and IL-8 expression, while it was not able to overcome the IFN-γ mediated downregulation of IL-10. This suggests that CD40 ligation by activated T cells in skin inhibits the IFN-γ directed repression of pro-inflammatory cytokine production, whereas it does not affect the repression of anti-inflammatory cytokines. Because IL-10 promotes T helper 2 development, the IFN-γ mediated repression of IL-10, even in the presence of CD40 ligation, may be in advantage for the maintenance of the T helper 1 (Th1) balance in inflamed skin. Additionally, Th1 development is promoted via CD40 ligation-induced IL-12 production, even in the absence of IFN-γ.

The mechanism of CD40 ligation leading to cytokine expression in normal skin is summarized in the following model (Fig. 3). During inflammation, T cell or NKT cell derived IFN-γ elevates CD40 expression. Subsequent CD40 ligation on skin resident cells by CD40L on activated T cells results in the secretion of IL-1 followed by the IL-1 induced IL-6 and

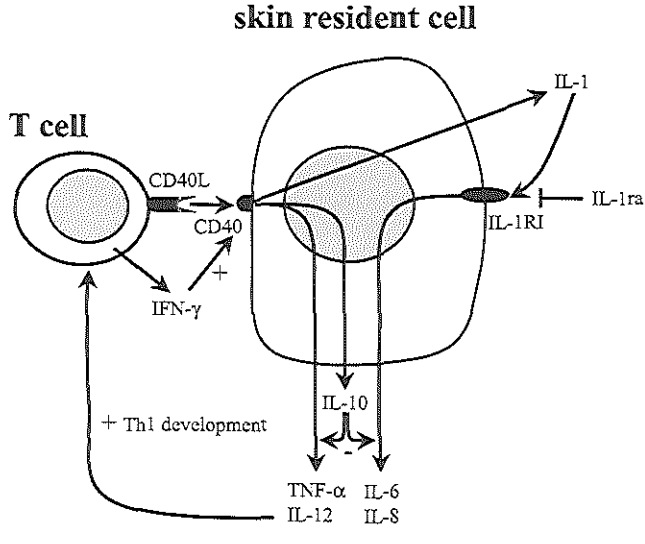

Fig. 3. CD40 ligation-induced expression of IL-6 and IL-8, but not TNF-α and IL-10, is mediated via IL-1. See text for explanation.

IL-8 production. TNF-α and IL-10 are directly mediated by CD40 ligation or via other factors induced after CD40 ligation. Additionally CD40 induces IL-12 expression, which is a critical promoter of Th1 development. Finally, IL-10 has a downregulating effect on the expression of TNF-α, IL-12, IL-6 and IL-8.

To our knowledge this is the first study integrating IL-1 and CD40 regulated immune responses in human skin. The data presented provide evidence that IL-1 is involved in CD40 mediated immune responses in the intact human skin. Since both IL-1 and CD40 expression are functionally implicated in inflammatory skin diseases like psoriasis, the present information adds to the understanding of the mechanisms involved in the onset and maintenance of inflammation in skin.

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Chapter 7

GENERAL DISCUSSION

The skin is continuously exposed to a broad array of environmental hazards such as pathogens or injury. In order to cope with these threats, human skin contains a tightly regulated network of pro-inflammatory cytokines which become active during infection or after trauma. Major pro-inflammatory cytokines that are known to be involved in such threats, are the members of the IL-1 family of proteins including IL-18, IL-1β and IL-1α. The aim of this thesis was to study the modulation of IL-18 and other members of the IL-1 family of pro-inflammatory cytokines, in normal and inflamed skin, with emphasis on psoriatic skin.

Four hypotheses were formulated in the general introduction of this thesis: (1) Epidermal cell derived IL-1β can be activated by proteases other than caspase-1; (2) Human keratinocytes express high levels of IL-18 and predominantly produce the unprocessed form; (3) IL-18 expression in psoriatic lesional skin is elevated compared to normal skin; and (4) Induction of IL-6 and IL-8 expression through CD40 stimulation is mediated via IL-1. The validity of the hypotheses is discussed here on the basis of the outcome of our studies and in the framework of relevant data in the literature.

This general discussion focusses on three themes that have become clear from the discussions of the previous chapters. The first theme concerns processing of IL-18 and IL-1 family members (hypotheses 1 and 2). Here, the data and discussions presented in chapters 2, 3 and 4 are integrated and presented in a broader perspective. The second theme concerns storage of IL-1 family members in which data presented in chapter 3 and 4 on the presence and amount of IL-18 and IL-1 in human skin are discussed (hypotheses 2 and 3). The third theme is on the biological functions of IL-18 and IL-1 isoforms (hypotheses 3 and 4). In this section the function of IL-18 in psoriatic skin (chapter 4) and the modulation of cytokine expression by IL-1 (chapter 5) are discussed together with the significance of IL-1 in CD40-CD40L interactions in skin (see chapter 6).

Processing of IL-1 family members

Processing of IL-18 and IL-1β in human skin

Activation of the pro-inflammatory cytokines IL-18 and IL-1β is dependent on the processing of the inactive proforms of these IL-1 family members by caspase-1 (interleukin-1 converting enzyme, ICE) (Dinarello, 1998). Active caspase-1 is associated with the cell membrane (Singer *et al.*, 1995) implying that conversion of pro-IL-18 or pro-IL-1β into their active forms is a process which takes place in or on the cell membrane. Although human keratinocytes can express caspase-1 (Shimizu *et al.*, 1999; Zepter *et al.*, 1997), the question remained whether they actually process IL-18 or IL-1β. In 1991 Mizutani *et al* (Mizutani *et al.*, 1991) showed that human keratinocytes produce but not process IL-1β. A few years later, however, Zepter *et al* (Zepter *et al.*, 1997) demonstrated that upon stimulation with urishiol, sodium lauryl sulfate (SDS) or PMA human keratinocytes could process pro-IL-1β. This process could be inhibited with specific inhibitors of caspase-1. Thus, these data indicate that keratinocytes under specific conditions process IL-1 via caspase-1.

It has been shown that blocking of caspase-1 by caspase-1 inhibitors *in vitro* results in secretion of IL-1 in its unprocessed form (Singer *et al.*, 1995). Considerable quantities of

IL-18 are released by normal human keratinocytes in the unprocessed 24 kD form into the extracellular environment (Mee *et al.*, 2000). However, since predominant Th1 skewing in skin diseases is observed, it is conceivable that, besides IL-12, biologically active IL-18 occurs in skin. It is therefore likely that IL-18 and IL-1β may be processed by other extracellularly located proteases (see general introduction, introduction to the chapters, hypothesis 1). Candidate proteases which have been shown to process IL-1β are the metalloproteases MMP-2, -3 and –9 (Schonbeck *et al.*, 1998). Similarly, proteinase-3 (PR-3) is capable of processing pro-IL-18 into bioactive IL-18 (Fantuzzi *et al.*, 1998). Also extracellular proteases produced by bacteria such as *Streptococcus pyogenes* are able to process IL-1β (Kapur *et al.*, 1993). At least MMP-2, -3 and –9 are present in human skin during inflammation (Han *et al.*, 2001; Varani *et al.*, 1998) but the presence of PR-3 in inflamed skin has not been confirmed yet. PR-3 is a serine protease, like caspase-1, and is implicated in the degrading process of matrix proteins including fibronectin, laminin and collagen type IV (Rao *et al.*, 1991). PR-3 is expressed by neutrophils which are common in psoriatic skin (Terui *et al.*, 2000). PR-3 is also produced by epithelial cells (Schwarting *et al.*, 2000). These data provide sufficient circumstantial evidence for the assumption that secreted pro-IL-18 in early psoriatic lesional skin may be processed by this protease.

In chapter 2 we showed that such alternative proteases do occur and that they are able to process pro-IL-1β, from plantar stratum corneum and psoriatic scales, into a bioactive molecule. Alternatively processed IL-1β has a biological activity comparable to recombinant mature IL-1β (see chapter 2). The alternative ICE in this case was the epidermis-specific stratum corneum chymotryptic enzyme (SCCE). *In vitro* experiments showed that SCCE is able to activate pro-IL-1β (Nylander-Lundqvist & Egelrud, 1997) and that SCCE is upregulated in psoriatic scales (Ekholm & Egelrud, 1999).

As stated in the second hypothesis, indeed normal human keratinocytes were found to express high levels of IL-18 intracellularly and to predominantly produce the 24 kD unprocessed form. These data independently obtained by Mee *et al.* and our group (Companjen *et al.*, 2000; Mee *et al.*, 2000) are, however, in contrast with observations by Kämpfer *et al* (Kampfer *et al.*, 1999). The latter showed that the human keratinocyte cell line HaCaT produces high amounts of mature IL-18 which is processed intracellularly. We present evidence that both normal human keratinocytes and HaCaT cells intracellularly express pro-IL-18 (Figs. 1A and B, respectively). Mee *et al.* showed that normal human keratinocytes secrete pro-IL-18 (Mee *et al.*, 2000), while we also observed that HaCaT cells spontaneously secrete unprocessed IL-18 in culture (unpublished data). Additionally we stimulated HaCaT cells with different stimuli (e.g. LPS, PMA, IL-1β) but also under these conditions did not observe IL-18 processing. This was also reported by Mee *et al* who stimulated primary normal human keratinocytes and used stimuli like TNF-α, PMA, $NiSO_4$ and IFN-γ. Our data (see chapter 3) might indicate that IL-18 is not processed by human keratinocytes at all. However, occasionally limited amounts of processed IL-18 could be detected in extracts of epidermal sheets but not in extracts of trypsinized single epidermal cells (chapter 4, Fig. 3 vs. chapter 3, Fig. 5). This suggests that secreted pro-IL-18 might be processed by alternative

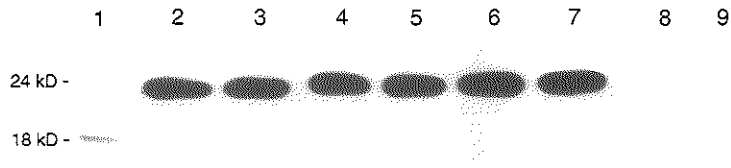

Figure 1A. IL-18 protein expression in unstimulated HaCaT cells. Detection on Western blot with an anti-IL-18 specific monoclonal antibody (MAB318, R&D systems, Minneapolis, MN). Lane 1: 1 ng rhuIL-18, lane 2-7: respectively: 3, 6, 12, 24, 48 and 72 hours of culture, lane 8: 1 ng rhuIL-18, omission of primary antibody and lane 9: HaCaT cells 3 hours of culture, omission of primary antibody. Pro-IL-18: 24 kDa and mature IL-18: 18 kDa.

proteases present in the inter- or extracellular space of intact skin or other epidermal cells.

The fact that secretion of unprocessed IL-1 isoforms by human keratinocytes occurs, might indicate that natural caspase-1 inhibitors may be active. A candidate natural caspase-1 inhibitor in this system may be ICEBERG (Humke *et al.*, 2000). This natural caspase-1 inhibitor has been shown to inhibit the release of IL-1β from monocytes and is upregulated upon LPS or TNF-α stimulation. However, production of unprocessed IL-18 or IL-1β due to inhibition of caspase-1 with ICEBERG and also its presence in human skin have not yet been proven.

Based on the studies described above the following model is presented (Fig. 2): in human keratinocytes IL-1β can be processed by caspase-1 as shown by Zepter *et al* (Zepter *et al.*, 1997). Although no conclusive data are available yet, it is possible that in keratinocytes IL-18 is also processed by caspase-1. Alternatively, IL-18 and IL-1β are secreted as unprocessed isoforms. These unprocessed forms may then be processed by PR-3 (IL-18) and/or SCCE or MMP's (IL-1β).

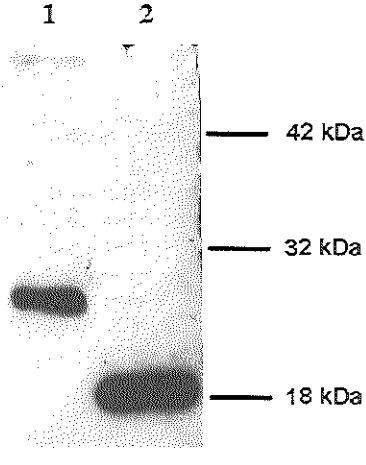

Figure 1B. IL-18 protein expression in unstimulated primary human keratinocytes. Western blot was probed with a goat anti-human IL-18 polyclonal antibody (sc6177, Santa Cruz, Santa Cruz, CA). Lane 1: unstimulated keratinocyte lysate; lane 2: rhuIL-18 (5 ng). Photograph courtesy of R. Groves.

Storage of IL-1 family members

Storage of IL-18

In chapter 3 we showed that normal human keratinocytes constitutively express both IL-18 mRNA and protein. In analogy to the other members of the IL-1 family, IL-1α and IL-1ra, IL-18 protein is abundantly present. However, in keratinocytes, IL-18 is mainly present in its inactive unprocessed form. The question remains why keratinocytes produce and store such high amounts of IL-18. Since IL-18 is a potent pro-inflammatory cytokine its acti-

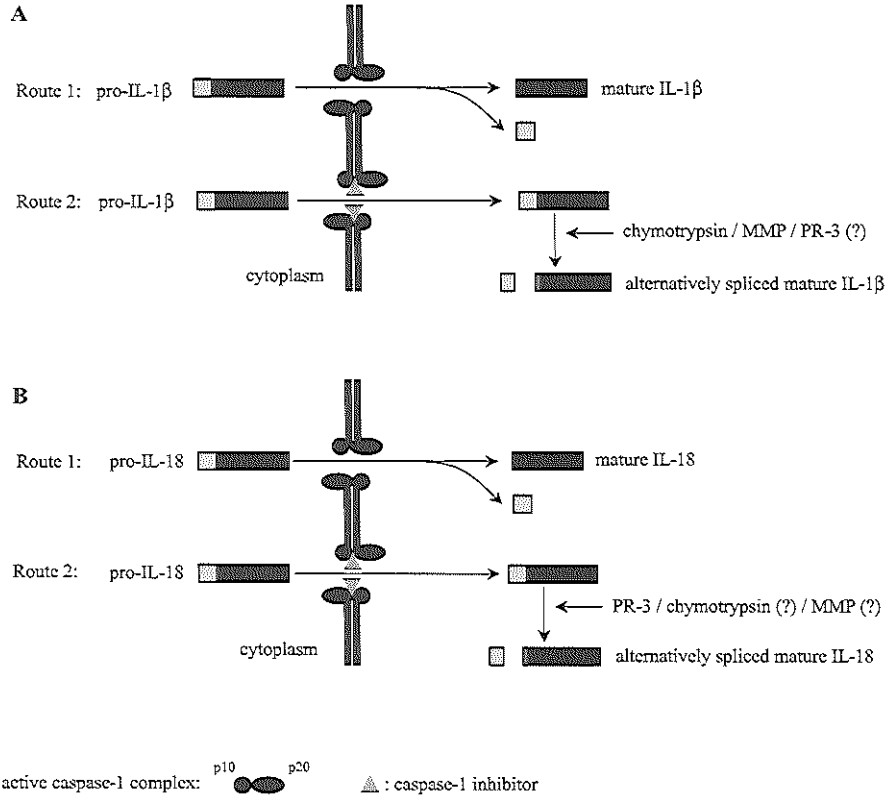

Figure 2. model for processing of IL-1β and IL-18 by caspase-1 or alternative proteases. A: Processing of pro-IL-1β takes place in the membrane. Pro-IL-1β enters the active complex of caspase-1 located in the plasma membrane. Subsequently pro-IL-1β is processed resulting in the secretion of mature active IL-1β (route 1). During inhibition of caspase-1 by caspase-1 inhibitors pro-IL-1β can be secreted without being processed. Extracellular pro-IL-1β might subsequently be processed by proteases other than caspase-1 such as chymotrypsin, MMP's and possibly PR-3 (route 2). **B:** Processing of IL-18 may be similar to IL-1β processing (route 1). Pro-IL-18 is known to be secreted by human keratinocytes. Proteases like PR-3, chymotrypsin and MMP may be responsible for the extracellular activation of IL-18 (route 2).

vation in skin might have undesirable effects. One contributing factor to these high levels could be the relatively high stability of IL-18 mRNA compared to mRNA of other cytokines (Okamura *et al.*, 1995). Considering its high pro-inflammatory activity, IL-18 activation in human skin must be tightly regulated. The high concentration of IL-18, stored in human keratinocytes may have important implications in the determination of the Th cell balance in skin. It is well known that the IL-1 family of proteins are part of a very old and conserved system which plays an important role in innate immunity. Since IL-18 is part of this IL-1 family, it might also have a function in innate immune responses in human skin. For example, bacterial products (such as peptidoglycan (PG), toxins or LPS), UVB radiation and trauma may induce the secretion and release of IL-18 by keratinocytes resulting in the induction of

inflammatory cytokines like for example IFN-γ and TNF-α. Taken together, the high level of IL-18 might reflect the rapid pro-inflammatory response properties of human skin.

Storage of IL-1α and IL-1ra in human skin

It has been shown that normal human keratinocytes are major producers of IL-1α and IL-1ra (Arend *et al.*, 1998; Kupper & Groves, 1995). Under normal circumstances a high amount of IL-1α is stored in basal keratinocytes (Debets *et al.*, 1997). Upon wounding or infection this IL-1α is released resulting in the initiation of an inflammatory response. In this thesis we show that compared to normal epidermis, epidermal sheets of psoriatic lesional skin contain significantly lower amounts of IL-1α (chapter 4). These low levels may be due to a) degradation of IL-1α in psoriatic lesional skin or b) receptor binding or internalisation. IL-1ra is one of the most abundant cytokines present in human keratinocytes. Indeed high amounts of total IL-1ra protein (about 0.6 μg/mg total protein) were detected in extracts from both normal and psoriatic lesional epidermis (unpublished data). The explanation for this huge amount of epidermal IL-1ra might be that the activity of released IL-1α after injury or infection should immediately be counteracted because the high activity of IL-1α might have undesirable effects.

Other less well known biological properties of IL-1α and IL-1ra, which might explain the high amount of these cytokines in skin, have also been reported. IL-1α and IL-1ra were implicated in the control of growth and differentiation (Hammerberg *et al.*, 1992; Maier *et al.*, 1990) of keratinocytes. In the latter case they exert their function completely intracellularly via nuclear localization (Dinarello, 1996).

Function of IL-1 family members

Contribution of IL-18 to the IFN-γ expression in psoriatic lesional skin

Stimulation of T cells by IL-18 in an environment containing IL-12 results in the development of Th1 cells. This is because IL-12 stimulates the upregulation of the IL-18R on naive T cells (Yoshimoto *et al.*, 1998), while ligation of the IL-18R results in the production of IFN-γ. Recent literature, however, indicates that in the absence of IL-12, IL-18 is also able to stimulate IL-4 and IgE expression on B cells and thus Th2 development (Yoshimoto *et al.*, 2000). These observations directly point to the importance of the microenvironment in which IL-18 is expressed and becomes activated. Hence IL-12 and not IL-18 seems the most likely determinant for the development of Th1 cells in skin (Fig. 3).

Human keratinocytes have been shown to produce IL-12p70 protein (Yawalkar *et al.*, 1998; Yawalkar *et al.*, 1996). We also demonstrated the expression of IL-12 protein in both normal and psoriatic lesional skin using immunohistochemistry and ELISA (chapter 4). Thus in general, human skin is an environment which favours Th1 development.

However, the development of elevated IgE serum levels and a Th2 phenotype was shown in mice overexpressing active caspase-1 in their keratinocytes (Yoshimoto *et al.*, 2000). Skin and serum levels of IL-18 were also elevated in these mice (Yamanaka *et al.*, 2000). Conversely, IL-1β has been shown to induce IL-9 in human eosinophils (Gounni *et al.*,

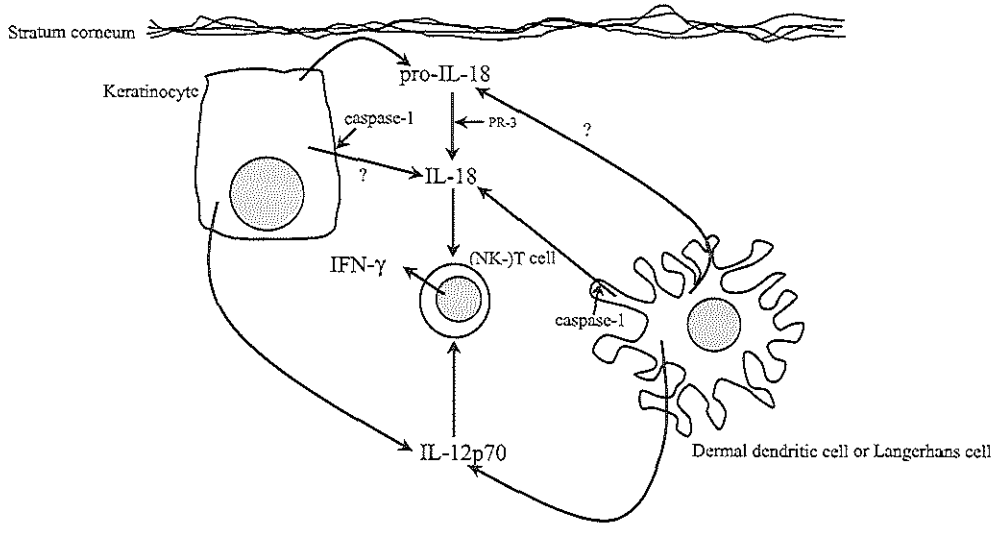

Figure 3. Stimulation of IFN-γ production by Th cells in skin is dependent on processing of IL-18 and the presence of IL-12p70. A high amount of pro-IL-18 is stored in human keratinocytes. IL-18 can be processed by membrane-bound caspase-1 or extracellular proteases like PR-3. Whether activated IL-18 can induce or upregulate IFN-γ expression in Th1 cells is dependent on the activity of IL-12 present during inflammation. SC: stratum corneum; KC: keratinocyte; DDC: dermal dendritic cell; LC: Langerhans cell; ?: intermediates unknown.

2000) and IL-9 can induce IgE production in B cells. Caspase-1 transgenic mice probably also produce elevated levels of IL-1β which might be resposible for the elevated IgE production observed rather than IL-18. With all limitations of extrapolating animal studies to the human situation, this observation does not imply that this keratinocyte-derived IL-18 solely induces Th2 skewing. It may be that IL-18 has differential effects locally (elevation of IFN-γ expression in skin, thus Th1 development) and systemically (elevation IgE expression in serum, thus Th2 development). This is illustrated by the fact that in atopic dermititis IFN-γ producing cells occur in the skin (Grewe *et al.*, 1998).

An important hallmark of psoriasis is the increased expression of IFN-γ in lesional skin (Bjerke *et al.*, 1983; Livden *et al.*, 1989). Because IL-18 is involved in the production of IFN-γ, the expression of this cytokine was determined in psoriatic lesional skin.

The third hypothesis underlying our studies was that IL-18 expression in psoriatic lesional skin is elevated compared to normal skin. However, IL-18 mRNA and protein expression or IL-18 processing was not elevated. Conversely, IFN-γ and IL-12p40 expression were dramatically increased in lesional skin compared to normal skin suggesting an important contribution of IL-12 in the regulation of IFN-γ production. The lack of an increased concentration of processed IL-18 in stable plaque type lesions of psoriatics does not necessarily rule out a role for IL-18 in the production of IFN-γ. It is possible that IL-18 exerts its effects in early stages before the development of stable lesions. Indeed preliminary data showed that total IL-18 protein expression was elevated in active and progressive psoriatic lesions (chap-

ter 4).

Stimulation of IFN-γ expression is also possible via other routes than IL-12/IL-18. For example Yang *et al* (Yang *et al.*, 1999) and Bucy *et al* (Bucy *et al.*, 1994) showed that IFN-γ expression can also be triggered via stimulation of the T cell receptor only, in the absence of IL-12 and IL-18. This mechanism may also be operational in stable lesions of psoriatic patients.

Modulation of the immune response in skin by IL-1

The function of IL-1 in skin has been investigated using *in vivo* and *in vitro* models. However, all these models have some disadvantages. The *in vivo* transgenic mouse model usually provides information on the function of IL-1 in mouse skin which might be different from the human situation. A new model which might provide information on IL-1 function in intact human skin is the SCID-human xenogenic transplantation model (reviewed by Boehncke (Boehncke, 1999)). In this system human skin is transplanted onto SCID mice in order to investigate different parameters of the immune system in intact skin (see chapter 5). However, the SCID-hu xenogenic transplantation model also has some disadvantages. It is time-consuming, requires specific expertise and facilities, and furthermore, although the SCID mouse does not have functional T and B cells, it possesses other immunocytes that might be of influence.

The main drawback of in vitro models, in which human skin cells are used in single cell suspensions, is that the microenvironment of the skin is disturbed which might yield an altered picture of the "real" situation *in vivo*. To investigate the function of IL-1 in skin without disturbing its microenvironment we developed an *ex vivo* culture system in which full thickness normal human skin explants were used (see chapter 5). We showed that an inflammatory reaction can be induced in normal skin biopsies by treatment with bacterial LPS, an inducer of inflammation. LPS induced the expression of IL-6, IL-8 and markers relevant in the immune response such as CD40, CD86 and ICAM-1. Further experiments showed that LPS also induced the expression of IL-1β and TNF-α (unpublished data). Direct effects of IL-1 were observed by stimulating normal human skin with IL-1β. IL-1β induced IL-6 and IL-8 expression and the upregulation of CD40, CD86 and ICAM-1 expression (chapter 5).

Additional experiments demonstrated that stimulation of psoriatic lesional skin biopsies with IL-1β induces TNF-α production (unpublished data). These data indicate that in this system IL-1 modulates key players in skin inflammation. IL-1 initiates the attraction of T cells and neutrophils by inducing the expression of IL-8, and induces the production of pro-inflammatory cytokines like IL-6 and TNF-α. IL-1 is also important during later stages of the inflammatory response when T cells are present. In this stage IL-1 facilitates co-stimulation through upregulation of CD86 and ICAM-1 expression.

All these effects stress the key function of IL-1 in the formation of the immunological synapse in skin. However, it should be mentioned that IL-1 is also involved in non-immunological responses in skin such as during UV radiation (Krutmann & Grewe, 1995), wound healing (Sauder *et al.*, 1990) and keratinocyte growth (Maas-Szabowski *et al.*, 2000).

CD40 is an other co-stimulatory molecule positively regulated by IL-1β in skin (Companjen *et al.*, 2001). Recently it was shown that human keratinocytes and fibroblasts express CD40 (Denfeld *et al.*, 1996; Fries *et al.*, 1995). The function of keratinocyte CD40 has recently been determined. The results indicate that CD40 stimulation affects the cell cycle and upregulates Bcl-x, IL-8, IL-6 and ICAM-1 (Denfeld *et al.*, 1996; Gaspari *et al.*, 1996; Grousson *et al.*, 2000; Peguet-Navarro *et al.*, 1997). The upregulation of these molecules leads to a prolonged protection against apoptosis, recruitment of neutrophils and T cells, development of memory by cytolytic T cells, increase of cytolytic activity of natural killer cells and increased adhesion of LFA-1^+ cells.

Because the expression of IL-6 and IL-8 in skin has been shown to be mediated via IL-1 (chapter 5), we hypothesized that the induction of IL-6 and IL-8 expression through CD40 stimulation is mediated via IL-1. Data supporting this hypothesis are presented in chapter 6. There we showed that stimulation of CD40 in normal human skin biopsies with agonistic anti-CD40 antibodies in the presence of IFN-γ resulted in the upregulation of several pro-inflammatory cytokines. Inhibition of IL-1 activity during CD40 stimulation by culturing in the presence of IL-1ra showed that IL-6 and IL-8 expression induced by CD40 ligation in normal human skin is partly mediated via IL-1. The fact that CD40 induced TNF-α production was not significantly affected by blocking with IL-1ra, suggests that IL-1 is not involved in this pathway. This suggests that CD40 stimulation directly induces TNF-α production. Alternatively, TNF-α production via CD40 stimulation may be mediated via factors other than IL-1, for example P-selectin which has been shown to induce TNF-α production in monocytes (Koike *et al.*, 2000).

Stimulation of CD40 in normal skin biopsies also affects the expression of IL-10, an anti-inflammatory cytokine. In skin biopsy cultures devoid of IFN-γ, stimulation of CD40 clearly induced the expression of this cytokine, which could not be inhibited by IL-1ra. This illustrates that the induction of pro-inflammatory cytokines is counteracted by the expression of anti-inflammatory cytokines via the same route of stimulation. Elevation of IL-10 production after CD40 triggering was also observed by others using *in vitro* systems. Stimulation of CD40 on PBMC in combination with IL-4 resulted in the induction of IL-10 expression (Itoh *et al.*, 2000). The same was observed after CD40 stimulation of dexamethasone treated DC (Rea *et al.*, 2000). Our CD40 ligation experiments suggested a more direct effect on IL-10 production, although we cannot exclude the contribution of additional unknown factors operative in skin.

A rather surprising observation was that IFN-γ suppressed the IL-6 and IL-8 secretion by 70 to 80% compared to the secretion in medium devoid of IFN-γ. IFN-γ also inhibited IL-10 secretion, which was previously reported by Kooy *et al* (Kooy *et al.*, 1999), whereas it had no effect on TNF-α production.

The pro-inflammatory effects of IFN-γ are well documented (Boehm *et al.*, 1997). Only occasionally inhibitory effects of IFN-γ were observed. In some cases, IFN-γ can inactivate IL-6 (Jernberg-Wiklund *et al.*, 1991). Also the IL-8 and IL-10 expression can be inhibited by IFN-γ (Galy & Spits, 1991; Suzuki *et al.*, 2001). Using the ex vivo skin organ culture

system we showed that IFN-γ has a dual effect in skin. An anti-inflammatory effect is exerted by the downregulation of pro-inflammatory cytokines like IL-8 and IL-6, whereas it has a pro-inflammatory effect by downregulating IL-10 production.

It is conceivable that IFN-γ has differential effects over time during an inflammatory response. T cells present in skin contain pre-stored IFN-γ mRNA and can produce IFN-γ protein rapidly during the initiation of an inflammatory response (Hassan-Zahraee *et al.*, 1998). IFN-γ expression during the early inflammatory response might upregulate IL-6 resulting in the activation of lymphocytes. After a prolonged exposure to IFN-γ, during later stages of the inflammatory response, IL-6 and IL-8 expression are downregulated, possibly via the induction of inhibitory factors. Thus prolonged exposure to IFN-γ leads to the inhibition of a selection of cytokines resulting in the downregulation of the immune response. To get full insight into the effects of IFN-γ on the regulation of cytokine expression in skin in time, more extensive kinetic studies should be done using microarrays for the analysis of the spectrum of cytokines induced.

Conclusion

In chapter 1, four hypotheses were postulated. Subsequently these hypotheses were tested using *in vivo*, *in vitro* and *ex vivo* assays. The validity of these hypotheses has been discussed in the preceding sections.

The results of the experiments described in chapters 2 and 3 showed that the first and the second hypothesis, on the activation of epidermal IL-1β by proteases other than caspase-1 and on the expression of high levels of unprocessed IL-18, respectively, are valid. Experiments described in chapter 4, however, showed that the third hypothesis, on elevated IL-18 expression in psoriatic lesional skin, is not valid at least in the case of stable psoriatic lesions. Finally, the fourth hypothesis, that the induction of IL-6 and IL-8 expression by CD40 stimulation is mediated via IL-1, does appear to be valid, with the marginal note that CD40 induced IL-6 and IL-8 expression is not completely dependent on IL-1.

Taken together, the data presented in this thesis result in the following general model for regulation of pro- and anti-inflammatory cytokines during inflammation in skin (Fig 4):

1. Upon stimulation of skin cells by bacterial products (LPS, toxin, peptidoglycan, etc.), the expression of IL-1β is induced, resulting in the production of pro-IL-1β which is subsequently processed by either caspase-1 or alternative proteases.
2. IL-1β induces IL-8 secretion which leads to the attraction of neutrophils and T cells, and IL-6 secretion resulting in leukocyte activation. Additionally IL-1β upregulates CD40 expression and other co-stimulatory molecules.
3. Cutaneous T cells produce IFN-γ under the influence of IL-18 and IL-12 resulting in the amplification of CD40 expression. T cells stimulate CD40 on skin resident cells through CD40 ligation.
4. CD40 ligation results in the upregulation of IL-1 and consequently in the amplification of CD40, IL-6 and IL-8 expression. Additionally, CD40 ligation leads to increased TNF-α secretion which is not mediated via IL-1, and IL-10 expression which can

inhibit the expression of pro-inflammatory cytokines. Finally, the CD40-induced IL-1 production is counteracted by the induced IL-1ra, which is also upregulated by IFN-γ.

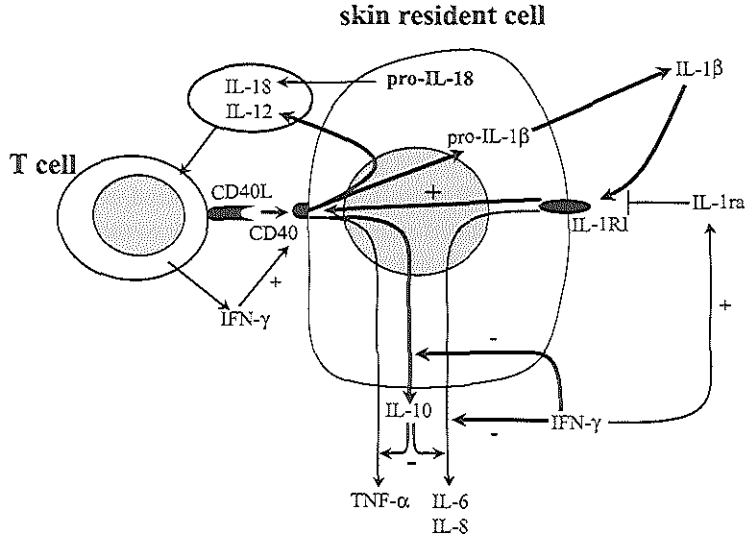

Figure 4. Regulation of pro- and anti-inflammatory cytokines during inflammation in skin. The data presented in this thesis are represented in this global model of the regulation of cytokines during skin inflammation (see text for details). Newly observed interactions presented in this thesis are indicated in bold. For reasons of simplicity, cells resident in the epidermis and the dermis, such as keratinocytes, Langerhans cells, fibroblasts and melanocytes, are represented as one cell.

Future directions

Investigating the induction and development of inflammatory responses in skin using the skin organ culture system

The skin organ culture system (described in chapter 5 and 6) has proven to be a valuable tool for investigating inflammatory responses in human skin. In this thesis, experiments have been described analysing the effects of LPS and IL-1 on the expression and modulation of cytokine and cell membrane markers after the induction of an inflammatory response at a fixed time point. The initial dose-finding and time course experiments were only focussed on optimising of the expression of specific cytokines and markers. The overall experiments, however, do not give sufficient information on the development and maintenance of inflammation in skin in time and in the role and site of cellular sources of cytokine expression herein. Therefore, more extensive studies on the kinetics and cellular source(s) of cytokines after LPS or IL-1 stimulation should be done. These studies will provide a better view on the dynamics or the development of inflammation in skin in time and the role and site or cellular sources of specific cytokines.

Novel molecules like the Toll like receptors (TLR) have recently be implicated in the

pathology of inflammation. TLR are pattern recognition receptors which can be triggered by several molecules like LPS, PG and CpG (Hemmi *et al.*, 2000; Takeuchi *et al.*, 1999). Signal transduction proteins exploited by the members of the IL-1 family of proteins are also shared by TLR (Medzhitov & Janeway, 2000) and should therefore be included in studies on IL-1 and LPS orchestrated inflammation in skin.

Similar experiments as described in this thesis could be done using normal appearing (non-involved) psoriatic skin. The development of inflammation in normal skin could be compared with that in normal appearing psoriatic skin. This will generate information on differences in the regulation of inflammatory responses between normal and psoriatic skin.

Based on the information about the role of CD40 in skin (chapter 6), a new anti-psoriatic therapy using CD40 blocking antibodies might be developed. In fact, a broad array of antibodies directed against cytokines and co-stimulatory molecules known to be involved in the pathophysiology of psoriasis are currently under investigation. For example, CD80 blocking antibodies and Ig-CTLA-4 fusion proteins were able to clearly improve psoriasis. One drawback of these new therapeutic agents, however, are the high costs which might hamper clinical application.

The role of IL-18 in skin

The data presented in this thesis do not give definitive information on the role of IL-18 in skin. To gain more insight in the function of IL-18 in skin, skin specific IL-18 transgenes and skin specific IL-18 transgenes/IL-12 knock outs (KO) should be generated. These mouse models should provide information about the role of skin-derived IL-18 in the induction of IFN-γ and the development of Th1 or Th2 cells in skin.

Additionally the role of IL-18 in this system could be investigated using antibodies neutralising IL-18 or using IL-18 binding protein (IL-18BP). IL-18BP is an important factor in the regulation of the IL-18 system. To investigate the regulation of IL-18 activity in normal and inflamed skin, the expression of IL-18BP isoforms should be analyzed. The regulation of active and inactive IL-18BP isoforms in normal and psoriatic skin might be different and could contribute to the elevated IFN-γ levels in psoriatic skin.

Expression and regulation of novel IL-1 isoforms in skin

Recently new IL-1 isoforms have been described (see chapter 1). At least two of these (IL-1δ and IL-1ε) are expressed in human skin and upregulated in psoriatic lesional skin (Debets *et al.*, 2001). The function of these two IL-1 isoforms is currently unknown but considering their expression and the important role of other IL-1 isoforms in skin they may have important implications in skin biology. Finally the investigation of novel IL-1 isoforms and their function in skin could yield new perspectives on the regulation of inflammatory mediators in normal and inflamed skin.

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ABBREVIATIONS

AD	:	Atopic dermatitis
Ag	:	Antigen
APC	:	Antigen presenting cell
apPP	:	Active and progressive psoriatic lesional skin
BAL	:	Bronchoalveolar lavage
BMT	:	Bone marrow transplantation
CD	:	Cluster of differentiation
CD40L	:	CD40 ligand (CD154)
CLA	:	Cutaneous lymphocyte antigen
DC	:	Dendritic cell
DDC	:	Dermal dendritic cell
DTH	:	Delayed type hypersensitivity
EAE	:	Experimental autoimmune encephalomyelitis
EC	:	Epidermal cells
ECS	:	Epidermal cell suspension
EGF	:	Epidermal growth factor
ELISA	:	Enzyme linked immunosobent assay
FAF	:	Fibroblast activating factor
HBSS	:	Hank's buffered saline solution
HLA	:	Human leukocyte antigen
HPRT	:	Hypoxantine phosphatidyl ribosyltransferase
HRP	:	Horse radish peroxidase
HUVEC	:	Human umbilical vein endothelial cell
ICAM-1	:	Intercellular adhesion molecule-1
ICE	:	Interleukin-1β converting enzyme
IDC	:	Interdigitating cell
IFN-γ	:	Interferon-γ
IGIF	:	Interferon-γ inducing factor
IL	:	Interleukin
IL-18BP	:	Interleukin-18 binding protein
IL-1R	:	Interleukin-1 receptor
IL-1ra	:	Interleukin-1 receptor antagonist
IRF	:	Interferon regulating factor
KC	:	Keratinocyte
KGF	:	Keratinocyte growth factor
KO	:	Knock out
LC	:	Langerhans cell
mAb	:	Monoclonal antibody
MAPK	:	Mitogen-activated protein kinases
MECLR	:	Mixed epidermal cell leukocyte reaction
MHC class II	:	Major histocompatibility class II
MMP	:	Matrix metalloproteinase
MS	:	Multiple sclerose
NF-κB	:	Nuclear factor-κB
NK	:	Natural killer
NN	:	Normal skin
pAb	:	Polyclonal antibody
PBMC	:	Peripheral blood mononuclear cell
PBS	:	Phosphate buffered saline
PG	:	Peptodoglycan
PGE_2	:	Prostaglandin E_2
PMA	:	Phorbol 12-myristate 13-acetate
PN	:	Psoriatic non-lesional skin
PP	:	Psoriatic lesional skin
PR-3	:	Proteinase-3
RANTES	:	Regulated upon activation, normal T cell expressed, and presumably secreted
SC	:	Stratum corneum
SCCE	:	Stratum corneum chymotryptic enzyme
SCID	:	Severe combined immunodeficiency
SDS	:	Sodium dodecyl sulphate
sptPP	:	Stable plaquetype psoriatic lesional skin
STAT-3	:	Signal transducers and activators of transcription-3
TAF	:	T cell activating factor
TBS	:	Tris buffered saline
TCR	:	T cell receptor
Th1	:	T helper 1
TLR	:	Toll-like receptor
TNF-α	:	Tumor necrosis factor-α
UVB	:	Ultraviolet B
VCAM	:	Vascular cell adhesion molecule-1

SUMMARY

To cope with infection or trauma, the skin possesses a tightly regulated immune system. Key players in this skin immune system are Langerhans cells, keratinocytes and melanocytes located in the epidermis, and fibroblasts, dermal dendritic cells and endothelial cells in the dermis. T cells also occur in skin and are located mainly in the dermis. During inflammation, cells resident in the skin communicate via cytokines and surface molecules. Aberrant expression of pro-inflammatory cytokines like IL-1, IL-6, IL-8, IFN-γ and TNF-α is thought to play a central role in the course of inflammatory skin diseases such as psoriasis. In order to understand the mechanism of cytokine dysregulation in inflammatory skin diseases, the expression and function of pro-inflammatory cytokines in normal and inflamed skin should be investigated.

The IL-1 system comprises several agonists (i.e. IL-1β, IL-1α and IL-18), antagonists (IL-1ra, IL-1RII) and receptors (i.e. IL-1RI, IL-1RAcP, IL-18Rα and AcPL). Several IL-1 isoforms are known to be expressed in skin (e.g. IL-1α, IL-1ra and IL-1β) to orchestrate the initiation and maintenance of skin inflammation. The aim of the studies described in this thesis was to define the role of IL-1β and IL-18 in normal and inflamed skin. IL-1β and IL-18 are two members of the IL-1 family which are structurally homologous, but functionally different. Both IL-1β and IL-18 are produced as inactive proforms (pro-IL-1β and pro-IL-18 both 24 kD) which need processing by the serine protease caspase-1 (ICE) to become biologically active. Both IL-1β and IL-18 activate the same signal transduction pathway, but transduce their signal via different receptor complexes. The most well known function of IL-18 is the induction of IFN-γ secretion by Th1 cells and NK cells. Interleukin-1β is involved in the induction of many inflammatory mediators, including pro-inflammatory cytokines such as IL-6, IL-8 and TNF-α.

Activation of IL-1β is thought to be dependent on processing by caspase-1. In chapter 2 we show that in normal and psoriatic lesional skin, IL-1β activation is not solely dependent on caspase-1 but may also be processed and activated by other proteases.

Since it was not known whether normal human keratinocytes produce IL-18, we asked whether IL-18 is expressed in normal skin. In chapter 3 we show that IL-18 mRNA and protein is constitutively expressed in normal human skin. We report that keratinocytes are major producers of IL-18. IL-18 is present intracellularly, mainly in its unprocessed form, but is also released. Interestingly, the amount of IL-18 in normal keratinocytes significantly exceeded the expression levels in other cell types like monocytes and bronchial epithelial cells. This high level of IL-18 present in normal human skin might reflect the rapid pro-inflammatory response properties of human skin.

Because IL-18 is involved in the induction of IFN-γ expression and since IFN-γ levels in psoriatic skin are significantly increased, we hypothesized that IL-18 expression in psoriatic lesional skin is elevated compared to normal skin (chapter 4). However, IL-18 protein expression and processing was not elevated in stable plaque type epidermis. The lack of an increased IL-18 expression in psoriatic stable plaque type lesions does not rule out its

involvement in the IFN-γ production in psoriatic skin. It may be that IL-18 induces IFN-γ expression in early stages of the lesion, while IL-18 expression is normalized in stable lesions. Preliminary experiments indeed show that IL-18 total protein expression is elevated in active and progressive psoriatic lesions.

Current knowledge of the function of IL-1 in skin is based mainly on *in vivo* or *in vitro* models. To study the function of IL-1 in intact human skin, we developed a skin organ culture system, which approaches the *in vivo* situation by maintaining the normal skin architecture without spontaneous induction of regenerative epidermal markers (chapter 5). In this system IL-1β upregulated the expression of skin-derived cytokines like IL-6 and IL-8 in the medium and different surface molecules, like ICAM-1, CD40 and CD86 on cells. Elevation of both cytokine and cell marker expression could be blocked by dexamethasone and by IL-1ra which specifically counteracts IL-1β.

CD40-CD40L interactions play an important role in various immunological responses such as isotype switching and activation of antigen presenting cells and T cells. Data from *in vitro* culture systems show that CD40 ligation on human skin cells leads to the induction of pro-inflammatory cytokines like IL-6, IL-8 and TNF-α. The skin organ culture system described in chapter 5 was used to investigate the function of IL-1 in CD40 mediated responses in normal skin (chapter 6). We confirm observations obtained using *in vitro* model systems by showing that stimulation of CD40 in normal human skin explants also results in the induction of these cytokines. Additionally we show that IL-10 expression is enhanced upon CD40 stimulation, and that IL-1 is involved in the CD40 ligation-induced secretion of IL-6 and IL-8, but not TNF-α.

In conclusion the data presented in this thesis show that pro-IL-18 is produced in high quantities in normal skin but that IFN-γ expression in stable plaque type psoriatic epidermis is not accompanied by an elevated IL-18 expression. However, IL-1 isoforms in the epidermis like IL-1β can be processed by alternative proteases suggesting that also other IL-1 isoforms like IL-18 might be processed extracellularly. Furthermore we show that the expression of pro-inflammatory cytokines in intact skin is directly induced by IL-1 or indirectly via CD40 stimulation. Overall these studies extend the conceptual framework for the rational design of IL-1 targeted therapies for inflammatory skin diseases.

SAMENVATTING

De huid bezit een strak gereguleerd immuunsysteem om ons te verweren tegen micro-organismen en andere agentia. Cellen die hierbij een centrale plaats innemen zijn Langerhans cellen, keratinocyten en melanocyten in de epidermis en fibroblasten, dermale dendritische cellen en endotheelcellen in de dermis. T-cellen bevinden zich ook in de huid en zijn voornamelijk gelokaliseerd in de dermis. Gedurende een ontstekingsreactie communiceren cellen in de huid met elkaar via cytokinen en oppervlaktemoleculen. Aangenomen wordt dat een verstoorde expressie en regulatie van pro-inflammatoire cytokinen zoals IL-1, IL-6, IL-8, IFN-γ en TNF-α een centrale rol spelen bij het verloop van huidziekten gekenmerkt door ontsteking, zoals psoriasis. Om het mechanisme van cytokine disregulatie bij inflammatoire huidziekten te kunnen ontrafelen, moet men inzicht hebben in de expressie en functie van pro-inflammatoire cytokinen in normale en ontstoken huid.

Het IL-1 systeem omvat verschillende agonisten (bijv. IL-1β, IL-1α en IL-18), antagonisten (IL-1ra en IL-1RII) en receptoren (bijv. IL-1RI, IL-1RAcP, IL-18Rα en AcPL). Verschillende hiervan, zoals IL-1α, IL-1ra en IL-1β, komen tot expressie in de huid en orkestreren de initiatie en het in stand houden van de ontsteking.

Het doel van het onderzoek zoals beschreven in dit proefschrift was het verkrijgen van informatie over de rol van IL-1β en IL-18 in normale en ontstoken huid. IL-1β en IL-18 maken beide deel uit van het IL-1 systeem en zijn structureel homoloog, maar functioneel verschillend. Zowel IL-1β en IL-18 worden geproduceerd als biologisch inactieve precursors (pro-IL-1β en pro-IL-18; beide hebben een molecuulmassa van 24 kD), die worden geactiveerd door verwerking via de serine protease caspase-1 (ICE). IL-1β en IL-18 activeren dezelfde signaaltransductieroute, maar via verschillende receptorcomplexen. De meest bekende functie van IL-18 is de inductie van IFN-γ secretie door T helper 1 en Natural Killer-cellen. Interleukine-1β is betrokken bij de inductie van vele ontstekingsmediatoren waaronder pro-inflammatoire cytokinen zoals IL-6, IL-8 en TNF-α.

In hoofdstuk 2 wordt aangetoond dat in normale en aangedane huid van psoriasis patiënten IL-1β niet alleen door ICE, maar ook door alternatieve proteasen kan worden geactiveerd, zoals het epidermis specifieke enzym chymotrypsine.

Omdat het nog niet bekend was of humane keratinocyten in staat zijn IL-18 te produceren, werd de vraag gesteld of IL-18 ook tot expressie komt in normale huid. In hoofdstuk 3 wordt aangetoond dat zowel IL-18 mRNA als IL-18 eiwit constitutief tot expressie komt in normale huid. We laten zien dat keratinocyten zeer belangrijke producenten zijn van IL-18. IL-18 is voornamelijk intracellulair aanwezig als precursormolecuul, maar wordt ook uitgescheiden. We tonen aan dat de hoeveelheid IL-18 die aanwezig is in normale keratinocyten, significant hoger is vergeleken met de hoeveelheid in andere cellen zoals monocyten en bronchiale epitheelcellen. Dit hoge IL-18 expressieniveau is mogelijk nodig voor een snelle inflammatoire response in de huid.

Omdat IL-18 betrokken is bij de inductie van IFN-γ en omdat INF-γ expressie ver-

hoogd is in laesionale huid van psoriasispatiënten, werd de hypothese gesteld dat de IL-18 expressie in psoriasis lesionale huid in vergelijking met normale huid verhoogd is (hoofdstuk 4). Echter, de IL-18 expressie en IL-18 verwerking waren niet verhoogd in laesies van het stabiele plaque type. Het ontbreken van een verhoogde IL-18 expressie in stabiele laesies betekent niet dat IL-18 niet betrokken is bij de IFN-γ productie in psoriatische huid. Het is mogelijk dat IL-18 IFN-γ induceert in vroege actieve laesies, terwijl de IL-18 expressie is genormaliseerd in stabiele laesies. In voorbereidende experimenten is inderdaad aangetoond dat de totale IL-18 eiwitexpressie verhoogd is in actieve laesies.

De huidige kennis van de functie van IL-1 in de huid is voornamelijk verkregen via *in vivo* of *in vitro* modellen. Om de functie van IL-1 in intacte huid te kunnen bestuderen hebben we een huidkweeksysteem ontwikkeld, dat de *in vivo* situatie benadert door het behoud van de normale huidstructuur (hoofdstuk 5). In dit systeem verhoogt IL-1β de secretie van cytokinen zoals IL-6 en IL-8 in het medium. IL-1β verhoogt ook de expressie van verschillende oppervlaktemoleculen zoals ICAM-1, CD40 en CD86 op cellen in het weefsel. De verhoging van zowel cytokine- als celmarkerexpressie kan worden geblokkeerd door dexamethason en IL-1ra.

CD40-CD40L interacties spelen een belangrijke rol in verschillende immunologische reacties zoals "isotype switching" en activatie van antigeenpresenterende cellen en T-cellen. Informatie verkregen via *in vitro* kweeksystemen laat zien dat binding van CD40 aan humane huidcellen leidt tot de verhoogde expressie van pro-inflammatoire cytokinen zoals IL-6, IL-8 en TNF-α. Het huidkweeksysteem dat wordt beschreven in hoofdstuk 5, werd gebruikt om de functie van IL-1 in CD40 gemedieerde reacties in de huid te onderzoeken (hoofdstuk 6). In dit onderzoek konden we eerdere waarnemingen via *in vitro* kweeksystemen bevestigen door te laten zien dat stimulatie van CD40 in biopten van humane huid leidt tot de verhoogde expressie van deze cytokinen. Tevens laten we zien dat de IL-10 expressie is verhoogd na CD40 stimulatie, en dat IL-1 is betrokken bij de CD40 binding-geinduceerde IL-6 en IL-8 expressie, maar niet bij de door CD40 binding-geinduceerde TNF-α expressie.

Concluderend kan worden gezegd dat in dit proefschrift wordt aangetoond dat pro-IL-18 in grote hoeveelheden wordt geproduceerd in de normale huid, maar dat IFN-γ expressie in stabiele laesies van psoriasispatiënten niet vergezeld gaat van een verhoogde IL-18 expressie. IL-1 isovormen zoals IL-1β kunnen echter worden verwerkt door alternatieve proteasen, wat zou kunnen betekenen dat ook andere IL-1 isovormen zoals IL-18 extracellulair kunnen worden geactiveerd. Verder laten we zien dat de expressie van pro-inflammatoire cytokinen in de intacte normale huid direct via IL-1 stimulatie of indirect via CD40 stimulatie wordt geïnduceerd. Over het geheel verbreden deze studies de basis voor de ontwikkeling van IL-1 gerichte therapieën voor inflammatoire huidziekten.

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Arjen

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren in Berg en Dal (gem. Groesbeek) op 15 december 1966. Hij behaalde in 1984 het LBO diploma en na het volgen van een vooropleiding voor hoger beroeps onderwijs (diploma behaald in 1987) begon hij met een opleiding voor microbiologisch analist aan de Laboratorium Hogeschool "Larenstein" te Wageningen. In 1992 werd het diploma voor microbiologisch analist behaald en in hetzelfde jaar begon hij met de studie biologie aan de Landbouw Universiteit Wageningen. In 1995 heeft de schrijver stage gelopen bij de universiteit van Aberdeen, Schotland. In hetzelfde jaar studeerde hij af als bioloog binnen de afstudeerrichting Celbiologie met als specialisatie Immunologie. Op de afdeling Immunologie van de Erasmus Universiteit Rotterdam (hoofd: Prof. dr. R. Benner) werd in 1996 begonnen met het promotie-onderzoek dat in dit proefschrift wordt beschreven. Dit onderzoek werd verricht onder leiding van Dr. J.D. Laman en Dr. E.P. Prens.

LIST OF PUBLICATIONS

1. **Companjen A.R.**, van der Wel L.I., Boon L., Prens E.P. and Laman J.D. 2001. CD40 ligation-induced cytokine production in human skin explants is partly mediated via IL-1. *Submitted*

2. **Companjen A.R.**, van der Wel L.I., Laman J.D., Prens E.P. 2001. IFN-γ Expression in Psoriatic Stable Plaque Type Skin is not Accompanied by Elevation of IL-18. *Re-submitted*

3. **Companjen A.R.**, van der Wel L.I., Wei L., Laman J.D., Prens E.P. 2001. A modified *ex vivo* skin organ culture system for functional studies. *Arch Dermatol Res.* **293**: 184-190

4. **Companjen A.R.**, Prens E., Mee J.B. and Groves R.W. 2000. Expression of IL-18 in Human Keratinocytes [Letter]. *J Invest Dermatol* **114**: 598

5. **Companjen A.R.**, van der Velden V.H.J., Vooys A., Debets R., Benner R., Prens E.P. 2000. Human keratinocytes are major producers of IL-18: Predominant expression of the unprocessed form. *Eur Cytokine Netw* **11**: 383-390

6. Schrijver I.A., Melief M.J., van Meurs M., **Companjen A.R.**, Laman J.D. 2000. Pararosaniline fixation for detection of co-stimulatory molecules, cytokines, and specific antibody. *J Histochem Cytochem* **48**: 95-103.

7. Lundqvist E.N., **Companjen A.R.**, Prens E.P., Egelrud T. 1998. Biological activity of human epidermal interleukin-1beta: comparison with recombinant human interleukin-1beta. *Eur Cytokine Netw* **9**: 41-6.

8. Rombout J.H., van de Wal J.W., **Companjen A.R.**, Taverne N., Taverne-Thiele J.J. 1997. Characterization of a T cell lineage marker in carp (*Cyprinus carpio* L.). *Dev Comp Immunol* **21**: 35-46.