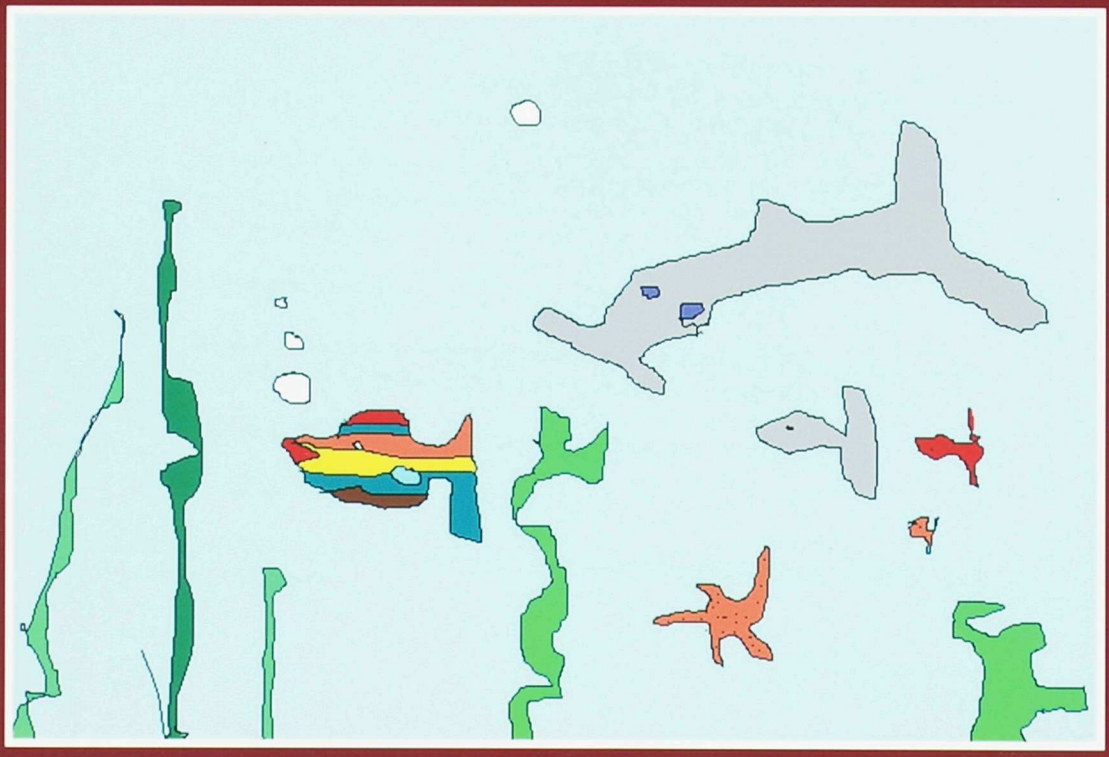
Initiation and regulation of specific immune responses by keratinocytes and dendritic cells

Role of cytokines and chemokines linking innate and specific immunity



M. Cristina Lebre

STELLINGEN

Behorend bij het proefschrift:
"Initiation and regulation of specific immune responses by keratinocytes and dendritic cells. Role of cytokines and chemokines linking innate and specific immunity"

1. Keratinocytes initiate immune responses. By expressing several Toll-like receptors, keratinocytes can detect the presence of distinct pathogens that invade the skin (dit proefschrift).

2. Keratinocytes communicate with dendritic cells: TNF-α, IFN-α and IL-18 produced by double-stranded RNA-activated keratinocytes induce maturation and polarization of dendritic cells (dit proefschrift).

3. Mature dendritic cells type 1 (DC1) play a role in peripheral tissues. They produce inflammatory chemokines locally and attract distinct cell populations (dit proefschrift).

4. BDCA1$^+$ dendritic cells and BDCA4$^+$ dendritic cells are aberrant in atopic dermatitis patients. Compared to healthy controls, BDCA1$^+$ dendritic cells produce less IL-12p70 and TNF-α, while BDCA1$^+$ dendritic cells produce less IFN-α (dit proefschrift).

5. (...) the origin of your current Langerhans cell population reflect your distance from the equator and the efficacy of your sun blockers. Lucas A and MacPherson G. Nat. Immunol 3:1125 2002.

6. Research is an addiction.

7. Tomorrow is the permanent address of scientists, as they bring things unseen here and now, into existence tomorrow. "Elsewhere" is Science's country. Maria de Sousa.

8. Keratinocytes and dendritic cells, more than an attraction.

9. Immune system? It is all about migration...

10. There is always an easy solution to every human problem – neat, plausible, and wrong. L. Mencken.

M. Cristina Lebre
May 2003

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Initiation and regulation of specific immune responses by keratinocytes and dendritic cells

Role of cytokines and chemokines linking innate and specific immunity

ACADEMISCH PROEFSCHRIFT

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The human being is a part of the whole, which we call Universe, limited both in time and space. He sees himself, his thoughts and emotions as if being separated from the rest, in a kind of optical illusion of consciousness. This illusion is a form of prison that restricts our personal desires and limits the affection of some of those around us. Our task should be to release ourselves from this prison, widening our sympathy circles, so that we can embrace all living creatures and the breadth of nature with all its beauty.

Albert Einstein

To my parents
To Ronald
In memory of Susana

Abbreviations

AD	atopic dermatitis
Ag	antigen
APC	Ag-presenting cell
CCR	chemokine receptor
CD	cluster of differentiation
CD40L	CD40 ligand
CPM	counts per minute
DC	dendritic cells
DNA	deoxyribonucleic acid
dsRNA	double stranded ribonucleic acid
GM-CSF	granulocyte/macrophage colony-stimulating factor
HLA	human leukocyte antigen
iDC	immature dendritic cell
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LC	Langerhans cells
LN	lymph node
LPS	lipopolysaccharide
LTA	lipoteichoid acid
mAb	monoclonal antibody
MF	maturation-inducing factors (TNF-α/IL-1β)
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NF	nuclear factor
PDC	IL-3Rα^+ plasmacytoid dendritic cells
PGN	peptidoglycan
PMA	phorbol 12-myristate 13-acetate
poly I:C	polyriboinosinic polyribocytidylic acid
R	receptor
RT-PCR	reverse transcriptase polymerase chain reaction
SAC	*Staphylococcus aureus* Cowan strain I
SEB	*S. aureus* enterotoxin B
TCR	T cell receptor
TGF	tumor growth factor
Th1/Th2	type-1/type-2 T helper cell
TLR	Toll-like receptor
TNF	tumor necrosis factor
PAMP	pathogen-associated molecular pattern
PRR	pathogen recognition receptor

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CHAPTER 1

General Introduction

THE KERATINOCYTE, AN IMPORTANT COMPONENT OF THE SKIN IMMUNE SYSTEM (SIS)

The skin's first line of defense against invasion by microbial agents is the stratum corneum, a nonviable, desiccated layer of the epidermis.[1] However, this physical barrier is susceptible to injuries that allow the entry of opportunistic microbial agents into the skin. Since human skin is constantly in contact with a wide range of microbial agents it is not surprising that many highly efficient and complementary defense mechanisms are present in both epidermal and dermal compartments. Some of the cellular constituents of the skin are resident, and others can be recruited. Although the main function of resident cells is to support the skin structure these cells may also contribute to immune defense. In addition, there is an army of cells with the mere function of host defense. These cells are present in low numbers in steady state conditions but can be massively recruited to the skin by the alarm signals provided by resident cells, in particular keratinocytes, or occasional immune cells. Innate immunity is the first line of defense and is supported by nonspecific events and lacks immunological memory. Cellular constituents of the innate immune system in the skin include keratinocytes, Langerhans cells (LC), dermal dendritic cells (DC), macrophages, natural killer (NK) cells, and granulocytes.[2] The second line of defense, specific immunity, is indispensable when innate immunity fails and is supported by antigen-specific T and B cells. The generation of this response is determined by signals provided by highly specialized antigen-presenting cells (APC), the DC.

The epidermis is mainly constituted by keratinocytes and in non-inflamed skin immature LC are present as a three-dimensional network in suprabasal layers where they can monitor the environment as sentinel cells.[2] Melanocytes are also present in the epidermis forming a monolayer along the basement membrane. The fibroblasts are the main constituent of the dermis. Tissue macrophages, monocytes, interstitial (dermal) DC (intDC), small numbers of T cells, granulocytes (e.g. eosinophils, basophils and neutrophils), endothelial cells and mast cells are also present in the dermis under steady-state conditions (Fig. 1).

Keratinocyte-derived cytokines and their role in the skin immune system

Keratinocytes, the principle epidermal cells, are major contributors to epidermal cytokine and chemokine production.[3] Keratinocyte-derived cytokines are produced either constitutively or upon induction by various stimuli. Once secreted, these cytokines may act in an autocrine, juxtacrine, or paracrine fashion to influence multiple resident and recruited cell types within

the skin, including endothelial cells, macrophages, DC, neutrophils, lymphocytes, fibroblasts, LC, and keratinocytes themselves,[3] contributing to local inflammatory reactions within the skin. Moreover, these cytokines may function as innate immune molecules initiating specific immunity. For instance, the presence of pathogens can be sensed by keratinocytes, which results in the release of inflammatory cytokines, such as IL-1β[4] and tumor necrosis factor (TNF)-α,[5] that activate immature LC/DC and induce their maturation and migration from the skin to the local lymph nodes where specific immune responses are initiated.[6] In addition, keratinocytes can produce various other cytokines, such as inflammatory (i.e. IL-6[7]) or anti-inflammatory (i.e. IL-10[8]) cytokines, growth factors (i.e. IL-7,[9] IL-15[10] and granulocyte macrophage-colony stimulating factor (GM-CSF)[11]), type I interferons ((IFN)-α, -β[12] and -κ[13]), and various others (i.e. IL-18[14] and IL-20[15]). Keratinocyte-derived cytokines may therefore influence a considerable range of homeostatic and inflammatory events. In particular, keratinocyte-derived GM-CSF,[16] TGF-β[17] and IL-15[18] are important for LC survival and LC generation from monocyte precursors *in vitro*. In addition, TNF-α produced by keratinocytes in response to endotoxin[5] or contact allergens[19] is an important factor that induces LC maturation and migration.

Expression of cell surface molecules by keratinocytes

The skin is frequently subject to immune-mediated disease processes that involve prominent infiltration by T lymphocytes.[20] The knowledge of the molecular basis for T cell trafficking in normal and diseased skin has increased considerably during the past years, with particular emphasis on the role of adhesion molecules. With the recognition of skin-homing T cell subsets[21] and the migration pattern of T cells into the skin mediated by adhesion molecules[22] and chemokines,[23,24] attention was directed at exploring how T cells become activated to proliferate once they enter epidermal and dermal compartments.[25] T cells are activated by ligation of their T cell receptors upon recognition of Ag-derived peptides presented in the context of MHC class I (HLA-ABC) or MHC class II (HLA-DR) expressed by APC (signal 1).[26] Optimal T cell activation requires a second costimulatory signal (signal 2) provided by, amongst others, the B7 family members CD80 and/or CD86, intercellular adhesion molecule-1 (ICAM-1), and CD40 which are expressed by activated APC.[26] In steady-state skin, keratinocytes do not express, or express very low levels of HLA-DR, HLA-ABC, ICAM-1, FasR and CD40.[27] However, in inflammatory conditions characterized by T cell

infiltration.[28,29] T cell-derived soluble factors induce in keratinocytes the expression of molecules that are particular helpful for effective interaction with T cells.[30,31] For instance, keratinocytes start to express HLA-ABC and HLA-DR molecules required for Ag presentation, and ICAM-1 required for adhesion between APC and T cells during Ag presentation. Indeed, keratinocytes-expressing HLA-DR and ICAM-1 can function as nonprofessional APC by presentation of bacterial superantigens to epidermal T cells.[32] Moreover, skin-infiltrating T cells induce the expression of CD40, allowing the keratinocytes to interact with CD40-ligand (CD40L) expressed by these T cells. Ligation of CD40 in the keratinocytes results in a boost of the production of inflammatory mediators, which amplifies the inflammatory response.[33] In addition, in these conditions, keratinocytes will express Fas receptor, a molecule that makes these cells susceptible to apoptosis. Fas-mediated keratinocyte apoptosis caused by skin-infiltrating T cells is proposed to be a key event in the pathogenesis of allergic contact dermatitis[34] and atopic dermatitis (AD).[35] Altogether, these accessory functions of keratinocytes are important for the amplification of the cutaneous inflammation.

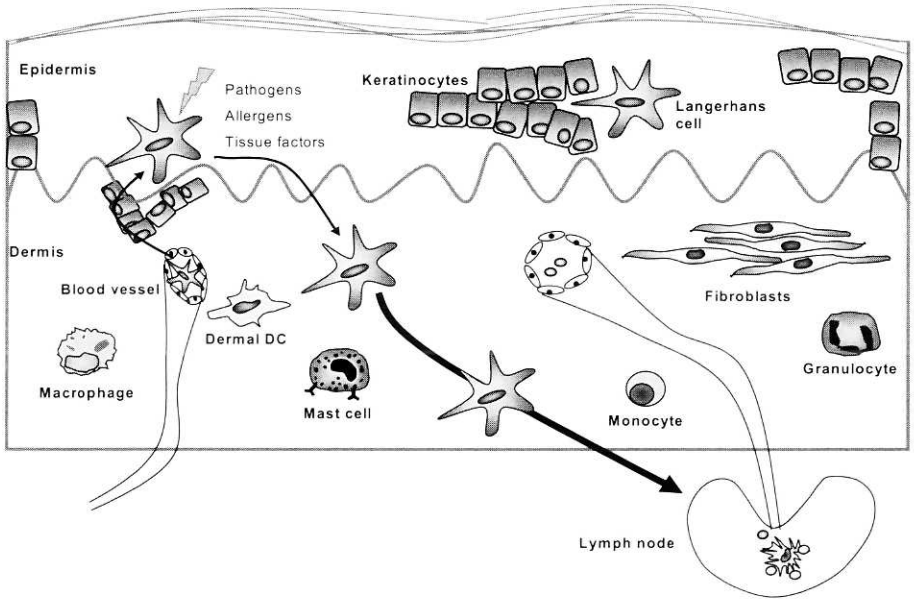

Figure 1. Cellular constituents of the skin immune system. The epidermis includes a variety of specialized cell types, such as Langerhans cells (LC), however the major cell type is the epidermal keratinocyte. Fibroblasts are the major constituent of the dermis, where dermal DC, endothelial cells, mast cells and granulocytes are also present. Upon activation with allergens, pathogens or keratinocyte-derived factors, LC migrate towards draining lymph nodes where they present Ags to naive T cells. Precursors of LC are at the same time recruited to inflamed skin to replenish the migrated LC population.

THE ROLE OF TOLL-LIKE RECEPTORS IN CONTROLLING ADAPTIVE IMMUNE RESPONSES

As the skin is constantly exposed to a wide variety of microbial agents the host must detect the pathogen and display mechanisms of defense in order to eradicate efficiently the invading pathogen. As stated above, the innate immune system detects the presence and the nature of infection, provides the first line of host defense, and controls the initiation and determination of the effector class of the specific immune response.[36] Innate immune recognition is based on the detection of the constitutive and conserved products of microbial metabolism that are called pathogen-associated molecular patterns (PAMPs).[37] PAMPs are recognized by so-called pattern recognition receptors (PRRs)[37] that are expressed on the cell surface, in intracellular compartments, or secreted into the blood stream and tissue fluids. The Toll-like receptors (TLRs) comprise a class of PRRs that belong to the family of type I transmembrane receptors, which are characterized by an extracellular leucine-rich repeat domain and an intracellular Toll/IL-1 receptor domain.[38] In mammalian species there are at least ten TLRs, and each seems to have a distinct function in innate immune recognition (Fig. 2). TLR1 recognizes microbial lipoproteins from *Mycobacterium tuberculosis*,[39] TLR2 a variety of bacterial components, such as peptidoglycan (PGN),[40] bacterial lipoproteins,[41] atypical LPS produced by *Porphyromonas gingivitis*,[42] glycosylphosphatidylinositol lipid from *Trypanosoma cruzi*[43] and mycobacterial cell-wall lipoarabinomannan,[44,45] although the latter can also be recognized by the C-type lectin DC-SIGN.[46] This unusual broad range of ligands recognized by TLR2 is explained, in part, by cooperation between TLR2 and TLR1 or TLR6.[47,48] TLR3 recognizes double-stranded (ds)RNA, polyriboinosinic polyribocytidylic acid (poly I:C).[49] TLR4 is the main receptor for LPS,[50] TLR5 recognizes bacterial flagellin[51] and TLR6, together with TLR2, coordinate activation by zymosan, a cell wall component of the yeast *Saccharomyces cerevisiae*.[47] Lipoteichoic acid (LTA), a cell wall component from Gram-positive bacteria, is recognized by both TLR2[52] and TLR4,[53] and both TLR7 and TLR8 recognize anti-viral compounds, the imidazoquinolines.[54,55] TLR9[56] has recently been shown to mediate signals from unmethylated CpG motifs present in bacterial DNA. The ligand that is recognized by TLR10 is still unknown.[57] TLRs transduce signals leading to the activation of nuclear factor (NF)-κB via a MyD88-dependent and -independent pathway.[58] NF-κB will in turn regulate series of genes involved in the production of inflammatory mediators and the expression of cell surface molecules in a variety of innate immune cells, including keratinocytes and DC.

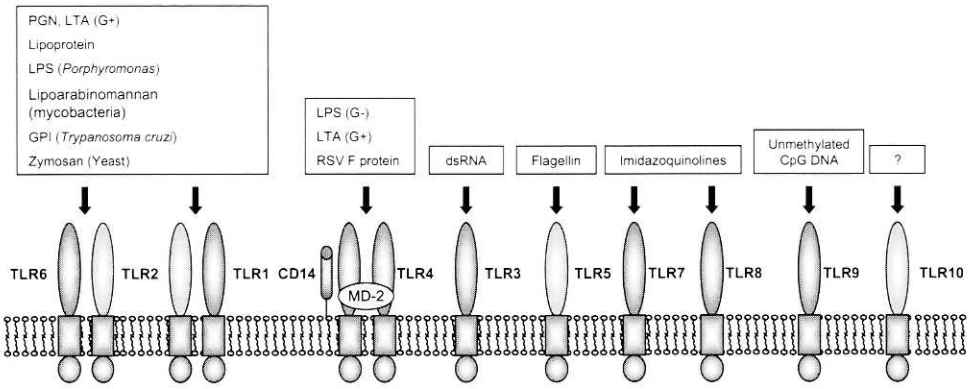

Figure 2. Ligand specificities of Toll-like receptors (TLRs). TLRs recognize a variety of pathogen-associated molecular patterns (PAMPs). Recognition of lipopolysaccharide (LPS) by TLR4 is aided by two accessory proteins: CD14 and MD-2. Adapted from Medzhitov[36].

THE ROLE OF DENDRITIC CELLS IN IMMUNITY

The immune system has evolved different types of adaptive immunity each specialized for the elimination of particular classes of pathogens.[59] In response to intracellular pathogens, such as viruses and most bacteria, $CD4^+$ T cells differentiate into Th1 cells, which produce IFN-γ. IFN-γ is a potent activator of natural killer cells, cytotoxic T cells and macrophages. In contrast, helminths induce the differentiation of Th2 cells, whose cytokines, principally IL-4, IL-13, IL-5, induce IgE- and eosinophil-mediated destruction of these pathogens.[60,61] To recognize Ags, T cells need to establish contact with APC.

DC are APC with a unique ability to induce primary immune responses. Immature myeloid DC reside in epithelia tissues and are unable to stimulate T cells, since they lack the requisite accessory signals for T cell activation, such as CD40, and the costimulatory molecules CD80 and CD86. However, they are well equipped to capture an Ag, a key event in the induction of immunity.[26] Immature myeloid DC capture Ag via macropinocytosis, receptor-mediated endocytosis via C-type lectin receptors (mannose receptor, DEC-205)[62,63] or Fcγ receptors type I (CD64) and II (CD32), and phagocytosis of particles such as apoptotic and necrotic cell fragments,[64] whole bacteria[65] and intracellular parasites.[66] Upon activation by pathogens or their products, or by tissue-derived factors myeloid DC upregulate costimulatory molecules and the chemokine receptor CCR7 a process defined as DC maturation. The process of maturation can be initiated by pathogens or their products, or by tissue-derived inflammatory products. Then, maturing DC migrate to secondary lymphoid organs which is crucial for the

initiation of specific immunity.[67] There, myeloid DC attract T and B cells by releasing chemokines[68] and maintain the viability of recirculating T lymphocytes.[69]

DC subsets

The DC of epidermal skin are named LC, whereas dermal skin contains (dermal) intDC, as stated above, and they display unique phenotypes. While LC are characterized by the presence of Birbeck granules[70] and langerin,[71] dermal DC express the intracytoplasmatic transglutaminase clotting factor XIIIa[72] and DC-SIGN.[73,74] At present, five subsets of DC - cells that are lineage (CD3, CD14, CD20 and CD56) negative and HLA-DR positive - have been described in human peripheral blood. Four subsets are myeloid DC, namely CD1b/c$^+$ (also know as BDCA1$^+$ DC subset[75]), CD16$^+$, BDCA3$^+$[75] and CD34$^+$ DC, and one subset is a lymphoid DC, namely CD123$^+$ DC (also know as BDCA4$^+$ DC subset[75] or plasmacytoid DC, PDC).[76] The precise role of each of these subsets in immunity is unclear, to date. The best described subset is the BDCA1$^+$ DC subset, the cell that picks up Ag in peripheral tissues, such as in dermal skin, and carries this Ag to draining lymphoid tissue. This BDCA1$^+$ myeloid DC subset is the major producer of IL-12, in response to CD40L or bacterial products.[77] IL-12 is one of the most potent factors driving T helper type 1 (Th1) responses.[78] However, efficient IL-12 production requires two signals,[79] and it was recently shown that also human PDC may produce high levels of IL-12 in response to the combination of bacterial CpG motifs and CD40 ligation.[77,80] PDC express some molecules of the lymphoid lineage[81] and have the capacity to produce large amounts of type I IFN in response to bacterial CpG motifs[82] or in response to several viruses.[83,84] PDC are also found in lymphoid organs (thymus, bone marrow, spleen, tonsils and LN), and are increased in inflamed tissues such as in cutaneous lupus erythematosus lesions[85] or in allergen-challenged nasal mucosa.[86]

Polarizing capacity of DC

It has been proposed that the development of different types of T cell-mediated immune responses depends on the DC lineage.[87] In this model myeloid DC are considered as potent inducers of Th1 responses while PDC are inducers of Th2 responses.[87] However, recent studies support the hypothesis that skewing towards either type of response is not exclusively lineage commitment, and that the same DC subset can promote either a Th1 or Th2 response according to the dose and type of Ag, including different pathogens, and the environmental milieu present during their differentiation.[77,88] In addition, the presentation of Ags by DC

during steady-state conditions can contribute to the establishment of non-responsiveness to self Ags.[89] In humans, DC show different T cell-polarizing capacities, which depend on multiple factors. In this respect, when immature myeloid DC (iDC) are matured in the presence of IFN-γ,[90] *Bordetella pertussis* toxin[91] or double-stranded (ds)RNA[91,92] they become type 1 effector myeloid DC (DC1) which drive the development of Th1 cells. In contrast, factors that upregulate intracellular cAMP such as prostaglandin E_2 (PGE_2),[93] histamine[94,95] or the toxin from *Vibrio cholerea*,[91,96] or factors derived from extracellular pathogens such as *Schistosoma mansoni* egg antigen (SEA)[91] induce the generation of myeloid DC2 which drive the development of Th2 cells. Moreover, myeloid DC (DCr) are capable of inducing T regulatory 1 cells (Tr1), which produce high levels of IL-10, when activated with filamentous hemagglutinin (FHA) from *B. pertussis*,[97] or schistosomal lyso-phosphatidylserine (PS).[98] The effects of these factors on iDC polarization are summarized in Fig. 3.

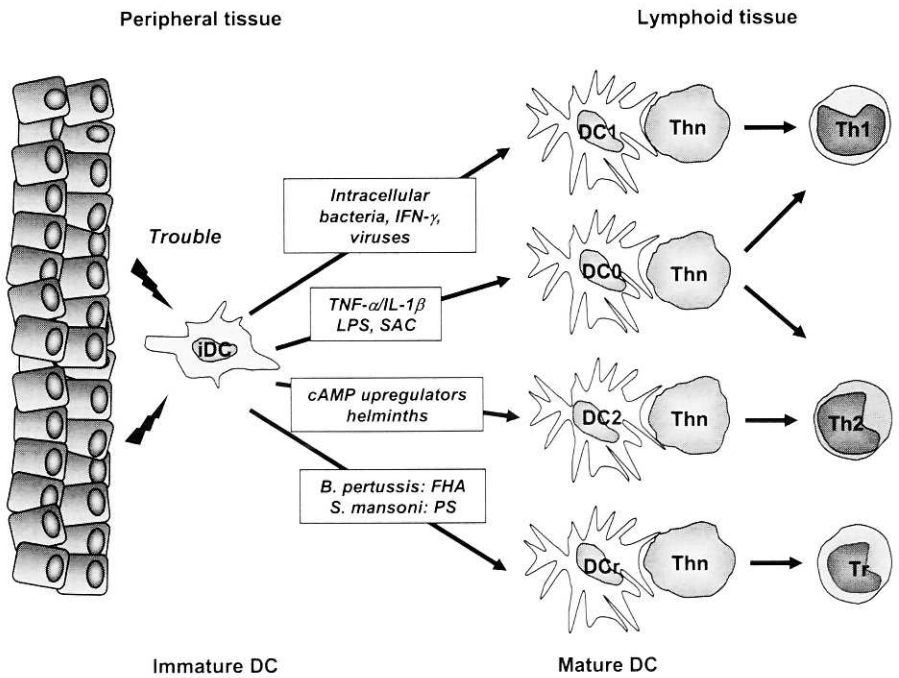

Figure 3. Functional polarization of myeloid DC. When type 1 factors such as IFN-γ or intracellular pathogens are present during DC maturation, DC acquire the capacity to induce Th1 cells (DC1). Conversely, type 2 factors, such as PGE_2, SEA, and *V. cholerea* toxin induce the development of DC2, which drives the development of Th2 cells. Factors such as filamentous hemagglutinin (FHA) from *B. pertussis* or schistosomal lyso-phosphatidylserine (PS) induce DCr that drive the development of T cells with suppressor activity (Tr).

A similar DC1/DC2 dichotomy is applicable to PDC. While iPDC acquire the capacity to induce the development of Th1 responses upon viral stimulation, CD40L in the presence of IL-3-activated PDC induce Th2 responses.[84,99] These findings demonstrated that a given DC subset has a remarkable plasticity in directing different types of T cell responses.[88]

Although the signals from DC that govern a specific T cell cytokine production profile have not all been determined, the production of the Th1-inducing cytokine IL-12 is an important factor.[100] The nature of the DC-derived factors that induce Th2 responses is less clear. However, the costimulatory molecule OX40L is upregulated *in vitro* on SEA-primed DC2 and is a possible candidate for skewing Th2 responses.[91]

DC/pathogen interactions

The molecular response of DC to microbes represents one of the most critical steps in the induction of protective specific immunity. Immature DC, present at the site of infection, can sense microbes directly by recognizing molecular patterns within microbial carbohydrates, lipids, proteins, nucleic acids and many others, using highly conserved PRRs.[101,102]

Studies with DC subsets have suggested that specialized functions of human myeloid DC and PDC are partly the result of a different pattern of TLRs expressed on their surface. In particular, PDC, but not myeloid DC, express TLR9. By contrast, myeloid DC, but not PDC, express TLR2, TLR4 and TLR6.[103,104] The differential TLR expression pattern permits DC to initiate responses to a large variety of microbes and to generate different immune responses to a single microbe, thus increasing the chances of successfully controlling the microbial invasion.[105] The overall type of response against the whole microorganism may well be an integration of all microbial signals through all receptors and all APC subsets.[106] Thus, DC link these evolutionary conserved pattern-recognition systems of the innate immune system to the variable receptors of the adaptive immune response: the T cell receptors.[107]

CHEMOKINES

Chemokines are a superfamily of small (6-14 kDa) structurally related proteins that regulate the traffic of various leukocytes.[108] To date, more than 50 human chemokines have been characterized. In table I, a summary is given of the known human chemokines, their most commonly used names, and their receptors. Most chemokines have four characteristic cysteines, and depending on the motif displayed by the first two cysteines, they have been classified into CXC or alpha, CC or beta, C or gamma, and CXXXC or delta chemokine

classes. Chemokines exert their effects by signaling through specific 7-transmembrane G-protein-coupled receptors on the surface of many leukocytes (Fig. 4).

Table I. Human chemokines and their respective receptors

Family	Systematic name	Current name	Receptor(s)
CXC	CXCL1	Growth-related oncogene (GRO)α	CXCR1, CXCR2
	CXCL2	GROβ, macrophage inflammatory protein (MIP)-2α	CXCR2
	CXCL3	GROγ	CXCR2
	CXCL4	PF4	Unknown
	CXCL5	ENA-78	CXCR2
	CXCL6	GCP-2	CXCR1, CXCR2
	CXCL7	NAP-2	CXCR2
	CXCL8	Interleukin (IL)-8	CXCR1, CXCR2
	CXCL9	Monokine-induced by IFN-γ (Mig)	CXCR3
	CXCL10	IFN-induced protein of 10 kDa (IP-10)	CXCR3
	CXCL11	IFN-induced T cell α-chemoattractant (I-TAC), IP-9	CXCR3
	CXCL12	Stromal-derived factor (SDF)-1	CXCR4
	CXCL13	B cell activating chemokine (BCA)-1, BLC	CXCR5
	CXCL14	Breast and kidney chemokine (BRAK)	Unknown
	CXCL15	Lungkine	Unknown
	CXCL16	SR-PSOX	Unknown
CC	CCL1	I-309	CCR8
	CCL2	Monocyte chemotactic protein (MCP)-1	CCR2
	CCL3	MIP-1α	CCR1,CCR5
	CCL4	MIP-1β	CCR5
	CCL5	Regulation and activated normal T cell-expressed and -secreted (RANTES)	CCR1, CCR3, CCR5
	(CCL6)	Unknown	Unknown
	CCL7	MCP-3	CCR1, CCR2, CCR5
	CCL8	MCP-2	CCR3
	CCL9/10	Unknown	Unknown
	CCL11	Eotaxin-1	CCR3
	CCL12	Unknown	CCR2
	CCL13	MCP-4	CCR2, CCR3
	CCL14	HCC-1	CCR1
	CCL15	HCC-2	CCR1, CCR3
	CCL16	HCC-4	CCR1
	CCL17	Thymus- and activation-regulated chemokine (TARC)	CCR4
	CCL18	Dendritic cell-derived CC chemokine-1 (DC-CK1), PARC	Unknown
	CCL19	MIP-3β, ELC	CCR7
	CCL20	MIP-3α, LARC	CCR6
	CCL21	Secondary lymphoid tissue (SLC), 6Ckine	CCR7
	CCL22	Macrophage-derived chemokine (MDC)	CCR1
	CCL23	MPIF-1	CCR4
	CCL24	Eotaxin-2	CCR3
	CCL25	Thymus-expressed chemokine (TECK)	CCR9
	CCL26	Eotaxin-3	CCR3
	CCL27	Cutaneous T cell-attracting chemokine (CTACK)	CCR10
	CCL28	Mucosa-associated epithelial chemokine (MEC)	CCR10
C	XCL1	Lymphotactin, ATAC	XCR1
	XCL2	SCM-1β	XCR1
CXXXC	CX3CL1	Fractalkine	CX3CR1

Adapted from Sebastiani *et al.*[122].

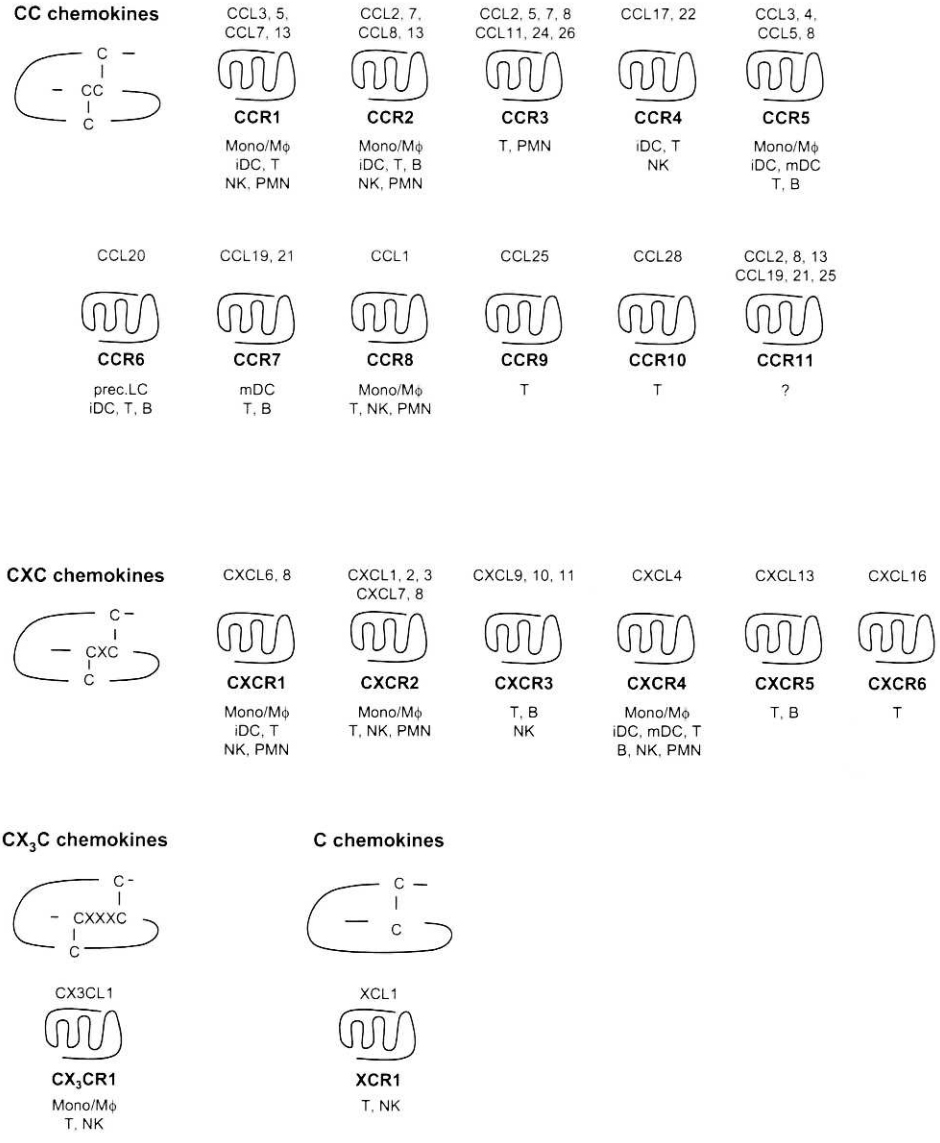

Figure 4. Chemokines and their receptors. The four main chemokine families (CC, CXC, CX_3C and C) are shown, as classified by the arrangement of amino-terminal cysteine residues. The 7-transmembrane G-protein-coupled receptors are depicted; the names of the receptors for each group of chemokines and the cellular expression of each receptor is indicated below each group. Mono, monocytes; Mϕ, macrophages; iDC, immature dendritic cells; mDC, mature DC; NK, natural killer cells; PMN, polymorphonuclear neutrophils. Adapted from Hancock *et al.*[154].

Functionally, chemokines can be classified into inflammatory and homeostatic chemokines. Inflammatory chemokines are expressed in inflamed tissues by resident and

infiltrated cells on stimulation by proinflammatory cytokines or during contact with microbial agents. In contrast, homeostatic chemokines are produced within lymphoid or nonlymphoid tissues such as the skin and mucosa. These constitutively produced chemokines are involved in maintaining physiological traffic and positioning of cells that mainly belong to the specific immune system, during hematopoiesis, Ag sampling in secondary lymphoid tissue and immune surveillance.[109] The distinction between inflammatory and homeostatic chemokines is not absolute; several chemokines, such as CCL17/thymus and activation-regulated chemokine (TARC), CCL20/macrophage inflammatory protein (MIP)-3α and CCL22/monocyte-derived chemokine (MDC) have been ascribed to both families.[109]

Chemokines and chemokine receptors in primary immune responses

In the course of a primary immune response, the rare Ag-specific T and B lymphocytes present in lymphoid tissues first need to interact with DC and follicular DC (FDC), respectively, and subsequently with each other. In case of infection, DC and their precursors migrate at an enhanced rate from the blood into the tissues up to the site of pathogen entry. Here, the cells are exposed to inflammatory stimuli that redirect them to the lymphatics and subsequently into T cell areas of secondary lymphoid organs, where they present Ag to T cells.[67] For instance, following stimulation with LPS, DC have an initial burst of CCL3/MIP-1α, CCL4/MIP-1β, and CXCL8/IL-8 production, chemokines that are specialized for the recruitment of effector cells, including monocytes, granulocytes and effector T cells.[110] At later time points DC produce mainly homeostatic chemokines such as CCL17/TARC, CCL18/dendritic cell-derived CC chemokine-1 (DC-CK1), CCL19/MIP-3β, and CCL22/MDC.[111] These chemokines are involved in maintaining homeostatic leukocyte traffic and cell compartimentalization within secondary lymphoid tissues.[108] Maturing DC upregulate receptors for homeostatic chemokines such as CXCR4, CCR4, and especially CCR7.[108] CCR7 upregulation results in responsiveness to CCL19/MIP-3β and CCL21/6Ckine, which are produced in the T cell areas of the secondary lymphoid organs by stromal cells.[112,113] In addition, mature DC-derived CCL19/MIP-3β and CCL21/6Ckine,[112,113] and CCL18/DC-CK1[68] attract naive T cells thus promoting the initiation of immune responses through co-localization of naive T cells with DC. To initiate a humoral immune response, the recruited and subsequently activated T cells have to interact with Ag-specific B cells. After Ag binding, B cells move to the boundary of B and T zones to interact with Th cells[114,115]

guided by DC-derived CXCL13/B cell-attracting chemokine-1 (BCA-1)[116] and CCL18/DC-CK1.[68,117]

Chemokine receptor expression by polarized T cell subsets

T cell recirculation in peripheral tissues is a complex event regulated by the local release of chemotactic stimuli and by the differential expression of receptors on distinct T cell subsets. Two chemokine receptors are preferentially expressed on Th1 cells: CCR5 and CXCR3. The chemokines they bind are produced in numerous Th1-driven inflammatory lesions, including rheumatoid arthritis, atherosclerosis and multiple sclerosis. Conversely, Th2 cells preferentially express CCR3, CCR4 and CCR8[118,119] and the chemokines they bind are expressed, for instance, in lesional skin of AD patients[120] and in the airway mucosa of atopic asthmatics patients.[121]

Keratinocyte-derived chemokines

Keratinocytes are an important source of chemokines. For instance, resting keratinocytes release low levels of CXCL8/IL-8 and express CCL27/cutaneous T cell-attracting chemokine (CTACK).[122,123] Moreover, constitutive expression of CCL20/MIP-3α by keratinocytes is of particular importance for the basal recruitment of LC in the epidermis.[124] Following activation, keratinocytes rapidly upregulate CXCL8/IL-8 and CCL27/CTACK, and synthesize CXCL10/IFN-induced protein of 10 kDa (IP-10), CXCL9/monokine-induced by IFN-γ (Mig), CXCL11/IFN-induced T cell α-chemoattractant (I-TAC), CCL5/regulation and activated normal T cell-expressed and -secreted (RANTES), CCL2/monocyte chemotactic protein (MCP)-1, and CCL20/MIP-3α.[125] CXCL8/IL-8 attracts neutrophils[126] and CCL27/CTACK is chemokine that is selectively produced by keratinocytes and attracts skin-homing memory T cells.[123] The production of CCL17/TARC by keratinocytes is still controversial. While some authors have demonstrated the production of CCL17/TARC by keratinocytes[127] we (M.C. Lebre *et al.*, manuscript submitted) and others could not detect CCL17/TARC mRNA expression neither protein release.[128] CCL22/MDC production by keratinocytes is very low.[128] CXCL10/IP-10, CCL2/MCP-1 and CXCL8/IL-8 are abundantly released by activated keratinocytes.[125,128] By producing this large array of chemokines, keratinocytes can participate in skin inflammation through the regulated expression of chemokines attracting various leukocyte subsets.

ATOPIC DERMATITIS (AD)

Allergy is a major health problem in most modern societies. The prevalence and severity of allergic diseases has increased in many industrialized countries during the past few decades. This phenomenon is observed for the so-called atopic diseases, namely bronchial allergic asthma, allergic rhinoconjunctivitis ("hay fever") and AD. AD is a chronic skin disease manifested by eczematous skin lesions that affects more than 10% of children.[129] A complex interplay between genetic predisposition, immune dysregulation, skin barrier dysfunction, lifestyle, and environmental factors is thought to be important for the development of AD.[130-132] The skin lesions are characterized by the presence of lichenification, pruritic excoriations, severely dry skin and a susceptibility to cutaneous infections.[133] Keratinocytes in AD exhibit a particular cytokine[134,135] and chemokine[136] secretion profile, a phenomenon that has a major role in promoting and maintaining inflammation. Keratinocytes cultured from AD patients show enhanced production of GM-CSF[134] and are hyperresponsive to IFN-γ in terms of cytokine production.[135] Moreover, keratinocytes from AD patients express high levels of CCL5/RANTES compared to healthy controls.[136]

Acute or early lesions of AD are characterized by spongiosis of the epidermis, and a sparse epidermal infiltrate of T lymphocytes may be observed. Increased numbers of LC are present within the epidermis.[137] The LC in the skin of AD patients possess an abnormal phenotype. They have an increased capacity to capture allergens because of their expression of receptors for IgE (namely, FcεRI) on their cell surface. In addition, LC in AD express large amounts of the costimulatory molecule CD86.[138,139] In the dermis, an increased number of mononuclear cells (e.g., monocytes and macrophages) is observed compared to normal healthy subjects. Mast cells are also present in the dermis in normal numbers but in a degranulated state. The dermal lymphocytic infiltrate is composed predominantly of allergen-specific $CD4^+$ T cells with a smaller number of $CD8^-$ T cells.[140,141] Most skin-infiltrating T cells express cutaneous lymphocyte-associated Ag (CLA), a carbohydrate-modified surface glycoprotein which is known to be important for the homing of T cells to the skin.[142,143] In AD, circulating allergen-specific memory/effector T cells expressing CLA have been demonstrated to be activated *in vivo*, to regulate IgE by an IL-13-dominated cytokine pattern, and to delay eosinophil apoptosis by IL-5.[143,144] Allergen-specific T cells from skin and peripheral blood have shown a profound dysregulation of IL-4 and IFN-γ secretion, with an overproduction of IL-4 and reduced IFN-γ secretion in AD.[145,146] However, studies that focused on the cytokine production in skin revealed higher IFN-γ levels than observed in T cells from peripheral

blood.[147,148] A sequential activation of Th2 and Th1 cells in the pathogenesis of AD was therefore proposed. In this concept, the initial phase is predominated by Th2 cells that produce high levels of IL-4. Later on, this Th2 cytokine pattern switches into the second, eczematous phase predominated by the Th1 cytokine IFN-γ, leading to the ongoing inflammation of the skin lesions.[149,150]

There is accumulating evidence that IL-12 production by APC in allergic disorders such as allergic asthma and AD is deficient.[151,152] Monocytes from atopic patients have reduced IL-12 levels[151] and the expression of IL-12 mRNA in biopsies of allergen-challenged patients with allergic asthma is increased after steroid therapy.[152] In addition, Soumelis *et al.*[153] demonstrated that in inflamed AD skin keratinocytes produce thymic stromal lymphopoietin (TSLP) that amplifies DC-mediated allergic inflammation. TSLP induces in human $CD11c^-$ myeloid DC the production of Th2-attracting chemokines CCL17/TARC and CCL22/MDC. Moreover, TSLP-activated DC primed naive Th cells to produce Th2-associated cytokines IL-4, IL-5 and IL-13. In summary, although it was shown that nonprofessional APC from allergic individuals have reduced bioactive IL-12 production[151,152] there is no data on the IL-12 expression levels in DC. In addition, the molecular mechanisms underlying the preferential Th2 type immune response observed in these patients are poorly understood.

SCOPE OF THIS THESIS

The scope of this thesis is to get more insight in the role of both keratinocytes and myeloid DC in the initiation and regulation of the class of the immune response, focusing on cytokines and chemokine produced by these distinct cells.

It is well established that the expression of PRRs by innate immune cells is of particular importance to recognize PAMPs. As the skin is the first line of defense against invasion by microbial agents we questioned whether keratinocytes, as the main producers of soluble factors in the skin, do express TLR and if so, which TLR types. **Chapter 2** describes the expression of TLR by human keratinocytes and addresses the role of these PRRs in chemokine and cytokine production in response to different PAMPs.

As in the skin environment iDC reside in the close vicinity of keratinocytes we questioned in **Chapter 3** whether keratinocytes may play a role in the activation and polarization of iDC into DC1 or DC2. We studied whether and how factors produced by poly I:C-activated keratinocytes activate and/or polarize DC.

Although the DC1- and DC2-derived cytokines, involved in T cell polarization, have been subject of intense investigation, the production of chemokines by these DC subsets is unknown, thus far. We investigated in **Chapter 4** whether human monocyte-derived DC0, DC1, or DC2 may also display differential production of inflammatory and homeostatic chemokines.

Three DC subsets in human blood can be distinguished on the basis of the expression of three novel DC Ags: BDCA1, BDCA3 and BDCA4. While BDCA1 and BDCA3 Ags are characteristic of two different subsets of myeloid DC, BDCA4 Ag is exclusively present in PDC. In **Chapter 5** the BDCA3$^+$ DC subset is characterized in more detail, such as the expression of TLR and their responsiveness to different pathogens. In addition, the localization of this subset in lymphoid and nonlymphoid tissues is addressed.

AD is a skin disease characterized by the preferential development of Th2 cells, which may be based on an aberrant function of DC. At present, little is know about the function of DC in AD. Therefore, we investigated (**Chapter 6**) the functional characteristics of peripheral blood DC subsets (BDCA1$^-$ and BDCA4$^-$ DC) in AD patients with respect to their phenotype, cytokine production and their capacity to induce a particular Th response. In addition, the *in vivo* localization of BDCA1$^+$ and BDCA4$^+$ DC in human skin is addressed.

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CHAPTER 2

The role of Toll-like receptors in chemokine and cytokine production by human keratinocytes in response to different pathogen-associated molecular patterns

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ABSTRACT

Keratinocytes are constantly in contact with external stimuli and have the capacity to produce several soluble mediators constitutively or upon induction. Pathogens express pathogen-associated molecular patterns (PAMPs) that are recognized, amongst others, by Toll-like receptors (TLRs). The functional responses of human keratinocytes to different PAMPs have not yet been fully established. We show here that keratinocytes constitutively express mRNA for TLR1, 2, 3, 4, 5, 6, 9 and 10, but not for TLR7 and 8. Stimulation of keratinocytes with different TLR ligands resulted in differential expression patterns of cell surface molecules and, in particular, of chemokines. CXCL8/IL-8 was significantly enhanced in response to all TLR ligands tested, except for the TLR9 ligands CpG-ODN 2006 and 2216. A similar pattern was evident in the production of CCL2/MCP-1 that, however, could not be induced by both CpG ODNs and the TLR2/4 ligand lipoteichoic acid (LTA). CCL20/MIP-3α production was selectively induced upon stimulation with the TLR3 ligand poly I:C, the TLR5 ligand flagellin and the TLR2/6 ligand zymosan A. CCL27/CTACK production was selectively induced by poly I:C and flagellin. The expression of the Th1-attracting chemokines, CXCL9/Mig, CXCL10/IP-10 and CXCL11/I-TAC was exclusively induced by poly I:C that is known to induce a response type characterized by a predominance of Th1 cells. Furthermore, TNF-α mRNA was induced by CpG-ODN 2006, whereas CpG-ODN 2116 induced both TNF-α and IFN-α mRNA. The ability CpG-ODN to activate keratinocytes was underscored by the translocation of the NF-κB subunits p50 and p65 to the nucleus. Altogether, these findings stress an unexpectedly multifaceted role of keratinocytes in innate immunity as evident by their differential, TLR-mediated responses to PAMPs associated with different classes of pathogens.

INTRODUCTION

Keratinocytes, the main constituent of the skin, not only form a passive barrier between external environment and internal organs, but upon external stimuli, such as trauma, bacterial and viral infections, chemical substances, or ultraviolet irradiation, they produce various cytokines and chemokines.[1,2] Therefore, keratinocytes may represent the first line of defense against microbial pathogens in the skin.

The innate immune system has evolved to recognize a broad spectrum of pathogens and is often extremely successful as a first line of defense. Innate immune cells also provide

specific immune cells with information required for a most effective second line of immunity, which is indispensable when innate immunity fails.[3] Recognition of pathogens by innate immune cells is mediated by pattern-recognition receptors (PRRs) that recognize conserved pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLR), amongst others, function as PRRs and are of particular importance for translating the recognition of pathogen components to activation of the immune system.[4,5] TLRs transduce signals leading to the activation of NF-κB[6] that subsequently drive the transcriptional induction of several cytokine, chemokine and adhesion molecule genes.[7] In particular TLR1, together with TLR2, recognizes microbial lipoproteins from *Mycobacterium tuberculosis*,[8] TLR2 a variety of bacterial components, such as peptidoglycan (PGN),[9] and TLR3 double-stranded (ds)RNA polyriboinosinic polyribocytidylic acid (poly I:C).[10] TLR4 is the main receptor for LPS,[11] TLR5 recognizes bacterial flagellin[12] and TLR6, together with TLR2, coordinate activation by zymosan, a cell wall component of the yeast *Saccharomyces cerevisiae*.[13] Lipoteichoic acid (LTA), a cell wall component from Gram-positive bacteria, is recognized by both TLR2[14,15] and TLR4,[9] and both TLR7 and TLR8 recognize anti-viral compounds, the imidazoquinolines.[16,17] TLR9[18] has recently been shown to mediate signals from unmethylated CpG motifs present in bacterial DNA. The ligand that is recognized by TLR10 is still unknown.[19] Immune responses to different types of pathogens, or to their products, are associated with different classes of immune responses. The variety of PRRs suggest that the discrimination of the pathogen by TLRs, and the subsequent production of a specific subset of cytokines and chemokines, may be the first point at which the immune system tailors its response to different classes of pathogens.

Chemokines are small, secreted proteins that regulate leukocyte migration.[20,21] Members of this superfamily share structural similarities, including four conserved cysteine residues that form disulphide bonds that are crucial for the tertiary structures.[20] Chemokines can be subdivided into four classes, the C-C, C-X-C, C and C-X3-C chemokines, depending on the location of the first two cysteines in their protein sequence.[20] In general, CXC-chemokines have chemotactic properties for neutrophils, T and B lymphocytes whereas CC-chemokines represent rather selective chemoattractants for monocytes, macrophages, dendritic cells (DC), different T lymphocyte subsets, basophils and eosinophils.[22] Numerous *in vitro* studies have documented the increased expression of mRNA and/or protein for several chemokines by human keratinocytes.[1,2] In particular, keratinocytes produce in response to proinflammatory cytokines, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α, the CXC

chemokines IFN-inducible protein-10 (IP-10; CXCL10),[23] monokine-induced by IFN-γ (Mig; CXCL9),[24] IFN-inducible T cell α-chemoattractant (I-TAC; CXCL11),[25] interleukin-8 (IL-8; CXCL8)[26] and the CC chemokines monocyte chemoattractant protein-1 (MCP-1; CCL2),[27] macrophage inflammatory protein-3α (MIP-3α; CCL20),[28] and cutaneous T-cell-attracting chemokine (CTACK; CCL27).[29] The specific effects of chemokines on target cell types are mediated by specific 7-transmembrane G-protein-coupled receptors (GPCR).[30] In this respect, CXCL9/Mig, CXCL10/IP-10 and CXCL11/I-TAC chemokines specifically attract activated $CXCR3^+$ T helper type 1 (Th1) cells[31] and CXCL8/IL-8 selectively attracts $CXCR1^+2^+$ neutrophils.[32] CCL2/MCP-1 attracts monocytes,[33] DC and Langerhans cells (LC),[27] memory T cells[34] and natural killer cells[35] via CCR2.[36] CCL20/MIP-3α attracts both memory T cells[37] and epidermal LC into the skin[28] via CCR6 receptor. CCL27/CTACK is exclusively and constitutively expressed by human keratinocytes and binds to the receptor CCR10. This chemokine preferentially attracts skin-homing cutaneous lymphocyte-associated antigen (CLA^+CCR10^+) memory T cells.[29,38]

Although it has long been recognized that keratinocytes are the main producers of cytokines and chemokines in the skin,[1,2] the knowledge of the mechanisms of this secretion in response to microbial stimuli remained fragmented. Therefore, we examined the expression of TLRs by normal human keratinocytes and questioned whether these PRRs played a role in cytokine and chemokine production in response to different PAMPs. We demonstrate for the first time that, in addition to TLR4[39] human keratinocytes constitutively express mRNA for TLR1, 2, 3, 5, 6, 9 and 10, but not for TLR7 or 8. The fact that keratinocytes respond to TLR2, 3, 4, 5, 6 and 9 ligands, indicates that keratinocytes may play a critical role in alerting the immune system to the presence of pathogens so that an immediate response can be mounted to contain the infection. This suggests that keratinocytes function as a link between the innate and specific immunity.

MATERIAL AND METHODS

Keratinocyte cultures

Primary keratinocyte cultures were prepared from plastic surgery skin obtained from healthy subjects. Briefly, The skin was incubated with Dispase (0.3%; Boehringer, Mannheim, Germany) for 16 h at 4°C. Epidermal sheets were removed from the dermis and single cell suspensions were obtained by placing epidermal sheets in trypsin (0.025%; Life

Technologies, Paisley, U.K.) for 5 min at 37°C. After neutralizing with equal volume of FCS, (HyClone, Logan, UT) stratum corneum debris was removed and then thieved through sterile nylon gauze to obtain a single cell suspension. Isolated epidermal cells were seeded at the density of 8-10 x 10^4 cells/cm^2. At 70-80% confluence, keratinocytes were detached with 0.025% trypsin, 2 mM EDTA for 5 min at 37°C, and subcultured or frozen. Keratinocyte cultures were maintained in Keratinocyte SFM medium (Gibco, Paisley, UK).

RNA isolation and cDNA synthesis

Keratinocytes were plated at the concentration of $1.5x10^5$ cells/3 ml (6-well plates, Costar, Cambridge, MA), and cultured at least for 48 h before the cells were lyzed. Keratinocyte total RNA was purified by using the NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany) according to manufacture's instructions. cDNA was generated using the First strand cDNA synthesis kit for RT-PCR (MBI Fermentas, St Leon-Rot, Germany). To anneal the primer to the RNA, 9 µl of total RNA, 1 µl oligo$(dT)_{18}$ and 1 µl $D(N)_6$ were added. This mix was then heated for 5 minutes at 94°C.

PCR analysis

The primers sequences used are shown in Table I. Complementary (c)DNAs were amplified for 45 cycles and were analyzed on a 1% agarose gel containing ethidium bromide. A 100 bp DNA ladder standard (MBI Fermentas) was used as a size marker.

Keratinocyte stimulation for analysis of cell surface expression

Keratinocytes were plated at the concentration of $1.5x10^5$ cells/3 ml (6-well plates, Costar, Cambridge, MA), and cultured at least for 48 h. Keratinocytes were cultured in the presence of peptidoglycan (PGN) from *Staphylococcus aureus* (100 µg/ml; Sigma-Aldrich, St. Louis, MO), or synthetic dsRNA poly I:C (50 µg/ml; Sigma-Aldrich), or lipoteichoic acid (LTA) from *S. aureus* (100 µg/ml; Sigma), or LPS from *Escherichia coli* (100 µg/ml; Sigma-Aldrich), or flagellin (100 ng/ml; a kindly gift from Dr. A.T. Gewirtz), or zymosan A from the yeast *Saccharomyces cerevisiae* (100 µg/ml; Sigma-Aldrich), or the CpG-oligodeoxynucleotides (ODN) 2006 (5'-GGGGGGACGATCGTCGGGGGG-3')[40] or CpG-ODN 2216 (5'-TCGTCGTTTTGTCGTTTTGTCGT-3')[41] (5 µM; Biosource International, Nivelles, Belgium). After 48 h the cells were detached from the wells by incubating them

with 1 ml of the cell dissociation solution (Sigma-Aldrich) for 15 min. at 37°C. Trypsin neutralization was performed by adding equal volume of FCS. After spinning for 10 min at 800 rpm the single cell suspension was analyzed for the expression of cell surface molecules by FACS (see next).

Table I. Primer sequences

Gene	Forward primer	Reverse primer
TLR1	AAAAGAAGACCCTGAGGGCC	TCTGAAGTCCAGCTGACCCT
TLR2	AACCCTAGGGGAAACATCTCT	GGAATATGCAGCCTCCGGAT
TLR3	AAATTGGGCAAGAACTCACAGG	GTGTTTCCAGAGCCGTGCTAA
TLR4	TACAAAATCCCCGACAACCTC	AGCCACCAGCTTCTGTAAACT
TLR5	TGCATTAAGGGGACTAAGCCTC	AAAAGGGAGAACTTTAGGGACT
TLR6	TTGACAGTTTTGAGACTTTCCC	TGGACCTCTGGTGAGTCCTG
TLR7	TCCAGTGTCTAAAGAACCTGG	TGGTAAATATACCACACATCCC
TLR8	TAATAGGCTGAAGCACATCCC	TCCCAGTAAAACAAATGGTGAG
TLR9	GTGCCCCACTTCTCCATG	GGCACAGTCATGATGTTGTTG
TLR10	TGACCACAATTCATTTGACTACTC	TTGAATACTTTTGGGCAAGCACC
CXCL9/Mig	TGGTGTTCTTTTCCTCTTGGGCA	GACGAGAACGTTGAGATTTTCGAA
CXCL10/IP-10	TCTAAGTGGCATTCAAGGAGTACC	CAGTAAATTCTTGATGGCCTTCGA
CXCL11/I-TAC	GCTATAGCCTTGGCTGTGATATTG	GATTTGGGATTTAGGCATCGTTGT
TNF-α	GGCTCCAGGCGGTGCTTG	CAGATAGATGGGCTCATACCA
IFN-α2	AGTCAAGCTGCTCTGTGGGC	GTGAGCTGGCATACGAATCA
IFN-β	GATTCATCTAGCACTGGCTGG	CTTCAGGTAATGCAGAATCC
β2μ	AAGATTCAGGTTTACTCACGTC	TGATGCTGCTTACATGTCTCG

Analysis of cell surface molecules expression

The expression of cell surface molecules by keratinocytes was determined using mouse anti-human monoclonal antibodies (mAbs) against the following surface molecules: ICAM-1 (15.2, IgG1; Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, CLB, Amsterdam, The Netherlands), HLA-DR (L234, IgG2a; Becton Dickinson, San Jose, CA), HLA-ABC (IgG2a; Halan Sera-Lab Ltd, Belton, UK), FasR/CD95 (ZB4, IgG1; Immunotech, Marseille, France) and CD40 (5D12, IgG1; ATCC, Manassas, VA). FITC-coupled goat $F(ab')_2$ anti-mouse IgG and IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as a secondary reagent. Samples were analyzed on a FACScan (Becton Dickinson).

Keratinocyte stimulation for cytokine/chemokine measurements

Keratinocytes were plated at the concentration of $0.1x10^4$ cells/200 µl (96-well plates, Costar), and cultured at least for 48 h before conducting experiments. To induce cytokine and chemokine production, keratinocytes were cultured for 48 h in the presence of PGN, or poly I:C, or LTA, or LPS, or flagellin, or zymosan A, or the CpG-ODN 2006 or CpG-ODN 2216 at the concentrations above mentioned.

Determination of cytokine and chemokine production by ELISA

Measurements of CXCL8/IL-8 and TNF-α were performed by ELISA using pairs of specific mAbs and recombinant standard obtained from BioSource International (Camarillo, CA). CCL2/MCP-1 was determined using the Ab pair, rat polyclonal 20521D for coating and rabbit polyclonal 20532D for detection and recombinant CCL2/MCP-1 19781T (BD Pharmingen, San Diego, CA). CCL20/MIP-3α was determined using a DuoSet Elisa purchased by R&D Systems. CXCL10/IP-10, CXCL9/Mig and CCL27/CTACK were determined using pairs of specific Abs and recombinant standard obtained from R&D Systems. The limits of detection of these ELISA are as follows: CXCL8/IL-8, 30 pg/ml; TNF-α, 20 pg/ml; CCL2/MCP-1, 40 pg/ml; CCL20/MIP-3α, 60 pg/ml; CXCL10/IP-10, 80 pg/ml; CXCL9/Mig, 40 pg/ml and CCL27/CTACK, 80 pg/ml.

Detection of NF-κB subunits (p50, and p65) expression by immunohistochemistry

Keratinocytes were plated at the concentration of $0.1x10^4$ cells/200 µl (Lab-Tek® chamber slides; Life Technologies), and cultured at least for 48 h before conducting experiments. To induce nuclear translocation of p50 and p65, keratinocytes were cultured for 4 h in the presence of poly I:C, or CpG-ODN 2006, or CpG-ODN-2216, at the concentrations stated above. After removal of the culture medium, the cells were fixed with acetone (-20°C) for 10 min. Blocking of non-specific staining was performed with 10 % human serum (Biowittaker, Walkersville, MD) in PBS, for 20 min., followed by an incubation (1 h at 37°C) with the following primary antibodies (diluted in 0.1% BSA plus 0.1% saponin (Sigma-Aldrich)): p50 (2 µg/ml, goat polyclonal G079, Santa Cruz Biotechnology, Santa Cruz, CA) and p65 (2 µg/ml, rabbit polyclonal E069, Santa Cruz Biotechnology). The cells then were washed with PBS containing 1% FCS and incubated (1 h at 37°C) with the following secondary antibodies (diluted in PBS containing 10% human serum): HRP-conjugated rabbit anti-goat (1:400,

Dakopatts, Glostrup, Denmark) or HRP-conjugated goat anti-rabbit (1:400, Nordic Immunological Laboratories, Tilburg, The Netherlands). After washing the cells with PBS, the peroxidase activity was revealed using 3-amino-9-ethylcarbazole substrate (AEC, Sigma). Slides were analysed with a wide field upright microscope (Leica DMRA, Wetzlar, Germany) coupled to a CCD camera and Image-Pro Plus software (Media Cybernetics, Dutch Vision Components, Breda, The Netherlands).

Statistical analysis

Data are expressed as mean ± SD. Data were analyzed for statistical significance with the GraphPad InStat® software (version 3.00; GraphPad InStat, Inc., San Diego, CA) using ANOVA followed by Dunnett's multiple comparisons test. A P value <0.05 was considered as the level of significance.

RESULTS

Human keratinocytes express TLR1, 2, 3, 4, 5, 6, 9 and 10, but not TLR7 and 8

TLRs play a central role in innate immunity by mediating recognition of PAMPs.[4] To gain insight into keratinocyte expression of TLRs, we examined the mRNA expression of all known TLRs (TLR1-TLR10) by RT-PCR. The purity of the keratinocytes was verified by the expression of the epithelial marker, cytokeratine, and the fibroblast-specific marker (ASO2) (data not shown), and was 99% (data not shown). As depicted on Fig. 1, keratinocytes constitutively express mRNA for TLR1, 2, 3, 4, 5, 6, 9, and 10, but not for TLR7 and 8.

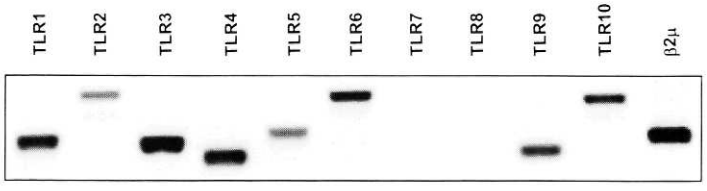

Figure 1. Detection of TLR mRNA by RT-PCR in cultured human keratinocytes. Human keratinocytes express TLR1, 2, 3, 4, 5, 6, 9, and 10, but not TLR7 and 8. Keratinocyte purity was verified by the expression of the epithelial cell marker, cytokeratine to be 99%. Keratinocytes were plated in 6-well plates and cultured for at least 48 h before the cells were lyzed. cDNAs were amplified for 45 cycles and were separated on a 1 % agarose gel containing ethidium bromide. The data shown are representative of four independent experiments.

The TLR ligands PGN, poly I:C, LPS and flagellin induce different patterns of surface molecule expression by keratinocytes

To study the effects of different TLR-ligands on the expression of surface molecules by keratinocytes, keratinocytes were cultured for 48 h in the absence or in the presence of TLR-ligands that were selected according to the documented TLR expression: PGN (TLR2), LTA (TLR2/4), poly I:C (TLR3), LPS (TLR4), flagellin (TLR5), zymosan A (TLR2/6), CpG-ODN 2006 or CpG-ODN 2216 (both TLR9). As shown in Fig. 2, poly I:C and flagellin were the only ligands that induced the expression of ICAM-1 and MHC class I molecules (HLA-ABC). In contrast, MHC class II (HLA-DR) expression was induced by PGN, poly I:C and LPS. None of the TLR ligands induced significant changes in FasR and CD40 expression, except for poly I:C that moderately, but not significantly, upregulated the expression of these two surface molecules. These results demonstrate that human keratinocytes are able to respond to selected TLR ligands by enhancing the expression of certain surface molecules.

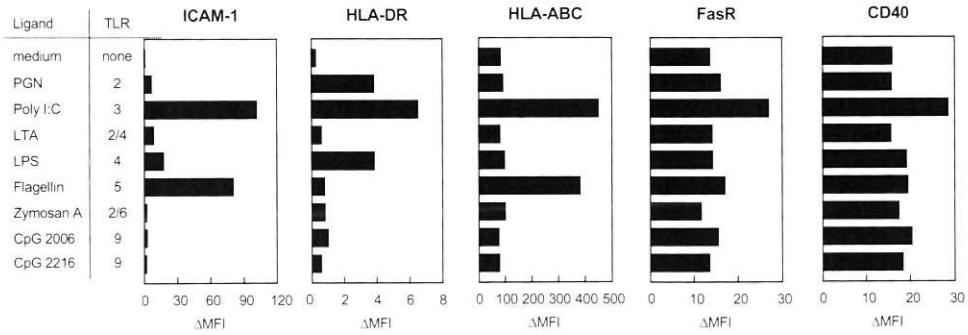

Figure 2. Keratinocytes modulate the expression of cell surface molecules in response to different stimuli. Keratinocytes were stained with antibodies to ICAM-1, HLA-DR, HLA-ABC, FasR or CD40, 48 h after exposure to the indicated TLR ligands or to medium alone, and analyzed by FACS. Results are expressed as mean of duplicate cultures. ΔMFI represents the difference between the various stainings and the isotype control. Data are representative of four experiments with similar results.

Human keratinocytes respond to different TLR ligands/PAMPs with differential chemokine production patterns

As the defense against different invading pathogens requires different types of immune responses, we questioned whether PAMPs that trigger different TLRs, induce different patterns of expression of chemokines that are associated with the attraction of different types of immune cells. To this aim, human keratinocytes were exposed for 48 h to different TLR ligands/PAMPs and the contents of different chemokines in culture supernatants was

analyzed using specific ELISAs. As shown in Fig. 3, all the TLR ligands, except the TLR9 ligands CpG-ODN 2006 and 2216, induced significantly large amounts of CXCL8/IL-8. The same was observed for CCL2/MCP-1 production except for the fact that this chemokine was also not produced in response to the TLR2/4 ligand LTA. CCL20/MIP-3α was induced exclusively by TLR3 ligand poly I:C, TLR5 ligand flagellin and TLR2/6 ligand zymosan A. CCL27/CTACK production was exclusively induced by poly I:C and flagellin. Altogether, these data demonstrate that human keratinocytes respond to different TLR ligands/PAMPs by showing different patterns of chemokines that are associated with the attraction of different immune cells.

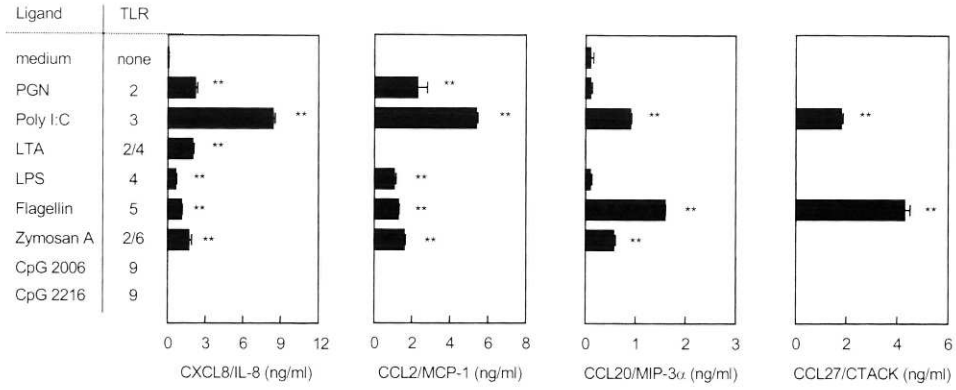

Figure 3. CXCL8/IL-8, CCL2/MCP-1, CCL20/MIP-3α, and CCL27/CTACK production by keratinocytes stimulated with microbial molecules that trigger different TLR signalling. Keratinocytes were cultured with PGN (100 μg/ml), poly I:C (50 μg/ml), LTA (100 μg/ml), LPS (100 μg/ml), flagellin (100 pg/ml), zymosan A (100 μg/ml), CpG-ODN 2006 or 2216 (5 μM), for 48 h, and the concentrations of chemokines in the cell-free supernatants were measured with specific ELISA. Results, expressed as mean ± SD of triplicate cultures are from one experiment representative of four. Data were analysed for statistical significance using ANOVA followed by Dunnett's multiple comparisons test. * $P<0.01$, ** $P<0.001$.

The expression of the Th1-attracting chemokines CXCL9/Mig, CXCL10/IP-10 and CXCL11/I-TAC is exclusively induced by the TLR3 ligand poly I:C

The selective recruitment of T helper (Th) cell subsets to an inflammatory site contributes strongly to the class of the specific immune responses. Therefore, we questioned whether the activation of human keratinocytes by the different TLR ligands would result in differential patterns of Th type 1 (Th1)- (CXCL9/Mig, CXCL10/IP-10 and CXCL11/I-TAC) or Th2- (CCL22/MDC, CCL17/TARC) -associated chemokine production.[31] We could not detect CCL22/MDC and CCL17/TARC mRNA expression by human keratinocytes, in response to

the TLR ligands tested (data not shown). In addition, the levels of CCL22/MDC and CCL17/TARC were below the detection limit of the ELISAs (data not shown), as previously reported.[42] Interestingly, only the TLR3 ligand poly I:C induced production (Fig. 4A) and expression (Fig. 4B) of the Th1-associated chemokines CXCL9/Mig, CXCL10/IP-10 and CXCL11/I-TAC.

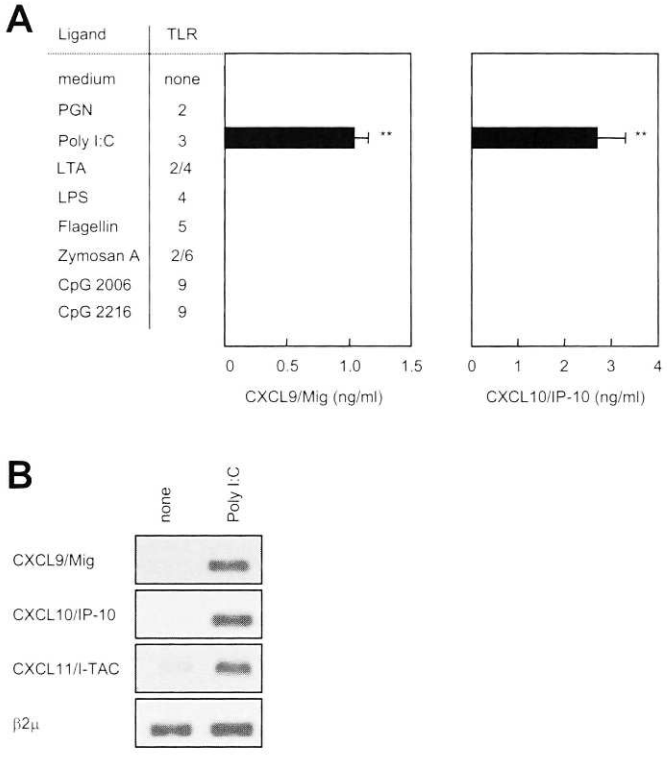

Figure 4. The TLR3 ligand poly I:C selectively induces the production and expression of Th1-attracting chemokines. (A) Keratinocytes were cultured for 48 h with PGN, poly I:C, LTA, LPS, flagellin, zymosan A or CpG-ODN 2006 or 2216 and the concentrations of chemokines in the cell-free supernatants were measured with specific ELISAs. Results, expressed as mean ± SD of triplicate cultures are from one experiment representative of four. Data were analysed for statistical significance using ANOVA followed by Dunnett's multiple comparisons test. * $P<0.01$, ** $P<0.001$. (B) Keratinocytes were cultured for 4 h with the stimuli stated above, and the cells lyzed for RT-PCR analysis. The data shown are representative of four experiments.

Cytokine release by TLR-triggered keratinocytes

Several bacterial compounds induce in keratinocytes the release of the proinflammatory cytokine TNF-α, which contribute to local inflammatory reactions within the epidermis.[43] Therefore, we analyzed the TNF-α production by ELISA in the supernatants of keratinocytes

that were stimulated in the absence or in the presence of the different TLR ligands. TNF-α was only found in the supernatants of poly I:C- and flagellin-stimulated keratinocytes (Fig. 5A).

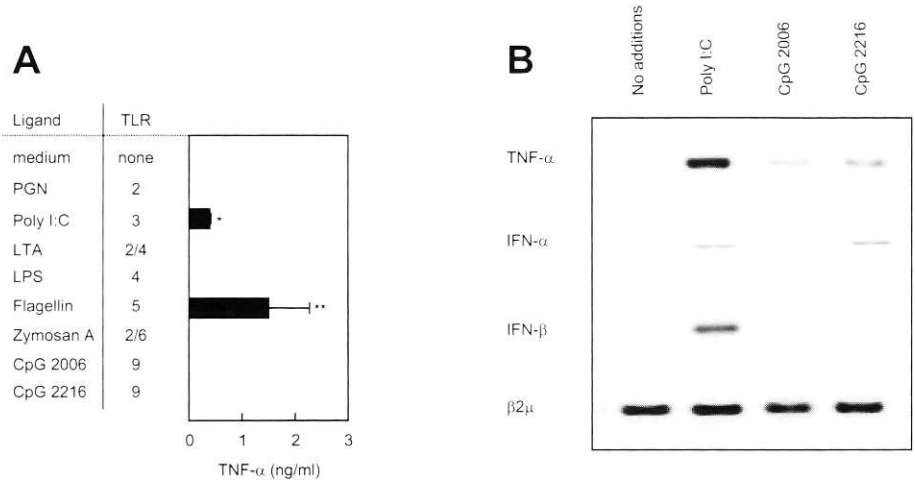

Figure 5. Cytokine release by TLR-triggered human keratinocytes. (A) TNF-α production by keratinocytes is selectively induced by the TLR3 ligand poly I:C and TLR5 ligand flagellin. Keratinocytes were cultured for 48 h with the stimuli stated above, and the concentrations of TNF-α in the cell-free supernatants were measured with a specific ELISA. Results, expressed as mean ± SD of triplicate cultures are from one experiment representative of four. Data were analysed for statistical significance using ANOVA followed by Dunnett's multiple comparisons test. * $P<0.01$, ** $P<0.001$. (B) CpG-ODN 2006 induces TNF-α mRNA expression while CpG-ODN 2216 induces both TNF-α and IFN-α mRNA expression. Poly I:C induces both TNF-α, IFN-α, and IFN-β mRNA expression by keratinocytes Keratinocytes were cultured for 4 h with the stimuli stated above, and the cells lyzed for RT-PCR analysis. The data shown are representative of four experiments.

Recent reports indicate that in humans TLR9 is primarily expressed in plasmacytoid DC[44] and B cells.[40] We showed for the first time that human keratinocytes constitutively express TLR9 mRNA (Fig. 1). However, culture of keratinocytes in the presence of the TLR9 ligands, CpG-ODN 2006 or 2216 did not result in the production of any of the chemokines and/or cytokines when tested by ELISA. Therefore, we investigated whether these two CpG-ODNs were able to induce keratinocyte mRNA expression for TNF-α, IFN-α, and IFN-β, by RT-PCR. As positive control, the TLR3 ligand poly I:C, was used. Indeed, both TLR9 ligands CpG-ODN 2006 and 2216 induced the expression of TNF-α mRNA in 4 h-stimulated keratinocytes (Fig. 5B), indicating that keratinocytes are able to respond to CpG motifs, consistent with the presence of TLR9. Since CpG-ODN 2216 is known to induce the expression of type I IFNs, IFN-α and IFN-β in plasmacytoid DC,[41] we investigated whether

these cytokines are also expressed by keratinocytes upon activation with CpG-ODNs. Type I IFNs were undetectable by standard ELISA in all groups (data not shown). However, RT-PCR analysis readily revealed mRNA expression for IFN-α and IFN-β, only in CpG-ODN 2216-activated keratinocytes. Poly I:C also induced expression of mRNA for TNF-α, IFN-α and IFN-β (Fig. 5B).

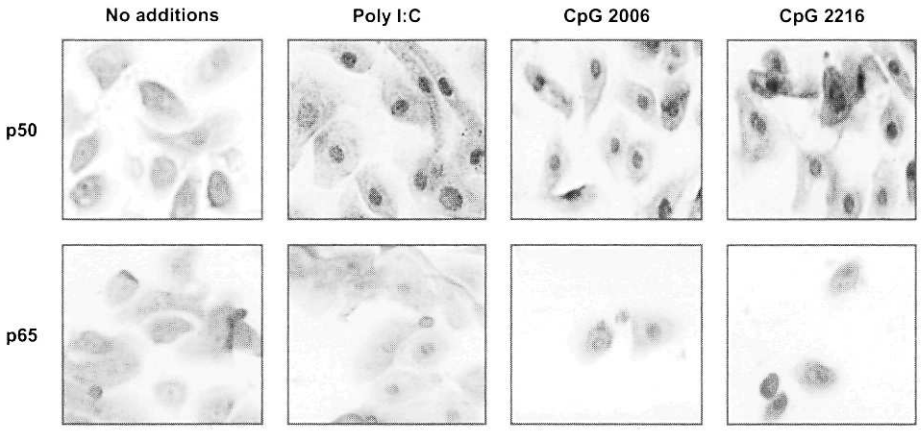

Figure 6. Detection of NF-κB expression in poly I:C-, or CpG-ODN 2006-, or CpG-ODN 2216-stimulated keratinocytes by immunohistochemistry. The NF-κB subunits, p50 and p65 immunoreactivity in unstimulated keratinocytes was primarily in the cytoplasm, whereas after stimulation with the different microbial compounds a more pronounced nuclear immunoreactivity was observed (original magnification 100x). The data shown are representative of four experiments. (☞ page 162).

Activation of TLR9 leads to nuclear translocation of the NF-κB subunits p50 and p65

To further substantiate the activation of keratinocytes by CpG-ODN, we have tested whether these compounds activate transcription factors of the NF-κB family. The NF-κB family of transcription factors play an important role in regulating inflammatory and immune-response genes[7]. TLR activate NF-κB and induce the expression of a variety of immune defense genes.[6] To examine the molecular mechanisms of cytokine production in CpG-ODN-activated human keratinocytes, these cells were cultured in slide-chambers in the presence or in the absence of poly I:C, CpG-ODN 2006 or CpG-ODN 2216. After 4 h the cells were washed, fixed in acetone and stained for the following NF-κB subunits p50 and p65, using Abs as described in Material and Methods. Fig. 6 shows that in unstimulated keratinocytes the staining for the all the NF-κB subunits are mainly visible in the cytoplasm. In contrast,

when keratinocytes are stimulated with poly I:C or the CpG-ODN 2006 or 2216, a clear nuclear translocation of p50 and p65 subunits is observed, indicating that these NF-κB subunits participates in TLR9 signalling in response to CpG-ODNs.

DISCUSSION

Here we have shown for the first time that human keratinocytes constitutively express mRNA for TLR1, 2, 3, 5, 6, 9, and 10, but not for TLR7 and 8. Activation of keratinocytes with different PAMPs resulted in differential expression patterns of cell surface molecules, chemokines and cytokines. These data indicate that signals emanating from different TLRs are not equivalent and that these PRRs may differentially contribute to the innate immunity.

A major challenge to innate immune cells is the discrimination of foreign pathogens. Innate immune cells possess germline-encoded PRRs that recognize and are triggered by evolutionary conserved molecules essential to pathogen function but absent in the host.[4,5] The activation of TLRs by innate immune cells has been extensively studied in monocytes, in macrophages and in different subsets of DC.[4,44] Recently, it was reported that non-immune cells also express TLRs. In particular, tracheobronchial epithelial cells express TLR1, 2, 3, 4, 5, and 6,[45] intestinal epithelial cell lines express TLR2, 3, and 4,[46,47] and renal tubular epithelial cells express TLR1, 2, 3, 4, and 6, but not TLR5 or 9.[48] These findings also indicate that the expression of TLRs is tissue-specific. Thus far, in keratinocytes only the expression of TLR4 has been documented.[39] Here we show that, in addition to TLR4, human keratinocytes also express mRNA for TLR1, 2, 3, 5, 6, 9, and 10, but not for TLR7 and 8.

Stimulation of keratinocytes with the different TLR ligands resulted in differential patterns of cell surface molecules expression. These data suggest that by expressing different TLRs, human keratinocytes can discriminate different PAMPs. Ligation of these TLR may evoke different signaling cascades, subsequently, to a particular pattern of surface molecule expression. The expression of ICAM-1, HLA-DR and CD40 is of particular importance since these molecules participate in the contact between skin infiltrating T cells and keratinocytes. This contact may result in the triggering of the release of a second wave of chemokines and cytokines by both T cells and keratinocytes, which will further amplify the inflammatory response contributing to efficient elimination of the pathogen. In addition, MHC class I-expressing keratinocytes that have been activated with the TLR3 ligand poly I:C or the TLR5 ligand flagellin may be target for lysis by $CD8^+$ cytotoxic T cells (CTL), which recognize microbial antigens expressed on the epithelial cell surface. This is particularly efficient in

case of poly I:C, since dsRNA-bearing pathogens are viruses (that are inside cell cytoplasm).[49] In case of flagellin this is less clear, however this widely and conserved PAMP is present in many intracellular bacteria.[50] The fact that FasR expression by keratinocytes was selectively induced by poly I:C may not be surprising since this molecule is necessary for the Fas-mediated apoptosis of virally-infected cells by CTLs.[51]

Keratinocytes secrete various soluble mediators that orchestrate the immune response.[1,2] Thus, chemokines released from keratinocytes may determine whether and which types of immune cells are attracted to the skin epithelium. We found that all the TLR ligands, except for the TLR9 ligands CpG-ODN 2006 and 2216, induced significantly high levels of CXCL8/IL-8. The neutrophil is the major cell type accumulating early in subcutaneous tissue in response to inflammatory signals induced by various pathogen types.[52,32] Therefore, the migration of these cells to sites of pathogenic exposure is considered as a key event in the immune response.[32] The current data indicate that CXCL8/IL-8 production is driven by ligands of most TLRs, i.e. TLR2, 3, 4, 5 and 6. The induction of CCL2/MCP-1 production by keratinocytes showed an almost similar pattern as the induction of CXCL8/IL-8 production. However, CCL2/MCP-1 could also not be induced by TLR2/4 ligand LTA, in contrast to the other cell-wall component from Gram-positive bacteria, PGN, which did induce CCL2/MCP-1. The broad induction profile of CCL2/MCP-1 expression will promote the immigration of monocytes,[33] DC and LC,[27] memory T cells[34] and natural killer cells[35] into the skin in case of most pathogen infections. Optimal LTA recognition requires the expression of both TLR2 and 4.[9] The observation that LTA does not induce CCL2/MCP-1 whereas TLR2 or TLR4 ligands do, may be due to differences in the function of TLR2 as the PGN and LTA receptor associated with differential intracellular signal transduction pathways by PGN and LTA,[53] or with an exclusive function of TLR2 as an PGN receptor, as proposed recently.[9]

The recruitment of immature DC into the epidermis is a key step in the development of specific immunity. It was recently reported that keratinocyte-derived CCL20/MIP-3α is important not only in recruiting precursors of LC to the epidermis,[28] but also memory T cells.[37] The expression of CCL20/MIP-3α by keratinocytes was observed previously in response to the proinflammatory cytokines TNF-α and IL-1β.[54] Our data demonstrate that this chemokine can also be release by keratinocytes in response to the TLR3 ligand poly I:C, the TLR5 ligand flagellin, and the TLR2/6 ligand zymosan A. In addition, our data confirm the specificity of flagellin as one of the major bacterial PAMPs that induces the production of CCL20/MIP-3α by epithelial cells, in contrast to LPS.[55] The migration of LC could be

essential for the uptake of the pathogens followed by antigen processing and presentation necessary for the induction of specific immune responses in the skin.

CLA identifies a skin-associated population of memory T cells that preferentially extravasate and migrate into normal or inflamed skin.[56] CCL27/CTACK is a chemokine that is skin-restricted and has an important role in both skin homeostasis and the initiation of skin inflammation.[29,57] We show that CCL27/CTACK production by keratinocytes was selectively induced by the TLR3 ligand poly I:C and the TLR5 ligand flagellin. $CCR10^{+}CLA^{+}$ T cells that will be attracted to the skin by keratinocyte-derived CCL27/CTACK, in response to poly I:C or flagellin, may encounter their specific antigen and release effector mediators, which induce more and different chemokines to sustain a state of inflammation that finally leads to efficient clearance of the virus or bacteria, respectively.

Effector T cells are characterized by their responsiveness to numerous inflammatory chemokines that are produced at the site of pathogen entry and tissue injury.[22] The selective recruitment of T helper (Th) cell subsets to an inflammatory site is of particular importance in determining the character of the immune responses. CXCL9/Mig, CXCL10/IP-10 and CXCL11/I-TAC are CXCR3 ligands that attract mainly activated Th1 cells. In the skin these chemokines are mainly produced by keratinocytes.[24] Recently, we have reported that poly I:C-activated promote Th1 responses by inducing a type-1 polarized phenotype in DC, DC1 (M.C. Lebre *et al.*, in press). Here we show that poly I:C further promotes local Th1 responses by inducing the expression of Th1-attracting chemokines in keratinocytes. The experiments with poly I:C as model for virus infection, thus suggest that keratinocytes may be important players in cutaneous anti-viral responses by participating in both initiation phase of the immune response, by inducing the DC1 phenotype and, in the effector phase, by attracting polarized Th1 cells to the site of virus entry. When these Th1 cells interact with keratinocytes via surface molecules[58] and cytokine release,[42] keratinocytes will become an important source of chemotactic factors that direct the recruitment of additional specific leukocyte populations and thus regulate the quality, magnitude and duration of the inflammatory response.[42]

The release of TNF-α protein by keratinocytes was exclusively induced by poly I:C and flagellin, viral and bacterial PAMP, respectively. Our findings are in line with previous studies that demonstrated that this cytokine is required for the host defense against intracellular pathogens such as viruses and bacteria.[59,43] Moreover, TNF-α is the main

proinflammatory mediator produced by several cell types in response to dsRNA, poly I:C (M.C. Lebre *et al.*, *in press*) or flagellin.[60]

The immune system uses TLR9 to detect the presence of unmethylated CpG motifs.[18] In humans, the expression of TLR9 is thought to be restricted to B cells and plasmacytoid DC.[40,44] Here we show for the first time that TLR9 is also expressed by human keratinocytes. Remarkably, the TLR9 ligands CpG-ODN 2006 or 2216 did not induce detectable protein levels of any of the chemokines and/or cytokines tested. However, mRNA analysis revealed that CpG-ODN 2006-activated keratinocytes show upregulated mRNA expression for TNF-α, whereas CpG-ODN 2116-activated keratinocytes have both upregulated TNF-α and IFN-α mRNA, which is consistent with the type I IFN-inducing capacity of this CpG motif.[44] Compared to TNF-α,[1,2] type I IFNs are rapidly expressed by keratinocytes[61] and participates in the innate immune response both as a signal for the presence of bacterial and viral infections and as an effector molecule inhibiting the spread of infection.[62] In addition, our data indicate that TLR9 present in human keratinocytes is functional.

TLR activation results in activation of NF-κB[6] that induces a genetic program that is essential for host defense, including the induction of a subset of surface molecules expression, cytokines and chemokines.[7] NF-κB is present in the cytoplasm as an inactive complex, which is rapidly translocated into the nucleus upon stimulation.[7] To confirm that TLR9 expression by human keratinocytes is functional, we investigate whether its ligation result in NF-κB translocation. Indeed, we demonstrate that the NF-κB subunits p50 and p65 are translocated to the nucleus upon keratinocyte activation with the TLR9 ligands, CpG-ODN 2006 and 2216, indicating that signaling via these receptors led to NF-κB activation. These findings support the notion that NF-κB is one of the most important cellular factors involved in the regulation of the host innate antimicrobial response.[7] Moreover, these data point to an also important role of NF-κB in epidermal biology.

The early expression of certain cell surface molecules, chemokines and cytokines is essential in shaping the innate and specific immune responses. The present study strongly suggests that keratinocytes are not merely a barrier but actively contribute to the induction of the immune response through the differential activation of TLRs resulting in differential patterns of expression of genes involved in the inflammatory responses.

ACKNOWLEDGMENTS

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CHAPTER 3

Double-stranded RNA-exposed human keratinocytes promote Th1 responses by inducing a type-1 polarized phenotype in dendritic cells. Role of keratinocyte-derived TNF-α, type I IFNs and IL-18

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ABSTRACT

Dendritic cells (DC) play a key role in establishing the class of immune response against invading pathogens. Upon engagement with double-stranded RNA (dsRNA), a major bioactive constituent of many virus types, immature DC (iDC) develop into type 1 immunostimulatory DC (DC1) that promote Th1 responses. Immature DC reside in the epithelia and are in close contact with keratinocytes. We studied to what extent DC can also adopt a DC1 phenotype indirectly, as a result of the interaction with keratinocytes responding to dsRNA. In contrast to supernatants from keratinocytes activated by the combination of TNF-α and IL-1β, supernatants from keratinocytes activated by synthetic dsRNA, polyriboinosinic polyribocytidylic acid (poly I:C) comprised TNF-α and type I IFNs, which induced maturation of human monocyte-derived iDC. In addition, DC matured in the presence of these supernatants strongly biased the development of Th1 cells from naive Th cells. This bias was dependent on keratinocyte-derived IFN-α/β and IL-18, since neutralization of both IFN-α/β and IL-18 in the keratinocyte culture supernatant reduced the development of IFN-γ-producing Th cells. These findings suggest that keratinocytes can contribute to the development of selective Th1/Th2 responses through the induction of maturation and functional polarization of DC, indicating a novel role for keratinocytes as initiators and regulators of cutaneous T cell-mediated inflammation. In addition, these results support the concept that, in addition to direct interaction with pathogens, DC may also be activated and primed by pathogen indirectly, via the effect of resident tissue cells responding to pathogen.

INTRODUCTION

The skin is the primary interface between the body and the environment and provides the first line of defense against microbial and chemical agents. The most damaging consequence of the disruption of skin is invasion by pathogenic microorganisms.[1] Epidermal epithelial cells, keratinocytes, represent the major constituent of the skin and participate actively in the skin immune system by producing, constitutively or upon stimulation, various soluble mediators, such as cytokines, chemokines, eicosanoids and growth factors.[2,3] For instance, several bacterial and viral compounds induce in keratinocytes the release of the proinflammatory cytokine TNF-α, which contribute to local inflammatory reactions within the epidermis.[4,5]

Specific skin inflammation, such as infections, contact hypersensitivity and skin graft rejection, is regulated by a complex and sequential mechanism involving dendritic cells (DC),

keratinocytes and T cells, and a variety of soluble mediators they produce.[6,7] Immature dendritic cells (iDC) are located in peripheral tissues (e.g., epidermal Langerhans cells and dermal DC) in close contact with keratinocytes. DC are the only antigen-presenting cells that can prime naive Th cells and initiate immune responses. After exposure to antigens, activated DC migrate from peripheral tissues to T-cell areas of the draining lymph nodes. During this migration, iDC undergo maturation from cells that have the capacity to take up antigen into potent immunostimulatory effector cells.[8,9]

DC maturation can be induced in vitro by several factors including the combination of the proinflammatory cytokines IL-1β and TNF-α, type I IFNs, various microbial compounds, or CD40L.[10-15] During their maturation, DC may also gain the capacity to polarize naive Th cells into Th1 or Th2 cells. This ability is determined in peripheral tissues by the type of invading pathogen or their bioactive compounds, and is established either upon the direct activation of iDC by pathogen,[16-18] or upon the indirect activation by mediators of the local inflammatory response of tissue cells in response to pathogen.[19,20]

Double-stranded RNA (dsDNA) is an intermediate within the replication cycle for RNA viruses and some DNA viruses, and is their major bioactive component.[21,22] Polyriboinosinic polyribocytidylic acid (poly I:C) is a synthetic dsRNA that is often used as a model of viral infection.

Considering the fact that iDC reside in the close vicinity of keratinocytes, these cells may play an important role in the activation and polarization into Th1 (DC1)- or Th2 (DC2)-promoting effector DC. This is corroborated by the study of Pastore *et al.*[23] in which it was demonstrated that GM-CSF produced by keratinocytes enhanced the survival of DC. Therefore, we studied whether and how factors produced by keratinocytes, upon activation by the proinflammatory cytokines TNF-α/IL-1β or by a mimic for a virus infection, activate and/or polarize DC.

MATERIALS AND METHODS

Culture of human keratinocytes

Primary cultures of normal human keratinocytes were prepared from neonatal foreskins or adult skin undergoing plastic surgery. The skin was incubated with thermolysin (500 μg/ml; Sigma-Aldrich, St. Louis, MO) for 16 h at 4°C. Epidermal sheets were removed from the dermis and single cell suspensions were obtained by placing epidermal sheets in trypsin

(0.025%; Life Technologies, Paisley, U.K.) for 5 min at 37°C. After neutralizing with equal volume of FCS, (HyClone, Logan, UT) stratum corneum debris was removed and then sieved through sterile nylon gauze to obtain a single cell suspension. Isolated epidermal cells were seeded at the density of 8-10x10^4 cells/cm^2. At 70-80% confluence, keratinocytes were detached with 0.025% trypsin, 2 mM EDTA for 5 min at 37°C, and subcultured or frozen. Keratinocyte cultures were maintained in Keratinocyte SFM medium (Gibco, Paisley, UK).

Stimulation of human keratinocytes

Human keratinocytes were plated at the concentration of 2.5x10^4 cells/ml (6-well plates, Costar, Cambridge, MA) and after 48 h the cells were pulsed for 2 h with poly I:C (200 µg/ml; Sigma-Aldrich). Keratinocytes were then washed extensively (5 times in 5 ml of PBS, i.e. > 1x10^6 times diluted), fresh medium without hydrocortisone (5 ml) was added and the cells were cultured for additional 46 h. Keratinocytes were also pulsed with the proinflammatory cytokines recombinant human (rhu)TNF-α (50 ng/ml, PBH, Hannover, Germany) and rhuIL-1β (100 ng/ml; PBH). The culture supernatants were collected after the indicate time, centrifuged to remove cells and store at 4°C before use or stored at -20°C for cytokine measurements. The concentrations of IL-8, TNF-α, IFN-α and IL-18 in 46 h supernatants were determined by ELISA (see below). Keratinocytes were also stimulated for 4 h with the stimuli stated above, the cells were lyzed for examination of IFN-α and IFN-β gene expression by RT-PCR (see below).

RNA isolation and cDNA synthesis

Keratinocyte total RNA was purified by using the NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany) according to manufacture's instructions. Complementary DNA was generated using the First strand cDNA synthesis kit for RT-PCR (MBI Fermentas, St Leon-Rot, Germany). To anneal the primer to the RNA, 9 µl of total RNA, 1 µl oligo$(dT)_{18}$ and 1 µl $D(N)_6$ were added. This mix was then heated for 5 minutes at 94°C.

PCR analysis

The primers sequences were as follows: IFN-α2: sense, 5'-AGTCAAGCTGCTCTGTGGGC-3'; anti-sense, 5'GTGAGCTGGCATACGAATCA-3', defining a 571-bp product; HPLC-treated IFN-β: sense, 5'-GATTCATCTAGCACTGGCTGG-3'; anti-sense, 5'-

CTTCAGGTAATGCAGAATCC-3', defining a 186-bp product. Both primers have an annealing temperature of 60 °C and 45 cycles. PCR products were analyzed on a 1% agarose gel containing ethidium bromide. A 100 bp DNA ladder standard (MBI Fermentas) was used as a size marker.

Generation of immature DC from peripheral blood monocytes

Immature DC were generated from monocytes in cultures performed in Iscove's modified Dulbeccos's medium (IMDM; Life Technologies) containing 1% FCS (HyClone), rhuGM-CSF (500 U/ml; Schering-Plough, Uden, The Netherlands) and rhuIL-4 (250 U/ml; PBH), as described .[24,25]

Effect of keratinocyte supernatants on DC

In order to study the effect of keratinocyte-derived soluble mediators on DC, supernatants from non- or TNF-α/IL-1β- or poly I:C-activated keratinocytes were added to iDC at a concentration of 50% (v/v) of the culture. As controls, iDC were exposed to keratinocyte culture medium or LPS plus TNF-α+IL-1β (LPS+MF, maturation factors). After 48 h, DC were harvested and analyzed for their maturation status by cell surface expression determined by FACS, their cytokine production upon CD40 ligation, their T cell stimulatory capacity, and their capacity to induce the development of Th1 or Th2 cell responses in naive precursors. When indicated, the following were added to keratinocyte supernatants before addition to iDC: neutralizing antibodies against TNF-α (10 μg/ml, IgG1; Diaclone Research, Besançon, France), or two neutralizing sheep antisera to human type I IFN were used: Iivari (450.000 neutralizing U/ml anti-IFN-α + 3.000 U/ml anti-IFN-β) and Kaalepi (30.000 U/ml anti-IFN-α + 30.000 U/ml anti-IFN-β)[26] or IL-18 binding protein (IL-18bp; 10 μg/ml, Amgen, Thousand Oaks, CA). The following antibodies or serum were used as negative control Ig: sheep serum (Sigma-Aldrich) and mouse IgG1 (10 μg/ml, MOPC-21; Sigma-Aldrich).

Analysis of cell surface expression

The expression of cell surface molecules associated with maturation of DC was determined using mAb against the following surface markers: CD1b (IgG1; Diaclone Research), CD86 (BD Pharmingen, San Diego, CA), CD80 (Pharmingen) CD83 (HB15a, IgG2b; Immunotech, Marseille, France). FITC-coupled goat $F(ab')_2$ anti-mouse IgG and IgM (Jackson

ImmunoResearch Laboratories, West Grove, PA) was used as a secondary reagent. Samples were analyzed on a FACScan (Becton Dickinson).

IL-12 p70 production by immature DC

Immature DC were washed and stimulated ($2x10^4$ cells in 200 µl) in 96-well flat-bottom culture plates (Costar) in IMDM containing 10% FCS with either LPS (250 ng/ml; Sigma-Aldrich) and IFN-γ (10^3 U/ml) or with irradiated (2500 Gy) CD40L-transfected J558 plasmacytoma cells (J558-CD40L, $2x10^4$ cells/well; a gift of Dr. P. Lane, Birmingham, UK) in the absence or in the presence of 50% (v/v) keratinocyte supernatants. Supernatants were harvested after 24 h, and the concentrations of IL-12p70 were measured by ELISA (see below).

IL-12 p70 production by DC matured in the presence of keratinocyte supernatants

As IL-12 production by DC is dependent on their maturation status,[27] DC were matured by the addition of keratinocyte supernatants (50% v/v) in the presence of a combination of rhuIL-1β (10 ng/ml; PBH), rhuTNF-α (50 ng/ml; PBH) (maturation factors, MF) and LPS (250 ng/ml; Sigma-Aldrich) to iDC. As a control iDC were mature in the presence of IL-1β plus TNF-α plus LPS. On day 8, DC were washed and $2x10^4$ cells/well were stimulated in 96-well flat-bottom culture plates (Costar) in IMDM containing 10% FCS in a final volume of 200 µl with irradiated (2500 Gy) CD40L-transfected J558 cells ($2x10^4$ cells/well), which were previously shown to induce IL-12p70 in an IFN-γ-independent way.[14] Supernatants were harvested after 24 h, and the concentrations of IL-12p70 were measured by ELISA.

Isolation of $CD4^+CD45RA^+CD4RO^-$ naive Th cells (ThN)

ThN were isolated from peripheral blood leukocytes with the negative selection human $CD4^+/CD45RO^+$ column kit (R&D Systems, Minneapolis, MN). This method yielded highly purified (>98%) $CD4^+CD45RA^+CD45RO^-$ ThN as assessed by flow cytometry (data not shown).

Mixed lymphocyte reaction (MLR)

Mature DC where also tested for their ability to stimulate allogeneic ThN in a MLR. ThN ($2.5x10^4$ cells/200 µl) were cocultured in 96-well flat-bottom culture plates with different

concentrations of mature DC. After 5 days, cell proliferation was assessed by the incorporation of [^{3}H]thymidine ([^{3}H]-TdR, Radiochemical Centre, Amersham, Little Chalfont, U.K.) after a pulse with 13 KBq/well during the last 16 h, as measured by liquid scintillation spectroscopy.

Induction of Th1 or Th2 cell responses by mature DC

ThN ($2x10^4$ cells/200 µl) were cocultured in 96-well flat-bottom culture plates with heterologous DC ($0.5x10^4$ cells/200 µl) matured in the different conditions stated above and coated with the superantigen *Staphylococcus aureus* enterotoxin B (SEB, 100 pg/ml; Sigma-Aldrich). On day 5, IL-2 (10 U/ml; Cetus, Emeryville, CA) was added and the cultures were further expanded. After 14 days, resting memory Th cells were harvested, washed, and restimulated for 6 h with PMA (10 ng/ml; Sigma-Aldrich) and ionomycin (1 µg/ml; Sigma-Aldrich) in the presence of brefeldin A (10 µg/ml; Sigma) in order to analyze Th cell cytokine production profile at the single cell basis. The cells were fixed in parafolmadehyde (2%; Sigma-Aldrich), permeabilized with saponin (0.5%; Sigma-Aldrich), and labeled with FITC-coupled IFN-γ mAb (Becton Dickinson) and PE-coupled IL-4 mAb (Becton Dickinson). The cells were evaluated by FACScan (Becton Dickinson). When indicated, DC were co-cultured with naive T cells in the presence of antibodies against IL-12 (10 µg/ml, rabbit polyclonal; a gift of Dr. P. van der Meide, U-CyTech, Utrecht, The Netherlands).

Evaluation of cytokine production by ELISA

Determination of IL-12p70 concentrations in culture supernatants was performed by specific solid-phase sandwich ELISA as previously described.[25] To determine IL-8, TNF-α and IFN-α in culture supernatants, pairs of specific monoclonal antibodies and recombinant cytokine standards were obtained from BioSource International (Camarillo, CA). The detection limits of these ELISA are as follows: IL-8, 30 pg/ml, TNF-α, 20 pg/ml, IFN-α, 100 pg/ml, IL-12p70, 3 pg/ml. To determine IL-18 in culture supernatants, an IL-18 ELISA kit was obtained from Diaclone Research (detection limit 62,5 pg/ml).

Statistical analysis

Data are expressed as mean ± SD. Data were analyzed for statistical significance with the GraphPad InStat® software (version 3.00; GraphPad InStat, Inc., San Diego, CA) using

ANOVA followed by Dunnett's multiple comparisons test. A P value <0.05 was considered as the level of significance.

RESULTS

Poly I:C-activated, but not TNF-α/IL-1β-activated, keratinocytes induce DC maturation: role of TNF-α and type I IFNs

To evaluate whether keratinocyte-derived products can contribute to DC maturation, iDC were exposed for 48 h to supernatants (50% v/v) from keratinocytes that were pulsed for 2 h with poly I:C or TNF-α/IL-1β and cultured for further 46 h. The purity of the keratinocytes, as verified by the expression of the epithelial marker cytokeratine, and the fibroblast-specific marker (ASO2) was more than 99% (data not shown). DC maturation was monitored by testing for the induction of cell surface expression of CD83 and for the upregulation of expression of the costimulatory molecules CD80 and CD86. In a comparative assay with iDC matured in control conditions with the combination of LPS and maturation factors (MF, consisting of TNF-α and IL-1β), we found that exposure of iDC to either supernatants from non-activated or TNF-α/IL-1β-activated keratinocytes did not induce their maturation (Fig. 1). This immature phenotype was similar to the phenotype of cultured iDC, left untreated or treated with keratinocyte medium. In contrast, poly I:C-activated keratinocyte supernatants induced DC maturation in the majority of DC, as evident from the induction of CD83 expression, accompanied by upregulation of the costimulatory molecules CD80 and CD86 (Fig. 1).

Next, we questioned which factor(s) exclusively produced by poly I:C-activated keratinocytes are responsible for the DC maturation. Since TNF-α[28] and type I IFNs[2] can be readily expressed by keratinocytes and these soluble factors can induce DC maturation,[10-12] the role of TNF-α and type I IFNs in keratinocyte-induced DC phenotypical changes was evaluated by adding neutralizing antibodies, or their respective control Ig or serum, to keratinocyte supernatants before their addition to the DC cultures. Surprisingly, neutralization of either of these soluble factors strongly reduced the induction of CD83, CD80 and CD86 expression by DC (Fig. 1), suggesting that both TNF-α and IFN-α/β produced by poly I:C-activated keratinocytes are involved in the induction of DC maturation. Addition of both antibodies against TNF-α and IFN-α/β to keratinocyte supernatants did not result in further

inhibition of CD83, CD80 and CD86 expression by DC treated with poly I:C-activated keratinocyte supernatants.

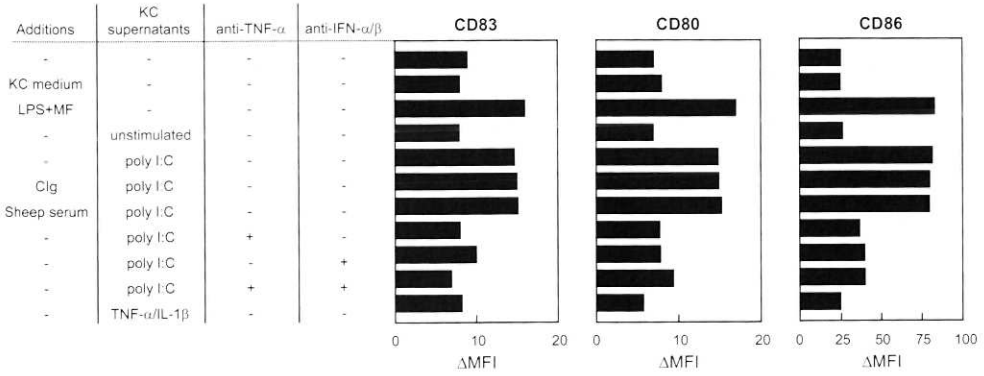

Figure 1. Supernatants from poly I:C-activated, and not TNF-α/IL-1β-activated, keratinocytes induce DC maturation. Immature DC were cultured for 48 h in the presence of 50 % (v/v) supernatants from non-activated, poly I:C- or TNF-α/IL-1β-activated keratinocytes and assayed for maturation by determining the expression of CD83, CD80 and CD86 by FACS analysis. As a control, DC were matured in the presence of the combination of LPS and the maturation factors TNF-α and IL-1β (MF). In contrast to no additions, keratinocyte medium, non-activated or TNF-α/IL-1β-activated keratinocyte supernatants, exposure of iDC to poly I:C-activated keratinocyte supernatants enhanced the expression of CD83, CD80 and CD86 to levels comparable to control DC. The role of TNF-α and IFN-α/β in DC maturation was tested by adding neutralizing antibodies to TNF-α (10 μg/ml) or IFN-α/β (two neutralizing sheep antisera to human type I IFN were used: Iivari (450.000 neutralizing U/ml anti-IFN-α + 3.000 U/ml anti-IFN-β) and Kaalepi (30.000 U/ml anti-IFN-α + 30.000 U/ml anti-IFN-β),[26] or their respective control Ig (CIg and sheep serum, respectively), to the supernatants from poly I:C-activated keratinocytes, prior to the administration of these supernatants to the DC cultures. Addition of these antibodies to poly I:C-activated keratinocyte supernatants blocked the induction of CD83, CD80 and CD86 expression by DC. ΔMFI represents the difference between the various stainings and the isotype control. Results are one representative experiment out of three.

The role of TNF-α and type I IFNs from supernatants of poly I:C-activated keratinocytes in DC maturation was further confirmed by studying the expression of these cytokine by differently stimulated keratinocytes. Therefore, keratinocytes were pulsed for 2 h with either poly I:C or TNF-α/IL-1β, cultured for additional 46 h and the supernatants analyzed by ELISA. Whereas poly I:C- and TNF-α/IL-1β-activated keratinocytes produced similar levels of IL-8 (Fig. 2B), only keratinocytes activated by poly I:C produced TNF-α (Fig. 2A). Type I IFNs were undetectable by standard ELISA in all groups (data not shown). However, RT-PCR analysis readily revealed that only poly I:C-activated keratinocytes were able to upregulate mRNA expression for IFN-α and IFN-β (Fig. 2D).

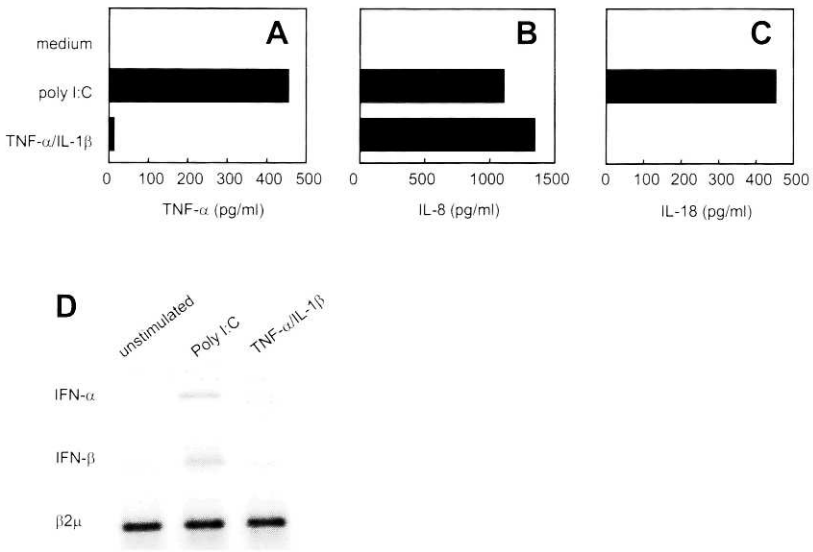

Figure 2. Poly I:C-activated, and not TNF-α/IL-1β-activated, keratinocytes produce significant amounts of TNF-α and IL-18, and express IFN-α and IFN-β. Normal human keratinocytes were pulsed for 2 h with either poly I:C or TNF-α/IL-1β, washed extensively (5 times in 5 ml) and cultured for additional 46 h in the presence of fresh medium. Supernatants were harvested and TNF-α (A), IL-8 (B), IL-18 (C) and IFN-α (data not shown) were determined by ELISA (see Material and Methods). Fig. D shows the expression of IFN-α and IFN-β mRNA in 4 h stimulated keratinocytes. RNA was extracted for RT-PCR analysis. Each figure shows one representative out of three independently performed experiments.

DC matured in the presence of poly I:C- or TNF-α/IL-1β-activated keratinocyte supernatants exhibit similar capacity to support naive Th cell proliferation

We subsequently studied whether the exposure of maturing DC to keratinocyte supernatants affects their ability to stimulate allogeneic naive Th cells in MLR conditions. As the capacity of DC to induce a particular Th development is dependent on their maturation status we added LPS+MF to all DC cultures to synchronize their maturation. As shown in Fig. 3, maturing DC exposed to non-activated, or poly I:C- or TNF-α/IL-1β-activated keratinocyte supernatants were as effective in inducing Th cell proliferation as DC matured with LPS+MF only. As expected, iDC were less effective in inducing Th cell proliferation. While the proliferative response of iDC compared to all mature DC groups was statistically significant, the comparison of the proliferative response between the mature DC groups with each other did not reach the level of significance. These results indicate that the exposure of DC to keratinocyte supernatants does not affect their ability to stimulate naive Th cell proliferation.

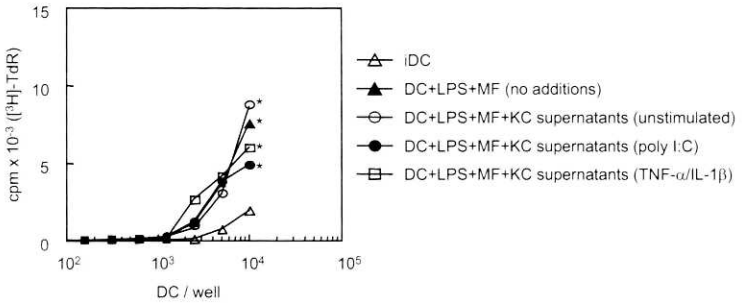

Figure 3. DC matured in the presence of poly I:C- or TNF-α/IL-1β- activated keratinocyte supernatants exhibit similar capacity to support naive Th cell proliferation. Maturation of DC was induced by either a combination of keratinocyte supernatants (50% v/v) and LPS+MF or LPS+MF alone. Immature DC or differentially mature DC were cultured at different numbers with $2.5x10^4$ allogeneic naive ($CD45RA^+$) $CD4^+$ Th cells. After 5 days, the cells were pulsed during the last 16 h with [^{3}H]thymidine ([^{3}H]-TdR). Immature DC (open triangles), DC+LPS+MF (close triangles), DC+LPS+MF+keratinocyte supernatants: unstimulated (open circles), poly I:C (close circles), TNF-α/IL-1β (open squares). Results are expressed in counts per minute (cpm). Data were analyzed for statistical significance using ANOVA followed by Dunnett's multiple comparisons test using iDC as control. * $P<0.05$, ** $P<0.01$. Results are representative of three independent experiments.

DC matured in the presence of poly I:C-activated keratinocyte supernatants acquire the ability to induce Th1-like responses: role of type I IFNs and IL-18

To test whether the presence of keratinocyte supernatants during DC maturation primes these cells for the capacity to bias for the development of Th1 or Th2, synchronized mature DC were cocultured with naive Th cells in the presence of superantigen, as previously described.[25] After 14 days, the percentage of cells producing IL-4 and/or IFN-γ was evaluated by stimulation of effector cells with PMA/ionomycin and analysis of intracellular cytokine expression at the single cell basis by FACS. While the priming of naive Th cells with DC matured in control conditions (LPS+MF) or DC cultured in the additional presence of supernatants from non-activated or TNF-α/IL-1β-activated keratinocytes resulted in the development of a mixed population of Th1 and Th2 cells after restimulation, DC matured in the presence of poly I:C-activated keratinocyte supernatants induced a strong bias toward the development of Th1 cells (Fig. 4).

Type I IFNs and IL-18 have been implicated in the virus-induced development of Th1-type immune responses.[29] Indeed, we found that poly I:C-activated keratinocytes not only produce type I IFNs (Fig. 2D) but also IL-18 (Fig. 2C). In addition, neutralization of type I IFNs or IL-18 in the supernatants of poly I:C-activated keratinocytes partially inhibited Th1 polarization (Fig. 5A). However, when the two soluble factors were neutralized we observed no further decrease in the percentage of IFN-γ-producing T cells to levels close to neutral conditions.

These data indicate that poly I:C-activated keratinocytes produce both type I IFNs and IL-18 that prime for DC inducing the development of Th1 cells.

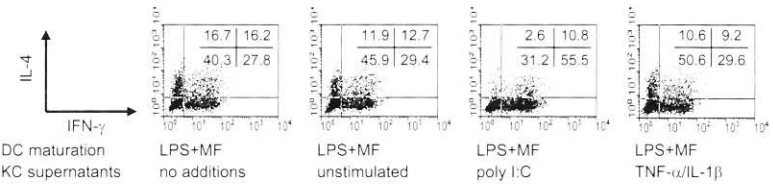

Figure 4. DC matured in the presence of supernatants from poly I:C-activated keratinocytes have the ability to induce Th1-like responses. Maturation of DC was induced as indicated in Fig. 3. Differentially matured DC were loaded with SEB and cocultured with naive Th cells. After 14 days, resting memory Th cells were restimulated for 6 h with PMA+ionomycin in the presence of brefeldin A. The expression of IL-4 and IFN-γ was assessed by intracellular staining. Results are expressed as the percentage of cells in each population. Data are representative of six independent experiments.

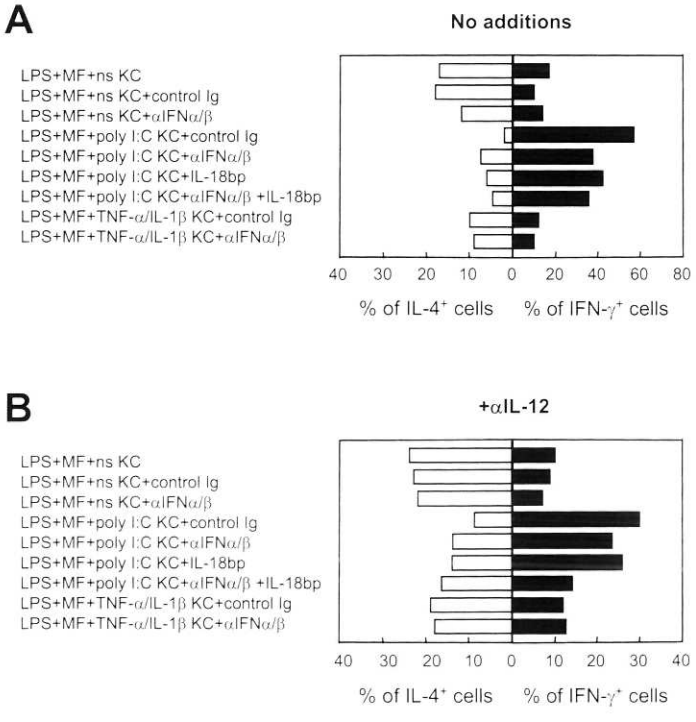

Figure 5. Both IFN-α/β and IL-18 produced by poly I:C-activated keratinocytes and IL-12 produced by mature DC contribute to the development of Th1 cells. DC were matured as described in Fig. 3. After 48 h, DC were thoroughly washed, loaded with SEB and cocultured with naive Th cells. After 14 days, responding Th cells were analyzed as described in Fig. 4. (A) Blockage of both IFN-α/β and/or IL-18 in poly I:C-activated keratinocyte supernatants reduced the frequency of Th cells expressing IFN-γ. This effect was more evident when neutralizing antibodies against IL-12 were added to DC/Th naive cocultures (B). This figure shows one representative out of four independently performed experiments.

Th1 polarization induced by poly I:C-activated keratinocytes is partially mediated by DC-derived IL-12

IL-12 is a major Th1-driving cytokine, promoting cell-mediated immunity[30] and can be produced by DC and other antigen presenting cells upon CD40 ligation by CD40L expressed by activated T cells.[14] An indication for a role of IL-12 in the enhanced Th1 polarization by poly I:C-activated keratinocytes was the finding that the presence of such supernatants, and not supernatants from TNF-α/IL-1β-activated keratinocytes, enhanced the production of IL-12p70 in CD40L-stimulated iDC (Fig. 6A). This enhanced production was blocked entirely by the additional presence of neutralizing antibodies against IFN-α/β in the keratinocyte supernatants (Fig. 6A), indicating that keratinocytes enhance IL-12p70 production by CD40L-activated iDC via the release of IFN-α/β.

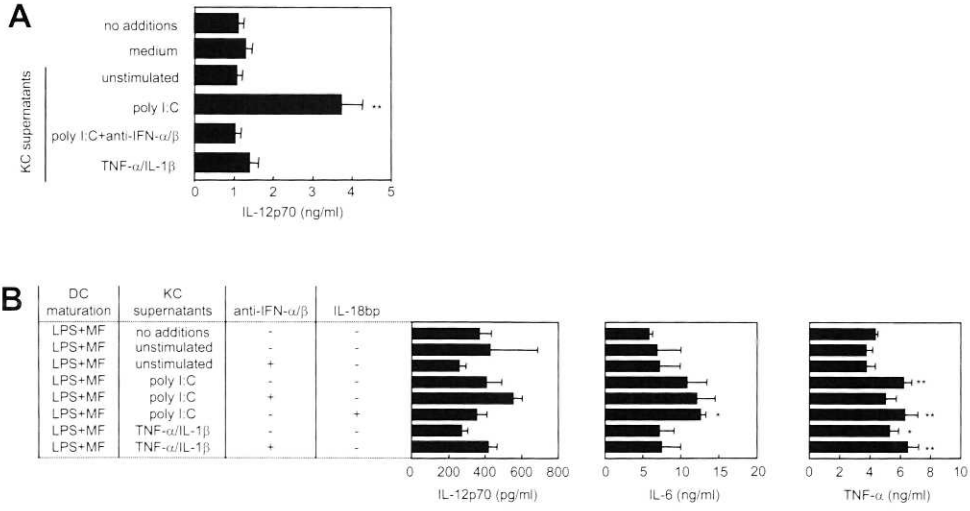

Figure 6. Poly I:C-activated keratinocyte supernatants enhanced IL-12 production by iDC upon CD40 ligation whereas it did not prime for high IL-12 production in mature DC. (A) Immature DC were stimulated with CD40L-transfected J558 cells (each 20.000 cells/well) in the absence or in the presence of poly I:C- or TNF-α/IL-1β-activated keratinocytes supernatants (50%, v/v). In addition, supernatants of poly I:C-activated keratinocytes were incubated with neutralizing antibodies against IFN-α/β were added to iDC. Poly I:C-activated keratinocytes enhance bioactive IL-12 production by CD40L-stimulated immature DC via the release of type I IFNs. (B) IL-12p70, IL-6 and TNF-α production by mature DC. Differentially activated keratinocyte supernatants do not modulate the bioactive IL-12 production of mature DC. Maturation of DC was induced by either a combination of keratinocyte supernatants (50% v/v) and LPS+MF or LPS+MF alone. After 48h DC were thoroughly washed and stimulated with CD40L-transfected J558 cells. IL-12p70, IL-6 and TNF-α concentrations in 24 h supernatants were determined by ELISA. Results, expressed as mean ± SD of triplicate cultures, are from one representative experiment out of three with similar results. Data were analyzed for statistical significance using ANOVA followed by Dunnett's multiple comparisons test using DC mature in the presence of LPS+MF+unstimulated KC supernatants as control. * $P<0.05$, ** $P<0.01$.

However, it remained to be established to what extent keratinocyte-derived supernatants were able to prime for enhanced IL-12 production, i.e. do DC matured in the presence of keratinocyte-derived supernatants show enhanced IL-12 in response to CD40L. As the maturation status of DC may readily determine their ability to produce IL-12,[27] these experiments were performed with DC with a synchronized maturation status by adding LPS and MF to the all iDC groups, which does not alter the ability to produce IL-12 in the mature DC.[25] After 48 h, the mature DC were extensively washed and restimulated for 24 h with CD40L. In contrast to the above-mentioned experiments with iDC, the CD40L-induced IL-12p70 production by DC matured in the presence of poly I:C-activated keratinocytes supernatants was not significantly different from DC matured in the presence of supernatants from non-activated or to TNF-α/IL-1β-activated keratinocytes (Fig. 6B). In addition, the capacity of IL-12p70, IL-6 and TNF-α production by differently matured DC upon CD40 activation was not, or only marginally, altered upon neutralization of type I IFNs or IL-18 in the keratinocyte supernatants (Fig. 6B).

In order to analyze the contribution of the moderate levels of T cell-induced DC-derived IL-12 in the enhanced Th1 polarization, neutralizing antibodies against IL-12 were added to cocultures of DC and naive Th cells. As depicted in Fig. 5B, neutralization of IL-12 inhibited Th1 polarization in any condition, including Th1 polarization induced by DC matured in the presence poly I:C-activated keratinocyte supernatants. This effect was more evident when simultaneously type I IFNs and/or IL-18 were neutralized in the keratinocyte supernatants, indicating that both soluble factors produced by poly I:C-activated keratinocytes and IL-12p70 produced by matured DC are involved in the observed Th1 polarization.

DISCUSSION

In the present study it is demonstrated for the first time that soluble mediators from pathogen-activated keratinocytes can profoundly affect DC function and consequently the class of the immune response by demonstrating that supernatants from poly I:C-activated keratinocytes, but not from TNF-α/IL-1β-activated induce iDC to mature into effector DC, and that these DC appeared to be type 1 DC (DC1) that bias the development of Th1 cells from naive T cells.

Keratinocytes and viral infections

Human keratinocytes are target of several viruses such as, herpes simplex virus,[31,32] human papillomavirus (HPV),[33] and varicella-zoster virus.[34] In particular, it was reported that infection of human keratinocytes with HPV type 16[35] or herpes simplex virus[31] induces the secretion several soluble mediators. Moreover, it was suggested that herpes simplex virus does not directly infect DC but epithelial cells[36] indicating a crucial role for keratinocyte-derived factors in this viral infection.

DC maturation induced by keratinocyte-derived TNF-α and IFN-α

Synthetic dsRNA (poly I:C) is often used as a model of viral infection. It has been reported that poly I:C induces maturation of human DC[13,37] via binding to Toll-like receptor 3.[38] In addition, poly I:C-matured DC acquired the capacity to trigger naive T cells and drive polarized Th1 responses,[13] a phenomenon which is partially dependent of IL-12.[39] Our current experiments extended these observations by demonstrating that poly I:C can also modulate DC function via the activation of keratinocytes. Indeed, in a separate study we found that keratinocytes express various TLR types, including high levels of TLR3 (Lebre *et al.*, manuscript submitted). Experiments with neutralizing antibodies indicated that poly I:C-activated keratinocytes induce DC maturation through the production of TNF-α and IFN-α/β, two well-known DC-maturation inducing factors.[10] The importance of TNF-α-dependent maturation of local DC in activating adaptive immune response to virus infection is supported by a study showing that DC from TNF-deficient mice cannot mature in response to virus infection.[40]

IFN-α- and IL-18-dependent Th1 response

In a search for the soluble factors driving the type 1 phenotype of DC matured in response to the supernatants from poly I:C activated DC, we identified the cytokines type I IFNs and IL-18. Upregulation of expression of type I IFNs is one of the earliest cellular responses upon contact with infectious agents, in particular viruses. The rapid induction of type I IFNs reflects the crucial role that these cytokines play in the inhibition of viral spread before the generation of a specific immune response.[41] In addition, because of this early expression, the type I IFNs are ideal signaling molecules alerting the specific immune system to the presence of viral infection which requires protective Th1 cell responses. Thus, type I IFNs have a

potent antiviral function which is not only due to their direct effects on infected cells (i.e. by inhibiting virus replication), but also by their indirect effects (i.e. by stimulating of iDC maturation resulting in Th1-biased responses).[42] Consistently, keratinocyte-derived type I IFNs belong to the molecules linking innate and adaptive immunity.

Importantly, the DC maturation observed is not due to carry over of poly I:C present in keratinocyte supernatants since the addition of supernatants derived from paraformaldehyde-fixed keratinocyte, after poly I:C activation, did not result in upregulation of CD83, CD80 and CD86 by DC (data not shown). Moreover, after exposure to poly I:C, keratinocytes were washed 5 times in 5 ml of PBS, i.e. > $1x10^6$ times diluted. When the last wash (PBS) was added to iDC for 48 h, no induction of DC maturation was observed (data not show). Altogether, these data suggest that keratinocyte secreted factor(s), rather than poly I:C carry-over, induce(s) DC maturation.

IL-18 is widely expressed by both leukocyte and non-leukocytes, and IL-18 expression in keratinocytes was shown before.[43,44] One of the most well known activities of IL-18 is the stimulation of IFN-γ production during inflammation, in particular the IL-12-induced IFN-γ production by Th1 cells.[45,46] Our data are in line with an earlier report proposing that keratinocyte-derived IL-18 is involved in the cutaneous Th1-type immune response.[47]

Role of DC-derived IL-12

We found that Th1 polarization induced by poly I:C-activated keratinocytes is partially mediated by DC-derived IL-12. IL-12 is an important factor in the induction of Th1 responses,[48] although DC-derived IFN-α and IL-18 have also been implicated.[29] IL-12 is produced by both iDC and mature DC upon CD40 ligation[14,49-51] and is regulated by cytokines and PGE_2.[20,52,53] Here, we have shown that type I IFNs from poly I:C-activated keratinocytes enhance CD40L-induced IL-12 production in iDC. Surprisingly, these keratinocytes did not prime for enhanced IL-12 production in CD40L-activated mature DC. This discrepancy is also found when DC are directly activated by poly I:C, which induces high levels of IL-12 in iDC[13,37] but not does not prime for high IL-12 in mature DC.[39] The enhanced IL-12 production by iDC, may be primarily important for the optimal and rapid activation of local effector cells, e.g. primed Th cells, MHC class I-restricted cytotoxic T lymphocytes or NK cells, to eliminate virus-infected keratinocytes. The lack of enhanced IL-12 production in DC primed by poly I:C-activated keratinocytes and the partial inhibition of the development of Th1 cells indicate that additional DC-derived factors have contributed to the development of

these Th1 cells. Since it has been suggested that dsRNA and influenza virus can induce DC maturation and confer the capacity to prime a Th1 response through the production of both IL-12 and type I IFNs[13] and that DC-derived IL-18 enhances the IL-12-induced production of IFN-γ by Th1 cells,[45,54] type I IFNs and IL-18 are likely candidates. Clearly, the direct activation of iDC by pathogen will contribute to the initiation and regulation of the specific immune response to this pathogen by committing the functional phenotype of the DC after their maturation.

DC polarizing capacity: role of microenvironment

However, it has been proposed that tissue-derived "danger" signals can also be vital in this respect.[55] The nature of these signals is dependent on the type of tissue and type of pathogen involved. The present data suggest that viruses can commit DC indirectly to become DC1 by the activation of keratinocytes located in the vicinity of DC. It may be speculated that other stromal cells of the skin, e.g. fibroblasts that are also capable to produce TNF-α or IFN-α in response to virus infection, may also be able to initiate maturation and polarization of DC. Recently, Soumelis *et al.*[56] reported that thymic stromal lymphopoietin (TSLP) is elevated in human keratinocytes from atopic dermatitis (AD) patients and that TSLP can induce the development of a Th2 cell-promoting DC phenotype DC2, characterized by the capacity to bias naive Th cells into Th2 cells. The authors, therefore, proposed that enhanced levels of TSLP may contribute to the allergic inflammation in AD. However, in contrast to our study, this study does not show direct evidence that TSLP present in keratinocyte culture supernatants is a factor crucial in inducing the effector DC2 phenotype. Our data and the data from the study of Soumelis *et al.*[56] were obtained by targeting monocyte-derived DC and peripheral blood $CD11c^+$ DC, respectively, and it was hypothesized that such DC comprise precursors of epidermal DC.[57] Although it remains to be established in full detail, Langerhans cells may also be responsive to keratinocyte-derived signals, since all DC populations assayed so far, have shown a high degree of plasticity in response to environmental stimuli.[58]

Concluding remarks

In summary, the present study demonstrates that pathogen-induced keratinocyte-derived factors can activate DC and modulate their function. These data support the notion that iDC as sentinels of specific immunity can respond to pathogen-associated molecules not only directly

via pattern recognition receptors,[59] but also indirectly, as a result of their reactivity to factors produced by tissue cells in response to those pathogens.[55]

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CHAPTER 4

Differential expression of inflammatory chemokines by Th1- and Th2-cell promoting dendritic cells (DC1 and DC2). A role for different mature DC populations in attracting appropriate effector cells to peripheral sites of inflammation

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ABSTRACT

Protective immunity to pathogens depends on efficient immune responses adapted to the type of pathogen and the infected tissue. Dendritic cells (DC) play a pivotal role in directing the effector T cell response to either a protective T helper (Th) type 1 (Th1) or Th2 phenotype. Human monocyte-derived DC can be differentiated into Th1- (DC1) and Th2-promoting (DC2) DC *in vitro* upon activation with microbial compounds or cytokines. DC that induce both Th1 or Th2 cells are DC0. Furthermore, host defense is highly dependent on mobile leukocytes. This cell trafficking is largely mediated by interactions of chemokines with their specific G-protein coupled receptors on the surface of leukocytes. The production of chemokines by effector DC0, DC1 or DC2 remains elusive. Here we assess the differential production of both inflammatory and homeostatic chemokines by monocyte-derived DC0, DC1, or DC2. We show that DC0 and DC1, but not DC2, selectively express elevated levels of the inflammatory chemokines CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β and CCL5/RANTES, as well as the homeostatic chemokine CCL19/MIP-3β. CCL21/6Ckine is preferentially expressed by DC2. Production of the Th1-attracting chemokines, CXCL9/Mig, CXCL10/IP-10 and CXCL11/I-TAC, is restricted to DC1. In contrast, the expression of Th2-associated chemokines does not strictly correlate with the DC2 phenotype, except for CCL22/MDC which is preferentially expressed by DC2. Since all the inflammatory chemokines tested are expressed constitutively by mature DC, we propose a novel role for mature DC present in inflamed peripheral tissues in orchestrating the immune response by recruiting appropriate leukocyte populations to the site of pathogen entry.

INTRODUCTION

Chemokines are small secreted proteins (6-14 kDa) that regulate leukocyte trafficking.[1,2] The chemoattractant signals are integrated by the interaction of chemokines with their specific 7-transmembrane G-protein-coupled receptors on the surface of leukocytes.[3] Specific routes of migration are thought to be determined by combinatorial expressions of receptors and sequential encounters with different chemotactic gradients.[4]

Chemokines can be functionally divided into homeostatic and inflammatory chemokines.[2,5] Homeostatic chemokines are constitutively produced mainly within secondary lymphoid organs in the T or B-cell areas. In the latter, CCL19/MIP-3β and CCL21/6Ckine mediate the encounters between mature dendritic cells (DC) and the rare antigen (Ag)-

specific T and B cells.[6,7] In addition, homeostatic chemokines are also produced in discrete microenvironments of non-lymphoid tissues such as the skin and mucosa.[4] In contrast, inflammatory chemokines such as CXCL1/growth-related oncogene (GRO)α, CXCL2/macrophage inflammatory protein (MIP)-2α, CXCL8/IL-8, CCL2/monocyte chemoattractant protein (MCP)-1, CCL3/MIP-1β, and CCL5/regulation and activated normal T cell-expressed and -secreted (RANTES) are rapidly induced in peripheral tissues upon pathogenic challenge and regulate the influx of inflammatory cells.[4,8] One example is neutrophil infiltration via CXCL1/GROα, CXCL2/MIP-2α, and CXCL8/IL-8 production.[9] While most chemokines fall into either the inflammatory or homeostatic category, some may play a dual role depending on the context in which they are produced. CCL17/thymus and activation-regulated chemokine (TARC), CCL20/MIP-3α, and CCL22/monocyte-derived chemokine (MDC) are examples of chemokines that are produced both in peripheral tissues and secondary lymphoid organs.[4,10]

DC are professional antigen-presenting cells (APC) that have the unique capacity to initiate specific immune responses.[11] Capture of Ag and initiation of immunity are distinct and spatially segregated functions carried-out by DC at different stages of development. Thus, a fundamental aspect of DC function is their capacity to migrate from inflamed tissues to the T cell areas of secondary lymphoid organs, i.e. lymph nodes (LN).[12] DC are a major source of chemokines. Immature DC (iDC) constitutively release CCL22/MDC and CCL17/TARC.[13] At early stages of maturation, DC produce high levels of inflammatory chemokines, such as CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CXCL8/IL-8, and CXCL10/IP-10, that sustain the recruitment of circulating iDC, DC precursors, and T cells to inflamed tissue.[10,14] Homeostatic chemokines, including CCL18/DC-CK1, CCL19/MIP-3β, CCL17/TARC, and CCL22/MDC, are produced or upregulated later during DC maturation,[14,15] providing chemotactic signals for mature DC and for T cells in secondary lymphoid organs. Mature DC-derived CCL18/DC-CK1, CCL19/ELC, CCL22/MDC and CCL17/TARC attract naive, recently activated and central-memory T cells expressing the cognate receptors CCR7 and CCR4.[14,16,17] $CXCR5^+$ B lymphocytes[18] and follicular homing T helper (Th) cells[19,20] are attracted by DC-derived CXCL13/B cell-attracting chemokine-1 (BCA-1).[21]

Likewise, T lymphocyte circulation in peripheral tissues is a complex event regulated by the local release of chemotactic stimuli and by differential expression of receptors on distinct T cell subsets. Th type 1 (Th1) cells are rich in CXCR3 and CCR5 receptors, whereas T

helper type 2 (Th2) cells express CCR3, CCR4 and CCR8.[8,22,23] Moreover, XCL1/lymphotactin (Lptn) is chemotactic for $CD8^+$ and $CD4^+$ T cells, but only modestly for natural killer cells.[24] The recently identified T regulatory (Tr) cells, producing high amounts of IL-10, coexpress both Th1- and Th2-associated receptors, with high levels of CCR8 and moderate amounts of CCR4[25,26] responding to the chemokines CCL1/I-309, CCL17/TARC, and CCL22/MDC.[27]

However, the production of chemokines by DC0, DC1 or DC2 has not yet been addressed. Therefore, we investigated whether human monocyte-derived DC0, DC1, or DC2 display differential production of inflammatory and homeostatic chemokines. We show that DC1 and DC0 preferentially express the inflammatory chemokines CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β and RANTES, and homeostatic chemokine CCL19/MIP-3β. In contrast, CCL22/MDC and CCL21/6Ckine are preferentially expressed by DC2. We propose a novel role for mature DC present in peripheral tissues in recruiting appropriate effector leukocyte populations to the site of pathogen entry since all inflammatory chemokines tested were expressed constitutively by mature DC.

MATERIAL AND METHODS

Generation of human monocyte-derived DC from peripheral blood

Monocytes were isolated from peripheral blood, as previously described.[28] To obtain immature DC, monocytes ($2.5x10^6$ cells/well/5 ml) were cultured for 6 days in 6-well plates (Costar, Cambridge, MA) in Iscove's modified Dulbeccos's medium (IMDM; Life Technologies, Paisley, U.K.) containing 1% FCS (HyClone, Logan, UT), supplemented with recombinant human (rhu) GM-CSF (500 U/ml; a gift of Schering-Plough, Uden, The Netherlands) and rhuIL-4 (250 U/ml; Pharma Biotechnologie Hannover (PBH), Hannover, Germany) as described.[29,30] At day 6, the cultures consisted of uniformly $CD14^-$, $HLA\text{-}DR^+$, and $CD83^-$ iDC. Cell-surface phenotype was analyzed by flow cytometry, after labelling of cells with appropriate mAb as described.[29,30]

Induction of DC maturation under polarizing conditions

To induce DC maturation, day 6 DC were cultured for additional 2 days in the presence of a combination of LPS (100 ng/ml; Sigma-Aldrich, St. Louis, MA), rhuIL-1β (10 ng/ml; PBH) and rhuTNF-α (50 ng/ml; PBH) (maturation factors, MF) to induce DC0, or a combination of

LPS+MF and IFN-γ (10^3 U/ml; a gift of P.H. van der Meide, U-CyTech, Utrecht, The Netherlands) to induce DC1, or polyriboinosinic polyribocytidylic acid (poly I:C) alone (20 μg/ml; Sigma-Aldrich) to induce DC1, or a combination of MF and PGE_2 (10^{-6} M; Sigma-Aldrich) to induce DC2.

Induction of IL-12p70 production by mature DC with different polarizing capacities

On day 8, mature DC were washed extensively and $2x10^4$ cells/well were stimulated in 96-well flat-bottom culture plates (Costar) in IMDM containing 10% FCS in a final volume of 200 μl with CD40L-transfected J558 cells ($2.5x10^6$ cells/well; a gift of Dr. P. Lane, Birmingham, UK). Supernatants were harvested and the concentrations of IL-12p70 were measured by ELISA (see next) as previously described.[31]

Real-time PCR

Differentially matured effector DC were washed extensively and $1x10^6$ DC were stimulated (6-well plates, Costar) with irradiated (2500 Gy) L cells transfected with CD40L (a kind gift from Dr. C. van Kooten, Dept. of Nephrology, LUMC. Leiden, The Netherlands) for 24 h. The CD40L-stimulated DC were then gently harvested and lyzed.

Total RNA was prepared from cells by a single step extraction method using RNA STAT-60 (TelTest, Inc) according to the manufacturer's instructions. Each RNA preparation was treated with Qiagen DNase I. DNase treatment was determined to be complete if the sample required at least 38 PCR amplification cycles to reach a threshold level of fluorescence using β-2 microglobulin for human samples as an internal amplicon reference. Agarose gel electrophoresis and ethidium bromide staining confirmed the integrity of the RNA samples following DNase treatment. Complementary DNA was prepared from the RNA sample using Multiscribe Reverse Transcription Kit (Applied Biosystems, PE, Norwalk, CT) following the manufacturer's instructions. A negative control of RNA without reverse transcriptase was mock reverse transcribed for each RNA sample. Gene expression was measured by TaqMan® quantitative PCR (Applied Biosystems) in cDNA prepared from the cells. PCR Probes and primers were designed utilizing PrimerExpress software (Applied Biosystems). The designed primer and probe sequences are shown in Table I. Each target gene probe was labelled using FAM (6-carboxyfluorescein). The internal reference probe, human β2-microglobulin (β2μ),

was labelled with a different fluorescent dye, VIC. The differential labelling of the target gene and internal reference gene thus enabled measurement in the same well. Forward and reverse primers and the probes for both the internal reference control and the target gene were added to the TaqMan® Universal PCR Master Mix (Applied Biosystems). Each reaction contained 200 nM of forward and reverse primers plus 100nM probe for β2-microglobulin and 600 nM forward and reverse primers plus 200 nM probe for the gene of interest. TaqMan® experiments were carried out on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The thermal cycler conditions were as follows: hold for 2 min at 50°C and 10 min at 95°C, followed by two-step PCR for 40 cycles of 95°C for 15 sec followed by 60°C for 1 min. The PCR cycles needed for FAM and VIC fluorescence to cross a threshold where a statistically significant increase in change in fluorescence (Ct = Threshold Cycle) was measured using Sequence Detector software (Applied Biosystems) according to their recommendations. Relative target gene expression was determined using the formula Rel Exp $= 2^{-(\Delta\Delta CT)}$ where ΔΔCT = (Ct Target Gene – Ct Internal Reference Control in experimental sample) – (Ct Target Gene – Ct Internal Reference Control in no template control sample).

Table I. Primer sequences

Gene	Forward primer	Reverse primer	Probe
CCL2/MCP-1	Applied Byosystems premade prime/probe mix		
CCL3/MIP-1α	CAACCAGTTCTCTGCATCACTTG	GCCGGGAGGTGTAGCTGAA	CACGCCGACCGCCTGCTG
CCL4/MIP-1β	TGTTCCTGAGCCACCCTACTG	TGTTTCCTTGGGCATGAAGAG	CCCTCCGCACATCCAATGCTGG
CCL18/DC-CK1	CCTGGAGGCCACCTCTTCTAA	TCAAAATGAAATATGATGCATAAACTAAGAT	TCCCATCTGCTATGCCCAGCCAC
CCL19/MIP-3β	CATCGTGAGGAACTTCCACTACCT	CCCTCAGTGTGGTGAACACTACAG	CAAGGATGGCTGCAGGGTGCCT
CCL20/MIP-3α	TGCTTTGATGTCAGTGCTGCTA	GTATCCAAGACAGCAGTCAAAGTTG	TGCTGCTTCTGATTCGCCGCA
CCL22/MDC	TGGCGCTTCAAGCAACTG	GACGTAATCACGGCAGCAGA	AGGCCCCTACGGCGCCAACA
CXCL2/MIP-2α	CAGAAGGAAGGAGGAAGCTTATTG	GCTGTGTCTCTCTTTCCTCTTCTGTT	CCTGAAGGAGGCCCTGCCCTTACA
CXCL9/Mig	CCCCAACACCCCACAGAAG	GGTTTATTTAAGAGGCAGGACTTGAAC	TCCCAATTCATCCTCACTCAGTCCAGC
CXCL10/IP-10	AAAATCGATGCAGTGCTTCCA	TTGACATATACTCCATGTAGGGAAGTG	ACCACACAGAGGCTGCCTCTCCCA
CXCL11/I-TAC	GTCTTCCTGAATGAATGACAATCAGA	CGATGGTAACCAGCCTTTCTTAA	CCACTGCCCAAAGGAGTCCAGCA
CXCL13/BCA-1	CCTAGACGCTTCATTGATCGAAT	TTCTTCCAGACTATGATTTCTTTTCTTG	TCTTGCCCCGTGGGAATGGTTG
β2μ	CACCCCCACTGAAAAAGATGA	CTTAACTATCTTGGGCTGTGACAAAG	ATGCCTGCCGTGTGAACCACGTG

Array-based ELISAs

In order to measure the levels of various chemokines in mature DC-derived supernatants, we used a novel technique, array-based ELISA, which allows the simultaneous analysis of 15 chemokines in 50 μl of supernatants.[32] The limits of detection of each chemokine are as follows: CCL1/I-309, 0.78 pg/ml; CCL2/MCP-1, 0.78 pg/ml; CCL3/MIP-1α, 1.56 pg/ml; CCL4/MIP-1β, 0.78 pg/ml; CCL5/RANTES, 0.39 pg/ml; CCL17/TARC, 0.39 pg/ml; CCL19/MIP-3β, 0.39 pg/ml; CCL20/MIP-3α, 0.50 pg/ml; CCL22/MDC, 0.39 pg/ml;

CXCL1/GRO-α, 0.39 pg/ml; CXCL8/IL-8, 0.39 pg/ml; CXCL9/Mig, 1.56 pg/ml; CXCL10/IP-10, 1.37 pg/ml; CXCL11/I-TAC, 0.55 pg/ml; and XCL1/Lptn, 1.56 pg/ml.

Stimulation of iDC with microbial compounds

Immature DC were stimulated at the concentration of $2x10^4$ cells/200 µl (96-well plates, Costar) and cultured for 48 h in the absence or in the presence of the following stimuli: LPS from *Escherichia coli* (250 ng/ml; Sigma-Aldrich), or synthetic dsRNA poly I:C (20 µg/ml).

Determination of cytokine and chemokine production by ELISA

Determination of IL-12p70 concentrations in culture supernatants was performed by specific solid-phase sandwich ELISA as previously described.[31] Measurements of CXCL8/IL-8 were performed by ELISA using pairs of specific mAbs and recombinant standard obtained from BioSource International (Camarillo, CA). CCL2/MCP-1 was determined using the Ab pair, rat polyclonal 20521D for coating and rabbit polyclonal 20532D for detection and recombinant CCL2/MCP-1 19781T (BD Pharmingen, San Diego, CA). CCL20/MIP-3α was determined using a DuoSet ELISA purchased by R&D Systems. The limits of detection of these ELISA are as follows: CXCL8/IL-8, 30 pg/ml; CCL2/MCP-1, 40 pg/ml; and CCL20/MIP-3α, 60 pg/ml.

RESULTS

Phenotype of DC0, DC1 and DC2

Monocyte-derived DC0, DC1, and DC2 were generated as previously described.[31,33,34] Monocyte-derived iDC were then cultured for 48 h in the presence of TNF-α and IL-1β (maturation-inducing factors: MF) plus LPS to induce DC0, or MF+LPS+IFN-γ, or poly I:C to induce DC1, or MF+LPS+PGE_2 to induce DC2 differentiation. DC maturation was comparable within the different DC groups as analyzed by the upregulation of surface expression of CD83, and the costimulatory molecules CD80 and CD86 (data not shown). To confirm whether the different effector DC subsets display the phenotype previously described,[33,34] the DC0, DC1 and DC2 were stimulated for 24h with CD40L-transfected cells, thereby mimicking local engagement with T cells (Fig. 1A). DC0 and poly I:C-induced DC1 produced similar levels of bioactive IL-12p70, whereas IFN-γ-induced DC1 produced higher levels of IL-12p70. In contrast, DC2 produced low levels of IL-12p70 upon CD40 ligation.

As CCR7 is upregulated during DC maturation,[14] the expression of this chemokine receptor was measured by real-time PCR analysis. No differences were found in CCR7 expression levels between the different DC groups (Fig. 1B).

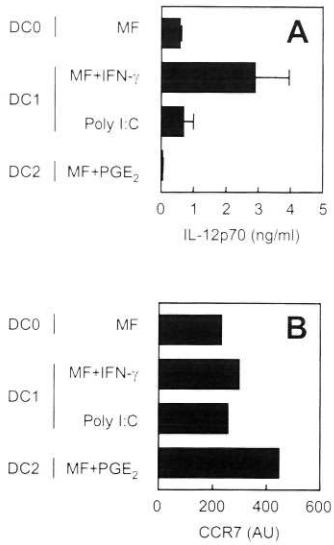

Figure 1. Phenotype of DC0, DC1 and DC2 populations. Monocyte-derived iDC were cultured for 48 h in the presence of TNF-α and IL-1β (maturation-inducing factors: MF) plus LPS (DC0), or MF+LPS+IFN-γ or poly I:C (DC1), or with MF+LPS+PGE_2 (DC2). (A) The different effector DC types were stimulated for 24h with CD40L-transfected cells and IL-12p70 contents were analyzed in culture supernatants by specific ELISA. Data are expressed as mean±SD of triplicate cultures. (B) CCR7 mRNA expression levels was analyzed by real-time PCR analysis. All the DC groups expressed similar levels of CCR7 mRNA. The data are from one representative experiment out of three.

Preferential expression of inflammatory chemokines by DC1

Chemokines can be divided into two different groups: inflammatory and homeostatic being responsible for the movement of leukocytes under inflammatory conditions or in the steady state, respectively.[2,5] Real-time PCR analysis revealed that the inflammatory chemokines CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β and CXCL2/MIP-2α (Fig. 2A), were constitutively expressed by all the mature DC groups and that their expression was downregulated in response to CD40L. With the exception of CCL20/MIP-3α, which instead was upregulated upon CD40 ligation (Fig. 2A). CCL2/MCP-1, CCL3/MIP-1α, and CCL4/MIP-1β were preferentially expressed by DC0 and DC1. To verify if protein production followed the same pattern as the mRNA expression, array-based ELISAs were performed, to simultaneously measure 15 different chemokines.[32] Fig. 2B shows that the inflammatory chemokines CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CCL20/MIP-3α, CXCL1/GROα and CXCL8/IL-8 were produced constitutively by the different mature DC subsets and, with the exception of CCL2/MCP-1, their production was enhanced upon CD40 ligation. In addition, XCL1/lymphotactin production was not affected

upon CD40 ligation. DC2 produced lower levels of CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β and CCL5/RANTES compared to DC0 and DC1, confirming the mRNA data. The pattern of CXCL1/GROα production by the different DC subsets was similar, whereas CXCL8/IL-8 production was higher in DC0 and IFN-γ-matured DC1. These data demonstrate that the expression profile of inflammatory chemokines, both at the mRNA and protein level, reflect the microenvironment of DC maturation.

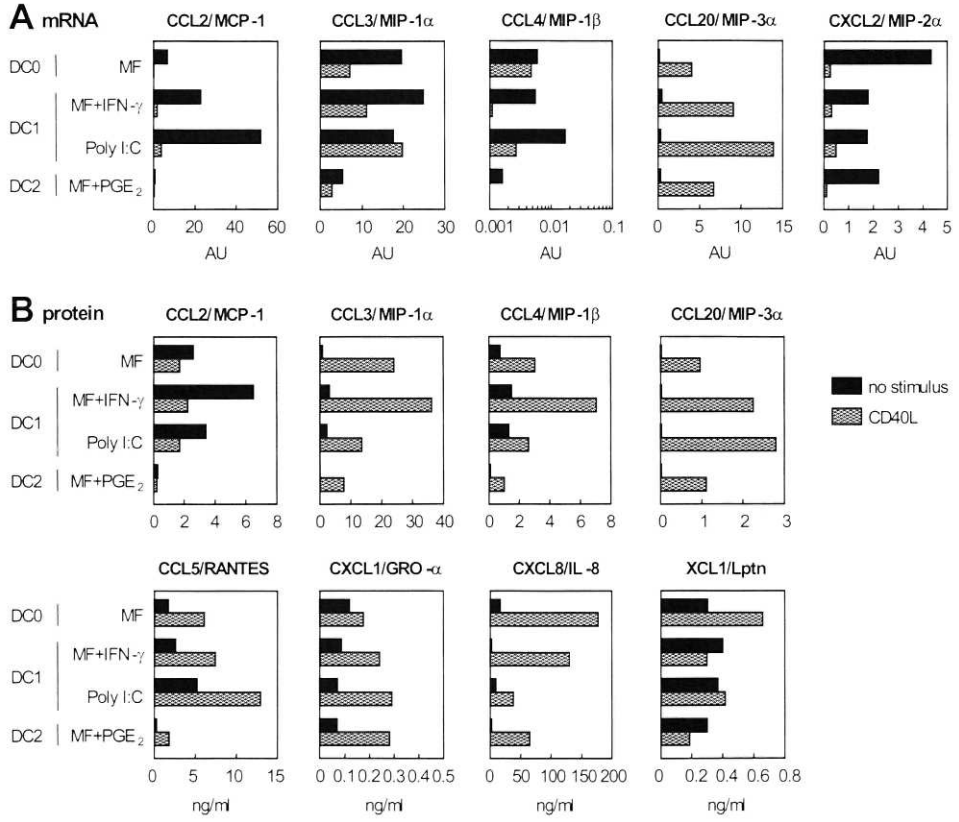

Figure 2. DC0 and DC1 express higher levels of inflammatory chemokines. mRNA expression (A) and protein release (B) of inflammatory chemokines by DC0, DC1 and DC2 following stimulation with CD40L. mRNA levels were measured after 24 h by quantitative real-time PCR analysis and the array-based ELISA was used to measure the amount of chemokine production in culture supernatant. Protein data are expressed as mean of duplicate cultures.The data are from one representative experiment out of three.

DC0 and DC1 preferentially express CCL19/MIP-1β whereas DC2 preferentially express CCL21/6Ckine

Although, the homeostatic chemokines CCL18/DC-CK1, CCL19/MIP-3β, CCL21/6Ckine and CXCL13/BCA-1 were constitutively expressed by all the DC groups, CCL18/DC-CK1 was preferentially expressed by DC0, CCL19/MIP-3β preferentially expressed by DC0 and DC1, and CCL21/6Ckine was preferentially expressed by DC2 (Fig. 3A). CD40 ligation downregulated the expression of CCL18/DC-CK1, CCL21/6Ckine and CXCL13/BCA-1, while it upregulated the expression of CCL19/MIP-3β. Analysis of the production of CCL19/MIP-3β protein, by the different mature DC confirmed the mRNA data (Fig. 3B).

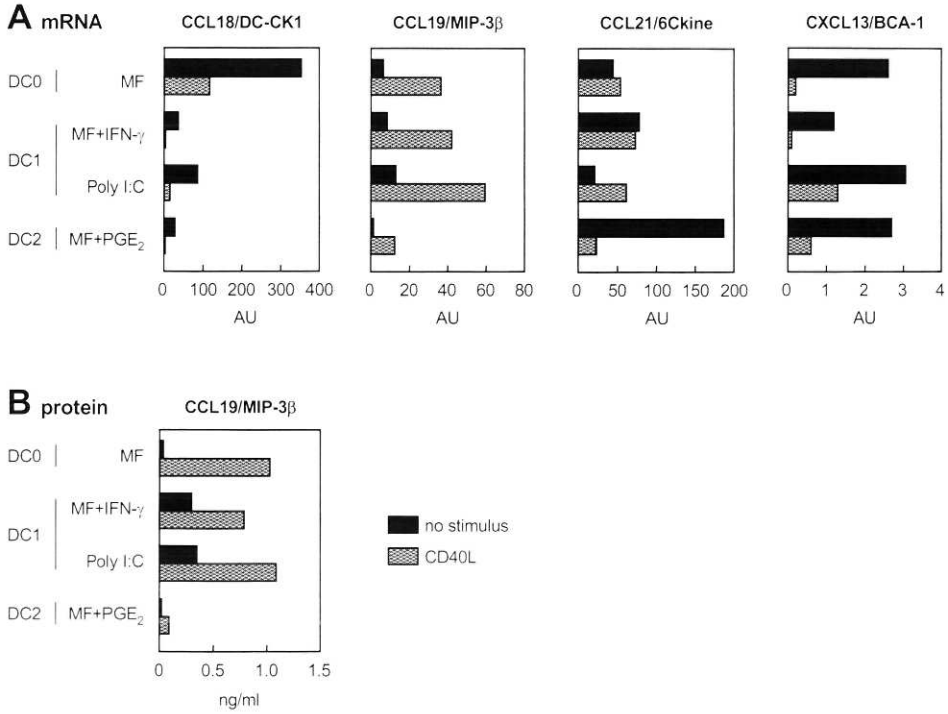

Figure 3. Preferential expression of CCL19/MIP-3β and CCL21/6Ckine by DC0 and DC1, and DC2 respectively. mRNA and protein release by J558-CD40L-stimulated DC0, DC1 and DC2 subsets was performed as indicated above. Protein data are expressed as mean of duplicate cultures.The data are from one representative experiment out of three.

Preferential expression of Th1-associated chemokines by DC1

Th1 and Th2 cells differ in their expression of chemokine receptors allowing them to migrate toward distinct types of chemokine gradients. The receptors CXCR3 and CCR5 are highly

expressed on Th1 cells (ligands: CXCL9/Mig, CXCL10/IP-10, CXCL11/I-TAC; and CCL5/RANTES, respectively), whereas CCR3, CCR4, and CCR8 are preferentially expressed by Th2 cells (ligands: CCL11/eotaxin; CCL17/TARC and CCL22/MDC; and CCL1/I-309, respectively).[22,23] Therefore, we investigated whether the profile of Th1- and Th2-associated chemokines produced by DC0, DC1, and DC2 was associated with their effector phenotype. Indeed, CXCL10/IP-10 and CXCL11/I-TAC were constitutively and preferentially expressed by both DC1 subsets. However, CXCL9/Mig was preferentially expressed by IFN-γ-matured DC1 (Fig. 4A and 4B). The Th2-associated chemokine CCL22/MDC was constitutively expressed by all the DC groups albeit at higher levels in DC2. Although the expression levels were low (pg/ml), CCL1/I-309, was preferentially produced by DC0 and IFN-γ-matured DC1. Production of CCL17/TARC was similar in all the DC groups, except for DC1 matured in the presence of IFN-γ, which produced lower levels of this chemokine. The pattern of the Th2-associated chemokines CCL1/I-309 and CCL17/TARC did not correlate with the DC effector phenotype except for CCL22/MDC (Fig. 4B). The protein data are in agreement with the mRNA expression, except for the fact that upon CD40 ligation the production of CXCL9/Mig, CXCL10/IP-10 and CXCL11/I-TAC was not affected or only marginally enhanced. Together, these data demonstrate a strict correlation of expression of Th1-associated chemokines and Th1-promoting effector DC1, while this link appears to be less stringent for Th2-associated chemokines and DC2.

Chemokine production by iDC upon stimulation with different pathogen-associated molecular patterns (PAMPs)

As effector DC1 preferentially express Th1-associated chemokines we asked whether tissue-type sentinel DC stimulated with a Th1-eliciting microbial compound, poly I:C, would lead to a similar profile of chemokine production. As shown in Fig. 5 both LPS and poly I:C were able to induce the production of the inflammatory chemokines CCL2/MCP-1 and CXCL8/IL-8 by iDC. However, we found that the production of Th1-associated chemokines CXCL9/Mig and CXCL10/IP-10 was exclusively induced in poly I:C-activated iDC. These findings confirmed the data obtained with mature DC1 (mature in the presence of LPS+MF+IFN-γ or poly I:C) which selectively produced CXCL9/Mig and CXCL10/IP-10.

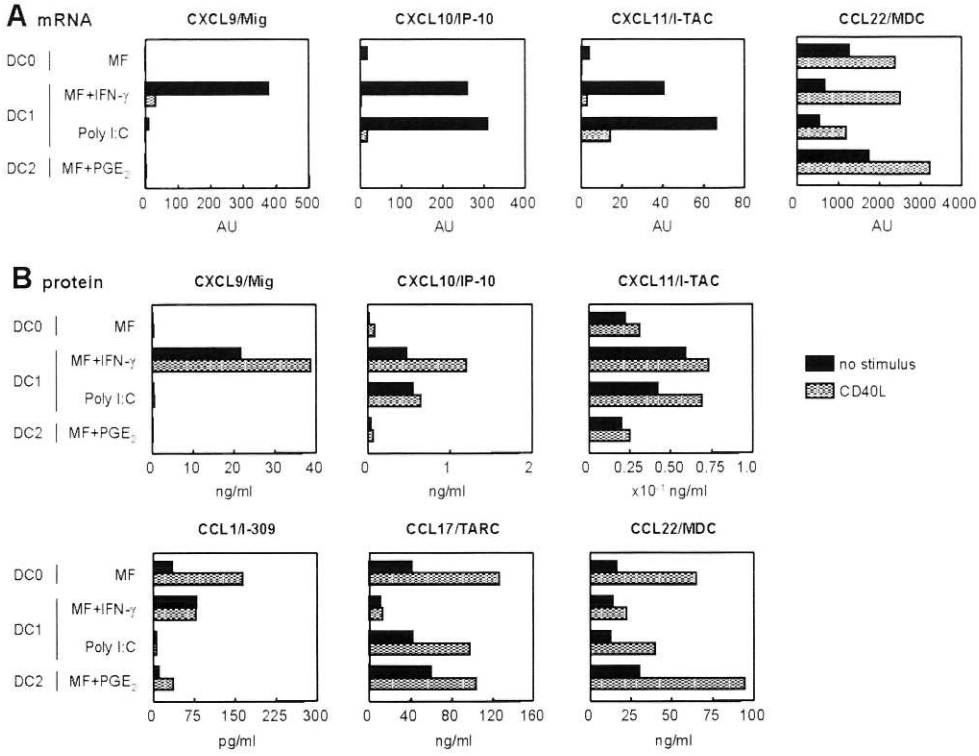

Figure 4. DC1 preferentially express mRNA for Th1-associated chemokines: downregulation by CD40 ligation. The Th2-associated chemokine CCL22/MDC was constitutively expressed by all the DC groups albeit at higher levels in DC2. CD40 ligation upregulated CCL22/MDC expression on all DC populations. mRNA and protein release by J558-CD40L-stimulated DC0, DC1 and DC2 subsets was performed as indicated above. Protein data are expressed as mean of duplicate cultures. The data are from one representative experiment out of three.

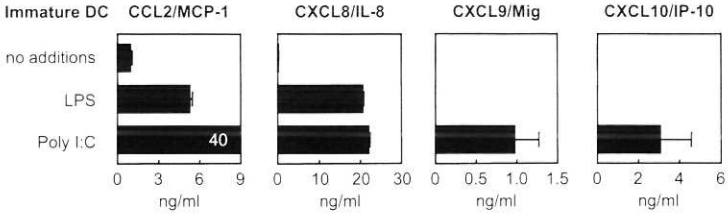

Figure 5. Chemokine production iDC upon stimulation with different microbial compounds. Monocyte-derived-iDC were stimulated for 48 h in the absence or in the presence of LPS or poly I:C and the contents of the different chemokines in culture supernatants was assessed by commercial ELISAs. Data are expressed as mean±SD of triplicate cultures. The data are from one representative experiment out of three.

DISCUSSION

The present study demonstrates that Th1-promoting (DC1) and Th2-promoting (DC2) effector DC constitutively express distinct patterns of inflammatory chemokines. Moreover,

the expression of Th1-attracting chemokines is strictly restricted to the DC0 and DC1 populations. The homeostatic chemokines CCL19/MIP-3β and CCL21/6Ckine were preferentially expressed by the polarized DC1 and DC2, respectively.

DC are highly specialized APC that recognize pathogens, or their products, and mature into potent inducers of T cell immunity. A critical event in the initiation of specific immune responses is DC migration from the infected peripheral tissue to secondary lymphoid organs.[11,35] In the T cell areas of LN or spleen, mature DC not only present pathogen-derived peptides to naive T cells, but also adapt the class of immune response to the type of invading pathogen by driving the development of appropriate effector Th cell subsets.[36,37] To what extent chemokines produced by mature DC contribute to the development and/or maintenance of Th1 or Th2 responses remains unknown. We demonstrate that Th1-promoting effector DC (DC1) are the main producers of inflammatory chemokines such as CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β and CCL5/RANTES. The production of these chemokines may sustain the recruitment of circulating CCR1-, CCR2-, CCR5- and CXCR1-positive iDC, and DC precursors to inflamed tissues important to replenish the loss of maturing DC due to their mobilization and migration to the LN.[10,14] In addition, the selective expression of the CCR5 ligands CCL3/MIP-1α, CCL4/MIP-1β and CCL5/RANTES by DC1 is in agreement with the preferential attraction of $CCR5^+$ Th1 cells by these chemokines.[38] However, our data provide the first evidence that DC1 themselves may recruit Th1 effector cells to inflamed tissues. Since mature DC-derived inflammatory chemokines are downregulated upon CD40 ligation, it may point at a primary role to support ongoing Th1 responses in peripheral tissues while inflammatory chemokines should not be expressed in lymphoid organs where mature DC interact and activate naive T cells.

A prerequisite for efficient Ag presentation to naive T cells is the swift migration of mature DC into the draining LN. Effective activation of the rare Ag-specific T cell may be further promoted by the expression of T cell-attracting chemokines by the mature DC themselves once they are positioned in the T cell are of the LN. To this extent, expression of CCL19/MIP-3β and CCL21/6Ckine (CCR7 ligands) and CCL18/DC-CK1 have been detected on a fraction of DC in human LN.[14,15] In agreement with this hypothesis, we could detect the homeostatic chemokines CCL18/DC-CK1, CCL19/MIP-3β, CCL21/6Ckine and CXCL13/BCA-1 in all mature DC populations analyzed. These chemokines regulate the encounters between DC, T and B cells in secondary lymphoid organs.[6] To our surprise, CCL19/MIP-3β was preferentially expressed by DC0 and DC1, whereas DC2 expressed

higher levels of CCL21/6Ckine. CCR7-positive cells entering the draining LN, e.g. mature DC and naive T cells, move into the T cell area in response to CCL19/MIP-3β and CCL21/6Ckine.[14] Our data provide evidence that production of these chemokines by mature DC may amplify the CCR7-mediated chemotactic response in an autocrine fashion. However, it is interesting to note that DC1 and DC2 take preferentially advantage of CCL19/MIP-3β and CCL21/6Ckine production, respectively.

Activated effector T cells are characterized by their responsiveness to numerous inflammatory chemokines that are produced at the site of pathogen entry and tissue injury.[4] The expression patterns of chemokines and their receptors determine the selective recruitment of Th1 and Th2 cells.[2,39,40] Our study demonstrates the selective production of the Th1-attracting chemokines, CXCL9/Mig, CXCL10/IP-10 and CXCL11/I-TAC, by Th1-promoting effector DC. Secretion of these chemokines by mature DC may contribute to the attraction of CXCR3$^+$ Th1 cells to the inflammatory site prior to DC mobilization into afferent lymphatics. Alternatively, it may point at a new function for mature DC that fail to leave the inflamed tissue in sustaining the appropriate (or pathologic) polarized Th1 response in the periphery. This is corroborated by the observation that only 30% of epidermal Langerhans cells migrate to the draining LN after chemical (contact allergen),[41] cytokine[42] or pathogenic challenge.[43] On the other hand, upregulation of expression of these chemokines in response to CD40 ligation may also support the DC:T-cell interaction in lymphoid organs. Indeed, it was demonstrated that DC-derived CXCL10/IP-10 regulates DC-Th1 cell cluster formation and retention of CD4$^+$ T lymphocytes during their polarization towards Th1 in the T cell areas of draining LN.[44] This concept of Th1 retention may provide a new insight into the mechanism of polarized effector T cell generation.[44] In addition, the data obtained with iDC stimulated with different microbial compounds confirm the notion that the preferential production of the Th1-associated chemokines CXCL9/Mig and CXCL10/IP-10 is associated with a Th1-eliciting microbial compound, namely poly I:C.

In contrast to chemokines linked to Th1 immunity, the expression of Th2-associated chemokines was not strictly associated with the Th2-promoting effector DC phenotype. Except for CCL22/MDC that was preferentially and constitutively expressed by mature DC2. In agreement with previous reports, its expression was upregulated upon CD40 ligation.[13] CCL22/MDC-expressing DC with a mature phenotype have been detected *in situ* in peripheral tissues, such as the skin.[45] Together with our data these studies suggest a role for mature DC2-derived chemokines in maintaining Th2 responses locally in the periphery.

The current findings suggest that diverse DC populations produce different repertoires of homeostatic and inflammatory chemokines. The production of inflammatory chemokines by mature DC, and its downregulation upon CD40 ligation, led us to propose a novel role for mature DC that are present in peripheral tissues in recruiting appropriate effector leukocyte populations to the site of pathogen entry.

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CHAPTER 5

BDCA3^{hi} dendritic cells: a novel subset with distinct phenotypical characteristics

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ABSTRACT

Specific immunity to pathogens is initiated by the activation and maturation of sentinel dendritic cells (DC) upon pathogen recognition by pattern recognition receptors, including Toll-like receptors (TLRs). It has been previously reported that subsets of the two distinct lineages of DC, e.g. myeloid $CD11c^+/BDCA1^+$ DC and plasmacytoid $CD123^+/BDCA4^+$ DC, strongly differ in their TLR expression profile, responsiveness to pathogens, and production of cytokines. In the present study we addressed, in comparative assays using these previously defined DC populations, the phenotypical and functional characteristics of a novel subset of myeloid DC expressing high levels of BDCA3 antigen ($BDCA3^{hi}$ DC), a DC population with the lowest frequency in human peripheral blood. We found that, in addition to TLR1 and TLR3, $BDCA3^{hi}$ DC uniquely express TLR10 mRNA, of which the ligands are presently unknown. $BDCA3^{hi}$ DC, cultured in GM-CSF, mature upon stimulation by the combination of IL-1β and TNF-α, or by the TLR3 ligand double-stranded RNA (dsRNA). Upon activation by dsRNA, $BDCA3^{hi}$ produce low levels of IL-12 and IFN-α and acquire the capacity to promote Th1 responses, albeit less potently compared to $BDCA1^+$ DC that produce higher levels of IL-12 in response to dsRNA. In contrast to $BDCA1^+$ DC, $BDCA3^{hi}$ DC produce no or very low levels of cytokines in response to TLR4 ligand LPS and to SAC. Immunohistochemical analysis of human tissue sections revealed the presence of $BDCA3^+$ cells with dendritic morphology in both lymphoid (tonsil, spleen and thymus) and non-lymphoid tissues (skin). In the skin, $BDCA3^+$ cells can be found in both epidermis and dermis. This study suggests that $BDCA3^{hi}$ DC, due to their narrow TLR expression profile, has a limited capacity to recognize pathogens, but are as flexible in their capacity to bias the development of Th1/Th2 cells, as other DC subsets.

INTRODUCTION

Protective immunity to a variety of pathogens depends on immune responses adapted to the type of pathogen. Dendritic cells (DC) play a pivotal role in the initiation of specific immune responses. DC reside in an immature form (iDC) in epithelial tissues where they constantly sample the environment for incoming pathogens.[1] Recognition of pathogens is mediated by pattern-recognition receptors (PRRs) that recognize conserved pathogen-associated molecular patters (PAMP). Toll-like receptors (TLRs) form a major group of PRRs that translate the recognition of a PAMP into activation of the immune system.[2,3]

Upon activation by pathogens, iDC undergo a programme of maturation and migrate through the lymphatics towards secondary lymphoid organs. During maturation, DC upregulate the expression of MHC class II (HLA-DR) and CD83, and the costimulatory molecules, CD80 and CD86, to become potent immunogenic antigen-presenting cells (APC) for naive T helper (Th) cells.[1] In the T cell areas of secondary lymphoid organs, mature DC activate naive Th cells and adapt the class of the immune response to the type of invading pathogen by driving the polarization of naive Th cells into effector Th type 1 (Th1) or Th2 cells.[4-6] For example, iDC activated with virus or the viral mimic double-stranded RNA (dsRNA) polyriboinosinic polyribocytidylic acid (poly I:C), develop into a mature DC type 1 (DC1) that potently induces the development of Th1 cells.[7,8]

Two distinct lineages of human DC, myeloid DC and plasmacytoid DC (PDC) can be distinguished. These lineages differ in their expression of TLRs, responsiveness to pathogens, and the production of signature molecules that drive the development of Th1 or Th2 cells.[9,10] Myeloid CD11c$^+$ DC, similar to monocyte-derived DC, express TLR1, 2, 3, 4, 5, 6 and 7, whereas CD123$^+$ (IL-3Rα) PDC express TLR6, 7, 8, and 9.[9,10] Myeloid DC recognize several bacterial components via TLR1, 2, 4, 5 and 6[11-15] and viral dsRNA via TLR3.[16] In contrast, PDC selectively recognize bacterial DNA-containing CpG motifs via TLR9[17] and are of particular importance in innate immunity against several viruses.[18] While IL-12 is produced at high levels by myeloid DC, PDC have the capacity to produce high levels of type I interferons (IFN-α and IFN-β).[9,10]

Peripheral blood contains several DC subsets[19-21] of which the smallest population of DC, approximately 2.7% of the MHC class II positive and lineage negative cells (lin$^-$, CD3, CD14, CD16, CD19, CD20, CD34 and CD56), is characterized by the expression of the blood dendritic cell antigen 3 (BDCA3).[21] BDCA3 is a molecule of which the function and ligand are still unknown. The present study describes the phenotypic and functional characteristics of BDCA3^{hi} DC subset and compared them to BDCA1$^-$ myeloid and BDCA4$^+$ plasmacytoid DC. We isolated this BDCA3^{hi} DC subset from peripheral blood and analyzed their TLR expression, their capacity to take up antigen, the responsiveness to certain PAMPs, their cytokine secretion profile and their capacity to induce a particular Th1 or Th2 response. In addition, we analyzed the distribution of BDCA3$^+$ DC in lymphoid and non-lymphoid tissues by immunohistochemistry.

MATERIALS AND METHODS

Isolation and culture of BDCA1$^+$, BDCA3^{hi} and BDCA4$^+$ DC

Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation with Lymphoprep (Nycomed, Torshov, Norway). Myeloid DC, BDCA1$^+$ and BDCA3$^+$, and plasmacytoid, BDCA4$^+$ DC were isolated from PBMC by magnetic cell sorting (MACS) using a high gradient MACS device (Mini-MACS^R; Miltenyi Biotec, Bergisch-Gladbach, Germany). Briefly, CD19$^+$ B cell-depleted PBMC were incubated with PE-labeled mouse monoclonal antibodies (mAbs) against BDCA1, BDCA3 or BDCA4 (all from Miltenyi Biotec) followed by anti-PE magnetic beads (Miltenyi Biotec). In addition, BDCA3^{hi} DC were isolated with anti-BDCA3 microbeads (Miltenyi Biotec). To analyze the purity of the obtained BDCA1$^+$, BDCA3$^+$, BDCA3^{hi}, BDCA4$^+$ DC populations, the cells were incubated with a cocktail of FITC-conjugated mAb specific for CD3, CD14, CD16, CD19, CD20, CD34 and CD56 receptors (lineage, lin) (IgG1; BD Biosciences, San Jose, CA). The purity of each cell population was >90%. Purified DC were cultured in Iscove's modified Dulbeccos's medium (IMDM; Life Technologies, Paisley, U.K.) containing gentamycin (86 μg/l; Duchefa, Haarlem, The Netherlands) and 10% FCS (HyClone, Logan, UT). In addition, DC cultures were supplement with the following growth factors (GF): BDCA1$^+$ and BDCA3^{hi} DC with GM-CSF (500 U/ml; Schering-Plough, Uden, The Netherlands) and BDCA4$^+$ DC with IL-3 (10 ng/ml, Strathmann Biotech, Hannover, Germany).

Analysis of cell surface molecules expression by FACS

To analyze the phenotype of circulating DC, cell suspensions (PBMC, isolated BDCA1$^+$, BDCA3^{hi} and BDCA4$^+$ DC) were incubated with the following mouse mAbs: anti-CD86-FITC (IgG1; BD Pharmingen, San Diego, CA), anti-CD83-allophycocyanin (APC) (IgG2b; Caltag Laboratories, Barlingame, CA), anti-HLA-DR-PerCP (IgG2b; BD Biosciences, San Jose, CA), anti-CD11c-APC (IgG1; BD Biosciences), anti-CD123-biotin (BD Pharmingen), streptavidin-PerCP (BD Biosciences), anti-BDCA1-biotin (Miltenyi Biotec), streptavidin-APC (BD Biosciences), anti-BDCA4-APC (Miltenyi Biotec) and anti-lin-FITC (BD Pharmingen). As controls, cells were stained with corresponding isotype matched control mAb. Stained cells were analyzed using a four-color cytometer (FACSCalibur, BD Biosciences). The cells were cultured overnight in medium containing maturation factors (MF) IL-1β (10 ng/ml; Strathmann Biotech) plus TNF-α (50 ng/ml; Strathmann Biotech), or

poly I:C (20 μg/ml, Sigma-Aldrich, St. Louis, MA) or CpG-oligodeoxynucleotide (ODN) 2006 (5'-GGGGGGACGATCGTCGGGGGG-3')[22] (0.5 μM; Biosource International, Nivelles, Belgium).

Quantification of TLR and β2-microglobuline mRNA levels by real-time PCR

In order to obtain highly purified DC subsets, DC were isolated by MACS as described above followed by further purification using FACS sorting (BD Biosciences). This method yielded highly purified (>99%) BDCA1$^+$, BDCA^{hi} and BDCA4$^+$ DC. Subsequently, purified cells were lyzed and total RNA was purified by using the NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany) according to manufacture's instructions. Complementary (c)DNA was generated using the First strand cDNA synthesis kit for RT-PCR (MBI Fermentas, St Leon-Rot, Germany). To anneal the primer to the RNA, 9 μl of total RNA, 1 μl oligo$(dT)_{18}$ and 1 μl $D(N)_6$ were added. This mix was then heated for 5 minutes at 94°C. Real-time PCR was performed in a LightCycler (Roche Diagnostics, Mannheim, Germany) based on specific primers (see Table I) and general SYBR green fluorescence detection. PCR products were analyzed on a 1% agarose gel containing ethidium bromide. A 100 bp DNA ladder standard (MBI Fermentas) was used as a size marker.

Table I. Primer sequences

Gene	Forward primer	Reverse primer
TLR1	AAAAGAAGACCCTGAGGGCC	TCTGAAGTCCAGCTGACCCT
TLR2	AACCCTAGGGGAAACATCTCT	GGAATATGCAGCCTCCGGAT
TLR3	AAATTGGGCAAGAACTCACAGG	GTGTTTCCAGAGCCGTGCTAA
TLR4	TACAAAATCCCCGACAACCTC	AGCCACCAGCTTCTGTAAACT
TLR5	TGCATTAAGGGGACTAAGCCTC	AAAAGGGAGAACTTTAGGGACT
TLR9	GTGCCCCACTTCTCCATG	GGCACAGTCATGATGTTGTTG
TLR10	TGACCACAATTCATTTGACTACTC	TTGAATACTTTTGGGCAAGCACC
β2μ	AAGATTCAGGTTTACTCACGTC	TGATGCTGCTTACATGTCTCG

Antigen uptake assay

BDCA1$^+$, BDCA3^{hi} and BDCA4$^-$ DC were incubated in culture medium for 30 min at 37°C in the presence of 0.4 μg/ml bovine serum albumin (BSA)-FITC (1:12 molar ratio, Sigma-Aldrich) or α-D-mannosylated-BSA-FITC conjugate (Man-BSA, 1:2.5 molar ratio; Sigma-Aldrich). Negative controls were incubated with the antigen for 30 min at 4°C. Antigen

uptake was stopped by extensive cold washing and cell surface fluorescence was quenched with trypan blue (Sigma). Antigen uptake was evaluated by flow cytometry.

Stimulation of BDCA1$^+$, BDCA3^{hi} and BDCA4$^+$ DC

BDCA1$^+$, BDCA3^{hi} BDCA4$^-$ DC were stimulated at the concentration of $2x10^4$ cells/200 µl (96-well plates, Costar, Cambridge, MA) with LPS from *Escherichia coli* (100 ng/ml; Sigma-Aldrich), fixed *Staphylococcus aureus* Cowan strain I (SAC, 75 µg/ml; Calbiochem, San Diego, CA), polyriboinosinic polyribocytidylic acid (poly I:C, 20 µg/ml; Sigma-Aldrich), CpG-ODN 2006 (0.5 µM). Culture supernatants were harvested after 24 h and the levels of IL-12p70, IFN-α, TNF-α, IL-6 and IL-10 were measured by ELISA (see below).

Isolation of CD4$^+$CD45RA$^+$CD4RO$^-$ naive Th cells (ThN)

ThN were isolated from peripheral blood leukocytes with the negative selection human CD4$^+$CD45RO$^-$ column kit (R&D Systems, Minneapolis, MN). This method yielded highly purified (>98%) CD4$^+$CD45RA$^+$CD45RO$^-$ ThN as assessed by flow cytometry (data not shown).

Analysis of proliferation of T cells cocultured with BDCA1$^+$, BDCA3^{hi} or BDCA4$^+$ DC in a mixed lymphocyte reaction (MLR)

IL-1β/TNF-α (maturation factors, MF)-matured DC subsets where tested for their ability to stimulate allogeneic ThN in a MLR. ThN ($2.5x10^4$ cells/200 µl) were cocultured in 96-well flat-bottom culture plates with different concentrations of matured DC. After 5 days, cell proliferation was assessed by the incorporation of [^{3}H]thymidine ([^{3}H]-TdR, Radiochemical Centre, Amersham, Little Chalfont, U.K.) after a pulse with 13 KBq/well during the last 16 h, as measured by liquid scintillation spectroscopy.

Induction of Th1 or Th2 cell responses by mature BDCA1$^+$, BDCA3^{hi} or BDCA4$^+$ DC

Isolated DC subsets ($1x10^4$ cells/200 µl) were cultured in the presence of MF or poly I:C before cocultured with ThN ($4x10^4$ cells/200 µl) in 96-well flat-bottom culture plates. After 24 h, superantigen *Staphylococcus aureus* enterotoxin B (SEB, 100 pg/ml; Sigma-Aldrich)

and naive Th cells were added to DC cultures. On day 5, IL-2 (10 U/ml; Cetus, Emeryville, CA) was added and the cultures were further expanded. After 14 days, resting memory Th cells were harvested, washed, and restimulated for 6 h with PMA (10 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) in the presence of brefeldin A (10 μg/ml; Sigma-Aldrich) in order to analyze Th cell cytokine production profile at the single cell basis. The cells were fixed in parafolmadehyde (2%; Sigma-Aldrich), permeabilized with saponin (0.5%; Sigma-Aldrich), and labeled with FITC-coupled IFN-γ mAb (Becton Dickinson) and PE-coupled IL-4 mAb (Becton Dickinson). The cells were evaluated by FACScan (Becton Dickinson).

Evaluation of cytokine production by ELISA

Determination of IL-12p70 concentrations in culture supernatants was performed by specific solid-phase sandwich ELISA as previously described.[23] Pairs of specific monoclonal antibodies and recombinant cytokine standards were obtained from BioSource International (Camarillo, CA) for determinations of IL-6, TNF-α and IFN-α, and from BD Pharmingen for IL-10 determination. The detection limits of these ELISAs are as follows: IL-6, 20 pg/ml, TNF-α, 20 pg/ml, IFN-α, 100 pg/ml, IL-12p70, 3 pg/ml, IL-10, 20 pg/ml.

Immunohistochemical analysis of BDCA1$^+$, BDCA3$^+$ and BDCA4$^+$ cells in human tissues

For immunohistochemical staining, tissues specimens of human tonsils, spleen, thymus and skin, were embedded in Tissue-Tek (Sakura Finetek, Torrance, CA), cryopreserved in liquid N_2 and stored at -20°C. Acetone-fixed cryosections (6 μm) were prepared and incubated with specific mouse mAbs against BDCA1 (IgG2a), BDCA3 (IgG1) or BDCA4 (IgG1) (1:5, Miltenyi Biotec) for 1 h at 37°C after blocking endogenous peroxidase activity with 0.3% H_2O_2 and 0.1% sodium azide (NaN_3). An IgG1 isotype mAb was used as negative control. After incubation with biotinylated rabbit anti-mouse IgG (Dakopatts, Glostrup, Denmark) for 1 h at room temperature, skin sections were rinsed with PBS and incubated with streptavidin horseradish peroxidase-conjugated (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, CLB, Amsterdam, The Netherlands). After washing, 3-amino-9-ethylcarbazole (AEC, Sigma-Aldrich) as used as chromogen. With this procedure BDCA1-,

BDCA3- and BDCA4-positive cells stained red. Other cellular elements were counterstained with hematoxylin (Cellpath, Newton Powys, UK).

For double immunostainings, tonsil and skin sections were incubated first with normal goat serum (10%; Dakopatts) for 15 min followed by anti-BDCA1 mAb (IgG2a, 1:5; Miltenyi Biotec) for 1 h at 37°C. Thereafter, FITC-coupled goat $F(ab')_2$ anti-mouse IgG and IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) was added to the sections followed by an incubation with rabbit anti-FITC (Dakopatts) and by an incubation with alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Dakopatts); all incubation were performed for 30 min at 37°C. Next, normal mouse serum (10%, Dakopatts) was added to the sections, followed by biotin-conjugated BDCA3 mAb (IgG1, 1:5; Miltenyi Biotec) overnight at 4°C. Then the sections were incubated with streptavidin horseradish peroxidase-conjugated (CLB). AP staining was developed using naphtol AS-MX phosphate (20 mg, Sigma-Aldrich) plus levamisole (15 mg, Sigma-Aldrich) plus Fast blue BB salt (1 mg, Sigma-Aldrich) in 100 mM Tris-HC buffer, pH 8.5. With this procedure $BDCA1^+$ DC stained blue. AEC staining was performed as stated above. With this procedure $BDCA3^+$ DC stained red. The sections were dried and mounted with glycerol/gelatin. Slides were analyzed with a wide field upright microscope (Leica DMRA, Wetzlar, Germany) coupled to a CCD camera and Image-Pro Plus software (Media Cybernetics, Dutch Vision Components, Breda, The Netherlands).

RESULTS

Peripheral blood contains several subsets of DC that express BDCA3 antigen

Although $BDCA3^+$ DC were previously reported to occur in human blood,[20,21] it is unclear whether BDCA3 expression is heterogeneous or restricted to one subset. Therefore, PBMC were stained with PE-labelled antibodies directed against BDCA3 antigen in combination with FITC-labeled mAbs against lineage (CD3, CD14, CD16, CD19, CD20, CD56, CD34) and anti-CD123-biotin (followed by streptavidin-PerCP), or anti-CD11c-APC, or anti-BDCA1-biotin (followed by streptavidin-APC). As shown in Figure 1A, BDCA3 antigen is expressed on lineage-negative cells at two different intensities, $BDCA3^{lo}$ and $BDCA3^{hi}$ expression. The $BDCA3^{lo}$ DC population consists of a mixed population of subsets of both plasmacytoid $CD123^+$ and myeloid $CD11c^+$ DC, as demonstrated by the coexpression of CD123 or CD11c and, BDCA4 or BDCA1, respectively. In contrast, the $BDCA3^{hi}$ DC population is homogenous and does not express CD123, BDCA1 or BDCA4, and is characterized by CD11c expression (Fig. 1A).

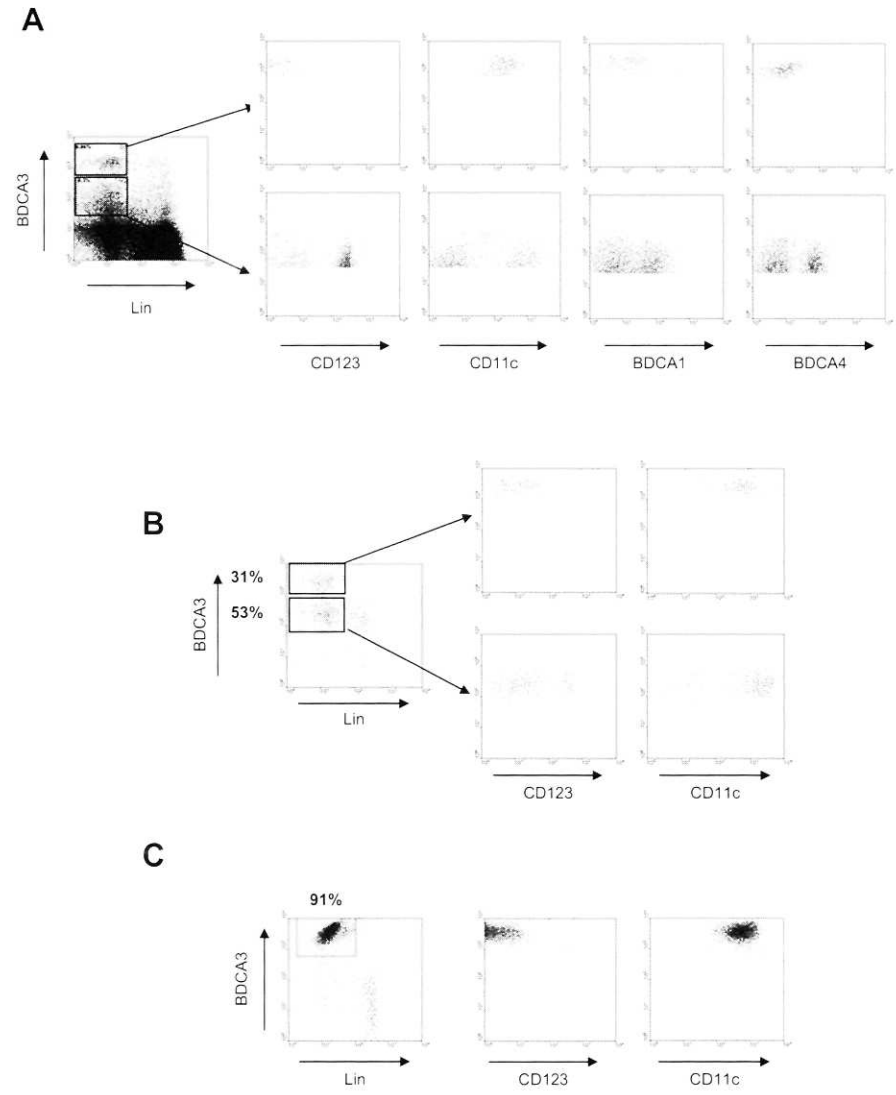

Figure 1. Peripheral blood contains several subsets of DC that express BDCA3 antigen. (A) PBMC were stained with anti-BDCA3-PE and anti-Lin-FITC mAbs in combination with anti-CD123-biotin (followed by streptavidin-PerCP) or anti-CD11c-APC or BDCA1-biotin (followed by streptavin-APC) or BDCA4-APC mAbs. Cells were analyzed using a four-color flow cytometry. The gates were set on Lin$^-$/BDCA3^{hi} and Lin$^-$/BDCA3^{lo} and the expression of CD123, CD11c, BDCA1 and BDCA4 are depicted. (B) BDCA3$^+$ cell isolation with anti-BDCA3-PE mAb yielded two populations that express the BDCA3 antigen, BDCA3^{hi} and BDCA3^{lo}. The BDCA3^{hi}-expressing population expresses CD11c but not CD123, whereas the BDCA3^{lo}-expressing population expresses both CD11c and CD123. (C) BDCA3 isolation with anti-BDCA3 microbeads yielded a single BDCA3^{hi}-expressing population characterized by CD11c expression but not CD123. These results are from a representative experiment out of five.

Next, BDCA3$^+$ DC were purified by using two different methods, with PE-labeled BDCA3 mAb (Fig. 1B) or with anti-BDCA3 microbeads (Fig. 1C). Figure 1B shows that the isolation PE-labeled BDCA3 mAb leads to a mixed population of DC. In contrast, a single population of BDCA3^{hi} DC was obtained using anti-BDCA3 microbeads (Fig. 1C). Therefore, for the next set of experiments this last methodology was used.

BDCA3^{hi} DC show fluid phase but not mannose receptor-mediated endocytosis

As the uptake of antigens in peripheral tissues is a prerequisite of the antigen-presenting cell function of DC,[1] we studied the endocytic capacity of the BDCA3^{hi} DC subset and compared to BDCA1$^+$ and BDCA4$^+$ DC subsets. To this aim, the different BDCA subsets were incubated with either FITC-labeled bovine serum albumin (BSA) or with FITC-labeled mannosylated BSA (Man-BSA) at 37°C or, at 4°C as a negative control. After 30 min, the capacity of the antigen uptake was measured by flow cytometry. As shown in Fig 2., BDCA3^{hi} DC are capable of taking up BSA, however, not as efficient as BDCA1$^+$ DC. In contrast, BDCA4$^+$ DC are not able to take up Man-BSA, which is in accordance with the observation that these blood-derived DC subsets do not express the mannose receptor or DC-SIGN (data not shown).

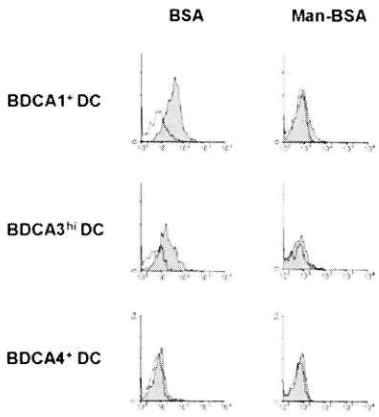

Figure 2. BDCA3^{hi} DC show fluid phase but not mannose receptor-mediated endocytosis. BDCA1$^+$, BDCA3^{hi} and BDCA4$^+$ DC subsets were incubated with BSA-FITC or Man-BSA-FITC for 30 min at 30°C (grey profiles) or at 4°C (open profiles) as negative control. Surface binding of BSA or Man-BSA was quenched with trypan Blue. Cells were analyzed by flow cytometry. One representative experiment out of three is shown.

BDCA3^{hi} DC selectively express TLR1, TLR3 and TLR10

DC are able to recognize different PAMPs using highly conserved PRRs, which include the TLRs. To date, 10 TLRs have been described (TLR1-10) and studies with human DC subsets have shown that plasmacytoid DC express TLR7, 8 and 9. In contrast, myeloid DC express TLR1, 2, 3, 4, 5, 6 and 7.[9,10] Therefore, we questioned whether BDCA3^{hi} DC show a particular TLR expression profile. To this aim, highly purified (FACS-sorted, >99% pure) freshly isolated BDCA1^{+}, BDCA3^{hi} and BDCA4^{+} DC were analyzed for their expression of TLR1, 2, 3, 4, 5, 9 and 10 by real-time PCR. Figure 3 shows that BDCA3^{hi} DC selectively expressed TLR1, 3 and 10. In addition BDCA1^{+} DC selectively expressed TLR1, 2, 3, 4 and 5 mRNA, BDCA4^{+} DC selectively express TLR9, confirming previous reports.[9,10]

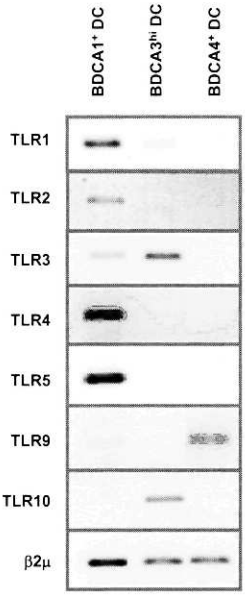

Figure 3. BDCA3^{hi} DC selectively express TLR1, TLR3 and TLR10 mRNA. BDCA1^{+}, BDCA3^{hi} and BDCA4^{+} DC subsets were isolated with MACS followed by FACS-sorting in order to obtain highly purified (>99%) DC populations. After lysis, RNA was extracted and mRNA for TLR1, 2, 3, 4, 5, 9, and 10 was detected by real-time PCR using a Light Cycler. PCR products were visualized on a 1% agarose gel containing ethidium bromide.

BDCA3^{hi} DC mature after culture, stimulation with TNF-α/IL-1β poly I:C

Upon stimulation by pathogens or pathogen-induced tissue factors, DC undergo a process of maturation that is characterized by the enhanced expression of the costimulatory molecules CD80 and CD86, HLA-DR, CD40 and CD83.[1] To study the capacity of BDCA3^{hi} DC to

mature, the expression of CD86, HLA-DR and CD83 was analyzed directly after isolation of the cells, as well as after overnight (o/n) culture in the presence of growth factors (GF) GM-CSF (for BDCA1$^+$ and BDCA3^{hi} DC) or IL-3 (for BDCA4$^+$ DC), or with maturation factors IL-1β/TNF-α (MF), or with poly I:C. BDCA4$^+$ DC were additionally stimulated with the TLR9 ligand CpG-ODN. Culture of BDCA3^{hi} DC readily upregulates CD86 and CD83 expression (Fig. 4B). However, additional priming with MF or poly I:C resulted in higher levels of CD86 and CD83 (Fig. 4C and D). The same expression pattern was observed in BDCA1$^+$ DC (Fig. 4). In contrast, additional priming with MF or poly I:C in BDCA4$^+$ DC did not result in higher levels of CD86 and CD83 expression. However, priming with CpG-ODN 2006 did induce the upregulation of CD86 (Fig. 4E). All DC subsets expressed high levels of HLA-DR directly after isolation and no further upregulation was observed after culture either in the absence or in the presence of GF or stimuli (data not shown).

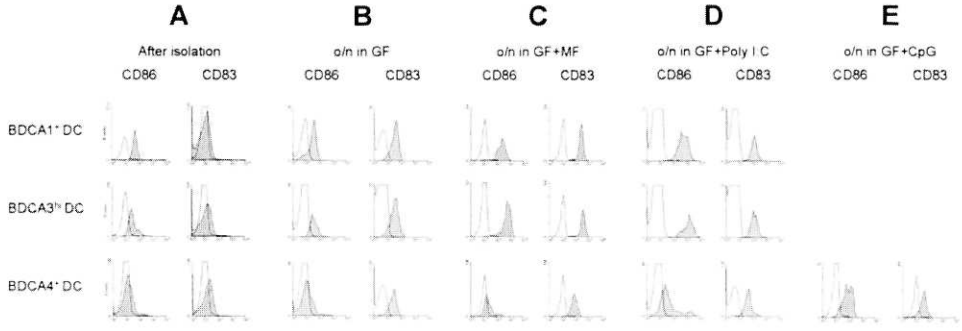

Figure 4. BDCA3^{hi} DC mature after culture, stimulation with IL-1β/TNF-α or with poly I:C. BDCA1$^+$, BDCA3^{hi} and BDCA4$^+$ DC subsets were analyzed for their expression of CD86 and CD83 by flow cytometry directly after isolation (A), after overnight (o/n) culture in their respective GF, GM-CSF (500 U/ml) for BDCA1$^+$ and BDCA3^{hi} cells and IL-3 (10 ng/ml) for BDCA4$^+$ cells (B), or with GF plus maturation factors (MF) IL-1β (10 ng/ml) and TNF-α (50 ng/ml) (C) or with GF and poly I:C (20 μg/ml) (D). Additionally, BDCA4$^+$ DC were stimulated with CpG-ODN 2006 (0.5 μM) (E). One representative experiment out of four is shown.

BDCA3^{hi} DC have the capacity to induce naive T cell proliferation

Since the stimulation of BDCA3^{hi} DC resulted in the upregulation of CD86 and CD83, we next analyzed to what extent these cells are potent activators of naive Th cell proliferation. To this aim, BDCA1$^+$, BDCA3^{hi} and BDCA4$^+$ DC were matured with MF for 24 h and subsequently cocultured with allogeneic naive Th cells. After 5 days the proliferation of the Th cells was measured by ^{3}H-thymidine incorporation. As shown in Fig. 5, both BDCA1$^+$ and BDCA3^{hi} DC have the capacity to induce proliferation in naive Th cell precursors. In contrast,

BDCA4$^+$ DC and control monocytes were poor naive Th cell stimulators. As expected, freshly isolated BDCA1$^+$, BDCA3^{hi}, or BDCA4$^+$ DC did not induce naive Th cell proliferation, probably since they express low levels of costimulatory molecules (data not shown).

Cytokine production by BDCA3^{hi} DC

An important function of DC is the activation and polarization of naive Th cells.[1] Several DC-derived cytokines may promote the development of Th1 or Th2 cells. In particular, the cytokines IL-12p70 and IFN-α are potent inducers of Th1 development.[24] Previously, it has been demonstrated that myeloid BDCA1$^+$ DC are the main producers of IL-12p70, whereas plasmacytoid BDCA4$^+$ DC predominantly produce IFN-α.[10,25] Therefore, we addressed the question whether BDCA3^{hi} DC as well show a particular cytokine secretion profile in response to the PAMPs e.g. the TLR4 ligand LPS, the TLR3 ligand dsRNA (poly I:C) or the TLR9 ligand CpG-ODN 2006. Although the TLR specificity is unknown, fixed *Staphylococcus aureus* Cowan strain (SAC) was included in these experiments, since preliminary experiments have shown that SAC potently activates both BDCA1$^+$ and BDCA4$^+$ DC. Whereas BDCA1$^+$ DC are the main producers of IL-12p70, IL-6, TNF-a and IL-10 and BDCA4$^+$ DC produce high levels of IL-6 and IFN-α, BDCA3^{hi} DC produce IL-6 and only low levels of IL-12p70 in response to dsRNA (Fig. 6). Similar low levels of IL-6 and TNF-α were detected in BDCA3^{hi} DC in response to LPS or SAC, whereas no cytokines were produced in response to CpG-ODN. Strikingly, BDCA4$^+$ DC, which do not express TLR3 (Fig. 3), produce in response to the TLR3 ligand poly I:C high levels of IFN-α and IL-6 (Fig. 6). In addition, both SAC and CpG-ODN 2006 stimulation of BDCA4$^+$ DC induced the production of IFN-α, IL-6 and TNF-α at various levels. LPS-stimulated BDCA4$^+$ DC produce low levels of IL-6 and TNF-α, but no IFN-α (Fig. 6). IL-10 is selectively produced by BDCA1$^+$ DC in response to LPS, SAC or poly I:C (Fig. 6). Except for IL10, the cytokine secretion pattern of BDCA3^{hi} DC in response to the different PAMPs tested is comparable to the secretion pattern observed in BDCA1$^+$ DC, albeit the cytokines are produced at lower levels.

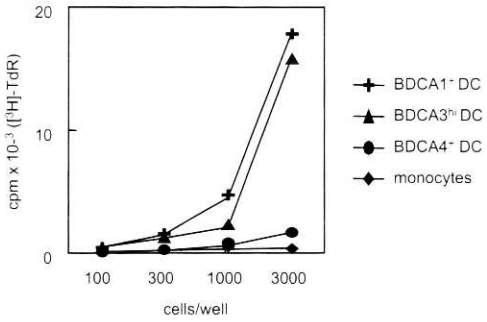

Figure 5. BDCA3^{hi} DC have the capacity to induce naive T cell proliferation. To analyze the capacity of the three DC subsets and monocytes (used as negative control) to induce proliferation in allogeneic naive Th cells, the different DC subsets were cultured for 24 h in MF (IL-1β, 10 ng/ml and TNF-α, 50 ng/ml). Subsequently, the DC subsets and monocytes were cocultured at different numbers with naive Th cells (25.000 cells/well). After 5 days, the cells were pulsed during the last 16 h with [^{3}H]thymidine ([^{3}H]-TdR). Data are expressed as counts per minute (cpm). One representative experiment out of two is shown.

BDCA3^{hi} DC have the capacity to polarize naive Th cells

It has been proposed that distinct DC lineages differentially induce Th1 and Th2 cell responses in default conditions, e.g. in the absence of pathogen- or tissue-derived molecules that affect the Th cell-polarizing capacity of DC.[25] However, the functions of these DC lineages are rather flexible, since their ability to induce the development of particular Th cell subsets may depend on, amongst others, the microenvironment and on the type of invading pathogen.[4,6] Therefore, we determined which Th cell cytokine profile is induced by BDCA3^{hi} DC matured in response to MF or poly I:C. As reported before,[23] priming of BDCA1$^+$ DC in default maturation conditions (in the present experiments priming by MF), compared to priming of BDCA4$^+$ DC, results in the predominant development of IFN-γ-producing Th cells (Fig. 7). Interestingly, MF-primed BDCA3^{hi} drive an intermediate Th1/Th2 cell profile.

The priming of the three DC subsets with poly I:C always resulted in the development of DC that promote the development of IFN-γ-producing Th1 cells, although the frequency of the Th1 cells is different. BDCA1$^+$ DC is the most potent inducer of Th1 responses, followed by BDCA3^{hi} DC and BDCA4 DC (Fig. 7). The DC-derived factors responsible for the observed Th1 development are currently being investigated.

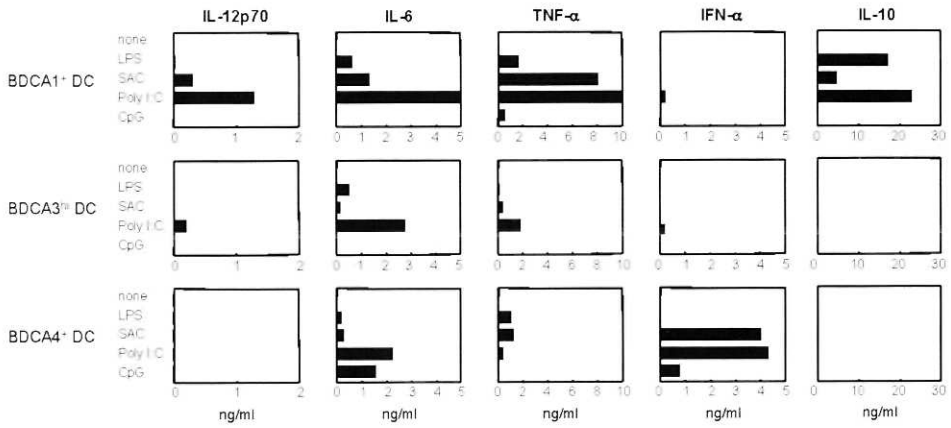

Figure 6. Cytokine production by BDCA1$^+$, BDCA3^{hi} and BDCA4$^+$ DC subsets in response to various stimuli. BDCA1$^+$, BDCA3^{hi} and BDCA4$^+$ DC were left unstimulated or stimulated with LPS (100 ng/ml), SAC (75 μg/ml), poly I:C (20 μg/ml or CpG-ODN (0.5 μM). After 24 h, supernatants were harvested and analyzed for the presence of IL-12p70, IL-6, TNF-α, IFN-α and IL-10 by specific ELISAs. Results are from a representative experiment out of four.

In vivo localization of BDCA3$^+$ cells: preliminary data

Although BDCA3^{hi} DC are present in peripheral blood in low frequencies,[21] the localization of these cells in lymphoid and peripheral tissues remains to be established. Therefore, we analyzed the distribution of the three DC subsets by immunohistochemistry of human tonsils, spleen, thymus and skin by using specific antibodies directed against BDCA1, BDCA3 and BDCA4 antigens. As show in Figure 8A (upper panel), BDCA3$^+$ DC are present close to the mantle zone (MZ) of the tonsil follicles in contact with the B cell area, but the staining pattern does not clearly reveal a dendritic morphology. Brightly stained BDCA1$^+$ cells with dendritic morphology are located in the T cell-rich interfollicular areas (Fig. 8A, upper panel). BDCA4$^+$ cells with dendritic morphology are detected in human tonsils as well, in a scattered pattern in the T cell-rich interfollicular areas (Fig. 8A, upper panel), as previously described for BDCA2$^+$ cells.[26] However, their exact distribution is difficult to establish, since high endothelial venules are stained with BDCA4 antigen as well (HEV and see arrows, Fig. 8A, upper panel).

In the spleen, BDCA1 expression was mainly found in the B cell area, although some staining was detected in the periarteriolear lymphoid sheath (PALS, Fig. 8A, middle panel). In contrast, chain-like patterns of BDCA3$^+$ cells were found in the PALS, as well as near the marginal zone around the B cell area. In addition, similar patterns of intensive BDCA3

staining were detected in the red pulp (RP) where the sinuses are (Fig. 8A, middle panel). BDCA4 staining was detected in the PALS and near the marginal zone (Fig. 8A, middle panel). Negative controls using isotype control antibodies resulted in negative staining (data not shown).

All three BDCA antigens are also detected in the thymus, and again different staining patterns are observed. Scattered BDCA1$^+$ cells were found within the inner medulla of the lobuli, which contains the mature T cells. In addition, intensive staining was detected in the outer cortex, in a pattern that suggests the weak staining of the immature thymocytes and the bright staining of occasional DC (Fig. 8A, lower panel). Occasional BDCA1$^+$ cells were found in the connective tissue between the lobuli of the thymus as well (Fig. 8A, lower panel), which may represent true DC. In contrast to the widely spread BDCA1 antigen expression, only few BDCA3$^+$ DC with dendritic morphology are found in the thymus. These cells are located both in the connective tissue between the lobuli and within the lobuli (Fig. 8A, lower panel). BDCA4 antigen was expressed in a similar pattern, albeit that higher numbers of cells were found (Fig. 8A, lower panel). No red staining was found in sections stained with control mAb (data not shown).

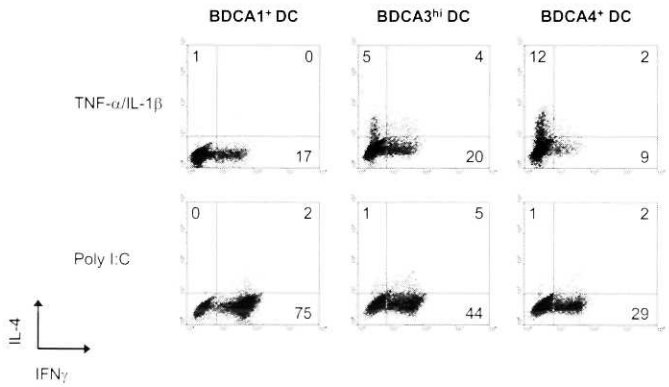

Figure 7. BDCA3^{hi} DC have the capacity to polarize naive Th cells. MF or poly I:C-mature BDCA subsets ($1x10^4$ cells/well) were loaded with SEB (100 pg/ml) and cocultured with naive Th cells ($4x10^4$). After 14 days, resting memory Th cells were stimulated for 6 h with PMA (10 ng/ml) and ionomycin (1 μg/ml) in the presence of brefeldin A (10 μg/ml). The expression of IL-4 and IFN-γ was assessed by intracellular staining. Results are expressed as the percentage of cells in each population. Data are representative of three independent experiments.

Analysis of the three BDCA antigens in human skin revealed that BDCA1$^+$ and BDCA3$^+$ cells are present in both the epidermis and in the dermis (Fig. 8B). By contrast, BDCA4$^+$ cells

are only detected in the dermis. Double staining of BDCA1 (in blue) and BDCA3 (in red) antigens in human tonsils and skin clearly shows that these two antigens are not expressed on the same cell and distinguish two different cell populations (Fig. 8C).

Further identification of the BDCA$^+$ cells as genuine DC is currently performed by double staining using antibodies against langerin, HLA-DR and the myeloid marker CD11c and the plasmacytoid marker IL-3Rα.

DISCUSSION

Human peripheral blood contains several subsets of DC.[19,21] While myeloid BDCA1$^+$ and plasmacytoid BDCA4$^+$ DC subsets have been extensively studied,[9,10] the function of the other subsets remains elusive. Here, we describe the functional characteristics of the blood myeloid DC subset with a high expression of BDCA3 antigen (BDCA3^{hi}), and compared this subset to the characteristics of BDCA1$^+$ and BDCA4$^+$ DC. We show that BDCA3^{hi} DC selectively express mRNA for TLR1, 3 and 10, they have the capacity to mature in response to poly I:C and TNF-α/IL-1β (MF), and produce only low levels of IL-12p70 and IFN-α in response to poly I:C. When matured in neutral conditions, BDCA3^{hi} DC do not bias the development ofTh1/Th2 cells. In contrast, when they are primed by poly I:C, they promote Th1 responses, indicating their flexibility to adopt a Th1-polarizing phenotype. In addition, a preliminary immunohistochemical analysis demonstrated the presence of BDCA3$^+$ cells with dendritic morphology in both lymphoid as non-lymphoid tissues. However, in the thymus only few BDCA3$^+$ cells could be detected. In human skin, BDCA3$^+$ cells were found in both epidermis and dermis.

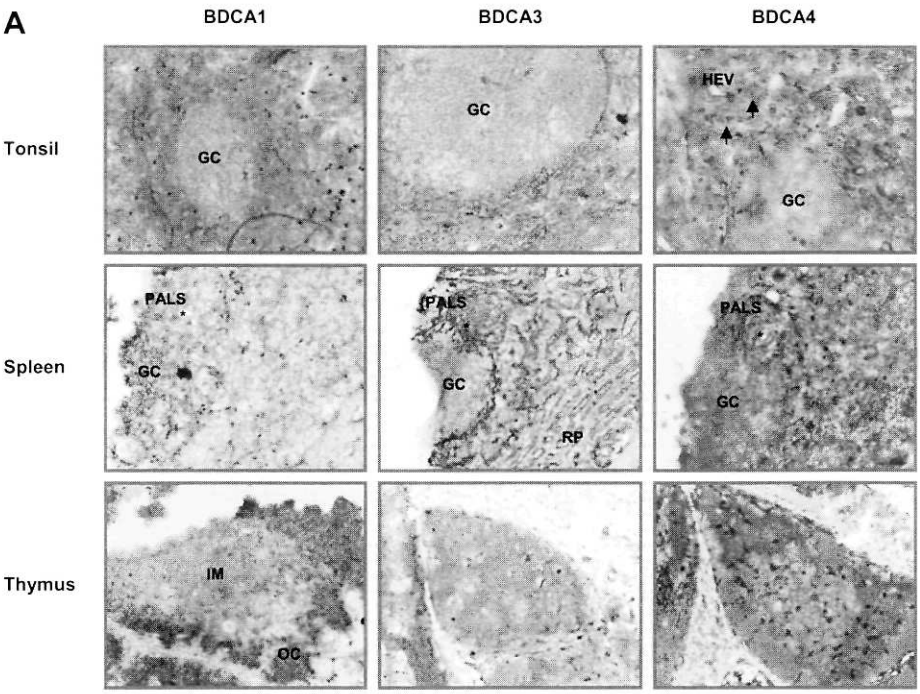

Figure 8. *In vivo* localization of BDCA3$^+$ cells. Cryosections of human tonsil, spleen, thymus (A) and skin (B) (see next page) were stained with mAb directed against BDCA1, BDCA3 and BDCA4 antigens. Staining was detected by AEC and counterstained by hematoxylin. (C) (see next page) Double staining of BDCA1 (in blue) and BDCA3 (in red). The magnifications used are indicated in the respective figures. GC, germinal center; HEV, high endothelial venules; PALS, periarteriolar lymphoid sheath; B, B cell area; RP, red pulp; *, central arteriole; IM, inner medulla; OC, outer cortex. See next page for Figures B and C. (☞ page 162).

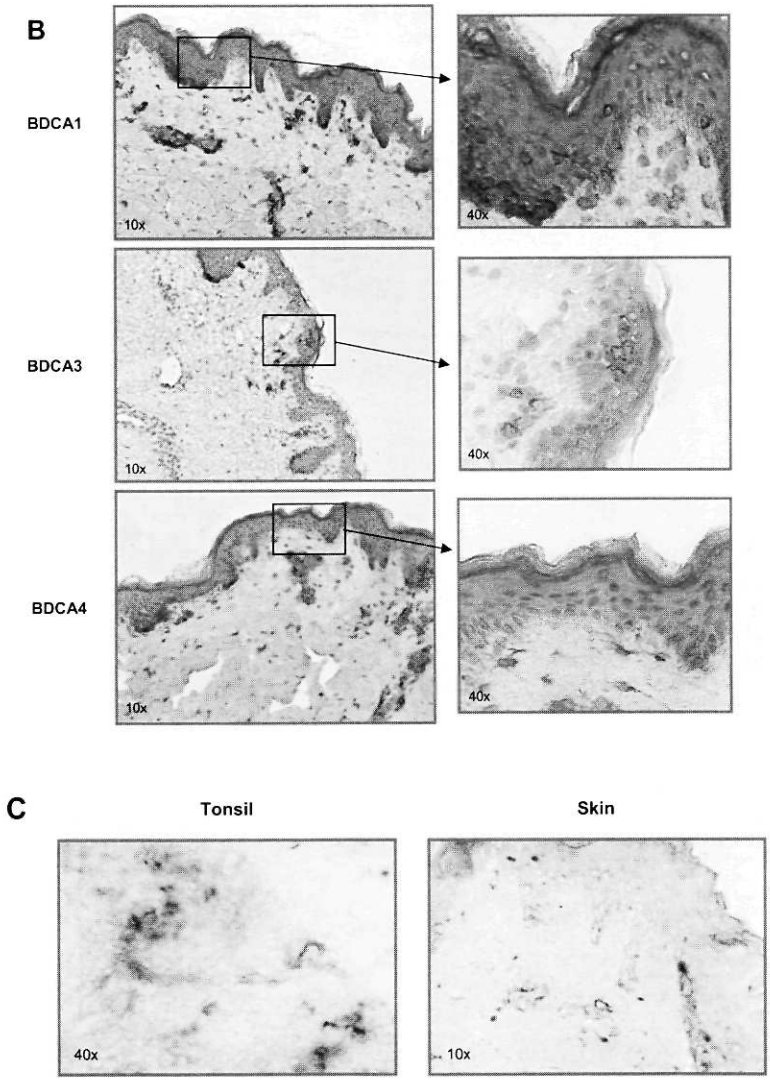

Figure 8. *In vivo* localization of BDCA3$^+$ cells (cont.). Cryosections of human tonsil, spleen, thymus (A) (see previous page) and skin (B) were stained with mAb directed against BDCA1, BDCA3 and BDCA4 antigens. Staining was detected by AEC and counterstained by hematoxylin. (C) Double staining of BDCA1 (in blue) and BDCA3 (in red). The magnifications used are indicated in the respective figures. GC, germinal center; HEV, high endothelial venules; PALS, periarteriolar lymphoid sheath; B, B cell area; RP, red pulp; *, central arteriole; IM, inner medulla; OC, outer cortex. (☞ page 163).

Although BDCA3$^+$ DC are also of myeloid origin[20] the preliminary data show that this subset is characterized by a distinct anatomical localization, suggesting a distinct function. Such a different function was underscored by the fact that these cells express a TLR profile

different from the other DC subsets, indicating a specialized role in the defense against invading pathogens. The exclusive expression of TLR10, of which the ligands are unknown,[27] by BDCA3^{hi} DC is intriguing. The identification of TLR10 ligands will offer the opportunity to selectively target BDCA3^{hi} DC without affecting other myeloid or plasmacytoid DC. Stimulation of BDCA3^{hi} DC with poly I:C resulted in high levels of IL-6, indicating a normal susceptibility of these cells to poly I:C, but only low levels of IL-12, IFN-α and TNF-α. The pattern of cytokine production is in real contrast to the pattern of cytokines produced by BDCA1^{-} DC, that these cells readily produce high levels of IL-10, whereas no IL-10 could be detected in BDCA3^{hi} DC. Since only BDCA1^{-} DC are capable of producing high levels of IL-10 in response to the stimuli tested, it may be speculated that these cells have a role in the IL-10-dependent maintenance of peripheral tolerance by regulatory T cells.[28] It should be noted here that, while IL-10 may have a role in the maintenance of tolerance, IL-10 is probably not the major factor in the induction of regulatory T cells[29] (and H.H. Smits *et al.*, unpublished observations), allowing BDCA1^{+} DC to be very efficient in the induction of protective Th1 or Th2 cells. A surprising finding in these comparative experiments was that the TLR3 ligand poly I:C could induce considerable levels of IL-6 and IFN-α production in BDCA4^{+} DC, whereas these cells lack the expression of TLR3[9,10] (and Fig. 3), suggesting that poly I:C may signal via other PRR, at least in this DC subset.

Stimulation of BDCA3^{hi} DC with poly I:C resulted in their development into effector DC1 that potently promote the development of IFN-γ-producing Th cells from naive Th precursors. These data indicate that BDCA3^{hi} DC may express Th1-promoting factors, depending on the way they have been primed. The DC-derived factors that may drive these Th1 responses are currently being investigated and may be IL-12,[24] IFN-α,[30] IL-18,[31] IL-23[32] or ICAM-1,[33] since they all have been implicated in inducing Th1 responses.

Previously it has been proposed that a bias to the development of Th1 or Th2 responses may also depend on the DC lineage.[34] In this respect, myeloid DC promote the development of Th1 cells whereas plasmacytoid DC promote the development of Th2 cells when tested in conditions of default maturation, i.e. maturation induced by non-polarizing compounds. Here we show that BDCA3^{hi} DC maturing in neutral conditions initiate an Th cell response that is only slightly Th1-biased, when compared to their myeloid counterpart, the BDCA1^{+} DC. In a recent report, the differences in the capacity of these DC subsets in default conditions to bias the Th1/Th2 development could be related to their capacity to present antigen, since their Th cell polarizing capacity was equalized by adjusting the antigen dose.[35] Both myeloid and

plasmacytoid DC are highly plastic in their responses to infection and soluble factors.[4,5,8] Upon maturation by poly I:C also the BDCA3^{hi} DC subset show an enhanced induction of Th1 responses as well, indicating a similar plasticity for this DC subset. However, in these priming conditions, the percentages of IFN-γ-producing Th cells are lower in BDCA3^{hi} DC compared to BDCA1^{+} DC. This data is in line with the lower capacity of BDCA3^{hi} DC to produce IL-12 and IFN-α compared to BDCA1^{+} and BDCA4^{+} DC respectively.

The observation that both BDCA1^{+} and BDCA3^{+} DC are present in both epidermis and dermis during steady-state conditions suggests that both subsets play a role in immune surveillance. It is tempting to speculate that BDCA1^{+} and BDCA3^{+} DC present in the dermis may represent a pool of LC in steady-state conditions, as recently described,[36] or two distinct dermal DC populations. It would be interesting to know whether the culture of these two myeloid subsets in the presence of TGF-β[19] or IL-15[37] will lead to the acquisition LC characteristics, such as the expression of langerin.[38] This issue is currently being investigated. BDCA4^{+} DC were also detected in the skin but exclusively in the dermal compartment. Altogether, these observations suggest the existence of three subsets of dermal DC that may be important in cutaneous immune responses.

In summary, the present comparative study suggests that BDCA3^{hi} DC, due to their narrow TLR expression profile, has a more limited capacity to recognize pathogens, but are as flexible in their capacity to bias the development of Th1/Th2 cells. Their tissue distribution, which is probably slightly different from the distribution of the other DC subsets, may further support a distinct role in the protection against invading pathogens and the maintenance of homeostasis.

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CHAPTER 6

Reduced production of bioactive IL-12 and IFN-α by peripheral blood dendritic cells in atopic dermatitis patients

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Manuscript in preparation

ABSTRACT

Atopic dermatitis (AD) is a chronic inflammatory skin disorder associated with a generalized bias of Th2 cells producing the cytokines IL-4, IL-5 and IL-13. As the Th1/Th2 balance depends on signals provided by dendritic cells (DC) we questioned whether circulating DC in AD patients, compared to DC from healthy controls, differ in their phenotype and function. Purified BDCA1$^+$ myeloid DC from AD patients showed a selective and dramatic reduction of IL-12p70 release, a cytokine that promotes the development of Th1 cells. Message (m)RNA analysis indicated that the decreased ability of BDCA1$^+$ DC to produce IL-12p70 is likely to be due to a defective expression of both IL-12p35 and IL-12p40 subunits. Accordingly, even after maturation in the presence of an extremely potent Th1 stimulus (double stranded (ds)RNA plus IFN-γ), BDCA1$^+$ DC from AD patients induced considerably less IFN-γ-producing Th cells, compared to BDCA1$^+$ DC from healthy controls. This reduced capacity of BDCA1$^+$ DC could neither be attributed to a deficiency in the expression of class II MHC or costimulatory molecules nor to a reduced capacity of these cells to induce T cell proliferation. Moreover, analysis of peripheral blood BDCA4$^+$ plasmacytoid DC from AD patients demonstrated that BDCA4$^+$ DC produce significantly lower levels of IFN-α, which is essential in natural immunity to virus and bacterial infections. The defective IL-12 and IFN-α production by DC may contribute to the maintenance of the allergic state in AD patients, as well as their increased susceptibility to skin infections with pathogens that require protective IFN-α and Th1 cell responses.

INTRODUCTION

Atopic dermatitis (AD) is a eczematous skin disease that is characterized by elevated levels of total and antigen-specific IgE and IgG4, by increased numbers of blood eosinophils, increased numbers of Langerhans cells (LC) and T cells in lesional skin.[1,2] AD has been associated with the activation of peripheral blood T cells that secrete T helper (Th) type 2 (Th2) cytokines (IL-4, IL-5 and IL-13) favoring the differentiation of eosinophilic granulocytes and supporting the production of high amounts of IgE.[1,2] In addition, a biphasic model for AD skin has been proposed, in this model a shift from a Th2 type response in the initiation phase to a Th1/Th0 response in the late and chronic phase (when IFN-γ production predominates) is observed.[3] A major issue of current study is the definition of molecular and histophysiological mechanisms underlying the preferential development of Th2 cells in AD, taking into account

that the development of a particular Th response is mediated by signals derived from antigen presenting cells (APC).

Dendritic cells (DC) are professional APC that have a central role in the initiation and regulation of immune responses both in lymphoid and non-lymphoid tissues. There is however considerable intra- and inter-tissue variation in the phenotype, morphology, function, and tissue localization of different DC populations.[4] Human blood DC have been recently divided into five distinct subsets, CD1b/c$^+$, CD16$^+$, BDCA3$^+$, CD123$^+$ and CD34$^+$ DC.[5] In particular, the so-called myeloid DC (mDC) which are CD1c$^+$(BDCA1)CD11c$^+$CD45RO$^+$CD123^{low} (IL-3R α-chain) have the ability to produce IL-12 in response to bacterial compounds or CD40L, and require GM-CSF for survival.[6] Conversely, plasmacytoid DC (PDC) are BDCA4$^+$CD11c$^-$CD45RA$^+$CD123^{high} and require the presence of IL-3 for survival.[7,8] Upon herpes or influenza virus infection, PDC, but not the mDC, produce high amounts of type I IFNs, IFN-α and IFN-β.[9,10] These cells are of major importance during viral infections, when they are massively recruited to the site of infection and serve as an important source of antiviral type I IFNs.[11] Host defense molecules such as type I IFNs play a vital role in innate resistance to a wide variety of infectious agents through the induction of direct, cell-autonomous resistance to viral and microbial pathogens.[12] In addition, type I IFNs have been increasingly recognized as essential early warning molecules signaling the presence of pathogens, providing a pivotal function at the interface between innate and adaptive responses.[12]

Although it has been described that IFN-α therapy decreases IL-4-mediated IgE synthesis in AD patients[13,14] it remains to be established whether IFN-α production levels in AD patients are similar or not compared to healthy controls.

The production of IL-12 by DC, triggered by their exposure to microbial products or their interaction with activated T cells, is known to play a critical role in the induction of Th1-mediated immune responses.[15] Bioactive IL-12 is a heterodimeric cytokine composed of two subunits, p35 and p40, encoded by different genes. Both subunits must be expressed in the same cell to generate the bioactive form, p70, of the cytokine.[16] The notion that dysregulated IL-12 levels may be important in allergy is supported by several studies of human asthmatic and AD patients. It was demonstrated that the number of IL-12p40-expressing cells in bronchial biopsy specimens from allergic asthmatics was significantly less then that found in the lungs of normal control subjects.[17] Moreover, it was shown that bioactive IL-12 production in whole blood cultures of patients with allergic asthma[18] and in monocytes of

patients AD[19] is significantly lower compared to control subjects, and is associated with decreased IFN-γ production by Th cells.[18] These studies may indicate an aberrant function of APC in allergic diseases, but to date no studies have been performed with the most relevant APC, the DC.

Dysfunction of DC subsets in AD patients could be associated with aberrant expression of cytokines/cell surface molecules that in turn may be responsible for the preferential development of Th2 cells observed in this skin disease. To gain insight into the *in vivo* characteristics of DC, we investigated whether peripheral blood DC, the stage in the DC life cycle that immediately precedes recruitment into the skin or into other non-lymphoid or lymphoid tissues, are different in AD patients compared to healthy, nonatopic subjects. Here we show that $BDCA1^+$ myeloid DC from AD patients expressed significant lower levels of IL-12 and TNF-α, in response to double-stranded RNA, poly I:C plus IFN-γ, and induced the development of T cells with reduced capacity to produce IFN-γ. In addition, $BDCA4^+$ plasmacytoid DC from AD patients produced lower levels of IFN-α in response to the bacterial product *Staphylococcus aureus* Cowain strain I (SAC). These data suggest that in AD, blood DC may also critically contribute to the development of the atopic phenotype by producing reduced levels of the Th1-associated cytokines, IL-12 and IFN-α.

MATERIAL AND METHODS

Subjects

Peripheral heparinized blood samples were obtained from 17 patients with AD (see table I for characteristics), who were diagnosed on the basis of the Millennium criteria for AD.[20] None of the patients studied had been treated with systemic glucocorticoids and no topical corticosteroids were used for 1-2 weeks before their blood donation. As control group 16 healthy subjects aged-matched (total serum IgE <50 kU/L) and with no history of AD, allergic rhinitis, or asthma were studied. Donation of blood and skin biopsies by patients and healthy controls followed approval by the ethical committee of the Academic Medical Center (Amsterdam, The Netherlands). All patients and normal subjects gave written informed consent.

Isolation and culture of BDCA1+ and BDCA4+ DC

Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation with Lymphoprep (Nycomed, Torshov, Norway). Myeloid DC, BDCA1$^+$ and plasmacytoid, BDCA4$^+$ DC were isolated from PBMC by magnetic cell sorting (MACS) using a high gradient MACS device (Mini-MACS^R; Miltenyi Biotec, Bergisch, Gladbach, Germany). Briefly, CD19$^+$ B cell-depleted PBMC were incubated with BDCA1-PE monoclonal antibody (mAb) and BDCA4-PE mAb followed by anti-PE magnetic beads (Miltenyi Biotec). To analyze the purity of the obtained BDCA1$^+$ and BDCA4$^+$ cell populations, the cells were incubated with a cocktail of FITC-conjugated mAb specific for CD3, CD14, CD16, CD19, CD20, CD34 and CD56 receptors (lineage, lin; BD Bioscience). The purity of each cell population was >90%. Purified BDCA1$^+$ and BDCA4$^+$ DC were cultured in Iscove's modified Dulbeccos's medium (IMDM; Life Technologies, Paisley, U.K.) containing 10% FCS (HyClone, Logan, UT). While BDCA1$^+$ DC were cultured in medium supplement with GM-CSF (500 U/ml; Schering-Plough, Uden, The Netherlands), BDCA4$^+$ DC were cultured in the presence of IL-3 (10 ng/ml; Strathmann Biotech, Hannover, Germany).

Table I. Characteristics of patients with AD

Patient	Age	Sex	Sum of affected area (%)	Other allergies	Prick test	total IgE
1	50	M	11.4	No	+	151
2	26	M	NA	Asthma	+	NA
3	20	F	NA	No	+	NA
4	20	F	NA	Asthma, rhinitis	+	NA
5	26	F	9.7	No	+	NA
6	44	M	9.5	Asthma	+	2566
7	40	M	11.8	Rhinitis	+	134
8	31	M	63	No	+	1143
9	34	M	10.5	Hay fever	+	NA
10	48	M	16.5	Asthma	+	NA
11	52	M	63	Asthma	+	9572
12	31	M	23.1	Hay fever, asthma	+	999
13	36	M	5.8	Hay fever, rhinitis	+	5082
14	29	M	42	Hay fever, asthma	+	49535
15	45	M	56.6	Asthma	+	NA
16	29	M	7.7	Asthma	+	9000
17	24	M	27.5	Asthma, rhinitis	+	2747

Stimulation of BDCA1+ and BDCA4+ DC

BDCA1$^+$ and BDCA4$^+$ DC were stimulated ($2x10^4$ cells/200 µl; 96-well plates, Costar, Cambridge, MA) by fixed *Staphylococcus aureus* Cowan strain I (SAC, 75 µg/ml; Calbiochem, San Diego, CA), synthetic double-stranded (ds)RNA polyriboinosinic

polyribocytidylic acid (poly I:C, 20 μg/ml; Sigma-Aldrich, St. Louis, MA), CD40L-transfected J558 cell line ($2x10^4$ cells; a gift of Dr. P. Lane, University of Birmingham, Birmingham, U.K.) alone or in combination with rhuIFN-γ (1000 U/ml; a gift of Dr. P.H. van der Meide, U-CyTech, Utrecht, The Netherlands). Supernatants were harvested after 24 h. The levels of IL-12p70, IL-12p40, IFN-α, TNF-α, IL-6 and IL-10 were measured by ELISA (see below).

Analysis of cell surface molecules expression by FACS

To analyze the phenotype of circulating DC, freshly isolated or matured BDCA1$^+$ DC were incubated with FITC-conjugated mAb against CD86 (mouse IgG1; BD Pharmingen, San Diego, CA), allophycocyanin (APC)-conjugated anti-CD83 mAb (mouse IgG2b; Caltag Laboratories, Barlingame, CA) and PerCP-conjugated anti-HLA-DR mAb (mouse IgG2b; BD Bioscience, San Jose, CA). As controls, cells were stained with corresponding isotype-matched control mAb (values of their mean fluorescence intensity (MFI), were subtracted to each value of the appropriate fluorochrome-conjugated mAb, delta (Δ)MFI). Stained cells were analyzed using a four-color cytometer (FACSCalibur, Becton Dickinson).

Isolation of CD4$^+$CD45RA$^+$CD4RO$^-$ naive Th cells (ThN)

ThN were isolated from peripheral blood leukocytes with the negative selection human CD4$^+$/CD45RO$^-$ column kit (R&D Systems, Minneapolis, MN). This method yielded highly purified (>98%) CD4$^+$CD45RA$^+$CD45RO$^-$ ThN as assessed by flow cytometry (data not shown).

Analysis of proliferation of T cells cocultured with BDCA1$^+$ DC in a mixed lymphocyte reaction (MLR)

Poly I:C plus IFN-γ-matured BDCA1$^+$ myeloid DC where tested for their ability to stimulate allogeneic ThN in a MLR. ThN ($2.5x10^4$ cells/200 μl) were cocultured in 96-well flat-bottom culture plates with different concentrations of matured DC. After 5 days, cell proliferation was assessed by the incorporation of [^{3}H]thymidine ([^{3}H]-TdR, Radiochemical Centre, Amersham, Little Chalfont, U.K.) after a pulse with 13 KBq/well during the last 16 h, as measured by liquid scintillation spectroscopy.

Induction of Th1 or Th2 cell responses by mature BDCA1+ DC

ThN ($8x10^4$ cells/200 µl) were cocultured in 96-well flat-bottom culture plates with allogeneic BDCA1+ DC ($2.0x10^4$ cells/200 µl), matured for 24 h in the presence of poly I:C plus IFN-γ, which were coated with the superantigen *Staphylococcus aureus* enterotoxin B (SEB, 100 pg/ml; Sigma-Aldrich). On day 5, IL-2 (10 U/ml; Cetus, Emeryville, CA) was added and the cultures were further expanded. After 14 days, resting memory Th cells were harvested, washed, and restimulated for 6 h with PMA (10 ng/ml; Sigma-Aldrich) and ionomycin (1 µg/ml; Sigma-Aldrich) in the presence of brefeldin A (10 µg/ml; Sigma-Aldrich) in order to analyze Th cell cytokine production profile at the single cell basis. The cells were fixed in parafolmadehyde (2%; Sigma-Aldrich), permeabilized with saponin (0.5 %; Sigma-Aldrich), and labeled with FITC-coupled IFN-γ mAb (Becton Dickinson) and PE-coupled IL-4 mAb (Becton Dickinson). The cells were evaluated by FACScan (Becton Dickinson).

Generation of monocyte-derived DC and their stimulation

Immature monocyte-derived DC (mo-DC) were generated from monocytes in cultures performed in IMDM (Life Technologies) containing 1% FCS (HyClone), rhuGM-CSF (500 U/ml; Schering-Plough, Uden, The Netherlands) and rhuIL-4 (250 U/ml; PBH), as described.[21,22] Immature DC were stimulated ($2x10^4$ cells/200 µl; 96-well plates, Costar) by fixed SAC, poly I:C or CD40L-transfected J558 cell line alone or in combination with rhuIFN-γ, as stated above. Supernatants were harvested after 24 h and the levels of IL-12p70 were measured by ELISA (see below).

Quantification of IL-12 p35, p40 and β2-microglobuline mRNA levels by real-time PCR

BDCA1- DC ($4.0x10^5$) were stimulated for 6h in the presence or in the absence of the following stimuli: poly I:C plus IFN-γ or SAC. After lyzing the cells total RNA was purified by using the NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany) according to manufacture's instructions. cDNA was generated using the First strand cDNA synthesis kit for RT-PCR (MBI Fermentas, St Leon-Rot, Germany). To anneal the primer to the RNA, 9 µl of total RNA, 1 µl oligo$(dT)_{18}$ and 1 µl $D(N)_6$ were added. This mix was then heated for 5 minutes at 94°C. Quantification of IL-12p35 and IL-12p50, and as a control β2-microglobulin (β2µ) transcripts, real-time PCR was performed in a LightCycler (Roche Diagnostics,

Mannheim, Germany) based on specific primers ands general SYBR green fluorescence detection. The primer sequences were the following: β2m sense: 5'-AAGATTCAGGTTTACTCACGTC-3'; β2μ anti-sense: 5'-TGATGCTGCTTACATGTCTCG-3' (melting temperature (Tm) 62°C); IL-12p35 sense: 5'-CTTCACCACTCCCAAAACCT-3'; IL-12p35 anti-sense: 5'-AGCTCATCACTCTATCAATAGT-3' (Tm 60°C); IL-12p40 sense: 5'-ATTGAGGTCATGGTGGATGC-3'; IL-12p40 anti-sense: 5'-AATGCTGGCATTTTTGCGGC-3' (Tm 60°C), resulting in the amplification of PCR products of 294 bp (β2μ), 532 bp (p35) and 297 bp (p40). IL-12 mRNA (p35 or p40) levels were expressed as the absolute number of copies normalized against β2μ mRNA. This was achieved by generating standard curves from serial dilutions of standards. These standards consisted in PCR products that included the IL-12p35 or IL-12p40 amplicon and that were purified following standard procedures.

Evaluation of cytokine production by ELISA

Determination of IL-12p40 and IL-12p70 concentrations in culture supernatants was performed by specific solid-phase sandwich ELISA as previously described.[22,23] Pairs of specific monoclonal antibodies (mAbs) and recombinant cytokine standards were obtained from BioSource International (Camarillo, CA) for determinations of IL-6, TNF-α and IFN-α, and from BD Pharmingen for IL-10 determination. The detection limits of these ELISA are as follows: IL-6, 20 pg/ml, TNF-α, 20 pg/ml, IFN-α, 100 pg/ml, IL-12p70, 3 pg/ml, IL-12p40, 100 pg/ml.

Immunohistochemical analysis of BDCA1$^+$ and BDCA4$^+$ DC in skin sections

For immunohistochemical staining, tissues specimens were embedded in Tissue-Tek (Sakura Finetek, Torrance, CA), cryopreserved in liquid N_2 and stored at -20°C. Acetone-fixed cryosections (6 μm) were prepared and incubated with specific mouse mAbs against BDCA1 (IgG2a, Miltenyi Biotec) or BDCA4 (IgG1, Miltenyi Biotec) Ags for 1 h at 37°C after blocking endogenous peroxidase activity with 0.3% H_2O_2 and 0.1% sodium azide (NaN_3), and after preincubation (15 min) with 10% normal goat serum (Dakopatts). An IgG1 or IgG2a isotype mAbs were used as negative controls. After incubation with biotinylated rabbit anti-mouse IgG (Dakopatts, Glostrup, Denmark) for 30 min at room temperature, skin sections

were rinsed with PBS and incubated for 30 min with streptavidin horseradish peroxidase-conjugated (Strep-HRP, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, CLB, Amsterdam, The Netherlands). After washing, 3-amino-9-ethylcarbazole (AEC, Sigma-Aldrich) as used as chromogen. With this procedure BDCA1$^+$ and BDCA4$^+$ DC stained red. Other cellular elements were counterstained with hematoxylin (Cellpath, Newton Powys, UK). Then the sections were dried and mounted with glycerol/gelatin.

For double immunostainings slides were incubated with 0.3% H_2O_2 and 0.1% NaN_3 followed by a preincubation (15 min) with 10% normal goat serum (Dakopatts). Then the sections were incubated for 1 h with the primary mAb against BDCA1 (IgG2a, Miltenyi Biotec) followed by incubation with FITC-coupled goat F(ab')$_2$ anti-mouse IgG and IgM (Jackson ImmunoResearch Laboratories, West Grove, PA). Then the sections were incubated with rabbit anti-FITC (Dakopatts) followed by incubation with alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Dakopatts). After blocking with normal mouse serum (10%, Dakopatts), the biotinylated primary mAb against BDCA4 (IgG2a, Miltenyi Biotec) was added to the sections followed by an incubation with Strep-HRP (CLB). AP staining was developed using naphtol AS-MX phosphate (20 mg, Sigma-Aldrich) plus levamisole (15 mg, Sigma-Aldrich) plus Fast blue BB salt (1 mg, Sigma-Aldrich) in 100 mM Tris-HC buffer, pH 8.5. With this procedure BDCA1$^+$ DC stained blue. AEC staining was performed as stated above. With this procedure BDCA4$^+$ DC stained red. Slides were analyzed with a wide field upright microscope (Leica DMRA, Wetzlar, Germany) coupled to a CCD camera and Image-Pro Plus software (Media Cybernetics, Dutch Vision Components, Breda, The Netherlands).

Statistical analysis

Data are expressed as mean ± SEM. Data were analyzed for statistical significance with the GraphPad InStat® software (version 3.00; GraphPad InStat, Inc., San Diego, CA). The nonparametric unpaired Student *t* test was used to compare variables between two groups. A *P* value <0.05 was considered as the level of significance.

RESULTS

BDCA1$^+$ and BDCA4$^+$ DC frequencies in peripheral blood from AD patients do not differ from healthy controls

To study the mechanisms underlying decreased cell-mediated immunity and IL-12 production in patients with AD, we examined the frequencies of BDCA1$^+$ and BDCA4$^+$ DC in these patients and compared to DC from healthy controls. To this aim, PBMC from healthy controls (n=16) and AD patients (n=16) were analyzed by flow cytometry after staining with mAbs against BDCA1 or BDCA4 Ags and lineage (Lin; CD3, CD14, CD16, CD19, CD20, CD34 and CD56) markers. Flow cytometry analysis of PBMC from AD patients demonstrated no significant differences in the frequency of both BDCA1$^+$ and BDCA4$^+$ DC from healthy controls compared to DC from AD patients (Fig. 1A and B).

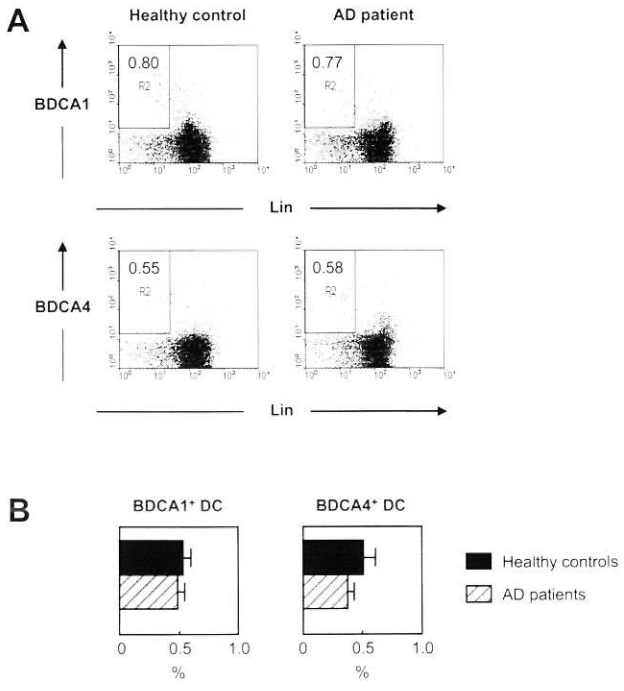

Figure 1. Frequencies of BDCA1$^+$ and BDCA4$^+$ DC in PBMC from healthy controls and AD patients. PBMC were isolated from healthy controls or from AD patients and analyzed by FACS after two-color staining with a combination of FITC-labeled mAbs against lineage markers (Lin) and PE-labeled BDCA1 or BDCA4. (A) An example of the frequency of BDCA1$^+$ and BDCA4$^+$ DC in one healthy donor and in one AD patient is shown. The numbers indicate the percentage of cells in the respective quadrant. (B) Frequency (%) of BDCA1 and BDCA4 in PBMC from healthy controls (n=16) and AD patients (n=16). Results are expressed as mean±SEM. Data were analyzed for statistical significance using a two-tailed Student's *t*-test. $*P<0.05$, $**P<0.01$, $***P<0.001$ compared to healthy controls.

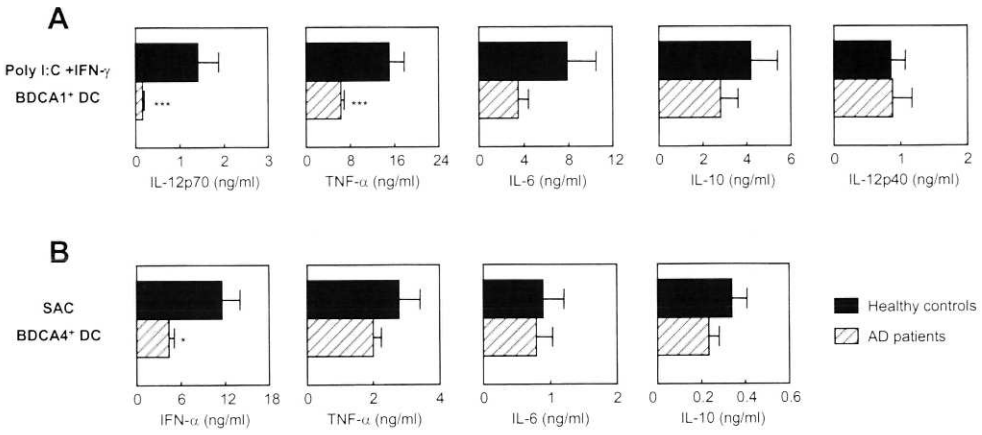

Figure 2. Cytokine production by isolated BDCA1⁺ and BDCA4⁺ DC from healthy controls and AD patients after *in vitro* stimulation with poly I:C+IFN-γ or SAC, respectively. BDCA1⁺ and BDCA4⁺ DC were isolated from PBMC by indirect magnetic labeling with PE-conjugated primary mAbs and anti-PE mAb-conjugated microbeads followed by enrichment of labeled cells by MACS. After 24 h stimulation the contents of IL-12p70, IL-12p40, TNF-α, IL-6, IL-10 or IFN-α were analyzed by specific ELISAs. (A) Purified BDCA1⁺ DC stimulated with poly I:C+IFN-γ from AD patients show reduced capacity to produce IL-12p70 and TNF-α, compared to healthy controls. (B) Purified BDCA4⁺ DC from AD patients show reduced capacity to produce IFN-α compared to healthy controls. Results represent the mean ± SEM of cytokine concentrations contained in the culture supernatants obtained from different healthy controls and AD patients BDCA1, n=10; BDCA4, n=16. Data were analyzed for statistical significance using a two-tailed Student's *t*-test, $*P<0.05$, $**P<0.01$, $***P<0.001$ compared to healthy controls.

AD patients show impaired IL-12p70 and IFN-α production by BDCA1⁺ and BDCA4⁺ DC, respectively

AD is a skin disease associated with the preferential development of Th2.[1,2] As the development of a particular Th response is dependent on DC-derived signals[24] we questioned whether the Th2 phenotype observed in AD patients may result from a deficiency in the production of Th1-associated cytokines by peripheral blood DC. To this aim, purified BDCA1⁺ (Fig. 2A) and BDCA4⁺ (Fig. 2B) DC were stimulated with poly I:C plus IFN-γ or with SAC, stimuli that optimally induce IL-12p70 or IFN-α respectively. After 24 h, the cytokines present in culture supernatants were analyzed by specific ELISAs. Poly I:C plus IFN-γ-stimulated BDCA1⁺ DC from AD patients show selectively and significantly ($P<0.001$) lower levels of IL-12p70 and TNF-α compared to BDCA1⁺ DC derived from healthy controls. The production of IL-6, IL-10 and IL-12p40 by BDCA1⁺ DC from AD patients was not significantly different from healthy controls (Fig. 2A). While the levels of IFN-α production by SAC-stimulated BDCA4⁺ DC from AD patients were selectively and

significantly ($P<0.05$) low compared to healthy controls, the levels of TNF-α, IL-6 and IL-10 production were similar (Fig. 2B). SAC-stimulated BDCA4$^+$ DC did not produce detectable levels of IL-12p70 (data not shown).

BDCA1$^+$ DC from AD patient express similar levels of HLA-DR, CD83 and CD86 compared to healthy controls

Purified BDCA1$^+$ DC from healthy controls and AD patients were stimulated *in vitro* with poly I:C, or SAC, or CD40L, and HLA-DR, CD83 and CD86 expression was measured after 24 h by FACS. Freshly isolated BDCA1$^+$ DC were used as control. Fig. 3 shows that irrespective of the stimuli tested, BDCA1$^+$ DC upregulated the expression of HLA-DR, CD83 and CD86 and there were no significant differences between DC derived from healthy controls and AD patients. Addition of IFN-γ to the BDCA1$^+$ DC cultures did not change the expression of these mature phenotype markers studied (data not shown). Freshly isolated BDCA4$^+$ DC from both healthy controls and AD patients also show similar levels of HLA-DR, CD83 and CD86 expression, albeit low in this DC subset (data not show).

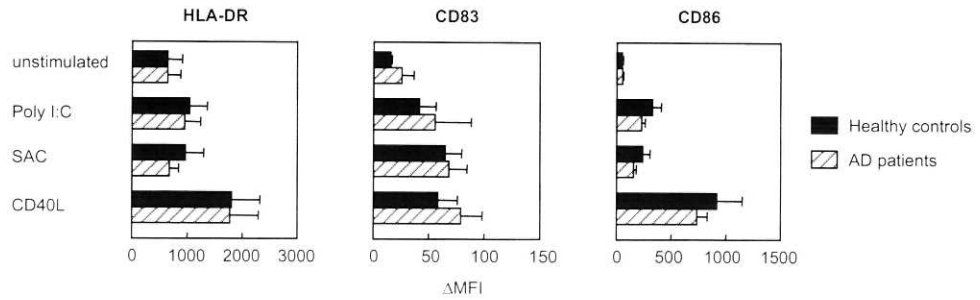

Figure 3. Freshly isolated and activated BDCA1$^+$ DC from AD patients express similar levels of HLA-DR, CD83 and CD86 compared to healthy controls. Freshly isolated or cultured (24 h after exposure to poly I:C, or SAC, or CD40L-transfected J558 cells) BDCA1$^+$ DC were stained with mAbs to HLA-DR, CD83 or CD86, and analyzed by FACS. ΔMFI represents the difference between the various staining and the isotype control. Results represent the mean ± SEM of ΔMFI from different healthy controls (n=6) and AD patients (n=6). Data were analyzed for statistical significance using a two-tailed Student's *t*-test, $*P<0.05$, $**P<0.01$, $***P<0.001$ compared to healthy controls.

Poly I:C-matured BDCA1+ DC from AD patients exhibit reduced capacity to induce IFN-γ-producing T cells compared to healthy controls, albeit their similar capacity to support naive Th cell proliferation

To evaluate the consequences of a reduced capacity to produce IL-12p70 by BDCA1+ DC from AD patients, we subsequently studied the capacity of mature BDCA1+ DC to stimulate naive Th cells in allogeneic MLR, and to induce a particular Th1/Th2 secretion profile. Fig. 4A shows that poly I:C-matured BDCA1+ DC from AD patients were as effective as BDCA1+ DC from healthy controls in inducing naive Th cell proliferation. These results are in line with the similar expression of mature phenotype markers by BDCA1+ DC from both healthy controls and AD patients.

To test whether poly I:C plus IFN-γ-matured BDCA1+ DC from AD patients have a different capacity to bias the development of Th1 or Th2 cells, mature BDCA1+ DC were cocultured with naive Th cells in the presence of superantigen (SEB). After 14 days, the percentage of cells producing IL-4 and/or IFN-γ was evaluated by stimulation of effector T cells with PMA/ionomycin and analysis of intracellular cytokine expression at the single cell basis by FACS (Fig. 4B). Although the priming of BDCA1+ DC from both healthy controls and AD patients with poly I:C plus IFN-γ result in DC that biased for the development of Th1 cells, the percentage of IFN-γ-producing Th cells induced by mature BDCA1+ DC from AD patients was lower. In addition, a higher percentage of IL-4-producing Th cells induced by these cells was observed. Fig. 4B shows a representative experiment out of ten with similar results. These findings indicate that BDCA1+ DC from AD patients have a reduced capacity to induce Th1 responses compared to healthy controls, in accordance with their strongly reduced IL-12p70 production

BDCA1+ DC and monocyte-derived DC (Mo-DC) from AD patients produce low levels of IL-12p70 in response to different stimuli

To analyze whether the reduced capacity of BDCA1+ DC to produce IL-12p70 was not a consequence of the particular stimulus tested, BDCA1+ DC were stimulated with poly I:C, or SAC, or CD40L in the absence or in the presence of IFN-γ. In addition, we verified whether the reduced capacity of BDCA1+ DC from AD patients to produce IL-12p70 was also observed in Mo-DC, generated from peripheral blood monocytes. Irrespective to the stimuli used both BDCA1+ DC and Mo-DC from AD patients produced significantly lower levels of

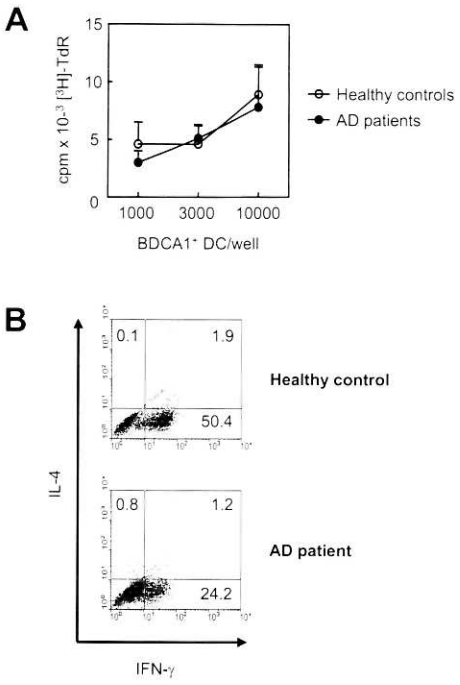

Figure 4. Poly I:C+IFN-γ-matured BDCA1$^+$ DC from healthy controls and AD patients exhibit similar capacity to support naive Th cell proliferation (A). BDCA1$^+$ DC were cultured for 24 h in the presence of poly I:C+IFN-γ. Then matured BDCA1$^+$ DC were cultured at different numbers with allogeneic naive CD45RA$^+$CD4$^+$ Th cells. After 5 days, the cells were pulsed during the last 16 h with [^{3}H]thymidine ([^{3}H]-TdR). Mature BDCA1$^+$ DC from healthy controls (○, open circles), mature BDCA1$^+$ DC from AD patients (●, close circles). Data are expressed as the mean ± SEM of counts per minute (cpm) from different healthy controls (n=6) and AD patients (n=6). Data were analyzed for statistical significance using a two-tailed Student's *t*-test, $*P<0.05$, $**P<0.01$, $***P<0.001$ compared to healthy controls. Poly I:C+IFN-γ-matured BDCA1$^+$ DC from an AD patient have reduced capacity to induce IFN-γ-producing Th cells compared to healthy control (B). Matured BDCA1$^+$ DC were loaded with SEB and cocultured with naive Th cells. After 14 days, resting memory Th cells were stimulated for 6 h with PMA+ionomycin in the presence of brefeldin A. The expression of IL-4 and IFN-γ was assessed by intracellular staining. Results are expressed as the percentage of cells in each population. A representative experiment out of ten with similar results is shown.

IL-12p70 compared to healthy controls (Fig. 5 shows a representative experiment out of six with different AD patients and controls). These results suggest that decreased IL-12p70 in AD patients may be due to an intrinsic deficiency of APC to produce this Th1-promoting cytokine.

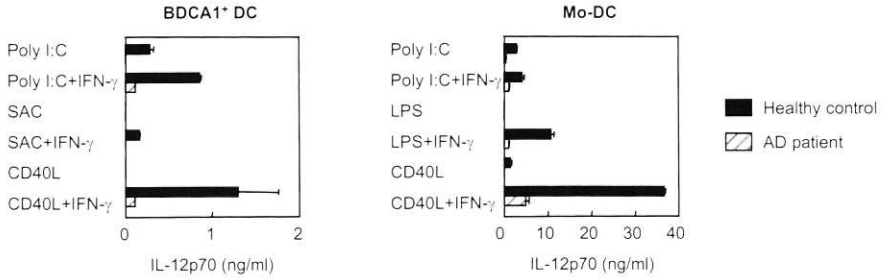

Figure 5. Reduced capacity of BDCA1$^+$ DC and Mo-DC to produce IL-12p70 is independent of the type of stimuli. Purified BDCA1$^+$ DC and Mo-DC from both healthy controls and AD patients were stimulated for 24 h with poly I:C, or SAC or CD40L-transfected J558 cells in the absence or in the presence of IFN-γ. IL-12p70 levels were measured by ELISA in culture supernatants. Data are mean±SEM of a representative experiment out of six with similar results. Data were analyzed for statistical significance using a two-tailed Student's *t*-test, $*P<0.05$, $**P<0.01$, $***P<0.001$ compared to healthy controls.

IL-12 p35 and p40 gene expression are defective in stimulated BDCA1$^+$ DC from AD patients

Since the secretion of bioactive IL-12p70 requires the production of both IL-12 subunits: p35 and p40,[16] we investigate the mechanisms of IL-12p70 deficiency observed in BDCA1$^+$ DC from AD patients by analyzing the mRNA expression of these subunits by quantitative real-time PCR. Fig. 6 shows a representative experiment out of five with similar results. Both p35 and p40 mRNA expression by poly I:C plus IFN-γ-stimulated BDCA1$^+$ DC from the AD patient was lower compared to the healthy control. BDCA1$^+$ DC stimulation with SAC resulted in the selective induction of p40 in the absence of p35 induction. Also in this case p40 expression of stimulated BDCA1$^+$ DC was lower compared to the healthy control.

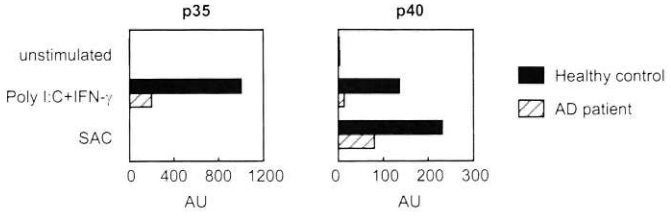

Figure 6. Reduced expression of IL-12(p35) and IL-12(p40) mRNA levels in BDCA1$^+$ DC from AD patients. BDCA1$^+$ DC from healthy controls or AD patients were incubated in the absence or in the presence of poly I:C+IFN-γ or SAC. After 6 h, mRNA was extracted, reverse transcribed, and amplified by quantitative real-time PCR using specific primers for IL-12(p35), IL-12-(p40), or β2-microglobulin (β2μ). IL-12(p35) and IL-12-(p40) mRNA levels were normalized against β2μ mRNA levels. Data show one representative experiment out of six with similar results.

BDCA1$^+$ and BDCA4$^+$ DC are present in elevated numbers in lesional skin of AD patients

Since AD is characterized by the accumulation of DC in the dermis[2] we analyzed by immunohistochemistry whether BDCA1$^+$ and BDCA4$^+$ DC are present in non-lesional and lesional skin of AD patients and compared to normal healthy controls. BDCA1 (Fig. 7A) and BDCA4 (Fig. 7D) staining in normal healthy skin (five separate samples) was detected in the upper part of dermis close to the border epidermis/dermis. BDCA1$^+$ DC were also detected in the epidermis (Fig. 7A, arrows) while BDCA4$^+$ DC were absent. In non-lesional skin from three different AD patients both BDCA1$^+$ (Fig. 7B) and BDCA4$^+$ cells (Fig. 7E) were observed in the dermis. However, BDCA1$^+$ DC were absent in the epidermis of non-lesional AD skin. Lesional skin of AD patients is characterized by the thickness of the epidermis and a massive infiltration of activated CD4$^+$ T cells and APC namely DC, monocytes and macrophages [2]. Indeed, elevated numbers of BDCA1$^+$ DC (Fig. 7C) are observed in the epidermis and dermis of lesional AD skin. BDCA4$^+$ DC (Fig. 7F) were also present in the epidermis (arrow) at elevated numbers and in the dermis. Isotype control stainings (Fig. 7G) gave a negative staining.

Since both BDCA1$^+$ and BDCA4$^+$ DC are expressed in lesional epidermis of AD patients we confirmed the specificity of BDCA1 and BDCA4 mAbs by performing double stainings. Figure 8 shows single positive BDCA1 (blue cells) and BDCA4 (red cells) DC present in epidermis of lesional AD skin, suggesting that BDCA1 and BDCA Ags are not co-expressed. Further double stainings are currently being performed using antibodies against BDCA1 and 4 antigens in combination with antibodies against the myeloid DC marker CD11c and the PDC marker CD123 (IL-3Rα).

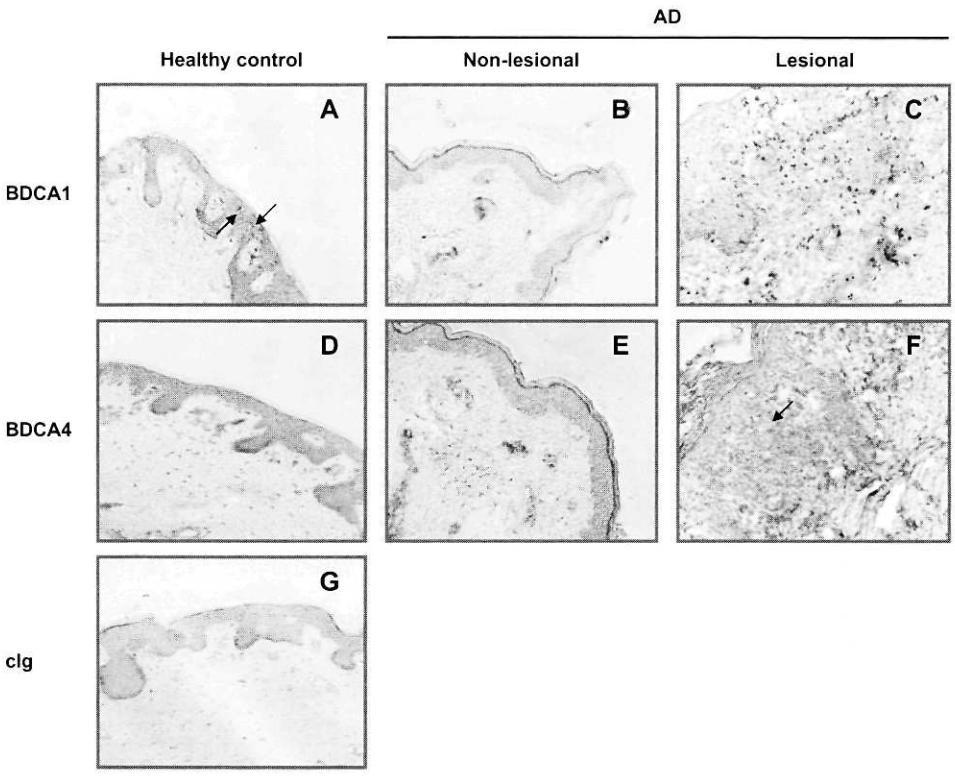

Figure 7. Immunohistochemical analysis of BDCA1$^+$ and BDCA4$^+$ DC in skin. Cryosections of human skin from healthy controls (n=5) (A, D, G), non-lesional (n=3) (B, E) and lesional (n=3) (C, F, H) AD patients were stained (red) with specific mAbs against BDCA1 (A, B, C) or BDCA4 (D, E, F), and counterstained with hematoxylin (blue). BDCA1$^+$ and BDC4$^+$ DC were present in the dermis of healthy controls (A and B, respectively) and BDCA1$^+$ DC were also present in the epidermis (arrows). BDCA1$^+$ (B) and BDC4$^+$ DC (E) were also present in the dermis of non-lesional AD skin whereas BDCA1$^+$ DC were absent in the epidermis. An increased number of BDCA1$^+$ DC is observed in the epidermis and dermis of lesional AD skin (C). BDCA4$^+$ DC accumulated in the epidermis in lesional AD skin (F, arrow) although some cells were also present in the dermis in contact with infiltrated cells. Isotype control antibody gave negative staining (G). Original magnification 10x. (☞ page 165).

DISCUSSION

The present study shows that both BDCA1$^+$ myeloid DC and BDCA4$^+$ plasmacytoid DC (PDC) from AD patients exhibit aberrant functions compared to healthy controls. BDCA1$^+$ DC from AD patients are characterized by a significant decreased capacity to produce bioactive IL-12p70 in response to various stimuli as a result of a defective IL-12p35 and IL-12p40 mRNA gene expression. Consistent with their reduced capacity to produce IL-12, BDCA1$^+$ DC from AD patients induce the development of Th cells with a decreased

frequency of IFN-γ-positive cells. Moreover, BDCA4$^+$ DC-derived IFN-α in AD patients is lower compared to healthy controls.

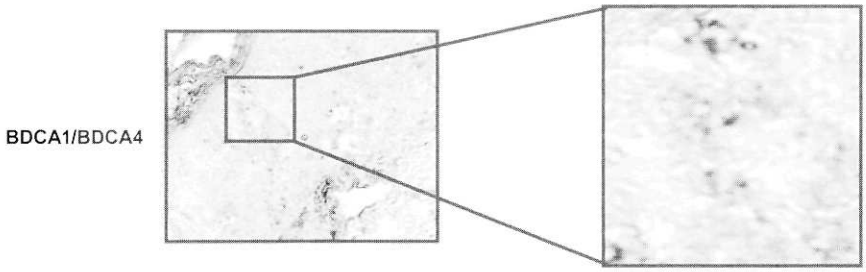

Figure 8. BDCA1 and BDCA4 Ags are not co-expressed in lesional AD skin. Double immunostainings revealed that both BDCA1$^+$ (blue) and BDCA4$^+$ (red) DC are localized in the epidermis from lesional AD skin (H). Original magnifications 10x and 40x. (☞ page 165).

These aberrant functions observed in the two blood DC subsets in AD patients could be caused by a decrease or increased frequencies of specific cell types. In fact, it has been demonstrated that patients suffering from systemic lupus erythematosus,[25] myeloid leukemia,[26] Sézary syndrome[27] or human immunodeficiency virus (HIV)[28] exhibit reduced numbers of peripheral blood DC. There are contradictory reports concerning the frequencies of peripheral blood DC in atopic patients compared to healthy controls. In one report[29] patients with atopy (asthma and AD) have a slightly enhanced percentage of PDC, while the percentage of CD11c$^+$ myeloid DC did not differ. In the other report the frequency of Lin$^-$ HLA-DR$^+$CD123$^+$ PDC in patients suffering from AD or allergic rhinoconjunctivitis was not statistically significantly different, whereas the frequency of Lin$^-$HLA-DR$^+$CD123$^-$ myeloid DC was significantly reduced compared to healthy subjects.[30] In addition, Upham *et al.*[31] demonstrated that the number of myeloid DC is not different between normal controls and atopic asthmatics in the absence of relevant allergen challenge. These discrepancies are likely to be due to the differential characterization of atopic patients and differential characterization of both myeloid and PDC subsets. In addition, from these data it is not clear which myeloid subpopulation present in peripheral blood (CD16$^+$, or BDCA1$^+$ or BDCA3$^+$ DC [5]) is responsible for the increased or decreased cell frequency in atopic patients. Our study, which defines two blood DC subsets, demonstrates that the frequencies of both BDCA1$^+$ and BDCA4$^+$ DC present in blood from AD patients did not differ from healthy controls. However, it remains to be established whether the frequencies of the other two CD11c$^+$ DC

present in peripheral blood, CD16$^+$ and BDCA3$^-$ DC, are different in AD patients compared to healthy controls.

IL-12 is a proinflammatory cytokine that is produced by APC, such as macrophages, B and DC cells.[32] The biologically functional form of IL-12 is a 70-kDa heterodimer (IL-12p70), which consists of disulfide-bounded 40-kDa (p40) and 35-kDa (p35) subunits, which plays a key role in the induction of cellular immunity by promoting the proliferation of NK and T cells and the differentiation towards Th1 cells.[32] AD patients are characterized by a biased polarization towards Th2 cells.[1,2] In addition, the response to mycobacterial antigens, although dominated by IFN-γ, is less polarized towards Th1 cells as compared to healthy controls.[33] Previously it has been shown that APC from atopic patients e.g., monocytes[18,19] and monocyte-derived DC,[30] produced less bioactive IL-12 compared to healthy controls. Here we show for the first time that BDCA1$^+$ DC present in peripheral blood of AD patients have a decreased capacity to produce bioactive IL-12 in response to various stimuli compared to healthy controls. Moreover, poly I:C plus IFN-γ (a potent Th1-inducing stimulus) induced BDCA1$^+$ DC maturation that subsequently induce the development of Th cells with low capacity to express IFN-γ compared to healthy controls. These findings are of particular importance since DC are the most potent APC in the initiation of specific immune responses.[24] In addition, our results indicate that the polarization towards Th2 observed in AD patients may be due to a deficient IL-12 production by BDCA1$^+$ DC but not due to a reduced number of these circulating DC in peripheral blood neither due to a decreased capacity of these cells to induce proliferation of T cells.

Analysis of the IL-12p35 and p40 gene expression (mRNA) suggested that the reduced IL-12p70 production observed in AD patients might be due to a decreased mRNA expression of both subunits. In addition, although IL-12p40 gene expression in AD BDCA1$^+$ DC was defective, the protein synthesis of this subunit was similar compared to healthy controls, suggesting the existence of posttranscriptional regulation.

PDC are the main producers of type I IFNs, IFN-α and IFN-β, innate immune molecules that are crucial after bacterial and viral challenge.[34] The *in vivo* effects of type I IFNs are associated with promoting an antiviral and antibacterial state, including a broad spectrum of cellular targets.[12] For instance, by producing high levels of IFN-α response to viruses, PDC (BDCA4$^+$) may protect myeloid DC (BDCA1$^-$) from the cytopathic effect of the virus[35] and may exert an adjuvant effect on antibody responses.[36] The disease AD is complicated by recurrent infections of skin lesions by bacterial, viral, and fungal pathogens.[37] About 30% of

patients with AD have bacterial or viral infections in the skin.[38] The decreased secretion of IFN-α by AD BDCA4$^+$ DC, and BDCA1$^+$ DC-derived IL-12 and TNF-α, may have serious consequences on the induction of antibacterial and antiviral immune response and may account for the susceptibility of patients suffering from AD to skin infections with bacteria and viruses.[37]

Our results established a clear difference in the function of myeloid circulating DC from AD patients compared with their normal counterparts, however is still unknown whether other atopic disease, such as atopic asthma may also display similar abnormalities, or whether these differences are associated with the inflammatory status of the skin. Our preliminary data show that BDCA1$^+$ DC from atopic asthma patients have a reduced capacity to produce bioactive IL-12, and in contrast to BDCA1$^+$ DC from psoriasis patients which release similar levels of IL-12p70 compared to healthy controls (data not shown).

In situ immunohistochemical studies revealed that BDCA1$^+$ and BDCA4$^+$ DC are present in the skin under steady-state conditions (healthy controls) and the number of both DC subsets is increased in both dermis and epidermis in lesional AD skin. These data suggest that these subsets may contribute to the cutaneous inflammation observed in these patients.

A recent study indicated that skin epithelial cells, keratinocytes may provide the initial trigger of the allergic immune cascade in AD.[39] Epithelial-derived thymic stromal lymphopoietin (TSLP) not only potently activates DC, but also endows DC with the ability to polarize naive T cells to produce Th2 cytokines. Moreover, TSLP is highly expressed by keratinocytes from patients with both acute and chronic AD *in situ*.[39] It is, therefore, tempting to speculate that TSLP skin concentrations in AD may reach the bone-marrow and peripheral blood and prime DC progenitors and precursors, respectively. Moreover, it is unclear whether TSLP can trigger the development of AD or this molecule plays a role in the amplification of the disease since TSLP is mainly detected in lesional AD skin. However, it remains to be determined whether TSLP may reduce the production of IL-12 or IFN-α by stimulated BDCA1$^+$ or BDCA4$^+$ DC, respectively, and whether the treatment of AD patients (e.g. glucocorticoids, tacrolimus) may restore the capacity of BDCA1$^+$ and BDCA4$^+$ DC to produce IL-12 or IFN-α, respectively.

In summary, in AD patients, both circulating DC subsets exhibit qualitative, but not quantitative, intrinsic abnormalities. Our observations may be relevant to the increased susceptibility of AD patients to intracellular pathogens and viruses but also may have

important implications for the development of new strategies for the treatment of this chronic skin disease.

ACKOWLEDGMENTS

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CHAPTER 7

General Discussion

The Chemokine Connection

INTRODUCTION

An important question in immunology is how organisms defend themselves against the wide variety of pathogens they encounter. The immune response to pathogens has innate and specific components that synergize in the clearance of pathogens. The innate, non-specific, immune response is crucial in limiting the early replication and spread of infectious agents. By contrast, the specific immune response is mediated by T and B cells and it is characterized by specificity and memory. In addition to these complementary activities, there is a fundamental connection between innate and specific immunity, since the magnitude and class of the specific immune response is dependent on signals derived from the innate immune response.[1] This chapter focuses on how keratinocytes and immature dendritic cells (iDC), as innate immune cells, support specific immunity by the expression of soluble factors, with special emphasis on the role of chemokines herein. In addition, it is discussed how DC-derived chemokines amplify antigen (Ag)-specific lymphocyte responses.

Skin-derived chemokines link innate and specific immunity

As stated above, innate immune cells are capable of recognizing a wide variety of pathogens. Toll-like receptors (TLRs) play a critical role in innate immunity by recognizing structurally conserved bacterial, fungal, parasitic and viral components, termed pathogen-associated molecular patterns (PAMPs).[2] At present, ten human Toll-like receptors (TLRs) have been cloned.[3] Upon penetration of the skin, pathogens activate innate cells, such as keratinocytes, by selectively ligating TLRs (TLR1, 2, 3, 4, 5, 6, 9 and 10) (**Chapter 2**). Triggering of different TLRs on keratinocytes induces the expression of different sets of cytokines and chemokines (Fig. 1 and **Chapters 2 and 3**). Except for CpG motifs ligating TLR9, all the PAMPs indicated in Figure 1 induce the production of CXCL8/IL-8 by human keratinocytes (**Chapter 2**). It is well documented that during the initiation of an acute, local inflammatory response, CXCL8/IL-8 attracts peripheral blood $CXCR1^+$ neutrophils, the first immune cells to arrive in significant numbers at the site of inflammation.[4] Their major role is to phagocytose and eliminate bacteria, fungi, protozoa and viruses.[5] Another chemokine produced by keratinocytes upon pathogenic stimuli, is CCL2/MCP-1 (**Chapter 2**). This chemokine attracts CCR1-positive monocytes and iDC, which contributes to the skin inflammation.[6] Recently it was demonstrated that CCL2/MCP-1 produced by inflamed skin cells (e.g. keratinocytes (**Chapter 2**) or, iDC and DC type 1, DC1 (**Chapter 4**)) may as well be transported via the lymph to the luminal surface of high endothelial venules (HEV), where

it triggers integrin-dependent arrest of rolling monocytes.[7] In this way, inflamed tissues project their local chemokine profile to HEVs in draining lymph nodes (LNs) and thereby exert "remote control" over the composition of leukocyte populations that home to these organs from the blood. As monocytes are leucocytes possessing a high plasticity for further differentiation into macrophages or DC,[8] this study suggest that within the LNs these cells may acquire the ability to process and present Ags and stimulate a vigorous specific immune response.

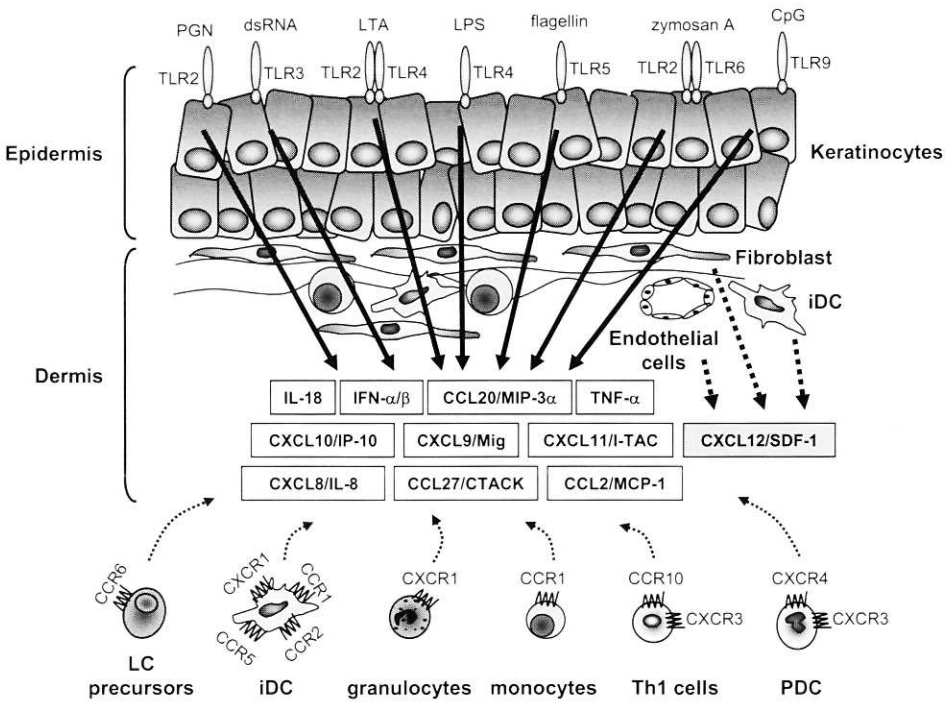

Figure 1. Schematic representation of cytokines and chemokines released by keratinocytes upon TLR triggering. Pathogens or their PAMPs (PAMPs are shown here) can induce the production of soluble mediators by keratinocytes by hitting their TLRs. Such pathogen-induced chemokines may induce the selective recruitment of LC precursors (CCL20/MIP-3α), iDC (amongst others, CXCL8/IL-8 and CCL2/MCP-1), granulocytes that include the neutrophils (CXCL8/IL-8), Th1 cells (CCL27/CTACK and CXCL9/Mig, CXCL10/IP-10, and CXCL11/I-TAC). PDC may also be recruited to the skin by responding to CXCL9/Mig, CXCL10/IP-10, and CXCL11/I-TAC, however this responsiveness is conferred by additional signals provided by CXCL12/SDF-1, which is produced in inflamed skin by fibroblasts, iDC and endothelial cells. Abbreviations: LC, Langerhans cells; iDC, immature dendritic cells; Th1, T helper type 1 cells; PDC, plasmacytoid dendritic cells.

In addition to chemokines, keratinocytes release cytokines upon pathogenic stimuli (Fig. 1 and Fig. 2). For example, upon activation by double-stranded RNA (poly I:C, a viral compound), keratinocytes produce various soluble mediators that induce maturation (TNF-α

and IFN-α/β) and polarization (IFN-α/β and IL-18) of DC (Fig. 2 and **Chapter 3**). In the skin environment, these factors will induce the migration of both epidermal LC and dermal DC via lymph towards secondary lymphoid organs. In most cases, pathogen-induced migration is accompanied by maturation of LC/DC from a processing stage into a more mature, costimulatory stage.[9] In addition, inflammatory stimuli, such as TNF-α and IL-1β, and microbial agents can recruit LC/DC precursors from peripheral blood to infected tissues to replace the migrated populations. In this respect, it has been demonstrated that $CD1a^+$ LC precursors express CCR6 and respond selectively to keratinocyte-derived CCL20/MIP-3α (**Chapter 2**).[10,11] Within the lymph nodes, the matured DC present pathogen-derived peptides to T cell receptor (TCR) in naive T cells in the context of MHC (signal 1) and provide costimulation (signal 2).[12] Additionally, these DC provide naive T cells with another signal that determines the class of the immune response towards T helper type 1 (Th1) or type 2 (Th2) development, proposed to be the T cell polarizing signal 3.[13] For example, poly I:C-activated keratinocytes induce the development of protective Th1-promoting DC (DC1) via the production of type 1 IFNs and IL-18 (Fig. 2 and **Chapter 3**). Once activated, effector Th cells subsets selectively migrate to the sites of infection, which is determined by the differential production of chemokines by pathogen-activated tissue cells and differential expression of chemokine receptors by Th subsets. While Th1 cells selectively express CXCR3 and CCR5, Th2 cells selectively express CCR3, CCR4 and CCR8.[14] Their immigration in infected tissue is determined by the particular chemokines induced by the pathogen type that infects the tissue. While Th1 cells are selectively attracted by CXCL9/Mig, CXCL10/IP-10, CXCL11/I-TAC, CCL3/MIP-1α, CCL4/MIP-1β and CCL5/RANTES, Th2 cells are selectively attracted by CCL1/I-309, CCL11/eotaxin, CCL17/TARC and CCL22/MDC.[15]

For example, stimulation of keratinocytes by dsRNA, results in the production of CXCR3 ligands (CXCL9/Mig, CXCL10/IP-10 and CXCL11/I-TAC) and CCL27/CTACK (binding to skin-homing CCR10-positive T cells[16]) (**Chapter 2**), which will lead to the recruitment of CXCR3/CCR10-positive anti-viral Th1 cells into the skin. In addition to attracting Th1 cells, CXCR3 ligands derived from dsRNA-activated keratinocytes may attract CXCR3-positive precursor plasmacytoid DC (prePDC),[17] the main producers of IFN-α, which limit the replication and spread of viruses. However, migration of prePDC in response to CXCR3 ligands may require additional signals provided by CXCL12/SDF-1 (binding to CXCR4[17]) that is produced by iDC, fibroblasts and endothelial cells[18] (Fig. 1). Since CXCL12/SDF-1

expression was demonstrated in the high endothelial venules (HEVs) in lymph nodes[17] it was postulated that CXCL12/SDF-1 only played a role in the extravasation of PDC to the HEVs of secondary lymphoid organs.[17,19] The observation that this chemokine is also expressed in inflamed skin[18] indicates that CXCL12/SDF-1 may also play an important role in the recruitment of PDC to the skin. Indeed, the concept that PDC are also able to migrate from blood to peripheral non-lymphoid tissues, and not only from blood to secondary lymphoid organs is supported by recent observations demonstrating that PDC may also accumulate in cutaneous lupus erythematosus lesions.[20]

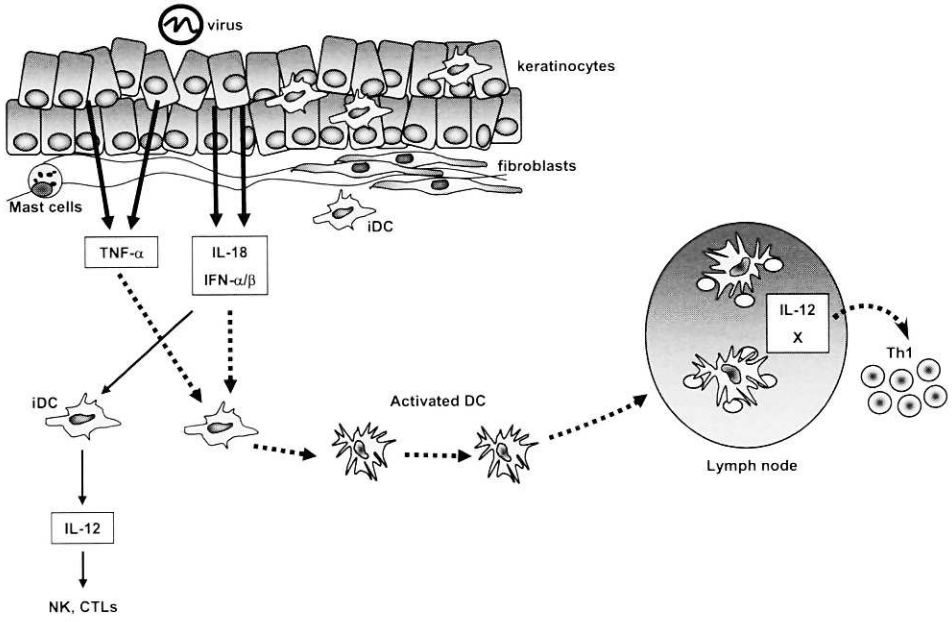

Figure 2. The role of the microenvironment on DC maturation and function. When a pathogen invades the skin (e.g. a virus is shown here), pathogen-activated keratinocytes will release an array of soluble mediators (TNF-α, IFN-α/β and IL-18) that have profound effects on resident DC. TNF-α and IFN-α/β induce maturation of DC and trigger their migration to lymphoid tissues (e.g. lymph node, as shown here). Concomitantly, DC are primed by polarizing signals (IFN-α/β and IL-18) resulting in their maturation into effector DC that promote the development of Th1 cells. IL-12 production by sentinel iDC is IFN-α/β-dependent. In addition, when both IFN-α/β and IL-18 are blocked, DC induce the development of a mixed population of Th1 and Th2 cells.

While the production of Th1-associated chemokines by human keratinocytes is well documented, the production of Th2-associated chemokines is not fully established. Strikingly in this respect, keratinocytes cultured from non-lesional skin of atopic dermatitis (AD) patients, a Th2-associated disease,[21] show, compared to healthy controls and patients with

psoriasis,[22] elevated levels of CCL5/RANTES, a chemokine that preferentially attracts Th1 cells.[14] However, in this study the production of Th2-associated chemokines by keratinocytes was not addressed. Normal human keratinocytes release very low amounts of CCL17/TARC, CCL22/MDC and CCL1/I-309, albeit only after additional IFN-γ stimulation, suggesting that keratinocytes may attract Th2 cells after prior activation by Th1-derived cytokines.[23] Altogether these findings suggest that LC/DC (to be discussed below), rather than keratinocytes, are the major source of Th2-associated chemokines in the skin environment. In summary, both keratinocyte- and LC/DC-derived chemokines are key factors providing a pivotal function at the interface between innate and specific responses.

DC-derived chemokines and the initiation of the immune response

Immature DC or DC precursors (monocytes) express receptors for inflammatory chemokines (e.g. CCL2/MCP-1, CCL3/MIP-1α and CCL5/RANTES), such as CXCR1, CCR1, CCR2 and CCR5, to extravasate and migrate into inflamed tissues (Fig. 3, 2a). CCL2/MCP-1, CCL3/MIP-1α and CCL5/RANTES and, CXCR3 ligands are produced by iDC upon pathogenic stimuli[15] (Fig. 3, 2b and **Chapter 4**) and are of particular importance for the replacement of the LC/DC population that have migrated out of the skin towards secondary lymphoid organs. LC/DC maturation (Fig. 3, 3) and migration (Fig. 3, 4a) can be triggered directly by pathogens or their products,[9] or indirectly, by the inflammatory cytokines they induce in resident cells (**Chapter 3**). Moreover, a prerequisite for LC/DC migration is the upregulation of CCR7[24] (Fig. 3, 4a) that guides them to the T and B cells areas of the draining lymph nodes where CCR7 ligands (CCL19/MIP-3β and CCL21/6Ckine) are produced.[25,26] However, not all DC migrate to the secondary lymphoid organs upon inflammatory stimuli.[27] The observation that mature DC1 constitutively express the inflammatory chemokines CCL2/MCP-1, CCL3/MIP-1α, CCL5/RANTES and CXCR3 ligands (Fig. 3, 4b and **Chapter 4**), may imply a new role for pathogen-primed mature DC in peripheral tissues, since these chemokines are mainly produced at the site of pathogen entry. The initiation of a specific immune response takes place in the draining lymphoid tissues and not in the tissue itself. Within the lymph nodes, mature Ag-loaded DC use CCR7- or CXCR4-mediated mechanisms to respectively enter the T or B cell zones of the lymph nodes (Fig. 3, 5), where they initiate a specific immune response by activating either naive T (Fig. 3, 6a and 6b) or B. Depending on the nature of eliciting stimulus, mature DC will display a particular pattern of chemokines

(**Chapter 4**) that may be released in secondary lymphoid organs, or in the peripheral tissues when they fail to migrate. DC1 and DC2 preferentially express the CCR7 ligands CCL19/MIP-3β and CCL21/6Ckine,[25,26] respectively (Fig. 3, 6a and **Chapter 4**). Moreover, the expression of Th1- (CXCL9, 10 and 11) or Th2-associated (CCL22/MDC) chemokines by DC is correlated with the expression of DC1 or DC2 effector phenotype (Fig. 3, 6b and **Chapter 4**). The functional significance of Th1- and Th2-attracting chemokines produced by effector DC1 or DC2 in lymphoid tissues is unclear, but it cannot be ruled out that they control Th1/Th2 responses by mechanisms that are unknown to date. While some T cells upregulate CXCR5[28] and then become directed toward the B-cell follicle to deliver help to B cells (Fig. 3, 7), other activated T cells leave lymphoid tissue and find their way back to the site of inflammation[29] (Fig. 3, 8) to deliver the specific effector response. This recruitment to the site of inflammation may be the result of chemokines released by keratinocytes (**Chapter 2**), iDC and non-migratory mature DC (**Chapter 4**). In this respect, dsRNA-activated keratinocytes (**Chapter 2**) and iDC (Fig. 3, 2b and **Chapter 4**) and dsRNA-primed mature DC (Fig. 3, 4b and **Chapter 4**) are an important source of CXCR3 ligands (CXCL9, 10 and 11) that recruit CXCR3-positive Th1 cells to the periphery (Fig. 3, 8b).

Peripheral blood DC in health and disease: migration from blood to peripheral tissues or to LNs

Since their original identification, there has been increasing evidence that DC represent a heterogeneous population of cells with distinct origins, stages of differentiation/maturation, and specific functions.[9] The characterization of different DC subsets both in peripheral blood and tissues is based on a panel of different cell surface markers. In particular, BDCA1, BDCA3 and BDCA4 Ags define three peripheral blood DC subsets.[30] BDCA1$^+$ DC are of myeloid origin and represent a CD11c$^+$HLA-DR^{high} population in blood[31]).[30] Although BDCA3$^+$ DC are of myeloid origin as well,[30] this subset is characterized by a distinct anatomical localization and function (**Chapter 5**). The BDCA4$^+$ DC subset is of lymphoid origin (also known as precursor PDC, or type I IFN-producing cells).[32,33] The distribution of these three DC subsets in nonlymphoid and lymphoid tissues is of particular interest. In steady-state conditions BDCA1$^+$, BDCA3$^+$ and BDCA4$^+$ DC are all present in human dermal skin. While BDCA1$^+$ and BDCA3$^+$ DC are abundant in the epidermis, BDCA4$^+$ DC are absent (**Chapter 5**) in this skin compartment. It is, therefore, tempting to speculate that BDCA1$^+$, BDCA3$^+$ and BDCA4$^+$ DC may represent three DC subsets in the dermis that may

play an important role in cutaneous immune responses. In addition, since BDCA1$^-$ and BDCA3$^-$ DC are found in the epidermis, they may represent a pool of LC in steady-state conditions, as recently described.[34] The homing of these three subsets from the blood to the skin is not surprising due to their expression of the skin-homing Ag,[35] cutaneous lymphocyte-associated Ag (CLA).[30] While the BDCA1$^+$ and BDCA4$^+$ DC subsets have been extensively studied,[36] the functional roles of the BDCA3 subset are still emerging. In fact, BDCA3$^+$ DC subset consists of two subpopulations, BDCA3^{hi} and BDCA3^{low}, the latter with highest frequency in PBMC[31] (**Chapter 5**). It is interesting to note that BDCA3$^-$ DC subset expresses significantly higher levels of CD40 and DEC205/CD205 but the lower levels of CD86 than other myeloid populations.[31] DEC205 is an endocytic receptor with 10 membrane-external, contiguous C-type lectin domains[37] which mediates the efficient processing and presentation of Ags on MHC class II molecules *in vivo*[38] suggesting a major role for BDCA3$^+$ DC in Ag-uptake both in the skin and lymphoid organs (**Chapter 5**). Moreover, BDCA-3^{hi} DC selectively express TLR1, 3 and 10 (**Chapter 5**) which may imply a highly specialized function in the recognition of pathogen-derived signature molecules.

To date, there are no reports on chemokine receptors expression profile of the BDCA3$^+$ DC subset. In contrast, freshly isolated BDCA1$^+$ and BDCA4$^+$ DC subsets express relatively high levels of CCR2, CCR5, and CXCR4.[19] On the other hand, CCR7 and CXCR3 expression is diverged. CCR7 is negligible on BDCA1$^+$ DC but very high on BDCA4$^+$ DC, whereas CXCR3 expression is weak on BDCA1 and relatively high on BDCA4$^+$ DC.[19] In contrast to the overall similar pattern of chemokine receptor expression, with the exception of CCR7, circulating BDCA1$^+$ and BDCA4$^+$ DC exhibited profoundly different migration capacity in response to these chemokines. While BDCA1$^+$ DC were able to migrate in response to CCL2/MCP-1, CCL5/RANTES and CXCL12/SDF-1, BDCA4$^+$ DC migrated only in response to CXCL12/SDF-1.[19] The demonstration that BDCA4$^+$ DC subset is able to respond to CXCR3 ligands in the presence of CXCL12/SDF-1,[17] is consistent with their important role in during viral infections. In response to viral infection, CXCR3 ligands that are produced locally by keratinocytes (**Chapter 2**) and, iDC and DC type 1 (**Chapter 4**) will attract BDCA4$^+$ DC, the precursor DC that are able to produce extremely large amounts of type I IFNs.[32] The selective capacity of BDCA1$^+$ DC to migrate in response to inflammatory chemotactic signals may imply a role for this subset in the replacement of migrated DC populations from peripheral tissues. Thus, the divergent migration programs of BDCA1$^+$ and

BDCA4$^+$ DC are consistent with their specialized roles in the induction and regulation of the immune response.

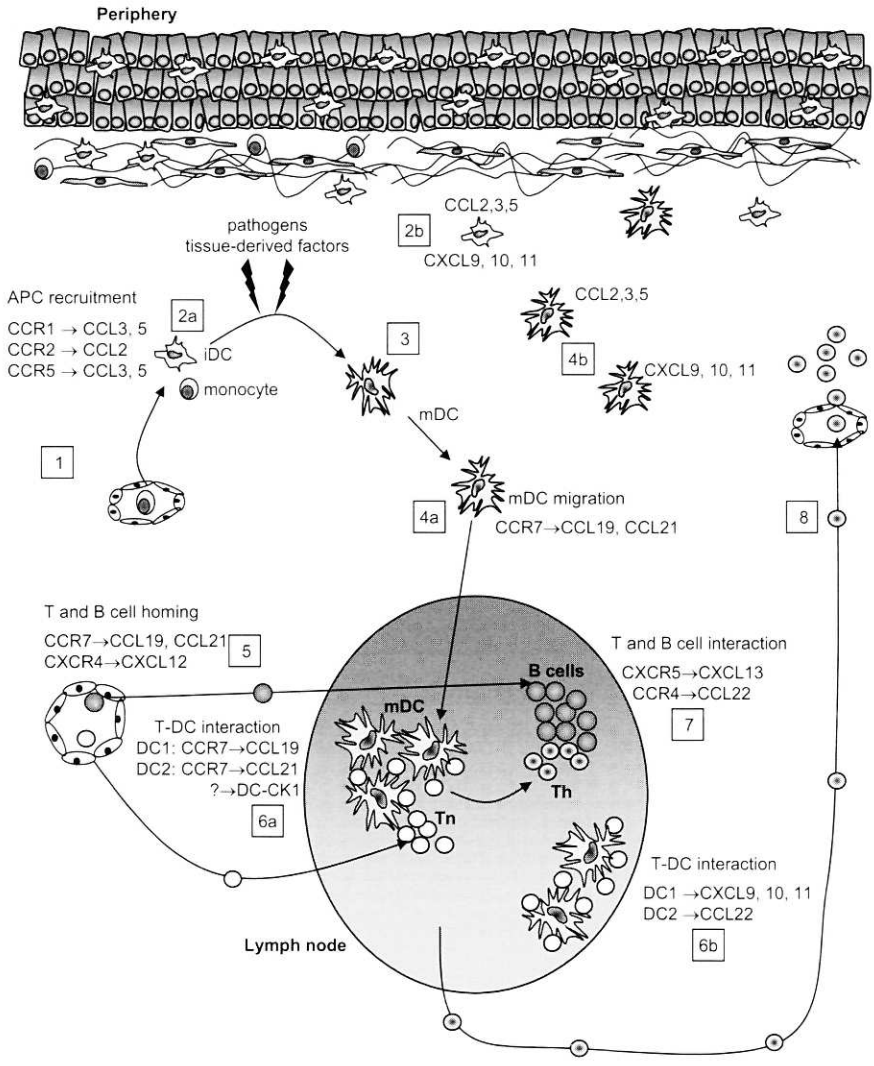

Figure 3. Selective expression of chemokines and chemokine receptors orchestrates the afferent and effector phases of the immune response. The critical migratory steps and cellular interactions in the immune response are highlighted, together with some of the molecules involved. (1) Monocytes and iDC are recruited by inflammatory chemokines to sites of pathogen entry. (2) Pathogens or tissue-derived factors induce DC maturation (3) and the release of additional inflammatory chemokines by iDC (2b). Maturation of DC results in a switch in chemokine receptor expression (4a) allowing mature DC to enter lymphatic vessels and to be actively drained to lymphoid tissues, where they home to the T cells areas. (4b) At the same time, mature DC that failed to migrate to the lymph nodes produce an array of inflammatory chemokines. (5) Naive T (Tn) and B cells home to the T- and B-cell areas of the lymph nodes (LN). T cells interact with differently polarized DC (6a and 6b) and are activated. (7) Effector Th cells migrate to B-cell areas where they stimulate Ag-specific B cells and (8) Th1 or Th2, migrate to peripheral tissues.

An interesting question is whether these DC subsets exhibit phenotypical and/or functional differences in pathological states. As BDCA1$^+$ and BDCA4$^+$ DC subsets in AD patients show different cytokine profiles compared to from healthy control DC (**Chapter 6**), it is likely that they express different chemokine secretion and chemokine receptor expression patterns as well. It has been recently proposed that the selective recruitment of the different DC subsets to the epidermal skin lesions is dependent on the type of skin disease.[39] In this respect, only a few PDC (BDCA4$^+$ DC) could be detected in epidermal single cell suspensions of both normal healthy controls and lesional AD patients. In contrast, patients suffering from psoriasis, contact dermatitis or lupus erythematosus exhibit elevated numbers of epidermal PDC.[39] At the site of inflammation, BDCA1$^+$ DC along with their plasmacytoid counterparts BDCA4$^+$ DC (PDC) may conceivably capture Ags, while receiving additional stimuli, which promote their maturation (e.g. TNF-α, herpes simplex virus). Once mature, both BDCA1$^+$ and BDCA4$^+$ DC upregulate CCR7 expression allowing them to migrate to LN where they can participate in the initiation of Ag-specific T cell responses.[19] In addition, since BDCA1$^+$ and BDCA4$^+$ DC accumulate in lesional skin from AD patients (**Chapter 6**), they may have the capacity to stimulate memory T cells directly. Besides their roles as APCs, the cytokines and chemokines produced locally by BDCA1$^+$ and BDCA4$^+$ DC may be of great importance. In healthy controls, BDCA1$^+$ and BDCA4$^+$ DC were shown to produce CXCL10/IP-10, CCL3/MIP-1α, and CCL5/RANTES in response to influenza virus.[17] In AD patients, the capacity of BDCA1$^+$ and BDCA4$^+$ DC to produce virus-specific chemokines may be deficient and, together with the defective production of IL-12 and IFN-α, respectively (**Chapter 6**), these aberrant phenotypes may account for the survival of the opportunistic herpes virus simplex, which is observed in these patients. Moreover, the susceptibility of these patients to skin infections may be the result of combinatorial factors that were stated above and in addition, defective skin barrier,[40] deficiency in the expression of antimicrobial peptides in the skin.[41] It remains to be investigated whether the increased susceptibility to *Staphylococcus aureus* infections observed in AD patients may be due to the presence of a polymorphism in TLR2 gene as described by Lorenz *et al.*[42] as well. Moreover, the abnormal expression of certain molecules by keratinocytes may also contribute to the pathogenesis of AD, such as the exaggerated GM-CSF.[43] This local GM-CSF production may account for the generation/survival of BDCA1$^+$ and BDCA3$^+$ DC subsets that are present in normal skin (**Chapter 5**), since these two subsets require GM-CSF for their survival.[30]

Concluding remarks

The initiation and maintenance of an acute inflammatory response is driven by a multifactorial system in which chemokines play an important role. In normal situations, the response of resident cells to an initial stimulus rapidly sets in motion a sequence of leukocyte-recruiting events, which culminates in the clearance of the pathogen and the resolution of the response. The sustained recruitment and activation of leukocytes found in pathological conditions, such as in multiple sclerosis,[44] rheumatoid arthritis,[44] atopic asthma,[45,46] psoriasis,[47,48] allergic contact dermatitis[48,49] and AD,[48,50] is associated with a dysregulation of this process.

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Summary

Protective immunity to pathogens depends on efficient immune responses adapted to the type of pathogen and the infected tissue, which is mediated by polarized T helper type 1 (Th1) or Th2 cells. The skin is constantly exposed to microbial pathogens and other stressful stimuli. Since the primary function of the skin is to provide an effective barrier against the outside world, it harbours a rapid and efficient host defense system exists that is triggered once the physical barrier has been broken. Keratinocytes are the major constituent of the skin and may act as natural immune cells capable of pathogen recognition, and they have an important role in initiating and perpetuating the activation of both innate and specific immune responses. Immature dendritic cells (DC) are strategically located in tissues that represent pathogen entry routes (the skin and mucosal surfaces), where they continuous monitor the environment. Upon contact with pathogens, or tissue-derived inflammatory factors induced by these pathogens, DC undergo a program of maturation that, amongst others, is associated with the acquisition of surface expression of T cell costimulatory molecules and migration through the lymph, toward secondary lymphoid organs. In this way, DC become the most potent antigen-presenting cells, the only ones capable of activating naive T lymphocytes and of initiating specific immune responses.

In this thesis, studies are described on the role of two distinct cell types, keratinocytes and DC, in the initiation and regulation of the class of the immune response.

Pathogens express pathogen-associated molecular patterns (PAMPs) that are recognized, amongst others, by Toll-like receptors (TLRs) present on innate immune cells. In **Chapter 2** we examine the expression profile of TLRs by primary human keratinocytes and address the question whether these pathogen-recognition receptors play a role in cytokine and chemokine production by keratinocytes in response to different PAMPs. We demonstrate that human keratinocytes constitutively express mRNA for TLR1, 2, 3, 4, 5, 6, 9 and 10, but not for TLR7 or 8. Keratinocytes respond to TLR2, 3, 4, 5, 6 and 9 ligands, which indicates that they may play a critical role in alerting the immune system to the presence of pathogens and forming a link between innate and specific immunity.

DC play a key role in establishing the class of immune response against invading pathogens and they reside, in an immature state, in the epithelia in close contact with keratinocytes. **Chapter 3** addresses whether and how soluble factors derived from pathogen-activated keratinocytes induce maturation and functional polarization of DC. Based on the

analysis of monocyte-derived DC, we found that double-stranded (ds)RNA-activated keratinocytes release TNF-α and IFN-α, that induce the maturation of DC. Moreover, the IFN-a and IL-18 released by these keratinocytes induce mature DC that strongly biased the development of Th1 cells from naive Th cells. These findings suggest that keratinocytes can contribute to the development of selective Th1/Th2 responses through the induction of maturation and functional polarization of DC. They also point to a novel role for keratinocytes as initiators and regulators of cutaneous T cell-mediated inflammation.

Upon activation with microbial compounds of cytokines, human monocyte-derived DC may mature into effector DC that promote the development of Th1- (DC1) or Th2- (DC2) *in vitro*. These functionally polarized DC populations, and nonpolarized DC0, may vary in their capability to attract other immune cells. **Chapter 4** investigates the differential production of both inflammatory and homeostatic chemokines by these different DC populations. It is shown that DC0 and DC1, but not DC2, selectively express elevated levels of inflammatory chemokines. In addition, we show that the production of Th1-attracting chemokines is restricted to DC1. Since all inflammatory chemokines tested are expressed constitutively by mature DC, we propose a novel role for mature DC present in inflamed tissues in orchestrating the immune response by recruiting appropriate leukocyte populations to the site of pathogen entry.

Peripheral blood contains several DC subsets. The smallest population of DC is characterized by the expression of the blood DC antigen 3 (BDCA3) of which the function and ligand are still unknown. **Chapter 5** describes the phenotypic and functional characteristics of $BDCA3^{hi}$ DC subset and compared them to $BDCA1^{+}$ myeloid and $BDCA4^{+}$ plasmacytoid DC. We show that, in addition to TLR1 and TLR3, $BDCA3^{hi}$ DC uniquely express TLR10 and upon activation with the TLR3 ligand dsRNA acquire the capacity to promote Th1 responses, albeit less potently compared to $BDCA1^{+}$ DC. Immunohistochemical analysis of human tissue revealed the presence of $BDCA3^{+}$ cells in both lymphoid and non-lymphoid tissues. This study suggests that $BDCA3^{hi}$ DC, due to their narrow TLR expression, have a limited capacity to recognize pathogens, but are as flexible in their capacity to bias the development of Th1/Th2 cells, as other DC subsets.

Atopic dermatitis (AD) is an eczematous skin disease associated with a generalized bias of Th2 cells. In **Chapter 6** we questioned whether circulating DC in AD patients, compared to healthy controls, differ in their phenotype and function. We show that $BDCA1^{+}$ DC from AD patients have a selective and dramatic reduced capacity to produce IL-12p70, whereas

BDCA4$^+$ DC show reduced IFN-α. Accordingly, even after maturation in the presence of an extremely potent Th1 stimulus (dsRNA+IFN-γ), BDCA1$^-$ DC from AD patients induced considerably less IFN-γ-producing Th cells, compared to BDCA1$^+$ DC from healthy controls. This study suggests that the defective IL-12 and IFN-α production by DC may contribute to the maintenance of the allergic state in AD patients, as well as their increased susceptibility to skin infections with pathogens that require protective IFN-α and Th1 cell responses.

In **Chapter 7** we discuss how keratinocytes and immature DC, as innate immune cells, support specific immunity by the expression of soluble factors, with special emphasis on the role of chemokines herein. In addition, it is discussed how DC-derived chemokines amplify antigen-specific lymphocyte responses.

Nederlandse samenvatting

Het menselijk lichaam wordt voortdurend blootgesteld aan allerlei pathogenen zoals bacterieën, virussen, schimmels en parasieten, die via de huid, luchtwegen en darmstelsel proberen binnen te dringen. Als bescherming tegen deze invasie hebben we een efficiënt afweersysyteem, ook wel immuunsysteem genoemd.

Het immuunsysteem kent grofweg twee type responses, namelijk een niet-specifiek en een specifiek respons. De niet-specifieke respons treedt direct op na het binnendringen van een pathogeen en is er vooral op gericht om de verspreiding van het pathogeen zoveel mogelijk te beperken. De specifieke immuunrespons komt langzamer op gang maar zet een heel leger van cellen aan om het binnen gedrongen pathogeen op te ruimen.

Het immuunsysteem bestaat uit zeer veel verschillende typen cellen die zich overal in ons lichaam bevinden. Een van de meest centrale cellen van het immuunsysteem is de dendritische cel. Deze cellen bevinden zich precies daar waar pathogenen binnen komen dus in de huid, darm en luchtwegen. De functie van dendritische cellen is om continu deze weefsels te surveilleren en om als er een bacterie, virus, schimmel of parasiet binnen komt daarop te reageren en specifieke immuuncellen te activeren. Dendritische cellen beschikken namelijk over receptoren, Toll-like resceptoren (TLR) genaamd, die verschillende soorten pathogenen herkennen en opnemen. Na herkenning raken dendritische cellen geactiveerd en migreren naar de drainerende lymfeknopen. Tijdens deze migratie ondergaan de dendritische cellen een aantal veranderingen waardoor zij optimaal T helper (Th) cellen kunnen activeren.

Th cellen vormen een andere groep van cellen van het immuunsysteem. Er zijn twee duidelijke typen van Th cellen te onderscheiden, Th1 en Th2 cellen. Th1 cellen spelen een belangrijke rol bij het opruimen van intracellulaire microben (bacterieën en virussen), terwijl Th2 cellen betrokken zijn bij de verdediging tegen parasieten. Een verstoring van de balans tussen Th1 en Th2 cellen kan leiden tot de ontwikkeling van een ziekte. Zo is allergie (zoals astma of eczeem) geassocieërd met Th2 cellen terwijl een aantal autoimmuunziekten (zoals multiple sclerose of reuma) geassocieërd zijn met Th1 cellen.

Dendritische cellen en Th cellen ontmoeten elkaar in de secundaire lymphoid organen (zoals lymfeknopen of milt). Afhankelijk van het voor soort pathogeen dat de dendritische cel eerder heeft herkent in bijvoorbeeld de huid, zullen vooral Th1 of Th2 cellen zich ontwikkelen. De balans tussen Th1 en Th2 cellen wordt bepaald door de door de dendritische cellen uitgescheide moleculen, cytokinen genaamd. Zo stimuleert de productie van IL-12 of

IFN-α de ontwikkeling van Th1 cellen. War precies Th2 cellen stuurt is niet geheel duidelijk. Deze Th cellen zullen vervolgens migreren naar de plek van infectie.

De studies beschreven in dit proefschrift gaan over verschillende aspecten van dendritische cellen en keratinocyten. Dendritische cellen bevinden zich in de huid tussen de keratinocyten, de grootste celcomponent van de huid.

In **hoofdstuk 2** werd bestudeert in hoeverre keratinocyten een rol spelen bij het binnen dringen van een pathogeen. Het blijkt dat ook keratinocyten receptoren (TLRs) hebben om verschillende pathogenen te herkennen. Bovendien scheiden keratinocyten in reactie op het herkennen van bepaalde pathogenen verschillende moleculen (chemokinen en cytokinen) uit, die heel specifiek Th1 of Th2 cellen naar de huid kunnen aantrekken. Deze studie toont aan dat keratinocyten een belangrijke rol spelen in het alarmeren van het immuunsysteem en een link vormen tussen het niet-specifieke en specifieke immuunsysteem.

In **hoofdstuk 3** is onderzocht of de keratinocyten de dendritische cellen ook werkelijk beinvloeden. Uit de studie blijkt dat door virale bestanddelen geactiveerde keratinocyten in staat zijn om dendritische cellen te activeren, die vervolgens de uitgroei van Th1 cellen stimuleren.

Hoofdstuk 4 beschrijft dat ook dendritische cellen chemokinen kunnen uitscheiden. Daarbij bleek dat dendritische cellen die de uitgroei van Th1 stimuleren, en dendritische cellen die de uitgroei Th2 stimuleren, verschillende patronen van chemokines laten zien.

De populatie van dendritische cellen in ons lichaam is zeer heterogeen. Er bestaan verschillende soorten dendritische cellen. In **hoofdstuk 5** wordt de functie van een recent ontdekt type dendritisch cellen, $BDCA3^{hi}$ cellen, beschreven. Deze cellen bevinden zich niet alleen in het bloed maar ook in verschillende organen die tot het immuunsysteem behoren zoals lymfeknopen, milt en zwezerik (thymus), maar ook in de huid. $BDCA3^{hi}$ cellen brengen zeer specifieke TLRs tot expressie waardoor deze cellen bepaalde (nog onbekende) pathogenen kunnen herkennen.

Allergisch eczeem is een huidaandoening waarbij de patiënt heftig reageert op onschuldige stoffen in onze omgeving, zoals huisstofmijt. Omdat deze patienten een zeer sterke Th2 respons hebben, was de vraag of er in dendritische cellen soms iets mis is met de productie aan IL-12 en/of IFN-α, die beiden de uitgroei van Th1 ondersteunen. Dit is in **hoofdstuk 6** onderzocht. De studie toont duidelijk aan dat de dendritische cellen van patienten zich zeer afwijkend gedragen in vergelijking met dendritische cellen van gezonde personen. Zo kunnen de cellen van patiënten bijna geen IL-12 of IFN-α meer maken waardoor ze meer Th2-achtige

cellen induceren. Dit verschijnsel zou kunnen bijdragen aan de ontwikkeling, of instandhouding, van hun ziekte. Bovendien zouden ze hierdoor minder goed beschermd kunnen zijn tegen bepaalde infecties.

Samenvattend beschrijft dit proefschrift enkele van de processen zich kunnen afspelen als een pathogeen de huid binnendringt. Hierbij zijn verschillende celtypen betrokken, zoals dendritische cellen en T cellen maar ook keratinocyten, de cellen die de grootste component van de huid vormen.

Sumário

Uma das questões mais estudadas em Imunologia é como o sistema imunitário é capaz de reagir de uma maneira eficaz a infecções provocadas por vírus, bactérias e fungos. Essa resposta imunitária é efectuada por um exército de células, células T auxiliadoras (T "helper" - Th) que dependendo do tipo de invasor são do tipo 1 (Th1) ou do tipo 2 (Th2) (imunidade adquirida). Para combater de uma maneira eficiente infecções provocadas por vírus ou por bactérias intracelulares são necessárias as células Th1. Pelo contrário, o combate a infecções provocadas por parasitas requerer a presença de células Th2. Este cenário é observado em situações normais em que existe um equilíbrio entre células Th1 e Th2. Em caso de doença, há o desenvolvimento exagerado destas populações. As doenças autoimunes (por exemplo a esclerose múltipla) são caracterizadas pela expansão exagerada de células Th1. Por outro lado, doenças alérgicas (asma, dermatite atópica, rinite) são caracterizadas pela expansão exagerada de células Th2.

Mas como é que as células T imaturas sabem se irão diferenciar-se em Th1 ou Th2? Este fenómeno é iniciado pelas células apresentadoras de antigénios. Dentro de diversos tipos celulares, as células dendríticas são células apresentadoras de antigénios especializadas porque são capazes de detectar organismos patogénicos, de se tornarem maduras, sendo as únicas capazes de estimular as células T imaturas e iniciar deste modo uma resposta imunitária específica. As células dendríticas, que num estado imaturo participam na imunidade inata, estão posicionadas estratégicamente em tecidos periféricos não linfóides (pele, pulmões e intestinos) onde patrulham constantemente o meio ambiente onde se encontram. Após infecção ou devido à acção de produtos produzidos pelas células que formam os tecidos, as células dendríticas são activadas, amadurecem e migram dos tecidos periféricos em direcção à área das células T nos tecidos linfóides secundários (nódulos linfáticos por exemplo). As células dendríticas podem ser vistas como mensageiros. Elas trazem a informação dos tecidos (por exemplo, que tipo de infecção) às células T imaturas iniciando e regulando as respostas imunes. Existem dois factores produzidos pelas células dendríticas que fazem com que as células T imaturas se transformem em Th1: interleucina-12 (IL-12) e interferão-α (IFN-α), e são deste modo denominados de factores tipo 1. Quanto a factores tipo 2, que induzem células Th2, estes não são tão conhecidos. As células dendríticas que induzem células Th1 são denominadas de tipo 1 (DC1) e as que induzem células Th2 são denominadas de tipo 2 (DC2).

A pele funciona como a primeira linha de defesa contra a invasão do corpo humano por agentes infecciosos provenientes do meio exterior. A pele é dividida em duas partes, a camada exterior é denominada de epiderme e a camada interior de derme. A epiderme é constituída na sua maioridade por células denominadas de queratinócitos e a derme na sua maioria por fibroblastos, que formam o tecido conjuntivo. Na epiderme e na derme também se encontram células dendríticas em contacto com os queratinócitos ou com os fibroblastos. Contudo, na pele os queratinócitos produzem uma grande quantidade de factores que podem contribuir para iniciar ou prolongar uma resposta imune.

Esta tese descreve a importância de duas populações de células distintas, queratinócitos e células dendríticas, na iniciação de uma resposta imune e o papel que os factores produzidos por essas células (quimiocinas e citocinas) poderão ter na imunidade inata e adquirida.

No segundo capítulo desta tese é descrito a presença de receptores ("Toll-like receptors" - TLRs) nos queratinócitos que reconhecem moléculas que estão presentes nos organismos patogénicos. Deste modo os queratinócitos também podem detectar a presença de agentes infecciosos na pele e informar/alertar outras células do sistema imune funcionando deste modo como a ligação entre imunidade inata e adquirida.

As células dendríticas estão localizadas na pele em contacto com os queratinócitos. No terceiro capítulo é estudada a interacção entre os queratinócitos e as células dendríticas através da análise das citocinas produzidas pelos queratinócitos que vão induzir a maturação e polarização das células dendríticas.

No quarto capítulo é estudada a produção de quimiocinas pelas células dendríticas tipo 1 e tipo 2 (DC1 e DC2). Quimocinas são moléculas produzidas por vários tipos de células que induzem a migração específica de outras células em direcção ao local onde são produzidas. É assim demonstrado, neste capítulo, que a produção de certas quimiocinas é dependente do tipo de células dendríticas que as produzem.

No quarto capítulo é estudada a função de uma nova população de células dendríticas, BDCA3, que foi originalmente descrita no sangue periférico. É demonstrado que as células BDCA3 não só se encontram localizadas no sangue mas também na pele, baço, amígdalas e nódulos linfáticos. Estas células também expressam especificamente certos TLR o que as torna muito específicas no que respeita ao reconhecimento de certos organismos.

No sexto capítulo é estudada a função de outros dois tipos de células dendríticas, BDCA1 e BDCA4 numa doença alérgica da pele, dermatite atópica. Esta doença inflamatória da pele é caracterizada pela proliferação exagerada de células Th2. É demonstrado que as células

BDCA1 nos doentes não são capazes de induzir tão eficazmente células Th1 o que está relacionado com a sua baixa capacidade de produzir IL-12. Por outro lado, é também demonstrado que as células BDCA4, que são extremamente importantes para o combate a vírus e bactérias, produzem níveis bastante baixos de IFN-α (factor essencial no combate contra vírus e bactérias). Estes resultados poderão explicar porquê que os doentes com dermatite atópica sofrem de infecções virais e bacterianas mais frequentemente do que indivíduos saudáveis.

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Thanks!!
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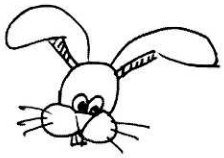
Cristina



Curriculum vitae

The author of this thesis was born in Lubango, Angola, on the 15th of October of 1970. She graduated in Biology from the University of Oporto, Portugal in September 1993. The graduation research project aimed at studying "The role of ultraviolet B rays on the expression of proteins and pigments in maize plants (*Zea mays*)". In October 1993, she teached Microbiology at the Santa Maria Nursing superior School, Oporto, Portugal. In November 1994, she started a two-year postgraduate training in Immunology coordinated by Prof. Dr. Maria de Sousa at the Department of Pathology and Molecular Immunology, Abel Salazar Institute for the Biomedical Sciences, University of Oporto, Portugal. During the second year of the Immunology program she went to the Netherlands for a short term training at the Department of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, to study "The role of matrix metalloproteinases in activation and migration of epidermal Langerhans cells in a human contact allergy model" under the supervision of Prof. Dr. Martien L. Kapsenberg (head Cellular Immunology) and Dr. Paweł Kaliński. In 1998 she started her PhD training at the same laboratory and studied the "Initiation and regulation of specific immune responses by keratinocytes and dendritic cells". Since April of 2003 she joined the group of Prof. Dr. Paul-Peter Tak (head Clinical Immunology and Rheumatology), Academic Medical Center, University of Amsterdam, as a postdoc to study the "Functional properties of chemokines and chemokine receptors in inflammatory joint disease (rheumatoid arthritis).

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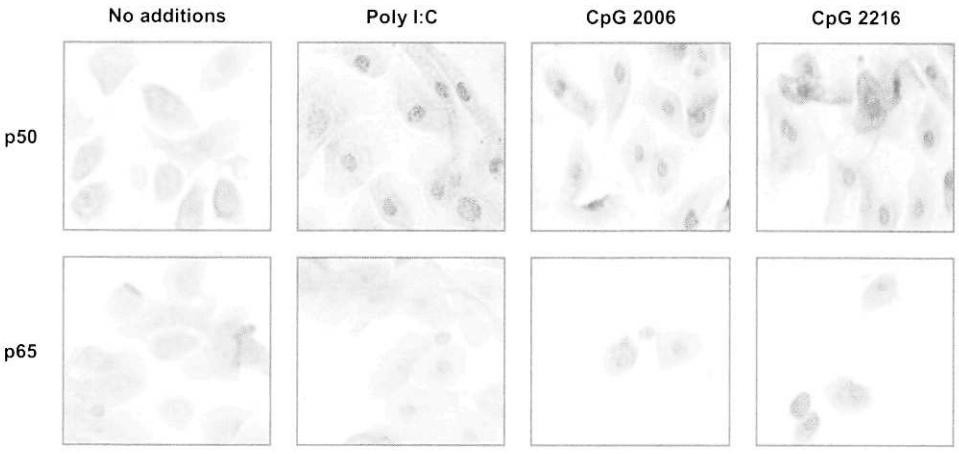
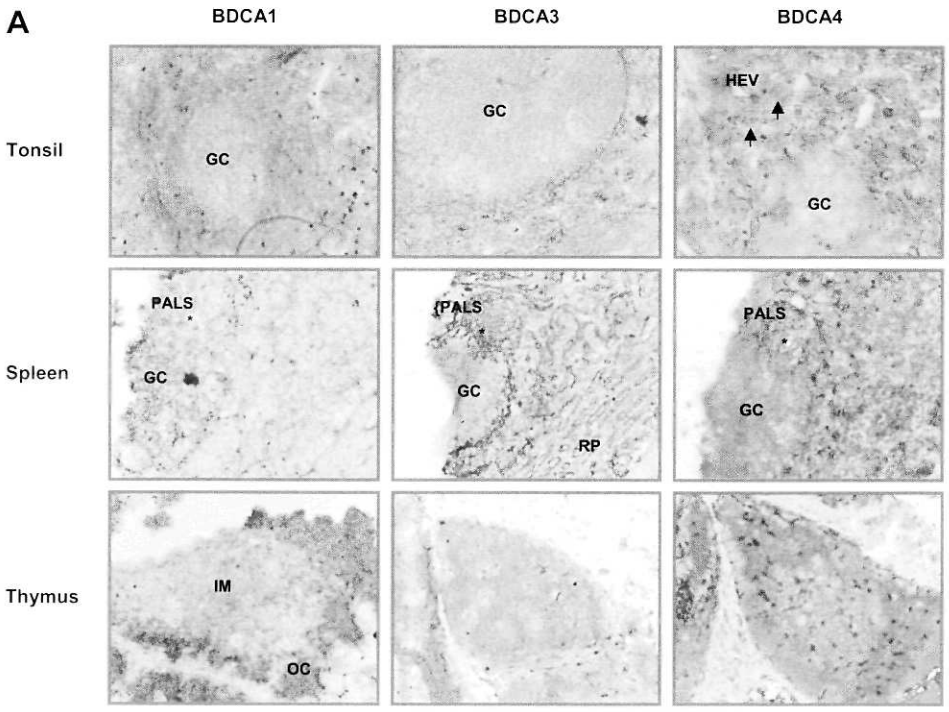
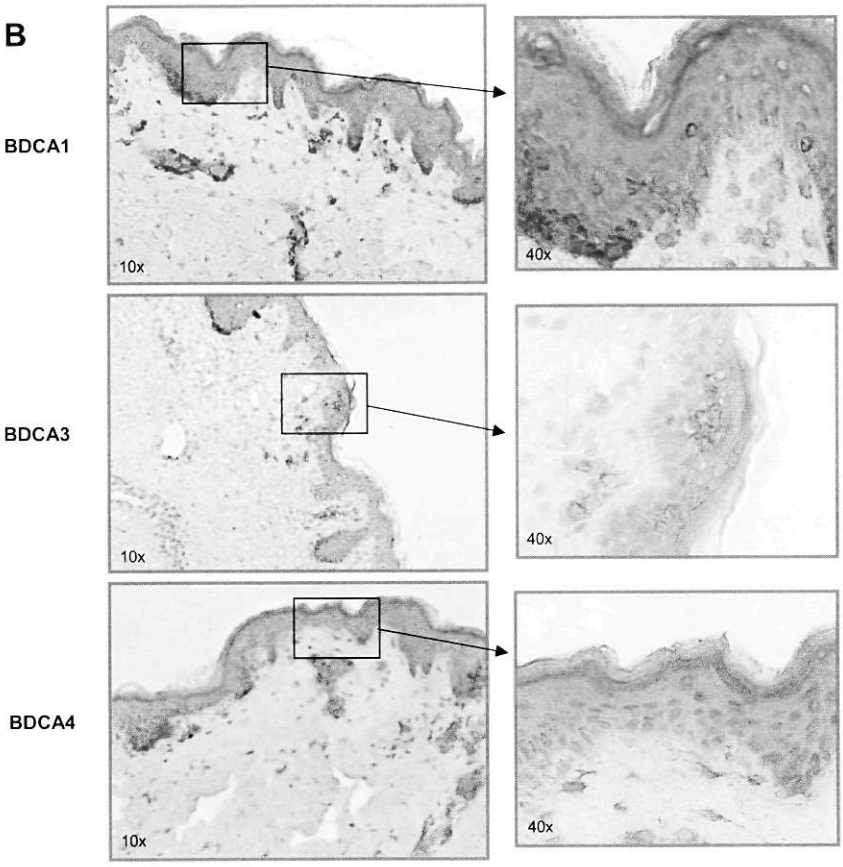
Color Figures



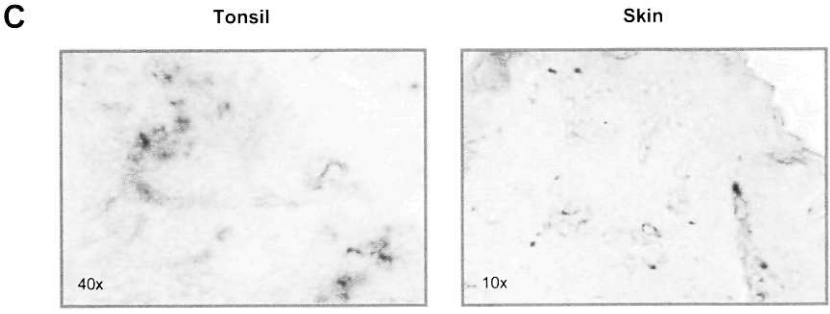
Chapter 2, Fig. 6

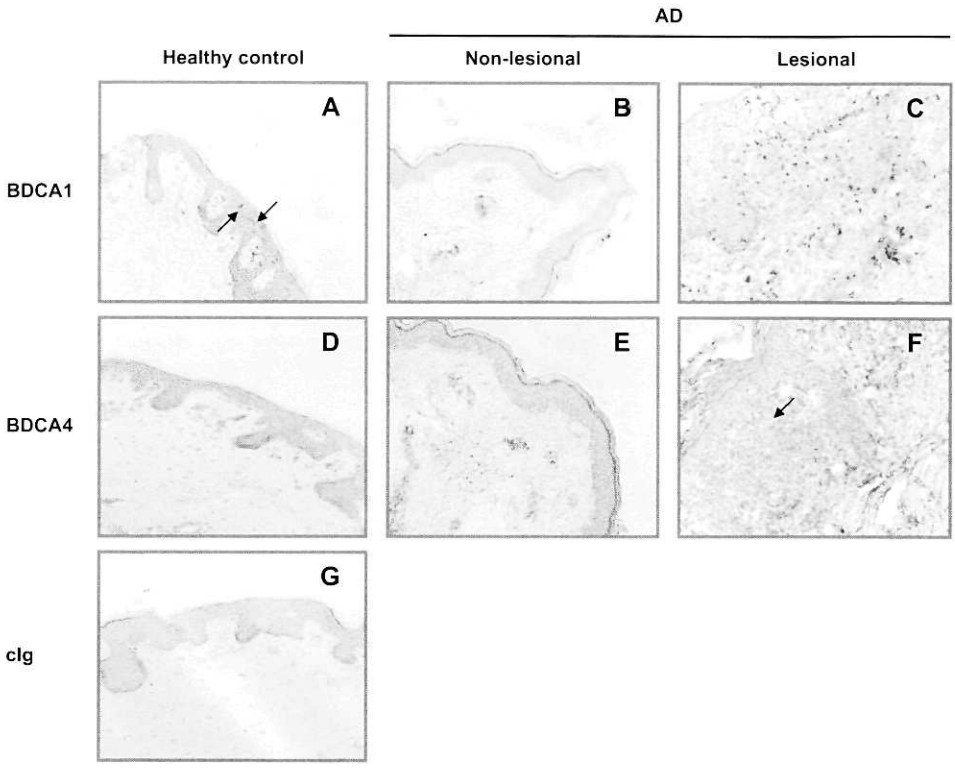


Chapter 5, Fig. 8A

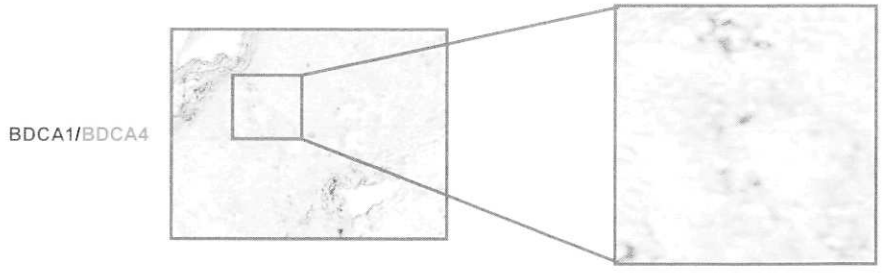


Chapter 5, Fig. 8B





Chapter 6, Fig. 7



Chapter 6, Fig. 8

A Rosa

E a Rosa estava lá
vermelha, como a rosa
que se esqueceu de enrubescer.

E rósea estava lá
as faces, como a rosa
que se esqueceu de amanhecer.

E claro estava lá
o gesto, como a rosa
que se esqueceu de aparecer.

E firme estava lá
a mente, como a Rosa
que se esqueceu de perceber.

E a cair estava lá
a lágrima, como a rosa
que se esqueceu que ia chover.

E a pensar estava lá
a vida, como a rosa
que se esqueceu de viver.

E a dizer estava lá
a Rosa, como a rosa
que se esqueceu de não dizer.

E a sonhar estava lá
o sonho, como a rosa
que se esqueceu de esquecer.

Leonardo Dias