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Verdijk, Pauline

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Pauline Verdijk

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PROMOTOR:	**Prof. dr. R. Willemze**
COPROMOTOREN:	**Dr. A.M. Mommaas**
	Dr. C.P. Tensen
REFERENT:	**Prof. dr. M.L. Kapsenberg**
	Academisch Medisch Centrum, Amsterdam
OVERIGE LEDEN:	**Prof. dr. M.R. Daha**
	Prof. dr. J. Neefjes
	Dr. F. Koning
	Prof. dr. R. Leurs
	Vrije Universiteit Medisch Centrum, Amsterdam

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Dit proefschrift is opgedragen aan:

oma Verdijk

In herinnering aan mijn moeder

Contents

List of Abbreviations

$[Ca^{2+}]_i$	increase in intracellular calcium concentrations
2DE	two-dimensional gel-electrophoresis
APC	antigen-presenting cells
BG	Birbeck granules
BisI	bisindolylmaleimide I
BSA	bovine serum albumin
CaMK	Ca^{2+}-calmodulin dependent kinase
CaMKK	CaMK kinase
CLA	cutaneous lymphocyte-associated antigen
CLL	chronic lymphocytic leukemia
CRAC	calcium release activated calcium channel.
CRD	carbohydrate recognition domain
CTACK	cutaneous T cell-attracting chemokine
CTL	cytotoxic T cells
DAG	diacylglycerol
DC	dendritic cells
DTE	dithioerythritol
EGTA	ethylene glycol bis ([beta]-aminoethyl ether)-N, N, N ', N '-tetraacetic acid
ER	endoplasmic reticulum
F-actin	filamentous actin
GFP	green fluorescent protein
GPCR	G protein–coupled receptor
ICAM	intercellular adhesion molecule
IFN-γ	interferon γ
IL	interleukin
IP10	IFN-γ-inducible protein 10
IP_3	inositol-1,4,5-triphosphate
IP_3R	IP_3 receptor
IP-9	IFN-inducible protein 9
ITAC	IFN-γ-inducible T cell α-chemoattractant
LC	Langerhans cells
LFA	lymphocyte function-associated antigen
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinases
MDC	macrophage-derived chemokine
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIG	monokine induced by IFN-γ
MIIC	MHC class II rich compartment
MS	mass spectrometry
PAGE	polyacrylamide gelectrophoresis
PBMC	peripheral blood mononuclear cells

PCR	polymerase chain reactions
PFA	paraformaldehyde
PI3K	phosphatidylinositol-3-kinase
PI4K	phosphatidylinositol-4-Kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PTX	pertussis sensitive
RT	room temperature
Ser3	serine at position 3
SERCA	sarcoplasmatic/endoplasmatic reticulum calcium ATPase
SOC	store operated calcium channels
SDF	stromal derived factor
TAP	transporter associated with antigen processing
TARC	thymus and activation-regulated chemokine
TBST	0.05% Tween-20 in TBS
TCR	T cell receptor
TGF	transforming growth factor
TLR	toll-like receptors
VDCC	voltage dependent Ca^{2+} channels
WASp	Wiscott-Aldrich syndrome protein

Chapter 1:

General introduction

General introduction

The immune system

The immune system is an organization of cells and molecules with specialized roles in the defense against pathogens. The immune system can be divided in two compartments: The first is the innate immunity (also called the natural or native immunity), which represents the first line of defense against invading microorganisms and removes antigens relatively non-specifically. The second is the adaptive immunity (also known as specific or acquired immunity), which is highly antigen-specific. Another important quality of the adaptive immunity that distinguishes it from innate immunity is the generation of immune memory resulting in an enhanced and accelerated response upon a second encounter with the same antigen (Chaplin, 2003; Delves and Roitt, 2000b; Delves and Roitt, 2000a). The cells of the innate and adaptive immune system are illustrated in figure 1. Broadly defined, the innate immune system includes all aspects of the host defense mechanisms that are encoded in the germ-line genes of the host. These include barrier mechanisms, soluble proteins and bioactive small molecules, such as complement, antimicrobial peptides and cytokines, produced by resident cells, and migratory cells of the immune system. The cellular compartment of the innate immunity encompasses phagocytic cells, including macrophages and dendritic cells, and effector cells, such as NK cells and granulocytes (Chaplin, 2003; Delves and Roitt, 2000a). Unlike the innate mechanisms of host defense, the adaptive immune system manifests exquisite specificity for its target antigens. The cells of the adaptive immune system are T cells that recognize foreign antigens that are presented by antigen presenting cells (APC) and or tissue cells, and B cells, which are responsible for the production of antigen-specific antibodies. Adaptive immunity functions through presentation of small antigenic fragments from infectious agents by molecules encoded in the major histocompatibility complex (MHC) genetic locus on the cell surface of APC (Delves and Roitt, 2000a). There are two classes of MHC molecules. Class I molecules are expressed by all nucleated cells and present peptides derived from cytosolic microorganisms and endogenous proteins. Class II MHC molecules present peptides from extracellular antigens and are solely expressed on antigen presenting cells, like dendritic cells, macrophages and B lymphocytes. T lymphocytes, of the adaptive immune system, express antigen-receptors that recognize specific antigenic peptides in the context of either MHC class I or class II molecules (Watts and Powis, 1999). In response to infection, T cell receptor (TCR) interactions with pathogen-derived peptide-MHC complexes will trigger the differentiation of antigen-specific lymphocytes into effector

cells and memory cells (Delves and Roitt, 2000b). The effector cells are then instantly engaged in the immune response. Memory cells, the hallmark of the adaptive immunity, are long-lived cells and possess the capacity to respond more quickly and efficiently upon repeated exposures to the same antigen (Campos and Godson, 2003).

This thesis will focus on two types of immune cells, which are indissolubly linked together: the dendritic cell (**chapter 2 and 3**) of the innate immune system and the T cell of the adaptive immune system (**chapter 4-6**).

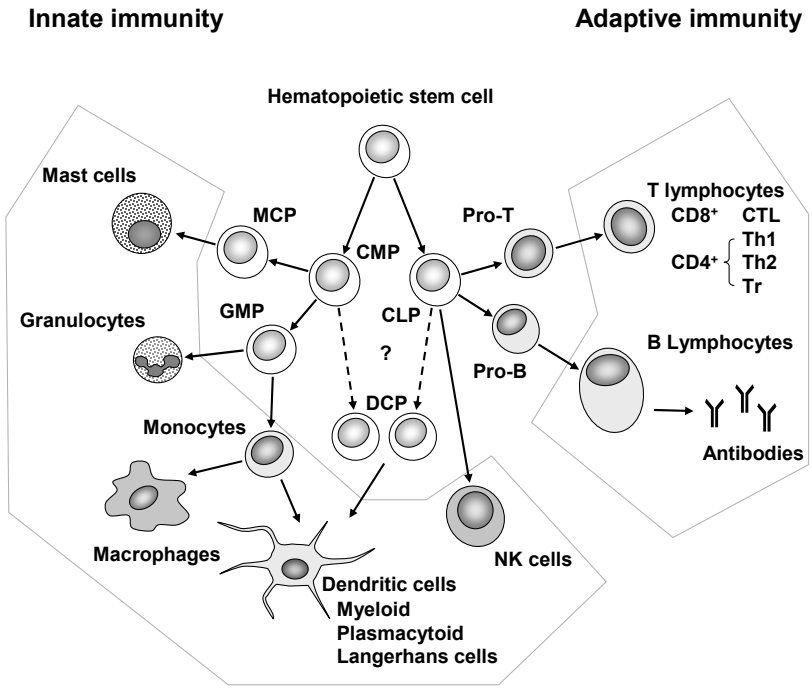

Figure 1: Schematic overview of the development of immune cells. CMP: Common myeloid precursor, CLP: Common lymphoid precursor, DCP: DC progenitors, MCP: Mast cell progenitor, GMP: granulocyte-macrophage precursor.

Dendritic cells

Dendritic cells (DC) play a critical role in the immune system as they serve as a link between the innate and adoptive immune responses and are particularly skilled in inducing the differentiation of naïve T cells into effector cells. Immature DC reside in the tissues or circulate through the blood, where they function as the sentinels of the immune system. Immature DC are phagocytic

cells with a high capacity for fluid-phase endocytosis and receptor-mediated endocytosis. When exposed to pathogens this capacity is transiently enhanced. Activated DC process the captured antigens and differentiate into mature DC. Meanwhile, the cells migrate out of the tissue via the efferent lymph to the draining lymph node. Mature DC have lost their capacity to endocytose, but have become excellent APC, high in costimulatory molecules and MHC-peptide complexes and with a dendritic morphology. In the lymph node DC migrate into the T cell-rich area where they encounter T cells (Banchereau et al., 2000; Austyn, 1998).

Origin and subsets

DC reside in small numbers in tissues that are potential sides of pathogen entry. They are continuously replenished by precursors in the blood that are generated from hematopoietic stem cells in the bone marrow and may include blood precursors as well as monocytes (fig. 1). Precursors that have entered the tissue differentiate into tissue-specific immature DC, e. g. dermal dendritic cells and Langerhans cells in the skin and gut DC in the intestine. Two major populations of immature DC have been identified: the $CD11c^{high}$ myeloid DC and the $CD11c^{low}$ plasmacytoid DC (Kapsenberg, 2003; Cella et al., 1997; Ardavin et al., 2001; Shortman and Liu, 2002). Many more subpopulations of DC have been characterized, however discussing all subclasses would go beyond the scope of this thesis. Most functional experiments have been carried out with classical myeloid DC derived from monocytes that are cultured with IL-4 and GM-CSF (Sallusto and Lanzavecchia, 1994). In the study described in **chapter 2** monocyte-derived DC were used to study the maturation of DC.

Activation

Different types of signals can induce the maturation of DC. The first is the pathogen itself (or parts of it) that binds to pathogen recognition receptors on the cell surface of the DC such as toll-like receptors (TLR1-9) (Kadowaki et al., 2001). For example, the bacterial wall substance lipopolysaccharide (LPS) binds to TLR4 and induces activation of DC (Chow et al., 1999; Rescigno et al., 1998). Secondly, mediators, that are produced by cells in the infected tissue can activate DC, such as cytokines, like TNFα and IL-1, and prostaglandins (Steinbrink et al., 2000; Jonuleit et al., 1996; Gallucci and Matzinger, 2001). In addition, cross-linking of CD40 molecules on the cell membrane of the DC can induce the maturation of DC. CD40 cross-linking occurs by the binding with its ligand CD40L, which is mainly expressed on activated T helper cells and is thought to be crucial for full maturation of DC and optimal activation of cytotoxic T cells (Cella et al., 1996; Schoenberger et

al., 1998). In **chapter 2** the maturation of dendritic cells is studied using stimuli from each category.

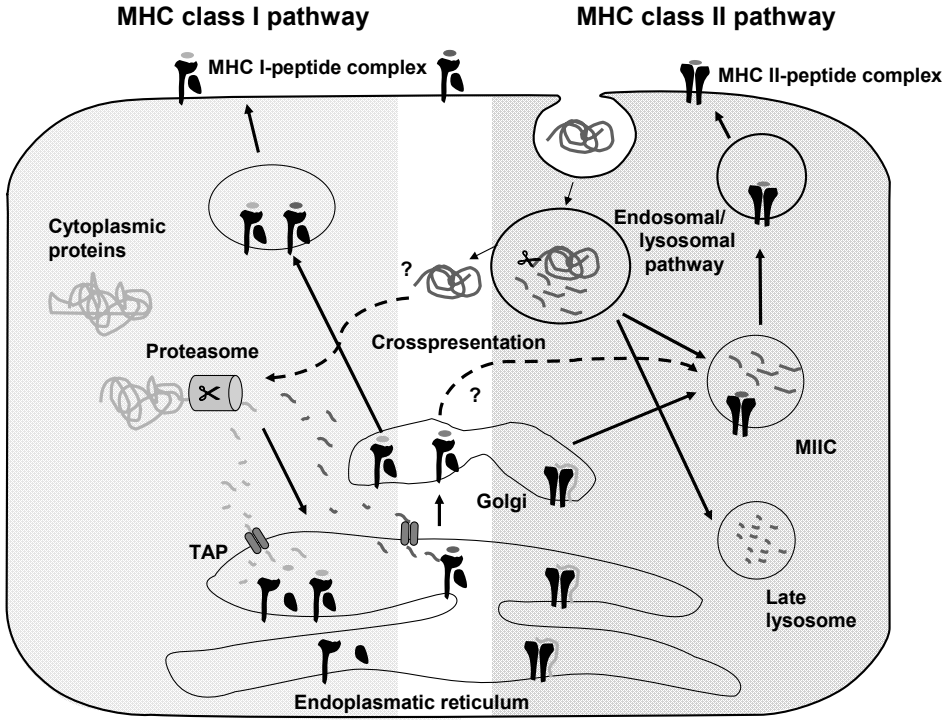

Figure 2. Antigen processing and presentation in MHC class I and II molecules. Cytosolic proteins, both self and non-self like viral proteins, are degraded by the proteasome. Antigenic peptides are transported via the transporter associated with antigen processing (TAP) into the endoplasmic reticulum (ER). In the ER antigenic fragments can form complexes with the MHC class I heavy chain and light chain. MHC class I-peptide complexes are then transported via the trans-Golgi network to the plasma membrane. Exogenous peptides are internalized in the endosomal/lysosomal pathway. In endosomes and lysosomes degradation of the antigens takes place by various proteases. MHC class II molecules are synthesized in the ER and are protected from endogenous peptides by the invariant chain. Vesicles containing MHC class II molecules fuse with late endosomes/early lysosomes and form the MHC class II rich compartment (MIIC). In the MIIC the invariant chain is replaced by antigenic peptides from the endosome. MHC class II-peptide complexes are then transported to the plasma membrane. DC are the only cells that are capable of presenting exogenous antigens in the context of MHC class I molecules, also called cross-presentation. The mechanisms for cross-presentation are yet poorly understood. Exogenous antigens may be released into the cytoplasm and processed by the proteasome and/or endocytosed MHC class I molecules may be loaded in the MIIC together with MHC class II molecules.

Antigen capturing and processing

Immature DC internalize antigens from its environment by fluidphase

endocytosis and receptor-mediated endocytosis (Lanzavecchia, 1996). DC express a wide range of receptors for recognition of glycoproteins, such as C-type lectins (mannose receptor, DC-SIGN, Langerin), conserved microbial patterns, such as toll-like receptors, and binding of antigen-antibody complexes via Fc receptors, that mediate internalization of antigens (Geijtenbeek et al., 2004; Gallucci and Matzinger, 2001). Depending on their tissue localization and differentiation state, DC are specialized to respond to specific microbes, by expressing distinct sets of TLR and C-type lectins (Kaisho and Akira, 2003). Antigen capturing is maximal shortly after activation of the DC and is down-regulated in mature DC to ensure that primarily antigens that were present at the time of infection will be processed and presented to T cells (Granucci et al., 1999). After uptake, antigens traffic through the endosomal/lysosomal pathway to be degraded (Pieters, 1997). Endosomes containing internalized antigens fuse with the so called MHC class II rich compartment (MIIC) where antigens are degraded into antigenic fragments and loaded onto MHC class II molecules (Neefjes, 1999; Watts, 2001). DC are exclusive in that they can also pass exogenous-peptides on to the MHC class I pathway (Thery and Amigorena, 2001; Heath et al., 2004; den Haan and Bevan, 2001; Gromme et al., 1999), enabling them to activate both $CD4^+$ and $CD8^+$ T cells (discussed later in this chapter). More details on antigen presentation by MHC class I and II by DC can be found in figure 2.

DC maturation

The functional modifications during maturation transform immature DC with low T cell stimulating capacity into potent T cell stimulating mature DC (fig. 3). In the first place, maturation of DC leads to a selective decrease of antigen and pathogen internalization activity and the redistribution of MHC molecules from intracellular endocytic compartments to the cell surface. In addition, mature DC show surface expression of several integrins and chemokine receptors, which enables them to migrate out of the tissue via the efferent lymph nodes to present captured antigens to T cells in the local draining lymph node. Mature DC increase the surface expression of costimulatory molecules, like CD80 and CD86, and adhesion molecules for the activation of T cells (Banchereau et al., 2000; van der Merwe and Davis, 2003). Maturation of DC also entails the re-organization of their cytoskeleton. In mature DC cytoskeletal rearrangements are involved in high cell motility typical for mature DC, the formation of dendrites and veils, and the establishment of stable immunological synapses with antigen-specific T cells (Banchereau et al., 2000; Al-Alwan et al., 2001b; Al-Alwan et al., 2001a). Furthermore, mature DC secrete chemokines to attract T cells and cytokines to influence their function (Banchereau et al., 2000). DC produce distinct sets of cytokines

dependent on the encountered stimuli and the maturation state of the cell (Kapsenberg, 2003). Different cytokine profiles can polarize T helper cells to Th1, Th2, or T regulatory cells (see *Effector T cells*). For full activation of cytotoxic T cells, DC have to interact first with antigen-specific T helper cells via CD40-CD40L (Cella et al., 1996; Schoenberger et al., 1998). The DC system is very efficient as one single DC can influence the function of between 300 and 1000 T cells. The study presented **in chapter 2** describes the change in morphology of DC after activation with different stimuli and identifies a potential key role player in the cytoskeletal rearrangements during DC maturation.

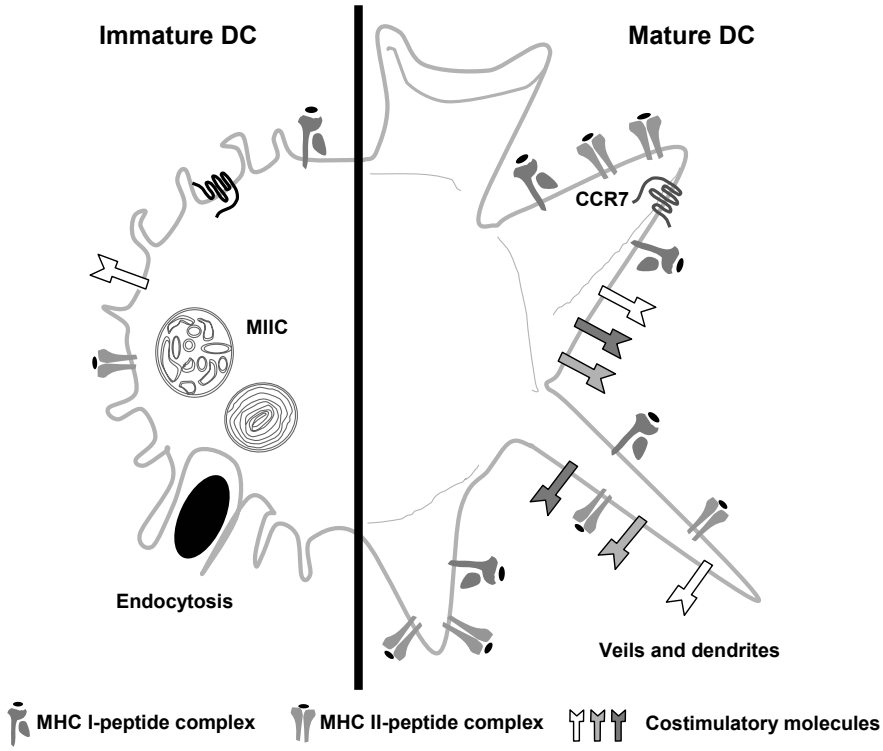

Figure 3. Functional changes during DC maturation. Immature DC are characterized by a high endocytic capacity, low expression of costimulatory molecules and well-developed MHC class II rich compartments (MIIC). After maturation the endocytic capacity and MIICs are lost. Instead, mature DC have high expression levels of MHC class II and costimulatory molecules. Furthermore, mature DC develop dendrites and veils and are highly motile. Moreover, CCR7, the chemokine receptor for lymph node homing, is up-regulated, while other chemokine receptors, such as CCR1, CCR5 and CCR6, are down-regulated.

T cells

T cell differentiation

Like DC, T cells are derived from hematopoietic stem cells in the bone marrow (Akashi et al., 2000). Early in the development precursor T cells migrate to the thymus, where they further differentiate into mature T cells (Delves and Roitt, 2000b). Central in the differentiation is the production of a functional T cell receptor that is specific for foreign antigens. The expression of T cell antigen receptors (TCR) is initiated by somatic recombination of gene segments that code for the variable regions of the receptors. Diversity is created during this process, so that every T cell expresses an unique antigen receptor. Subsequently, T cells are selected for the recognition of self-MHC molecules, but not self-antigenic peptides. Naïve T cells leave the thymus and circulate through the peripheral blood (Spits et al., 1998; Berg and Kang, 2001). Through the expression of the chemokine receptor CCR7 they home to the lymph nodes (Sallusto et al., 1998b; Dieu et al., 1998), where they will interact with DC and after recognition of the right peptide-MHC complex, will be activated into effector or memory T cells. T cells are divided into $CD4^+$ and $CD8^+$ subsets (Chaplin, 2003; Singer et al., 1999). $CD4^+$ T cells usually act as helper cells and can be further subdivided in Th1, Th2 and T regulatory cells (Tr; also called suppressor T cells or Th3) on the basis of their cytokine profiles. T helper cells recognize antigen-derived peptides presented by MHC class II and act primarily to regulate the cellular and humoral immune responses (Delves and Roitt, 2000b; Kidd, 2003). $CD8^+$ T cells are usually cytotoxic and recognize antigenic peptides in the context of MHC class I (Delves and Roitt, 2000b).

T cell activation

When traveling through the T cell areas of the lymph node, T cells will encounter DC that present foreign antigens in the context of both MHC class II and I. To recognize antigen, T cells need to establish a stable contact with APC by the formation of an immunological synapse, where T cell receptors and costimulatory molecules are congregated in a central area surrounded by a ring of adhesion molecules (Kupfer and Kupfer, 2003). The quality and duration of the contact determines the outcome of the interaction; differentiation into effector or memory cells or the development of tolerance, and is dependent on three factors: (1) the level of peptide-MHC complexes that initiate signal transduction (2) the level of costimulatory molecules that amplify the signaling process, and (3) the stability of the synapse (Lanzavecchia and Sallusto, 2001). The efficiency of the synapse as a signal transducing machinery varies with the nature of the APC and the

developmental stage of T cell. In naive T cells, TCR are inefficiently coupled to down-stream signal transduction pathways and are highly dependent on costimulatory molecules expressed on the APC. In contrast, in effector, and memory T cells, TCR triggering is efficiently coupled to signal transduction pathways so that the cells can rapidly respond to low doses of antigen even in the absence of costimulation.

Effector T cells

The two principal subsets of T cells are the $CD4^+$ and the $CD8^+$ positive T cells. After activation the majority of the $CD8^+$ T cells mature into cytotoxic T cells (CTL), that can kill autologous cells that are infected with intracellular bacteria or viruses. $CD4^+$ T cells differentiate mainly into T helper cells upon activation (Chaplin, 2003; Delves and Roitt, 2000b). T helper cells can be subdivided into Th1, Th2 and Tr cells on the basis of their cytokine secretion profile. The Th1 pathway primarily acts against intracellular pathogens, particularly viruses and bacteria, by helping the activation of cytotoxic T cells and stimulating CTL, NK cells and macrophages. Th2 cells are believed to emphasize protection against extracellular pathogens, by stimulation of antibody production of B cells, and of mast cells, basophils and eosinophils. T regulatory cells (Tr) comprise of 5 to 10 percent of the total peripheral T cells pool and are potent immunosuppressors (Kidd, 2003). Tr are required for the tolerance against self and harmles environmental proteins. Furthermore, they prevent excessive inflammation during protective immunity.

The array of cytokines produced by Th1 and Th2 cells varies greatly. In general, Th1 cells secrete far more interferon (IFN) -γ and interleukin (IL) -2 than Th2 cells and Th2 cells produce far more IL-4 (and possibly IL-5) than Th1 cells (Kidd, 2003). Tr cells typically secrete large amounts of either IL-10 or transforming growth factor (TGF) -β. T cell polarization is likely determined under control of APC (Kapsenberg, 2003; Moser and Murphy, 2000; Kidd, 2003), via costimulatory molecules and the secretions of cytokines and/or chemokines (Kapsenberg, 2003; Kidd, 2003). However, also factors produced by NK cells, mast cells and eosinophils and other cells may contribute to the differentiation into T cells (Kidd, 2003). Not only their cytokine profile, but also the expression of chemokine receptors distinguishes Th1 or Th2 cells. The chemokine receptors CCR5 and CXCR3 are preferentially expressed on Th1 and CCR3, CCR4, CCR8 are mainly found on Th2 cells (Zlotnik et al., 1999). Commitment to the Th1 or Th2 type appears to be final (Kidd, 2003). After activation, $CD8^+$ and $CD4^+$ effector/memory T lymphocytes migrate out of the lymph node and into the circulation. The expression of chemokine receptors will lead them to different tissues and eventually to the site of inflammation.

Skin immune system

Skin architecture and immune cells

With a surface of 1.5-2 m^2 the skin is the largest organ of the human body. It has a thermoregulation and a barrier function and protects against external physical, chemical and biological aggressions (Wysocki, 1999). The skin consists of three layers, the epidermis, the dermis and the subcutaneous tissue or subcutis (fig. 4). The epidermis is the most superficial layer of the skin and provides the first barrier against the invasion of foreign substances into the body. The principal cells of the epidermis are the keratinocytes, which are arranged in continuous layers, comprising: the statum basale, stratum spinosum (5-15 layers of cells), stratum granulosum (1-3 layers), and the stratum corneum (5-10 layers). Besides keratinocytes, the epidermis contains melanocytes and Merkel cells (mechano receptors), but also immune cells like Langerhans cells (3-6%) and a small percentage of lymphocytes (<1.3%) with a T memory/effector phenotype. The dermis and the epidermis are separated by a basement membrane that supports the basal keratinocytes and regulates exchanges of metabolic products. Through the basement membrane immune cells can migrate from and into the epidermis. The dermis consists mainly of supportive, compressible and elastic connective tissue and contains fibroblasts, next to vessels, nerve endings and sweat glands. The upper layer of the dermis contains scattered DC, macrophages and mast cells and sporadic T cells. The deepest layer of the skin is the subcutis, which is mainly made of fatty tissue. Adipocytes are arranged in primary and secondary lobules, separated by connective tissue septa containing fibroblasts and also DC and mast cells (fig 4) (Kupper and Fuhlbrigge, 2004; Kanitakis, 2002).

Immune function of the skin

The first barrier against the invasion of microorganisms is a physical one formed by the stratum corneum, which is made of flattened, anucleate cornified cells that are tightly packed in a lipid matrix. The second line of defense are the cells resident in the skin, like keratinocytes and immune cells, which function as sentinels for danger signals. Infiltration of pathogens results in the release of mediators, such as cytokines, chemotactic proteins, and antimicrobial peptides (Kupper and Fuhlbrigge, 2004; Yang et al., 2004). These proteins can act either directly on the cause of stress, like antimicrobial peptides, or activate residing immune cells and/or attract leukocytes form the peripheral blood. In addition, stress can also directly lead to the activation of skin-residing DC. Both the dermis and the epidermis contain DC: the dermal DC and the Langerhans cells, respectively. These cells remain in the skin for

longer periods of time as resting cells until they are activated by stress factors. Sporadic cutaneous lymphocyte-associated antigen (CLA) positive memory T cells are found throughout the skin and probably have a role in immune surveillance. Inflammation will attract great numbers of effector T cells, neutrophils, NK cells and macrophages and increase the number of DC that migrate into the skin (Kupper and Fuhlbrigge, 2004).

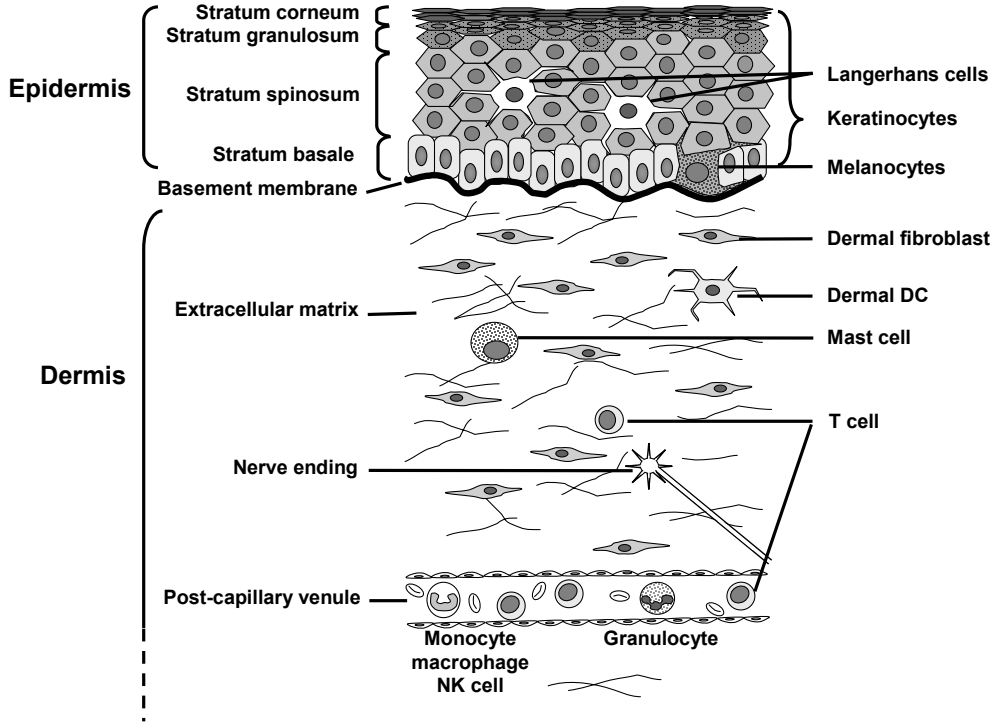

Figure 4. Architecture of the epidermis and the upperlayer of the dermis. Immune cells are present throughout the skin. In the epidermis mainly Langerhans cells are found and sporadic T cells (not shown). In the dermis, Dermal DC are present alongside mast cells and T cells. The dermis is easily accessable for leukocytes via microvessels in the case of inflammatory stimuli.

Langerhans cells

Langerhans cells (LC) represent the dendritic cells of the epidermis. In LC, a cytosolic organelle called the Birbeck granule is found which is exclusively expressed in immature LC. This organelle consists of superimposed and zippered membranes (Birbeck et al., 1961) that are positive for the C-type lectin Langerin (Valladeau et al., 1999). Langerin is specifically expressed by Langerhans cells and not only functions as a carbohydrate receptor but is also involved in the biogenesis of Birbeck granules (Valladeau et al., 2000). The

function of these granules is still poorly understood, but there are indications that they are actively involved in receptor-mediated endocytosis and participate in the antigen-processing/presenting function of LC (McDermott et al., 2002). The importance of Birbeck granules in the immune system can be questioned as in 1994 an healthy white man was identified whose LC completely lacked this organelle (Mommaas et al., 1994). Based on morphological criteria, the expression of characteristic LC markers such as MHC class II and CD1a, and antigen-presenting capacity, these LC appeared otherwise normal and functional, suggesting that Birbeck granules are not a prerequisite for normal LC function. In the Birbeck granule deficient person no labeling was detected with antibodies that are now known to recognize Langerin. **Chapter 3** describes the identification of a genetic defect that may cause the lack of Birbeck granules and the absence of antibody staining in the Langerhans cells of this person.

Homing of T lymphocytes in the skin

Homing

For homing of circulating leukocytes, they have to extravasate through a series of steps through the endothelium of blood vessels in the relevant organ site and migrate into the tissue (Fig. 5) (Thomsen et al., 2003). Homing to specific tissues is controlled by the combined expression of different chemokine receptors and specific adhesion molecule receptors. The earliest step in the recruitment of leukocytes is the tethering and rolling on selectins expressed by post-capillary venules. In addition, endothelial cells express high levels of adhesion molecules and can bind chemokines on their cell surface. Chemokine-binding to receptors on leukocytes is critically involved in activating integrins on the leukocyte, resulting in increasing avidity to vascular-endothelial-integrin ligands (Cinamon et al., 2001a; Thomsen et al., 2003). Subsequently, integrin-binding to its ligand on the endothelium leads to the arrest of leukocytes. For transendothelial migration leukocytes are dependent on chemokines bound to the vessel wall (Cinamon et al., 2001a).

Homing to the skin is established by the co-expression of CLA with specific chemokine receptors. CLA on the T cells will interact with E-selectin on the dermal post-capillary venules and initiate rolling and tethering of the cells. Chemokine binding to receptors on the T cell activates lymphocyte function-associated antigen-1 (LFA-1), which will interact with intercellular adhesion molecule (ICAM) -1 on the endothelial cell. The interaction with integrins and endothelial-bound chemokines will then lead to the arrest of the T cell and to transmigration into the tissue. T cells are then further directed along the chemokine gradient towards the site of inflammation.

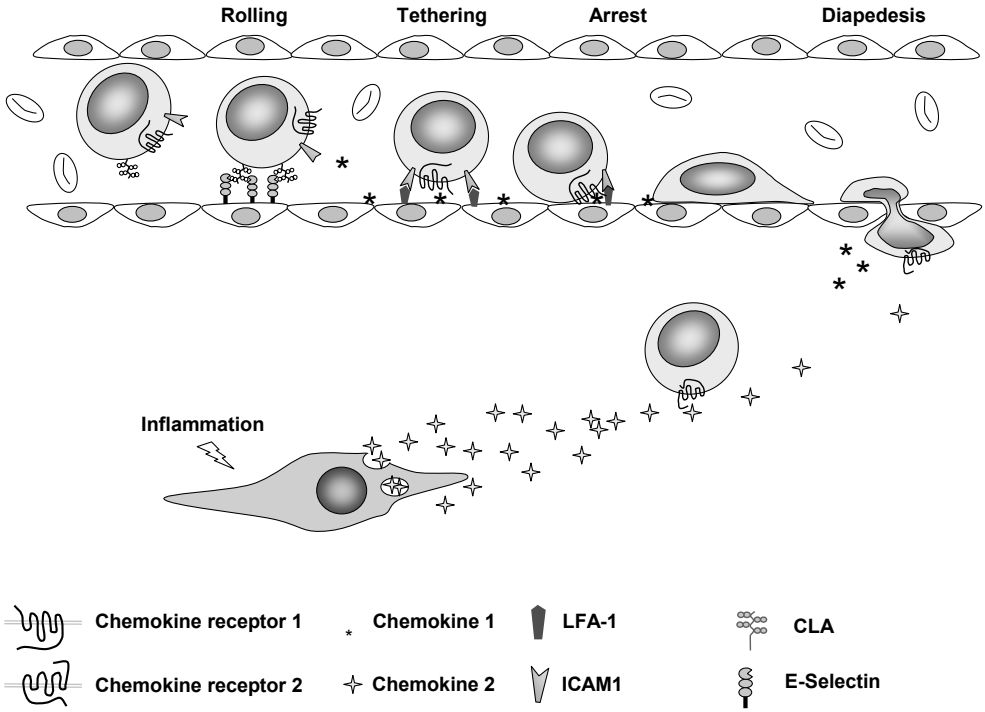

Figure 5. Schematic overview of the recruitment of leukocytes from microvessels into the tissue. Circulating leukocytes bind to adhesion molecules and chemokines bound to surface receptors on the endothelial cells of the inflamed tissue. Leukocytes then transmigrate through the endothelial layer and the basement membrane into the surrounding tissue. Chemokine gradients will then lead the cells to its destination. The arrest of leukocytes in the microvessel and the attraction of these cells in the tissue may be regulated by different chemokines and receptors (modified from Thomsen et al. 2003).

Chemokines and receptors

Although selectins and integrins are important in the arrest of leukocytes at specific sites to allow transmigration, it is essentially the chemokines that specifically target leukocytes to tissues and sites of inflammation. Nowadays, more than 45 chemokines have been identified in humans, binding to at least 18 different receptors. Chemokines are small proteins involved in the recruitment and homing of leukocytes. These proteins are subdivided into four families based on the relative position of conserved cysteine residues: CC, CXC, CXXXC, and XCL, with the CC and CXC groups being the far most common ones (Zlotnik and Yoshie, 2000; Murphy et al., 2000). Chemokines are produced by tissue cells and leukocytes and act through corresponding groups of CCR, CXCR, CX3CR and XCR receptors of the seven-transmembrane,

G protein-coupled receptor family. Several chemokines can bind the same receptor and one chemokine can bind to several receptors creating multiple combinations and, therefore, multiple biological outcomes. Functional consequences of chemokine receptor activation are not limited to locomotion. Granule exocytosis, gene transcription, mitogenic effects and apoptosis are also affected by chemokines (Thelen, 2001; Whiting et al., 2004). In addition, chemokine receptors may be expressed by cells other then leukocytes, including endothelia, stromal cells, smooth muscle cells, epithelia and neurons (Rollins, 1997). Thus, in addition to localization of cells of the immune system to particular compartments, chemokines may be involved in other aspects of tissue homeostasis.

The chemotactic function of chemokines can be classified into two categories: constitutive and inducible chemokines. Constitutive chemokines are involved in the development of lymphatic tissues, basal leukocyte trafficking and immune surveillance. Inducible chemokines are produced upon infection or other stress signals and regulate the recruitment of leukocytes to sites of inflammation (Zlotnik et al., 1999). The production of inducible chemokines is stimulated by early pro-inflammatory cytokines, such as IL-1 and TNF-α, bacterial products, such as lipopolysaccharide, and viral infection (Baggiolini, 1998). In addition, IFN-γ and IL-4, products of Th1 and Th2 lymphocytes, respectively, can induce the production of chemokines (Garcia-Zepeda et al., 1996a; Garcia-Zepeda et al., 1996b), thereby reinforcing the type 1 or type 2 immune response.

Skin homing, CLA^+ T cells typically co-express CCR4. Its ligands CCL17 (thymus and activation-regulated chemokine, TARC) and CCL22 (macrophage-derived chemokine, MDC) are associated with T cell migration into the skin. Both ligands are constitutively expressed on cutaneous venules and are produced during inflammatory conditions by skin fibroblasts and keratinocytes (Yu et al., 2002; Horikawa et al., 2002; Chong et al., 2004). The expression of CCR4 ligands, E-selectin and ICAM1 by endothelial cells is constitutive and is up-regulated upon inflammation. CCL27 (formerly known as cutaneous T cell-attracting chemokine; CTACK) is constitutively produced by keratinocytes, but can also be induced upon stimulation with tumor necrosis factor α (TNF-α) and IL-1β (Homey et al., 2000; Homey et al., 2002) and selectively recruits CLA^+ memory T cells to the skin (Morales et al., 1999). Also, CCL27 appears to be involved in local retention of $CCR10^+/CD4^+$ T cells, likely memory T cells, to the skin (Moed et al., 2004). Leukocytes that have been recruited to the site of inflammation can also stimulate the chemoattraction of more immune cells by the secretion of cytokines that promote the production of chemokines by surrounding tissue cells (fig. 5). For example, IFN-γ, produced by Th1 cells, can stimulate keratinocytes to produce a range of products, including the

chemokines CXCL9, CXCL10, and CXCL11 (Boorsma et al., 1998; Albanesi et al., 2000). The secretion of these chemokines then results in the recruitment leukocytes that express the chemokine receptor CXCR3 (Kupper and Fuhlbrigge, 2004). CXCR3 expression and/or its ligands are associated with atopic dermatitis, contact dermatitis, mycosis fungoides, psoriasis and other types of skin inflammation (Shimada et al., 2004; Serra et al., 2004; Yamaguchi et al., 2003; Kallinich et al., 2003; Flier et al., 2001; Tokuriki et al., 2002).

CXCR3

CXCR3 is expressed mainly on activated T cells (both $CD4^+$ and $CD8^+$ T cells), but can also be found on B cell plasmablasts and NK cells (Inngjerdingen et al., 2001; Loetscher et al., 1998). Expression of CXCR3 on $CD4^+$ T cells is associated with a Th1 phenotype, although it is also expressed by a small subset of Th2 cells (Kim et al., 2001). The ligands of CXCR3, CXCL9 (monokine induced by IFN-γ, MIG), CXCL10 (IFN-γ-inducible protein 10, IP10) and CXCL11 (IFN-inducible protein 9, IP-9; IFN-γ-inducible T cell α-chemoattractant, ITAC) are produced under the influence of IFN-γ, secreted by Th1 cells. In the skin, large amounts of CXCL9-11 are secreted by fibroblasts in the dermis and keratinocytes in the epidermis when stimulated with IFN-γ (Villagomez et al., 2004; Boorsma et al., 1998; Albanesi et al., 2000). Secretion of CXCL9-11 is associated with infiltrates of Th1 cells and CTL in both normal inflammation and in inflammatory diseases and is often found in type 1 immune responses.

CXCR3 and its ligands CXCL9-11 are not restricted to inflammatory skin diseases, but are also found during inflammation in a wide range of other tissues, including liver, brain and intestine. Expression of CXCR3 ligands and infiltration of $CXCR3^+$ cells have been demonstrated in rheumatoid arthritis (Qin et al., 1998; Patel et al., 2001), multiple sclerosis (Balashov et al., 1999), hepatitis C–infected liver (Shields et al., 1999), and artherosclerosis (Mach et al., 1999). The association of CXCR3 with a wide range of immune disorders makes it an attractive target for therapeutic applications. Blocking of CXCR3-ligand interactions in inflammatory disorders may attenuate cell-mediated immune reactions by preventing recruitment of $CXCR3^+$ CTL and Th1 cells. In addition, blocking CXCR3 or its ligands, may delay graft rejection (Hancock et al., 2001; Meyer et al., 2001; Baker et al., 2003; Belperio et al., 2002). On the other hand, expression or injection of CXCR3-ligands in tumors may enhance anti-tumor reactivity. For the development of therapies specifically acting on CXCR3 it is of importance to know the mechanisms of CXCR3-mediated cell activation.

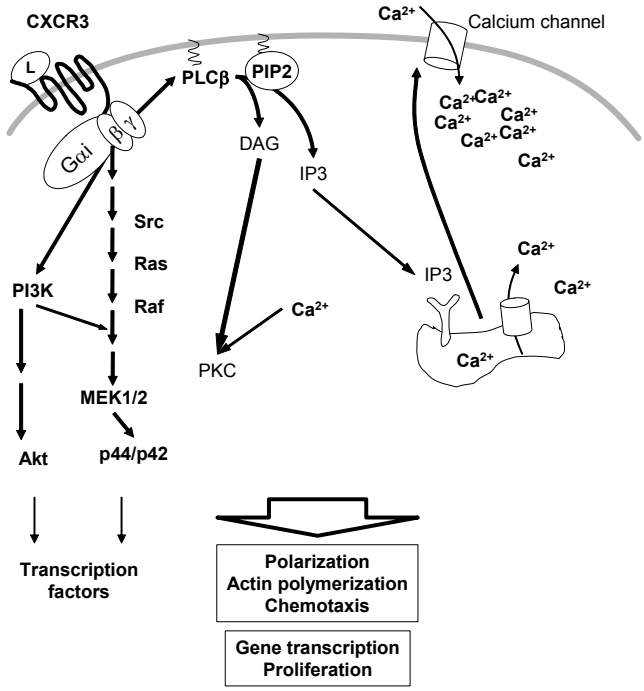

Figure 6. Schematic overview of a selection of signal transduction pathways activated by chemokine receptors. Chemokine receptor triggering leads to the activation of Gi-proteins. The βγ subunits subsequently activate PI3Kγ and PLCβ2 and β3. Activation of PLC results in the formation of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). DAG is a potent activator of protein kinase C (PKC), while IP_3 binds to the IP_3 receptor on intracellular $Ca2^+$ stores, resulting in the release of calcium into the cytosol. This release subsequently induces the influx of calcium via store operated calcium (SOC) channels. Some subsets of PKC are also activated by increased levels of calcium. The α subunit of the Gi protein activates Src-like kinases, which in turn can activate PI3K and mitogen activated protein kinases (MAPKs), like the MEK1/2-p44/p42 pathway. Activation of PI3K leads to activation of Akt.

Signal transduction

A great number of activities of the cell are regulated by ligands binding to receptor on the plasma membrane. The outcome of receptor triggering depends on the signal transduction pathways that are linked to the receptor. Signaling can lead to direct effects, such as activation of integrins, cytokine secretion and/or activation of cytoskeletal rearrangements, and indirect effects by activation of gene transcription (Thelen, 2001; Whiting et al., 2004). Knowledge of transcription pathways used by certain receptors and their effects may reveal leads for the development of specific intervention therapies and will aid the evaluation of the effectiveness of specific receptor antagonists.

The family of chemokine receptors belongs to the seven-transmembrane domain, G-protein coupled receptors. They signal predominantly via Gi proteins and regulate Ca^{2+} fluxes. After activation, the α and $\beta\gamma$ subunits of the Gi-protein each activate a cascade of signaling events. This paragraph will describe the some of the best studied signal transduction pathways that are involved in chemokine signaling and are depicted in figure 6. Following receptor triggering, the $\beta\gamma$ subunits of Gi activate phosphatidylinositol-3-kinase (PI3K) -γ and phospholipase C (PLC) β1 and β2 isoenzymes. Several studies show crucial roles for PI3K in leukocyte migration. Activation of PLC leads to the formation of inositol-1,4,5-triphosphate (IP_3) and a transient rise in the concentration of intracellular calcium. This increase in intracellular calcium has been widely used to test the responsiveness of chemokine receptors to different chemokines. Another product of PLC is diacylglycerol (DAG), which is a potent activator of protein kinase C (PKC). Some subsets of PKC are also activated by increased levels of calcium. The α subunit of the Gi protein activates Src-like kinases, which in turn can activate PI3K and mitogen activated protein kinases (MAPKs) (Thelen, 2001).

CXCR3-triggering also elicits Ca^{2+} mobilization in CXCR3-transfected cells (Loetscher et al., 1996; Tensen et al., 1999) and in peripheral blood T cells and NK cells (Rabin et al., 1999). Furthermore, binding of CXCL10 to CXCR3 on pericytes obtained from human liver activates the PI3K/Akt pathway and the ras/raf/Mek/p44/p42 pathway, probably via Src. In these pericytes, PI3K and MEK are involved in CXCR3-mediated migration (Bonacchi et al., 2001). Which signal transduction pathways are used by CXCR3 on activated T cells and how they are involved in CXCR3-mediated effects is largely unknown. In **chapter 4** the functional importance of the PI3K/Akt pathway and the MEK/p44/p42 pathway in T cell migration is studied. **In chapter 5** the characteristics and importance of Ca^{2+} mobilization following CXCR3-triggering is determined. Stimulation of $CXCR3^+$ cells not only induces chemotaxis, but also stimulates proliferation of T cells and the production of IFN-γ by activated $CD4^+$ T cells (Whiting et al., 2004). Knowledge of all signal transduction pathways involved in CXCR3-signaling and their functional importance may facilitate the development and evaluation of new therapeutic agents that are aimed at $CXCR3^+$ T cells. Development of these agents may be focused on neutralization of CXCR3 ligands, by administration of blocking antibodies (Xie et al., 2003), or on the receptor itself, either by blocking the ligand binding site or by intervening in the relevant ligand-induced signaling pathways. In collaboration with the dept. of Pharmacochemistry, Free University, Amsterdam an antagonist for CXCR3 is now being developed and tested in biological assays (described in **chapter 6**).

Chapter 2:

Morphological changes during dendritic cell maturation correlate with cofilin activation and translocation to the cell membrane

Pauline Verdijk[1], Peter A. van Veelen[2], Arnoud H. de Ru[2], Paul J. Hensbergen[1], Kensaku Mizuno[4], Henk K. Koerten[3], Frits Koning[2], Cornelis P. Tensen[1], A. Mieke Mommaas[3]

[1]Department of Dermatology, LUMC
[2]Department of Immunohaematology and Bloodtransfusion, LUMC
[3]Center for Electron Microscopy, LUMC
[4]Department of Biomolecular Sciences, Graduate School of Life Sciences, Tohoku University, Aoba Sendai, Japan

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Abstract

Upon activation, tissue residing immature dendritic cells (DC) start to migrate towards the draining lymph node and mature into efficient antigen-presenting cells. During maturation DC loose their capacity to endocytose antigens, change their surface expression of adhesion molecules, chemokine receptors, and costimulatory molecules, and change morphology. We employed 2D-PAGE and mass spectrometry to identify additional differentially expressed proteins in immature and mature DC. Human monocyte-derived DC were matured with LPS and protein expression profiles were compared before and after maturation. One of the proteins differentially expressed between immature and mature DC was identified as the actin-binding protein cofilin. We show here that cofilin is dephosphorylated in response to several maturation stimuli (i. e. CD40 ligand, LPS or a combination of TNF-α and prostaglandin E_2). Moreover, dephosphorylated cofilin translocated towards the plasmamembrane during maturation. Importantly, this correlated with an increase in filamentous actin and the appearance of veils, suggesting a role for cofilin in cytoskeletal rearrangements during maturation.

Introduction

Dendritic cells are professional antigen-presenting cells that have a central role in the initiation of both innate and adaptive immune responses {reviewed in (Banchereau et al., 2000; Cella et al., 1997; Gallucci and Matzinger, 2001; Hartgers et al., 2000)}. DC are essential for the induction of efficient T cell responses as they are the only cell type able to prime naive T cells and to cross-present exogenous peptides in MHC class I molecules. The majority of DC in the periphery are in an immature state. Immature DC are poor T cell stimulators and reside in non-lymphoid tissues or circulate through the peripheral blood. They are specialized in the uptake of antigen and function as the sentinels of the immune system. After the encounter with inflammatory signals, such as LPS, DC enhance their uptake of foreign antigens and activate the machinery involved in processing and presenting them (Granucci et al., 1999). Simultaneously, DC start to mature and migrate towards the draining lymph node, where they interact with T cells and initiate an immune response. During maturation, DC undergo many phenotypical and morphological changes. Mature DC have lost the ability to capture antigen and have up-regulated MHC class II and costimulatory molecules. Inflammatory stimuli also induce a change in the expression of chemokine receptors and adhesion molecules that are involved in migration. Furthermore, changes in morphology of the cell, such as the formation of veils and dendrites, may augment the establishment of DC-T cell contacts and improve the DC-T cell interaction.

Differences between immature and mature DC have been studied extensively. Most studies focus on mRNA expression patterns, with techniques such as DNA microarrays (Ahn et al., 2002; Granucci et al., 2001; Huang et al., 2001; Le Naour et al., 2001; Pulendran et al., 2001). However, these techniques do not provide information on protein expression, activity and post-translational modifications.

We have now compared protein expression profiles acquired by two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) to identify novel proteins putatively involved in the differentiation from immature to mature DC. With this approach, several proteins were found to be differentially expressed between immature and mature monocyte-derived DC. By mass spectrometry one of these proteins was identified as cofilin, an actin-binding protein involved in the turnover and severing of actin filaments {reviewed in (Bamburg, 1999; Chen et al., 2000; Holt and Koffer, 2001; Weber, 1999)}. Cofilin is inactivated by phosphorylation on its serine at position 3, which blocks its activity by interfering with the binding to actin (Agnew et al., 1995; Moriyama et al., 1996). We show here that cofilin phosphorylation is regulated during DC maturation. Furthermore, we observed translocation of cofilin to the cell membrane concomitant with an increase in the concentration of filamentous actin (F-actin) near the cell membrane, suggesting a role for cofilin in cytoskeletal rearrangements during DC maturation.

Materials and methods

Antibodies and reagents

Purified antiserum against phosphorylated cofilin used for fluorescence microscopy was prepared as described previously (Toshima et al., 2001). The antiserum against phosphorylated cofilin usedf or immunoblot detection was generously donated by J. R. Bamberg (Colorado State University, Fort Collins, CO). Anti-cofilin and -profilin were purchased from Cytoskeleton, rhodamine-phalloidin, and secondary antibodies labeled with fluorochromes were obtained from Molecular Probes (Leiden, The Netherlands), goat anti-rabbit peroxidase-conjugated secondary antibody was from Pierce (Rockford, IL) Antibodies against CD40, CD80, CD83 and HLA-DR and isotype controls labeled with FITC or phycoerythrin were from Becton Dickinson (Alphen a/d Rijn, The Netherlands). Human cofilin was obtained from Tebu-bio (Heerhugowaard, The Netherlands).

DC culture

Monocytes were purified from human PBMC by positive selection with CD14 microbeads (Miltenyi Biotec, CLB, Amsterdam, The Netherlands). Monocytes (>95% pure) were cultured in RPMI 1640 with 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin and 10% FCS (all from Gibco-BRL, GB), 800 U/ml GM-CSF (Leukomax; Novartis Pharmaceuticals, East Hanover, NJ), and 500 U/ml IL-4 (PeproTech, Rocky Hill, NJ). After 6 days, cells developed into typical immature DC being $CD14^-$, $CD1a^+$, $CD11c^+$, CD80-, CD86low, HLA-DRintermediate, and CD83- (data not shown). For maturation, DC were incubated with 10 mg/ml LPS from Escherichia coli (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 24 or 48 h. After 24-h stimulation with LPS, DC showed a typical mature phenotype, being CD1ahigh, HLA-DR^{high}, $CD80^{high}$, $CD86^{high}$, and $CD83^+$. Alternatively, DC were matured with L-cells transfected with human CD40L (Garrone et al., 1995) (L-CD40L) in a 1:4 ratio to DC or with a combination of 100 nM prostaglandin E_2 (PGE_2; Sigma) and 50 ng/ml TNF-α (R and D systems, Abingdon, GB) (Lee et al., 2002). Mouse fibroblast L-cells, non-transfected (L-orient) or stably transfected with CD40L (L-CD40L) were grown in RPMI, 10% FCS and penicillin/streptomycin. For co-culture experiments, L-cells were irradiated (80 Gy) before addition to the DC culture. Expression of HLA-DR, CD86, CD83, and CD40 was analyzed by flow cytometry (FACScan, BD Biosciences, Mountain View, CA). Relative expression was determined by the ratio $MFI_{t=x}/MFI_{t=0}$.

Two-dimensional polyacrylamide gel electrophoresis

Cell pellets, consisting of 1×10^6 cells, were lysed directly in 500 μl of 8 M urea containing 2% (w/v) CHAPS, 0.5% (v/v) IPG buffer pH 3-10 (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) and 10 mM dithioerythritol (DTE). After 60 min the samples were centrifuged to sediment insoluble cell components. Lysates were then applied onto isoelectric focusing immobiline DryStrips with a linear gradient from pH 3 to 10 (Amersham Pharmacia Biotech). After rehydration of the IPG-strips for 12 h, proteins were focused for 50,000 Vh at room temperature. Prior to the second dimension, strips were equilibrated in 2% w/v DTE, followed by 2.5% w/v iodoacetamide, both in 6.0 M urea, 2% SDS, 30% glycerol in 50 mM Tris-HCl (pH 6.8) for 15 min. After this procedure, the strips were placed on top of a 12% polyacrylamide gel (2.6% C piperazine diacryl, 375 mM Tris/HCl pH 8.8) and electrophoresed overnight. Proteins were visualized using silver staining as described previously (Aizawa et al., 1997).

Protein identification by mass spectrometry

Protein spots of interest were excised out of the 2D gels. Gel pieces were successively washed with distilled water, 50% acetonitrile in water and 100% acetonitrile. After freeze-drying, the gel pieces were equilibrated by incubating with 0.1 M DTT in 0.1 M sodium bicarbonate for 45 min at 56° C and 55 mM iodoacetamide in 0.1 M sodium bicarbonate for 30 min at room temperature. The gel slices were then washed with 50% acetonitrile in 0.1 M sodium bicarbonate and 100% acetonitrile in succession. Tryptic digestion was performed overnight at 37° C with 10 ng/ml trypsin (Promega Benelux, Leiden, The Netherlands) in 50 mM sodium bicarbonate. Peptides were extracted by first adding 50 ml of 0.1% TFA for 15 min and then 50 ml acetonitrile for another 15 min. The supernatant was then transferred to a glass tube for mass spectrometry and the procedure was repeated. The supernatant was dried down in a speedvac and samples were analyzed by mass spectrometry.

Mass spectrometry

Electrospray ionization mass spectrometry was performed using a Q-TOF1 hybrid mass spectrometer (Micromass, Manchester, GB). Precursor ions were selected with the quadrupole window set to 2-Daand fragments were collected with high efficiency with the orthogonal time of flight mass spectrometer. The collision gas applied was argon (pressure 4×10^{-5} mbar) and the collision voltage ~30 V. The program PeptideSearch was used for pattern searches

(http://www. mann. embl-heidelberg. de/GroupPages/PageLink/peptide-search/Services/PeptideSearch/FR_PeptidePatternFormG4.html) (Mann and Wilm, 1994).

Western blotting

Immature and mature cells were resuspended in lysis buffer (50 mM Tris/HCl pH 7.5, 10 mM EDTA, 80 mM KCl, 1% NP40), supplemented with protease and phosphatase inhibitors [10 mg/ml leupeptin, 10 mg/ml aprotinin, 50 mM PMSF, sodium orthovanadate and serine/threonine phosphatase inhibitor cocktail I (1:100), all obtained from Sigma]. After at least 30 min on ice, insoluble fragments were removed by centrifugation (850 $\times$ g, 4° C). Proteins were separated on 10% SDS-PAGE and transferred onto nitrocellulose membranes. Nonspecific binding was blocked by incubating the membranes with 3% w/v non-fat dry milk and 0.05% Tween-20 in TBS (TBST). Membranes were incubated with the first antibody in the appropriate dilution for 1 h at room temperature or overnight at 4° C. After incubation, membranes were rinsed with distilled water and washed three times in wash buffer (0.25 % non-fat dry milk in TBST). Secondary antibody conjugated to horseradish peroxidase was added to the membranes in 3% non-fat dry milk in TBST in the appropriate dilution. After 1 h of incubation at room temperature membranes were washed three times and developed using an enhanced chemiluminescence kit (Tebu-bio).

Scanning electron microscopy

Immature and mature DC were fixed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer for at least 60 min. Cells were then allowed to attach to poly-L-lysine coated coverslips for 1 h. Cells were postfixed in the same fixative for another 0.5 h. After a short rinse in PBS, specimens were dehydrated in ascending concentrations of ethanol (50-100%). Samples were then critical point dried, mounted on aluminum stubs and coated with a layer of gold in a sputtering device (Emitech, Ashford, GB). Specimens were viewed with a JSM 6700F scanning electron microscope (Jeol, Peabody, MA) at 2.5 kV.

Fluorescence microscopy

Immature and mature DC were fixed by adding an equal volume of 4% paraformaldehyde in PBS to the culture for at least 15 min. Cells were spun onto slides and air-dried before staining. Cells were permeabilized with 0.1% Triton X-100 and then blocked with 1% BSA in PBS. Cells were incubated with

phalloidin-rhodamine or with primary antibody, followed by Alexa-fluor-labeled secondary antibody. Coverslips were mounted with glycerol, containing 100 ng/ml DAPI. Specimens were viewed on a Leica fluorescence microscope.

Actin polymerization assay

Cells were cultured in 24- or 28-well culture plates and stimulated with different stimuli as indicated. After 24-48 h of stimulation cells were resuspended in the culture medium and fixed by adding an equal volume of 4% paraformaldehyde in PBS for at least 15 min. Cells were permeabilized in 0.1% Triton X-100 in PBS for 10 min, washed with 0.25% BSA in PBS and blocked in 1% BSA in PBS for 5 min, prior to incubation with 0.5 mM rhodamine-phalloidin in 1% BSA in PBS for 1 h at room temperature. After three washes cells were resuspended in 0.25% BSA/PBS. MFI was measured by flow cytometry (FACScan). Relative F-actin was determined by the ratio $MFI_{t=x}/MFI_{t=0}$.

Results

Cofilin expression is regulated during maturation

Monocyte-derived DC cultured with LPS were used to study the differences in protein expression in immature and mature DC. LPS-stimulated DC exhibited a typical mature phenotype compared to non-stimulated cells, as evidenced by the expression of maturation markers (CD80, CD86, CD83; data not shown), and by their morphology (fig. 1). Membrane morphology of DC changed from microvilli in immature cells (fig. 1a) into veils in mature cells (fig. 1b, c) with the magnitude of the veils increasing with time of LPS exposure. Protein expression profiles of immature DC and mature DC were analyzed by 2D-PAGE. Proteins in the cell lysates from these cells were first separated on the basis of isoelectric point and molecular weight (fig. 2A). Protein spots of interest (down- or up-regulated during DC maturation) were excised and analyzed by mass spectrometry. Based on the combined information of peptide fingerprints and the sequence of selected peptides, one of the proteins that was down-regulated after 24 h of LPS, was identified as the 18-kDa actin-binding protein cofilin. The intensity of the cofilin spot was decreased in mature cells compared to immature cells, and was located in the gel at about pH 6.5. Upon phosphorylation of an N-terminal serine, cofilin is known to be deactivated. The theoretical isoelectrical point of non-phosphorylated cofilin is 8.2 (www. expasy. org). We hypothesized that the cofilin isolated from the gel might be the phosphorylated form, as phosphorylation of proteins can cause a shift to a more acidic isoelectric point. Indeed, a fragment was found within the tryptic digest of cofilin that corresponded to the expected mass of the phosphorylated N terminus of cofilin. The identity of this fragment was confirmed by MS/MS analysis (fig. 2B). When commercially available cofilin was analyzed with 2D-PAGE two forms of cofilin could be detected: one at pH 6.5 and one at ca. pH 8 (data not shown). The latter could not be distinguished in gels derived from whole cell lysates, as the resolution of the 2D gels was not adequate in the region from pH 8 to 10. Overall, our results clearly show that in response to LPS, cofilin activity in DC is regulated by dephosphorylation.

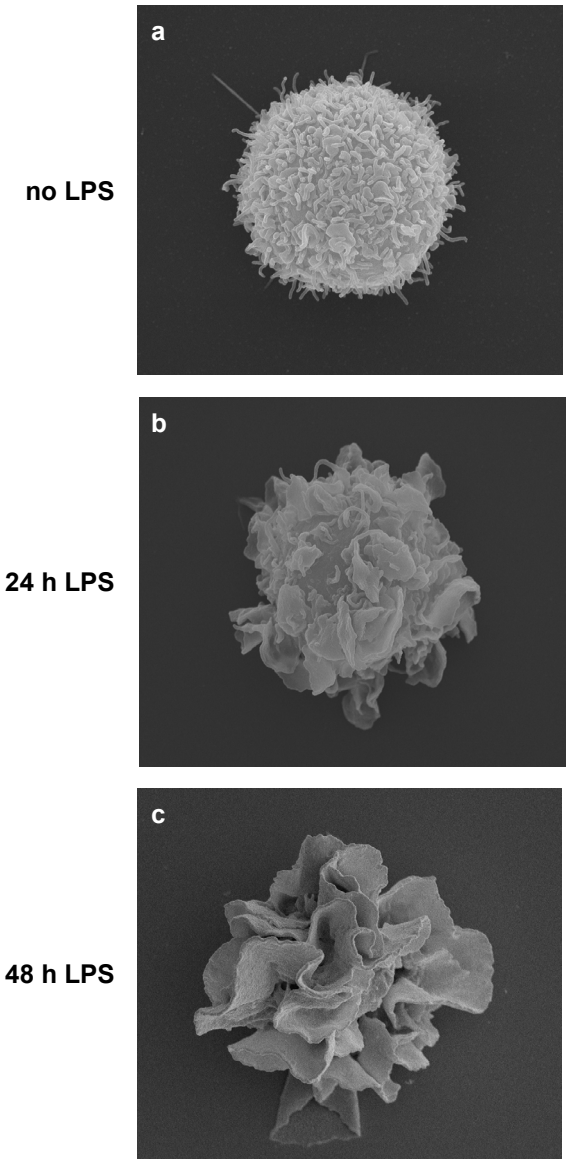


Figure 1. Immature versus mature DC. Scanning electron micrograph of immature and mature DC. Monocyte-derived DC were cultured without (a) or with LPS for 24 h (b) or 48 h (c). Cells were fixed and allowed to adhere to poly-L-lysine-coated coverslips before preparation for scanning electron microscopy.

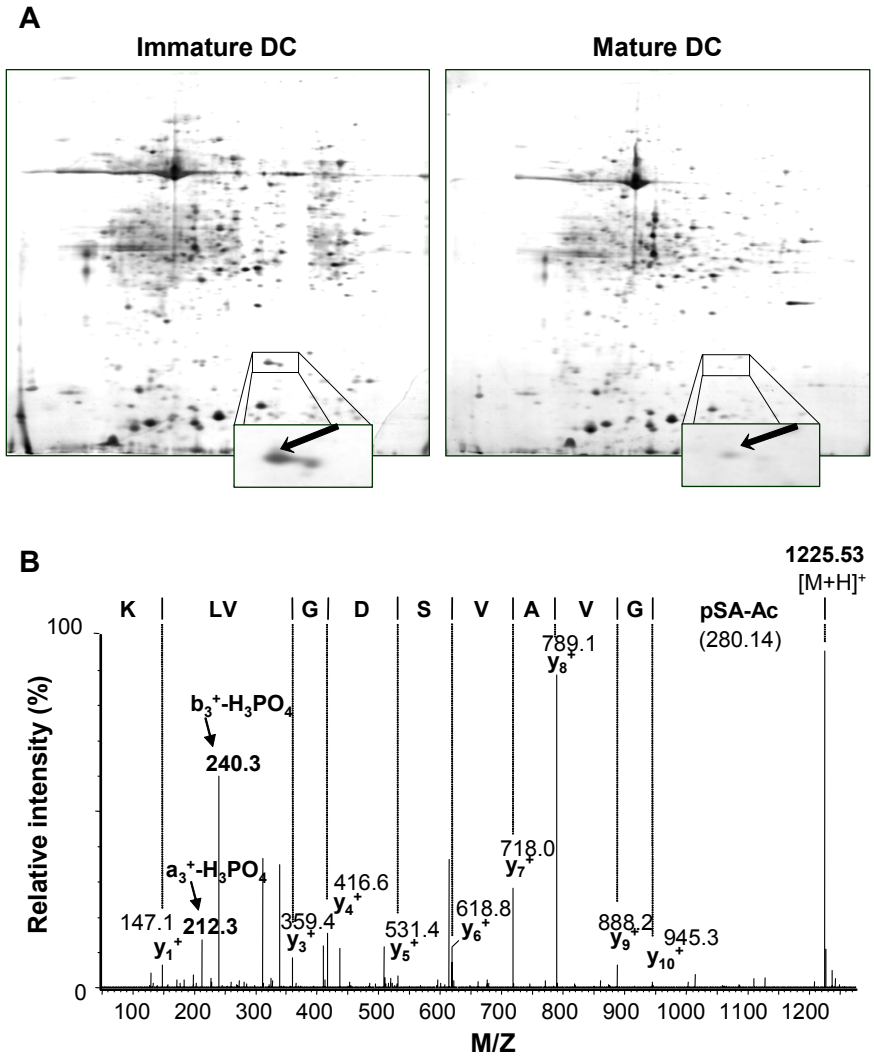


Figure 2. A. Two-dimensional PAGE of cell lysates of immature and mature DC. Monocyte-derived DC were cultured with or without LPS for 24 h. Proteins were separated by isoelectric focusing (pH range 3-10, linear) and SDS-PAGE. Inserts show magnifications of one of the differentially expressed proteins. The spot indicated with the arrow was identified as cofilin by mass spectrometry. **B.** MS/MS spectrum of phosphopeptide from cofilin. The spot indicated with an arrow in (A) was digested with trypsin and analyzed by mass spectrometry. In addition to several non-phosphorylated peptides from cofilin, a fragment of 1225.5 $[M+H]^+$ was identified. MS/MS analysis of this fragment revealed that it corresponded to the N-terminal tryptic fragment of cofilin, starting with an acetylated alanine (Ac-A) followed by a phosphorylated serine (pS, judged by neutral loss of H3PO4 from b3 and a3 ions).

Cofilin is dephosphorylated after LPS exposure

To confirm that cofilin was dephosphorylated during maturation and to study the time course in which this occurs, we studied the level of phosphorylation of cofilin in DC at different time points during maturation with an antibody specific for the phosphorylated form of cofilin. Monocyte-derived DC were stimulated with LPS, lysed at different time points ranging from 5 min to 24 h and analyzed by Western blot (fig. 3). In immature DC a basal level of phosphorylated cofilin was detected. Stimulation with LPS increased the level of cofilin phosphorylation within 5 min after stimulation, which then gradually decreased. After 24 h of LPS stimulation, when DC reached the mature phenotype, the level of phosphorylated cofilin was nearly undetectable, while no or little change was seen in the total expression of cofilin. In addition, levels of profilin, an actin-binding protein responsible for filament assembly, did not change during maturation. These results confirm that cofilin is dephosphorylated during maturation, and suggest a role for cofilin in cytoskeletal rearrangements occurring during maturation.

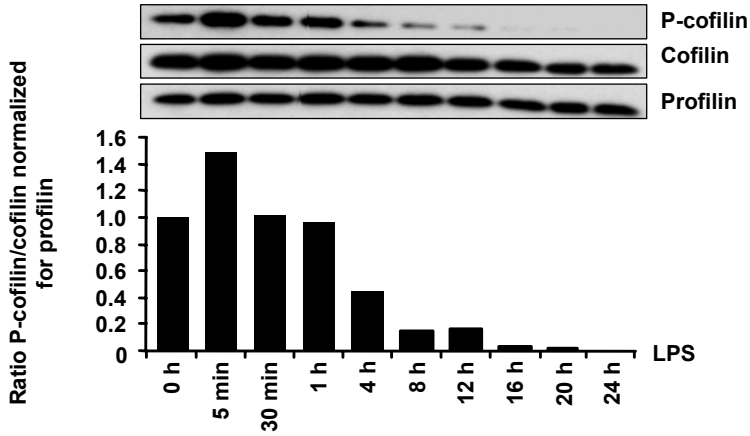


Figure 3. Effect of LPS-induced maturation on cofilin phosphorylation in DC. Immature monocyte-derived DC were stimulated with LPS for various times from 5 min to 24 h. Cells were lysed at indicated time points and analyzed by Western blot, with antisera specific for cofilin, phosphorylated cofilin (P-cofilin) and profilin. The ratio of phosphorylated cofilin versus total cofilin was calculated and normalized for the expression of profilin. Results given are representative for four experiments with cells from four different donors.

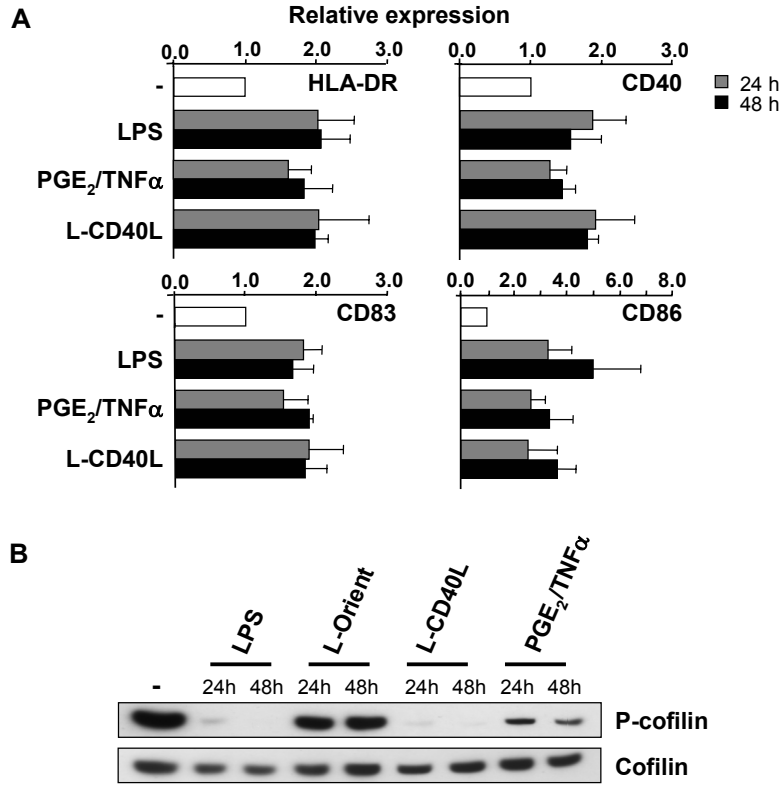


Figure 4. A. Phenotypic analysis of immature DC and DC matured with LPS, PGE_2/TNF-α or CD40L for 24 or 48 h. Expression of HLA-DR, CD40, CD83 and CD86 was measured by FACS analysis. Diagrams present data from three donors and show the relative expression, calculated as described in materials and methods. Error bars show the SEM. **B.** Different DC maturation stimuli induce cofilin dephosphorylation. Immature monocyte-derived DC were stimulated with either LPS, PGE_2/TNF-α, L-Orient, or L-CD40L for 24 or 48 h. Cell lysates were analyzed by Western blot, with antisera specific for cofilin and phosphorylated cofilin (P-cofilin). Results given are representative for experiments with five to eight different donors.

Cofilin dephosphorylation corresponds with DC maturation

To investigate whether the dephosphorylation of cofilin was a general effect of DC maturation, we studied the effect of other known inducers of DC maturation. In addition to LPS, cells were stimulated either with a combination of prostaglandin E_2 (PGE_2; 100 nM) and TNF-α (50 mg/ml) (PGE_2/TNF-α) (Lee et al., 2002) or by cross-linking of CD40 by co-cultured DC with irradiated L-cells transfected with human CD40 ligand (L-CD40L) (Garrone et al., 1995) for 24 and 48 h. Untransfected L-cells (L-Orient) were used as a control. DC stimulated with LPS, PGE_2/TNF-α or CD40L-expressing cells showed typical

mature morphology (e. g. clustering, dendrite formation) in culture within 24 h (data not shown), while unstimulated cells or cells co-cultured with L-Orient resembled immature DC (e. g. absence of clusters of cells, no dendrite formation). This was confirmed by phenotypic analysis (fig. 4A). DC cultured with LPS or with CD40L-expressing cells were highly positive for HLA-DR, CD86, CD40 and CD83. DC stimulated with PGE_2/TNF-α, however, showed an intermediate phenotype: CD86 and CD83 positive, but low expression of HLA-DR and CD40 (fig. 4A).

Similar to maturation with LPS both CD40 ligation and stimulation with PGE_2/TNF-α induced dephosphorylation of cofilin (fig. 4B). The degree of DC maturation as measured by FACS analysis correlated with the degree of cofilin dephosphorylation, CD40 ligation and LPS being more effective than PGE_2/TNF-α. The degree of dephosphorylation of cofilin increased with the time of maturation for all three stimuli. Thus, in addition to LPS, also CD40L and PGE_2/TNF-α induced activation of cofilin, and the level of dephosphorylation paralleled the degree of DC maturation.

Cofilin relocalizes during maturation

Next, we investigated whether cofilin indeed interacts with the DC cytoskeleton, by studying the localization of cofilin in immature and mature DC by immunofluorescence microscopy (fig. 5a-d). In immature DC, cofilin was localized throughout the cytoplasm and excluded from the nucleus (fig. 5a). Phosphorylated cofilin was also found in the cytoplasm, but was more concentrated towards the plasma membrane (fig. 5c). In mature cells the expression of phosphorylated cofilin was strongly diminished or absent (fig. 5d). In contrast, staining for total cofilin (fig. 5b) demonstrated higher expression near the cell membrane and in membrane protrusions of mature cells, while cofilin was absent from the cytoplasm. Thus, during DC maturation cofilin was dephosphorylated and the active form was concentrated below the plasma membrane and in veils, suggesting a translocation of cofilin towards, and interaction with, the actin cytoskeleton underneath the cell surface.

F-actin is increased in mature DC

Finally, we studied the dynamics of the actin cytoskeleton during DC maturation. Cofilin can bind to actin and is involved in actin depolymerization, polymerization and rearrangement of the actin cytoskeleton. The actin cytoskeleton is mainly localized below the plasma membrane and is

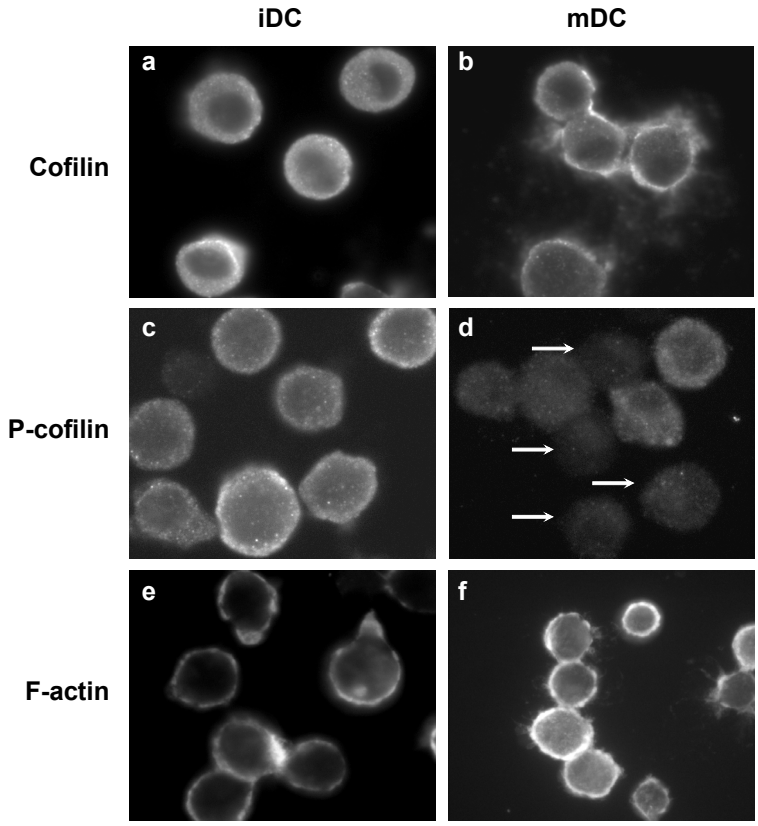
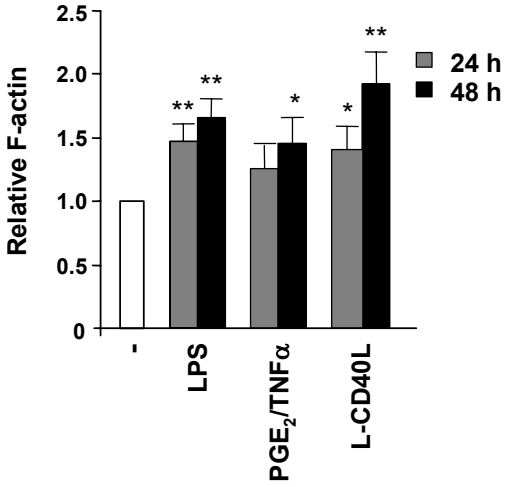


Figure 5. Effects of LPS-induced maturation on the localization and phosphorylation state of cofilin and on F-actin in DC. Monocyte-derived DC were stimulated for 24 h with LPS. Immature (iDC; a, c, e) and mature (mDC; b, d, f) DC were fixed and spun onto slides. Cells were stained for cofilin (a, b) or phosphorylated cofilin (P-cofilin) (c, d) with specific antisera, followed by a secondary antibody labeled with Alexa^{594}. Arrows indicate cells with very low expression of phosphorylated cofilin. Actin-filaments were visualized with rhodamine-phalloidin (e, f).

responsible for the shape, stability and motility of the cell surface. As cofilin is activated and translocated towards the cell membrane, it may be associated with and alter the amount of F-actin in the cell. Therefore, F-actin was visualized with rhodamine-labeled phalloidin for immunofluorescence microscopy. In immature DC, F-actin could be detected below the cell membrane (fig. 5e). After 24 h of LPS stimulation, the amount of F-actin was increased and seemed more concentrated at the cell membrane (fig. 5f). The increase in F-actin was even more pronounced after 48 h of LPS treatment. Levels of F-actin during DC maturation were quantified by staining actin filaments in the cell with rhodamine-labeled phalloidin and analyzing the mean

fluorescence intensity (MFI) by flow cytometry (fig. 6). The amount of F-actin increased during DC maturation independent of the stimulus used: LPS, CD40L-expressing cells or PGE_2/TNF-α. Co-culture with L-Orient control cells did not increase the F-actin content. Again, LPS and CD40 ligation were more effective in inducing an increase in F-actin ($p<0.01$, 48 h) than PGE_2/TNF-α ($p=0.052$, 48 h). Scanning electron microscopy revealed that after 48 h of stimulation with L-CD40L DC had an appearance similar to DC matured with LPS (not shown). Co-culturing with L-orient did not induce morphological changes. Thus, simultaneously with the dephosphorylation and translocation of cofilin, the amount of F-actin increased during DC maturation and this was paralleled by the appearance of veils.

Figure 6. Maturation induces an increase in F-actin content of DC. DC were stimulated with LPS, L-Orient, L-CD40L, or PGE_2/TNF-α and fixed with paraformaldehyde after 24 or 48 h. After staining with phalloidin-rhodamine, the amount of F-actin was measured by FACS analysis. The diagram represents data from 5 to 11 different donors and shows the relative F-actin, calculated as described in materials and methods. Error bars show SEM (Student's t-test; *; *; $p<0.01$, *; $p=0.05$).



Discussion

During maturation of DC a large number of genes and proteins are being regulated to establish the transition of the immature into the mature phenotype. In this study, we have used 2D-PAGE followed by mass spectrometry to compare protein expression profiles of immature and mature monocyte-derived DC. One of the differentially expressed proteins identified with this technique was cofilin, which appeared to be dephosphorylated during DC maturation. Stimulation with LPS induced translocation of the dephosphorylated (activated) form of cofilin towards the F-actin-rich area below the cell membrane. This shift correlated with an increase in F-actin and in the concentration of actin filaments at the cell membrane. Cofilin dephosphorylation was dependent on the degree of maturation, induced by LPS, CD40-cross-linking or PGE_2/TNF-α, and correlated with an increase in F-actin, suggesting a role for cofilin in rearrangements in the actin cytoskeleton. Moreover, this was paralleled by the appearance of veils appearing during DC maturation.

Cofilin is a small phosphoprotein of 18.5 kDa {reviewed in (Bamburg, 1999; Chen et al., 2000; Holt and Koffer, 2001; Weber, 1999)}, which can bind to both F-actin as well as free actin monomers (G-actin) and binds preferentially to actin-ADP. Binding of cofilin to F-actin increases the dissociation of actin monomers and can sever actin filaments. Hereby, cofilin can enhance the turnover of the actin cytoskeleton and increase the number of actin filaments and, thereby, increase the amount of potential sites of actin polymerization. The actin cytoskeleton is vital for the stability of the cell membrane and membrane-dependent processes such as migration (Albrecht-Buehler, 1977; Huttenlocher et al., 1996); reviewed in (Vicente-Manzanares et al., 2002), motility (Chen et al., 2000; Shutt et al., 2000), phagocytosis (Chen et al., 2000; Diakonova et al., 2002; Nishita et al., 2002) and division (Chen et al., 2000; Nagaoka et al., 1995). Moreover, in DC the actin-cytoskeleton is essential for the formation of its characteristic dendrites and veils and the immunological synapse (Al-Alwan et al., 2001b; Badour et al., 2003; Dustin and Cooper, 2000; Sechi et al., 2002; Thome, 2003; Vicente-Manzanares et al., 2002; Wetzel et al., 2002). Cofilin is capable of increasing the rate of actin depolymerization and polymerization, and the turnover of actin filaments. A role for cofilin has been described in processes involving the formation of membrane protrusions and podosomes, which have a part in phagocytosis, membrane ruffling, lamellipod extension and cell motility (Aizawa et al., 1996; Chan et al., 2000; Diakonova et al., 2002; Nishita et al., 2002; Svitkina and Borisy, 1999; Zebda et al., 2000). In line with this, cofilin may play a role in a great number of DC characteristics involving actin reorganization, such as

morphology, endocytosis, migration, 'capturing' and adherence of T cells and the formation of the immunological synapse. We show here that cofilin activity is regulated during DC maturation. Our study is the first to imply the involvement of a direct effector of actin rearrangements in dendritic cell maturation.

An actin-binding protein of a different category, fascin has been shown to be specifically expressed in mature DC and not in mature DC (Al-Alwan et al., 2001a; Ross et al., 2000). Fascin is an actin-bundling protein and is involved in the formation of long dendrites. The combined expression of active cofilin and fascin may contribute to the formation of the characteristic veils of mature DC; cofilin by increasing F-actin turnover and enabling rearrangements of the actin cytoskeleton and fascin by the formation of stable F-actin bundles. As DC are very motile cells, it is to be expected that cofilin activity is crucial to maintain motility of the cell and possibly also of its veils, enabling a highly flexible manner of antigen presentation.

Cofilin activity is regulated by phosphorylation via LIM kinases (Agnew et al., 1995; Moriyama et al., 1996). Cofilin is dephosphorylated by serine protein phosphatases. A specific phosphatase of cofilin, slingshot, has been identified only recently (Ambach et al., 2000). Additionally, the protein phosphatases PP1/PP2A can associate with and activate cofilin (Niwa et al., 2002). How these phosphatases are regulated is not well understood. We observed an initial rise in phosphorylated cofilin shortly after DC stimulation. Similarly, Nishita et al. (Nishita et al., 2002) showed cofilin phosphorylation in T cells as quickly as 1 min after stimulation with SDF-1a. This transient rise in phosphorylated cofilin may be involved in the initiation of the actin cytoskeleton reorganization to enable clustering of membrane-bound molecules involved in signaling (Samstag et al., 1996). After the initial rise in phosphorylation upon LPS-induced DC maturation cofilin was dephosphorylated and translocated towards the membrane. Maturation in DC may, thus, be accompanied by the activation of (one of) these serine/threonine phosphatases. We tried to inhibit the activity of PP1/PP2A with thephosphatase inhibitor okadaic acid, which has been described to inhibit dephosphorylation of cofilin (Ambach et al., 2000; Djafarzadeh and Niggli, 1997). During incubation with okadaic acid, the cell membranes were blebbing and after several hours cells were fragmented. After 16 h of okadaic acid (at 50 nM), most of the cells were disintegrated (data not shown). Possibly, this is the effect of inhibition of cofilin activity, causing instability of the cell membrane by affecting the actin cytoskeleton. However, it cannot be excluded that apoptotic cell death is induced, since okadaic acid is also an inducer of apoptosis (Samstag et al., 1996).

Cofilin dephosphorylation upon stimulation was also observed in T cells, neutrophils and polymorphic mononuclear leukocytes after aspecific activation (Ambach et al., 2000; Djafarzadeh and Niggli, 1997; Lee et al., 2000; Okada et al., 1996). These and other studies have demonstrated that after dephosphorylation cofilin translocates to the plasma membrane (Adachi et al., 2000; Chan et al., 2000; Djafarzadeh and Niggli, 1997; Nagaoka et al., 1996; Shevchenko et al., 1996), or, more specifically, to the actin cytoskeleton (Lee et al., 2000) and is essential for the formation of membrane protrusions like podosomes and filopodia (Adachi et al., 2000; Aizawa et al., 1997; Nagaoka et al., 1996). As mentioned before, cofilin activation can result in either depolymerization, polymerization or in a neutral turnover (tread milling) of F-actin. Several reports have demonstrated a positive effect of cofilin activation on actin polymerization in both cell-free (Ichetovkin et al., 2002) and cell-based studies (Aizawa et al., 1996; Aizawa et al., 1997; Chan et al., 2000; Kleijmeer et al., 2001; Zebda et al., 2000). Non-phosphorylated cofilin appeared to be essential for lamellipod extension, membrane ruffling and cell movement. Moreover, active cofilin is essential for polarization of cells and direction of lamellipodia during migration (Dawe et al., 2003). Translocation of dephosphorylated cofilin to the cell membrane can thus have a profound effect on the actin cytoskeleton. Consistent with these data, we found an increase in both F-actin and active cofilin in the F-actin-rich area below the cell membrane during DC maturation. Moreover, cofilin dephosphorylation correlated with both the increase in F-actin and the appearance of veils on mature DC. The decrease in phosphorylated cofilin was stronger after 48 h than after 24 h of DC maturation, as was the appearance of veils. Collectively, our results indicate that cofilin is involved in the morphological transformation of DC during maturation.

Acknowledgements

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Chapter 3:

A lack of Birbeck granules in Langerhans cells is associated with a naturally occurring point mutation in the human Langerin gene.

Pauline Verdijk[1], Remco Dijkman[1], Elsemieke I. Plasmeijer[1], Aat A. Mulder[1], Willem H. Zoutman[1], A. Mieke Mommaas[2] and Cornelis P. Tensen[1]

[1]Department of Dermatology, LUMC
[2]Department of Molecular Cell Biology, LUMC

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Abstract

A heterozygous mutation in the Langerin gene corresponding to position 837 in the Langerin mRNA was identified in a person deficient in Birbeck granules. This mutation results in an amino acid replacement of Tryptophane by Arginine at position 264 in the CRD domain of the Langerine protein. Expression of mutated Langerin in human fibroblasts induces tubular like structures that are negative for BG specific antibodie staining and do not resemble the characteristic stratified structure of BG.

Introduction

Birbeck granules (BG) are cytoplasmic organelles that are only found in Langerhans cells (LC). The function of BG, first described in 1961 (Birbeck et al., 1961), is still not completely understood, although most studies point towards an active role in receptor-mediated endocytosis and participation in the antigen-processing/presenting function of (McDermott et al., 2002). Recent studies demonstrated a crucial role for the C-type lectin Langerin, in the biogenesis of BG. Expression of human or mouse cDNA encoding Langerin, an endocytic receptor exclusively present in Langerhans cells, into fibroblasts results in the formation of BG (Valladeau et al., 2000; Valladeau et al., 2002).

About 10 years ago we were the first and thus far only group that identified a healthy white man whose LC completely lack presence of BG as determined by electron microscopic studies (Mommaas et al., 1994). The absence of BG in these LC was documented further by the lack of staining with a BG-specific antibody (DCGM4). Considering the role of Langerin in the formation of BG and the fact that DCGM4 recognizes Langerin (Valladeau et al., 1999), we hypothesized that alterations/mutations in the Langerin gene might be responsible for the lack of BG in this index person.

To test this hypothesis we performed DNA sequence analysis of all Langerin encoding exons from the index person lacking BG. We identified a hitherto unknown mutation resulting in an amino acid substitution (W > R) at position 264 in a conserved (carbohydrate recognition) domain of the Langerin protein. Expression of a wild type Langerin construct in dermal fibroblast resulted in BGs positive for DCGM4. Expression of the mutant Langerin construct, identical to wild type except for the single base pair substitution, resulted in irregular tubular structures, negative for DCGM4. From these data we conclude that W264 in the Langerin protein is essential for BG biogenesis.

Materials and methods

PCR

All polymerase chain reactions (PCR) were carried out using custom primers and Platinum Taq from Invitrogen (Breda, the Netherlands) in a PTC-200 Thermal cycler (MJ Research, Waltham, MA).

Langerin target		Primer sequence (5' > 3')	Product size (bp)
Exon 1	A	TGGGATAGGTTTGGGACAAG	405
	AS	GTGTGTTGAAGGAGCAGCAA	
Exon 2	A	TTGCTGCTCCTTCAACACAC	191
	AS	GCTAAGCCCAGACGATGAAA	
Exon3	A	CCTCAGCTGACCTCCTGACT	479
	AS	CCTCAGGTCTGGGACAGGTA	
Exon 4	A	CCAGCGTTCACTTTTACCTCTT	222
	AS	ATACGCCCCCTTCACAGAG	
Exon 5	A	CGCACCTCTGCTTATCCTGT	242
	AS	CCTGTCTCATGGGGAACATC	
Exon 6	A	GGACAAAAGCTTGGGTTGAG	1123
	AS	CCTGGACAACCAGAAATGAAA	
cDNA	A	CCAGGATAAGGGTGAGCACT	1049
	AS	CGTTGGAGCTCAAAGAGTGA	
Coding region	A	GGGGACAAGTTTGTACAAAAAAGCA GGCTTCAGGATGACTGTGGAGAAGGA	1051
	AS	GGGGACCACTTTGTACAAGAAAGCTG GGTGTCACGGTTCTGATGGGACATAGG	
Mutation 5' overlap	A	CCAGGATAAGGGTGAGCACT	826
	AS	CCACCCAGGACCGGTCCCCTT	
Mutation 3' overlap	A	GGAAGGGGACCGGTCCTGGGT	264
	AS	CGTTGGAGCTCAAAGAGTGA	

Table I. PCR-primers used in this study.

Sequencing of the Langerin gene

DNA was isolated from peripheral blood lymphocytes of the index person lacking Birbeck granules (Mommaas et al., 1994) and controls (obtained with informed consent) using the QIAamp DNA mini kit (Qiagen, Hilden, Germany).

Individual Langerin encoding exons were amplified by PCR (touchdown protocol, annealing temperature 65° C to 50° C over 15 cycli followed by 20 cycli at 50° C) using 6 primer sets (See table I) developed on the human DNA and mRNA sequences deposited in the Genbank under # AC007395 and NM_015717. PCR products were purified and directly sequenced from both 5' and 3' ends using an ABI 3700 automatic sequencer (Applied Biosystems, Foster City, CA).

Heterologous expression of mutant and wild type Langerin cDNA

In default of a frozen skin biopsy from the index person enabling mRNA extraction, wild type and mutant Langerin expression constructs were created as follows. cDNA synthesized from mRNA isolated from normal skin (obtained from plastic surgery with written informed consent from the donor) was used as a template for a Langerin specific PCR (Primers S1/AS1, see Table I; annealing at 50° C; 35 cycli). The resulting 1049 bp PCR product was cloned in pTOPO (Invitrogen) and sequenced. The identified mutation at position 837 (numbering according to NM_015717) was introduced in the Langerin cDNA sequence by overlap extension mutation PCR (Horton et al., 1990) using two mutation primers (See table I) in combination with the S1/AS1 primers at the 5' and 3' end and the pTOPO plasmid containing wild type Langerin cDNA as template. PCR products were cloned in pTOPO and sequenced.

Next, the coding region of both the wild type and mutant Langerin cDNA were amplified with PCR primers containing flanking sequences for Gateway cloning and pTOPO (harboring wild type or mutant sequences) as template. Resulting PCR products were cloned in the Gateway Cloning vectors (Invitrogen) pDEST53, linking Green Fluorescent Protein (GFP) to the N-terminus of Langerin, and pDEST 12.2. All Gateway constructs were sequenced to confirm the position of the insert and the absence of PCR errors.

Human primary dermal fibroblasts (cultured in DMEM, supplemented with L-glutamine (2mM, Gibco BRL, Breda, The Netherlands), 5% FCS (Hyclone/Perbio, Etten Leur, the Netherlands) and penicillin/streptomycin (Gibco BRL) were transfected with Gateway constructs containing the wild type or mutant Langerin cDNA using the Amaxa nucleofector (Amaxa, Cologne, Germany). The transfections were performed according to the Human Dermal fibroblast Nucleofector Kit (Amaxa) and protocol. The cells were cultured for a maximum of 40 hrs in a 37° C/ 5% CO2 incubator and prepared for immuno-electron microscopy.

Electron microscopy

For electron microscopy, cells were fixed in 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M PHEM buffer for 2 hours at room temperature. Part of the cells was processed further for routine electron microscopy and the other part for immuno-electron microscopy. For routine electron microscopy cells were post-fixed in 1% osmiumtetroxide in 0.1M phosphate buffer for 30 minutes at 4°C, dehydrated in a graded ethanol series and embedded in epon LX112. Ultrathin sections were stained with uranylacetate and lead salts. For immuno-electron microscopy, cells were pelleted in 12% gelatin, cryoprotected in 2.3M sucrose and snapfrozen in liquid nitrogen. Ultrathin cryosections were incubated with either mouse monoclonal anti-Langerin IgG1 (formerly known as DCGM4; Beckman Coulter, Mijdrecht, the Netherlands) (1/100), followed by a rabbit anti-mouse immunoglobulins as bridging antibody (DAKOcytomation, Heverlee, Belgium)(1/200) and 15 nm protein A-gold (1/200) or rabbit polyclonal anti-GFP IgG (Molecular Probes Europe, Leiden, the Netherlands)(1/200) followed by 15 nm protein A-gold. After incubation, the sections were embedded in methylcellulose and stained with uranylacetate. All specimens were viewed with a Philips 410 electron microscope (Philips, Eindhoven, The Netherlands).

Results and discussion

PCR and sequence analysis of Langerin encoding exons

PCR analysis of all Langerin encoding exons demonstrated no large differences in terms of deletions/insertions between the DNA of the index person and the

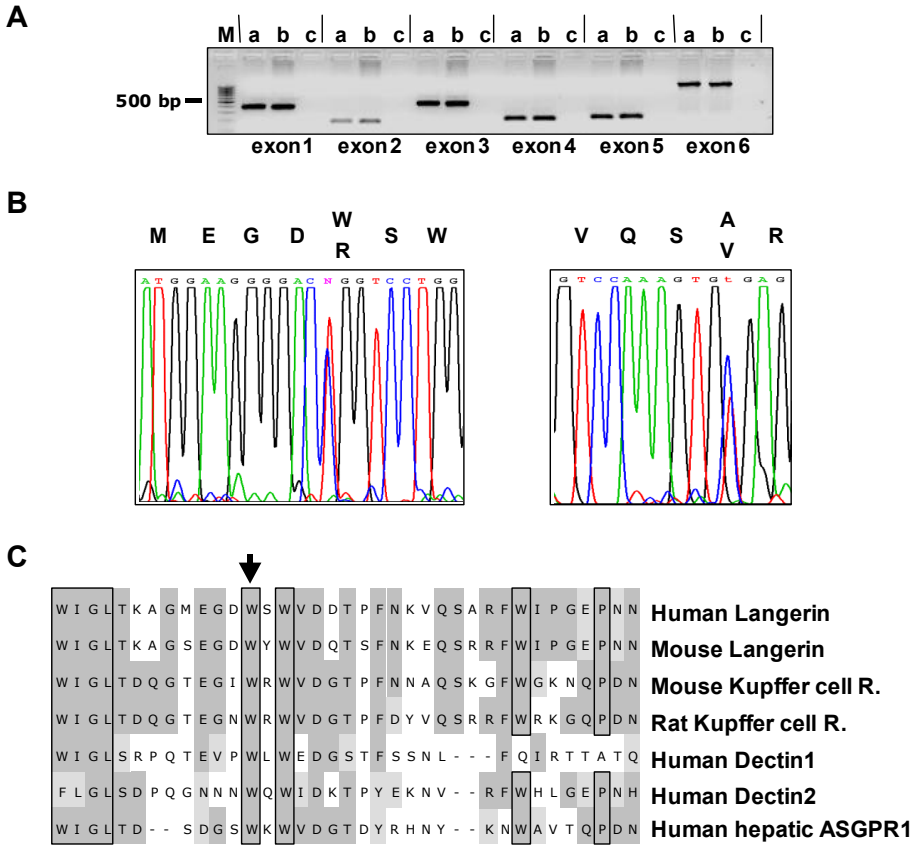

Figure 1. Analysis of the Langerin gene. A. Representative example of a PCR analysis using Langerin specific primers for exon 1-6 and as template genomic DNA of the index person lacking Birbeck granules (a), control DNA (b) or no template (c). Obtained PCR products were analyzed on a TBE based agarose gel and stained with ethidium bromide. M denotes DNA marker (Generuler 100 bp ladder; Fermentas, St. Leon-Rot, Germany), size in base pairs is given on the left. **B**. Fragment of a sequence chromatogram of a PCR product representing exon 5 of the index person lacking Birbeck granules. A double sequence (T/C) is detected at positions 837 and 880. Corresponding amino acids are given on top using single letter abbreviation. **C.** Alignment of a conserved domain within the type II C-type lectin family of proteins. Arrow indicates conserved tryptophane residue (W) at position 264 within this family.

control DNA (Fig. 1A). DNA sequence analysis of all obtained PCR products revealed that the exons encoding Langerin in our index person were heterozygous at four positions: 281, 837, 880 and 989. Two of these mutations were silent (pos. 281 C/T and 989 A/G), while the other two resulted in an amino acid change in the Langerin protein (Fig. 1B). Heterozygosity at pos 880, however, was also found in the control DNA (See Fig. 1B right panel) and in the cDNA of the second control that was used as a basis to create the Langerin expression constructs. Moreover, until recently, this nucleotide was indicated as a known SNP in Genbank; this annotation was removed after a recent update (July 2004). Heterozygosity at pos. 837 is of particular interest since it encodes a variant Langerin containing the basic residue Arginine (R) in stead of the aromatic amino acid Tryptophane (W) in a highly conserved region of the Langerin protein and related type II C-type lectins (Fig. 1C). As this mutation was located in the carbohydrate recognition domain (CRD), we reasoned that this mutation (W264R) might have functional consequences for the Langerin protein.

Expression of wild type and mutant Langerin cDNA

Expression of wild type Langerin with or without GFP in human fibroblasts resulted in the mass formation of Birbeck granules, as was revealed by transmission electron microscopy (Fig. 2), indicating that GFP did not interfere with the formation of Birbeck granules. Birbeck granules in GFP-wild type Langerin transfected cells were strongly positive for anti-GFP (Fig. 3A) and moderate positive for DCGM4-labeling (Fig. 3B). In contrast, expression of GFP-mutant Langerin did not induce the formation of BG demonstrating the

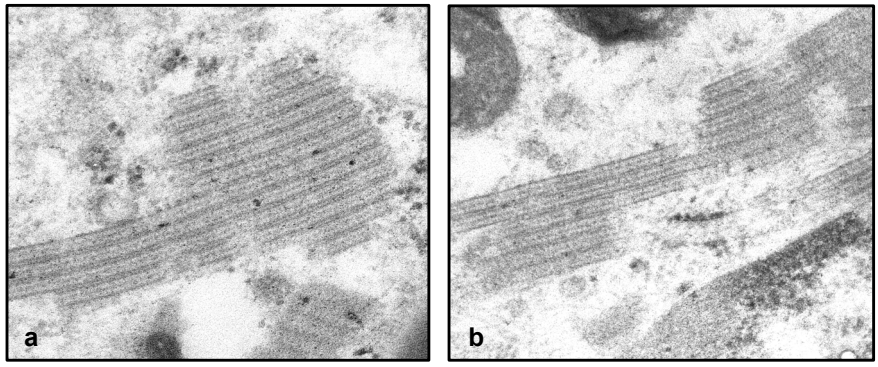

Figure 2. Detection of Birbeck granules in Langerin expressing fibroblasts by electron microscopy. Fibroblasts transfected with wildtype Langerin (A) of wildtype GFP-Langerin (B) show typical structures reminiscent of Birbeck granules.

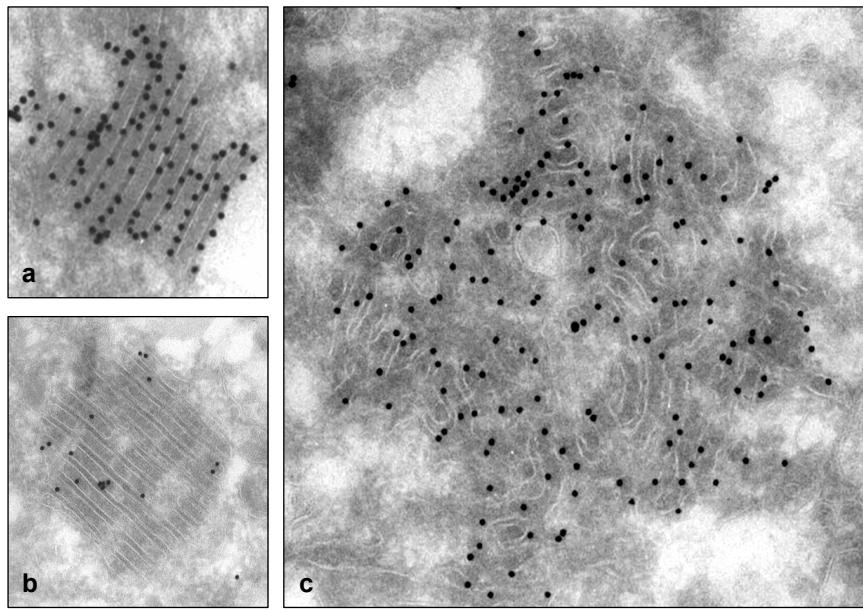

Figure 3. Immuno-electron microscopic analysis of GFP-Langerin expressing fibroblasts. A-B. Typical anti-GFP (A) or DCGM4 (B) immunostaining of fibroblasts transfected with GFP- wild type Langerin construct. **C.** Representative example of an anti-GFP staining of fibroblasts transfected with the GFP-mutant Langerin construct. Antibodies were detected with 15 nm protein A-gold.

functional consequences of the W264R conversion. Although lacking Birbeck granules, transfection of human fibroblasts with the GFP-mutant Langerin induced the formation of an assembly of GFP-positive tubular membrane structures seemingly consisting of lipid bilayers, but lacking the stratified organization found in Birbeck granules (Fig. 3C). The membrane structures induced by the mutant Langerin were negative for DCGM4 labeling, although expression of the protein was confirmed by staining for GFP. These results suggest that the W264R mutation is either located in the epitope recognized by DCGM4 or that it causes a conformational change preventing the binding of the antibody. Furthermore, these data demonstrate that the W264R amino acid replacement prevents the formation of the typical structure of Birbeck granules. Nonetheless, this mutant Langerin is still able to induce the formation of lipid bilayer membrane structures. The mechanisms by which this mutation prevents the formation of the superimposed and zippered membranes typical for Birbeck granules are currently under investigation and will expand our understanding of the role of Langerin in the formation of BG and in the function of Langerin and Birbeck granules in Langerhans cells.

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Chapter 4:

CXCR3-mediated chemotaxis of human T cells is regulated by a G_i- and phospholipase C-dependent pathway and not via activation of MEK/p44/p42 MAPK or Akt/PI3 kinase.

Martine J. Smit[1], Pauline Verdijk[2], Elisabeth M. H. van der Raaij-Helmer[2], Marjon Navis[1], Paul J. Hensbergen[2], Rob Leurs[1] and Cornelis P. Tensen[2]

[1]Leiden/Amsterdam Center for Drug Research, Vrije Universiteit, Division of Medicinal Chemistry, Amsterdam
[2]Department of Dermatology, LUMC, Leiden

Blood **102**:1959-1965, 2003

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Abstract

The chemokines CXCL9, 10 and 11 exert their action via CXC chemokine receptor-3 (CXCR3), a receptor highly expressed on activated T cells. These IFN-γ-induced chemokines are thought to be crucial in directing activated T cells to sites of inflammation. As such, they play an important role in several chronic inflammatory diseases, including ulcerative colitis, multiple sclerosis, artherosclerosis and delayed type hypersensitivity reactions of the skin. In this study, we first demonstrate that in COS-7 cells heterologously expressing CXCR3, CXCL11 is a potent activator of the pertussis toxin sensitive (PTX) p44/p42 MAPK and Akt/phosphatidylinositol-3-kinase (PI3K) pathways. Next, we show that these signal transduction pathways are also operative and PTX sensitive in primary human T cells expressing CXCR3. Importantly, abrogation of these signaling cascades by specific inhibitors did not block the migration of T cells towards CXCR3 ligands, suggesting that MAPK and Akt activation is not crucial for CXCR3-mediated chemotaxis of T cells. Finally, we demonstrate that CXCR3 targeting chemokines control T cell migration via PTX sensitive, phospholipase C pathways and phosphatidylinositol kinases other than class I PI3Kγ.

Introduction

Chemokines are a group of small, secreted and heparin-binding proteins that are not only active as chemotactic factors but also as end-point effectors of immune protective functions. These proteins are classified into CXC, CC, C and CX3C chemokines, based on the position of characteristic structure determining cysteine residues within their N-terminal part (Baggiolini, 2001; Luster, 1998; Murphy et al., 2000; Zlotnik et al., 1999). They are produced locally in the tissues and exert their action on leukocytes by binding to specific G protein–coupled receptors (GPCRs), embedded within the membrane of their respective target cells. Typically, the binding of a chemokine to its cognate GPCR triggers the activation of multiple signal transduction pathways, including a transient intracellular rise in Ca^{2+} (Baggiolini, 1998). To date about 50 different chemokines and at least 18 different receptors have been identified (Zlotnik et al., 1999).

For several chemokines it has been shown that receptor binding also activates the p44/p42 MAPK (extracellular signal-regulated kinase) pathway in multiple cell types (Boehme et al., 1999; Knall et al., 1997; Tilton et al., 2000; Dubois et al., 1996; Ganju et al., 1998; Coffer et al., 1998). Blockade of MAPK activation inhibited eotaxin-induced eosinophil rolling in vivo and chemotaxis in vitro (Boehme et al., 1999) as well as CXCL12 mediated chemotaxis of

$CD4^+$ T cells (Vlahakis et al., 2002) pointing towards a crucial role of MAPK activation in leukocyte migration.

The chemokines CXCL9, 10 and 11 (formerly known as resp. Mig, IP-10 and IP-9/I-TAC) exert their action via CXC chemokine receptor-3 (CXCR3), a receptor highly expressed on activated T cells (Loetscher et al., 1996; Qin et al., 1998). These IFN-g induced chemokines, are thought to be crucial in directing activated T cells to sites of inflammation, where they play an important role in Th1 mediated immune responses. Expression of the CXCR3 targeting chemokines have been demonstrated in several chronic inflammatory diseases, including ulcerative colitis (Uguccioni et al., 1999), multiple sclerosis (Balashov et al., 1999; Sorensen et al., 1999), hepatitis C-infected liver (Mach et al., 1999), artherosclerosis (Shields et al., 1999), delayed type hypersensitivity skin reactions and chronic skin inflammations (Flier et al., 1999; Flier et al., 2001). However, to date little is known about the intracellular events following agonist binding to CXCR3, except for Ca^{2+} mobilization, in T cells (Loetscher et al., 1996; Rabin et al., 1999; Tensen et al., 1999; Cole et al., 1998).

In this study we have delineated several signaling pathways of CXCR3 ligands, in particular of CXCL11, which appears to be the most potent agonist in all assays used. We show that CXCR3 ligands are able to activate the p44/p42 MAPK and Akt/phosphatidylinositol-3-kinase (PI3K) signaling cascades in CXCR3-transfected COS-7 cells and primary human T cells. Chemotaxis of T cells, however, is not mediated by these kinases, but controlled via G_i and phospholipase C dependent pathways and phosphatidylinositol kinases other than class I PI3Kγ.

Materials and methods

Materials

Cell culture media and supplements were obtained from GIBCO BRL. CXCL10 (IP-10) was obtained from Peprotech (Rocky Hill, NJ, U. S. A) and CXCL11 (IP-9/I-TAC) was from R & D systems Inc. (Minneapolis, MN, U. S. A.). The MEK inhibitors PD98059 and U0126 were obtained from New England Biolabs, Inc. (Beverly, MA, USA) and Promega (Leiden, The Netherlands) resp. The PLC inhibitor U73122, its inactive analog U73343, phosphatidylinositol kinase inhibitors wortmannin and LY294002 and pertussis toxin were obtained from Sigma (Sigma-Aldrich Chemie B. V. Zwijndrecht, The Netherlands). hCXCR3 in pcDNA III was a gift from Dr. B. Moser (Loetscher et al., 1996).

Cell culture and transfection

COS-7 cells were grown at 5% CO2 at 37˚C in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 mg/ml streptomycin. Transfection of COS-7 cells was performed with DEAE-dextran. The total amount of DNA in transfected cells was kept constant by addition of the empty vector. Transfected cells were maintained in serum-free medium. Isolations, activation and culturing of human T lymphocytes were performed as described previously (Tensen et al., 1999).

Chemotaxis assays

The assay for chemotaxis was performed in 24-well plates (Costar, Cambridge, MA) carrying Transwell permeable supports with 5-µm polycarbonate membrane. Cultured T cells were washed once, and resuspended at 5×10^6 cells/ml in RPMI 1640 containing 0.25% BSA. Medium alone or supplemented with chemokine was placed in the lower compartment, and cells were loaded onto the inserts at 0.5×10^6/100 µl for each individual assay. Chambers were incubated for 120 min in a 5% CO2 -humidified incubator at 37° C. After the incubation period, numbers of T cells migrating to the lower chamber were counted under a microscope using a hemocytometer. Viability was checked with trypan blue exclusion. All conditions were tested in at least in triplicate; statistic evaluation was performed using the Student's t-Test.

Cell stimulation

If not stated otherwise, IL-2–expanded activated T cells were serum starved overnight in medium supplemented with 400 U/ml IL-2 and 0.2% BSA. The cells were washed twice and resuspended in HBSS containing 20 mM Hepes, pH 7.4 (2×10^7 cells/ml). Aliquots of 2×10^6 cells for immunoblotting were incubated for 10 min at 37° C and then stimulated with the appropriate agonist at indicated time intervals. Incubations were terminated by centrifugation and pelleted cells were lysed as described under Western blot analysis. COS-7 cells were serum-starved and stimulated as indicated in the figures.

Western blot analysis

Transiently transfected COS-7 cells (48 h after transfection) and IL-2-expanded activated T cells were lysed in RIPA buffer (phosphate-buffered saline containing 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM phenylmethylsulphonylfluoride, 1 mM sodium orthovanadate and 2 mg/ml of aprotinin and leupeptin), sonicated, separated by SDS polyacrylamide gel electrophoresis, and blotted to polyvinylidene difluoride membrane. Antibodies recognizing p44/p42 MAPK, Akt, phospho-Akt (S473) (New England Biolabs, Inc, Beverly, MA, USA) were used in combination with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Biorad, Hercules, CA, USA). The antibody recognizing phospho-p44/p42 MAPK (T202/Y204) (New England Biolabs, Inc, Beverly, MA, USA) was used in combination with a goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA, USA). Protein bands were detected with ECL chemiluminescence and directly quantified using an Image station (NEN Life Science Products, Inc, Boston, MA, USA).

Actin polymerization assay

Cells were washed three times in RPMI 1640 and resuspended in assay medium (RPMI 1640 with 0.1% BSA [BSA, fraction V, Sigma]) in a concentration of 1×10^6 cells/ml. Prior to the experiment, the cells and chemokine were kept at room temperature for 10 minutes. Chemokine was subsequently added to the cell suspension (10 nM). At indicated time points, 100 ml of cell suspension was transferred to 100 ml of fixation solution (4% paraformaldehyde [PFA]). Cells were incubated in the fixation solution for at least 15 minutes. Thereafter, the cells were centrifuged and resuspended in 100 ml of permeabilization reagent (0.1% Triton-X 100). After 10 minutes of incubation in this solution, cells were washed, blocked with 1% BSA (5 min), washed and incubated with 0.5 mM rhodamine-phalloidin (Molecular Probes,

Leiden, The Netherlands) to visualize the F-actin. After 30 minutes, the cells were centrifuged and resuspended in PBS with 0.25% BSA. Mean fluorescence intensity was measured by FACS analysis (FACScan; BD Biosciences, Alphen aan den Rijn, The Netherlands).

Results

CXCR3 agonists induce activation of p44/p42 MAPK in CXCR3-transfected COS-7 cells

We first examined the relative potencies of CXCR3 agonists CXCL11 and CXCL10 to activate the p44/p42 MAPK signaling pathway. To this end, the human CXCR3 receptor was transiently expressed into COS-7 cells. Exposure of CXCR3-expressing COS-7 cells to (30 nM) CXCL11 (IP-9) for different periods of time resulted in a time-dependent increase in endogenous MAPK activity as detected by an increase in phosphorylation of p44/p42 MAPK, measured using an antibody recognizing the phosphorylated form of p44/p42 MAPK (fig. 1A). A maximal transient increase in phosphorylation of p44/p42 MAPK was observed after 15 min exposure of cells to CXCL11 at a concentration of 30 nM (fig. 1A). The other well-known CXCR3 agonist CXCL10 (IP-10) also induced an increase in phosphorylation of p44/p42 MAPK, albeit to a lesser extent (fig. 1B).

As expected, incubation of cells expressing CXCR3 with the specific MEK inhibitors U0126 (10 mM) or PD98059 (10 mM, data not shown) led to a complete abolishment of basal and CXCL11-induced phosphorylation of p44/p42 MAPK, confirming the involvement of the Raf-MEK pathway (fig. 1C). CXCR3 belongs to the class of G_i-coupled receptors, which are known to activate p44/p42 MAPK in COS-7 cells (Crespo et al., 1994; Luttrell et al., 1996) through the release of $G\beta\gamma$ subunits leading to activation of PI3K and Ras (Koch et al., 1994). In order to examine the involvement of the G_i-signaling pathway in CXCR3-mediated signaling to p44/p42 MAPK, cells were treated with pertussis toxin (PTX, 100 ng/ml) for 48 hrs. Pretreatment of cells with PTX resulted in a partial reduction of CXCR3-induced increase in MAPK phosphorylation (fig. 1D). Involvement of PI3K was examined by pretreatment of cells for 30 min with wortmannin (100 nM). Pretreatment of cells with wortmannin led to a complete inhibition of basal and CXCL11-induced phosphorylation of MAPK (fig. 1E).

CXCR3 agonists induce activation of p44/p42 MAPK in T lymphocytes

As previously shown, T lymphocytes treated for 10 days with IL-2 express CXCR3 (Loetscher et al., 1996; Tensen et al., 1999). In IL-2 expanded, activated T cells, CXCL11 induced a time- (data not shown) and concentration-dependent increase in phosphorylation of p44/p42 MAPK (fig. 2A). A maximal increase in phosphorylation of p44/p42 MAPK, ranging from 2 to 5 fold increase, was observed after 5-15 min stimulation of cells with

CXCL11, varying with the T cell donor. Analogous to the observations made in CXCR3-expressing COS-7 cells, the activation of p44/p42 MAPK by CXCL11 appeared to be transient, since no increase in phosphorylation of MAPK was detected after 60 min exposure of cells to CXCL11 (data not shown). A whereas CXCL9 and in particular CXCL10 were less potent in phosphorylating p44/p42 MAPK (fig. 2B).

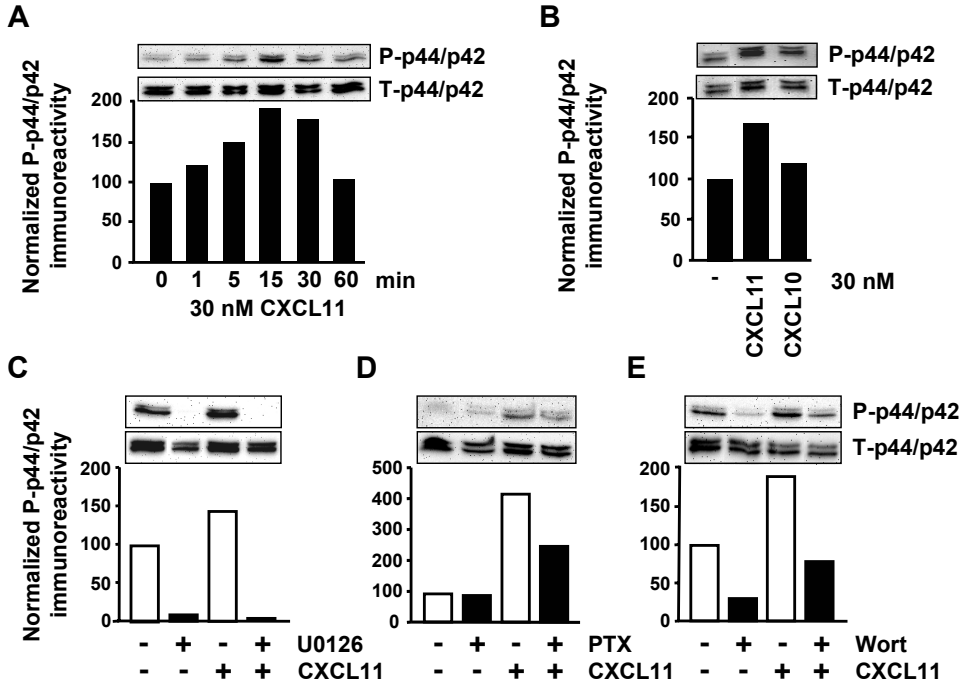

Figure 1. CXCR3 agonists stimulate activation of p44/p42 MAPK in CXCR3-transfected COS-7 cells A. COS-7 cells transiently transfected with CXCR3 (2.0 mg plasmid/106 cells) were stimulated for different periods of time with 30 nM CXCL11. Phosphorylation of p44/p42 MAPK was determined by Western blot analysis using specific anti-phosho-p44/p42 (P-p44/p42) antibodies. Phosphorylation was quantified by chemiluminescence and corrected for total MAPK (T-p44/p42) expression on stripped blots. **B.** CXCR3-expressing COS-7 cells were incubated with CXCL10 and -11 (30 nM) for 15 min before lysis of the cells. **C-E**. Involvement of Raf-MEK- (C), Gi- (D) and PI3K- (E) signaling pathway in CXCL11 (15 min, 30 nM) -induced p44/p42 MAPK phosphorylation. CXCR3-expressing COS-7 cells were grown in serum-free medium in the presence of U0126 (10 mM, 30 min; C) or PTX (100 ng/ml, 48 h; D) or wortmannin (wort; 100 nM, 30 min; E) before lysis and Western blot analysis. A representative experiment out of at least two independent experiments is shown.

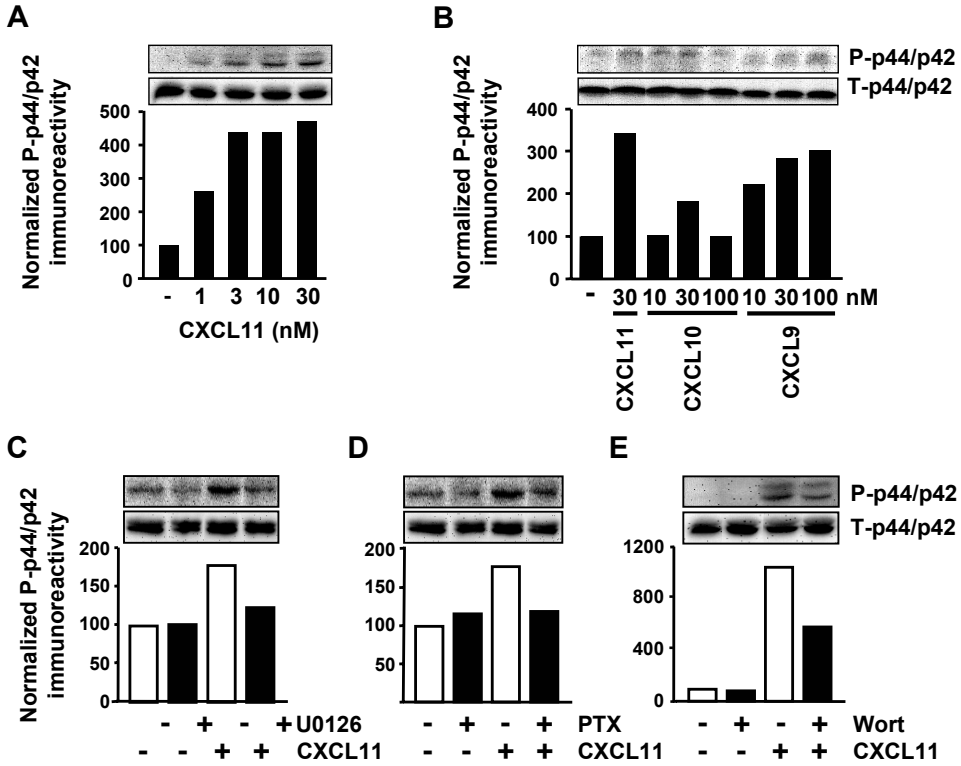

Figure 2. CXCR3 agonists stimulate activation of p44/p42 MAPK in T lymphocytes. A-B. Activated IL-2 expanded blood T cells were incubated for 15 min with various concentrations of CXCL11 (A), CXCL10 (B) or CXCL9 (B) before lysis of the cells. Phosphorylation of p44/p42 MAPK was determined by Western blot analysis using specific anti-phosho-p44/p42 (P-p44/p42) antibodies. Phosphorylation was quantified by chemiluminescence and corrected for total MAPK (T-p44/p42) expression on stripped blots. **C-E.** Involvement of Raf-MEK- (C), Gi- (D) and PI3K- (E) signaling pathway in CXCL11-induced p44/p42 MAPK phosphorylation. T cells were grown in serum-free medium in the presence of U0126 (10 mM, 30 min; C) or PTX (100 ng/ml, 48 h; D) or wortmannin (wort; 100 nM, 30 min; E) and exposed to CXCL11 (15 min, 30 nM) before lysis and Western blot analysis. A representative experiment out of at least two independent experiments is shown.

In activated T cells, CXCR3-mediated activation of p44/p42 MAPK appeared to be mediated by the Raf-MEK pathway, as the MEK inhibitor (U0126) completely inhibited the CXCL11-induced increase in MAPK phosphorylation (fig. 2C). The CXCL11-induced phosphorylation of p44/p42 MAPK in T cells appeared to be G_i- and partially PI3K mediated, as preincubation of cells with PTX (48 h, 100 ng/ml, fig. 2D) resulted in a complete, and preincubation of cells with wortmannin (30 min, 100 nM, fig. 2E) led to a partial attenuation of the CXCL11-induced phosphorylation of p44/p42 MAPK.

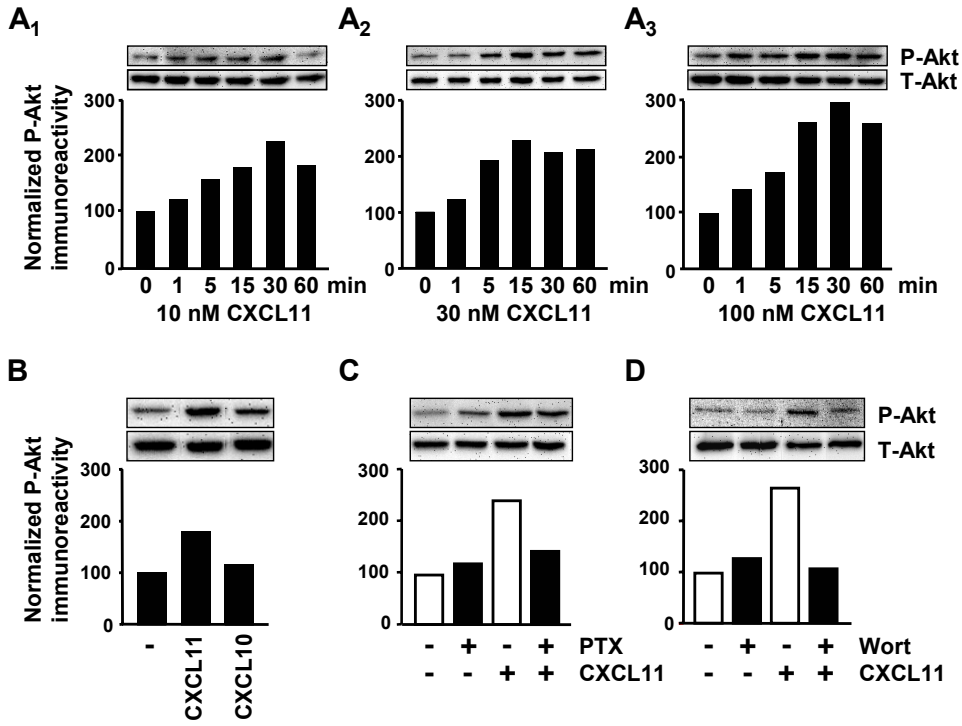

Figure 3. CXCR3 agonists stimulate activation of Akt in CXCR3-transfected COS-7 cells A. COS-7 cells transiently transfected with CXCR3 (2.0 mg plasmid/106 cells) were stimulated for different periods of time with 10 (A1), 30 (A2) and 100 (A3) nM CXCL11 and phosphorylation of Akt was determined by Western blot analysis using specific anti-phosho-Akt (P-Akt) antibodies. Phosphorylation was quantified by chemiluminescence and corrected for total Akt (T-Akt) expression on stripped blots. **B.** CXCR3-expressing COS-7 cells were incubated with 30 nM CXCL11 or CXCL10 for 15 min before lysing the cells. **C-D.** Involvement of G_i- (C) and PI3K- (D) signaling pathway in CXCL11-induced Akt phosphorylation. CXCR3-expressing COS-7 cells were grown in serum-free medium in the presence of PTX (100 ng/ml, 48 h; C) or wortmannin (wort) (100 nM, 30 min; D) and exposed to CXCL11 (15 min, 30 nM) before lysis and Western blot analysis. A representative experiment out of at least two independent experiments is shown.

CXCR3 agonists phosphorylate Akt in CXCR3-transfected COS-7 cells

The serine/threonine kinase Akt is known to be critical for cell survival, proliferation and gene expression (Chan et al., 1999). In addition, Akt has been shown to be implicated in cell migration (Haugh et al., 2000; Morales-Ruiz et al., 2000; Servant et al., 2000). Exposure of CXCR3-expressing COS-7 cells to CXCL11 resulted in a marked and sustained increase in phosphorylation of endogenous Akt in COS-7 cells, as evidenced by

immunoblotting with a phosphospecific antibody (fig. 3A). CXCL11 induced a time- and concentration-dependent phosphorylation of Akt, reaching a maximal effect with 10 nM after 15 to 30 min exposure (fig. 3A). CXCL11 induced a marked increase in phosphorylation of Akt at 30 nM, while at this concentration CXCL10 only induced a marginal increase in phosphorylation of Akt. Involvement of G_i-signaling pathways in the CXCR3-induced signaling to Akt was determined by treatment with PTX. As can be seen in fig. 3C, PTX treatment resulted in a marked inhibition of CXCL11-induced phosphorylation of Akt. To study the role of PI3K (a known key player in the activation of Akt (Burgering and Coffer, 1995)), CXCR3-expressing cells were treated with PI3K inhibitor wortmannin (30 min, 100 nM). As expected, inhibition of PI3K resulted in a total abolishment of CXCL11-induced phosphorylation of Akt (fig. 3D).

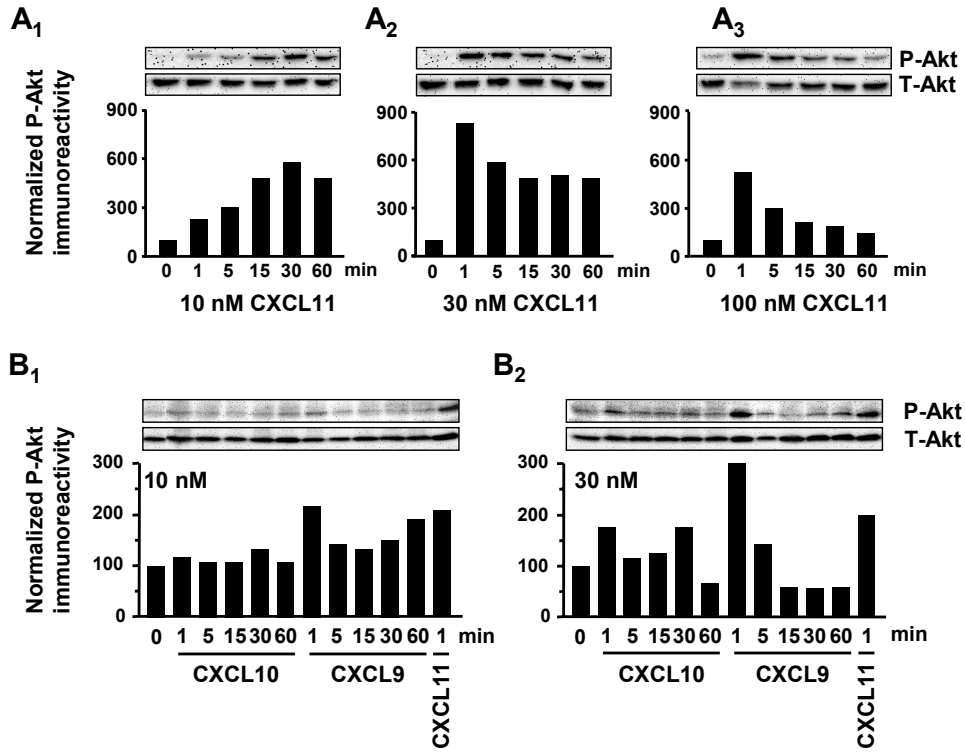

Figure 4. CXCR3 agonists stimulate activation of Akt in T lymphocytes A-C. T cells were stimulated for different periods of time with 10, 30 and 100 nM CXCL11 (A1-3), CXCL10 (B1-3), CXCL9 (B1-3) or CXCL12 (C) and phosphorylation of Akt was determined by Western blot analysis using specific anti-phosho-Akt (P-Akt) antibodies. Phosphorylation was quantified by chemiluminescence and corrected for total Akt (T-Akt) expression on stripped blots.

CXCR3 agonists phosphorylate Akt in T lymphocytes

Also in activated T cells, CXCL11 generated a time- and concentration-dependent increase in phosphorylation of Akt (fig. 4A1-3). At low concentrations of CXCL11 (10 nM) a maximal increase in phosphorylation of Akt was detected after 30 min incubation. Remarkably, at higher concentrations of CXCL11 (30 and 100 nM) already after 1 min exposure of cells a marked increase in Akt phosphorylation was observed. Akt phosphorylation levels remained at an elevated level for up to at least 60 min when cells were exposed to 30 nM, while at higher concentrations of CXCL11 (100 nM) a gradual decrease to basal levels was observed. Again, the ability of CXCL10 to induce an elevation of Akt phosphorylation was less pronounced when compared to CXCL11 at the different concentrations (fig. 4B1-3). CXCL9

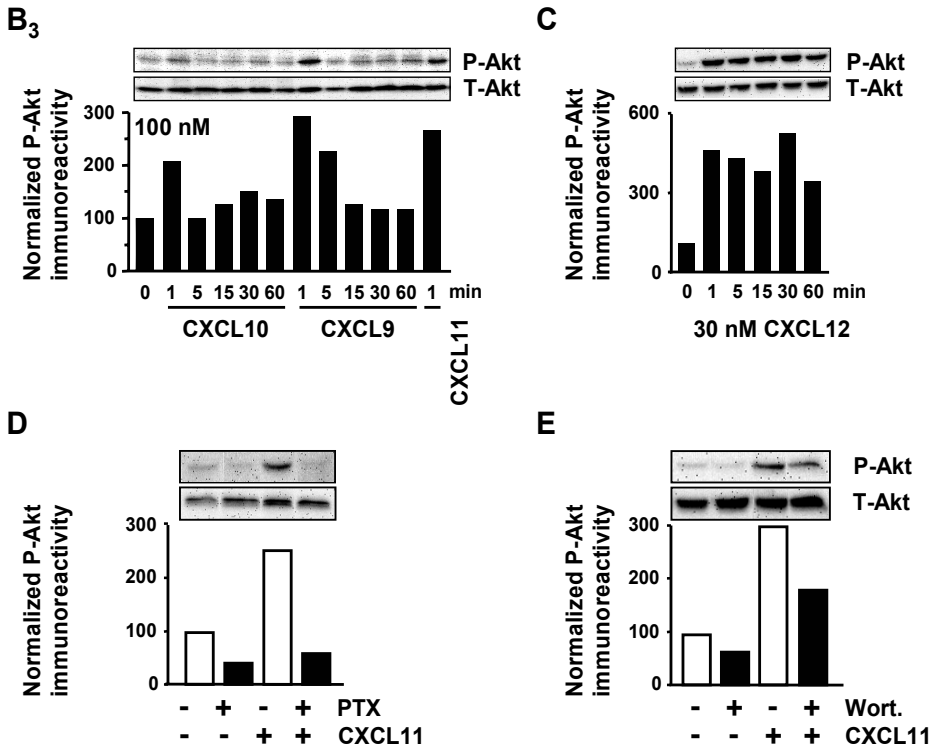

Figure 4 continued. D-E. Involvement of G_i- (D) and PI3K- (E) signaling pathway in CXCL11-induced Akt phosphorylation. T cells were grown in serum-free medium in the presence of PTX (100 ng/ml, 48 h; D) or wortmannin (wort; 100 nM, 30 min; E) and exposed to CXCL11 (1 min, 30 nM) before lysis and Western blot analysis. A representative experiment out of at least two independent experiments is shown.

appeared to exert a similar increase of Akt phosphorylation compared to CXCL11 after 1 min exposure (fig. 4B1-3). However, CXCL9 was not able to induce a sustained elevated level of Akt phosphorylation. As a control, activated T cells were also exposed to CXCL12 (SDF-1α), which binds to its cognate receptor CXCR4 on these cells. As previously shown by Tilton et al. (Tilton et al., 2000), CXCL12 (SDF-1α) induced a sustained increase in activation of Akt (fig. 4C). CXCL11-mediated phosphorylation of Akt appeared to be G_i-dependent as treatment of cells with PTX abrogated the CXCL11-induced increase in phosphorylation of Akt (fig. 4D). In addition, PI3K appeared to be involved as preincubation of cells with wortmannin markedly attenuated the CXCL11-induced phosphorylation of Akt (fig. 4E).

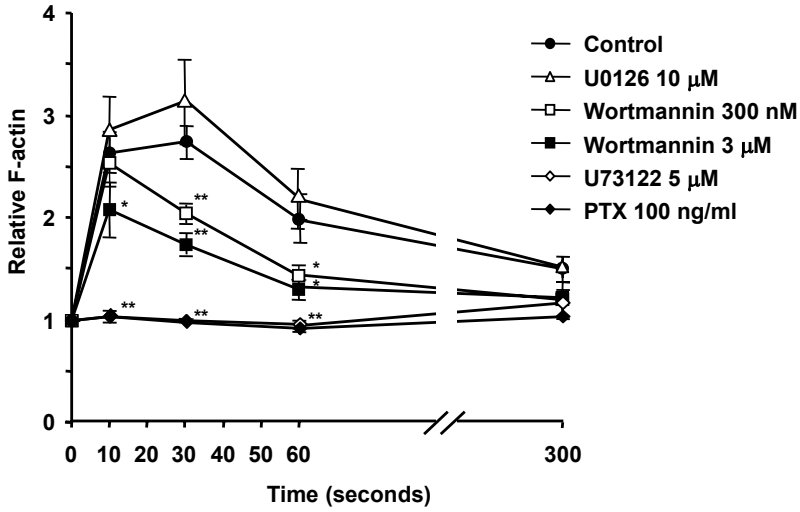

Figure 5. CXCL11–induced F-actin formation in primary human T cells and the effect of inhibitors. At 10, 30, 60 and 300 seconds after 10 nM CXCL11 stimulation (indicated in the panels), the levels of F-actin were determined by FACS analysis. Significantly lower F-actin levels were found after pre-treatment with PTX, U73122 and high concentrations of wortmannin. No effects were observed with the MEK inhibitor U0126 and the inactive analog of U73122 (U73433; data not shown). Data shown are the average of independent experiments with T cells from seven different donors. Error bars represent SEM. Asterisks indicate values significantly different from CXCL11-induced actin polymerization without inhibitor (Student's T-test; * $p < 0.05$, ** $p < 0.005$).

CXCR3-mediated actin polymerization and chemotaxis of T lymphocytes

Finally, the role of MEK-1, PI3K/PI4K, phospholipase C and G_i protein, was determined in CXCR3-induced actin polymerization and CXCR3-mediated chemotaxis of T cells. Addition of CXCL11 to CXCR3-expressing primary T cells induced a rapid and marked onset of F-actin polymerization (fig. 5). CXCL11-stimulated actin polymerization was not inhibited by pretreatment with the MEK inhibitor U0126 (fig. 5). Moderate inhibition of CXCL11-mediated actin polymerization was found after 30 seconds when cells were pretreated with 300 nM wortmannin, or earlier (10 seconds) when pretreated with higher MEK inhibitor U0126 (fig. 5). Moderate inhibition of CXCL11-mediated actin polymerization was found after 30 seconds when cells were pretreated with 300 nM wortmannin, or earlier (10 seconds) when pretreated with higher

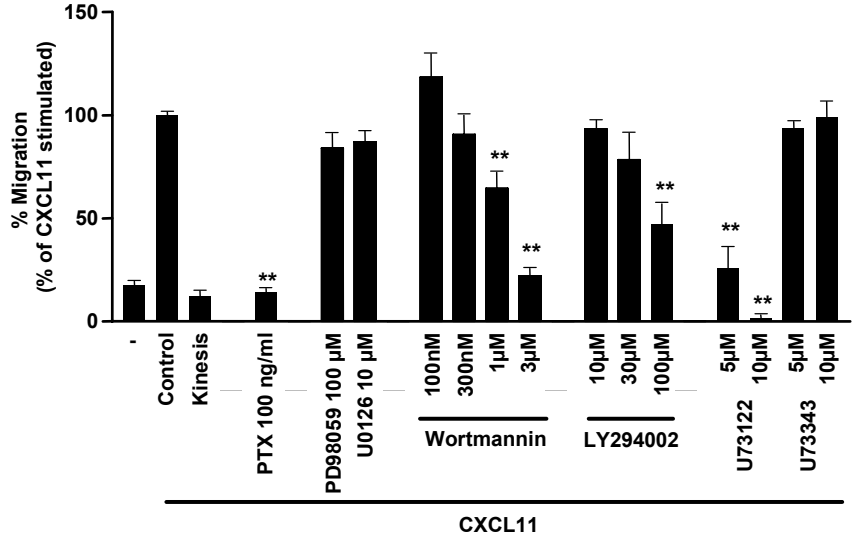

Figure 6. CXCR3-mediated chemotaxis of activated, primary human T-lymphocytes and the effect of inhibitors. The chemotactic activity of activated IL-2 expanded, blood derived human T cells towards 30 nM CXCL11 was determined by the Transwell migration system. Migration is reduced by pre-treatment with PTX, the PLC inhibitor U73122 and high concentrations of the kinase inhibitors wortmannin and LY294002. No significant effects on chemotactic activity were found with the inactive analog of U73122 (U73433), and the MEK inhibitors U0126 and PD98059. Data shown are the average of experiments with T cells from at least five different donors, except for the different LY294002 concentrations (n = 4). Chemotaxis is expressed as percentage of cells migrating in comparison to 30 nM CXCL11 (set at 100% for each individual donor). Error bars represent SEM. Asterisks indicate values significantly different from CXCL11-induced chemotaxis without inhibitor (Student's T-test; ** $p < 0.001$).

concentrations of wortmannin (3 mM). Complete inhibition, however, was achieved after pretreatment with pertussis toxin and the PLC inhibitor U73122 (5 mM). The inactive analog of U73122, U73343, had no effect (not shown).

The role of MEK-1 and p44/p42 MAPK in CXCR3-mediated T cell migration was studied using the MEK inhibitors PD98059 and U0126 (fig. 6). Neither PD98059, nor U0126 caused inhibition of CXCL11-induced chemotaxis. Low concentrations of wortmannin, selective for class I PI3K inhibition, did not block CXCR3-mediated T cell migration (fig 6). However, at high concentrations of wortmannin (>1 μM) significant inhibition of CXCR3-mediated T cell migration was observed. Similar results were obtained with the structurally distinct PI3/PI4K inhibitor LY294002. At concentrations selective for class I PI3K inhibition no effects were detected, while increased concentrations induced partial inhibition of CXCL11-induced T cell migration. Finally, CXCL11-induced migration was completely blocked by pertussis toxin pretreatment and the selective inhibitor of phospholipase C (U73122), while the inactive analogue of U73122 (U73343) had no effect (fig. 6). This confirmed the crucial role of G_i proteins and PLC in CXCR3-mediated migration of human primary T cells. None of the inhibitors except for the specific PLC inhibitor U73122 affected chemokinesis (data not shown).

Discussion

T cell migration into sites of inflammation is a complex process in which several cell surface molecules, e. g. adhesion molecules, integrins and their ligands, act together. Soluble mediators, mainly chemokines, are central in both activating and directing T cell subsets to target tissues. However, the signaling pathways involved in the regulation of these processes are still poorly defined. CXCR3 is predominantly expressed on activated T cells and activation by CXCL9, 10 and 11 leads to Ca^{2+} mobilization (Loetscher et al., 1996; Rabin et al., 1999; Tensen et al., 1999; Cole et al., 1998) and chemotaxis in T cells (Tensen et al., 1999). Recently, CXCL9 was shown to stimulate also the p44/p42 MAPK pathway in melanoma cells (Robledo et al., 2001) and both CXCL9 and -10 were reported to activate the Ras/ERK, Src, and the PI3K/Akt pathways (Bonacchi et al., 2001) in tissue-specific pericytes. However, CXCR3-signaling events in T cells as well as the relative effects of CXCL9, -10 and -11 on activation of the p44/p42 MAPK- and Akt-signaling pathways on cells heterologously expressing CXCR3 are thus far unknown.

CXCR3 induces activation of the p44/p42 MAPK and Akt/PI3K pathways in T cells

In CXCR3-transfected COS-7 cells and in activated primary human T cells, CXCL11, -10 and -9 induce activation of the p44/p42 MAPK- as well as the Akt-signaling pathways. However, considerable differences exist between these CXCR3 activating chemokines, with CXCL11 being a more potent ligand than CXCL9 and CXCL10. The potencies of CXCL9 and in particular CXCL10 to phosphorylate p44/p42 MAPK and Akt are lower than that observed for CXCL11 at different time points. These observations are in agreement with the reduced affinities reported for CXCL9 and -10 compared to -11 for CXCR3, with CXCL11 being unique as it interacts with two receptor states of CXCR3 (Cox et al., 2001). In both model systems, CXCR3 agonists activate p44/p42 MAPK and Akt in a G_i and PI3K sensitive manner. It should be noted that the efficacy of chemokines is subject to T cell donor variability, while their rank order of potency remains the same. As signaling induced by the different chemokines in CXCR3-expressing COS-7 is similar to those in activated T cells, CXCR3-expressing COS-7 cells may function as a useful alternative model system.

CXCR3 induces sustained activation of Akt in T cells

Interestingly, CXCR3 activation by CXCL11 leads to sustained phosphorylation of Akt in T cells which is similar to that previously observed for CXCL12 (SDF-

1α) and CXCR4, but distinct from other chemokine receptors. In case of CXCR4, persistent activation of Akt by CXCL12 was explained by the fact that CXCL12 and CXCR4 are involved in homeostasis rather than inflammation; sustained activation could protect $CXCR4^+$ cells from undergoing apoptosis, a process that is critical for the activation of T cells (Tilton et al., 2000). Our data not only reveal that persistent activation of the Akt-signaling pathway is not an exclusive property of the CXCL12/CXCR4 signaling pathway, but also demonstrate that sustained activation strongly depends on the concentration of the chemokine present in the environment of the receptor. At low concentrations of CXCL11 activation of Akt is sustained, whereas at higher concentrations CXCL11-mediated activation of Akt is transient. Loss of Akt activation might in turn be the signal for chemokine-induced apoptosis as recently demonstrated for CXCL12 and $CD4^+$ T cells (Loetscher et al., 1996). In a physiological context (e. g. inflammation) such processes might not only regulate and guide the migration of CXCR3-bearing T cells, but also be the trigger for T cell apoptosis in case of local high expression of CXCL11. Disturbance of such a balance might contribute to the presence of high numbers of CXCR3-positive T cells in certain inflammatory diseases e. g. chronic skin inflammations (Flier et al., 2001).

CXCR3-mediated T cell migration is not dependent on the PI3K/Akt or the p44/p42 MAPK pathway

Both the PI3K/Akt and the p44/p42 MAPK pathways have been reported to be involved in chemokine-induced migration of various cell types (Boehme et al., 1999; Loetscher et al., 1996; Qin et al., 1998; Vlahakis et al., 2002). Additional evidence for PI3Kγ as an important intermediary signal for chemotaxis came from genetic studies using PI3Kγ knock-out mice (Hirsch et al., 2000; Li et al., 2000; Sasaki et al., 2000). In our study the potent inhibitors of MEK1/2 (PD98059 and U0126) were unable to block CXCR3-mediated transmigration of T cells and CXCR3-induced actin polymerization. The inhibitors of PI3K showed a moderate attenuation of CXCR3-mediated transmigration of T cells and actin polymerization at relatively high concentrations. Both MEK1/2 and PI3K inhibitors were active in blocking the CXCL11-mediated phosphorylation of MAPK-p44/p42 and Akt at low concentrations.

Although wortmannin and LY294002 have been used extensively to study the physiological role of (class I) PI3Ks in various cellular responses (including chemotaxis) contradictory results have been obtained. For example, treatment with these PI3K inhibitors inhibited chemotaxis in some studies (Cox et al., 2001; Coffer et al., 1998) but not in others (Thelen, 2001; Neptune and

Bourne, 1997). These studies should be interpreted with caution, as it was shown that at higher concentrations (above 100 nM wortmannin or 10 μM LY294002) these compounds inhibit other signaling enzymes, including class II PI3K and Phosphatidylinositol-4-kinases (PI4K) (Fruman et al., 1998). As in our transmigration and actin polymerization assays only a moderate inhibition of T cell migration and actin polymerization is obtained with relative low and PI3K specific concentrations, we conclude that it is more likely that class II PI3K, PI4K or related enzymes other than PI3Kγ are involved in CXCR3-mediated T cell migration. To date limited information is available on the role of PI4K in cell migration. However, in neutrophils, a close association of PI4K, $\alpha 3\beta 1$ integrin, and transmembrane-4 superfamily (TM4SF) protein (CD151) was demonstrated (Yauch et al., 1998). From this observation, a functionally important role of this complex (including PI4K) in cell migration was suggested.

Our data on the possible involvement of class II PI3K or PI4K are in contrast with the general concept that chemotaxis is regulated via PI3Kγ (Rickert et al., 2000; Stephens et al., 2002; Weiner, 2002). This apparent discrepancy might result (aside from the fact that PI3K inhibitors at high concentrations also inhibited class II PI3K and PI4K; See above), also from the conception that signals involved in the regulation of cell migration act in a species, cell and chemokine/chemokine receptor dependent fashion (like the different roles of p44/p42 MAPK). The most convincing data pointing towards a crucial role for PI3Kγ in chemotaxis used PI3Kγ knock-out mice and tested CCR5/CXCR4/CXCR2 mediated migration of neutrophils/macrophages (Hirsch et al., 2000; Li et al., 2000; Sasaki et al., 2000). At this point one cannot exclude that CXCR3-mediated chemotaxis of human T cells is coordinated and regulated in a different way involving class II PI3K or PI4K rather than PI3Kγ.

An important role for MAPK in leukocyte migration came from studies in which blockade of MAPK activation by the MAPK kinase inhibitors lead to a decrease in eotaxin-induced eosinophil chemotaxis in vitro as well as inhibition of actin polymerization (Boehme et al., 1999). Similar observations were made for CXCL12-induced migration of $CD4^+$ T cells (Vlahakis et al., 2002). These pathways are clearly not employed in case of CXCR3-mediated T cell migration. Two structurally different MAPK inhibitors (PD98059 and U0126) were not able to inhibit CXCR3-induced actin polymerization assays or CXCR3-mediated in vitro transmigration, while fully inhibiting phosphorylation of p44/p42 MAPK. At this point a role for MAPK in the overall process of transendothelial T cell migration cannot be excluded. Recently, it was shown for eosinophils that under flow conditions CCR3-mediated MAPK-signals cause these cells to rapidly decrease their VCAM-1-mediated adherence while enhancing their ICAM-1 binding (Tachimoto et al., 2002). In analogy,

recruitment of T cells into sites of inflammations might require CXCR3-mediated MAPK activation for proper adherence during transendothelial migration. In addition, CXCR3 activation of MAPK pathways might be of importance for T cell function and development. As it was shown that MAPK-signaling is involved in T cell proliferation (DeSilva et al., 1998), the observed CXCL11-induced p44/p42 MAPK activation might induce long-term effects on T cells.

CXCR3-mediated T cell migration is dependent on Gαi, PLC and PI-Kinase signaling

The crucial role of G-proteins in chemotaxis of lymphocytes is well known. It is commonly accepted that all chemokine receptors couple to G_i although some chemokine receptors can also couple to $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$ and $G\alpha_{16}$ proteins (Wu et al., 1993; Kuang et al., 1996; Soede et al., 2001). As pretreatment of T cells with pertussis toxin completely blocks CXCR3-mediated chemotaxis, it can be concluded that CXCR3 is primarily coupled to $G\alpha_i$. The preference of CXCR3-coupling to $G\alpha_i$ over $G\alpha_{16}$, was also demonstrated in a CHO-cell line overexpressing human $G\alpha_{16}$ (Tensen et al., 1999). PTX treatment of these cells completely abrogates CXCL11-induced Ca^{2+} signaling, demonstrating that CXCR3 couples to endogenously $G\alpha_i$ and not to $G\alpha_{16}$.

The presence of the PLC-signaling pathways in T cells and the ability of chemokines to activate this system was previously demonstrated for CXCL8/CXCR2 (Bacon et al., 1995). Moreover, PLCβ activation of heterologously expressed chemokine receptors was shown for CCR1 and -2 (Kuang et al., 1996). The major role of PLC as determinant of chemokine-mediated chemotaxis of T cells (as described in this manuscript), however, is hitherto not recognized. Strikingly, the PLC pathway was thus far considered to play a role in down-modulation of chemotaxis (Li et al., 2000) rather than being crucial for T cell chemotaxis. The fact that the specific inhibitor of PLC attenuated chemokinesis, further stresses its pivotal role in T cell motility. It remains to be determined, which downstream signaling moieties of PLC are responsible for the control of the chemotactic process. Calmodulin kinase, myosin light chain kinase, Rho Kinase or other small GTPases are potential candidates, which are currently under investigation.

In summary, our experiments indicate that both PI3Kγ (resulting in Akt phosphorylation) and MEK1/2 (resulting in p44/p42 MAPK phosphorylation) are functionally active in translating CXCR3-triggered signals in human T-lymphocytes. However, these signals are not directly involved in CXCR3-mediated T cell migration, which is primarily regulated in a manner dependent on G_i, PLC, and phosphatidylinositol kinases other than class I PI3Kγ.

Chapter 5:

Significance of CXCR3-mediated calcium signaling in human primary T cells

Pauline Verdijk, Nynke M. S. van den Akker, Elisabeth M. H. van der Raaij-Helmer, Aat A. Mulder, Paul J. Hensbergen, Cornelis P. Tensen.

Department of Dermatologie, LUMC

Submitted

Abstract

The chemokines CXCL9, -10 and -11 are inflammation-induced chemokines and attract activated T cells via CXC chemokine receptor-3. We have previously shown that MAPK p44/p42 and Akt are activated via MEK1/2 and phosphatydylinositol-3-kinase, respectively, by agonist binding to CXCR3, but are not essential for CXCR3-mediated actin polymerization and migration in primary human T cells. In view of the importance of phospholipase C in CXCR3-regulated downstream signaling in T cells and its known role in inositol 1,4,5-trisphosphate-mediated intracellular calcium mobilization, we studied in detail the CXCR3-mediated calcium mobilization in relation to T cell migration, actin polymerization and downstream kinase activation. We demonstrate that in CHO cells transfected with human CXCR3 CXCL11-induced calcium mobilization CXCL11 occurred via store operated calcium channels. However, biological responses following CXCL11-stimulation of human peripheral T cells, such as actin polymerization, chemotaxis and phosphorylation of downstream kinases, were independent of entry of extracellular calcium. In contrast, T cell migration was dependent on CXCL11-induced release of calcium from intracellular stores. In addition, in the absence of intracellular calcium both actin polymerization and chemotaxis were severely diminished or abolished, while phosphorylation of the downstream kinase Akt, but not of p44/p42, was decreased. These novel observations expand our current understanding of the role of CXCR3 in T cell biology and trafficking.

Introduction

In T lymphocytes calcium (Ca^{2+}) plays a fundamental role as a second messenger in a wide variety of cellular responses, including T cell activation (van Leeuwen and Samelson, 1999), cytokine production (Kuklina and Shirshev, 2001), proliferation (Kotturi et al., 2003), and cell death (Medema and Borst, 1999). The cytosolic [Ca^{2+}] is maintained at relatively low levels by intracellular Ca^{2+}-binding proteins and by sequestration of Ca^{2+} in intracellular membrane-bound compartments such as the endoplasmic reticulum and mitochondria. After ligand binding to cell surface receptors, like chemokine receptors, intracellular [Ca^{2+}] can rapidly and transiently increase ([Ca^{2+}]$_i$). The information encoded in the transient [Ca^{2+}]$_i$ is converted by Ca^{2+}-binding proteins into specific biochemical changes involving Ca^{2+}-activated protein kinases, Ca^{2+}-dependent enzymes (Berridge et al., 2000; Berridge et al., 2003; Clapham, 1995) and transcription factors (Venkatesh et al., 2004; Lewis, 2003) Both calcium release from intracellular stores and influx through calcium channels contribute to [Ca^{2+}]$_i$, but influx dominates quantitatively and is required for subsequent gene expression (Negulescu et al., 1994).

Receptor-mediated calcium responses can be initiated by G protein coupled receptors (GPCR), such as chemokine receptors, or tyrosine kinase (-coupled) receptors, such as antigen-receptors in T and B cells, but are always induced via phospholipase C (PLC). Agonist-induced activation of PLC hydrolyses a minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). Following binding of IP_3 to its receptor (IP_3R) on intracellular calcium stores, calcium is released into the cytosol. Depletion of the intracellular Ca^{2+} stores induces the influx of extracellular Ca^{2+} via so-called store-operated Ca^{2+} channels (SOC). The only SOC described for T cells is the Ca^{2+} release activated Ca^{2+} channels (CRAC). Although SOC channels have been extensively studied, their molecular identity has not been identified to date. Neither is there a consensus about the mechanism by which the SOC channels are activated. It is generally believed that coupling between the release of calcium from intracellular stores and the influx of extracellular calcium does not occur via Ca^{2+}-binding to the calcium-channels in the plasma membrane. Alternative mechanisms for store operated calcium entry have been described of which two now are the most accepted: the conformational coupling model and the diffusible messenger hypothesis. For detailed reviews see Putney et al. (Putney, Jr. et al., 2001), Lewis (Lewis, 2001), and Prakriya and Lewis (Prakriya and Lewis, 2003).

The functional importance of calcium responses in lymphocytes has been studied for activation, proliferation and gene transcription. However, its role in lymphocyte migration remains unclear. We studied the nature of $[Ca^{2+}]_i$ following triggering of the chemokine receptor CXCR3 and its functional importance in biological processes involving T cell migration. CXCR3, highly expressed on activated T cells (Loetscher et al., 1996; Qin et al., 1998), is a $G\alpha i$ coupled seven-transmembrane receptor, which elicits calcium increases after activation by one of its ligands, CXCL9, -10 or -11 (formerly known as Mig, IP-10 and IP-9/I-TAC, respectively) (Scandella et al., 2002; Tilton et al., 2000; Kansra et al., 2001; Loetscher et al., 1996; Tensen et al., 1999). We have previously shown that agonist-induced activation of CXCR3 initiates multiple signal transduction pathways, including phosphorylation of MAPK p44p/p42 (via MEK 1/2) and Akt (via phosphatidylinositol-3-kinase; PI3K). CXCR3-mediated actin polymerization and migration were completely dependent on $G\alpha i$ and phospholipase C (PLC) (Smit et al., 2003). Importantly, we demonstrated that CXCR3-mediated actin polymerization and migration of human primary T cells is not dependent on PI3K or MEK1/2. Here, we describe the nature of the $[Ca^{2+}]_i$ in CXCR3-mediated signaling and determined its role in actin polymerization, migration and the activation of downstream kinases in human primary T cells and transfected cell lines.

Material and methods

Materials

Cell culture media and supplements were obtained from GibcoBRL (Carlsbad, CA). CXCL11 (IP-9/I-TAC) was from R & D systems Inc. (Minneapolis, MN). The MEK inhibitors PD98059 and U0126 were obtained from New England Biolabs Inc. (Beverly, MA) and Promega (Leiden, The Netherlands), respectively. The PLC inhibitor U73122, its inactive analog U73343, the phosphatidylinositol kinase inhibitors wortmannin and LY294002, pertussis toxin, thapsigargin, nifedipine, amiloride, latrunculin A, BAPTA-AM and Cytochalasin D were obtained from Sigma (Sigma-Aldrich Chemie B. V. Zwijndrecht, The Netherlands). Monoclonal antibodies against Akt, phospho-p44/p42 and p44/42 and polyclonal antibody against phospho-Akt (ser473) were purchased from New England Biolabs.

Cell culture

CHO cells heterologously expressing human CXCR3 were cultured as described previously (Tensen et al., 1999). Peripheral blood mononuclear cells (PBMC) were isolated from human peripheral blood by Ficoll density centrifugation. For the isolation of T lymphocytes, PBMC were passed through a nylon wool column. Monocytes were then removed by plastic adherence. T cells were activated by culturing in RPMI-1640 with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS (all from GibcoBRL) in the presence of 1 μg/ml phytohaemaglutinin (HA17, Murex Biotech Ltd, Dartford, UK) for 3 days and 200 U/ml IL-2 (Eurocetus, Amsterdam, The Netherlands) for an additional 7-14 days (Loetscher et al., 1996). The purity of T cells and CXCR3-expression were monitored by FACS analysis (FACScan; BD Biosciences, Alphen a/d Rijn, The Netherlands). The CXCR3 monoclonal antibody (1C6) was from BD Biosciences; Anti-CD3 antibody was purchased form Sanquin (Amsterdam, The Netherlands). The percentage of $CXCR3^+/CD3^+$ cells was typically about 95%.

Intracellular calcium assay

CHO-cells expressing human CXCR3 suspended in HEPES buffer (20 mM HEPES, 138 mM NaCl, 5.5 mM Glucose, 6 mM KCl, 1 mM $MgSO_4$, 1.1 mM $CaCl_2$, 1 mM NaH_2PO_4 and 0.1% BSA; pH7.4) were loaded with 2 μM of the calcium sensitive dye, Fura-2-AM (Molecular Probes, Leiden, The Netherlands) for 45 min at 37 °C. After incubation, excess dye was removed by washing the cells in HEPES buffer, and the cell suspension was placed in a stirred quartz

cuvette. Cytosolic calcium was measured using a Photon Technology International (Monmouth Junction, NJ) spectrofluorometer. Fluorescence was measured at 37°C at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Maximum and minimum calcium binding was determined by lysing the cells with 0.1% Triton and quenching with 25mM ethylene glycol bis ([beta]-aminoethyl ether)-*N*, *N*, *N* ', *N* '-tetraacetic acid (EGTA), respectively.

Actin polymerization assay

Cells were resuspended in RPMI-1640 or HEPES buffer with 0.25% BSA (fraction V, Sigma) in a concentration of 4×10^6 cells/ml. Prior to the assay cells were either incubated with 20 μM BAPTA-AM for 30 minutes and washed, to chelate intracellular calcium or washed and resuspended in Ca^{2+} free HEPES buffer. Cells were then stimulated with 10 nM CXCL11. At indicated time points, 25 μl of cell suspension was transferred to 25 μl of fixation solution (4 % paraformaldehyde [PFA]). Cells were fixed for at least 15 minutes. Thereafter, cells were washed and resuspended in 50 μl of permeabilization reagent (0.1% Triton-X 100). After 10 minutes, cells were washed, blocked with 1% BSA (5 min), washed and incubated with 0.5 μM rhodamine-phalloidin (Molecular Probes) to visualize filamentous actin (F-actin). After 45 minutes, cells were centrifuged and resuspended in PBS with 0.25% BSA. Mean fluorescence intensity was measured by FACS analysis.

Chemotaxis assays

The assay for chemotaxis was performed in 24 well plates (Costar, Cambridge, MA) carrying Transwell permeable supports with 5 μm pore size polycarbonate membrane. T cells were cultured overnight at 4×10^6 cells/ml in RPMI-1640 containing 0.25% BSA. Prior to the assay cells were either incubated with 20 μM BAPTA-AM for 30 minutes and washed, to chelate intracellular calcium or resuspended in Ca^{2+} free HEPES buffer or pretreated with 0.5 mM EGTA to remove extracellular Ca^{2+}. Medium alone or supplemented with chemokine was placed in the lower compartment, and cells were loaded onto the inserts at 0.4×10^6/100 μl. Chambers were incubated for 120 minutes in a 5% CO_2, humidified incubator at 37°C. After the incubation period, numbers of T cells migrated to the lower chamber were counted under a microscope using a hemocytometer. Viability was checked with trypan blue exclusion. All conditions were tested at least in triplicate; statistical evaluation was performed using the Student's t-test.

Cell Stimulation

If not stated otherwise, IL-2-expanded activated T cells were serum starved overnight in medium supplemented with 0.25% BSA in 2×10^7 cells/ml. Aliquots of 8-10 $\times$ 10^6 T cells were preincubated with BAPTA-AM for 30 minutes and washed three times. Other aliquots were pretreated with 0.5 mM EGTA, 1 μM latrunculin A or 2.5 μM cytochalasin D 5 minutes prior to stimulation. Cells were stimulated with 10 nM CXCL11 for 1 or 15 minutes or with 2.5 μM thapsigargin for 5 minutes. Following stimulation, cells were pelleted and frozen immediately.

Western blot

Cell pellets were resuspended in lysisbuffer (50 mM Tris/HCl pH 7.5, 10 mM EDTA, 80 mM KCl, 1% NP40), supplemented with protease and phosphatase inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, 50 μM PMSF, 1 mM sodium orthovanadate and serine/threonine phosphatase inhibitor cocktail I (1:100), all obtained from Sigma). After 30 minutes on ice, lysates were sonicated and insoluble fragments were removed by centrifugation (850 $\times$ g, 4° C). Proteins were separated on 10% SDS-PAGE and transferred onto nitrocellulose membranes. Non-specific binding was blocked by incubating the membranes with 3% w/v non-fat dry milk and 0.05% Tween-20 in TBS (TBST). Membranes were incubated with the first antibody in the appropriate dilution for 1 h at RT or overnight at 4°C. After incubation, membranes were rinsed with distilled water and washed three times in wash buffer (0.25% non-fat dry milk in TBST). Secondary antibody conjugated to horseradish peroxidase was added to the membranes in 3% non-fat dry milk in TBST in the appropriate dilution. After one hour of incubation at RT membranes were washed three times and developed using an enhanced chemiluminescence kit (Santa Cruz Biotechnology, Heerhugowaard, The Netherlands).

Results

Characterization of CXCR3-induced $[Ca^{2+}]_i$

Calcium influx in CHO cells heterologously expressing CXCR3 is mediated via store operated channels

To study the characteristics of CXCR3-mediated Ca^{2+} mobilization as well as testing the effectiveness of inhibitors and stimulators, we started with CHO cells stably expressing human CXCR3 [CHO-CXCR3 (Tensen et al., 1999)]. We previously demonstrated that CXCL11 is the most potent stimulator of Ca^{2+} mobilization (Smit et al., 2003; Tensen et al., 1999) which correlated with the highest potency in Akt and MEK activation compared to CXCL9 and -10 (Smit et al., 2003). Therefore, all experiments were performed with 10 nM CXC11.

We first studied the contribution of release of intracellular Ca^{2+} and influx of extracellular Ca^{2+} to the CXCR3-mediated $[Ca^{2+}]_i$. Under normal test conditions, CHO-CXCR3 showed a quick rise in intracellular calcium levels after encountering agonist, peaking within several seconds and returning to base line levels within 40-60 seconds (figure 1A). The removal of calcium from the extracellular medium, either by chelation with EGTA or by using calcium-free buffer, abrogated the $[Ca^{2+}]_i$ almost completely. However, a small increase in calcium was still detected, most likely representing calcium release from intracellular stores (figure 1A). Thapsigargin, an inhibitor of the re-uptake of calcium into intracellular stores, induced a rise in intracellular Ca^{2+}. Depletion of intracellular stores with thapsigargin prevented $[Ca^{2+}]_i$ induced by CXCR3 agonists (figure 1C), indicating that the mobilization of Ca^{2+} from intracellular stores is essential for the influx of extracellular calcium in CHO cells heterologously expressing CXCR3. These results suggest the involvement of store operated calcium channels (SOC) in CXCR3-mediated $[Ca^{2+}]_i$ in CHO cells. The cell-permeable calcium chelator BAPTA-AM (20 mM) used to study the role of intracellular Ca^{2+} in biological responses (see below) completely abrogate the Ca^{2+} signal (figure 1B).

A second type of Ca^{2+} channels is the voltage-dependent Ca^{2+} channel (VDCC). In CHO cells expressing CX3CR1, VDCC are involved in CX3CL1 (fractalkine)-induced $[Ca^{2+}]_i$ (Kansra et al., 2001), while T cell activation and proliferation is associated with L-type VDCC (Kotturi et al., 2003). Neither inhibition of the L-type VDCC with nifedipine (10 mM) nor inhibition of the T type VDCC with amiloride (100 mM) could block chemokine induced Ca^{2+} influx in CHO-CXCR3 cells (figure 1D). Thus, CXCR3-mediated Ca^{2+} influx in CHO cells did not occur via L-type or T type VDCC.

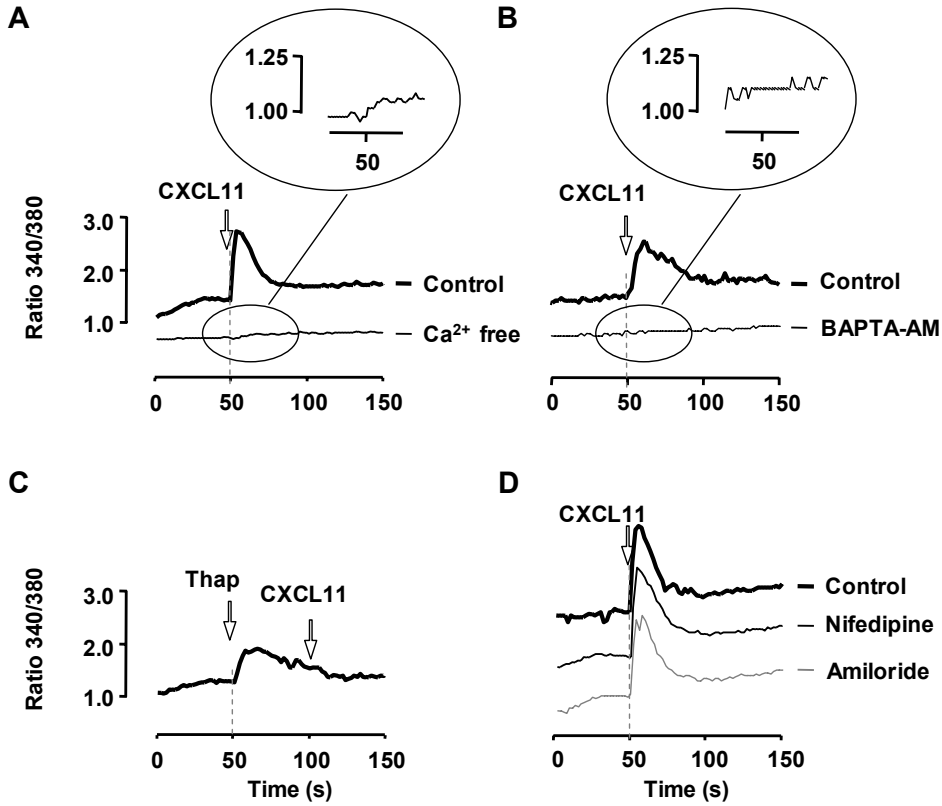

Figure 1. Capacitive Ca^{2+} entry in CHO-CXCR3 stimulated with CXCL11 and thapsigargin. CHO cells transfected with the human CXCR3 loaded with FURA-2AM were stimulated with 10 nM CXCL11 (**A-D**) and 2.5 μM thapsigargin (**C**). A. Cells were washed and resuspended in HEPES buffer with or without Ca^{2+} prior stimulation. **B**. Cells were pretreated with 20 μM BAPTA-AM, washed and resuspended in HEPES buffer before stimulation. **C**. Cells were first stimulated with thapsigargin (2.5 μM) and subsequently with CXCL11. **D.** Cells were incubated with an inhibitor of L-type (nifedipine, 10 μM) or T type (amiloride, 100 μM) voltage-dependent Ca^{2+} channels before stimulation with CXCL11. Intracellular concentration of Ca^{2+} is represented by the ratio of fluorescence intensity at 340 and 380 nm.

CXCR3-induced $[Ca^{2+}]_i$ is dependent on PLC and not on PI3K or MEK1/2

Gi-coupled receptors induce the activation of PLC resulting in the production of DAG and IP_3. Upon binding to its receptor, IP_3 triggers the opening of Ca^{2+} channels and the release of Ca^{2+} from the intracellular stores. We previously showed that CXCR3-mediated T cell migration is completely dependent on PLC (Smit et al., 2003). To examine the role of PLC in the calcium response after agonist binding we incubated CHO-CXCR3 cells with the PLC inhibitor U73122 and studied its effect on CXCL11-induced $[Ca^{2+}]_i$. As expected, U73122

completely blocked CXCL11-induced calcium mobilization, while its inactive homologue U73433 had no effect (figure 2A). In CHO cells transfected with CX3CR1, inhibition of PI3K, but not of MEK reduced the CX3CL1 (fractalkine)-induced Ca^{2+} influx (Kansra et al., 2001). We verified whether this was also true for CXCL11-stimulated CHO-CXCR3. Incubation with PI3K-inhibitors wortmannin (200 nM) and Ly294002 (50 mM) did not prevent the CXCL11-induced $[Ca^{2+}]_i$ in these cells (figure 2B). In addition, inhibition of MEK with U1026 (10 mM) had no effect on the CXCR3-mediated calcium response (figure 2C). Thus, CXCL11-induced $[Ca^{2+}]_i$ in CHO cells was dependent on PLC, but not on PI3K or MEK.

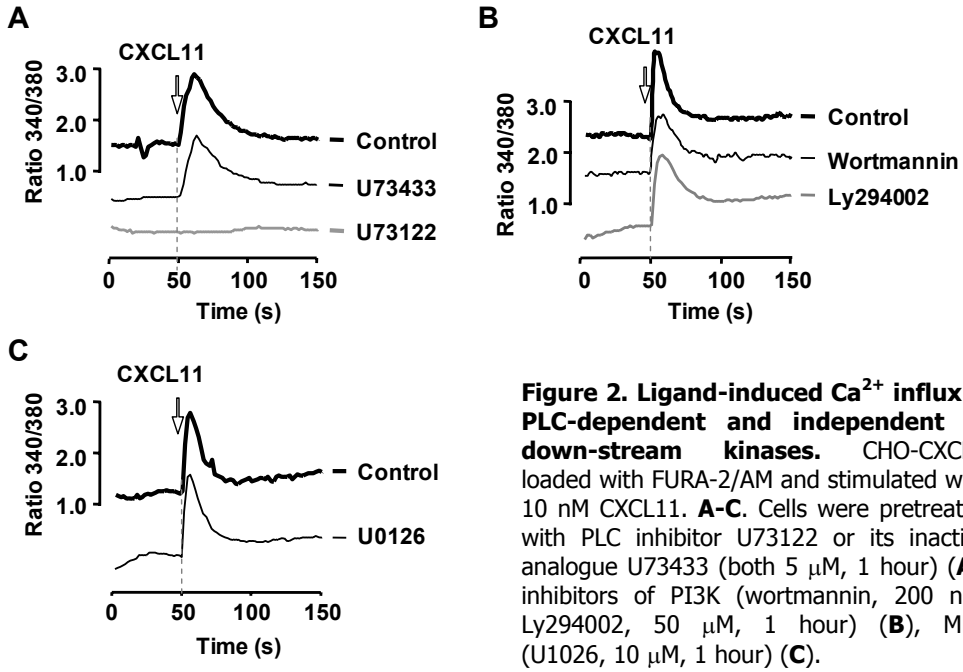

Figure 2. Ligand-induced Ca^{2+} influx is PLC-dependent and independent of down-stream kinases. CHO-CXCR3 loaded with FURA-2/AM and stimulated with 10 nM CXCL11. **A-C**. Cells were pretreated with PLC inhibitor U73122 or its inactive analogue U73433 (both 5 μM, 1 hour) (**A**), inhibitors of PI3K (wortmannin, 200 nM; Ly294002, 50 μM, 1 hour) (**B**), MEK (U1026, 10 μM, 1 hour) (**C**).

$[Ca^{2+}]_i$ and actin polymerization in human primary T cells

To determine the functional importance of [Ca2+]i, we examined the role of calcium signaling in biological responses following the encounter of CXCR3-chemokines by activated T cells. Human peripheral T cells were isolated from healthy donors, activated with PHA and expanded in the presence of recombinant human IL-2 as described previously, resulting in >95% CXCR3+/CD3+ cells (Tensen et al., 1999). One of the initial responses following chemokine receptor triggering is a fast and transient increase in

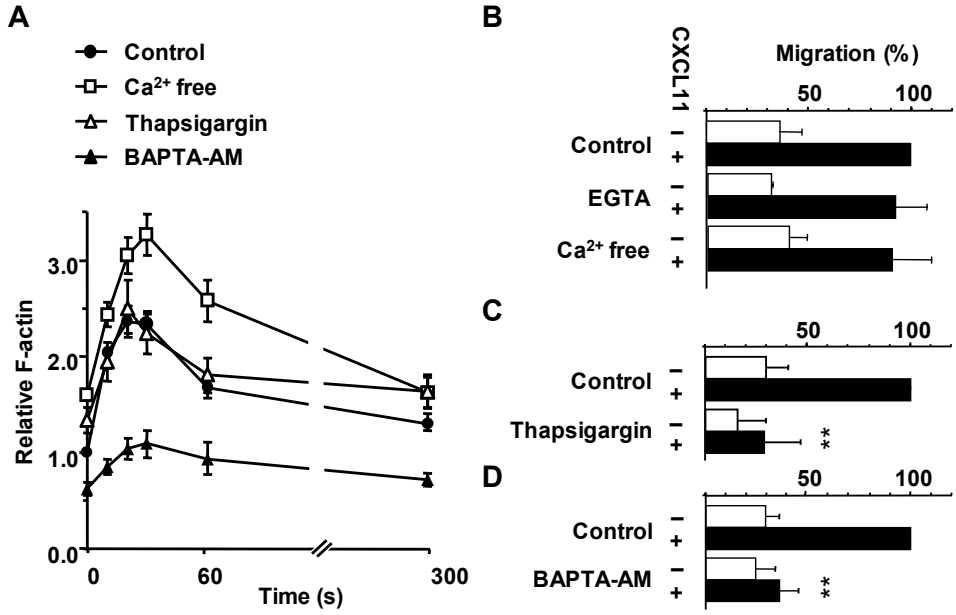

Figure 3. Role of Ca^{2+} in CXCL11-induced actin polymerization and migration in human activated T cells. Human primary T cells were cultured with PHA and IL-2 for 10 days. Cells were resuspended in buffer with or without Ca^{2+} or pretreated with 2.5 μM thapsigargin or 20 μM BAPTA-AM, before stimulation. **A.** Activated T cells were stimulated with 10 nM CXCL11 and fixed with paraformaldehyde at indicated time points. F-actin content of the cells was determined by staining with phalloidin-rhodamin. Fluorescence intensity was measured by FACS analysis. The graphs represent data from 4 to 15 different donors and show the relative F-actin, calculated as described in material and methods. Error bars show the standard error of the mean (Student's t-test). **B-D.** The chemotactic activity of activated human T cells towards 30 nM CXCL11 was determined in a Transwell migration system. The effect of Ca^{2+} mobilization in T cell migration was determined by performing the assay in Ca^{2+}-free HEPES buffer or by chelation of Ca^{2+} with 0.5 mM EGTA (**B**), depleting intracellular Ca^{2+} stores by pretreating the cells with thapsigargin (**C**) or by chelating intracellular Ca^{2+} by incubating the cells with BAPTA-AM before the start of the assay (**D**). Chemotaxis is expressed as percentage of cells migrating in comparison to 30 nM CXCL11 (set at 100 % for each individual donor). The figures show data from 3 to 8 different donors. Error bars represent the standard deviation. Asterisks indicate values significantly different from CXCL11-induced chemotaxis under normal conditions (control) (Student's t-test; ** $p < 0.01$).

filamentous actin. This response could be measured by labeling stimulated cells with phalloidin, which binds only to filamentous actin (F-actin) and not to monomeric actin. Thapsigargin and BAPTA-AM were used to discriminate between the mechanism of Ca^{2+} release from intracellular stores (and possible other signaling events that are generated via this mechanism) and the physical presence of cytosolic Ca^{2+}, respectively. Under normal conditions, a fast increase in F-actin was detected after stimulation with CXCL11, peaking within 30 seconds and returning to starting levels within 1 to 5 minutes. In the absence of extracellular calcium, the basal level of F-actin in the cell was increased and actin polymerization in response to CXCL11 was enhanced ($p<0.001$) (figure 3A). The level of F-actin in the cell was also increased by the thapsigargin-induced release of Ca^{2+} from intracellular stores ($p<0.01$). CXCL11-induced actin polymerization was still effective in thapsigargin-treated cells with an optimum in F-actin equal to control cells. Thus, thapsigargin-treatment of the cells prior to CXCL11-stimulation did not prevent the CXCR3-mediated response. On the other hand, chelation of intracellular Ca^{2+} by incubating cells with 20 mM BAPTA-AM severely reduced the level of F-actin in the cells. Furthermore, actin polymerization in response to CXCL11 was decreased, yet not completely abolished (figure 3A). Taken together, these results suggest that both the process of calcium release from intracellular stores and influx of extracellular Ca^{2+} are not necessary for the CXCL11-induced initiation of the transient increase in F-actin. However, intracellular Ca^{2+} concentrations are essential for the stability of the cytoskeleton in non-stimulated cells and for efficient actin polymerization in stimulated cells.

CXCR3-mediated $[Ca^{2+}]_i$ and migration of human primary T cells

We then studied the involvement of calcium in migration of human primary T cells, using a Transwell system. Spontaneous migration was not decreased in the absence of intra-or extracellular Ca^{2+} nor by emptying of calcium stores, indicating that basal cell motility was not affected. The migration of activated human T cells was not affected by the absence of extracellular Ca^{2+} (figure 3B). However, depletion of intracellular stores with thapsigargin (figure 3C) or chelation of cytosolic calcium by BAPTA-AM (figure 3D) abrogated migration towards CXCL11. Viability of the cells (determined by trypan blue exclusion) was not affected by any of the treatments described. Thus, although extracellular Ca^{2+} is not required, Ca^{2+} release from intracellular stores and the presence of intracellular calcium itself are essential for T cell migration.

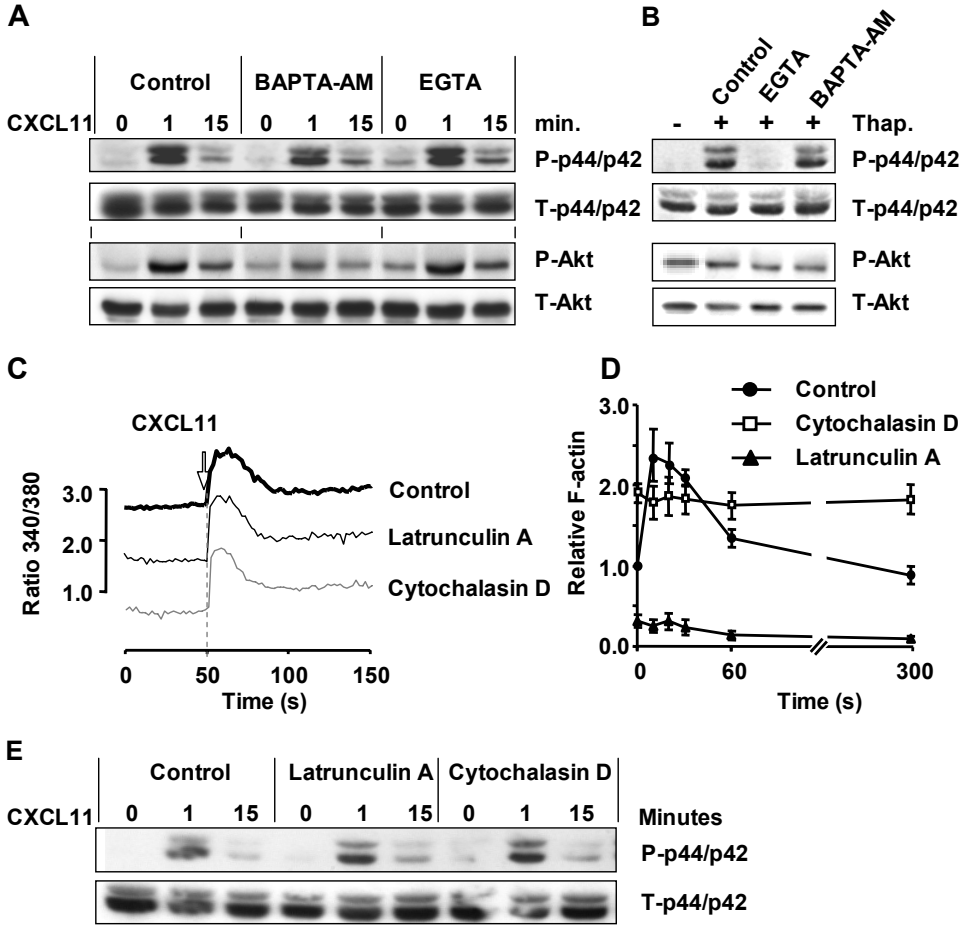

Figure 4. The role of Ca2+ mobilization and the cytoskeleton on CXCL11-induced phosphorylation of down-stream kinases p44/p42 and Akt. Activated human T cells were stimulated with CXCL11 for 1 and 15 minutes (A, C) or with thapsigargin for 5 minutes (**B**). Cells were lysed and analyzed by Western blot, with antibodies specific for phosphorylated p44/p42 (P-p44/p42) or phosphorylated Akt (Ser473) P-Akt. Membranes were subsequently stripped and reprobed with antibodies against total p44/p42 (T-p44/p42) and total Akt (T-Akt). **A, B.** Phosphorylation of p44/p42 and Akt was studied in normal medium, after chelation of extracellular Ca^{2+} with 0.5 mM EGTA or after depletion of intracellular Ca^{2+} with 20 μM BAPTA-AM. Results given are representative for 2 independent experiments with cells from 6 different donors. **C.** Pretreatment of CHO-CXCR3 cells with 2.5 μM cytochalasin D or 1 μM latrunculin A for 30 minutes does not influence CXCL11-induced Ca^{2+} mobilization. **D.** CXCL11-induced actin polymerization was abrogated activated human T cells in the presence of agents that disturb the actin cytoskeleton (cytochalasin D, 2.5 μM, latrunculin A, 1 μM, 5 minutes) **E.** Phosphorylation of p44/p42 and Akt was not affected in activated human T cells pretreated with 2.5 μM cytochalasin D or 1 μM latrunculin A.

Activation of down-stream kinases: dependency on Ca^{2+} and the actin cytoskeleton

Ca^{2+} signaling and phosphorylation of p44/42 and Akt

Next, we studied the role of $[Ca^{2+}]_i$ in the CXCR3-induced activity of MEK/p44/p42 and PI3K/Akt. Stimulation of T cells with CXCL11 resulted in phosphorylation of both p44/p42 and Akt (figure 4A). Phosphorylation was measured as early as one minute after stimulation and was decreased, but still detectable, after 15 minutes. In the absence of extracellular Ca^{2+}, CXCL11-induced phosphorylation of both p44/p42 and Akt was not changed. Chelation of intracellular calcium by BAPTA-AM had little or no effect on p44/p42 phosphorylation (figure 4A). However, Akt phosphorylation was partially diminished in the absence of intracellular calcium. Thus, CXCL11-stimulated phosphorylation of Akt, but not of p44/p42 was partly dependent on intracellular Ca^{2+}, while phosphorylation of both p44/p42 and Akt were independent of the influx of extracellular Ca^{2+}.

Phosphorylation of p44/p42 can also be induced in response to non-physiological calcium mobilizing agents such as thapsigargin and ionomycin (Qin et al., 1998). We observed that in primary human T cells thapsigargin-induced $[Ca^{2+}]_i$ resulted in the phosphorylation of p44/p42 (figure 4B). Phosphorylation of p44/p42 was prevented, in the absence of extracellular Ca^{2+}. Remarkably, chelation of intracellular calcium with BAPTA-AM had no effect (figure 4B). Thapsigargin-mediated Ca^{2+} mobilization had no effect on Akt phosphorylation. Taken together, these results suggest that $[Ca^{2+}]_i$, alone, does not activate Akt. However, for agonist-induced-activation of Akt, Ca^{2+} is necessary for an optimal response, perhaps by the action of Ca^{2+}-activated proteins. The opposite is true for p44/p42, which was activated by thapsigargin-induced $[Ca^{2+}]_i$, but can also be phosphorylated in response to chemokines in the absence of Ca^{2+}.

Activation of p44/p42 and Ca^{2+} mobilization through CXCR3 does not require receptor internalization or an intact cytoskeleton

We then studied the role of the cytoskeleton in $[Ca^{2+}]_i$ and the activation of p44/p42. The actin cytoskeleton may be involved in coupling the Ca^{2+} release from intracellular stores to the opening of calcium channels in the plasma membrane. We examined the role of the cytoskeleton in $[Ca^{2+}]_i$ signaling via CXCR3 in CHO-CXCR3 cells by disrupting the cytoskeleton with latrunculin A or cytochalasin D. Incubation of CHO-CXCR3 cells with 2.5 mM cytochalasin D or 1 mM latrunculin A had no effect on the CXCL11-mediated Ca^{2+} response (figure 4C), indicating that the cytoskeleton had no active role in coupling between intracellular Ca^{2+} release and Ca^{2+} entry.

Several reports suggest the requirement of receptor endocytosis for the activation of kinases, such as JNK (McDonald et al., 2000) and MEK/p44/p42 (Daaka et al., 1998; DeFea et al., 2000; Kranenburg et al., 1999). After treatment of human primary T cells with cytochalasin D or latrunculin A, the actin polymerization response to CXCL11 was absent (figure 4D). Incubation with latrunculin A for 5 minutes even caused a nearly complete depolymerization of the actin cytoskeleton. We then studied the influence of disruption of the actin cytoskeleton on CXCR3-mediated phosphorylation of p44/p42 in human primary T cells. Disruption of the actin cytoskeleton, had no effect on the phosphorylation of p44/p42 (figure 4E), indicating that CXCR3-internalization is not required for activation of the MEK/p44/p42 pathway.

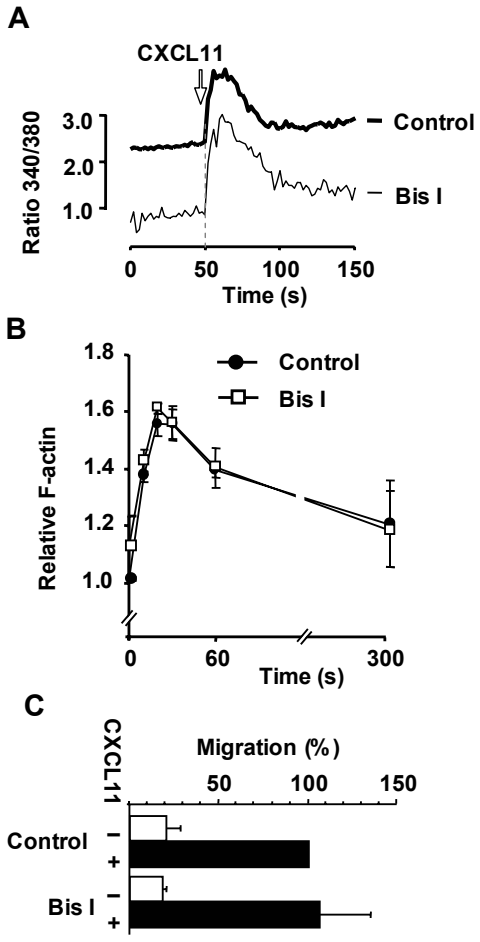

Figure 5. CXCR3-mediated Ca2+ mobilization, T cell migration and actin polymerization is PKC-independent. A. CHO-CXCR3 loaded with FURA-2/AM and stimulated with 10 nM CXCL11. Cells were pretreated with PKC (Bis I, 10 μM, 30 minutes). Intracellular concentration of Ca^{2+} is represented by the ratio of fluorescence intensity at 340 and 380 nm. **B, C.** Human primary T cells were cultured with PHA and IL-2 for 10 days. Activated T cells were stimulated with 10 nM CXCL11 and fixed with paraformaldehyde at indicated time points. (**B**) and chemotaxis assay (**C**). **B.** F-actin content of the cells was determined by staining with phalloidin-rhodamin. Fluorescence intensity was measured by FACS analysis. The graph represents data from 4 different donors and shows the relative F-actin, calculated as described in material and methods. Error bars show the standard error of the mean (Student's t-test). **C.** The chemotactic activity of activated human T cells towards 30 nM CXCL11 was determined in a Transwell migration system. Chemotaxis is expressed as percentage of cells migrating in comparison to 30 nM CXCL11 (set at 100 % for each individual donor). The figure shows data from 3 different donors. Error bars represent the standard deviation.

Discussion

Signaling by chemokine receptors occurs via Gi proteins, resulting amongst others in an increase in intracellular [Ca^{2+}] (Scandella et al., 2002; Tilton et al., 2000; Kansra et al., 2001; Loetscher et al., 1996). Ca^{2+} is an important signal in a wide variety of cellular responses, including cell proliferation, differentiation, and survival. Despite the fact that $[Ca^{2+}]_i$ in T cells have been broadly studied (Prakriya and Lewis, 2003; Putney, Jr. et al., 2001; Lewis, 2001), its functional importance in relation to chemokine receptors remains largely unknown. In this study, we investigated the role of $[Ca^{2+}]_i$ in the signal transduction and biological effects of T lymphocytes following agonist binding to the chemokine receptor CXCR3, a receptor highly expressed on activated T cells (Loetscher et al., 1996; Sallusto et al., 1998a; Qin et al., 1998).

CXCR3-triggered increase in intracellular Ca^{2+} in CHO-CXCR3 cells was mediated via store-operated Ca^{2+} channels. Incubation of cells with thapsigargin, the inhibitor of sarcoplasmatic/endoplasmatic reticulum calcium ATPase (SERCA) pumps on the endoplasmic reticulum (Lytton et al., 1991), blocks the re-uptake of Ca^{2+} into the intracellular stores and results in a rise in intracellular Ca^{2+} and the depletion of intracellular stores. Treatment of CHO-CXCR3 with thapsigargin prevented CXCL11-induced $[Ca^{2+}]_i$. Moreover, in the absence of extracellular Ca^{2+} only a small increase in Ca^{2+} was detectable likely representing release of Ca^{2+} from intracellular stores. It is generally believed that these influxes of Ca^{2+} occur via CRAC channels. However, some studies report the involvement of voltage-dependent Ca^{2+} channels, such as L-type Ca^{2+} channels in $[Ca^{2+}]_i$ following TCR/CD3-stimulation (Kotturi et al., 2003) or T type Ca^{2+} channels in CX3CR1-induced $[Ca^{2+}]_i$ (Kansra et al., 2001). We showed that, at least in CHO-cells, these channels were not involved in the CXCR3-mediated rise in intracellular Ca^{2+}. Furthermore, in contrast to other reports (Ribeiro et al., 1997) ligand–induced store operated Ca^{2+} entry in CHO-CXCR3 following the encountering of CXCL11 was not affected by disruption of the cytoskeleton. We also observed that in contrast to the related chemokine receptor CX3CR1 (Kansra et al., 2001), PI3K does not play a role in chemokine-induced Ca^{2+} mobilization, suggesting that mechanisms involved in chemokine signal transduction are receptor-specific.

Human activated T cells migrated effectively to CXCL11 both in the presence and in complete absence of extracellular Ca^{2+}, indicating that CXCR3-mediated T cell migration was independent of the influx of Ca^{2+}. Similar results were reported recently for the CCR7-mediated migration of monocyte-derived dendritic cells (Scandella et al., 2002). Although the depletion of intracellular Ca^{2+} stores did not greatly affect CXCR3-mediated actin polymerization, T cell migration towards CXCL11 was completely abrogated, indicating that actin

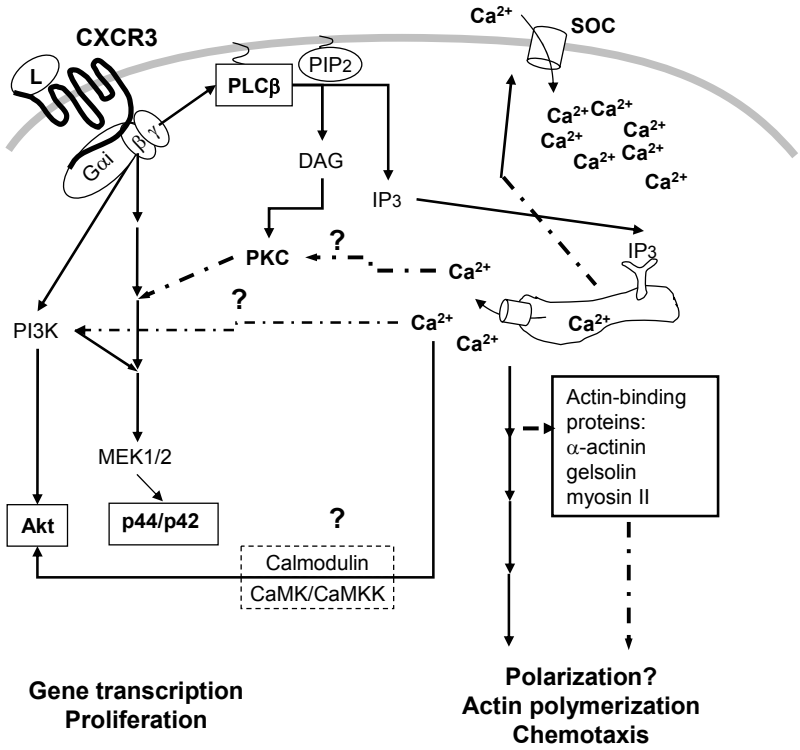

Figure 6: Schematic overview of a selection of components involved in the signal transduction pathways activated by CXCR3-ligand binding. Following ligand binding to CXCR3 the G_i protein is activated. The βγ subunits of the G-protein then activate PLCβ and PI3K. PLCβ hydrolyses PIP_2 into DAG and IP_3. DAG activates PKC, while IP_3 binds to its receptor on the intracellular Ca^{2+} stores, which causes the release of Ca^{2+} into the cytosol. The depletion of the intracellular stores activates store operated calcium (SOC) channels in the plasma membrane. Subclasses of PKC can also be activated by increased Ca^{2+} levels. The scheme is not meant to be complete. L: ligand. Lines composed of more than one arrow signify pathways in which more kinases /enzymes lie in between the indicated kinase/enzyme and the target. Broken lines represent possible connections and involvement of the indicated proteins.

polymerization responses are not fully representative of migratory responses. In contrast, the presence of intracellular Ca^{2+} was both essential for optimal CXCL11-induced actin polymerization and crucial for CXCR3-mediated T cell migration. Chemokine-induced local increases in Ca^{2+} could be necessary for polarization of T cells (Sanchez-Madrid and del Pozo, 1999) and migration towards a chemokine gradient. Intracellular Ca^{2+} signals have been associated with cell polarization in eosinophils, neutrophils and fibroblasts (Brundage et al., 1991; Mandeville and Maxfield, 1997; Hahn et al., 1992). We

demonstrated also that T cell migration was completely abrogated when cytosolic Ca^{2+} was chelated by BAPTA-AM indicating that Ca^{2+} itself is necessary for the migration of T cells. Although intracellular Ca^{2+}-levels seems to be essential for cytoskeletal rearrangements, via the activity of Ca^{2+} dependent actin binding proteins such as gelsolin (Arora and McCulloch, 1996), α-actinin (Bhatt et al., 2002) and myosin II (Eddy et al., 2000), the requirement of intracellular Ca^{2+} mobilization in CXCR3-controlled migration might be typical for T cells. Previous work showed that CXCR3-mediated migration of malignant B cells (Trentin et al., 1999) or transfected B cells (Jenh et al., 2001) occurs without eliciting a measurable calcium flux.

Interestingly, we observed that actin polymerization was independent of CXCL11-induced $[Ca^{2+}]_i$. In contrast, the presence of cytosolic Ca^{2+} was essential for actin polymerization. After depletion of free intracellular Ca^{2+} with BAPTA-AM F-actin levels were markedly lowered in non-stimulated cells, indicating that the intracellular $[Ca^{2+}]$ plays a role in the maintenance of the cytoskeleton. CXCL11-induced actin polymerization response was strongly decreased in BAPTA-AM treated cells, however not completely abolished. Calcium-dependence of the actin cytoskeletal organization was further demonstrated by the increased levels of F-actin after treatment of T cells with thapsigargin. Conversely, in Ca^{2+}-free medium basal F-actin levels were increased in non-stimulated cells and agonist-induced actin polymerization was enhanced. Thus, although Ca^{2+} is necessary for the maintenance and reorganization of the actin cytoskeleton, these data suggest that the $[Ca^{2+}]_i$ is not involved in the initiation of CXCR3-mediated actin polymerization. In conclusion, CXCL11-induced T cell migration, but not actin polymerization was dependent on $[Ca^{2+}]_i$. Importantly, Ca^{2+} release from intracellular stores is sufficient for these responses, rendering Ca^{2+} entry from the extracellular environment via SOC channels superfluous (See also figure 6).

Agonist binding to CXCR3 results in the phosphorylation of Akt which can be partially prevented by inhibition of PI3K (Smit et al., 2003). We here demonstrate that phosphorylation of Akt is dependent on intracellular Ca^{2+}. CXCL11-induced Akt phosphorylation was decreased when intracellular calcium was chelated with BAPTA-AM. Absence of extracellular Ca^{2+} did not affect Akt phosphorylation, indicating that intracellular Ca^{2+} was sufficient for an optimal response. On the contrary, induction of $[Ca^{2+}]_i$ in T cells with thapsigargin had no effect on Akt phosphorylation. One possible effect of Ca^{2+} after chemokine signaling may be the regulation of PI3K activity, however, this property of PI3K has not been described before. Agonist-induced receptor activation may also activate Ca^{2+} dependent kinases that can activate Akt [e. g. Ca^{2+}-calmodulin dependent kinase (CaMK) (Yano et al., 1998) and CaMK kinase (CaMKK) (Soderling, 1999)]. For example, in human neutrophils

inhibition of calmodulin attenuated fMLP-induced Akt phosphorylation (Verploegen et al., 2002). We tested the effect of the calmodulin inhibitor W7 on the migration and actin polymerization of human activated T cells. However, in our hands this compound was toxic for T cells at active concentrations (50 mM) (Verploegen et al., 2002; Zahalka et al., 2003). Taken together, our results suggest that CXCL11-induced phosphorylation of Akt occurs via both a PI3K-dependent pathway and a Ca^{2+}-dependent pathway (figure 6).

Phosphorylation of p44/p42 is known to be induced by increase in intracellular [Ca^{2+}]. Both thapsigargin and the Ca^{2+}-ionophore ionomycin are often used as a positive control for p44/42 phosphorylation (Anborgh et al., 1999). Indeed, thapsigargin induced the phosphorylation of this kinase in human primary T cells. Chelation of extracellular Ca^{2+}, but not of intracellular Ca^{2+}, completely abolished the response. These results suggest that for thapsigargin-induced activation of p44/42 the influx of Ca^{2+} across the plasma membrane, is crucial. In BAPTA-AM loaded cells the Ca^{2+} influx might still be activated by the processes induced by the Ca^{2+} release from intracellular stores.

In contrast to thapsigargin-induced phosphorylation, activation of p44/p42 by agonist binding to CXCR3 was almost completely independent of both intracellular and extracellular Ca^{2+}. The same phenomenon was found by Fierro et al. for TCR-crosslinking in T cells (Fierro et al., 2004). The discrepancy between Ca^{2+} dependent phosphorylation of p44/p42 by thapsigargin and Ca^{2+}-independent phosphorylation of the same kinases by CXCL11 may be explained by the multiple ways in which the Raf/Mek/p44/p42 pathway can be activated (Chong et al., 2003). Many different kinases can be activated in T cells following receptor triggering, some of which are dependent on Ca^{2+} [such as PKCβ (Carnevale and Cathcart, 2003), Pyk2 (Di, V et al., 2004; Rodriguez-Fernandez et al., 2002), CAMK1 (Schmitt et al., 2004)], while others are independent of Ca^{2+} [such as PKCδ (Shah and Catt, 2002), Ras (Jimenez-Sainz et al., 2003; Olson and Marais, 2000), Src pathway (Korade-Mirnics and Corey, 2000), PI3K (Sasaki et al., 2000)]. The dominant mechanism of MAP kinase activation varies significantly between receptor and cell type. Moreover, the mechanism of MAP kinase activation has a substantial impact on MAP kinase function and its location of action (Luttrell, 2003; DeFea et al., 2000). Thus, CXCL11 may trigger signal transduction routes that activate kinases, such as p44/p42, in a way that is specific for receptor and cell type, resulting in the desired effects, such as, in the case of CXCR3, cytokine production and proliferation (Luttrell, 2003; DeFea et al., 2000; Whiting et al., 2004). Which pathways determine the CXCL11-induced phosphorylation of p44/p42 and the functions of p44/p42 in CXCR3-stimulated cells are as yet unclear (figure 6).

Recent studies have pointed towards a role of receptor internalization and β-arrestin in MAP kinase activation. Several reports point to the requirement of endocytosis for the activation of MAPK, such as p44/p42 (Della Rocca et al., 1999; Daaka et al., 1998; DeFea et al., 2000; Kranenburg et al., 1999), while others contradict this (Blaukat et al., 1999; Schramm and Limbird, 1999; Budd et al., 1999). We show that in activated human T cells CXCR3 internalization is not essential for MEK1/2 activity since disruption of the actin cytoskeleton had no effect on the phosphorylation of p44/p42 in activated human T cells following stimulation with CXCL11, known to internalize CXCR3 (Sauty et al., 2001). This means either that complex formation of the receptor with β-arrestin is sufficient to activate MEK1/2-p44/p42 or that these kinases are activated through an β-arrestin independent alternative route, which is independent of internalization.

In monocytes, migration towards CCL2 (MCP-1) was dependent on the Ca^{2+}-dependent PKCβ (Carnevale and Cathcart, 2003). Similarly, CXCR4-mediated migration was prevented by inhibition of PKC (Wang et al., 2000). As CXCR3-mediated actin polymerization was PLC dependent (Smit et al., 2003) but independent of CXCR3-mediated $[Ca^{2+}]_i$, we studied the involvement of PKC in CXCR3–mediated actin polymerization and T cell migration. We show here that CXCR3-mediated actin polymerization and migration are independent of PKC. In conclusion, CXCR3-mediated actin polymerization and T cell migration both are independent of PKC.

In conclusion, CXCL11-induced $[Ca^{2+}]_i$ occurred in CHO cells transfected with human CXCR3 via store operated calcium influx. However, biological responses following CXCL11-stimulation of human peripheral T cells, such as actin polymerization, chemotaxis and phosphorylation of downstream kinases, were independent of influx of extracellular Ca^{2+}. Importantly, T cell migration was completely dependent on the chemokine-induced release of calcium from the intracellular store. In addition, actin polymerization and chemotaxis were severely diminished or abolished in the complete absence of intracellular Ca^{2+}. Also phosphorylation of the downstream kinase Akt, but not p44/p42, was decreased after chelation of intracellular Ca^{2+}.

Furthermore, we showed that CXCR3 signaling is distinct from other chemokine receptors: CXCR3-mediated Ca^{2+} mobilization in CHO cells, in contrast to other chemokine receptors, was independent of PI3K and not affected by disruption of the cytoskeleton. In addition, PKC was not involved in CXCR3-mediated calcium mobilization, actin polymerization and migration of human primary T cells. Also CXCL11-induced p44/p42 phosphorylation was not mediated via receptor internalization. Taken together, our results demonstrate that the repertoire of signal transduction proteins and

mechanisms involved in chemokine receptor signaling is highly dependent on the type of receptor and the cellular context.

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Chapter 6:

3-Phenyl-3H-quinazolin-4-one derivatives as CXCR3 antagonists

Pauline Verdijk[1], Stefania Storelli[2], Dennis Verzijl[2], Iwan J. P. De Esch[2], Rob Leurs[2], Martine J. Smit[2], and Cornelis P. Tensen[1]

[1]Department of Dermatology, LUMC, Leiden
[2]Leiden/Amsterdam Center for Drug Research (LACDR), Division of Medicinal Chemistry, Department of Pharmacochemistry, Vrije Universiteit, Amsterdam

Adapted from one submitted manuscript and one manuscript in preparation

Abstract

CXCR3-ligands CXCL9 (monokine induced by IFN-γ; MIG), CXCL10 (IFN-γ-inducible protein 10; IP10) and CXCL11 (IFN-γ-inducible T cell α-chemoattractant; ITAC) are inducible cytokines and are produced in response to exposure to interferon-γ (IFN-γ). Infiltration of $CXCR3^+$ cells and increased expression of CXCR3-ligands have been demonstrated in a number of type 1 inflammatory diseases, including rheumatoid arthritis, multiple sclerosis, hepatitis C–infected liver, artherosclerosis, delayed-type hypersensitivity skin reactions, and chronic skin inflammations. Its association with a wide variety of diseases makes CXCR3 an attractive target for therapeutic applications. Two small molecule CXCR3 antagonists containing a 3-phenyl-3H-quinazolin-4-one nucleus, have been reported (Schall et al., 2001). To attain antagonists with increased affinity for CXCR3, analogues were synthesized based on this scaffold, and tested in radioligand binding and functional assays. Of all analogues tested VUF5834 had the highest binding affinity for CXCR3. In functional assays with CXCR3-expressing cells, VUF5834 efficiently inhibited CXCR3 induced phospholipase C activity and calcium mobilization. Moreover, in CXCR3-expressing human primary T cells VUF5834 completely abrogated CXCR3-mediated actin polymerization and chemotaxis, while migration towards CXCR4-ligand CXCL12 was not affected. Thus, the small molecule VUF5834 specifically antagonizes CXCR3-mediated responses via interaction with the receptor. VUF5834 may serve as a lead to obtain a compound with improved affinity and potency for therapeutic applications and exploration of the role of $CXCR3^+$ T cells in type 1 inflammatory disorders.

Introduction

Chemokines are small proteins involved in the recruitment and homing of leukocytes. Chemokines are produced by tissue cells and leukocytes and are subdivided into four classes based on the relative position of conserved cysteine residues: CC, CXC, CXXXC, and XCL (Murphy et al., 2000). They act through corresponding groups of CCR, CXCR, CX3CR and XCR receptors that belong to the seven-transmembrane, G protein-coupled receptor family. The chemotactic function of chemokines can be classified into two categories: constitutive and inducible. Constitutive chemokines are involved in the development of lymphatic tissues, basal leukocyte trafficking and immune surveillance. Inducible chemokines are produced upon infection or other stress signals and regulate the recruitment of leukocytes to sites of inflammation (Zlotnik et al., 1999). For example exposure of tissue cells to interferon γ (IFN-γ) induced the production of the CXCR3-ligands CXCL9 (monokine induced by IFN-γ; MIG), CXCL10 (IFN-γ-inducible protein 10; IP10) and

CXCL11 (IP-9/IFN-γ-inducible T cell α-chemoattractant; ITAC). Secretion of these chemokines then results in the recruitment CXCR3-expressing leukocytes to the site of inflammation (Kupper and Fuhlbrigge, 2004). CXCR3 is expressed mainly on activated T cells (both $CD4^+$ and $CD8^+$ T cells), but can also be found on B cell plasmablasts and NK cells (Inngjerdingen et al., 2001; Loetscher et al., 1998). Increased expression of CXCR3 on $CD4^+$ T cells is associated with a Th1 phenotype (Kim et al., 2001). Infiltration of $CXCR3^+$ cells and increased expression of CXCR3-ligands have been demonstrated in a number of type 1 inflammatory diseases, including rheumatoid arthritis (Qin et al., 1998; Patel et al., 2001), multiple sclerosis (Balashov et al., 1999), hepatitis C–infected liver (Shields et al., 1999), artherosclerosis (Mach et al., 1999), delayed-type hypersensitivity skin reactions, and chronic skin inflammations (Tensen et al., 1999; Flier et al., 1999; Flier et al., 2001; Tokuriki et al., 2002). Moreover, infiltration of $CXCR3^+$ cells is associated with graft rejection (Hancock et al., 2001). The association of CXCR3 with a wide range of immune disorders makes it an attractive target for therapeutic applications.

So far only two small molecule CXCR3 antagonists containing a 3-phenyl-3H-quinazolin-4-one nucleus, have been reported (Schall et al., 2001). Based on this scaffold, analogues were synthesized and tested in radioligand binding and functional assays. Of all analogues, VUF5834 had the highest affinity for CXCR3. Preincubation with VUF5834 efficiently abrogated CXCR3-mediated PLC activation and Ca^{2+} mobilization in CXCR3 expressing COS-7-cells and blocked CXCR3-mediated actin polymerization and chemotaxis in human activated T cells in a dose-dependent manner. This novel small molecule CXCR3-antagonist may serve as an important tool to further unravel the role of CXCR3 in autoimmune and inflammatory diseases, and may serve as a lead to the development of therapeutic agents.

Materials and Methods

Cells and reagents

COS-7 cells transfected with the human CXCR3 (Smit et al., 2003) and HEK293 cells stably expressing CXCR3 (Dijkstra et al., 2004) were cultured as described previously. Human primary activated T cells (> 95% CXCR3+) were isolated and cultures as described by Verdijk et al. 2004 (Chapter 5). 3-Phenyl-3H-quinazolin-4-one derivatives, analogues of the small molecular antagonist for CXCR3 reported by Schall et al. (WO 01/16144 A2), were synthesized by the Leiden/Amsterdam Center for Drug Research (LACDR). ^{125}I-labeled CXCL1O (2200 Ci/mmol) was obtained from Perkin-Elmer Life Sciences (Boston, Mass.). CXCL10 was obtained from PeproTech (Rocky Hill, N.J.). CXCL11 was purchased from R&D Systems, Inc. (Minneapolis, Minn.). Poly-L-Lysine was from Sigma (St. Louis, Mo.).

Radioligand binding assay

In competition experiments, HEK293-CXCR3 cells were seeded in poly-L-Lysine-coated 48-well plates. After 24 hours the cells were incubated with ^{125}I-labeled CXCL10 in binding buffer (50 mM HEPES [pH 7.4], 1 mM CaCl2, 5 mM MgCl2, 0.5% BSA) containing increasing concentrations of the indicated compounds for 4 h at 4°C. After incubation, cells were washed three times with ice-cold binding buffer supplemented with 0.5 M NaCl. Subsequently, cells were lysed and counted in a Wallac Compugamma counter.

[^{3}H]Inositol phosphate production

COS-7 cells were transfected with CXCR3 and Gqi5 chimeric protein (Coward et al., 1999). 24 h after transfection cells were labeled overnight in inositol-free medium (modified Eagle's medium with Earle's salts) supplemented with 2 mM L-glutamine, L-cysteine, L-leucine, L-methionine, L-arginine, glucose, 0.2% bovine serum albumin, and 2 μCi/ml *myo*-[2-^{3}H]inositol (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). Subsequently, the labeling medium was aspirated, cells were washed for 10 min with Dulbecco's modified Eagle's medium containing 25 mM HEPES (pH 7.4) and 20 mM LiCl. Cells were preincubated for 10 minutes with/or without 3-Phenyl-3H-quinazolin-4-one derivatives and incubated for 2 h in the same medium in the absence or presence of 10 nM CXCL11 or 30 nM CXCL10. The incubation was stopped by aspiration of the medium and addition of cold 10 mM formic acid. After 90 min of incubation on ice, inositol phosphates were isolated by anion exchange

chromatography (Dowex AG1-X8 columns, Bio-Rad) and counted by liquid scintillation.

Ca^{2+} measurements

CXCL11-stimulated increases in $[Ca^{2+}]_i$ were quantified by monitoring the fluorescence of Fluo-4 AM-loaded HEK293-CXCR3 cells, using an automated NOVOstar microplate reader (BMG Labtech GmbH, Offenburg, Germany). Cells were loaded in Hanks' balanced salt solution containing 20 mM HEPES, 2.5 mM Probenecid, 2 µM Fluo-4 AM and 0.02 % Pluronic F-127, pH = 7.4. Cells were washed three times and fluorescence was measured (1 data point/second, excitation 485 nm, emission 520 nm) for 10 seconds to calculate the mean basal value. Cells were preincubated for 10 min with the indicated compounds (1 µM). After injection of the agonist CXCL11 (30 nM) fluorescence was recorded for 50 seconds. Changes induced by Triton X-100 (0.25% [v/v]) injection were recorded for further 10 seconds to determine the maximal fluorescence of the system. Basal and maximal values determined for each well were used to normalize the data. Results were expressed as percent age of the maximal stimulation induced by Triton X-100.

Actin polymerization assay

Cells were resuspended in RPMI-1640 with 0.25% BSA [BSA, fraction V, Sigma]) in a concentration of 4×10^6 cells/ml. 3-Phenyl-3H-quinazolin-4-one derivatives were added 10 minutes prior to the stimulation. Chemokines was added in a concentration of 10 nM. At indicated time points, 25 ml of cell suspension was transferred to 25 ml of fixation solution (4 % paraformaldehyde; PFA). Cells were fixed for at least 15 minutes. Thereafter, cells were washed and resuspended in 50 ml of permeabilization reagent (0.1% Triton-X 100). After 10 minutes, cells were washed, blocked with 1% BSA (5 min), washed and incubated with 0.5 mM rhodamine-phalloidin (Molecular Probes, Leiden, The Netherlands) to visualize filamentous actin (F-actin). After 45 minutes, cells were centrifuged and resuspended in PBS with 0.25% BSA. Mean fluorescence intensity was measured by FACS analysis.

Chemotaxis assays

The assay for chemotaxis was performed in 24 well plates (Costar, Cambridge, MA) carrying Transwell permeable supports with 5 µm pore size polycarbonate membrane. T cells were cultured overnight at 4×10^6 cells/ml in RPMI-1640 containing 0.25% BSA. Cells were incubated with 3-Phenyl-3H-quinazolin-4-one derivatives for 10 minutes. Medium alone or supplemented with

chemokine and/or 3-Phenyl-3H-quinazolin-4-one derivatives was placed in the lower compartment, and cells were loaded onto the inserts at 0.4×10^6/100 µl. Chambers were incubated for 120 minutes in a 5% CO2, humidified incubator at 37°C. After the incubation period, numbers of T cells migrating to the lower chamber were counted under a microscope using a hemocytometer. Viability was checked with trypan blue exclusion. All conditions were tested in at least in triplicate; statistic evaluation was performed using the Student's t-test.

Results and discussion

VUF5834 binds with high affinity to CXCR3

To attain a CXCR3 antagonist with increased affinity, various 3-Phenyl-3H-quinazolin-4-one derivatives, analogues of the small molecular antagonist for CXCR3 reported by Schall et al. (WO 01/16144 A2; compound 1c, figure 1A) were synthesized and there affinity for CXCR3 and ability to inhibit CXCR3-mediated signaling were determined. Variations were introduced in the adduct to the phenyl ring (X) and by removing or replacing the decanoyl chain with other groups (R) (Table 1). Radioligand binding assays using HEK293-cells heterologously expressing the human CXCR3 revealed that the decanoyl group was essential for binding to CXCR3. Removal or substitution of the decanoyl group resulted in a total loss of activity (Table 1). Varying the position and/or the nature of the phenyl ring adduct altered the affinity of the compound to the receptor (Table 1). Most effective was the replacement of the F at position 4 (4-F) of the lead compound by a cyano group (4-CN). Compound 1b (VUF5834: X = 4-CN, R = decanoyl, figure 1B) possessed the highest affinity for CXCR3 in the radioligand binding assay (pIC_{50}: 6.04 $\pm$ 0.09) and was selected for further research.

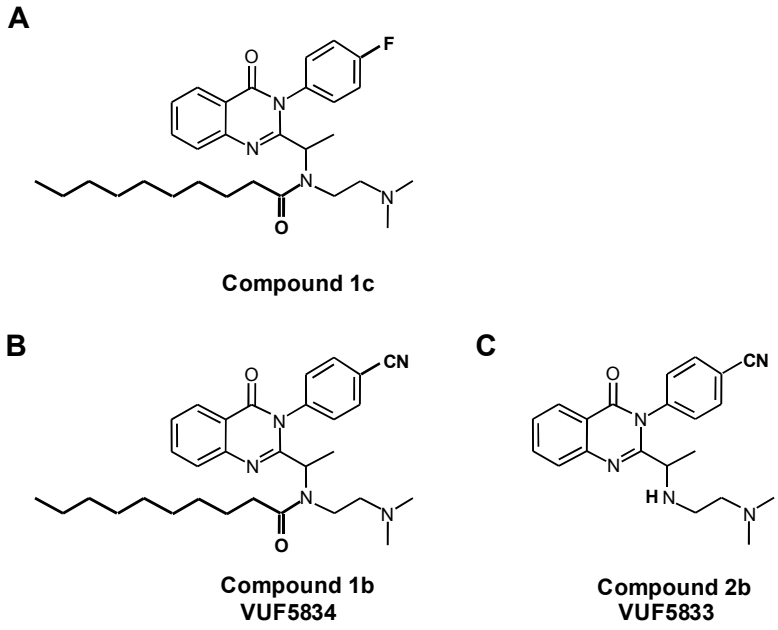
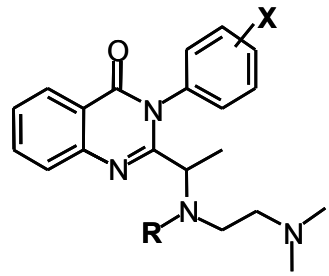


Figure 1: Molecular structure of **A**. the lead compound (Schall et al.; compound 1c), **B**. compound 1b (VUF5834) and **C.** compound 2b (VUF5833).



Compound	R	X	pIC_{50}	
1a	decanoyl	H	4.85 ± 0.15	
1b	decanoyl	4-CN	6.04 ± 0.09	➔ VUF5834
1c	decanoyl	4-F	5.51 ± 0.13	➔ Schall *et al.*
1d	decanoyl	4-OCH_3	5.51. ± 0.15	
1e	decanoyl	4-OH	3.94 ± 0.27	
1f	decanoyl	4-CH_3	5.54 ± 0.15	
1g	decanoyl	4-CF_3	< 4	
1h	decanoyl	3-CN	5.24 ± 0.05	
1i	decanoyl	2-CN	4.99 ± 0.08	
1j	cyclohexanoyl	4-CN	< 4	
1k	benzoyl	4-CN	< 4	
1l	naphtoyl	4-F	< 4	
2a	H	H	< 4	
2b	H	4-CN	< 4	➔ VUF5833

Table 1: Binding affinity of the different modifications of the reported scaffold. The binding experiments were carried out on HEK293 cells expressing the human CXCR3 receptor. The compounds were tested for their ability to displace [^{125}I]CXCL10. Compound **1c** represents the lead compound described by Schall et al. Compounds **1b** and **2b** were selected for follow up in biological assays.

VUF5834 abrogates CXCL11-induced Ca^{2+} mobilization and PI turnover in CXCR3-expressing COS-7 cells

The effect of VUF5834 on CXCL11- and CXCL10-mediated processes was studied in different signaling assays in human CXCR3 expressing cells and its effects were compared to a non-binding analogue (compound 2b/VUF5833: X = 4-CN, R = H, figure 1B). Agonist binding to CXCR3 results in the activation of PLC via G_i (Smit et al., 2003). The effect of VUF5834 on CXCL11-induced PLC activity was determined by measuring the production of IPx in COS-7-CXCR3 cells. A 10 minute preincubation of COS-7-CXCR3 with VUF5834 inhibited CXCR3–mediated PI turnover in a dose-dependent manner (IC_{50} [10 nM CXCL11]: 6 uM), while the non-binding analogue had no effect (figure 2A-B). IP_3, one of the products of PLC activity, induces an increase in intracellular Ca^{2+} concentrations, by mobilizing Ca^{2+} from intracellular stores and activating the influx of Ca^{2+} from the extracellular environment (Loetscher et al., 1996; Tensen et al., 1999; Rabin et al., 1999) and Verdijk et al. 2004, Chapter 5). Incubation of HEK293-CXCR3 cells with 1 μM of VUF5834, 10 minutes prior to chemokine stimulation completely abrogated the CXCR3-induced Ca^{2+} increase, while the non-binding analogue had no effect (figure 2C). These results demonstrate that VUF5834 efficiently blocks the CXCL11-induced PI turnover and subsequent Ca^{2+} mobilization interaction with CXCR3.

VUF5834 inhibits CXCR3-mediated actin polymerization and migration of human primary T cells

Agonist binding to CXCR3 on human T cells induces a rapid and transient increase in filamentous actin (F-actin) and chemotaxis towards the chemotactic gradient (Tensen et al., 1999; Smit et al., 2003). The fast and transient increase in F-actin content is one of the earliest measurable biological responses following binding of chemoattractants to their receptor and is considered as an early sign of lymphocyte migration (Voermans et al., 2001; Tenscher et al., 1997; Elsner et al., 1998). We investigated the effect of VUF5834 on both CXCR3-induced actin polymerization and migration of human activated T cells. Human peripheral blood T cells were isolated from healthy donors, activated with PHA and expanded in the presence of recombinant human IL2 as described previously, resulting in >95% $CXCR3^+/CD3^+$ cells (Tensen et al., 1999; Loetscher et al., 1996; Qin et al., 1998). CXCR3-induced increase in actin filaments was completely abrogated in the presence of 1 μM of VUF5834 (figure 4). Its non-binding analogue counterpart VUF5833 had no effect on CXCR3-agonist induced actin polymerization, indicating that inhibition was dependent on receptor binding.

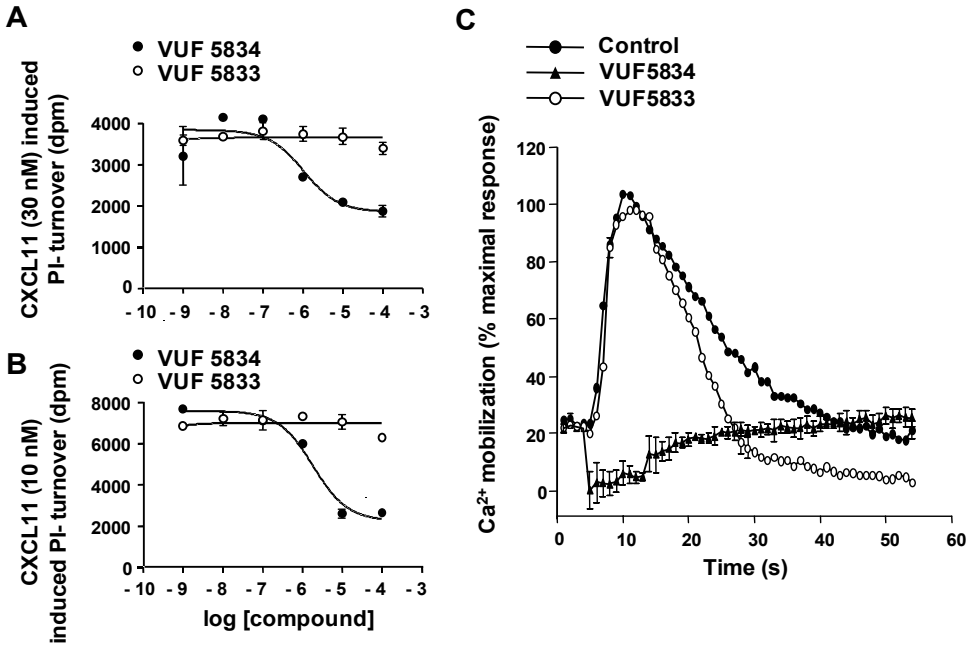


Figure 2: VUF5834 abrogates CXCR3-mediated IP3 production and [Ca^{2+}]i. A-B. Effect of VUF5834 and its non-binding analogue VUF5833 on CXCR3-mediated PLC activity by CXCL10 (A) and CXCL11 (B). PLC activity was measured by the production of [^{3}H]IPx from radioactive labeled inositol in COS-7-CXCR3. **C**. Ca^{2+} mobilization in response to 30 nM CXCL11 in HEK283-CXCR3 in the presence or absence of 1 μM of VUF5834 or the non-binding analogue VUF5833. Error bars show standard error of the mean.

We then investigated the effect of both compounds on CXCR3-mediated chemotaxis. Migration of activated T cells towards CXCR3-ligands CXCL11 and CXCL10 was dose-dependently inhibited by preincubation with VUF5834, but not with VUF5833 (figure 3 B, C). VUF5834 inhibited T cell migration induced by 30 nM CXCL11 with an IC_{50} of 0.5 μM. Viability of the cells in the presence of VUF5834 was determined by trypan blue exclusion and was not affected by concentrations up to 1 μM.

To confirm that the antagonistic action of VUF5834 on CXCR3-mediated processes occurred via binding to CXCR3, we studied its effect on T cell-migration towards CXCR4-ligand CXCL12 (formerly known as SDF-1a; 30 nM). In human peripheral blood the percentage of $CXCR4^+$ T cells is approximately 40%, which is increased to up to 80% upon culturing with PHA or IL2 (Bleul et al., 1997). VUF5834 did not affect the migration of activated T cells to CXCR4-agonist CXCL12 (figure 3C), indicating that VUF5834 acts specifically on CXCR3-mediated migration. Basal migration in the absence of chemoattractants was not changed by VUF5834, demonstrating that motility of the cells was not affected.

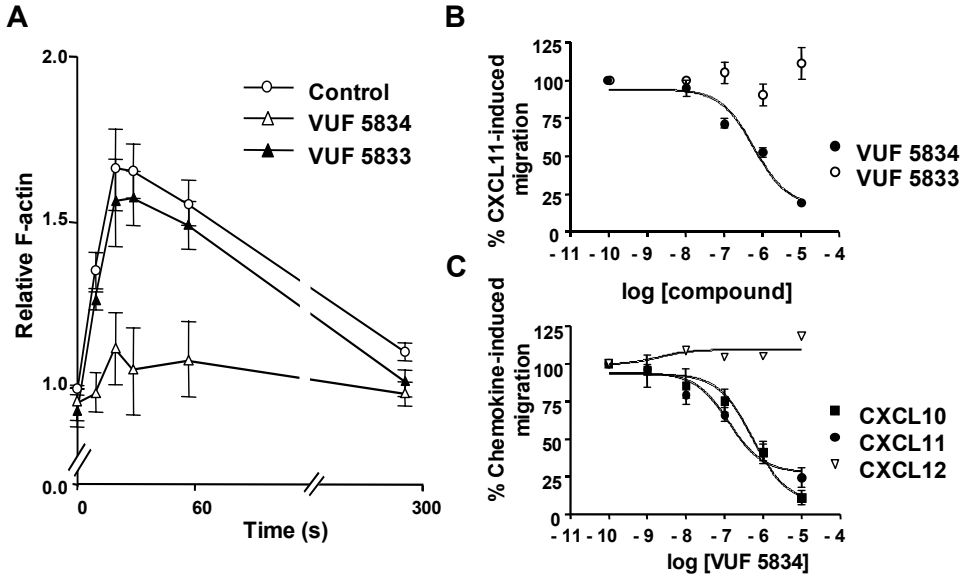


Figure 3: VUF5834 inhibits CXCR3-mediated actin polymerization and chemotaxis of human T cells. Human T cells were isolated form peripheral blood and activated with PHA and IL-2. **A.** Actin polymerization in response to CXCL11 was measured in the presence or absence of 1 μM of VUF5834 or its VUF5833. Results are representative for 3 donors. Error bars show the standard error of the mean. **B-C.** Chemotaxis of activated T cells towards CXCR3-ligands. **B.** Dose-response effect of VUF5834 and VUF5833 on chemotaxis of activated T cells induced by 30 nM of CXCL11. **C.** Dose-response effect of VUF5834 on CXCL11-, CXCL10- and CXCL12-induced chemotaxis (all 30 nM) of activated T cells. Results are representative for 3 donors. Error bars show the standard deviation.

Conclusion

In conclusion, binding of 3-Phenyl-3H-quinazolin-4-one derivatives to CXCR3 is determined by the decanoyl-group and the adduct on position 4 on the phenyl-ring. VUF5834 (X=4-CN, R= decanoyl) bound to CXCR3 with the highest affinity. Binding of VUF5834 to CXCR3 on COS-7-cells expressing the human CXCR3 inhibited activation of PLC and the mobilization of Ca^{2+}. Moreover, 1 μM of VUF5834 abrogated both CXCR3-mediated actin polymerization and chemotaxis. At this concentration no signs of toxicity were detected. Inhibition of CXCR3-signaling was dependent on receptor-binding, which was demonstrated by the lack of inhibition by the non-binding analogue VUF5833. The antagonistic effect of VUF5834 was specific for CXCR3, as CXCR4-mediated chemotaxis was not affected. All together VUF5834 may serve as a lead to obtain a compound with improved affinity and potency for therapeutic applications and exploration of the role of $CXCR3^+$ T cells in type 1 inflammatory disorders.

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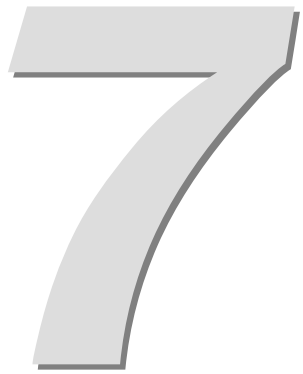
Chapter 7:



Summary and general discussion

Summary and general discussion

The immune system is a complex and well-organized organ. Knowledge of its function is crucial, not only for the treatment of infectious diseases and allergies, but also for many other disorders, such as autoimmunity and cancer, and for organ transplantation. In the skin, excessive infiltration of leukocytes are found in many inflammatory diseases. Two major players involved in acquired immune responses are the dendritic cell and the T cell. This thesis will focus on two aspects of this system; the maturation of DC and its antigen-presenting function and the trafficking of T cells, which can be considered as crucial steps in directing the acquired immune system.

The actin binding protein cofilin is dephosphorylated during DC maturation

The principal task of dendritic cells is to specifically direct T cells that are reactive with the antigens the DC has encountered and presents on its cell surface. In this process, the maturation state of the DC is of critical importance for T cell activation as it determines the outcome of the interaction between the DC with the T cell: activation or tolerance. The aim of the study described in **chapter 2** was to identify novel proteins involved in DC maturation that may have a role in T cell activation.

To accomplish this, total protein expression profiles of immature and mature DC were compared by the combined use of two-dimensional gel-electrophoresis (2DE) and mass spectrometry (MS). Although both 2DE and MS are no new techniques, recent advances in the technology and software make it an attractive and promising methodology for the identification of differentially expressed proteins. 2DE is based in the separation of proteins first on their iso-electric point and then on their molecular weight. Commercially available gel strips used for the first dimension make 2DE less time-consuming and more reproducible. Furthermore, the resolution and sensitivity of the latest generation of mass-spectrometers has greatly increased. New software programs and the availability of advanced protein databases on the internet have facilitate the analysis of the results. The value of 2DE lies in the detection of differences in both protein expression levels and protein modifications. Protein expression profiles are more informative than mRNA expression profiles, as mRNA levels not always reflect protein levels. Moreover, many processes in the cell are not regulated by protein expression levels, but by modulation of the protein activity. The bulk of protein modifications result either in a shift in mass or iso-electric point, or both and can be visualized by 2DE and identified with MS.

The proteome of a cell is very complex; the number of proteins expressed at the same time in a given cell type is estimated at 6000, which in addition can exist in different forms as a result of post-translational processing and chemical modifications. By 2DE only a proportion of proteins from total cell lysates can be identified. As a result of the low solubility of most proteins greater than approximately 100 kDa, the failure to resolve and separate most membrane proteins and the resolution of the gel (Mann et al., 2001; Hatzimanikatis et al., 1999), mainly soluble and high abundant, low molecular weight proteins will be detected with this technique. Prefractioning of the samples or the use of multiple first dimension gel strips with distinct, narrow pH ranges may increase the yield of this technique.

In spite of these shortcomings, the differential modification of an actin-binding protein during (cofilin) DC maturation was identified by proteomic profiling using 2DE and MS, and is described in **chapter 2.** Cofilin is a small protein of 18 kD, which is inactive when phosphorylated on its serine on position 3 and is involved in cytoskeletal rearrangements. During maturation the protein spot identified as phosphorylated cofilin by MS, was strongly reduced. Western blot analysis confirmed a gradual loss of cofilin phosphorylation during LPS-induced maturation. In addition, cofilin was translocated towards the plasma membrane, where the actin cytoskeleton is located. Cofilin dephosphorylation correlated with an increase in filamentous actin (F-actin) and the development of veils. These data suggest involvement of cofilin in the formation of veils that are characteristic for mature DC.

Activation of cofilin results in an increased turnover of F-actin, thereby enhancing cytoskeletal rearrangements and increasing cell motility (Lappalainen and Drubin, 1997; Moon and Drubin, 1995; Chen et al., 2000). Furthermore, through its severing capacity it can promote actin polymerization by increasing the number of filament ends (Ichetovkin et al., 2002). In addition, cofilin has been demonstrated to promote actin-bundling (Pfannstiel et al., 2001). Cofilin is most likely not the only player involved in the cytoskeletal rearrangements leading to the appearance of veils on mature DC. Expression of the actin-bundling protein Fascin has been demonstrated in mature, but not immature DC. Also the ARP2/3 complex may be important for the dendritic appearance of DC, through its capacity to initiate and nucleate new actin filaments, as deficiency in Wiscott-Aldrich syndrome protein (WASp), the activator of Arp2/3, prevents the formation of podosomes in DC (Binks et al., 1998; Burns et al., 2001). Moreover, mRNA levels encoding a cross-linker of F-actin, MARCK, were elevated early during DC maturation. Also the microtubular network might be involved, as mRNA levels for β-tubulin and dynein light chain were up-regulated (Huang et al., 2001). Other proteins that may be regulated during maturation are anchoring (ezrin/radixin/moesin,

α-actinin), actin-bundling (α-actinin) and severing (gelsolin) proteins (Otey and Carpen, 2004; Takubo et al., 1999; Barreiro et al., 2002; Chou et al., 2002).

The formation of veils by DC may be of great importance for its antigen-presenting function. First, the enlarged cell surface enables the expression of high levels of peptide-MHC complexes and costimulatory molecules. Second, the chance to encounter and interact with antigen-specific T cells is increased. And most importantly, when DC-T cells contact is established it may increase the contact area between DC and T cells and thereby strengthen the activation signal that is delivered to the T cell. In other leukocytes, cofilin has been demonstrated to be involved in migration (Matsui et al., 2001; Konakahara et al., 2004; Nishita et al., 2002) and phagocytosis (Matsui et al., 2002). It remains to be established whether cofilin plays a similar role in migration and endocytosis of dendritic cells.

A mutation in the carbohydrate recognition domain of Langerin protein prevents Langerin-induced formation of Birbeck granules

The Langerhans cell (LC) is the dendritic cell of the epidermis and distinguishes itself from dermal and other DC by the expression of Birbeck granules. The type II C-type lectin Langerin, which is uniquely expressed in LC, has been shown to induce the formation of Birbeck granules when heterologously expressed in murine fibroblasts (Valladeau et al., 2000). The function of Birbeck granules is still poorly understood, but many data now suggest a role for Langerin in the processing and presentation of exogenous antigens. Understanding their ontogenisis may give clues for the function of these organelles. In 1994 the department of Dermatology in Leiden, the Netherlands, encountered a healthy individual (E. N.) whose LC completely lacked Birbeck granules (Mommaas et al., 1994). The aim of the work described in **chapter 3** was to identify possible genetic defects underlying the deficiency in Birbeck granules.

Sequence analysis the Langerin gene of E. N. revealed a mutation in exon 5 in one of the Langerin alleles. This mutation was located in the area encoding the carbohydrate recognition domain (CRD) of Langerin and resulted in the replacement of a tryptophane at position 264 for an arginine residue (W264R) in a region that is highly conserved among related type II C-type lectins and among different species. Heterologous expression of the W264R form in human fibroblasts did not result in the formation of Birbeck granules, in contrast to the wildtype Langerin. The mutant Langerin could not be detected by the anti-Langerin monoclonal antibody DCGM4. However, linking green fluorescent protein (GFP) to the N-terminus of Langerin enabled the

visualization of the protein in transfected cells with an anti-GFP antibody. Although no Birbeck granules were formed in GFP-W264R Langerin transfected cells, GFP-W264R Langerin was detected in irregular tubular membrane structures. Recently, a mutation in mouse Langerin cDNA resulting from a cloning artifact was described, which gave rise to the same phenotype when transfected in fibroblasts (Valladeau et al., 2002). Interestingly, this mutation was also located in the region encoding the CRD domain. Together, these data indicate an important role of the CRD of Langerin in the formation of Birbeck granules.

Anti-GFP staining of cells expressing GFP-wildtype Langerin revealed a regular repetitive pattern of GFP-Langerin along the Birbeck granules. In contrast, staining with anti-DCGM4 resulted in a much lower labeling of Birbeck granules, suggesting that the epitope for DCGM4 is engaged in the formation of the Birbeck granules and therefore not available for antibody binding. In cells transfected with GFP-W264R Langerin, staining with anti-GFP revealed the absence of a repetitive pattern of Langerin expression. Conversely, GFP-W264R Langerin transfected cells demonstrated a tubule-like structure highly positive for GFP, that was reminiscent of cored tubules (Hopkins et al., 1994; McDermott et al., 2002; Valladeau et al., 2000). The spatial organization of Langerin in the Birbeck granule implicates an active role for Langerin in the ontogenesis of the typical zipper like structure and suggests that Birbeck granules arise through the interaction of Langerin molecules with one another and/or ligands, possibly via its CRD. This was also proposed by Mc Dermott et al, who described the formation of Birbeck granules like structures at sites where Langerin accumulates. They hypothesize that a "minimal zipping concentration" of these molecules is necessary in combination with preexisting tubular structure where close membrane apposition exists, like in the endosomal recycling compartment (Geissmann et al., 2002). This hypothesis is further strengthened by the observation that Langerin can bind mammalian high-mannosylated (endogenous) glycoproteins as well as glycoconjugates of microorganisms (Stambach and Taylor, 2003). This indicated that either Langerin interacts with high mannose structures on opposite Langerin molecules or opposite Langerin molecules might interact with the same glycosylated ligand that is present in cored tubules or the endosomal recycling compartment. A mutation in the CRD likely prevents the binding of glycoconjugates and thereby the zipping that is typical for the Birbeck like structures.

The next question was whether this mutation was unique or that it occurred more frequently in the population. The identified mutation could be detected with a simple assay consisting of PCR amplification of a small fragment of the Langerin gene followed by digestion with a restriction enzyme, specific for the

mutated allele. Using this method, 3 additional persons in a total of 219 screened were detected. Like the index person, genomic DNA of all three encoded for both wildtype and W264R-Langerin (unpublished data). From one of these persons a skin biopsy was obtained and analyzed for the presence of Birbeck granules. However, in contrast to E. N., the Langerhans cells of this person (M) did contain Birbeck granules in normal numbers (unpublished data).

These data suggest that heterozygosity for W264R Langerin is not correlated with the absence of Birbeck granules per se. The phenotype (Birbeck granules or not) may be influenced by other factors, such as mRNA expression levels, the translation of mRNA into protein or protein degradation. The Langerhans cells of E. N. not only lacked Birbeck granules, but were also negative for anti-Langerin staining (Mommaas et al., 1994). It is thus unclear, whether one or both Langerin alleles are expressed on the protein level in the LC. Alternatively, the level of Langerin expression and/or the ratio of W264R- and wildtype Langerin expression may differ between both persons. If equal levels of mutant and wild type Langerin are expressed, the minimal zipping concentration of functional Langerin molecules, hypothesized by Mc Dermott et al. may not be reached (McDermott et al., 2002). However, if the total expression level of Langerin is increased or if wildtype Langerin expression exceeds the expression of the mutant, the minimal zipping concentration needed for the formation of Birbeck granules may still be reached and Birbeck granules may be formed despite the presence of the mutant Langerin protein in the cell.

The functional significance of the lack of Birbeck granules can be questioned, as the Birbeck granule deficient person was healthy and showed normal mixed lymphocyte reactions (Mommaas et al., 1994). Moreover, no function has been discovered for Birbeck granules as yet. For the function of Langerhans cells the expression of functional Langerin may be of greater importance than the presence of Birbeck granules itself. The zipper-like conformation of Birbeck granules may only be a 'side effect' of the accumulation of Langerin in tubular structures and have no specific function in itself. Langerin has been associated with the uptake of glycolipids and the presentation of these antigens via CD1a (Hunger et al., 2004) to T cells. This way, Langerin may play a crucial role in the directing of immune responses via non-peptide antigens derived from invading pathogens.

Involvement of different signal transduction pathways in CXCR3 signaling and their role in T cell chemotaxis

After activation, T cells have to find their way to the site of pathogen invasion

or tissue trauma. Locally produced chemokines specifically direct leukocytes to distinct tissues and to sites of inflammation. In type 1 inflammatory responses of the skin CXCR3-ligands are involved in the recruitment of activated T cells. Many inflammatory disorders in the skin and throughout the body are associated with an excessive production of CXCR3-ligands and a massive infiltration of $CXCR3^+$ T cells. Therefore, CXCR3 is an attractive target for the development of intervention therapies. For the evaluation and development of specific therapies intervening in CXCR3-mediated processes it is of importance to unravel the signal transduction pathways used by the receptor and their functional importance. The aim of the studies described in **chapter 4 and 5** was to elucidate the pathways involved in CXCR3-mediated signaling and their functional importance for T cell migration and actin polymerization.

Beig typical for most Gi protein-coupled receptors, chemokine receptor triggering leads to an increase in intracellular calcium concentrations ($[Ca^{2+}]_i$). **Chapter 5** demonstrates that $[Ca^{2+}]_i$ in CXCR3-transfected CHO cells consists of the mobilization of Ca^{2+} from intracellular stores and the influx of extracellular Ca^{2+}, the latter making up the major part of the calcium signal. Although essential for T cell migration, $[Ca^{2+}]_i$ was not was needed for actin polymerization in T cells. Remarkably, CXCR3-mediated actin polymerization and T cell migration occurred completely independent of the influx of calcium into the cells, meaning that intracellular Ca^{2+} mobilization suffices for T cell migration. In experiments in T cells in which intracellular Ca^{2+} is depleted both T cell migration and actin polymerization were dependent on the presence of intracellular calcium and appeared to be regulated in a Ca^{2+}-dependent manner, both in resting and in activated cells. In conclusion, CXCR3-induced T cell migration is independent of Ca^{2+} influx, but is dependent on Ca^{2+} concentrations in the cell and CXCR3-induced mobilization of intracellular Ca^{2+}.

Receptor-mediated Ca^{2+} influx in T cells occurs predominantly via so-called store-operated calcium channels (SOC), which are activated through depletion of Ca^{2+} from intracellular Ca^{2+} stores. In T cells the only known SOC channel is the calcium release activated calcium (CRAC) channel. Ca^{2+} entry through CRAC channels is essential for T-cell activation and activation associated gene expression (IL-2 and -5 and IFN-γ) and proliferation (Ishikawa et al., 2003; Zitt et al., 2004; Fanger et al., 1995; Fischer et al., 2001). Voltage dependent Ca^{2+} channels (VDCC) were reported to be involved in T cell activation and proliferation (Kotturi et al., 2003) and in CX3CL1-induced $[Ca^{2+}]_i$ in CHO cells expressing CX3CR1 (Kansra et al., 2001). In CXCR3-mediated T cell migration, however, calcium channels whether CRAC or VDCC have no apparent function. Nonetheless, it is still unclear whether influx of calcium is essential for chemokine-mediated activities other than migration, actin polymerization and kinase activation, such as integrin activation, gene transcription, or T cell

proliferation (van Kooyk et al., 1991; Cinamon et al., 2001a; Cinamon et al., 2001b; Whiting et al., 2004; Lauffenburger and Horwitz, 1996).

In both **chapter 4 and 5** the involvement of different kinases in CXCR3-induced signaling was investigated. All three ligands of CXCR3 (CXCL9-11) were capable of activating the PI3K/Akt and MEK/p44/42 pathway in a dose-dependent manner in both activated T cells and COS-7 cells expressing human CXCR3, CXCL11 being the most potent. All CXCR3-induced processes were mediated via Gαi. CXCL11-induced phosphorylation of p44/p42 did not need a functional cytoskeleton, suggesting that receptor internalization was not involved in activation of this kinase. Activation of p44/p42 by CXCR3 relied completely on MEK, partially on PI3K and not on Ca^{2+}. Also Akt was partially regulated by PI3K and was decreased in the absence of intracellular calcium. Both CXCR3-mediated T cell migration and actin polymerization depended on PLC-linked signal transduction pathways. Furthermore, in contrast to several other reports describing chemokine signaling, PI3K, MEK (**chapter 4**), and PKC (**chapter 5**) were not involved in CXCR3-induced chemotaxis.

Until recently, PI3K was thought to be indispensable for chemoattractant-induced migration (Bonacchi et al., 2001; Knall et al., 1997; Sotsios et al., 1999; Sullivan et al., 1999; Turner et al., 1998; Vicente-Manzanares et al., 1999; Wain et al., 2002; Wang et al., 2000). However, more recently it has become clear that PI3K dependence is not universal for all chemokine receptors. Several reports describe migration of leukocytes that is either completely (Cinamon et al., 2001b; Cronshaw et al., 2004; Heit et al., 2002; Scandella et al., 2002; Verploegen et al., 2002; Fine et al., 2001; Ward, 2004) or partially independent (Wain et al., 2002; Reif et al., 2004) of PI3K. Heit et al. suggested that signaling via PI3K depends on the receptor responding to either intermediary chemoattractants or end target chemoattractants. Neutrophil migration towards intermediary chemoattractants depended on PI3K/Akt activity, whereas end target chemoattractants induced migration occurred via p38. Moreover, 'end target' chemokines were dominant over 'intermediary' chemokines (Heit et al., 2002). The notion that CXCR3 mediated migration is independent of PI3K fits in this theory. However, CCR4-medated migration (Cronshaw et al., 2004) and PBL transendothelial migration (Cinamon et al., 2001b) that occur independently of PI3K do not fit in this description of 'end targeting'. Perhaps, dividing chemokines in dominant and weak is a more appropriate classification. Strikingly, Bonnacchi et al report that CXCR3-mediated migration is dependent on PI3K (Bonacchi et al., 2001). However, these results were obtained from pericytes and not T cells, indicating that chemokine receptor signaling not only depends on the type of receptor, but also on the cell type in which they are expressed. Also on MEK and PKC involvement in leukocyte migration contradictory reports exist,

stating that chemokine induced chemotaxis is either independent or dependent on these kinases [MEK independency: (Stein et al., 2003; Knall et al., 1997; Bardi et al., 2003), PKC independency (Scandella et al., 2002; Fine et al., 2001; Choi et al., 2003); MEK dependency: (Hii et al., 1999; Sullivan et al., 1999), PKC dependency (Ko et al., 2002; Choi et al., 2003; Wang et al., 2000; Carnevale and Cathcart, 2003)]. These results indicate once more that receptor signaling may differ per chemokine receptor and cell type.

Thus, although different receptors may use the same components of signal transduction pathways, the exact combination and the repertoire of kinases differ per receptor and probably also per cell type. It is likely that distinct signaling pathways are involved in the diverse effects of chemokine triggering (chemotaxis, gene transcription and proliferation), which may also differ per receptor and cell type. Also, triggering of chemokine receptors by different ligands may result in diverse effects. For example, binding by CCR2 of CCL2 and the CCR3-ligand CCL11 both induce the activation of p44/p42, although via different pathways. However, where CCL2 mediates chemotaxis, CCL11 prevents CCL2-induced migration (Ogilvie et al., 2004). Unraveling all signal transduction routes and their effects on cell activity and cell signaling, will be indispensable for the evaluation of new pharmaceutics that target chemokine receptors.

The biological activity of a small-molecule CXCR3 antagonist

To study the role of $CXCR3^+$ infiltrating T cells in type 1 inflammatory disorders, specific CXCR3-antagonists may prove to be a valuable tool. In addition, the identification of high affinity CXCR3-antagonists may eventually lead to the development of intervening therapies. The aim of **chapter 6** was to synthesize a small molecule CXCR3-antagonist with high affinity that blocks CXCR3-ligand-mediated signal transduction and human T cell migration.

The design of small molecule antagonists may lead to compounds with higher stability than peptidic agents and may be suitable for oral administration. In addition, small molecule antagonist may be able to penetrate through the skin and be used for local administration. Recently, it was demonstrated that a small molecule that was originally designed as a murine CCR4-antagonist also antagonizes murine CXCR3 (Yang et al., 2002). For the human CXCR3, only two antagonistic small-molecules have been described with a moderate binding affinity (Schall et al., 2001). **Chapter 6** describes the modification of one of these compounds to increase its affinity to CXCR3. At the Department of Pharmacochemistry (Free University, Amsterdam), different compounds were synthesized based on the lead compound (Schall., et al. 2001) and tested in radio-ligand binding assays. Compound VUF5834 demonstrated the

highest affinity for CXCR3 and effectively prevented CXCR3-ligand-induced activation of phospholipase C and CXCR3-mediated $[Ca^{2+}]_i$ in cells transfected with the human CXCR3. Moreover, in T cells, 1 μM of VUF5834 completely abrogated CXCR3-mediated actin polymerization and T cell migration. This effect was specifically mediated via binding to CXCR3, as VUF5834 did not inhibit CXCR4-mediated T cell migration and a non-binding analogue of VUF5834 had no effect on CXCR3-mediated signaling and T cell migration. In conclusion, the small molecule VUF5834 specifically antagonizes CXCR3 via interaction with the receptor.

VUF5834 did not affect T cell chemotaxis induced by CXCR4-ligand CXCL12, indicating that the action of this antagonist is limited to CXCR3. Nevertheless, cross-reactivity of VUF5823 with other chemokine receptors remains to be determined, in particular with CCR3 and CCR5. Recently, the three ligands of CXCR3 were discovered to act as antagonists for CCR3 (Xanthou et al., 2003). In addition, CXCL11 is a natural antagonist of CCR5 (Petkovic et al., 2004). CXCR3 antagonist may therefore not only bind to CXCR3 but also to CCR3 and CCR5. Already, the murine CCR5 antagonist TAK-779 was shown to antagonize both CCR5 and CXCR3 (Yang et al., 2002).

For future studies on the in vivo effect of VUF5834 test animals will be indispensable. First, to asses toxicity and assemble information on the route of administration and bioavailability. Secondly, to study its effect on in vivo trafficking of $CXCR3^+$ T cells. In this respect, it would be preferable that the antagonist not only binds to the human receptor but also antagonizes CXCR3 of mammalian test-animals. The murine and human CXCR3 and their ligands share a high homology with each other. Murine CXCL11 also triggers human CXCR3, although with lower potency (Meyer et al., 2001). However, human CXCL11 does not activate the murine CXCR3 (unpublished data). In case mice are selected for in vivo experiments, the binding affinity of VUF5834 for the murine CXCR3 and its capacity to antagonize murine CXCR3 ligands should be determined.

For therapeutic applications, the route of administration may determine the effect of antagonists. Oral or intravenous administration will result in total body availability of the antagonist. If inhibition is complete this will prevent the recruitment of all $CXCR3^+$ T cells to site of inflammation producing CXCR3-ligands. Local administration, for example local injection or via penetration of the skin, will likely not prevent the initial recruitment of $CXCR3^+$ T cells, but may attenuate the immune response, by preventing the local activation of $CXCR3^+$ T cells (IFN-γ production and proliferation). Depending on the type of inflammatory disease either complete inhibition or attenuation of $CXCR3^+$ T-cell-mediated responses may be desirable.

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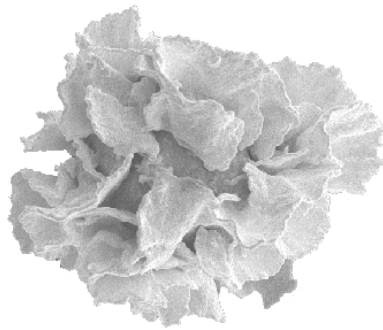
Samenvatting



Basale aspecten van verworven immuniteit: Dendritische cellen en T cellen in beweging

Samenvatting

Inleiding

Het lichaam wordt op verschillende manieren beschermd tegen infecties met ziekteverwekkers (pathogenen) zoals virussen, bacteriën en schimmels. Ten eerste belemmeren fysieke barrieres het binnentreden van pathogenen. Een voorbeeld hiervan vormt de huid; een haast ondoordringbare schild dat vocht en warmte van het lichaam binnenhoudt en vuil en pathogenen buiten. Als er toch op een of andere manier indringers binnen weten te komen, komt het immuunsysteem in actie. Het immuunsysteem is een zeer complexe organisatie, die door het hele lichaam heen vertegenwoordigd is. Hieraan doen ook weefselcellen mee (bijv. keratinocyten in de huid) door de productie van eiwitten die een antibacteriële of een signaalwerking hebben. De signaalstoffen activeren op hun beurt cellen die de bestrijding van infecties als hoofdfunctie hebben, zoals macrofagen, granulocyten, "natural killer" (NK) cellen en dendritische cellen. Deze reacties zijn niet-specifiek en vallen onder de eerste lijn van de verdediging, de aangeboren immuniteit. De tweede lijn wordt gevormd door de verworven immuniteit en bestaat uit T en B cellen. Deze cellen herkennen de pathogeen juist heel specifiek. Voor elke ziekteverwekker wordt een aparte set van cellen actief, die alleen reageert op die specifieke indringer. Een andere belangrijke eigenschap van de verworven immuniteit is dat het een geheugen ontwikkelt. Als het lichaam voor de tweede (of derde enz.) keer geïnfecteerd wordt met dezelfde bacterie of hetzelfde virus, zal het immuunsysteem veel sneller en effectiever kunnen reageren, waardoor het lichaam beter beschermd is.

Zoals gezegd zijn de T- en B cellen onderdeel van de verworven immuniteit. B cellen zijn verantwoordelijk voor de productie van antilichamen (antistoffen) tegen pathogenen. Antilichamen binden aan de ziekteverwekker, zodat deze herkenbaar wordt voor andere componenten van het immuunsysteem en opgeruimd kan worden. T cellen zijn er in verschillende soorten: 'killer' T cellen, die geïnfecteerde cellen kunnen herkennen en onschadelijk maken, helper T cellen, die o.a. de activatie van killer T cellen en B cellen helpen en het geheugen stimuleren en regulatoire T cellen, die ongewenste immuunreacties kunnen onderdrukken.

Dendritische cellen

Een belangrijke schakel tussen het eerste en het tweede leger is de dendritische cel (DC). Dendritische cellen hebben namelijk als hoofdtaak het activeren van naïeve T cellen (T cellen die nog niet eerder in aanraking zijn geweest met deeltjes van pathogenen [antigenen]) in geval van een infectie.

In weefsels die een barriere vormen tussen het lichaam en de buitenwereld, zoals de huid, de longen en de darmen, bevinden zich dendritische cellen. De eigenschappen van de DCs hangen af van het stadium waarin ze verkeren. In de weefsels zijn de DCs onrijp en zijn ze heel goed in het opnemen van alles wat in hun omgeving komt (antigenen). De opgenomen antigenen worden door de DCs in specifieke organellen (=orgaan van een cel) verwerkt om in kleine stukjes gepresenteerd te worden aan T cellen. Het presenteren van antigenen aan T cellen is een eigenschap van rijpe DCs. De rijping (maturatie) van DCs vindt plaats in reactie op een infectie. Tijdens de maturatie neemt de capaciteit om antigen op te nemen af en neemt de presentatie van antigenen sterk toe met als doel de activatie van specifieke T cellen. Tegelijkertijd migreren de DCs vanuit het weefsel via de lymfevaten naar de dichtstbijzijnde lymfeklier. Ook verandert het uiterlijk van de cel. Onrijpe DCs hebben een vrij rond uiterlijk, maar na maturatie vertonen ze op uitgebreide schaal dunne en langwerpige uitstulpingen als een soort sluier. Dit uiterlijk is nodig om zoveel mogelijk contact te kunnen maken met T cellen.

De lymfeklier is de ontmoetingsplaats voor dendritische cellen, T- en B cellen. Hier komen de DCs in aanraking met een heleboel T cellen. Elke T cell heeft echter een specifieke en heel eigen T cel receptor, waarmee hij unieke stukjes eiwit kan herkennen. Alleen de T cellen die een stukje herkennen dat gepresenteerd wordt door de DCs, worden geactiveerd. Een deel van de geactiveerde cellen zal achterblijven in de lymfklier om zich te ontwikkelen tot een geheugencel, maar het overgrote deel zal de klier verlaten en op zoek gaan naar de plaats van de infectie.

Migratie en chemokines

Het doel waar immuuncellen naartoe migreren wordt gereguleerd door eiwitten op het oppervlak van de immuuncellen en door eiwitten die daaraan binden en die gemaakt worden door weefselcellen. Een belangrijk onderdeel daarvan is het chemokine-receptor systeem. Chemokines zijn kleine eiwitten die als een soort lokstof dienen om bepaalde cellen aan te trekken. Welke cellen aangetrokken worden hangt af van het soort receptoren die de cel op zijn oppervlak heeft. Cellen die de juiste receptor hebben zullen in de richting van de hoogste concentratie van dat chemokine migreren. Op die manier kunnen cellen heel gericht naar de plaats van infectie migreren omdat daar de juiste chemokines gemaakt worden.

Dit proefschrift

In de hoofdstukken 2 tot en met 6 van dit proefschrift worden verschillende aspecten van verworven immuniteit belicht. Als eerste wordt de dendritische cel bestudeerd die zo'n belangrijke rol speelt bij de activatie van T cellen. In

hoofdstuk 2 worden de verschillen tussen de maturatiestadia van de DCs bestudeerd, terwijl hoofdstuk 3 zich richt op een speciale dendritische cel, namelijk de Langerhans cel. Als tweede wordt aandacht geschonken aan de migratie van T cellen en met name hoe chemokines via een bepaalde receptor inwerken op de geactiveerde T cel. In de hoofdstukken 4 en 5 wordt de manier waarop de T cel op chemokines reageert onderzocht. In hoofdstuk 6 wordt onderzocht of de werking van chemokines op de bijbehorende chemokinereceptor op T cellen geblokkeerd kan worden. Dit kan interessant zijn voor de ontwikkeling van nieuwe behandelmethodes voor een breed scala aan immuunziektes, waarbij een overmatige activiteit van geactiveerde T cellen aangetroffen wordt. Hieronder volgt een korte beschrijving van elk hoofdstuk.

Dendritische cellen

Hoofdstuk 2

In hoofdstuk 2 wordt de maturatie van DCs nader onderzocht. Er is al veel bekend over hoe een DC een T cel activeert, maar sommige aspecten zijn nog onderbelicht. In hoofdstuk 2 is gezocht naar verschillen tussen onrijpe en rijpe DCs in de eiwiten de ze aanmaken. Het aantal verschillende eiwitten dat een cel aanmaakt (eiwitexpressie) wordt geschat op rond de 6000. Eiwitten zijn opgebouwd uit aminozuren, waarvan er 20 verschillende bestaan, en bevatten soms ook suikers. De volgorde van de aminozuren bepalen de eigenschappen van het eiwit. Een groot aantal eiwitten kan in verschillende vormen voorkomen. Er kunnen bijvoorbeeld groepen, zoals fosfaat, aangehangen of verwijderd worden die de werking van het eiwit beinvloeden. Door de vorm van een eiwit te veranderen kunnen functies van een cel gereguleerd worden. Al deze aanpassingen leveren een een enorme variatie op. Om verschillen te kunnen vinden in het eiwitrepertoire in verschillende celstadia, moeten de eiwitten van elkaar gescheiden worden. Dit is mogelijk door gelelectroforese. Met deze methode kunnen eiwitten gescheiden worden op basis van hun eigenschappen. Om een zo groot mogelijke scheiding te krijgen, werden eiwitten eerst op basis van hun grootte en vervolgens op basis van hun zuurtegraad (pH) gescheiden. Daarna werden de eiwitten aangetoond met een zilverkleuring, waardoor een 2-dimensionaal patroon ontstaat. Van de eiwitten van zowel onrijpe als rijpe DCs zijn twee-dimensionele plaatjes gemaakt, die vervolgens met elkaar vergeleken werden. Hieruit kwam naar voren dat een van de eiwitten die verschilt tussen onrijpe en rijpe DCs Cofiline is. Dit eiwit was in beide celstadia in evengrote hoeveelheden aanwezig, echter in een andere vorm. Dit verschil in vorm zorgt ervoor dat in de rijpe cel veel meer cofiline actief is dan in de onrijpe cel. Cofiline speelt een belangrijke

rol in het skelet van een cel en daarmee in de vorm die een cel aanneemt. De verhoogde cofiline activiteit in rijpe DCs is waarschijnlijk belangrijk voor de verandering in vorm die waargenomen wordt tijdens de maturatie van DCs.

Hoofdstuk 3

Een speciaal type DC is de Langerhans cel. De Langerhans cel (LC) bevindt zich in de opperhuid en strekt lange tentakels uit tussen de huidcellen. Hiermee kan de LC de omgeving in de gaten houden en controlen op de invasie van ziekteverwekkers. Wat LCs onderscheidt van alle andere DCs is een organel (orgaan in een cel) dat de Birbeck granule genoemd wordt. De functie van dit organel is nog vrij onbekend, maar heeft mogelijk te maken met de presentatie van antigenen. Tien jaar geleden was bij toeval bij iemand vastgesteld dat deze persoon geen Birbeck granula had in zijn LCs. Deze indexpersoon was volkomen gezond en er konden geen afwijkingen gevonden worden in testen van verschillende immuunreacties. Onlangs is gebleken dat een bepaald eiwit dat alleen in Langerhans cellen voorkomt, genaamd Langerine, verantwoordelijk is voor de vorming van deze granula. Als dit eiwit kunstmatig in andere cellen die nooit Birbeck granula maken wordt gebracht, vertonen deze cellen ook Birbeck granula. Wij hebben onderzocht of er misschien een foutje zit in het DNA van de indexpersoon dat codeert voor Langerine, dat de afwezigheid van de Birbeck granula veroorzaakt. Wij hebben inderdaad een mutatie gevonden in het Langerine DNA, wat resulteerde in verandering van één aminozuur in het eiwit. Deze mutatie zat op een plaats in het eiwit met een belangrijke functie en is mogelijk de oorzaak van de afwezigheid van Birbeck granula in de indexpersoon. We hebben gecontroleerd of deze mutatie ook de aanmaak van Birbeck granula voorkomt. Daarvoor hebben we het DNA met en zonder de mutatie in cellen gezet en naar het effect gekeken. In cellen met het normale Langerine DNA ontstonden Birbeck granula. Echter in cellen met Langerine DNA met een mutatie gebeurde dat niet. Dit wijst erop dat deze mutatie inderdaad effect heeft op de vorming van Birbeck granula.

T cel migratie

Zoals boven vermeld, zijn chemokines kleine eiwitten die cellen kunnen aantrekken. De chemokines kunnen binden aan receptoren op het oppervlak van een cel. In de cel wordt daarop een signaal doorgegeven (signaaltransductie), dat verteld dat de cel zich moet gaan bewegen in de richting van de hoogste concentratie van dat chemokine. Er zijn veel verschillende receptoren en nog veel meer verschillende chemokines. Door middel van het selectief maken van chemokine receptoren kunnen immuuncellen zoals T cellen specifiek geleid worden naar verschillende

weefsels en zelfs nog specifieker, naar de plaats van de ontsteking. In de huid bijvoorbeeld worden onder invloed van bepaalde signaalstoffen de liganden (stoffen die aan een receptor binden en die activeren) van chemokinereceptor CXCR3 geproduceerd: CXCL9, CXCL10 en CXCL11. De receptor CXCR3 komt op een groot deel van alle geactiveerde T cellen tot expressie. Deze expressie van CXCR3 op T cellen en CXCL9-11 in de huid speelt waarschijnlijk een belangrijke rol bij een aantal huidziektes. Wanneer je in wilt grijpen op een bepaald mechanisme is het van belang alle betrokken processen en hun functie te kennen. In hoofdstukken 4 en 5 wordt daarom de signaaltransductie van CXCR3 in de T cel bestudeerd. In hoofdstuk 6 wordt vervolgens gezocht naar een stofje dat de binding van de chemokines CXCL9-11 aan CXCR3 kan blokkeren. Dit kan uiteindelijk leiden tot de ontwikkeling van een medicijn dat ziekteverschijnselen kan remmen in een groot aantal aandoeningen die gekenmerkt worden door een overmatige infiltratie van geactiveerde CXCR3-positieve T cellen.

Hoofdstuk 4 en 5

Wanneer een signaalstof als een chemokine bindt aan een cel, moet de cel deze informatie omzetten in actie. Dit proces wordt gereguleerd door een een verzameling eiwitten en andere moleculen, die op een of andere manier geactiveerd of juist geïnactiveerd moeten worden. Wanneer het chemokine aan zijn receptor op het oppervlak van de cel bindt, vindt er in de cel een cascade van reacties plaats die ervoor zorgen dat de benodigde eiwitten en moleculen in actie komen. De signalen hiervoor kunnen op heel veel manieren doorgegeven worden. Welke eiwitten en moleculen daarbij betrokken zijn, is afhankelijk van de receptor en het celtype. In hoofdstuk 4 en 5 wordt onderzocht welke signaal routes van belang zijn voor migratie van T cellen na stimulatie van de CXCR3-receptor.

In hoofdstuk 4 worden de betrokkenheid van verschillende eiwitten uitgetest door het toedienen van remmers. Hierbij werd gebruik gemaakt van zowel humane T cellen geïsoleerd uit bloed met de CXCR3-receptor op hun oppervlak, als cellijnen waar kunstmatig de CXCR3-receptor ingezet is. Uit de experimenten bleek dat het eiwit phospholipase C een cruciale rol heeft in de activatie van T celmigratie, maar dat de andere geteste eiwitten niet van belang zijn voor dit proces. Tevens werd aangetoond dat activatie van migratie niet in alle cellen en niet met alle chemokinereceptoren op dezelfde manier gaat. Een van de eiwitten, PI3K (fosfatidylinositol-3-kinase), waarvan door veel onderzoekers beschreven wordt dat het essentieel is voor migratie van cellen, bleek niet betrokken te zijn bij de migratie van T cellen na stimulatie van CXCR3. Ook werd bevestigd dat van de drie liganden het chemokine CXCL11 de beste activator van T cellen is in vergelijking met

CXCL9 en CXCL10.

In hoofdstuk 5 werd verder ingegaan op de rol van fosfolipase C in de signaal transductie van T cellen na stimulatie van CXCR3. Fosfolipase C zet een signaal route aan waardoor de concentratie van vrije calciumionen in de cel stijgt. Dit kan weer een reeks van processen activeren, maar het is nog niet duidelijk welke. Wij bestudeerden de rol van calcium in T cel migratie. Hieruit werd duidelijk dat cellen wel calcium nodig hebben, maar dat er daarvoor geen calcium vanuit de omgeving nodig is. Het calcium dat opgeslagen is in de cel is voldoende om de cel te laten migreren. Het is mogelijk dat de cel de signalering via calcium nodig hebben om de richting te bepalen waarin ze moeten migreren.

Hoofdstuk 6

In hoofdstuk 6 wordt onderzocht of de CXCR3-gedreven T cel migratie CXCR3 is af te remmen. Een aantal huidziekten wordt namelijk in verband gebracht met een verhoogde concentratie van CXCR3-dragende T cellen. Door een teveel aan actieve T cellen op een bepaalde plaats kunnen ongewenste reacties optreden. Daarnaast is gebleken dat CXCR3-dragende cellen een rol spelen in de afstoting van transplantaten (zoals hart en nier). Door middel van het blokkeren van de CXCR3-receptor, zou een chronische ontsteking verzacht of genezen kunnen worden doordat minder actieve T cellen naar de plaats van ontsteking worden aangetrokken. Door blokkade van de CXCR3-receptor bij transplantaties zou bovendien voorkomen kunnen worden dat afstoting plaatsvindt doordat actieve CXCR3-positieve T cellen naar het transplantaat migreren en de lichaamsvreemde cellen aanvallen. Voor verschillende chemokinereceptoren worden momenteel kleine moleculen ontwikkeld die binden aan de receptor en daardoor voorkomen dat de chemokines zelf binden en de cel activeren (antagonisten). Voor de CXCR3-receptor is onlangs een klein molecuul gemaakt dat deze functie heeft.

De afdeling Farmacochemie van de Vrije Universiteit van Amsterdam heeft deze antagonist zodanig weten te veranderen dat de binding aan de CXCR3-receptor verbeterd is en een mogelijke toepassing in de kliniek dichterbij komt. Met verschillende functionele test werd het effect op de cel bestudeerd. Hieruit bleek dat de antagonist de werking van chemokines op de cel kon blokkeren. In aanwezigheid van de antagonist reageerden de cellen niet op de relevante chemokines. De cellen migreerden niet naar de CXCR3-chemokines CXCL10 en -11, maar wel naar een ligand (CXCL12) van een andere chemokine receptor (CXCR4). Deze antagonist remt dus specifiek de migratie van T cellen die via CXCR3 gestimuleerd worden en niet via andere chemokine receptoren. Dit stofje is dus een aantrekkelijke kandidaat voor toepassing in de kliniek. Voordat deze antagonist als medicijn gebruikt kan worden is echter

nog veel aanvullend onderzoek nodig, bijvoorbeeld naar de toxiciteit. Als de studies uitwijzen dat het veilig is, kan een medicijn gemaakt worden dat bijvoorbeeld chronische ontstekingen verzacht of geneest of de acceptatie van transplantaten bevordert.

Conclusie

De verschillende hoofdstukken laten enkele aspecten zien van het immuunsysteem die onderling met elkaar verbonden zijn. De beschreven bevindingen zijn slecht kleine facetten van een complex systeem. Elk stukje nieuw opgedane kennis is echter waardevol voor een beter begrip van de werking van het immuunsysteem en betere behandelmethodes voor ziektes waarbij het immuunsysteem betrokken is.

Curriculum vitae

Pauline Verdijk werd geboren op zondag 20 juni 1976 te Voorburg. In 1994 behaalde ze haar VWO diploma aan het Christelijk Gymnasium Sorghvliet in Den Haag. In datzelfde jaar begon ze aan de studie Biomedische Wetenschappen aan de faculteit Geneeskunde van de Univerisiteit Leiden. Na stages op de afdeling Celbiologie (nu Moleculaire Celbiologie): sectie cytochemie en cytometrie begeleid door drs. S. Snaar en dr. R. Dirks en op de afdeling Infectieziekten onder toezicht van dr. P. Nibbering en dr. H. Beekhuizen, liep ze haar afstudeerstage op de afdeling Immunohaematologie en Bloedtransfusie, sectie Tumorimmunologie onder begeleiding van drs. J. Kessler, dr. R. Offringa en prof. dr. C. Melief. Aanvullend op haar opleiding heeft ze bovendien drie maanden stage gelopen in Cambridge onder supervisie van dr. S. Wagner en prof. dr. D. Fearon (Department of Medicine, Wellcome Trust Immunology Unit, Addenbrooke's Hospital) in het Verenigd Koninkrijk. In 2000 is zij afgestudeerd, waarna ze direct begonnen is als assistent in opleiding (AIO) aan de afdeling Dermatologie van het Leids Universitair Medisch Centrum (LUMC). Tijdens het promotieonderzoek werd samengewerkt met de afdeling Immunohaematologie en Bloedtransfusie, ook van het LUMC. Onder begeleiding van dr. M. Mommaas, dr. C.P. Tensen en dr. F. Koning werd het onderzoek uitgevoerd dat in dit proefschrift beschreven staat. Sinds 1 oktober 2004 is zij werkzaam als post-doc op de afdeling Tumorimmunologie aan het Nijmegen Centre for Molecular Life Science van het Universitair Medische Centrum St. Radboud te Nijmegen

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