Identification and Characterization of Genes with Specific Expression in Dendritic Cells

Dissertation zur Erlangung des
Doktorgrades der Naturwissenschaften (Dr. rer. Nat.)
der Naturwissenschaften Fakultät IV
– Chemie und Pharmazie –
der Universität Regensburg

vorgelegt von

Sven Heinz
aus Kassel

2002

The work presented in this thesis was carried out in the Department of Hematology and Oncology at the University Hospital Regensburg from October 1997 to December 2000 and Mai 2001 through December 2001.

Parts of this work have been published in:

Heinz, S., Krause, S.W., Gabrielli, F., Wagner, H.M., Andreesen, R., Rehli, M. (2002). Genomic Organization of the Human Gene HEP27: Alternative Promoter Usage in HepG2 Cells and Monocyte-Derived Dendritic Cells. *Genomics* **79**, 608-615.

Promotionsgesuch eingereicht am: 19. Juni 2002

Die Arbeit wurde angeleitet von: PD Dr. med. S.W. Krause

Prüfungsausschuß:

Vorsitzender: Prof. Dr. Pfitzner
1. Gutachter: Prof. Dr. Buschauer
2. Gutachter: PD Dr. med. Krause
3. Prüfer: Prof. Dr. Steinem

Tag der mündlichen Prüfung: 18. Juli 2002

To
Heather
my wife
and my
best friend

"Forty-two."
Deep Thought, on the question of life,
the universe and everything
in
"Hitchhiker's Guide to the Galaxy"
by Douglas Adams

aa	amino acid
AA	Acrylamide
AP	Ammonium Peroxodisulfate
ATCC	American Tissue Type Collection
BCA	Bicinchoninic Acid
BLAST	Basic Local Alignment Search Tool
BLOTTO	Bovine Lacto Transfer Technique Optimizer
BSA	Bovine Serum Albumine
cDNA	Complementary DNA
CNS	Central nervous system
CpG	Cytidine-Phosphate-Guanosine
cpm	Counts per Minute
dATP	deoxyadenosine triphosphate
DC	dendritic cell
dCTP	deoxycytidine triphosphate
DEAE	Diethylaminoethyl
DEPC	Diethyl Pyrocarbonate
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphates
DP	Difference Product
ds	double-stranded
DSM	Deutsche Sammlung für Mikroorganismen
DTT	Dithiothreitol
dTTP	deoxythymidine triphosphate
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
ELC	Epstein-Barr virus-induced molecule 1 (EBI-1) Ligand Chemokine
EPPS	4-(2-Hydroxyethyl)piperazine-1-propanesulfonic acid
ERV	Endogenous Retroviral Sequence
EtOH	Ethanol
FACS	Fluorescence-Activated Cell Sorting
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GTC	Guanidine Thiocyanate
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-Piperazineethane Sulfonic Acid
HOAc	Acetic Acid
HUGO	Human Genome Organization
HRP	Horseradish Peroxidase
IAA	Isoamyl Alcohol
IL	Interleukin
LAMP	lysosome-associated membrane glycoprotein
LARC	Liver and Activation-regulated Chemokine
LB	Luria Bertani
LPS	Lipopolysaccharide
MACS	Magnetic Cell Sorting
MBN	Mung Bean Nuclease
MCP	Monocyte Chemotactic Protein
MeOH	Methanol
MIP	Macrophage Inflammatory Protein
MLC	Mixed Leukocyte Culture
MLR	Mixed Leukocyte Reaction
MMLV-RT	Moloney Murine Leukemia Virus Reverse Transcriptase
MNC	Mononuclear Cells
MOPS	3-(N-Morpholino) Propanesulfonic Acid
mRNA	Messenger RNA
NaOAc	Sodium Acetate
NCBI	National Center for Biotechnology Information
NH_4OAc	Ammonium Acetate
NHDFC	Normal Human Dermal Fibroblast Cells
NP-40	Nonidet P-40
OD	Optical Density (Absorbance)
PAA	Polyacrylamide
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PEG	Polyethyleneglycol
PBS	Phosphate Buffered saline
PLB	Passive Lysis Buffer
PMSF	Phenylmethylsulfonic acid
PVDF	Polyvinylidene Difluoride
RACE	Rapid Amplification of cDNA Ends
RDA	Representational Difference Analysis
RNA	Ribonucleic Acid
rpm	Revolutions per Minute
rRNA	Ribosomal RNA
RT	Room Temperature
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SAGE	Serial Analysis of Gene Expression
SDS	Sodium Dodecylsulfate
SMART	Switching Mechanism At 5' end of RNA Transcript
SSC	Saline-Sodium Citrate
STBS	Suspension TBS
STTBS	Slimfast-TTBS
TAE	Tris-Acetate/EDTA Electrophoresis Buffer
TBS	Tris-Buffered Saline
TCA	Trichloracetic A
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethylenediamine
TGE	Tris-Glycine/EDTA
TNF	Tumor Necrosis Factor
Tris	Tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
TTBS	0.1% Tween-20/TBS
UV	Ultraviolet

1 Introduction

1.1 The Immune System

The immune system protects the body from infection and death. It comprises a set of cells and molecular products as well as a specialized infrastructure, the lymphoid organs. Most cells of the immune system patrol the body for activation signals. Recognition of pathogens by individual immune cells activates the immune system which leads to eradication of the pathogen during the ensuing immune response and eventually the return to the inactive state.

Cells of the innate immune system (granulocytes, macrophages, mast cells, NK cells and dendritic cells) react rapidly to a number of stimuli common to a wide range of pathogens or dangerous conditions, e.g. bacterial cell wall components, double-stranded RNA (e.g. from viral replication), unmethylated CpG-containing DNA (e.g. from lysed bacteria) or the loss of normally obligatory major histocompatibility complex (MHC) class I molecules on somatic cells (indicating immune escape of tumor or virus-infected cells). The elicited responses comprise phagocytosis and intracellular killing of pathogens, secretion of cytotoxic and microbicidal metabolites and proteins as well as production of messenger molecules, which are able to spread the activation information to other cells, both inside and outside of the immune system.

While the innate immune response does not have a “memory” of the previously encountered pathogens, one of the hallmarks of adaptive immunity is its ability to “remember” previous activation states and thus to react more rapidly and in a more specific fashion during a secondary immune response to the same pathogen. The immunological memory manifests itself in the somatic mutations found in previously activated, clonally selected memory B and T lymphocytes.

In contrast to B lymphocytes, which recognize their cognate antigen directly *via* a transmembrane version of the antibody they are clonal for, the T cell receptor is only able to identify short peptides bound to a cleft on self-MHC molecules on the surface of antigen presenting cells (APCs).

Subsets of T lymphocytes have been found to fulfill different functions: MHC class I-restricted $CD8^+$ T cells mediate cytotoxic responses, thereby inhibiting the replication of intracellular pathogens or tumor growth by killing infected or degenerated cells. Upon activation, MHC class II-restricted $CD4^+$ helper T (T_H) lymphocytes produce cytokines and important cell surface molecules. These supply the survival signals for recently activated naïve B and cytotoxic T cells, rescuing them from apoptotic death or paralysis (anergy), effectively validating the activation stimulus as non-self and enabling a lymphocyte response to the pathogen (Sornasse *et al.*, 1992; Ridge *et al.*, 1998).

1.2 Dendritic Cells: Antigen Presenting Cells Bridging Innate and Adaptive Immunity

Dendritic cells (DCs) are a heterogeneous cell population characterized by dendritic processes which generate a large interaction surface for efficient cell-cell contact. Due to their appearance, in his first description of DCs in the epidermis, Langerhans in 1864 erroneously identified DCs as nerve cells. Only much later, in 1973, Steinman and Cohn (Steinman and Cohn, 1973) realized that DCs are antigen presenting cells.

Until the emergence of DCs as essential APCs, activated B cells and macrophages were believed to be the main APCs, positioning the adaptive branch of immunity as a secluded entity of late evolutionary origin able to leverage the destructive capabilities of innate immunity as additional effector mechanisms without being dependent on it for antigen presentation.

With the discovery of DCs it became clear that this cell type is much better suited to initiate immune responses due to its constitutively high antigen presenting capacity, the broad distribution in almost all tissues and its migratory properties. Indeed, the presence of DCs has been shown to be crucial for priming of naïve T lymphocytes and the induction of primary immune responses (Ronchese and Hausmann, 1993).

1.2.1 Dendritic Cell Ontogeny

Like all leukocytes of the immune system, DCs are derived from common pluripotent stem cell precursors in the bone marrow. These give rise to myeloid and lymphoid progenitors which further differentiate and commit to the different myeloid and

lymphoid lineages. Myeloid progenitors can give rise to erythrocytes, megakaryocytes, granulocytes and monocytes/macrophages, the lymphoid progenitors have been shown to be the common precursors of B and T lymphocytes and NK cells.

Originally thought to be of myeloid origin, recent research suggested that both a myeloid and a lymphoid subset of DCs exist (Liu *et al.*, 2001). However, DC development seems to be characterized by high plasticity, since (in mice) both lymphoid-restricted and myeloid-restricted bone marrow precursors could be shown to produce all known mature splenic and thymic DC subsets *in vivo* (Manz *et al.*, 2001; Wu *et al.*, 2001). Consequently the final DC phenotype does not appear to be predetermined at the early myeloid/lymphoid developmental junction. Nevertheless, since mature DCs are non-proliferative, and *in vivo* BrdU-labeling in mice indicates that the different DC subpopulations have different life spans (Kamath *et al.*, 2000), some degree of DC sublineage commitment downstream of the early hematopoietic precursors can be assumed, probably at the level of monocytes and immature DCs found in blood (Shortman and Liu, 2002).

As the labels "myeloid" and "lymphoid" suit well to describe the fundamental differences between the DC subsets, they will be used throughout the rest of the text, bearing in mind that also in humans they possibly might not describe the situation adequately.

The myeloid DC set comprises interstitial DCs and epidermal Langerhans cells which have been shown to develop from a $CD34^+CD11c^+/CLA^-$ or CLA^+ (cutaneous lymphocyte-associated antigen) blood precursor, respectively (Strunk *et al.*, 1997). Interstitial DCs share a common progenitor with monocytes and macrophages and have been shown to spontaneously develop from a subset of inflammatory monocytes upon a phagocytosis stimulus both *in vitro* (human) and *in vivo* in mice (Randolph *et al.*, 1998; Randolph *et al.*, 1999). Langerhans cell development is dependent on TGF-β, and they are absent from TGF-β-deficient mice (Borkowski *et al.*, 1996).

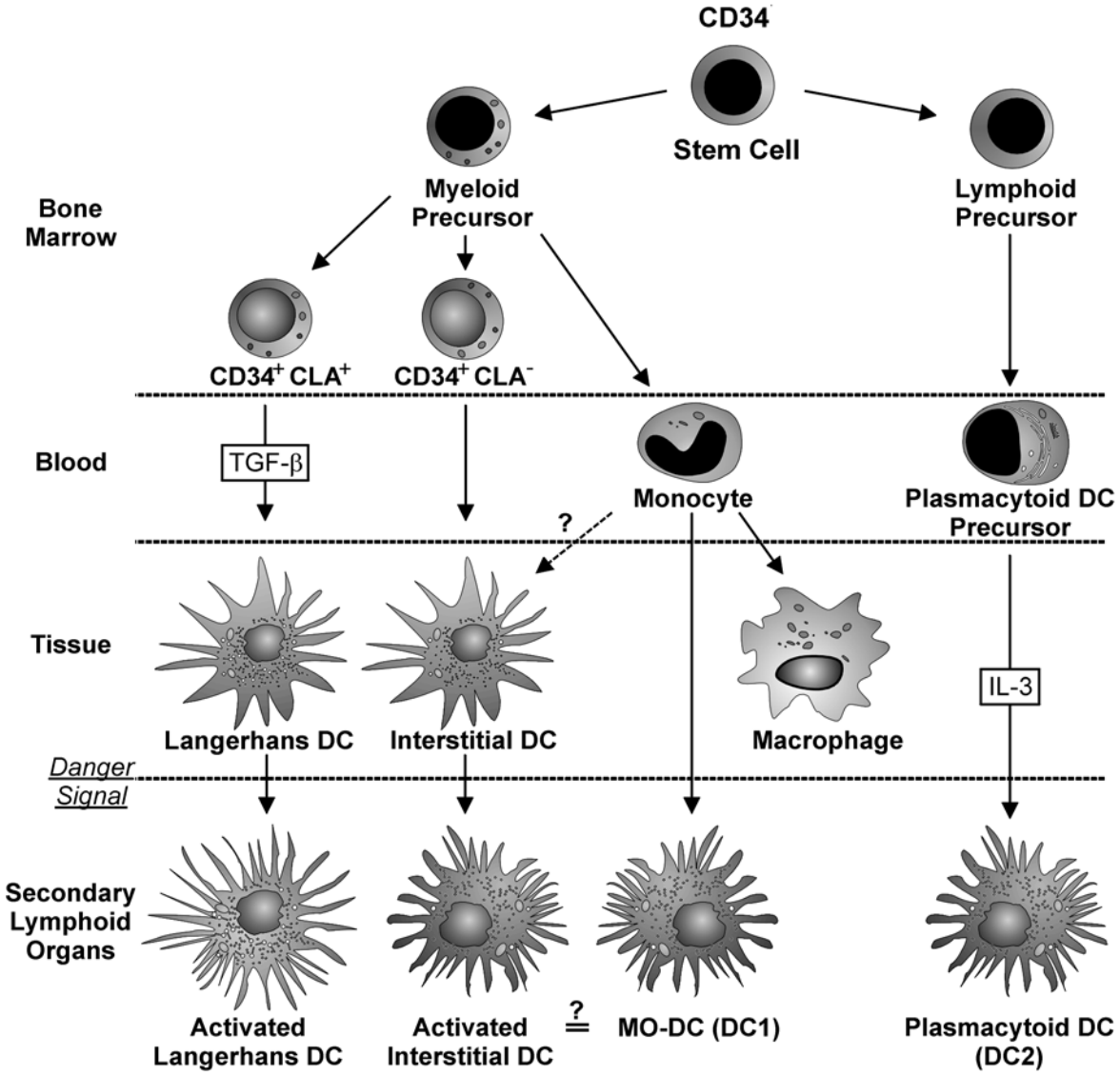


Figure 1.1 Pathways of human DC development.
Schematic representation of the DC differentiation pathways as deduced from *in vitro* studies. It is not yet clear whether the DCs generated from monocytes are equivalent to tissue DCs *in vivo*. Illustration modified after Shortman and Liu (2002).

The lymphoid DC subset contains the plasmacytoid DCs. Their name stems from the plasma cell-like morphology of their precursor cells which can be found in blood and many lymphoid tissues. They require IL-3 for survival, are only weakly phagocytic and secrete large amounts of type I interferons in response to viral stimulation (Grouard *et al.*, 1997). Plasmacytoid DCs acquire the dendritic morphology and functional competence upon CD40 ligation, enabling them to induce T_H2-biased T cell responses (see 1.3.4). They also seem to play a role in CTL tolerance by inducing $CD8^+$ regulatory T cells (Gilliet and Liu, 2002) (see also 1.3.5).

1.2.2 DC Model Systems

DC-like cells can be produced *in vitro* either from $CD34^+$ hematopoietic precursor cells from the bone marrow, peripheral blood or cord blood or from blood monocytes. $CD34^+$ cells acquire DC characteristics upon culture with GM-CSF and TNF (Caux *et al.*, 1992). Monocytes can be driven to a DC phenotype by IL-4 and GM-CSF (Peters *et al.*, 1993; Sallusto and Lanzavecchia, 1994; Zhou and Tedder, 1996).

The $CD34^+$ cells give rise to a mixture of immature and mature DCs. DCs derived from monocytes are immature with intermediate T cell activating capacity and mature to fully competent antigen presenting cells in response to a number of inflammatory stimuli such as TNF, LPS, CD40 ligation or necrotic cells (Sauter *et al.*, 2000).

To date, there are no known human DC cell lines. However, the CD34+ myelomonocytic cell line KG1 has been shown to differentiate into dendritic-like cells in response to GM-CSF plus TNF-alpha or PMA (St Louis *et al.*, 1999) and a number of myeloid cell lines have been reported to acquire a DC phenotype after treatment with calcium ionophore (Koski *et al.*, 1999).

1.3 Dendritic Cell Function

DCs originate from $CD34^+$ hematopoietic precursors and are seeded to the tissues *via* the bloodstream. An extensive network of interstitial DCs can be found in virtually all tissues except the brain, parts of the eye and the testes (Hart , 1997) but most prominently at sites of possible pathogen entry like epidermis and mucosal surfaces, e.g. intestinal tract (Pavli *et al.*, 1993) and respiratory tract (Schon-Hegrad *et al.*, 1991). In non-lymphoid tissues, DCs are present at an “immature” stage of development, where they perform sentinel functions, continuously sampling the environment for antigen (Sallusto *et al.*, 1995). In the absence of inflammation, i.e. under homeostatic conditions, low level turnover of immature DCs can be observed, which probably take up inhaled or ingested proteins and dying cells derived from normal cell turnover and take them to the draining lymph nodes (Huang *et al.*, 2000) where they presumably play a role in maintaining tolerance to self antigens (Steinman *et al.*, 2000; Roncarolo *et al.*, 2001). Encounters with “danger” signals such as microbial products like lipopolysaccharide (LPS), cytokines such as TNF or necrotic cells (Sauter *et al.*, 2000) or T cell signals like CD40L and interferon γ (IFN-γ)

initiate rapid DC maturation, accompanied by shutdown of endocytosis, loading of the antigenic sample taken at the time of “danger” onto MHC molecules for presentation on the cell surface (Steinman *et al.*, 2000) and emigration to the draining lymph nodes *via* the afferent lymphatic vessels, where DCs are recognized as “veiled” cells. Maturation also upregulates the expression of costimulatory molecules which are required for efficient interaction with T lymphocytes. In the lymph nodes, the matured “interdigitating” DCs efficiently activate T lymphocytes specific for the peptides presented on the MHC-peptide complexes. While the activated T lymphocytes recirculate through the efferent lymph and home to sites of inflammation through the bloodstream, DCs rapidly disappear after successful T cell clustering in vivo (Ingulli *et al.*, 1997) and very probably die in the lymph nodes, since they neither appear in the efferent lymph nor accumulate in the lymph node (Steinman, 1991).

1.3.1 Antigen Uptake

Immature DCs avidly sample their environment using several endocytic mechanisms: they take up small solid particles (< 0.5 µm) such as immune complexes and solutes by receptor-mediated endocytosis and fluid-phase micropinocytosis, respectively, both of which deliver antigen into clathrin-coated intracellular vesicles *via* triggering clathrin-coated pit-associated membrane receptors and are independent of actin polymerization (Aderem and Underhill, 1999).

Particles larger than ca. 0.5 µm such as bacteria and apoptotic cells are mostly picked up by phagocytosis which is usually clathrin-independent and involves actin polymerization-dependent extension of pseudopodia around the particle which then becomes engulfed into a cytosolic phagosome. Table 1.1 lists the most important receptors involved in antigen uptake and their ligands.

Table 1.1 Receptors involved in antigen uptake expressed on DCs

Receptor	Ligand/Function
Fcα-, ε-, γ- receptors[1,2]	immune complexes
macrophage mannose receptor[3]	mannosylated antigens on bacteria
CD36[4]	phosphatidylserine on apoptotic cells
$\alpha_V\beta_5$ integrin (CD51/β_5 integrin)[4]	necessary for engulfment

[1] (Geissmann *et al.*, 2001), [2] (Sallusto and Lanzavecchia, 1994), [3] (Sallusto *et al.*, 1995), [4] (Albert *et al.*, 1998a)

DCs have been shown to constitutively sample large volumes of extracellular fluid by macropinocytosis which is downregulated during DC maturation (Sallusto *et al.*, 1995). Macropinocytosis is related to phagocytosis in that it is also actin-dependent and clathrin-independent and proceeds by forming large fluid-filled vesicles through closure of plasma membrane lamellipodia generated primarily at ruffling, cholesterol-rich membrane domains (Nichols and Lippincott-Schwartz, 2001).

While macropinocytosis provides a mechanism for antigen-agnostic surveillance of the surroundings by DCs, receptor-triggered uptake mechanisms can induce further DC responses, including activation (Rodriguez *et al.*, 1999; Singh-Jasuja *et al.*, 2000) and facilitation of CTL induction (Schuurhuis *et al.*, 2002) or tolerogenization (Urban *et al.*, 2001).

1.3.2 Antigen Processing and Presentation

Antigen presentation on MHC molecules requires proteolytic degradation of proteins into short peptides of 9-11 residues (MHC class I) or 10-30 residues (MHC class II) which are small enough to bind to the clefts of the MHC molecules. The intracellular processing of antigen and the loading of class I *versus* class II molecules is strictly compartmentalized in most cells, resulting in MHC class I molecules which carry peptides derived from endogenously produced, cytosolic proteins and MHC class II molecules presenting peptides derived from endocytosed proteins.

Subsequent to its internalization, exogenous antigen is proteolytically degraded to short peptides in acidic endosomes/lysosomes. Fusion of the lysosomes with MHC class II-containing compartments enables the formation of peptide-MHC complexes which are then transported to the cell surface. In mature DCs, this process takes place very efficiently and together with the prolonged MHC surface retention time contributes to their antigen presentation proficiency (Cella *et al.*, 1997). In contrast, the relatively poor immunogenicity of immature DCs is caused by impaired vesicle fusion, impaired antigen processing, inefficient degradation of the invariant chain peptide that protects the cleft of MHC class II molecules from being loaded with endogenously produced peptides in the ER and the rapid re-endocytosis and degradation of peptide-loaded MHC class II (Steinman *et al.*, 2000; Pierre and Mellman, 1998; Villadangos *et al.*, 2001).

Cytosolic cellular proteins are degraded by the proteasome, a multi-subunit proteolytic complex. The ensuing peptides are then translocated into the ER where they are loaded onto MHC class I molecules and presented on the cell surface of all nucleated cells. In DCs, proteosomal degradation of ubiquitinated cytosolic proteins is characterized by immunoproteasomes which have been shown to produce peptides with proper motifs for efficient MHC binding. They account for half of the proteasomes in immature DCs and constitute all of the proteasomes in mature DCs, while in macrophages and several other cell types, the respective immunoproteasomal subunits are only produced after cellular activation by inflammatory mediators such as IFN-γ (Van den Eynde and Morel, 2001).

One outstanding feature of DCs is their ability to efficiently "cross-present" antigen, i.e. to present endocytosed antigen *via* the cytosolic pathway on MHC class I molecules to cytotoxic $CD8^+$ T lymphocytes (Kurts *et al.*, 2001). Apparently DCs possess an efficient way to transport small molecules (< 50 kDa) to the cytosol where they can gain access to the MHC class I presentation pathway (Rodriguez *et al.*, 1999). Cross-presentation plays a role in acquiring immunity to tumor antigens and to viruses, which do not replicate in DCs, as well as in tolerance induction.

In addition to presenting proteinaceous antigens, the DCs are able to present glycolipid antigens to NK T cells *via* CD1d molecules which are structurally highly related to MHC molecules (Kitamura *et al.*, 1999).

1.3.3 Costimulation

Initiation of productive immune responses by T cells requires a second signal in addition to TCR triggering, which is provided by membrane-bound costimulatory molecules on the APC. Compared to macrophages and B lymphocytes, DCs are distinguished by their constitutive expression of low levels of costimulatory molecules and their ability to rapidly upregulate their expression upon activation/maturation signals. The chief costimulatory molecules on DCs are the members of the B7 family of proteins, most notably B7.1 (CD80) and B7.2 (CD86) which interact with CD28 on T cells and lead to expression of CD40 ligand (CD40L), a member of the TNF family, on the activated T cells. CD40L in turn activates APCs through interaction with CD40 on their surface which is the most potent stimulus for upregulating B7 expression. Activation through CD40 ligation is necessary to confer to APCs the ability to prime

CTLs (Bennett *et al.*, 1998; Ridge *et al.*, 1998; Schoenberger *et al.*, 1998) and forms the basis of the feedback loop that validates an antigen as being non-self. Cell-cell contact is initiated and stabilized by interaction of adhesion molecules on the surfaces of both cell types, most importantly the members of the ICAM family on DCs which are ligands for LFA-1 (CD11a) on T cells.

Upon activation, DCs are able to secrete very high levels of soluble mediators, including various cytokines and chemokines. Most prominently, activated myeloid DCs produce large amounts of the T and B cell coactivator IL-12. Plasmacytoid DCs are only weak producers of IL-12 but rapidly synthesize large amounts of type I IFNs when activated, which has identified them as the natural type I IFN-producing cells in blood (Siegal *et al.*, 1999; Cella *et al.*, 1999).

1.3.4 Helper T cell polarization

Activation of $CD4^+$ helper T cells by DCs does not only lead to T cell proliferation. DCs also influence and perhaps dictate the subsequent development of the dividing T cells and thus the characteristics of the ensuing immune response.

Two subsets of activated helper T cells can be distinguished based on their cytokine expression profiles. The T_H1 subset produces large amounts of IFN-γ, T_H2 cells secrete IL-4, IL-5, IL-9, IL-10 and IL-13. The ensuing T_H1- and T_H2-type immune responses both include humoral and cell-mediated components, but the effector cells and antibody isotypes involved are distinct. T_H1 cells are responsible for the activation of macrophages to a microbicidal state, the induction of IgG antibodies that mediate opsonization and phagocytosis and the support of $CD8^+$ cytolytic T cells. By contrast, Th2 cells stimulate the growth and differentiation of mast cells and eosinophils, as well as the production of antibody isotypes, including IgE, which can mediate the activation of these cells.

Polarization of helper T cell depends on cytokines: IL-12 (Trinchieri, 1995) as well as IL-18 (Micallef *et al.*, 1996) and type I IFNs (Sareneva *et al.*, 1998) favour T_H1 differentiation while IL-4, OX40-ligation (Ohshima *et al.*, 1998) as well as other, not yet defined stimuli generate the T_H2 phenotype.

Due to their high level IL-12 expression upon activation, myeloid DCs were initially thought to induce T_H1 differentiation (Macatonia *et al.*, 1995) while lymphoid

(plasmacytoid) DCs did not express IL-12 and seemed to generate mainly T_H2 cells (Rissoan *et al.*, 1999), which led to the designation as DC1 and DC2, respectively. However, recent reports indicate that, depending on the DC activation stimulus, both myeloid and lymphoid DCs can be instructed to prime either T_H1 or T_H2 responses (Liu *et al.*, 2001; Guermonprez *et al.*, 2002). This concurs with the finding that the polarization of helper T cell responses *ex vivo* induced by DCs isolated from different tissues depends on the originating tissue, irrespective of the ratio of myeloid to lymphoid DCs, suggesting tissue-specific DC polarization and a high degree of DC functional plasticity.

1.3.5 Tolerance Induction

In addition to their stimulatory properties, DCs can modulate immune responses by inhibiting or modulating T cell activity. Immature DCs have been shown to anergize T cells in an antigen-specific fashion (Dhodapkar *et al.*, 2001). In vivo, constitutive trafficking of apoptotic cell-bearing immature DCs from non-inflamed tissues to the draining lymph nodes has been observed in rats (Huang *et al.*, 2000). This constant low level turnover has been suggested to play a role in maintaining peripheral tolerance to self antigens in the absence of danger signals (Steinman *et al.*, 2000).

Additionally, DCs treated with inhibitory stimuli are able to generate suppressive T cell populations. IL-10-treated myeloid and CD40L-activated lymphoid DCs have been reported to induce regulatory $CD4^+CD25^+$ (Jonuleit *et al.*, 2001) and $CD8^+$ non-cytotoxic T cells (Gilliet and Liu, 2002), respectively, which inhibit bystander T cell activation and expansion by secretion of IL-10.

1.3.6 Interactions with other Cells of the Immune System

DCs interact with a number of cells other than T lymphocytes, including B lymphocytes, NK T cells and NK cells.

DCs can induce and influence B cell proliferation and differentiation through secretion of IL-12 and IL-6R α-chain (Dubois *et al.*, 1997; Dubois *et al.*, 1998). They also play a role in heavy chain isotype switching to IgG (Gerloni *et al.*, 1998; Wykes *et al.*, 1998) and IgA (Fayette *et al.*, 1997). Due to their expression of T- and B cell-specific chemokines such DC-CK1 (Lindhout *et al.*, 2001) and BLC (Vissers *et al.*, 2001) and due to the fact that DCs are able to capture and retain unprocessed antigen for

transfer to naïve B cells (Wykes *et al.*, 1998), DCs probably serve to integrate encounters of the cells of adaptive immunity with one another and antigen.

Like DCs, NK cells belong to the innate arm of immunity and an intense cross-talk exists between both cell types, DCs inducing expansion and activation of NK cells and *vice versa*, NK cells activating or inhibiting DC activity (Guermonprez *et al.*, 2002). Upon activation, DCs rapidly produce NK cell-activating cytokines such as IL-12 or type I IFNs and recently myeloid DCs have been reported to secrete IL-2 immediately after receiving a maturation stimulus which could potentially stimulate NK cell proliferation in the periphery (Granucci *et al.*, 2001). In response to the DC-secreted cytokines, NK cells produce large amounts of IFN-γ well ahead of the appearance of T_H1 cells and are able to lyse aberrantly MHC class I molecule-expressing, potentially virus-infected or tumor cells.

Conversely, the ability of activated NK cells to mature and activate DCs would enhance the sensory repertoire of the latter for danger signals.

DCs have also been shown to interact with NK T cells through presentation of glycolipid antigens *via* CD1 molecules (Kitamura *et al.*, 1999). This in turn leads to IFN-γ as well as IL-4 production by adult NK T cells, irrespective of the activating DC population, with myeloid DCs acting through CD1d and plasmacytoid DC using a CD1d-independent pathway to activate the NK T cells (Kadowaki *et al.*, 2001).

1.4 DC Morphology and Function at the Different Stages of Differentiation

During *in vitro* differentiation of monocytes to DCs by IL-4 and GM-CSF, the initially round cells first undergo a macrophage-like adherent stage during which the cells enlarge and have a typical “fried-egg”-shaped appearance. After one to two days of culture most cells detach from the surface of the culture vessel in clusters which separate to give a single cell suspension around day three to four of culture. At this point, the generated DCs display an immature phenotype which is characterized morphologically by moderate amounts of dendritic processes that serve to greatly enhance the surface area of the cells as illustrated in Figure 1.2B.

In vivo, immature DCs form a network of interstitial cells in non-lymphoid tissues, possessing characteristic dendritic processes and constitutively expressing MHC class II molecules on their cell surface (see Figure 1.2A).

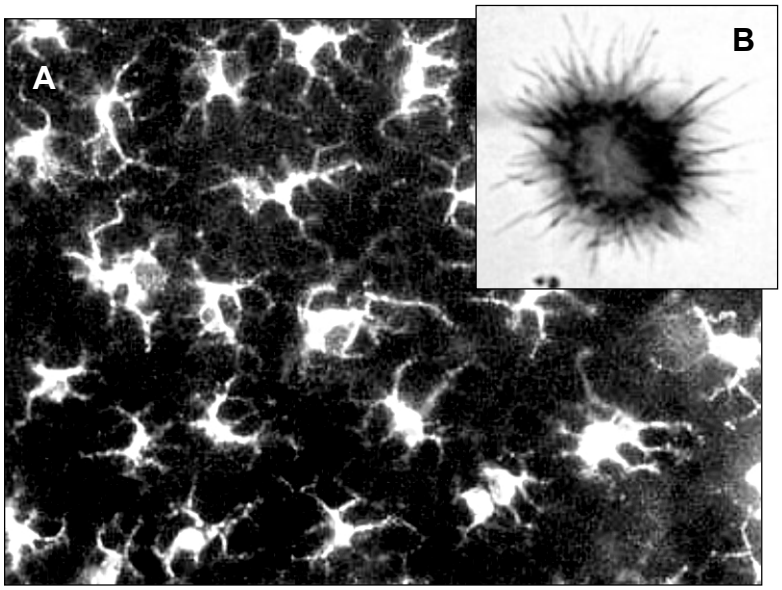


Figure 1.2 Dendritic cell morphology.
A, A network of dendritic cells *in vivo*. Fluorescence microscopical image of mouse Langerhans cells in an epidermal sheet prepared from murine ear skin, stained with an antibody against MHC class II. Image by courtesy of Dr. T. Jakob, Klinik und Poliklinik für Dermatologie und Allergologie, Technische Universität München. **B**, Morphology of a dendritic cell derived from human monocytes *in vitro*. Image by courtesy of Prof. Dr. Andreas Mackensen, Abteilung für Hämatologie und Onkologie, Regensburg.

Maturation of DCs by LPS or other danger signals leads to further ramification of the dendritic processes. *In vivo*, the maturing DCs migrate into the lymphatics and appear as veiled cells in the afferent lymph. Eventually the mature DCs arrive in the lymph nodes where they engage in cell-cell interactions with naïve lymphocytes and are recognized as interdigitating DCs.

Functionally, differentiation of monocytes to immature DCs increases the phagocytic/endocytic activity (Sallusto *et al.*, 1995) and the expression levels of antigen presenting, costimulatory and adhesion molecules, which result in improved antigen presentation capabilities compared to monocytes.

Danger signals such as microbial products (including LPS, dsRNA, CpG-DNA) and inflammatory cytokines (TNF, IFN-γ) lead to DC maturation, which is accompanied by a marked reduction of phagocytosis/endocytosis caused by diminished expression of Fc- and other antigen receptors and a lowered antigen processing activity. At the same time, the amounts of membrane molecules involved in antigen presentation

increase, most importantly MHC class II and the costimulatory molecules CD80 and CD86 as well as adhesion molecules such as CD54 (ICAM-1) and CD58 (LFA-3). The concomitant changes in chemokine receptor repertoire lets them emigrate towards the afferent lymphatics and eventually the regional lymph nodes in response to chemokines, e.g. SLC produced by lymphatic endothelium (Saeki *et al.*, 1999). DC maturation induces expression of the NK and lymphocyte co-activator IL-12 as well as a switch in chemokine expression which further enhances their efficacy in interacting with other immune cells. Upregulation of CD40 makes the maturing DCs more susceptible to activation by T cell-expressed CD40 ligand.

Ligation of CD40 activates the mature DCs and further upregulates expression of costimulatory molecules, maximizing DC antigen presentation capacity.

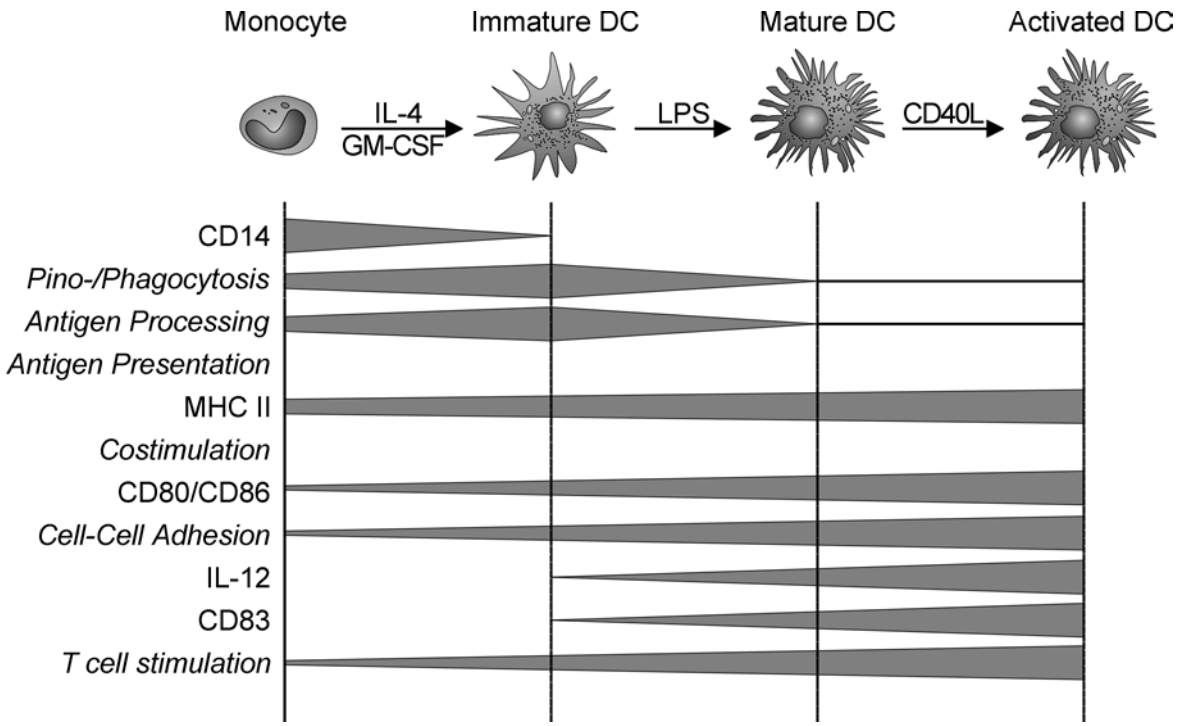


Figure 1.3 Schematic representation of the functional and morphological changes occurring during DC differentiation from monocytes *in vitro*.

1.4.1 Characteristic Molecules

Although the constitutive presence of MHC class II molecules on DCs has been widely used as a DC marker in non-lymphoid tissues in the absence of inflammation, MHC class II molecules as well as the other markers listed the first half of Table 1.2

are also expressed by other cells in secondary lymphoid tissues and during inflammation, mostly activated B cells and macrophages. Since DCs surpass the latter in antigen presentation, during the past decade considerable effort has been invested in finding molecules with specific expression in DCs that would account for their superior ability to present antigen and could serve as unique markers of DCs. The genes listed in the second half of Table 1.2 have been identified recently and can serve as reliable markers of DCs and DC subsets *in vivo*. DC-LAMP is a member of the lysosome-associated membrane glycoprotein family (LAMP), which is most similar (29% aa identity) to the D-type scavenger receptor CD68 (macrosialin) and may play a role in antigen processing. MADDAM (metalloprotease and disintegrin dendritic antigen marker) or ADAM 19 is a member of the ADAM (a disintegrin and metalloproteinase) family with unknown function. Langerin, DC-SIGN and BDCA-2, as well as several other genes with relatively specific expression in DC not listed, belong to the lectin family of carbohydrate-binding molecules and have been implicated in antigen capture. DC-CK1 is a CC chemokine with specificity for naïve T and B lymphocytes (Lindhout *et al.*, 2001). B7-DC belongs to the B7 family of proteins which also includes CD80 and CD86 (B7.1 and B7.2, respectively) and is a strong costimulator of T cell activation.

Table 1.2 Genes with specific expression in DCs

Name	Location	Family	Reference
S-100b	cytosol	S100/Ca-binding	Takahashi *et al.*, 1981
CD1a (Langerhans)	membrane	MHC homolog/Ig-SF	Chu *et al.*, 1982
CD83	membrane	Ig-superfamily (Ig-SF)	Zhou *et al.*, 1992
Fascin/p55	cytosol	Fascins/actin bundling	Mosialos *et al.*, 1996
DC-CK1	secreted	β-chemokine	Adema *et al.*, 1997
DC-LAMP (CD208)	lysosomal	D-type scavenger-R?	de Saint-Vis *et al.*, 1998
Langerin (CD207) (Langerhans)	membrane	C-type lectin	Valladeau *et al.*, 1999
MADDAM	membrane	ADAM family	Fritsche *et al.*, 2000
DC-SIGN (CD209)	membrane	C-type lectin	Geijtenbeek *et al.*, 2000
BDCA-2 (plasmacytoid)	membrane	C-type lectin	Dzionek *et al.*, 2000
B7-DC	membrane	B7 family	Tseng *et al.*, 2001

2 Research Objectives

The aim of this work was to gain further insight into the differentiation pathways and lineage-commitment steps of DCs *vs.* macrophages as well as to define new molecular DC markers by identifying genes with specific expression in DCs during differentiation from blood monocytes.

DC-specific mRNA transcripts were to be cloned by subtracting monocyte- and macrophage-expressed mRNAs from DC-derived mRNA transcripts using a PCR-based cDNA subtraction method, Representational Difference Analysis (RDA). The products were to be analyzed for DC-specificity on the RNA and protein level and their regulation was to be correlated with the DC differentiation status to allow for further investigation of their possible function in DCs.

To elucidate mechanisms governing the expression of the identified genes in DCs, the promoters of the most promising candidates were to be examined and analyzed for elements possibly involved in conferring DC-specificity by molecular biological techniques.

3 Materials and Methods

3.1 Equipment and Materials

3.1.1 Equipment

Autoclave	Technomara, Fernwald, Germany
Camera	Polaroid, Cambridge, USA
CCD-video documentation system	Intas, Göttingen, Germany
Centrifuges	Heraeus, Hanau; Eppendorf, Hamburg, Germany
Densitometer	Molecular Dynamics, Krefeld, Germany
Electrophoresis equipment	Biometra, Göttingen; BioRad, Munich, Germany
Elutriator J6-MC	Beckman, Munich, Germany
FACScan	Becton-Dickenson, San Jose, USA
Heat sealer (Fermant 400)	Josten & Kettenbaum, Bensberg, Germany
Incubators	Heraeus, Hanau, Germany
Laminar air flow cabinet	Heraeus, Hanau, Germany
Luminometer (Sirius)	Berthold Detection Systems, Pforzheim, Germany
Microscopes	Leitz, Heidelberg, Germany
PCR thermocyclers	Perkin Elmer, Überlingen, Germany and MJ Research, Hessisch Oldendorf, Germany
pH-Meter	Knick, Berlin, Germany
Power supplies	Biometra, Göttingen; Bachofer, Reutlingen, Germany
Pump, Masterflex 70 1600	Cole-Parmer, Chicago, USA
Spectrophotometer	Perkin Elmer, Überlingen, Germany
Stratalinker UV Crosslinker 1800	Stratagene, Heidelberg, Germany
Thermomixer	Eppendorf, Hamburg, Germany
TopCount microplate scintillation counter	Packard BioScience, Frankfurt, Germany
Ultracentrifuge Optima L-70	Beckman, Munich, Germany
Vacuum dot blot manifold	Schleicher und Schuell, Dassel, Germany
Water purification system	Millipore, Eschborn, Germany

3.1.2 Materials

Cell culture flasks	Costar, Cambridge, USA
U-shaped well multiwell cell culture plates	Greiner, Nürtingen, Germany
Pipettes	Costar, Cambridge, USA
Polystyrene tubes for flow cytometry (4 ml)	Falcon, Heidelberg, Germany
PCR, screw-top micro test tubes	Sarstedt, Nümbrecht, Germany
Cryo tubes	Nunc, Wiesbaden, Germany
Sterile micropore filters	Millipore, Eschborn, Germany
Nylon transfer membrane	MSI, Westboro, MA, USA
Nitrocellulose membrane (Protran)	Schleicher & Schuell, Dassel, Germany
PVDF membrane (Immobilon-P)	Millipore, Eschborn, Germany
KODAK X-OMAT AR autoradiography film	Eastman Kodak, , Germany
Micro test tubes (0.5, 1.5, 2 ml)	Eppendorf, Hamburg, Germany
Multiwell cell culture plates (6, 24, 96 well), cell culture and Petri dishes	Falcon, Heidelberg, Germany
Centrifuge tubes (15, 50, 225 ml)	Falcon, Heidelberg, Germany

3.1.3 Chemicals

Unless noted otherwise, chemicals where purchased either from Sigma Chemicals, Deisenhofen, Germany or from Merck, Darmstadt, Germany. Ready-made buffers and cell culture media were obtained from Biochrom, Berlin, Germany.

Water was generally of Millipore-purified/distilled quality. When denoted, ultra-pure, DEPC-treated H_2O_{USB}, purchased from USB Corp. through Amersham, Braunschweig, Germany, was used.

3.1.4 DNA Oligonucleotides

General	Sequence
18S rRNA-specific oligo	5'-ACG GTA TCT GAT CGT CTT CGA ACC-3'
M13 (-20) forward	5'-TTG TAA AAC GAC GGC CAG TG-3'
M13 reverse	5'-GGA AAC AGC TAT GAC CAT GAT-3'
β-ActinS	5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3'
β-ActinAS	5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3'
RDA	
R-Bgl-12	5'-GAT CTG CGG TGA-3'
R-Bgl-24	5'-AGC ACT CTC CAG CCT CTC ACC GCA-3'
J-Bgl-12	5'-GAT CTG TTC ATG-3'
J-Bgl-24	5'-ACC GAC GTC GAC TAT CCA TGA ACA-3'
N-Bgl-12	5'-GAT CTT CCC TCG-3'
N-Bgl-24	5'-AGG CAA CTG TGC TAT CCG AGG GAA-3'
pZeRO-2s	5'-GCA TCA AGC TTG GTA CCG-3'
pZeRO-2as	5'-GGC GGC CGT TAC TAG TG-3'
Primers for Nortern blot probe PCRs	
MCP-4s	5'-GCA GCT TTC AAC CCC CAG-3'
MCP-4as	5'-CCC ACA GGC ATG CTC TCA A-3'
Hep27e3s	5'-GAG CAG CAC CGG GAT AGA C-3'
C1qc_s	5'-ATC CGA GGA CCC AAA GGG-3'
C1qc_as	5'-ACA GCC AGC CAC ACC TCC T-3'
15LOXs	5'-ACG CCT GGT TCT GCA ACT G-3'
15LOXas	5'-CCC GAT TTC AGT GAT CTC TCG-3'
FR-βs	5'-CCT CAA TGT CTG TAT GGA TGC C-3'
FR-βas	5'-GCA GCT GGA GTG GGG AAG T-3'
MCP-4	
MCP-4RACE outer	5'-GCA GCA TCC CTT CAT GTC CAT GAC T-3'
MCP-4RACE inner	5'-ACA ACC CAC TGC CAG CAG CTC ATA G-3'
CpGsS	5'-TTG TTG TTA AAT AAA AGT TGA AAT TG-3'
CpGsAS	5'-ACA CAA AAA CAC TAC AAA AAC TTT C-3'
CpGs2S	5'-TTT GGT GAT AAA ATA TAA GTA GAT TAG –3'
CpGas2S	5'-AAT TTC ACA ATA TTT CTT TAC CTC-3'
CpGasS	5'-ACC AAC TTT CTC TTC TAA CTT TCC-3'
CpGasAS	5'-AGA TTT TTA TGT TGA AGG TTA AGA G-3'
CpGas2S	5'-TTC TCT TCT AAC TTT CCC TCT C-3'
CpGas2AS	5'-GGT TAA GAG TTG GAG ATT TTA TAA TG-3'

CpGi1sS	5'-TTT TGT GTT TGT TGT TTA TGA TAG TAG-3'
CpGi1sAS	5'-TCT ACC TCC AAC ATA AAC TTC AAC-3'
CpGi1asS	5'-CTA CTC ATA ACA ACA ACT TTC AAC C-3'
CpGi1asAS	5'-GAG GAG TTT TAT TAT TTT GGT GG-3'
MCP-4pS	5'-GAA CAG CTA GCA CGT GAA CAG AGT CCT TAG CAC AG-3'
MCP-4pAS	5'-AGT CTC CAG ATC TTT GCC TCT CTG CTC CTC-3'
MCP-4pPGL3s	5'-GAA CAG CTA GCA CGT GAA CAG AGT CCT TAG CAC AG-3'
CpG1s (-80 bp)	5'-CAT CAT GAC TTG GTC AAC GCC CTG CTC A-3'
CpG1as (-80 bp)	5'-CCT GAG CAG GGC GTT GAC CAA GTC ATG A-3'
mCpG1s (-80 bp)	5'-CAT CAT GAC TTG GTC AAmC GCC CTG CTC A-3'
mCpG1as (-80 bp)	5'-CCT GAG CAG GGmC GTT GAC CAA GTC ATG A-3'
CpG1M1s (-80 bp)	5'-CAT CAT GAC TTG GTC AAG CCC CTG CTC A-3'
CpG1M1as (-80 bp)	5'-CCT GAG CAG GGG CTT GAC CAA GTC ATG A-3'
CpG1M2s (-80 bp)	5'-CAT CAT GAC TTG GTC AAG AGC CTG CTC A-3'
CpG1M2as (-80 bp)	5'-CCT GAG CAG GCT CTT GAC CAA GTC ATG A-3'
CpG2s (-20 bp)	5'-CTC CCT ATA AAA GGC CGG CGG AAC AGC CAG-3'
CpG2as (-20 bp)	5'-CTC TGG CTG TTC CGC CGG CCT TTT ATA GG-3'
mCpG2s (-20 bp)	5'-CTC CCT ATA AAA GGC mCGG mCGG AAC AGC CAG-3'
mCpG2as (-20 bp)	5'-CTC TGG CTG TTC mCGC mCGG CCT TTT ATA GG-3'

Hep27

RACE inner	5'-CTT CCT GTC TAT CCC GGT GCT G-3'
Hep27GWouter	5'-TGT CTG GAG TGC AGT TTG GC-3'
Hep27GWinner	5'-AGC TGA GTA TGA GGA TGT GAG TAA G-3'
1s	5'-CCA GAC AGA CAG GTG CAC AGC-3'
a2s	5'-CTG CTC ACT CGT TGG GTC CG-3'
2s	5'-AAG ACC ACG AAT GCA CCG AGA G-3'
5as	5'-GAC GCC CCC ACA GTG CTC C-3'
8as	5'-CAG TTT ACC CGG ATG TCC TTG G-3'
10as	5'-CAC GAT TCC TGC ACA GTC CT-3'

3.1.5 Antibodies

monoclonal

CD1a (BL6)	Coulter, Krefeld, Germany
CD11c (LeuM5)	Dianova, Hamburg, Germany
CD14 (My4)	Coulter, Krefeld, Germany
CD40 (14G7)	Sanbio, Beutelsbach, Germany
CD54 (CBL 450F)	Immunotech, Marseille, France
CD80 (BB1)	Pharmingen, Hamburg, Germany
CD83 (HB15a)	Immunotech, Marseille, France
CD86 (Fun-1)	Pharmingen, Hamburg, Germany
HLA-DR (B-F1)	Diaclone, Besançon, France

polyclonal

rabbit anti-MCP-4	Pepro Tech EC, London, England
rabbit anti-mouse	DAKO, Hamburg, Germany
goat anti-rabbit, alkaline phophatase-conjugated	Dianova, Hamburg, Germany

3.1.6 Enzymes, Inhibitors and Kits

Restriction endonucleases, DNA polymerases and protease inhibitors were purchased from Boehringer Mannheim, Germany unless noted otherwise.

Enzymes, Inhibitors and Kits, *continued*

5'-end Labeling kit	Amersham, Braunschweig, Germany
SMART RACE kit	Clontech, Palo Alto, USA
GenomeWalker kit	Clontech, Palo Alto, USA
Oligotex mRNA kit	Qiagen, Hilden, Germany
RNeasy RNA extraction kit	Qiagen, Hilden, Germany
Plasmid preparation kits	Qiagen, Hilden, Germany
QIAEX II gel extraction kit	Qiagen, Hilden, Germany
ZAP Express cDNA synthesis kit	Stratagene, Heidelberg, Germany
MACS Blood DC isolation kit	Miltenyi, Bergisch Gladbach, Germany
Luciferase Assay System	Promega, Mannheim, Germany

3.1.7 Molecular Weight Standards

DNA molecular weight standards:
(all from Gibco, Eggenstein, Germany)
λ/*Hind*III digest

23130	9416	6557	4361	2322	2027	564	125 (bp)
(477)	(194)	(135)	(89)	(48)	(42)	(12)	(ng per band/µg DNA)

Φx174/*Hae*III digest

1353	1078	872	603	310	281/271	234	194	118	72	(bp)
(250)	(200)	(160)	(110)	(57)	(52/50)	(43)	(36)	(22)	(13)	(ng per band/µg DNA)

1 kB Plus DNA Ladder (synthetic)

12000-2000/1kb steps	1650	1000	850	650	500-100/100 bp steps (bp)

Protein molecular weight standards:
(BioRad, Munich, Germany)
Kaleidoscope Prestained Standard

202,000	133,000	71,000	41,800	30,600	17,800	6,900 (kDa)

Low Range Prestained Molecular Weight Standard (BioRad, Munich, Germany)

103,000	77,000	50,000	34,300	28,800	20,700 (kDa)

3.1.8 Primary Cells and Cell Lines

NHDFC	were kindly provided by Dr. Ulf Müller-Ladner, Internal Medicine Department, University Hospital, Regensburg
Buffy Coats	were obtained from the Bavarian Red Cross
THP-1	human acute monocytic leukemia (DSM No ACC 16)
HL-60	human acute myeloid leukemia (DSM No ACC 3)
Hep G2	hepatocellular carcinoma (ATCC No HB-8065)
HeLa	human cervix carcinoma (DSM No ACC 57)
CaCo-2	human colon adenocarcinoma (DSM No ACC 169)
HT-29	human colon adenocarcinoma (DSM No ACC 299)

3.1.9 Bacterial E.Coli Strains

TOP10	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G
TOP10F'	F′ {*lac*I^q Tn*10* (Tet^R)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G
DH10B	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G

3.1.10 Plasmid Vectors

pZeRO-2	Invitrogen, Karlsruhe, Germany
pCR II	Invitrogen, Karlsruhe, Germany
pCR2.1 TOPO	Invitrogen, Karlsruhe, Germany
pGL-3 Basic	Promega, Mannheim, Germany
pGL-3 CMV	Promega, Mannheim, Germany

3.2 Cell Isolation

3.2.1 Monocytes

Required buffers and chemicals:

Ficoll-Hypaque (Pharmacia, Freiburg, Germany)
PBS
6% H_2O_2/PBS
HBSS (Hank's Balanced Salt Solution) supplemented with 6% autologous blood plasma

Peripheral blood mononuclear cells (MNCs) were separated by leukapheresis (Graw, Jr. *et al.*, 1971) of healthy donors, followed by density gradient centrifugation over Ficoll/Hypaque (Johnson, Jr. *et al.*, 1977). Monocytes were isolated from MNCs by countercurrent centrifugal elutriation (Sanderson *et al.*, 1977).

Elutriation was performed in a J6M-E centrifuge equipped with a JE 5.0 elutriation rotor and a 50 ml flow chamber (Beckman, Munich, Germany). After sterilization of the system with 6% H_2O_2/PBS for 20 min, the system was emptied of air bubbles and washed with 500 ml PBS. Following calibration at 2500 rpm and 4°C with HBSS, MNCs were loaded at a flow rate of 52 ml/min. Fractions were collected and the flow rate was sequentially increased according to Table 3.1.

Table 3.1 Elutriation parameters and cell types.

Fraction	Volume (ml)	Flow rate (ml/min)	Main cell type contained
Ia	1000	52	platelets
Ib	1000	57	small (B) lymphocytes
IIa	1000	64	large (T) lymphocytes
IIb	500	74	
IIc	400	82	
IId	400	92	
III	800	111	monocytes

Representing the largest cells within the MNC input, monocytes were obtained in the last fraction and routinely were >85% pure as determined by morphology and expression of CD14 antigen. Monocytes were centrifuged (8 min, 300x*g*, 4°C), resuspended in RPMI and counted. Monocyte yields were donor-dependent and typically accounted for 10-30% of the MNC input.

3.2.2 Blood Dendritic Cell Isolation by MACS

Required buffers and equipment:

MACS buffer	1000 ml	RPMI1640 supplemented with 2% human pooled AB serum, penicillin/streptomycin and L-glutamine d*egassed for 20-30 min at RT in an ultrasonic water bath, then put on ice*
10% FCS/RPMI		
70% Ethanol		
MACS blood DC isolation kit		Miltenyi, Bergisch Gladbach, Germany

The principle of MACS sorting lies in the use of antibodies conjugated to magnetic particles which allow depletion or enrichment of antigen-bearing cells on a paramagnetic column mounted in a strong magnetic field. To isolate blood DCs, T cells, NK cells and monocytes were magnetically depleted by antibodies to the cell type-specific antigens CD3, CD56 and CD11b, respectively. In a positive selection step, $CD4^+$ blood DCs are collected by magnetically labeled anti-CD4 antibody.

Isolation of blood DCs was essentially performed as described in the manufacturer's product manual with the following modifications: As input, 10^9 cells from the pooled elutriation fractions IIc and IId, and, if necessary, IIb, were suspended in 1.5 ml MACS buffer, giving a total volume of 3 ml. MACS buffer was used throughout the procedure. After the second positive selection step, cells were eluted with 500 µl 10 % FCS/RPMI.

The purity of the blood DC preparation was assessed by 4-colour flow cytometry after staining of input cells and blood DCs with FITC-conjugated Lineage Cocktail (CD3, CD14, CD16, CD19, CD20, CD56) (lin 1, Becton Dickinson), CD123-PE, anti-HLA-DR-PerCP and CD11c-APC. Blood DCs were identified as $lineage^-$, $CD11c^+$, $CD123^+$, $HLA\text{-}DR^+$ cells and amounted to 92-96% of the sorted cells.

3.2.3 Isolation of Granulocytes from Buffy Coats

Required buffers:

0.2% NaCl (aq), *ice-cold*
1.6% NaCl (aq), *ice-cold*
PBS
Ficoll (Biochrom AG, Berlin, Germany)

Granulocytes were prepared from buffy coats by centrifugation through a Ficoll cushion and subsequent hypotonic erythrocyte lysis. The buffy coat was diluted with 2 volumes of PBS and 2x 30 ml were layered onto 20 ml Ficoll each in 50 ml

centrifuge tubes. After centrifugation (30 min, 700x*g*, 18°C) with the brake off, the supernatant containing MNC in a white interphase was discarded and the pellet, containing granulocytes and erythrocytes was washed once with 50 ml PBS (8 min, 300x*g*, 4°C). Red cells were lysed hypotonically by suspending the pellet in 20 ml ice-cold hypotonic 0.2% NaCl solution for 45 s and restoring isotonicity with 20 ml ice-cold 1.6% NaCl solution. After centrifugation, the lysis procedure was repeated once and the remaining cells were centrifuged (8 min, 300x*g*, 4°C) and washed with 50 ml PBS.

3.3 Cell Culture

3.3.1 Cell Culture Conditions and Passaging

If not noted otherwise, cells where cultured in RPMI 1640 supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM) , antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin), 2 ml vitamins, non-essential amino acids and 50 µM β-mercaptoethanol. Media supplements were purchased from Life Technologies, Karlsruhe, Germany.

Cells were cultured at 37°C, 5% CO_2 and 95% relative humidity in an incubator. FCS was heat-inactivated for 30 min at 56°C before use.

3.3.2 Assessing Cell Vitality by Trypan Blue Exclusion

Required solutions and materials:

Trypan blue solution	0.2% (w/v)	Trypan blue in 0.9% NaCl solution
Neubauer haemocytometer slide with coverslip		

The number of viable and dead cells was determined by Trypan blue exclusion. The cell suspension was diluted with trypan blue solution and the cells counted in a Neubauer haemocytometer. The concentration of viable cells was then calculated using the equation:

Number of viable cells/ml $C = N \times D \times 10^4$

with N: average of unstained cells per corner square(1 mm^2 containing 16 sub-squares)
D: dilution factor

3.3.3 Freezing and Thawing Cells

Freeze medium	50%	RPMI 1640
	40%	FCS
	10%	DMSO

Cells were harvested and suspended in ice-cold RPMI 1640 at 1-10 Mio cells/ml (following ATCC recommendations for cell lines), T lymphocytes at 25-50 Mio/ml, and 1 ml of cell suspension was added to 1 ml ice-cold freeze medium in cryo-vials which where closed and inverted twice to mix. To allow gradual freezing at a rate of -1°C/min, the vials were placed in Styrofoam containers or isopropanol-filled cryo-containers (Nalgene) and frozen at –80°C for 24 h. For long-term storage, the samples were then transferred to liquid nitrogen (-196°C).

To recover frozen cells, the cell suspension was thawed in a water bath at 37°C. To dilute the toxic DMSO, the suspension was transferred to 10-25 ml serum-containing medium as soon as thawed and the cells were spun down (8 min, 300x*g*, 4°C) and resuspended in fresh medium.

3.3.4 Primary Cells

3.3.4.1 Dendritic Cells

Immature monocyte-derived dendritic cells (DCs) were generated by culturing elutriated monocytes in 10% FCS/RPMI1640 supplemented with 500 U/ml each of recombinant human IL-4 (Schering-Plough, New Jersey, USA or Promocell, Heidelberg, Germany) and recombinant human GM-CSF (Essex, Munich, Germany) as described earlier (Meierhoff *et al.*, 1998). To generate mature DCs, immature monocyte-derived DCs were activated after five days of culture with 100 ng/ml LPS or 10 ng/ml recombinant human TNF (Knoll AG, Ludwigshafen, Germany) for two additional days.

3.3.4.2 Macrophages

In order to generate macrophages *in vitro*, monocytes were cultured in RPMI1640 in the presence of 2% human pooled AB-group serum. If the macrophages had to be detached for further experiments, the cells were cultured in Teflon bags on teflon foils (Biofolie 25; Heraeus, Hanau, Germany), from which they could be harvested after cooling to 4°C for 45 min (Andreesen *et al.*, 1983). Otherwise, cells were seeded into petri dishes at a density of 1-2x10^5 cells/cm^2.

3.3.5 Cell Lines

The cell lines THP-1, HL-60, HepG2 and HeLa were maintained in 10% FCS/RPMI1640 with the supplements detailed under 3.3.1.

NHDFC were cultured in Dulbecco's modified MEM (DMEM) supplemented with L-glutamine, antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 1 µg/ml amphotericin B), 10 mM HEPES and 10% FCS.

The colon carcinoma cell lines CaCo-2 and HT-29 were cultured in 10% FCS/DMEM supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM) , antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin), 2 ml vitamins and 5 ml non-essential amino acids.

Cells were split 1:3 to 1:8 into fresh medium every 2-4 days. Adherent cells were passaged by washing once with PBS and incubation with 3 ml 0.05% Trypsin/0.02% EDTA/PBS per 75 cm^2 culture vessel area at 37°C for 5-10 min. The detached cells were washed once with 5 ml medium containing FCS and resuspended and split 1:3 to 1:10 into 13 ml complete medium/75 cm^2.

3.3.6 Mycoplasma Assay

Cells were frequently checked for mycoplasma contamination by ELISA with a Mycoplasma Detection Kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions.

3.3.7 Mixed Leukocyte Culture

When cultured together, leukocytes from two unrelated individuals begin to proliferate. Bain and colleagues (Bain *et al.*, 1964) coined the term "Mixed Leukocyte Reaction" to describe this observation and suggested that this reaction might be useful as an indicator of compatibility between siblings prior to organ transplantation to assess the risk of rejection of the transplant. Indeed, later on it could be shown that the observed proliferation of the T lymphocytes is a response to the "allogenicity" of the two leukocyte populations and that an MLR is absent if the MLC is carried out between syngeneic populations which express the same MHC alleles. In a syngeneic MLC, the proliferative response depends on the presence of both non-self antigens and antigen presenting cells in the leukocyte populations. In an allogeneic setting, the

MHC incompatibility alone is sufficient to trigger T cell proliferation, the magnitude of the reaction being solely dependent on the costimulatory capacity of the antigen presenting cells.

To ascertain the superior antigen presenting capabilities of DCs compared to the monocytes the were generated from, the magnitude of the MLR induced by co-culture with allogeneic T lymphocytes was assessed as the amount of tritium-labeled thymidine incorporated during DNA synthesis by the proliferating T lymphocytes.

Ascending numbers of stimulators were used to induce proliferation of a fixed number of responder cells and the stimulatory capacity of the antigen presenting cells was assessed in terms of the amount of ^{3}H-thymidine incorporated by the proliferating T lympocytes.

T lymphocyte-rich elutriation fractions (IIa1) frozen previously from a different donor were used as responder cells. The cells were thawed as described under 3.3.3 and resuspended in 10%FCS/RPMI at a concentration of $1x10^{6}$ responder cells/ml. A fixed number of $1x10^{5}$ responders (100 µl) was seeded into sterile roundbottom 96-well plates, leaving one row empty for the stimulator-only control. As stimulator cells, either DCs prepared as described above (see 3.3.4.1) or their monocyte precursors were used, which had been frozen as above (see 3.3.3). To prevent cells in the stimulator population from proliferating, stimulators were irradiated with a single dose of 30 Gy from a ^{137}Cs source. Stimulators were washed with 10% FCS/RPMI and resuspended at $3x10^{5}$ cells/ml in 10% human pooled AB serum/RPMI and further dilutions of $1x10^{5}$, $3x10^{4}$, $1x10^{4}$, $3x10^{3}$ and $1x10^{3}$ cells/ml were prepared in the same medium. MLCs were set up as depicted in Figure 3.1:

One hundred milliliters quadruplicates of each concentration were added to the wells containing the responder cells, except one row for the responder-only control. The wells for the stimulator proliferation control were filled with 100 µl of the $3x10^{5}$ cells/ml suspension. The volume in single cell population-containing wells was adjusted to 200 µl by adding 100 µl of 10% AB serum/RPMI or 10% FCS/RPMI to FCS or AB serum-containing well, respectively and the microwell plates were centrifuged for 8 min at 300x*g* and RT.

	Stim. A	Stim. B	Stim. C	Stimulators	Responders
A	OOOO	OOOO	OOOO	$3x10^4$	none
B	OOOO	OOOO	OOOO	$3x10^4$	$1x10^5$
C	OOOO	OOOO	OOOO	$1x10^4$	$1x10^5$
D	OOOO	OOOO	OOOO	$3x10^3$	$1x10^5$
E	OOOO	OOOO	OOOO	$1x10^3$	$1x10^5$
F	OOOO	OOOO	OOOO	$3x10^2$	$1x10^5$
G	OOOO	OOOO	OOOO	$1x10^2$	$1x10^5$
H	OOOO	OOOO	OOOO	none	$1x10^5$

Figure 3.1 MLC pipetting pattern. Schematic drawing of a 96-well plate. The numbers indicate cell numbers per well in 200 µl final volume. The grey-underlaid wells are the stimulator-only and responder-only controls.

Following 5 days of incubation at 37°C, 5% CO_2 and 95% relative humidity, 1 µCi/well [methyl-^{3}H]thymidine was added (10 µl of 1 mCi/ml diluted 1:10 with RPMI 1640) and the cells were incubated as before for an additional 18 h. MLCs were harvested with onto H_2O-prewetted 96-well fiber glass filter plates using a FilterMate 196 harvester (Packard BioScience), washed three times with H_2O and air-dried briefly. The bottom of the glass filter plate was sealed, 50 µl scintillation cocktail were added to each well, the top of the plate sealed as well with transparent adhesive foil and the activity measured in a TopCount scintillation counter (Packard Bioscience).

3.4 DNA

3.4.1 Transient transfection of THP-1 cells with DEAE-Dextran

Required buffers and reagents:

STBS	3.029 g	**(25 mM)**	Tris/HCl, pH 7.5
	8.01 g	**(137 mM)**	NaCl
	0.373 g	**(5 mM)**	KCl
	0.161 g	**(0.6 mM)**	$Na_2HPO_4 \cdot 7\ H_2O$
	0.103 g	**(0.7 mM)**	$CaCl_2 \cdot 2H_2O$
	0.102 g	**(0.5 mM)**	MgCl2·6H2O
	add H2O to 1 l, *autoclave*		
DEAE-Dextran		**10 mg/ml**	in STBS
	filter sterilize (0.2 µm) and store at RT		
PBS			
1x PLB (Passive Lysis Buffer), Firefly Luciferase Substrate	Luciferase Reporter Assay System (Promega, Mannheim, Germany)		

The day before transfection, a sufficient number of THP-1 cells to allow duplicate transfection for each reporter plasmid was seeded at 250000 cells/ml into tissue culture flasks. On the following day, for each transfection 6 ml cell suspension were centrifuged (8 min. 300x*g*, 4°C) the cell pellet washed twice with 5 ml STBS each, and the supernatant was removed completely. For each transfection, 200 ng of reporter plasmid in 70 µl STBS were combined with 70 µl DEAE-Dextran (800 ug/ml),

mixed and immediately added drop-wise directly onto the THP-1 cell pellets. The cells were incubated at 37°C for 20 min, washed twice with 5 ml STBS each, resuspended in 6 ml 10% FCS/RPMI and cultured in ∅ 60 mm tissue culture dishes. Cells were harvested after 48h, the culture dishes washed once with 5 ml PBS at RT and the cells were pelleted (10 min , 400x*g*, 4°C). Pellets were washed once with 10 ml PBS and the PBS removed completely by decanting and briefly inverting the centrifuge tubes onto Kleenex paper towels. Cells were lysed in 1x PLB for 15 min at RT. After spinning down cellular debris (3', 16000x*g*, RT), luciferase activity of 20 µl lysate was assayed with 100 µl Luciferase substrate each (Promega) in a luminometer. Lysates were frozen at –20°C. Firefly luciferase activity of individual transfections was normalized for protein concentration as determined by a BCA protein assay (see 3.7.2).

3.4.2 Agarose Gel Electrophoresis

Required buffers:

TAE (50x)	252.3 g	**(2 M)**	Tris
	20.5 g	**(250 mM)**	NaOAc/HOAc, pH 7.8
	18.5 g	**(50 mM)**	EDTA
	add H_2O to 1 l		
EDTA (0.5 M)	18.6 g	**(0.5 M)**	EDTA/NaOH, pH 8.0
	add H_2O to 100 ml		
DNA loading dye	500 µl	**(50 mM)**	Tris/HCl, pH 7.8
DNA-LD (5x)	500 µl	**(1%)**	SDS (20%)
	1 ml	**(50 mM)**	EDTA (0.5 M), pH 8.0
	4 ml	**(40%)**	Glycerol
	10 mg	**(1%)**	Bromophenol blue
	add H_2O to 10 ml		

Table 3.2 Agarose gel composition

	1.0% Agarose	
TAE (1x)	50 ml	150 ml
Agarose	0.5 g	1.5 g
Ethidium bromide	2.5 µl	7.5 ml

Other concentrations accordingly.

Table 3.3 Agarose concentrations for different separation ranges.

Efficient range of separation (kb)	% [w/v] agarose in gel (%)
0.1-2	2.0
0.2-3	1.5
0.4-6	1.2
0.5-7	0.9
0.8-10	0.7

Taken from Sambrook *et al.*, 1989.

The required amount of agarose as determined according to Table 3.2 andTable 3.3 was added to the corresponding amount of TAE (1x) and approximately 10 ml of H_2O were added to make up for later volume loss. The slurry was heated in a microwave oven until the agarose was completely dissolved, and the ethidium bromide added after cooling the solution to 50-60°C. The gel was cast and mounted in the electrophoresis tank and covered with TAE (1x). DNA-containing samples were diluted 4:1 with DNA-LD (5x), mixed and loaded into the slots of the submerged gel. Depending on the size and the desired resolution, gels were run at 40-100 V for 30 min to 3 h.

3.4.3 Denaturing Alkaline Agarose Gels for Analysis of Single-Stranded DNA

Required solutions:

NaOH (10 M)	2 g add H_2O to 5 ml	**(10 M)**	NaOH
Loading buffer (5x)		**250 mM** **5 mM** **18%** **0.15%**	NaOH (10 M) EDTA Ficoll 400, Pharmacia Bromocresol green
	store at 4°C		
Electrophoresis buffer	2 ml 800 µl add H_2O to 400 ml	**(50 mM)** **(1 mM)**	NaOH (10 M) EDTA (0.5 M)
TCA (10%)	20 g in 180 ml H_2O	**(10%)**	Trichloroacetic acid

500 mg agarose were dissolved in 50 ml H_2O by boiling in a microwave oven, cooled to 60°C, 250 µl (50 mM) 10 M NaOH and 100 µl 0.5 M EDTA were added and the gel cast, mounted and overlaid with electrophoresis buffer. Samples were diluted 4:1 with 5x loading buffer, loaded into the gel slots and the gel was run in a precooled electrophoresis tank (-20°C) and precooled electrophoresis buffer (4°C) (Hoefer HE 33 Mini Submarine, Pharmacia) for 4-5 h at 30-50 V until the bromocresol blue had traversed half of the gel. As a molecular weight marker, 3000-14000 cpm of a λ/*Hind*III DNA ladder radioactively end-labeled with PNK (see 3.5.6) were used.

After the run had completed, the gel was rinsed in water for 3 min, fixed for 10 min in 10% TCA and pressed dry between two layers of Whatman 3MM filter paper on each side with a stack of cellulose wadding underneath (<1 cm) and on top (~4 cm), weighted with a ~4 kg weight for 1 hour.

The flattened gel was covered with plastic wrapping film and autoradiographed overnight with an intensifying screen at –80°C.

3.4.4 Purification of DNA Fragments by Gel Extraction

DNA fragments were purified by running on an ethidium bromide-containing agarose gel (3.4.2), excising the band containing the fragment under UV illumination and subsequent gel extraction using the QIAEX II Gel Extraction Kit (Qiagen) following the manufacturer's instructions. The amount of recovered DNA was estimated by running an aliquot on an agarose gel in parallel with a DNA ladder of known DNA band concentrations, such as λ/*Hind*III or Φ174/*Hae*III.

3.4.5 Representation Difference Analysis

Required buffers, reagents and enzymes:

5x PCR buffer		**(335 mM)**	Tris/HCl, pH8.8@25°C
		(20 mM)	MgCl2
		(80 mM)	NH2SO4
		(166 µg/ml)	BSA
3x EE buffer		**(30 mM)**	EPPS, pH8.0@20°C
		(3 mM)	EDTA
dNTP			dATP/dCTP/dGTP/dTTP (10 mM each)
TE	1 ml	**(10 mM)**	Tris/HCl (1 M), pH8.0
	0.2 ml	**(1 mM)**	EDTA (0.5 M), pH8.0
	add H_2O to 100 ml		
Phenol/Chloroform/ IAA	Buffered Phenol:Chloroform:Isoamyl Alcohol 25:24:1, saturated with 10 mM Tris, pH 8.0, 1 mM EDTA (Sigma)		
$CHCl_3$/IAA	Chloroform:Isoamyl Alcohol 49:1, see 3.5.1		
Ammonium acetate	7.71 g	**(10 M)**	NH_4OAc
	add H_2O to 10 ml		
3 M Acetate	see 3.5.2		
*Dpn*II	10 U/µl	New England Biolabs, Frankfurt, Germany	

Mung Bean nuclease (MBN) (New England Biolabs)
*Dpn*II (New England Biolabs)
Taq polymerase (5 U/µl) (Gibco)
T4 DNA ligase (400 U/µl, corresponding to 18 Weiss units)(New England Biolabs)

RDA of cDNA was performed as described by Hubank and Schatz (Hubank and Schatz, 1994).

3.4.5.1 cDNA-Synthesis

Synthesis of cDNA from mRNA by reverse transcription and nick translation was carried out using the ZAP express cDNA synthesis kit by Stratagene, Heidelberg, Germany according to the manufacturer's instructions.

Synthesis of cDNA was performed with 5 µg of poly A-selected mRNA prepared from macrophages and DCs as described under 3.5.3. To control for successful 1st and 2nd strand synthesis, one tenth of the reaction mixture and all of the 2nd strand reaction were labeled with radioactive [α-^{32}P]-dCTP and 0.75 µl of the radioactive 1st strand reaction and 3 µl of the second strand reaction were run on a denaturing alkaline agarose gel (see 3.4.3).

The 2nd strand reaction products (200 µl) were isolated by vortexing for 10 s with 200 µl buffered phenol/chloroform/IAA, centrifuging (2 min, 16000x*g*, RT) and extracting the supernatant with 200 µl chloroform. After overnight precipitation at –20C from the supernatant with 20 µl 3 M sodium acetate, pH5.2 and 400 µl 100% EtOH, centrifuging for 1 h at 16000x*g* and 4°C, the cDNA was washed twice with 500 µl each of ice-cold 70% EtOH, air-dried at RT for 15 min and dissolved in 20 µl H_2O.

3.4.5.2 Generation of Representations

For each representation, approximately 2 µg cDNA, prepared from 5 µg polyA$^+$ mRNA from monocytes/macrophages or DCs (see 3.5.3) (4/5th of the total amount) were digested with 15 U *Dpn*II (New England Biolabs) in 100 µl volume for 3½ h at 37°C and phenol-extracted with an equal volume (100 µl) of phenol/$CHCl_3$/IAA by vortexing for 10 s. After centrifugation (5 min, 16000x*g*, RT) and extraction of the supernatant with one volume (100 µl) $CHCl_3$/IAA, 2 µg glycogen were added to the supernatant as a carrier and the cut cDNA was ethanol-precipitated overnight at -20°C with 50 µl 10 M ammonium acetate and 200 µl 100% EtOH. The cDNA was pelleted (30 min, 16000x*g*, 4°C), washed once with ice-cold 75% EtOH (5 min, 16000x*g*, 4°C), dried and resuspended in 20 µl TE.

Approximately 1.2 µg (12 µl) cut cDNA was then ligated to the R-Bgl-12/24 adapter in a 60 µl reaction containing 4 µl desalted R-Bgl-24 oligo (2 mg/ml), 4 µl desalted R-Bgl-12 oligo (1 mg/ml), 6 µl 10x ligase buffer (New England Biolabs) and 31 µl H_2O. Oligonucleotides were annealed to each other and to the cDNA in a PCR cycler by

heating the ligation reaction to 50°C and then cooling to 10°C over a period of 1 h. Ligation was carried out by adding 3 µl (1200 U) T4 DNA ligase (corresponding to 18 Weiss units)(New England Biolabs) and incubating for 18 h at 14°C.

Ligations were diluted to 6 ng/µl by adding 140 µl TE and 25 parallel PCR reactions were set up to generate the initial representations. Each 200 µl reaction contained 2 µl diluted ligation and (final concentrations) 66 mM Tris/HCl, pH 8.8@25°C, 4 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 33 µg/ml BSA, 0.34 mM each dNTP and 2 µg R-Bgl-24 primer overlaid with two drops of mineral oil (Sigma) in 0.5 ml PCR tubes. After melting away the 12-mer for 3 min at 72°C, 1 µl (5 U) *Taq* polymerase (Gibco) was added, the 3' ends were filled in for 5 min at 72°C and 20 cycles of amplification (1 min, 95°C; 3 min, 72°C) and 10 min extension at 72°C were performed on a Perkin Elmer thermocycler 480. Four PCR products each were combined and extracted twice with 750 µl phenol/$CHCl_3$/IAA, once with 750 µl $CHCl_3$/IAA, precipitated for 30 min at –20°C with 75 µl 3 M acetate and 800 µl 100 % isopropanol, washed once with 70% EtOH, dried and resuspended in 30 µl H_2O_{USB}. The samples were pooled, DNA concentration was determined by measuring OD_{260} (OD_{260} of 1 corresponds to 50 µg/ml DNA) and adjusted to 0.5 mg/ml with H_2O_{USB}.

To remove the R-adapters, 300 µg (600 µl) of each amplification product were digested with 750 U *Dpn*II in 1.4 ml reactions for 4 h at 37°C. The digests were divided into two portions of 700 µl each and purified by extracting twice with phenol/$CHCl_3$/IAA and once with $CHCl_3$/IAA. After precipitation with 0.1 volumes (70 µl) 3M Acetate and 700 µl isopropanol for 30 min at –20°C, the driver representation was pelleted (30 min, 16000x*g*, 4°), washed twice with 1 ml ice-cold 70% EtOH each, dried and dissolved at a concentration of 0.5 mg/ml as determined on a spectrophotometer.

To generate the tester representation, 20 µg (40 µl) phenol-extracted DC-derived DNA digest were diluted with 40 µl TE and 20 µl 5x DNA loading dye and gel-purified from the R-adapters on a 1.2% TAE/agarose gel using QIAEX II resin (Qiagen) according to the manufacturer's instructions. Two micrograms of the gel extract were ligated to J-Bgl-12/24 adapter in a 60 µl reaction as described above for R-Bgl adapter and the mixture diluted with 120 µl TE to give a final concentration of ~10 ng/ml DNA.

3.4.5.3 Difference Analysis

For the first round of hybridization and subtractive amplification, a 100-fold excess of driver (40 µg in 80 µl) were mixed with 0.4 µg (40 µl) tester representation in a 0.5 ml PCR tube, the mixture extracted once with phenol and once with $CHCl_3$, precipitated with 30 µl 10 M ammonium acetate and 380 µl 100% EtOH for 10 min at –80°C, centrifuged (15 min, 16000x*g*, 4°C), washed twice with 70% EtOH and air-dried. The pellet was thoroughly resuspended in 4 µl 3x EE buffer, incubated for 5 min at 37°C, vortexed, spun down, overlaid with 35 µl of mineral oil (Sigma) and the DNA denatured in a PCR cycler for 5 min at 100°C. The mixture was cooled to 67°C, 1 µl 5 M NaCl was added to the solution and hybridization was performed at 67°C for 21½ h. After complete removal of the mineral oil, the DNA was diluted and mixed with 8 µl TE containing 5 µg/µl yeast tRNA, followed by 25 µl and 362 µl TE.

The first selective PCR amplification was performed in four parallel 200 µl reactions as above containing 20 µl diluted hybridization mix but initially omitting primers and *Taq* DNA polymerase. After melting away the J-Bgl-12 oligonucleotides for 3 min at 72°C, 5 U (1 µl), *Taq* DNA polymerase (Gibco) was added to fill in ends for 5 min at 72°C, 2 µg J-Bgl-24 primer were added and ten cycles (1 min, 95°C; 3 min, 70°C) of amplification and a final extension for 10 min at 72°C were performed. The four reactions were combined, extracted twice with an equal volume phenol/$CHCl_3$/IAA, once with an equal volume $CHCl_3$/IAA, precipitated for 30 min at -20°C together with 2 µg glycogen carrier by adding 75 µl 3 M acetate and 800 µl isopropanol, the pellet washed twice with 1 ml 70% EtOH, air-dried and resuspended in 40 µl 0.2xTE.

To remove DNA single strands, half of the PCR product was digested with 20 U Mung Bean nuclease (MBN) (New England Biolabs) for 35 min at 30°C in 40 µl volume. The reaction was stopped by adding 160 µl 50 mM Tris/HCl pH 8.9 and heating to 98°C for 5 min.

The first difference product was prepared by performing a second subtractive PCR using 20 µl of the MBN-treated DNA each as a template in four 200 µl reactions set up as above, this time including 2 µg (2 µl) J-Bgl-24 primer from the beginning, and hot-started by adding 1 µl (5 U) *Taq* DNA polymerase (Gibco) each after melting the DNA for 1 min at 95°C and cooling to 80°C. Following 18 cycles of amplification (1 min, 95°C; 3 min, 70°C) and 10 min extension at 72°C, the reactions were cooled to

4°C, pooled, DNA extracted twice with phenol/$CHCl_3$/IAA, once with $CHCl_3$/IAA, isopropanol-precipitated, washed and dried as described for the first subtractive PCR and dissolved in 500 µl TE (at 0.5 µg/µl as determined by measuring OD_{260}) to give the first difference product (DP1).

To change the adapters from J-Bgl to N-Bgl, the difference product was digested with 150 U *Dpn*II (New England Biolabs) for 3 h at 37°C in 300 µl reaction volume, twice phenol- and once $CHCl_3$-extracted as above, and ethanol-precipitated with 0.1 volume 3 M Acetate and 2.5 volumes (800 µl) 100% EtOH for 30 min at –20°C. The DNA was pelleted, washed and dried as above, and dissolved in 30 µl TE. The concentration was determined as OD_{260}, 200 ng *Dpn*II-digested DP1 were ligated to N-Bgl-12/24 adapters as described before and the DNA was diluted to 1.25ng/µl by adding 100 µl TE.

The second round of hybridization and subtractive amplification was carried out with an 800-fold excess of driver representation (40 µg in 80 µl) over N-Bgl-ligated DP1 (50 ng in 40 µl) using N-Bgl-24 primers for the subtractive PCRs as described above. In the final cut and ligation step, the N-adapters on the DP2 were changed back to J-adapters and the J-ligated DP2 was diluted to 1 ng/µl by adding 160 µl TE to the ligation reaction. 10 µl diluted ligation were diluted 1:100 with 990 µl TE containing 30 µg yeast tRNA carrier to give 10 pg/µl J-ligated DP2.

For generation of the third difference product a 400000-fold excess of driver (40 µg) was subtracted from 100 pg J-ligated DP2 (10 µl) by hybridization and two rounds of PCR with intermittent MBN digestion as described above, the second subtractive PCR being carried out for 22 cycles instead of 20 cycles.

To clone the final difference product , the adapters were removed from 25 µg DP3 by digesting with 75 U *Dpn*II in a 100 µl reaction for 4 h at 37°C and gel extraction after separation on a 2% agarose/TAE gel using QIAEX II resin (see 3.4.4).

DP3 was "shotgun"-cloned by ligating 3-fold molar excess (50 ng) of *Dpn*II-cut, gel-extracted DP3 with 14 ng *Bam*HI-restricted pZeRO-2 vector using 120 U (0.3 µl) T4 DNA ligase for 1½ h at 16°C in 10 µl volume and transforming chemically competent TOP10F' *E.Coli* (Invitrogen) with 2 µl ligation reaction.

3.4.6 Reverse Dot Blot

Required reagents and materials:

SSPE (20x)	2.76g	**(0.2 M)**	$NaHPO_4$/NaOH, pH 7.4
	0.74 g	**(20 mM)**	EDTA
	17.5 g	**(3 M)**	NaCl
	add H_20 to 100 ml, *autoclave*		
Prehybridisation buffer	15 ml	**(50%)**	formamide
	7.5 ml	**(5x)**	SSPE (20x)
	150 µl	**(0.1%)**	SDS (20%)
	600 µl	**(0.5 mg/ml)**	tRNA (25 mg/ml)
	300 µl	**(0.1 mg/ml)**	Herring sperm DNA (10 mg/ml)
	add H_2O to 30 ml		
Hybridisation solution	15 ml	**(50%)**	**F**ormamide
	7.5 ml	**(5x)**	SSPE (20x)
	150 µl	**(0.1%)**	SDS (20%)
	6 ml	**(5%)**	PEG 8000 (25%)
	600 µl	**(0.5 mg/ml)**	tRNA (25 mg/ml)
	300 µl	**(0.1 mg/ml)**	Herring sperm DNA (10 mg/ml)
	add H_2O to 30 ml		
Wash solution A	50 ml	**(2x)**	SSC (20x)
	2.5 ml	**(0.1%)**	SDS (20%)
	add H_2O to 500 ml		
Wash solution B	12.5 ml	**(0.5x)**	SSC (20x)
	2.5 ml	**(0.1%)**	SDS (20%)
	add H_2O to 500 ml		
Wash solution C	2.5 ml	**(0.1x)**	SSC (20x)
	2.5 ml	**(0.1%)**	SDS (20%)
	add H_2O to 500 ml		
Wash solution D	2.5 ml	**(0.1x)**	SSC (20x)
	25 ml	**(1%)**	SDS (20%)
	add H_2O to 500 ml		
3 M NaOH			
Nylon transfer membrane	Magna NT, 0.22 µm; MSI, Westborough, MA, USA		
Blot paper	Whatman GB003; Whatman, Göttingen, Germany		
Vacuum dot blot manifold	Schleicher und Schuell, Dassel, Germany		

To check for specificity of the difference product, cloned inserts of 85 randomly picked clones were PCR-amplified, blotted in duplicates onto two nylon transfer membranes and hybridized with radioactively labeled driver or tester cDNA. Utilizing pZeRO-2 vector-specific primers (pZeRO-2s/as), inserts were amplified by PCR from single bacterial colonies.

PCR mixture (100 µl)

10 µl	**(1x)**	PCR buffer (10x)
8 µl	**(0.2 mM)**	dNTP (2.5mM each)
0.8 µl	**(0.8 µM)**	pZeRO sense primer (100 µM)
0.8 µl	**(0.8 µM)**	pZeRO antisense primer (100 µM)
0.8 µl	**(4 U)**	*Taq* DNA polymerase (5 U/µl)
79.6 µl		H_20_{USB}

PCRs were on a Perkin Elmer 9600 PCR cycler equipped with a hot bonnet under the following conditions:

95°C, 2 min; 35x(93°C, 1 min; 60°C, 1 min; 72°C, 1 min); 72°C, 5 min; 4°C.

The PCR products were denatured with 10 µl of 3 M NaOH (0.3 M final) for 30 min at room temperature. Two dot blots with identical layout were prepared. For each blot, a transfer membrane was rinsed in distilled water, equilibrated for 1 min with 20x SSC, mounted on top of one layer blot paper soaked with 20x SSC and washed once by filling all wells with 500 µl of 20x SSC each and applying vacuum to the manifold. Wells were filled a second time with 500 µl 20x SSC each, half of each denatured PCR mix was directly added to the SSC in successive wells and blotted by applying vacuum. Wells were washed with 200 µl 20x SSC each, the membrane removed from the manifold, dried for 5 min on Whatman 3M paper and UV-crosslinked with 120 mJ/cm^2 at 254 nm wavelength.

After UV-crosslinking, membranes were washed for 30 min at 65°C in wash buffer D and prehybridized for 2 h at 52°C in 10 ml prehybridization buffer. Blots were hybridized overnight to radioactively labeled driver, i.e. monocyte/macrophage cDNA representation, or tester, i.e. dendritic cell cDNA representation, respectively, in 5 ml hybridization solution each at 52°C. The following day, blots were washed for 5 min in wash buffer A, and for 20 min each in wash buffers B and C at RT, followed by a final wash for 30 min at 50 °C in wash buffer D. Blots were heat-sealed in plastic bags and autoradiographed with an intensifying screen at –80°C for several days.

3.4.7 PCR

Required reagents:

DNA polymerase	**0.04-0.67 U/µl**	*Taq* DNA polymerase or Expand High Fidelity polymerase mix (Roche Biochemicals) with the supplied reaction buffer
Primers	**0.2-1 µM**	Sense-/antisense primers (10-100 µM)
dNTP	**0.2-0.25 mM**	dATP, dCTP, dGTP, dTTP (25 mM each)

The polymerase chain reaction (PCR) allows in-vitro synthesis of large amounts of DNA by primed, sequence-specific polymerization of nucleotide triphosphates catalyzed by DNA polymerase (Mullis *et al.*, 1986).

PCRs were generally performed in thick PCR tubes in 20-100 µl of reaction volume. If performed on a Perkin Elmer 480 (PE 480), reactions were overlaid with two drops or 30 µl mineral oil. On the MJ research PTC 200 thermocycler (Biozym), the "calculated temperature" feature was used to decrease temperature hold times. The nucleotide sequences of the utilized primersare given in 3.1.4. If not indicated otherwise, the primer annealing temperature was 65°C.

Typical reaction parameters for analytical PCR:

Action \ Cycler	**PE 480**	**PTC 200**
Initial Melting	95°C 2-5 min	93°C 30 s
20-35 Cycles { Melt	95°C 1 min	93°C 15 s
20-35 Cycles { Anneal	65°C 1 min	65°C 15 s
20-35 Cycles { Extend	72°C 1 min	72°C 45 s
Final Extension	72°C 10 min	72°C 5-7 min
Cool to	4°C	15°C

If PCRs were performed from bacterial colonies, on the PTC 200 cycler the initial melting step was prolonged to 2 min. To avoid generating unspecific products during the first heating phase on the PE 480, reactions were generally started by adding the *Taq* polymerase after the melting the template and annealing the primers at the beginning of the first extension step ("Hotstart"). For preparative and semi-quantitative PCRs on the PTC 200, the DNA polymerase or DNA polymerase mix was diluted 2:1 with TaqStart antibody (Clontech).

3.4.8 RT-PCR

Required additional reagents:

MMLV-RT	Superscript II (200 U/µl) (RNase H^- Moloney Murine Leukemia Virus reverse transcriptase, Gibco, Eggenstein, Germany) with supplied buffer and DTT (0.1 M)
Oligo dT	Oligo dT (100 µM) (Pharmacia)
H_2O_{DEPC}	

In RT-PCR, the cDNA PCR template is produced from RNA by reverse transcription with reverse transcriptase after priming with a poly dT oligonucleotide which is complementary to the poly A tail common to most mRNAs. For higher yields, mutated MMLV-RT devoid of the otherwise inherent 3'-5' RNase H activity was deployed.

One to two micrograms of total RNA were denatured for 5 min at 70°C in 12 µl H_2O_{DEPC} containing 100 pmol (1 µl) oligo dT (100 µM) and put on ice. After adding 4 µl MMLV-RT First Strand buffer, 1 µl dNTP (25 mM each) and 2 µl DTT (0.1 M), the primer was annealed for 2 min at 42°C, 1 µl MMLV-RT added and the reaction incubated for 1 h at 42°C. After inactivation of the enzyme for 10 min at 70°C, 0.5-1 µl of the cDNA were used as a template for PCR.

3.4.8.1 SMART-RACE

Rapid amplification of cDNA ends (RACE) allows to determine the sequence of mRNA ends by adding known sequences to the cDNA ends during or prior to reverse transcription. In conjunction with nested gene-specific primers and primers complementary to the added terminal sequence portions, the mRNA section between these primer pairs can be amplified by PCR, cloned and sequenced. To determine the 5'-ends of particular transcripts, and thus the transcription start site, SMART-RACE was performed according to the manufacturer's instructions. This RACE variant takes advantage of the 3'-terminal nucleotide transferase activity of MMLV-RT which adds 3-5 nucleotides of predominantly dC to the 3'-ends of the reverse transcripts. A "SMART" oligonucleotide, containing a terminal stretch of dG residues then can anneal to the 3' dC cDNA overhang and serve as additional template for the reverse transcriptase which switches template and extends the cDNA, adding a copy of the SMART oligo to its 3' end containing primer binding sites for subsequent nested PCRs. After carrying out nested PCRs with gene-specific primers vs. SMART-specific, the PCR products were cloned into the pCR TOPO 2.1 vector (see 3.6.4) and sequenced with M13 primers.

3.4.9 Precipitation of DNA using PEG

Required reagents:

PEG-mix	26.2 g	**(26.2 %)**	PEG 8000
	20 ml	**(0.67 M)**	NaOAc pH 5.2 (3 M)
	660 µl	**(0.67 mM)**	MgCl2 (1 M)
	add H_2O to 250 ml		

100% Ethanol

To precipitate DNA from small volumes, e.g. PCR reactions or endonuclease digestion, one volume of PEG-mix was added to the DNA-containing solution, vortexed and incubated for 10 min at RT. After centrifugation (10 min, 16000x*g*, RT), the supernatant was discarded and the precipitated DNA (often only visible as a faint

smear) was washed by carefully adding 200 µl 100% EtOH to the tube wall opposite of the (often invisible) pellet, centrifugation (10 min, 16000x*g*, RT) and careful removal of the supernatant. The pellet was dried and resuspended in H_2O at half to three-quarters of the initial volume.

3.4.10 PCR-based Site-Specific Mutagenesis

For site-specific mutagenesis of single sites in the MCP-4 promoter, two overlapping fragments containing the desired mutation at their ends were generated by PCR using sense/antisense mutant primers (PXS/PXAS=CpG1M1s/as, M2s/as), which contained the mutation in the center of the oligonucleotide together with fragment-specific (outer) primers (GS/GAS=MCP-4pPGL3s/MCP-4pAS) featuring *Nhe*I and *Bgl*II restriction sites for directional cloning in two separate reactions. As template, an MCP-4 promoter fragment ranging from –1121 bp to +8 bp previously cloned in pGL3-Basic using MCP-4pS/AS was used. The PCR products were gel-extracted together to give 20 µl of gel extract (GE) which served as template for a second PCR employing the outer primers to assemble the two fragments at their overlapping portion. The ensuing PCR product with the lowest cycle number as above, was PEG-precipitated, endonuclease-digested to create sticky ends, gel-extracted, cloned directionally into the *Bgl*II/*Nhe*I site of the pGL3-Basic reporter and sequenced. To ensure incorporation of the mutation into the PCR product, the first round of PCRs was performed with *Taq* polymerase which does not possess a 3'->5' proofreading activity. For maximum accuracy, the second PCR was carried out using a DNA polymerase mix (Expand High Fidelity, Roche) with proofreading activity.

PCR-Reactions:	**PCR 1**	**PCR 1'**	**PCR 2**
DNA template	1 µg	1 µg	20 µl GE PCR1+1'
10x Taq Buffer	10 µl	10 µl	-
10x HiFi Buffer	-	-	10 µl
dNTP (10mM each)	2 µl	2 µl	2 µl
GS (10 pmol/µl)	5 µl	-	5 µl
GAS (10 pmol/µl)	-	5 µl	5 µl
PXS (10 pmol/µl)	-	5 µl	-
PXAS (10 pmol/µl)	5 µl	-	-
H_20	to 98 µl	to 98 µl	to 98 µl
Taq+Start-Ab	2 µl	2 µl	-
HiFi+Start-Ab	-	-	2 µl

To avoid producing amplification errors due to high cycle numbers, PCRs were performed in quadruplicates for 6, 9, 12 and 15 cycles and the product generated by the lowest number of cycles was used for the rest of the procedure. For each PCR,

the 100 µl master mix was divided into four 25 µl aliquots, one of which was removed after each of the above numbers of cycles and later placed back in the cycler for the terminal elongation step. PCRs were performed on an MJ research PTC 200 thermocycler (Biozym) using the following program:

93°C, 30 s; (6/9/12/15)x(93°C, 15 s; 65°C, 15 s; 72°C, 45 s); 72°C, 5 min; 15°C, ∞.

3.4.11 Genome Walking

To obtain the sequence upstream of the Hep27 transcription start in DCs, genome walking was performed using the GenomeWalker Kit (Clontech) according to the manufacturer's instructions.

Genome walking enables cloning of genomic sequences adjacent to a known genomic site. The method encompasses PCR amplification of genomic fragments using a primer specific for the known site and a primer complementary to the adapters that have been ligated to both ends of genomic fragments prepared by restriction endonuclease-digestion of genomic DNA. Five genomic libraries prepared with a different enzyme were screened with nested PCRs.

The five primary PCRs employing the outer AP1 (supplied) and Hep27GWouter primers were performed on an MJ Research PTC-200 Thermal Cycler with a modified cycling program:

7x(94°C, 5 s; 72°C, 3 min); 32x(94°C, 5 s; 67°C, 3 min); 67°C, 5 min.

The five nested PCR were carried out with the inner AP2 (supplied) and Hep27GWinner primers as follows:

5x(94°C, 5 s; 72°C, 3min); 22x(94°C, 5 s; 67°C, 3 min); 67°C, 4 min.

The ensuing PCR products were visualised on a 1.5% agarose/TAE gel, PEG-precipitated and sequenced from both ends using the inner AP2 and Hep27GWinner primers.

3.4.12 Preparation of Genomic DNA and Bisulfite Sequencing

Required reagents:

NaOH (3 M) 1.2 g (3 M) NaOH
add H_2O to 10 ml, *prepare fresh before use*
Hydroquinone (0.4 M) 0.44 g (0.4 M) Hydroquinone
add H_2O to 10 ml, *prepare fresh before use*

Bisulfite (3.8 M $NaHSO_3$) 4.5 g (3.8 M) $Na_2S_2O_5$ (Metabisulfite)
dissolve in 5 ml H_2O, adjust pH to 5.0 with NaOH (3 M), add H_2O to 10 ml

Ammonium acetate (10 M)

Genomic DNA from various cell types was prepared using the Blood and Cell Culture DNA Midi Kit (Qiagen). Modification of DNA with sodium bisulfite, leading to conversion of unmethylated cytosine residues to uracil while leaving 5-methylcytosine residues intact (Frommer *et al.*, 1992) was performed as follows.

5 µg genomic DNA in 50 µl TE were denatured with 5.5 µl NaOH (3 M) at 37°C for 15 min. After the addition of 540 µl sodium bisulfite (3.8 M) and 15 µl hydroquinone (0.4 M), samples were mixed, divided into 6 aliquots and covered with mineral oil. Incubation was performed in a PCR cycler (95°C, 3 min, 55°C, 57 min, 5 cycles). Samples were combined after treatment, DNA was recovered in 100 µl H_2O using the Wizard DNA Clean-Up System (Promega) and desulfonated by the addition of 11 µl NaOH (3M) and subsequent incubation at 37°C for 15 min. The DNA was then precipitated using 44 µl ammonium acetate (10 M) and 390 µl ice-cold 100% ethanol and resuspended in 50 µl TE. 5 µl of DNA were amplified in individual nested PCR reactions for both strands using the following primers: sense strand, first round: sS, sAS; sense strand, second round: s2S, as2S; anti-sense strand, first round:, asAS; anti-sense strand, second round: as2S, as2AS.

Outer PCR mixture (50 µl each)

5 µl	**(1x)**	PCR buffer (10x)
1 µl	**(0.2 mM)**	dNTP (10 mM each)
2 µl	**(0.4 µM)**	sense primer (10 µM)
2 µl	**(0.4 µM)**	antisense primer (10 µM)
1 µl	**(3.3 U)**	*Taq* DNA polymerase (5 U/µl):TaqStart Ab (Clontech) 2:1
34 µl		H_20_{USB}
5 µl	**(0.5 µg)**	Bisulfite-treated genomic DNA as template

The nested PCRs were set up correspondingly with 0.5 µl outer PCR product as template and additional H_2O to give 50 µl/reaction.

PCRs were performed on an MJ research PTC 200 thermocycler (Biozym) under the following conditions:

93°C, 30 s; 35x(93°C, 15 s; 55°C, 15 s; 72°C, 70 s); 72°C, 5 min; 15°C.

Products from the nested PCR were PEG-precipitated (see 3.4.9) and sequenced using the nested PCR primers to prime the sequencing reaction.

3.4.13 DNA Sequencing and Sequence Analysis

Sequencing was performed by dideoxy termination (Sanger *et al.*, 1977) using fluorescent dye-labeled terminators (Dye Deoxy Terminator Cycle Sequencing Kit, Applied Biosystems, Weiterstadt, Germany) according to the manufacturer's instructions on an Applied Biosystems DNA sequencing system model 373A.

Database searches in GenBank were performed with BLAST v2.0 at the NCBI (URL: http://www.ncbi.nlm.nih.gov/BLAST/), to identify putative transcription factor the MatInspector software (Quandt *et al.*, 1995) from Genomatix Software GmbH (URL: http://genomatix.gsf.de/) was employed. DNA sequences were analyzed for known repetitive elements using the RepeatMasker program (Smit and Green, 2001) (URL: http://ftp.genome.washington.edu/cgi-bin/RepeatMasker).

3.5 RNA

3.5.1 RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction

Required solutions and materials:

Solution D	47.2 g	**(4 M)**	Guanidine thiocyanate (GTC)
	2.5 ml	**(25 mM)**	Sodium citrate 1 M / HCl, pH7.0
	1.67 ml	**(0.5 %)**	Sodium N-lauroylsarcosine solution (30%)
	add H_2O_{DEPC} to 100 ml, *store at 4°C for up to 3 months*		
	add 7.2 µl/ml β-mercaptoethanol (fume hood) directly before use		
2 M Acetate	16.4 g	**(2 M)**	$NaOAc{\cdot}3H_2O$
	dissolve in 40 ml H_2O_{DEPC}, adjust to pH 4.0 mit 2 M HAc,		
	add H_2O_{DEPC} to 100 ml		
Phenol	unbuffered, saturated with H_2O_{DEPC}:		
	melt redistilled phenol in water bath and add		
		0.1%	8-Hydroxyquinoline
	2x	**40%**	H_2O_{DEPC}
	shake well and let sit overnight (top water layer should be ¼ of the phenol phase)		

$CHCl_3$/IAA (49:1)	2 ml 98 ml	1 Vol 49 Vol	Isoamyl alcohol Chloroform
100% Isopropanol	*store at 4°C*		
80% Ethanol	80 ml add H_2O_{DEPC} to 100 ml, *chill on ice*	**(80 %)**	Ethanol

Up to $1.5x10^7$ cells were lysed per 1 ml of solution D, adherent cells directly in the culture vessel followed by scraping with a sterile rubber policeman, suspension cells after pelleting, discarding the supernatant and resuspending in the residual medium. The lysate was transferred to sterile 14 ml centrifuge tubes (volumes below 850 µl were processed in 2 ml micro centrifuge tubes), DNA sheared by passing 10 times through a 20G (0.9 mm x 40 mm) needle fitted to a syringe and 0.1 volumes 2M acetate were added to the lysate which at this point could be stored at -20°C. For each 1 ml solution D used, 1 ml phenol and 0.2 ml $CHCl_3$/IAA were added, and the RNA extracted into the aqueous phase by vigorous shaking, phase separation on ice for 15 min and centrifugation (20 min, 1000x*g*, 4°C). The upper aqueous phase was carefully aspirated without disturbing the protein-containing interphase and combined with an equal volume of cold 100% isopropanol in a fresh tube. Total RNA was precipitated by mixing thoroughly, incubation at –20°C for at least 1 h and centrifugation for 20 min at >10000x*g* and 4°C. The supernatant was discarded, the RNA pellet dissolved in 0.3 ml per 1 ml lysate of solution D, transferred to a sterile 1.5 ml screw-cap micro centrifuge tube and precipitated a second time by adding an equal volume of isopropanol, thorough mixing, incubation for > 1h at –20°C and centrifuging for 20 min at >10000x*g* and 4°C. The supernatant was discarded and the pellet was washed twice with 1 ml each of 80% EtOH (15 min, 10000x*g*, 4°C), air-dried and dissolved in H_2O_{DEPC}.

RNA yield and purification efficiency was determined on a spectrophotometer (an OD_{260} of 1 corresponds roughly to 40 µg/ml RNA, OD_{260}/OD_{280} < 1.8-2.0 indicates protein contamination, OD_{260}/OD_{230} < 2 indicates GTC carry-over), RNA integrity was assessed by running 0.5 µg of RNA on an agarose/formaldehyde gel (intact RNA has 28S:18S rRNA band intensity ration of 2:1, and the highest RNA density around 2 kb). Modified from Sambrook *et al.* (Sambrook *et al.*, 1989).

Alternatively, for Northern blots, RNA was prepared using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions.

3.5.2 CsCl purification of RNA

Required solutions and materials:

Solution D	β-*mercaptoethanol added directly before use,* see 3.5.1	
CsCl solution (d=1.69 g/ml)	96 g **(5.7M)**	CsCl
	2.0 ml **(0.01 M)**	EDTA (0.5 M), pH7.0
	add H_2O to 100 ml	
	add 100 µl **(0.1 %)** *overnight at RT, autoclave*	DEPC *(fume hood), shake well, allow to sit*
3 M Acetate	24.6 g **(3 M)**	NaOAc, pH 5.2 with 3 M HAc,
	dissolve in 40 ml H_2O_{DEPC}	
	add H_2O_{DEPC} to 100 ml	

80% Ethanol
Polyallomer ultracentrifuge tubes Beckman, Munich, Germany

For the construction of cDNA representations, RNA purified by CsCl gradient centrifugation proved to be superior to the RNA isolated by the standard acid phenol extraction method.

Up to 100x10^6 cells were lysed in 6 ml solution D (1-1.5 Mio cells/ml). Suspension cells were centrifuged (8 min, 300x*g*, 4°C), the supernatant discarded and the pellet resuspended in residual medium before adding solution D. Adherent cells were lysed directly in the tissue culture vessel in 2-3 ml solution D/160 cm^2 after medium removal and scraped with a sterile rubber policeman. Lysates were homogenized and nuclear DNA sheared by passing the lysates 10 times through a 20G needle. The samples were layered onto 5 ml CsCl solution in bottom-labeled ∅14 cm ultracentrifuge tubes pre-rinsed with solution D and the tubes were filled to within 4-5 mm of the top and weight-matched within 10 mg with solution D. After centrifugation for 18 h at 35000 rpm and 20°C in an SW40 Ti or SW 41 Ti swing bucket rotor with the brake off, supernatants were carefully aspirated with a fresh Pasteur pipette from the top to approximately 1 cm above the tube bottom and the remaining liquid was poured off by inverting the tubes onto a fresh Kleenex wipe. The tube bottoms containing the RNA pellets were cut off with a hot disposable scalpel ~1 cm above the bottom and place on ice. The RNA pellets were dissolved by rinsing the tube bottoms with four portions of 100 µl H_2O_{DEPC} which were transferred to RNAse-free 1.5 ml screw cap tubes. RNA was precipitated for at least 1 h at -20°C with 40 µl of 3 M acetate and 1 ml ice-cold 100% ethanol, centrifuged (30 min, 15000x*g*, 4°C), washed once with 1 ml ice-cold 80% ethanol (15 min, 15000x*g*, 4°C), air-dried at RT

and dissolved in 20-80 µl H_2O_{DEPC}. RNA was stored at –20°C. (modified from Sambrook *et al.*, 1989). RNA yield and integrity was assessed as described under 3.5.1.

3.5.3 Poly-A mRNA Isolation

Poly-A^+ mRNA was isolated from CsCl-purified total RNA using oligo-dT-conjugated polystyrene latex beads (Oligotex beads, Qiagen), following the supplied instructions.

3.5.4 Electrophoresis of RNA in Denaturing Formaldehyde Agarose Gels

Required buffers:

MOPS (20x)	42 g	**(0.4 M)**	MOPS/NaOH, pH 7.0
	4.1 g	**(100 mM)**	NaOAc
	3.7 g	**(20 mM)**	EDTA
	add H_2O_{DEPC} to 500 ml, *store in the dark*		
RNA loading buffer	10 ml	**(50%)**	Formamide, deionized
	3.5 ml	**(2.2 M)**	Formaldehyde (37%)
	1 ml	**(1x)**	MOPS (20x)
	0.8 ml	**(0.04%)**	Bromophenol blue (1% in H_2O)
	0.2 g	**(1%)**	Ficoll 400, Pharmacia (dissolve in 2 ml H_2O)
	add H_2O_{DEPC} to 20 ml, *store in 1 ml aliquots at –20°C*		
	add 5 µl/ml EtBr (10 mg/ml) before use		

The agarose was dissolved in MOPS/H_2O_{DEPC} by heating in a microwave oven, cooled to 60°C, formaldehyde was added while stirring the solution under a fume hood and the gel was cast, mounted in an electrophoresis tank and overlaid with 1x MOPS as electrophoresis buffer. RNA samples were prepared by diluting with four volumes RNA loading buffer (1:4), denaturing for 20 min at 65°C and brief incubation on ice. Samples were centrifuged and loaded into the gel slots. Gels were run at 40-60 V; for subsequent Northern blotting, gels were run overnight at 13-16 V.

3.5.5 Northern Blot – RNA Transfer

Required buffers:

SSC (20x)	88 g	**(0.3 M)**	$Na_3Citrate{\cdot}2H_2O$/HCl, pH 7.0
	175 g	**(3 M)**	NaCl
	add H_2O to 1 l, *autoclave*		

Following separation on formaldehyde/agarose gels, the RNA was transferred to nylon membranes by capillary elution.

A nylon membrane (Magna NT, MSI) was cut to gel size, wetted with H_2O, and briefly soaked in 20x SSC. The RNA gel was placed upside-down on two layers of 20x

SSC-soaked Whatman 3MM filter papers on a glass plate, the ends of which were reaching into a buffer reservoir placed below filled with 20X SSC. The membrane was laid on top of the gel, avoiding to trap air bubbles. One corner of the membrane was marked by cutting it of and plastic stripes were placed on the membrane borders to avoid short-circuiting the capillary flow past the membrane to the two layers of 20x SSC-soaked Whatman filters which were put on top of the membrane and the plastic covers. A 6-8 cm stack of cellulose wadding was placed on top to draw the 20x SSC through the gel and the membrane. The cellulose was weighed down with a glass plate (more weight was not necessary and only led to decreased transfer efficiency for higher molecular weight RNA).

After transfer overnight, completeness of the transfer was checked under UV illumination, the gel lanes and 18S and 28S rRNA bands were marked with a soft pencil and the RNA was fixed to the membrane by UV-crosslinking with 120 mJ/cm^2 at 254 nm in a Stratalinker.

3.5.6 Radioactive Labeling of DNA

Plasmid-excised or PCR-generated DNA fragments were radioactively labeled by second-strand synthesis with Klenow fragment either random-primed or primed with an end-specific antisense primer to reduce background. For random priming, either random hexamers (Boehringer Mannheim, Germany) and 50 µCi [α-^{32}P]-dCTP or random decamers (Strip-EZ DNA Kit, Ambion, Huntingdon, UK) and 50 µCi [α-^{32}P]-dATP were used according to the supplied instructions and purified by gel filtration as described below.

For antisense-primed labeling, 25 ng DNA fragment (200-1000 bp) were heat-denatured in 10 µl H_2O for 5 min at 95°C, chilled on ice or snap-frozen in liquid nitrogen, and combined with a 3-10-fold molar excess of antisense primer, 2.5 µl 10x Klenow buffer (InViTek, Berlin, Germany), 3 µl (0.5 pmol each) dA/G/TTP (133 µM each) (Pharmacia), 5µl (50 µCi) [α-32P]-dCTP (3000 µCi/mmol)(Amersham) and 1 µl (2 U) Klenow fragment in 25 µl reaction volume. In some experiments, instead of the above nucleotides, modified dCTP and dTTP/dGTP from the Strip-EZ DNA Kit (Ambion) were used together with 50 µCi [α-^{32}P]-dATP to produce easily strippable probes for multiple successive hybridizations of the same blot with different probes. Reactions were incubated for 20-45 min at 37°C and put on ice.

18S rRNA-specific oligonucleotide was 5'-end-labeled by phosphorylation with T4 polynucleotide kinase (PNK) and [γ-^{32}P]-ATP using the 5'-end Labeling Kit from Amersham according to the manufacturer's instructions.

Unincorporated nucleotides were removed by gel filtration on a TE-equilibrated G50 Sepharose NICK column (Pharmacia). The reaction volume of the labeling reaction was brought to 50 µl with TE buffer, one microliter of the reaction was removed for determining labeling efficiency and the remaining solution was applied to the column. 350 µl TE were added after the solution had entered the column, the flow-through was discarded and the labeled probe was collected by eluting with 400 µl TE. To determine the specific activity and the efficiency of the labeling reaction, 5 µl probe solution and the 1 µl aliquot taken from the diluted labeling reaction were added to water-filled scintillation tubes and the Cherenkov counts were measured in a β-counter (TRI-CARB 1600 TR, Canberra-Packard, Frankfurt, Germany). Labeling efficiencies were frequently between 10-30% of the radioactive input, ranging to up to 60% with random priming.

3.5.7 Northern Blot Hybridization

Required buffers:

Church buffer	250 ml	**(0.5 M)**	Phosphate buffer (1M), pH7.2
	175 ml	**(7%)**	SDS (20%)
	add H_2O to 500 ml		
Wash buffer	5 ml	**(0.1x)**	SSC (20x)
	50 ml	**(1%)**	SDS (20%)
	add H_2O to 1 l		
tRNA		**25 mg/ml**	tRNA from Baker's Yeast
	1 ml aliquots in H_2O, *heat-denature for 5 min in boiling water and put on ice*		
Herring Sperm DNA		**10 mg/ml**	Sonicated, Promega, Mannheim, Germany
	heat-denature for 5 min in boiling water and put on ice		
Denhardt's reagent	0.5 g	**(1%)**	Ficoll 400, Pharmacia
	0.5 g	**(1%)**	Polyvinylpyrrolidone
	0.5 g	**(1%)**	BSA (Fraction V), Sigma
	add H_2O to 50 ml		

Northern blot membranes were washed for 30 min at 65°C in wash solution and prehybridized for at least 45 min in Church buffer at the temperature later used for hybridization, i.e. 65°C for cDNA and 55°C for 18S rRNA oligonucleotide. To reduce background, 150 µg/ml tRNA and 100 µg/ml Herring sperm DNA were added to the pre-heated Church buffer. For Hep27 and MCP-4 cDNA hybridizations, the Church

buffer contained additional 1x and 5x Denhardt's reagent, respectively. The radioactive probe was melted for 10 min at 95°C and 0.5-5x10^6 cpm/ml were added directly to the prehybridization solution. Blots were hybridized overnight, rinsed once with wash buffer and washed with fresh wash buffer for 10-15 min each at increasing temperatures, starting at RT and gradually increasing the temperature from 42°C to up to 60°C in 5-6°C steps until the overall radioactivity of the blot was between 0.1-5 Bq/cm^2 as measured with a Geiger counter. In most cases, the final washing temperature was below 55°C. Blots were heat-sealed in plastic bags, fixed with adhesive tape in an X ray cassette with intensifying screen and exposed to autoradiography film at –80°C overnight to up to three weeks.

3.6 Molecular Cloning

3.6.1 Bacterial Culture

Required solutions:

LB medium	10 g	**(1%)**	NaCl
	10 g	**(1%)**	Bacto Tryptone (Difco)
	5 g	**(0.5%)**	Yeast extract
	add H_2O to 1 l, *autoclave*		
LB-agar	1.5 g	**(1.5%)**	Agar
	add LB medium to 100 ml, *dissolve by boiling,*		
	cool to 60°C and add antibiotic if required and pour into ∅10 cm Petri		
dishes.	*Store inverted at 4°C in the dark.*		
Antibiotics		**50 µg/ml**	Ampicillin (100 mg/ml)
	or	**30 µg/ml**	Kanamycin
80% Glycerol			
X-gal	40 mg	(**40 mg/ml)**	X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside)
	in 1 ml DMF, *store in a brown bottle at –20°C in the dark*		
IPTG	238 mg	(**100 mM)**	IPTG (isopropyl-β-D-thiogalactopyranoside)
	add H_2O to 10 ml, filter-sterilize and *store in 1ml aliquots at –20°C*		

E.Coli strains were streaked out on solid LB-agar with antibiotic, if necessary, were grown overnight and single colonies were picked and grown in liquid cultures overnight. For blue/white screening of insert-containing clones after transformation, 40 µl of X-gal (if TOP10F' cells were used, 40 µl of IPTG in addition to X-gal) were dispersed on the pre-warmed LB plates, plates incubated at 37°C for an additional 30 min prior to use and the transformation spread out using a Drigalski spatula. Liquid cultures were grown overnight at 37°C in LB medium with the necessary antibiotics.

Bacteria were stored in 20% glycerol by adding 500 µl liquid culture to 200 µl of 80% glycerol, mixing and freezing at –80°C.

3.6.2 Preparation of Chemically Competent *E.Coli*

Required buffers:

Ψ Broth	20 g	(2%)	Bacto Tryptone (Difco)
	5 g	(0.5%)	Bacto Yeast Extract (Difco)
	8,18 g	(0.4%)	$MgSO_4 \cdot 7H_2O$
	0.745 g	(10 mM)	KCl
	adjust to pH 7.6 with KOH		
	add H_2O to 1 l		
TfBI	6.045 g	(100 mM)	$RbCl_2$
	4.5 g	(50 mM)	$MnCl_2$
	1.472 g	(30 mM)	KOAc
	0.735 g	(10 mM)	$CaCl_2 \cdot 2H_2O$
	75 ml	(15%)	Glycerol
	adjust to pH 5.8 with 0.2 M HOAc		
	add H_2O to 500 ml, *filter sterilize and store at 4°C*		
TfBII	1.047 g	(10 mM)	MOPS/NaOH, pH 7.0
	0.605 g	(10 mM)	$RbCl_2$
	5.513 g	(75 mM)	$CaCl_2$
	75 ml	(15%)	Glycerol
	add H_2O to 500 ml, *filter sterilize and store at 4°C*		

The desired bacterial strain was streaked out on LB agar, grown overnight and a single bacterial colony was picked into 5 ml ψ Broth. Bacteria were grown at 37°C with shaking to an OD_{550} of 0.3, and the 5 ml culture was used to inoculate 100 ml ψ Broth prewarmed to 37°C. After growing to an OD_{550} of 0.48, the cells were chilled on ice and pelleted at 1300x*g* and 4°C for 5-10 min. The pellet was loosened up by vortexing and resuspended in 30 ml ice-cold TfBI. After incubation on ice for 0-5 min, cells were collected by centrifugation (5 min, 4000x*g*, 4°C) and resuspended in 4 ml ice-cold TfBII. The suspension was dispensed into 50 µl aliquots and frozen at –80°C.

3.6.3 Transformation of Chemically Competent *E.Coli*

Required:

SOC medium	20 g	(2%)	Bacto Tryptone (Difco)
	5 g	(0.5%)	Bacto Yeast Extract (Difco)
	0.6 g	(10 mM)	NaCl
	0.2 g	(3 mM)	KCl
	add H_2O to 1 l, *autoclave and add to the cooled solution:*		
	10 ml	(10 mM)	$MgCl_2$ (1 M), *sterile filtered*
	10 ml	(10 mM)	$MgSO_4$ (1 M), *sterile filtered*
	10 ml	(20 mM)	Glucose (2 M), *sterile filtered*

Chemically competent *E.Coli* (50 µl) were thawed on ice, 1-25 ng of plasmid DNA in 2-5 µl volume were added and the suspension was mixed gently and incubated on ice for 20 min. Cells were heat-shocked in a water bath at 42°C for 60 s, immediately cooled on ice for 2 min and 250 µl SOC medium were added. To express the resistance, bacteria were incubated for 1 h at 37°C with shaking and 50-150 µl of the transformation were plated and incubated overnight at 37°C on LB-agar containing the antibiotic necessary for selection of transformed cells.

3.6.4 Cloning

DNA fragments to be cloned were prepared by PCR from genomic DNA or cDNA. For directional cloning, restriction sites were introduced by adding the appropriate recognition sequences to the primer sequences, and, depending on the enzyme, an extra arbitrary overhang to allow for efficient endonuclease digestion.

The fragments were either cloned as described below and excised from the cloning vector after sub-cloning or PEG-precipitated and the precipitate as well as the cloning vector were digested with the necessary endonucleases in 10-30 µl reactions at 37°C. To suppress self-annealing of the vector, the vector ends were dephosphorylated by adding 1 µl (1 U) of calf intestinal phosphatase CIP to the digestion reaction and incubating for an additional 30 min at 37°C. The cut fragment and vector were gel-purified (see 3.4.4) , and combined in a 10 µl ligation reaction at a 3-fold molar excess of insert to vector, using 25 –50 ng of vector. To control for self ligation, an additional vector-only ligation reaction was included. Ligation was carried out overnight at 16°C with 1 U T4 DNA ligase in ligase buffer and 2 µl of the reaction were used to transform chemically competent *E.Coli*.

PCR products were alternatively cloned in TOP10F or TOP10F' cells using the TOPO-TA Cloning Kit (Invitrogen) with the supplied pCR2.1 or pCRII vectors according to the manufacturer's instructions.

Successful insertion of the fragment into the vector was checked either by performing PCR directly from single colonies with vector-specific primers such as M13 fwd/rev, or by preparing plasmid DNA from liquid cultures of insert-containing white colonies after blue/white screening. To check for correct insertion and sequence integrity, PCR products and plasmid constructs were sequenced using vector-specific primers.

3.6.5 Plasmid DNA Preparation

Plasmids were isolated from liquid cultures of transformed bacteria using QIAGEN Plasmid Kits (QIAGEN) according to the supplied instructions.

3.7 Protein Methods

3.7.1 Nuclear Extraction Procedure

Reagents (*prepare fresh before use, keep on ice*):

Buffer A (hypotonic)	100 µl	**(10 mM)**	HEPES (1 M)/KOH, pH 7.9 @4°C
	15 µl	**(1.5 mM)**	$MgCl_2$ (1 M)
	100 µl	**(10 mM)**	KCl (2 M)
	50 µl	**(0.5 mM)**	DTT (100 mM)
	100 µl	**(1.0 mM)**	PMSF (100 mM)
	10 µl	**(2 µg/ml)**	Aprotinin (2 µg/µl)
	25 µl	**(2.5 µg/ml)**	Leupeptin (1 µg/µl)
	10 µl	**(1 µg/ml)**	Pepstatin (1 µg/µl)
	5 µl	**(10 µg/ml)**	E46 (20 µg/µl)
	10 µl	**(20 µg/ml)**	Antipain (20 µg/µl)
	40 µl	**(80 µg/ml)**	Chymostatin (20 µg/µl in DMSO)
	100 µl	**(1 mM)**	Sodium o-vanadate (100 mM)
	add H_2O to 10ml		
	add protease and phosphatase inhibitors before use		
Buffer B	Buffer A		
		0.5 %	NP-40
	use lowest possible concentration to lyse cells		
Buffer C	20 µl	**(20 mM)**	HEPES (1 M) pH 7.9 @4°C
	1.5 µl	**(1.5 mM)**	$MgCl_2$ (1 M)
	84 µl	**(420 mM)**	NaCl (5 M)
	310 µl	**(25 %)**	Glycerol (80 %)
	0.4 µl	**(0.2 mM)**	EDTA (0.5 M) pH 8.0
	5 µl	**(0.5 mM)**	DTT (100 mM)
	10 µl	**(1.0 mM)**	PMSF (100 mM)
	1 µl	**(2 µg/ml)**	Aprotinin (2 µg/µl)
	5 µl	**(5.0 µg/ml)**	Leupeptin (1 µg/µl)
	1 µl	**(1 µg/ml)**	Pepstatin (1 µg/µl)
	1 µl	**(20 µg/ml)**	E46 (20 µg/µl)
	2.5 µl	**(50 µg/ml)**	Antipain (20 µg/µl)
	8.0 µl	**(160 µg/ml)**	Chymostatin (20 µg/µl in DMSO)
	5.0 µl	**(0.5 mg/ml)**	Pefabloc SC (100 µg/µl)
	10.0 µl	**(1 mM)**	Sodium o-vanadate (100 mM)
	add H_2O to 1ml,		
	add protease and phosphatase inhibitors fresh before use		

The employed nuclear extraction method was a modification of the method used by Osborn *et al.* (Osborn *et al.*, 1989): Cells are first suspended in a hypotonic buffer to osmotically enlarge the cells and lyse them by adding the weak detergent NP-40, which leaves the nuclei intact. Soluble nuclear proteins are subsequently extracted by applying a hypertonic buffer and high centrifugal force to "press out" the nuclei.

Cells were harvested, centrifuged (8 min, 300x*g*, 4°), washed three times in ice-cold PBS, resuspended at 10-20 Mio cells/ml in buffer A dispersed into eppendorf cups in 1 ml aliquots. After letting the cells swell for 3 min on ice, they were pelleted in a cooled microcentrifuge (6 min, 400x*g*, -9°C) and resuspended in 150-200 µl buffer A with a pipette. An equal volume of buffer B was added rapidly, the samples vortexed immediately for 1 s at 1400 rpm and incubated for 5-15 min on ice. When most of the nuclei had been released (as checked under a microscope), the nuclei were spun down (6 min, 600x*g*, -9°C), and washed once with 300-500 µl of buffer A, if necessary. After careful removal of the supernatant, depending on the size of the nuclear pellet 30-60 µl of buffer C were swiftly added and the tube was flicked until the nuclei were completely resuspended. The nuclei were incubated on ice for 8 min, centrifuged at maximal speed (10 min, 16000x*g*, -9°C) and the supernatant, representing the nuclear extract was aliquoted, snap frozen on dry ice and stored at –80°C.

3.7.2 BCA Protein Assay

Required reagents:

Working reagent	50 Volumes	Bicinchoninic acid (BCA) (Sigma)
	1 Volume	$CuSO_4 \cdot 5\ H_2O$ in H_2O (4% (w/v)) (Sigma)
	prepare fresh, stable for at least 1 day	
Protein standard	BSA, 6 concentrations from 8 mg/ml to 0.125 mg/ml, sequential 1:2 dilutions	

Duplicates of each protein sample were assayed in 96-well microtiter plates. Per well, 200 µl working reagent and 10 µl sample or protein standard were combined, incubated for 30 min at 37°C, cooled to RT for 10 min and the absorbance was measured at 540 nm on a plate reader. The protein concentration of each sample was calculated using the parameters obtained from linear regression of the protein standard values.

3.7.3 Electrophoretic Mobility Shift Assay

Required buffers and material:

10x Binding buffer	20 µl	(**200 mM**)	HEPES (1 M)/KOH, pH 7.9 at 4°C
(*freshly prepared*)	2 µl	(**20 mM**)	$MgCl_2$ (1 M)
	30 µl	(**600 mM**)	KCl (2 M)
	10 µl	(**10 mM**)	DTT (100 mM)
	2 µl	(**10 mM**)	EDTA pH 8.0 (500 mM)
	36 µl H_2O to give 100 µl		

10x Gel buffer	90.8 g add H_2O to 500 ml	(**1.5 M**)	Tris/HCl pH 8.8
10x electrophoresis buffer TGE (10x) (pH 8.3)	15.14 g 72.07 g 2 ml add H_2O to 500 ml	(**250 mM**) (**1.92 M**) (**2 mM**)	Tris Glycine EDTA pH 8.0 (500 mM)
Nucleotide mix	1 µl each 97 µl	(**1 mM each)**	dNTP-*dXTP* (100 mM) Pharmacia H_2O
dXTP(α-^{32}P)	50 µCi	(**3000µCi/mmol**) Amersham, Braunschweig, Germany	
NICK column	Sephadex G50 size exclusion column, Pharmacia, Freiburg, Germany		
Poly (dI-dC)	Poly(dI-dC) (1µg/µl)		
5% Acetic acid			

In the nucleus, gene expression is controlled by DNA-binding transcription factors which bind to characteristic DNA motifs to initiate or repress transcription.

The electrophoretic mobility shift assay (EMSA) is a powerful tool for evaluating DNA-protein interactions. It is based on the principle that when subjected to electrophoresis in native polyacrylamide gels, free DNA has a different electrophoretic mobility than a DNA-protein complex.

Double-stranded DNA probes were prepared by annealing 100 pmol complementary single-stranded synthetic oligonucleotides each (sense/antisense, featuring 5'-overhangs of 3-6 bp for labeling by fill-in with Klenow fragment) (1 µl of 100 µM solution each) in 20 µl H_2O. The mixture was heated for 10 min to 10-15°C above the calculated melting point of the double-stranded DNA oligonucleotides, annealed with high stringency for 45 min at 5°C below the melting point and then allowed to cool to RT to give a 5 µM (5pmol/µl) solution of double-stranded oligonucleotide. The probe was end-labeled with ^{32}P using Klenow fragment by incubating 5 pmol (1 µl) of 5' overhang-containing double-stranded oligonucleotide with radioactively labeled dXTP complementary to one or more bases of the overhang in a reaction mixture as given below for 45 min at 37°C.

Labeling Reaction (20 µl)

10 µl		H_2O
1 µl	(5 pmol)	double-stranded oligonucleotide (5 pmol/µl)
2 µl		Klenow buffer (10x)
1 µl		Nucleotide mix (1 mM each) without dXTP
5 µl	(50 µCi)	α-[^{32}P]-dXTP (3000 µCi/mmol)
1 µl	(2 U)	Klenow fragment

The reaction was diluted with 30 µl TE and the labeled probe was separated from unincorporated oligonucleotides by passing over a NICK column. The column was flushed with 350 and 400 µl of TE buffer and fractions of 3 drops were collected in micro-reaction tubes. After the initial 350 µl portion had run through, the radioactive probe typically eluted in the 2nd and 3rd fraction. The two fractions containing the highest amount of radioactivity were combined and directly used for gel shifts or stored at –20°C for up to two weeks.

The non-denaturing PAA gel was cast at least 2 h before the experiment as given below, AA concentrations ranging from 5-9% to achieve maximal resolution. The discontinuous buffer system with TGE as electrophoresis buffer and Tris buffer in the gel served to produce focussed bands and to further enhance resolution.

15 ml PAA gel (9 % AA, other concentrations accordingly)

1.5 ml		Gel buffer (10x)
4.5 ml	**(9%)**	Acrylamide (30%,)
9 ml		H_2O
75 µl		Ammonium peroxodisulfate (10%), *fresh*
15 µl		TEMED

Binding reactions were prepared by combining the following in 1.5 ml micro-reaction tubes:

Binding reaction (10 µl)

1.5 µl	**(12%)**	Glycerol (80%)
1 µl	**(1x)**	10x binding buffer
0.5 µl	**(0.05 µg/µl)**	Poly (dI-dC) (1µg/µl)
1 µl		Nuclear extract (~5 µg/µl)
1 µl	**(25 fmol)**	Labeled probe (~25 fmol/µl)
H_2O to 10 µl		

To check specificity of nuclear factor binding, cold competitor, i.e. unlabeled probe, was added (0.5 µl (2.5 nmol) double-stranded probe (5 pmol/µl)) prior to adding the labeled probe in control binding reactions. Purity of the labeled probe preparation was checked by omitting nuclear extract. After 15 min incubation at RT, samples were loaded into the wells, and 5 µl of a 0.02% bromophenol blue solution were loaded into a separate well to track the position of unbound probe. The gel was run with 1x TGE as electrode buffer for 2½ h at 160 V, until the bromophenol blue marker reached the bottom of the gel. The gel was removed, fixed in 5% HOAc for 10 min, mounted onto Whatman 3MM filter paper and dried under vacuum for 1 h at 80°C. Dried gels were autoradiographed overnight at RT without intensifying screen.

3.7.4 Immunoprecipitation

Required buffers and chemicals:

TBS (2×)	4.8 g	**(40 mM)**	Tris / HCl, pH 8.0
	18 g	(**0.3 M)**	NaCl
	0.74 g	**(2 mM)**	EDTA
	50 mg	**(0.05%)**	NaN3
	add H_2O to 1000 ml		
Protease inhibitors:			
PMSF	1.74 g	**(100 mM)**	PMSF
	dissolve in 100 ml Isopropanol		
Aprotinin	10 mg/ml	Aprotinin in H_2O	
	store 50 µl aliquots at –20°C		
Leupeptin	0.5 mg/ml	Leupeptin in H_2O	
	store 100 µl aliquots at –20°C		
Wash buffer	5 ml	**(1x)**	TBS (2x)
	100 µl	**(1 mM)**	PMSF
	2 µl	**(2 µg/ml)**	Aprotinin
	10 µl	**(0.5 µg/ml)**	Leupeptin
	add H_2O to 10 ml		
PGS	Protein G-Sepharose 4 Fast Flow; Amersham-Pharmacia, Freiburg, Germany		

Immunoprecipitation was performed with supernatants of $3x10^6$ DCs or teflon bag-cultured monocyte-derived macrophages after overnight culture in 6-well cell culture plates in 2 ml medium. The supernatants were supplemented with PMSF (1mM), aprotinin (2µg/ml) and leupeptin (0.5 mg/ml) and immunoprecipitation was performed by adding 30 µg rabbit polyclonal anti-MCP-4 antibody (15 µg/ml) (Pepro Tech EC, London, England) for 2 h at 4°C and precipitating with 30 µl Protein G Sepharose (Pharmacia) for 1 h at 4°C on a rotating mixer. Immunoprecipitates were washed 3 times with 1 ml wash buffer (8 min, 730x*g*, 4°C), diluted with 1 volume 2x SDS sample buffer, boiled for 5 min at 95°C and subjected to SDS-PAGE and subsequent Western blotting.

3.7.5 Discontinuous SDS-PAGE

Acrylamide stock	146 g		Acrylamide
solution AA (30 %)	4.0 g	**(%C=2.67 %)**	BIS
	add H_2O to 500 ml		
Separating gel buffer	90.83 g	**(1.5 M)**	Tris / HCl, pH 8.8
	add H_2O to 500 ml		
Stacking gel buffer	30 g	**(0.5 M)**	Tris / HCl, pH 6.8
	add H_2O to 500 ml		

SDS (10 %)	10 g	**(10 %)**	SDS
	add H_2O to 100 ml		
Tris buffer TP (1.25 M)	13 g	**(1.25 M)**	Tris / HCl, pH 6.8
	add H_2O to 100 ml.		
Sample buffer (2x)	10 ml	**(20 %)**	Glycerol
	5 ml	**(125 mM)**	TP (1.25 M)
	2 g	**(4 %)**	SDS
	5 ml	**(10 %)**	2-Mercaptoethanol
	10 mg	**(0.02 %)**	Bromophenol blue
	add H_2O to 50 ml		
Ammonium persulfate AP (10 %)	100 mg	**(10 %)**	Ammonium persulfate
	add H_2O to 1 ml		
	(frisch hergestellt)		
Isobutanol	saturated with H_2O		
Laemmli buffer (5×)	10 g	(40 mM)	Tris
	144g	(0.95 M)	Glycine
	10 g	(0.5 %)	SDS
	add H_2O to 2000 ml		
TEMED			

Table 3.1 SDS-PAGE gel mixtures

Stock solutions	**Separating gel**				**Stacking Gel**
Final AA concentration	7.5 %	10 %	12 %	15 %	5 %
Stacking gel buffer					25 ml
Separating gel buffer	25 ml	25 ml	25 ml	25 ml	
SDS (10 %)	1.0 ml	1.0 ml	1.0 ml	1.0 ml	
AA (30 %)	25 ml	33 ml	40 ml	50 ml	16.65 ml
H_2O	add H_2O to 100 ml				

The separating gel was cast the day before electrophoresis according to Table 3.1 and overlaid with water-saturated isobutanol until polymerized. The isobutanol was exchanged for separating gel buffer diluted 1:3 with water and the gel stored overnight at 4°C. The following day, the stacking gel was cast on top of the separating gel, polymerized for 1 h and the gel mounted in the electrophoresis tank, which was filled with 1x Laemmli buffer. Meanwhile, the protein samples were mixed with an equal volume 2x sample buffer, heated for 5 min to 95°C and loaded into the wells . Gels were run with 25 mA per gel for 3-4 h. (Shapiro *et al.*, 1967; Laemmli, 1970).

3.7.6 Western Blot

Required buffers and materials:

Buffer (Anode)	A	36.3 g 200 ml add H_2O to 1000 ml	**(0.3 M)** **(20 %)**	Tris (pH 10.4) Methanol
	B	3.03 g 200 ml add H_2O to 1000 ml	**(25 mM)** **(20 %)**	Tris (pH 10.4) Methanol
Buffer (Cathode)	C	5.20 g 200 ml add H_2O to 1000 ml	**(4 mM)** **(20 %)**	ε-amino-n-caproic acid pH 7.6 Methanol

Nitrocellulose membrane	Protran, Schleicher und Schuell
PVDF membrane	Immobilon P, Millipore

After separation by SDS-PAGE (3.7.5), proteins were blotted electrophoretically onto nitrocellulose or PVDF membranes using a semi-dry technique (Towbin *et al.*, 1979).

The membrane was cut to gel size, moistened with buffer B (PVDF membrane was first wet with methanol) and placed on top of three sheets each of Whatman 3MM filter paper soaked with buffer A (bottom, on the anode) and buffer B, respectively. The SDS-PAGE gel was removed from the glass plates, immersed in buffer B for 10-20 s and placed on top of the membrane. Another three sheets of Whatman paper soaked with buffer C were put on top of the gel, followed by the cathode. Protein transfer was conducted for 45 min at 0.8 mA/cm^2 gel surface area.

3.7.7 Immunostaining of Blotted Proteins

Buffers and reagents:

TTBS	1000 ml 2 ml add H_2O to 2000ml	 (0.1%)	TBS (2x) Tween-20
BLOTTO	5 g 50 ml add H_2O to 100 ml	(5%)	Low fat dried milk, Nesté, Frankfurt, Germany TBS (2x)
STTBS	required amount of (1-4%) in 50 ml TTBS		Slimfast "Schoko Royale", Sun Nutritional, Slough, England)

Blots were blocked overnight at 4°C either with BLOTTO (for MCP-4 staining) or 4% STTBS (for Hep27 immunostaining). Blots were washed three times for 10 min with TTBS and incubated at RT with primary antibody: MCP-4 was stained for 1 h with anti-MCP-4 (PeproTech) diluted 1:100 in BLOTTO, Hep27 was detected by staining

with anti-Hep27 antibody diluted 1:2000 in STTBS for 2h. After washing with three times for 10 min with TTBS, blots were developed using either HRP-conjugated goat anti-rabbit antibody (DAKO) diluted 1:800 with 5% BLOTTO for 1 h at RT (MCP-4) or HRP-conjugated goat anti-mouse antibody (DAKO) diluted 1:2000 with STTBS for 30 min at RT. Blots were washed three times for 10 min with TTBS and bound antibody was visualized using ECL (Amersham-Pharmacia, Freiburg, Germany). To control for equal protein loading of the Hep27 blots, gels run in parallel were stained with Coomassie blue (BioRad, Munich, Germany) according to the manufacturer's instructions. HepG2 whole-cell lysates were used as a positive control for Hep27 expression.

3.7.8 Flow Cytometry

Required buffers and reagents:

FACS buffer	5 ml	**(600 µg/ml)**	Immunoglobulins (60 mg/ml) (Sandoglobin, Sandoz, Basel)
	5 ml	**(0.1 %)**	Sodium azide (10 %)
	in 500 ml PBS		
Paraformaldehyde	1g	**(1 %)**	Paraformaldehyde
	in 500 ml PBS, *stir overnight at RT to clear solution*		

To characterize the phenotype of the different cell types, the cell surface expression of a number of membrane proteins was assessed by flow cytometry. For determination of surface expression, 1-5x10^5 cells per staining reaction were washed twice for 5 min with 500 µl each of ice-cold FACS buffer and immunostained for 30 min at 4°C with a selection of fluorescein-isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated antibodies chosen from a panel of mouse anti-human antibodies to CD1a, CD14, CD40, CD54, CD80, CD83, CD86, HLA-DR. Depending on the isotype, fluorochrome-labeled purified IgM or polyclonal IgG from normal mouse serum were used as isotype control. After two washing steps, cells were fixed in 500 µl 1% paraformaldehyde/PBS and flow cytometric analysis of 10000 cells per antigen was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

4 Results

4.1 Identification of genes with DC-specific expression by Representational Difference Analysis

To identify genes relevant to dendritic cell (DC) differentiation and function, the gene expression of monocyte-derived DCs versus monocytes and monocyte-derived macrophages were compared by Representational Difference Analysis (RDA) (Lisitsyn and Wigler, 1993). Originally described as a general method for finding the difference between two complex genomes, the adaptation by Hubank and Schatz (Hubank and Schatz, 1994) facilitates identification of differences in mRNA expression, i.e. analysis of the difference between the polyA$^+$ fractions of two transcriptomes. In principle, RDA modifies and extends classical subtractive hybridization (Lamar and Palmer, 1984) in which unique DNA (target) sequences present in one DNA population (tester) but not a second (driver) DNA population are enriched by depleting common sequences by mixed hybridization and removal of heteroduplexes of sequences present in both tester and driver DNA.

In RDA, enrichment is accomplished by suppression of PCR amplification of common sequences. Primer-ligated tester DNA is mixed and hybridized with an excess of primer-less driver DNA. Tester-specific target DNA with no counterpart in the driver population re-anneals and gives rise to double-stranded DNA with primer sequences on both ends, which is amplified at an exponential rate in the subsequent PCR step. Sequences common to both tester and driver population form mixed hybrids with the primer sequence only on one end and thus are only amplified at a linear rate, effectively resulting in enrichment of target sequences in the ensuing difference product. After three rounds of hybridization and amplification, 10^5-10^7-fold enrichment of target sequences can be achieved (Lisitsyn and Wigler, 1993). Figure 4.1 gives a schematic overview of the RDA principle.

For the driver cDNA representations, mRNA from freshly isolated monocytes and from monocytes after one and nine days culture towards a macrophage phenotype was pooled. To generate the tester representation, mRNA from DCs derived from monocytes of the same donor was used. RDA was performed as described under Materials and Methods.

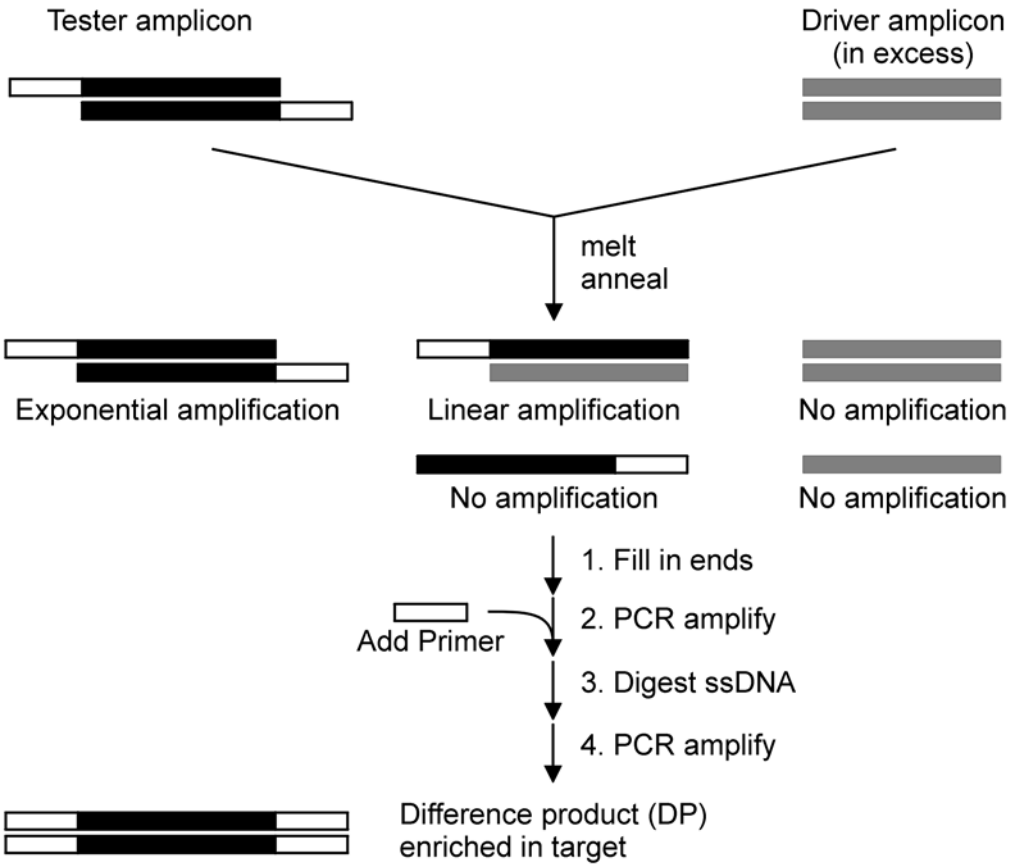


Figure 4.1 Schematic diagram of RDA.
The filled bars represent cDNA strands, tester cDNA in black, driver cDNA in gray. Open bars show the oligonucleotides which were ligated to the tester cDNA digest to facilitate suppressive amplification and the complementary primers used for subtractive PCR. ssDNA: single-stranded DNA

In parallel, the superior antigen presentation capability of the DC preparation compared to the original monocytes was confirmed by MLR. Figure 4.2 depicts the produced driver and tester representations as well the resulting difference products after each round of subtractive amplification.

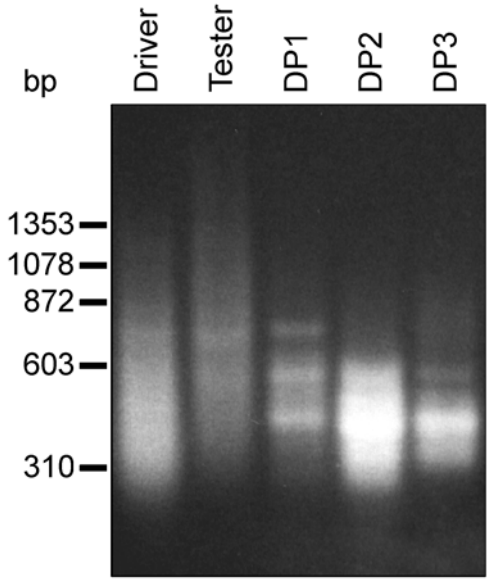


Figure 4.2 RDA of DCs *versus* monocytes and macrophages.
The driver and tester representation as well as the difference products (DP) after one, two and three rounds of hybridization and PCR amplification were separated on a 1.3% Agarose gel containing EtBr. The driver representation was derived from pooled fresh monocytes and monocytes cultured with 2% human pooled AB-serum for one or nine days. The tester representation was produced from nine day-old DCs derived from monocytes of the same donor.

The third difference product was shotgun-cloned and the DC-specificity of the inserts of 85 randomly picked clones was assessed by reverse dot blot analysis using the tester and driver representations as probes. 11 clones were excluded from further analysis because they were present in both representations (see Figure 4.3).

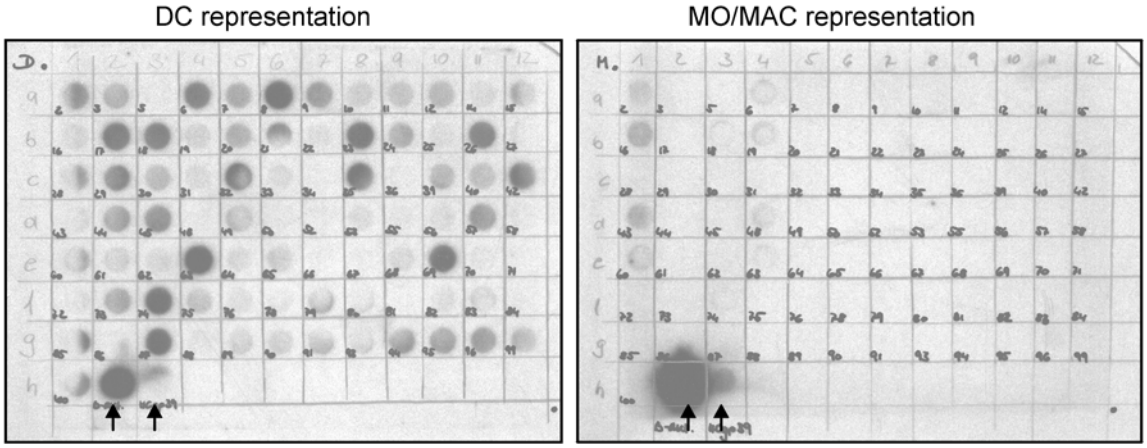


Figure 4.3 Reverse dot blot of cloned RDA fragments.
The inserts of randomly picked clones were PCR-amplified, denatured and blotted in duplicates onto two separate Nylon membranes. The indicated radioactively labeled representation was hybridized to each membrane. The arrow pairs indicate a β-actin fragment (left arrows) which served as a positive control and a fragment of human cartilage glycoprotein 39 (HCgp39) (right arrows) which is expressed late during macrophage differentiation and which was used to control for DC/macrophage phenotypes.

The remaining clones were sequenced and analyzed for homologies with known sequences available in GenBank using BLAST. Each of the 61 inserts proved to be derived from one out of six already known genes. Table 4.1 lists the number of clones mapping to each gene. The chemokine DC-CK1 has previously been described as a gene with specific expression in DCs (Adema *et al.*, 1997) and thus provided an internal positive control for the employed identification strategy.

Table 4.1 Overview of the DC-specific genes identified by RDA.

Gene	Number of fragments
15-lipoxygenase	35
DC-CK1	13
MCP-4	10
Hep27	1
C1q c-chain	1
folate-binding protein	1

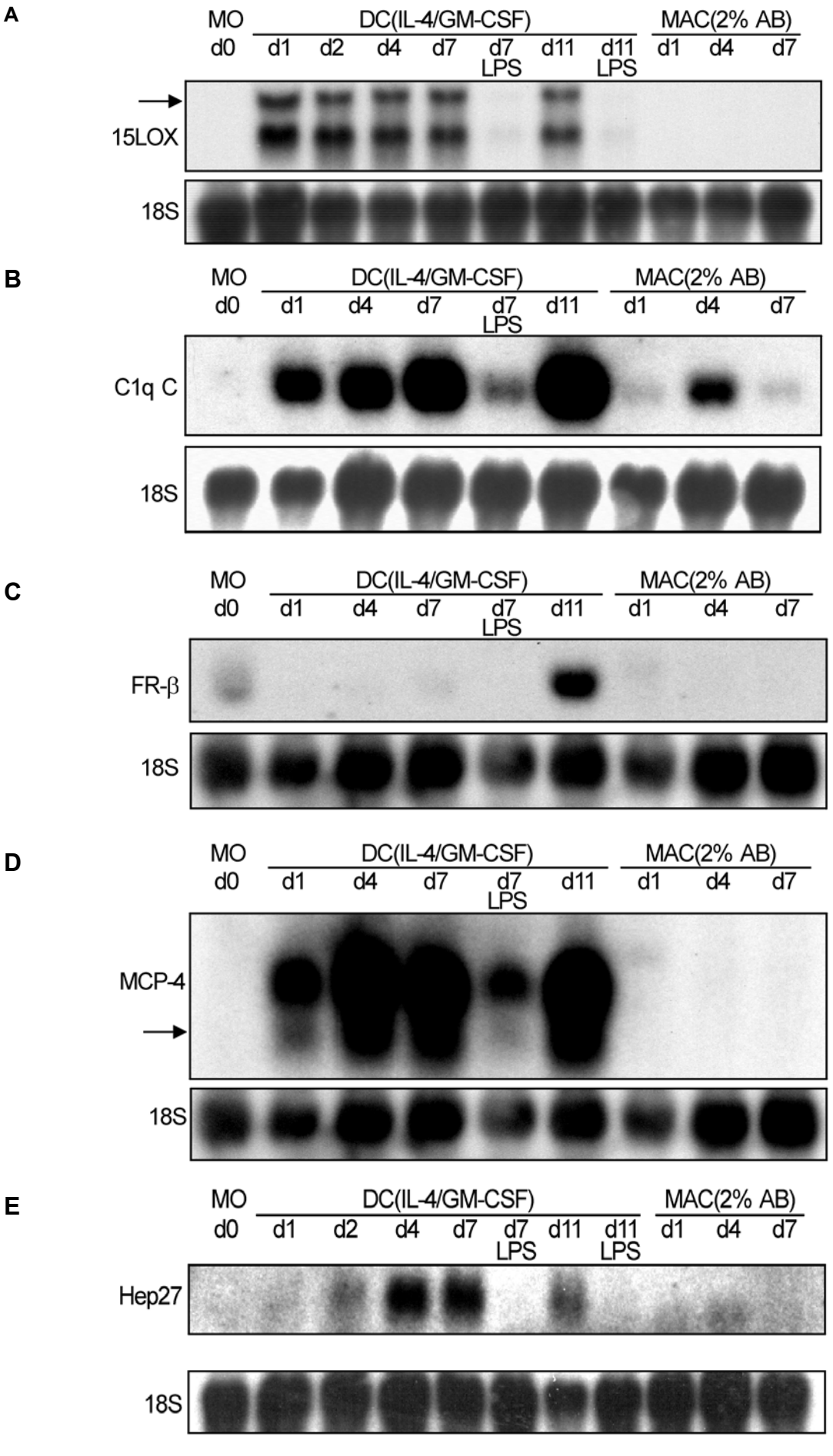
4.2 Expression analyses by Northern blot hybridization

To analyze the expression kinetics of the identified genes during monocyte to macrophage *versus* DC differentiation, Northern blots were hybridized with radioactive probes for the respective genes (see Figure 4.4).

DC-CK1 was excluded from further analysis since its exclusive expression and regulation in DCs had already been described (Adema *et al.*, 1997). Monocytic expression of 15-lipoxygenase in response to stimulation with IL-4 with or without GM-CSF had been described before (Conrad *et al.*, 1992) and could be confirmed to be expressed in the DC context while being absent from macrophages. C1q expression in humans had previously been ascribed to macrophages (Muller *et al.*, 1978) and interdigitating (Schwaeble *et al.*, 1995) and follicular DCs (Parmentier *et al.*, 1991). Within the monocytic lineage, C1q C-chain expression could be shown to be strongest in DCs while being expressed in a weaker fashion in macrophages. Interestingly, its weak expression in one and seven day-old macrophages with a peak on day four of culture mirrored the cell populations analyzed in the RDA assay which did not include four day-old macrophages.

Expression of MCP-4 and Hep27 was up-regulated during DC differentiation and restricted to DCs at an immature stage of differentiation. A maturation stimulus like LPS led to down-regulation of the respective gene products. FR-β was weakly expressed in freshly isolated monocytes but absent from macrophages. During DC differentiation it became detectable after four days of culture and reached maximal expression after more than seven days.

Figure 4.4 Northern blot analyses of the expression kinetics of the identified genes in monocytes, macrophages and dendritic cells.
Northern blots were hybridized with probes for (**A**) 15-lipoxygenase (15LOX), (**B**) complement C1q, C-chain (C1q C), (**C**) folate receptor β (FR-β), (**D**) monocyte chemotactic protein 4 (MCP-4) and (**E**) Hep27. Freshly isolated monocytes (MO) were cultured in the presence of IL-4, GM-CSF (500 U/ml each) and 10% FCS (DC) or 2% pooled human AB-serum (MAC). Cells were harvested and total RNA was extracted after the indicated time periods (d1-11). Where indicated, 100 ng/ml LPS was added on day 5 of culture (LPS) and DCs were matured for an additional two (d7) or four (d11) days. Blots were sequentially probed with a gene-specific cDNA probe and an 18S rRNA-specific oligonucleotide as loading control. Sequence variants of 15LOX and MCP-4 are indicated by arrows.


A
MO
DC(IL-4/GM-CSF)
MAC(2% AB)
d0
d1
d2
d4
d7
d7 LPS
d11
d11 LPS
d1
d4
d7
15LOX
18S
B
MO
DC(IL-4/GM-CSF)
MAC(2% AB)
d0
d1
d4
d7
d7 LPS
d11
d1
d4
d7
C1q C
18S
C
MO
DC(IL-4/GM-CSF)
MAC(2% AB)
d0
d1
d4
d7
d7 LPS
d11
d1
d4
d7
FR-β
18S
D
MO
DC(IL-4/GM-CSF)
MAC(2% AB)
d0
d1
d4
d7
d7 LPS
d11
d1
d4
d7
MCP-4
18S
E
MO
DC(IL-4/GM-CSF)
MAC(2% AB)
d0
d1
d2
d4
d7
d7 LPS
d11
d11 LPS
d1
d4
d7
Hep27
18S

4.3 MCP-4

Expression of the recently cloned CC-chemokine MCP-4 by DCs had not been described before. To confirm and further analyze MCP-4 expression in DCs, Northern blot analyses as well as Western blots were performed.

4.3.1 Analysis of MCP-4 expression during DC differentiation

MCP-4 mRNA was strongly induced during IL-4/GM-CSF-mediated differentiation of immature DCs while its expression was undetectable in monocytes and macrophages (Figure 4.4D). To assess the individual roles of IL-4 and GM-CSF in MCP-4 induction, monocytes were incubated with either cytokine alone. As shown in Figure 4.5, short term culture of freshly isolated monocytes readily induced robust MCP-4 mRNA expression after only four hours. The induction occurred regardless of whether human AB serum or FCS with or without GM-CSF was used and might be a result of the adherence stimulus. The presence of IL-4 led to reduced MCP-4 induction at four hours which was partially overcome by the additional presence of GM-CSF.

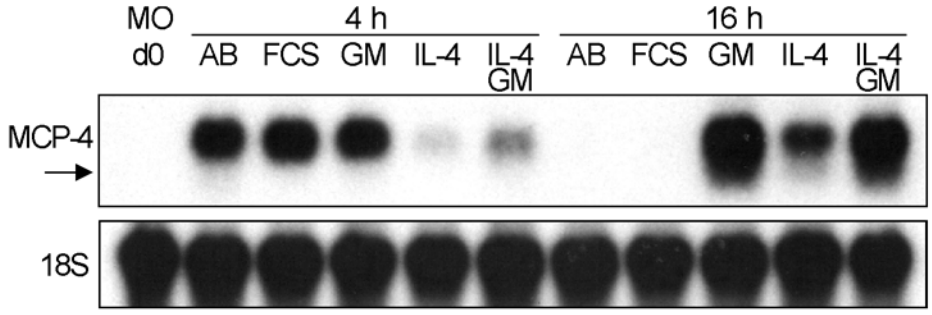


Figure 4.5 Northern blot analysis of the short time kinetics of MCP-4 expression by monocytes in response to various stimuli.
Freshly isolated monocytes (**MO 0 h**) were incubated for four (**4 h**) or 16 hours (**16 h**) in RPMI1640 with 2% human pooled AB serum (**AB**), 10% FCS (**FCS**) alone or together with 500 U/ml GM-CSF (**GM**), 500 U/ml IL-4 (**IL-4**) or both.

After 16 hours, the presumably adherence-induced initial MCP-4 expression completely disappeared under serum-only conditions but was further enhanced in the presence of GM-CSF. IL-4-induced MCP-4 expression was also stronger at 16 hours and was enhanced by GM-CSF, reaching a similar level as the GM-CSF-supplemented FCS culture. Interestingly, at 16 hours a shorter mRNA species could be observed which was absent after four hours of culture.

GM-CSF did not induce long-term MCP-4 expression (Figure 4.6A), while IL-4 alone was able to induce MCP-4 mRNA in monocytes (Figure 4.6B). However, maximal expression of MCP-4, which is associated with DC differentiation, required the synergistic effect of both GM-CSF and IL-4.

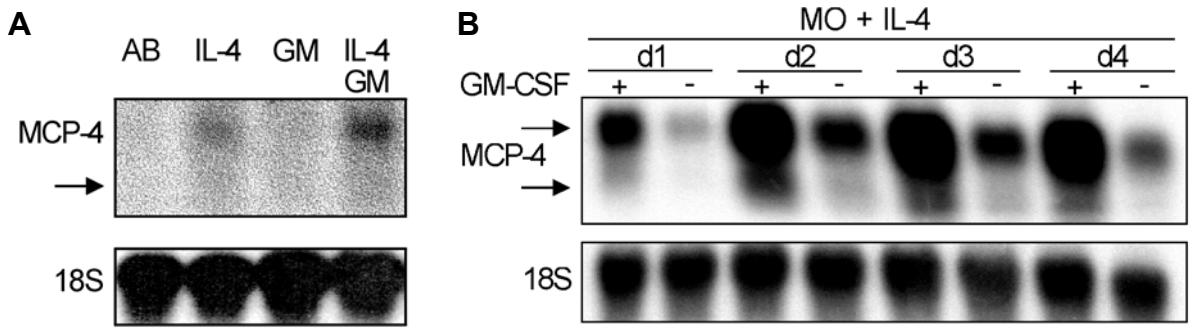


Figure 4.6 GM-CSF acts synergistically on IL-4-induced MCP-4 mRNA expression in monocytes.
Northern blots of total RNA of monocytes. **A**, Monocytes were cultured for seven days in 2%pooled human AB-positive serum/RPMI (**AB**) or 10%FCS/RPMI supplemented with 500 U/ml **IL-4**, **GM-CSF** or both. **B**, Monocytes were cultured in 10% FCS/RPMI with IL-4 (500 U/ml) with (**+**) or without (**-**) GM-CSF (500 U/ml) for the stated time periods.

Induction of terminal DC differentiation by LPS or TNF after 5 days of culture led to a decrease in MCP-4 expression, LPS exerting a more pronounced effect (see Figure 4.7). Activation of DCs by CD40 cross-linking with anti-CD40 antibody also caused down-regulation of MCP-4 mRNA and exhibited a stronger effect on LPS-primed DCs (data not shown).

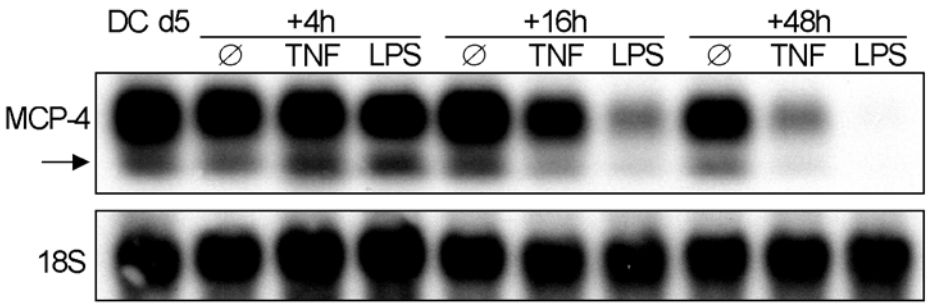


Figure 4.7 Terminal maturation of DC induced by TNF or LPS leads to down-regulation of MCP-4 mRNA.
On day 5 of culture, half of the medium was substituted for fresh medium (∅), or fresh medium supplemented with 20 ng/ml TNF or 200 ng/ml LPS and culture was continued for the indicated time periods. Blotting and hybridization was carried out as described before.

To investigate whether immature DCs also secrete MCP-4 protein, immunoprecipitations from cell culture supernatants of monocytes, macrophages and DCs were performed. As shown in Figure 4.8, immature DCs produce large amounts of MCP-4 protein (≥3 ng/(10^6 DCsx24 h)) as visualized by immunoblotting of the immunoprecipitates. Secretion of MCP-4 protein was reduced after terminal maturation by LPS or CD40 cross-linking (data not shown) which correlates with decreased mRNA expression in mature DCs. Supernatants of macrophages as well as supernatants of monocytes (data not shown) contained no detectable amounts of protein (detection limit: 1 ng/ml).

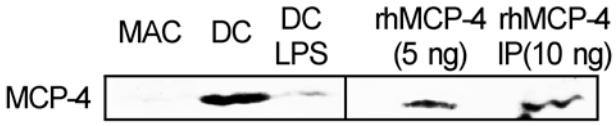


Figure 4.8 Immature DCs produce MCP-4 protein in large quantities.
Western blot of immunoprecipitates for MCP-4 from supernatants of macrophages (MAC) and DCs, either untreated (DC) or stimulated with LPS (DC/LPS) as described in Figure 4.7. As control for the immunoprecipitation, 10 ng rhMCP-4 were immunoprecipitated from 10% FCS/RPMI (MCP-4/IP). To control for blotting and staining, 5 ng MCP-4 with 5 µg BSA as carrier protein were applied directly to the gel (MCP-4).

4.3.2 Blood DC produce MCP-4 mRNA

Blood DC precursors represent an easily accessible sub-population of DCs and differentiate into functional DC during overnight culture. As shown in Figure 4.9, MCP-4 mRNA was detectable by RT-PCR in total RNA from blood DC precursors either when freshly isolated or after overnight culture. In contrast, MCP-4 was not detectable in freshly isolated blood monocytes by this technique. Overnight culture in 10% FCS transiently induced very low levels of MCP-4 mRNA in monocytes (compare also with the data obtained by Northern blot analysis after 16 h of culture with either 2% AB-serum or 10% FCS, shown in Figure 4.5). Blood DC preparations

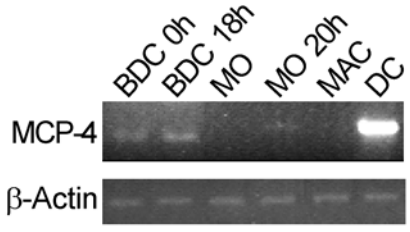


Figure 4.9 Blood DC express MCP-4 mRNA.
RT-PCR for MCP-4 with total RNA from blood DC precursors (**BDC)** directly after isolation (**0h**) and after overnight culture (**18h)**, monocytes (**MO**) after isolation and after overnight culture (**20h)**, as well as macrophages (**MAC**) and **DC**. The amount of cDNA used for PCR was normalized for β-actin. The reaction products are shown after 33 cycles (**MCP-4**) and 18 cycles (**β-actin**).

were 92-96% pure (lineage-negative (TCR, CD14, CD19, CD56)$^-$, HLA-DR$^+$, (CD123 v CD11c)$^+$) as assessed by flow cytometry.

4.3.3 DCs utilize the same MCP-4 promoter as dermal fibroblasts

To characterize the regulation of MCP-4 expression in DCs, the 5'-end of DC-expressed MCP-4 mRNA was determined by 5'-RACE followed by cloning and sequencing of the ensuing PCR products. As shown in Figure 4.10, 5'-RACE with an MCP-4-specific antisense primer yielded a homogenous amplification product of approximately 800 bp length. Sequence analysis of several cloned products revealed that transcription of MCP-4 is initiated at the same genomic site as in dermal fibroblasts which have previously been reported to express MCP-4 mRNA.

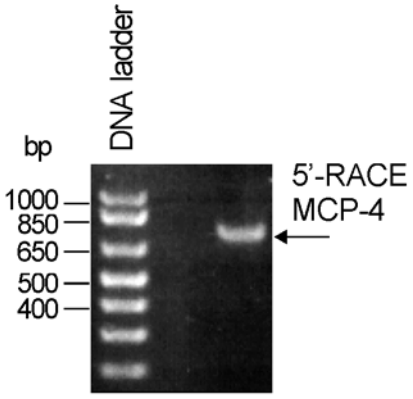


Figure 4.10 5'-RACE PCR for MCP-4 with total RNA from DCs.
EtBr-stained 1.8% Agarose/TAE gel. A primer located in exon 3 of the published MCP-4 genomic sequence was utilized (MCP-4 R5d).

4.3.4 Different regulatory pathways govern MCP-4 expression in DCs and dermal fibroblasts

Normal human dermal fibroblast cells (NHDFC) have been reported to produce MCP-4 mRNA after stimulation with IL-4, IFN-γ, or TNF. Comparison of DC with stimulated NHDFC by Northern blot hybridization demonstrated that DC produce far greater amounts of MCP-4 than dermal fibroblasts stimulated with either stimulus (seeFigure 4.11; data for TNF are not shown).

Whereas both IFN-γ and IL-4 induced MCP-4 mRNA in NHDFC, their effects adding when combined, only IL-4 but not IFN-γ caused MCP-4 mRNA expression in monocytes. On the contrary, IFN-γ counteracted the MCP-4-inducing effect of IL-4 in these cells, suggesting different regulation pathways of MCP-4 in monocytes and NHDFC. Western blot analyses of immunoprecipitates from supernatants of both cell types confirmed these findings on the protein level.

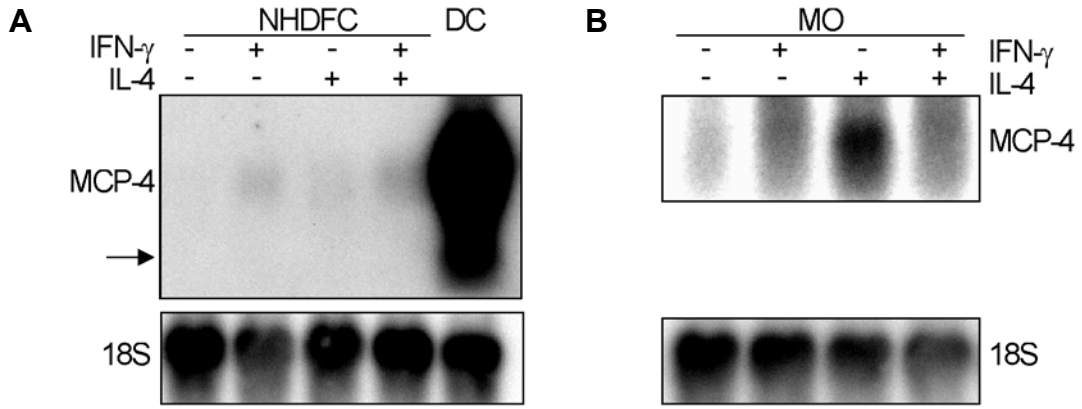


Figure 4.11 Comparison of MCP-4 mRNA expression in DC, stimulated NHDFC and monocytes by Northern blot analysis.
A, Confluent **NHDFC** were left untreated (∅) or stimulated for 26 hours with **IL-4** (500 U/ml), **IFN-γ** (200 U/ml), or IL-4 and IFN-γ after 1 hour of preincubation with IFN-γ. DC were generated as described above. **B**, In monocytes IFN-γ counteracts the induction of MCP-4 mRNA by IL-4. Freshly isolated monocytes were cultured for 40 hours in RPMI1640 with 2% pooled AB-group human serum. The stimulation pattern was as described in **A**. 10 µg of total RNA in each lane were probed sequentially with an MCP-4 cDNA probe and an 18S rRNA-specific oligonucleotide as loading control.

4.3.5 CpG methylation analysis of the proximal MCP-4 promoter

Currently only limited data is available on the mechanisms regulating gene expression in DCs. Due to the strong expression of MCP-4 in monocyte-derived DCs, we examined the MCP-4 promoter as a possible prototype for DC-specific promoters.

As shown in Figure 4.12, the proximal MCP-4 promoter sequence contains a number of CpG residues and putative binding sites for STAT factors, Ikaros 2, Oct-1, and NF-AT as well as for the NF-κB family member c-Rel which has been implicated in DC development. One major drawback in examining gene regulatory mechanisms in monocytes and derived cells is the fact that they can not be transfected efficiently, preventing promoter activity assays by transient transfection of reporter constructs. Although there are a number of monocyte/macrophage cell lines which can substitute for the primary cells in such assays, to date there are no human cell lines with DC characteristics available which could fulfil a similar role for the DC system. Recent reports by Koski et al. (Koski *et al.*, 1999) showed that calcium ionophore-treated myeloid cell lines like the promyelocytic HL-60 cell line display DC-like properties in terms of antigen presentation and expression of typical surface markers and thus might serve as a model for DCs. Indeed, Northern blot analysis of MCP-4 expression of HL-60 cells showed up-regulation of MCP-4 mRNA in response to calcium

ionophore treatment (data not shown). However, transfection of the cells with a wide array of methods was either unsuccessful (various lipid-based transfectants) or did not produce reproducible results (electroporation).

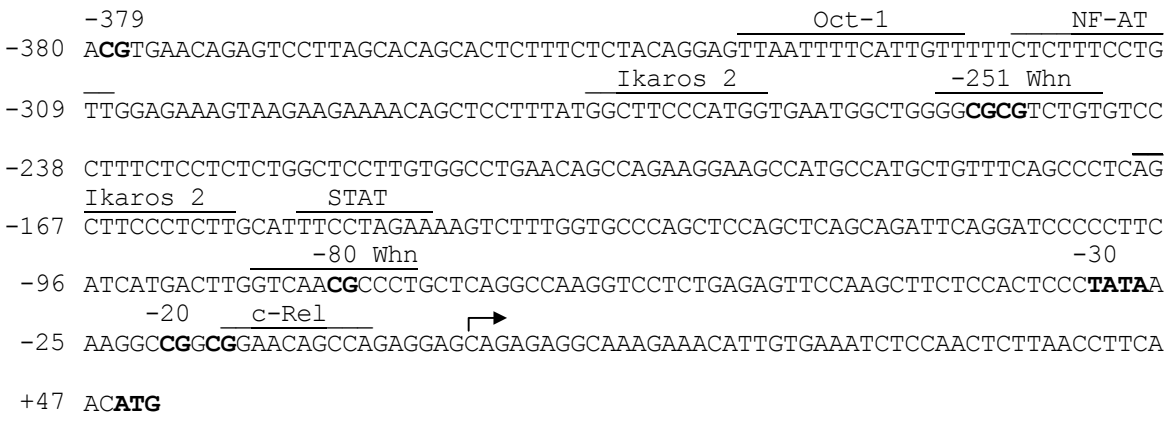


Figure 4.12 Sequence of the human proximal MCP-4 promoter.
CpG motifs, TATA box and the start codon are in *bold face*, potential transcription factor binding sites are *overlined*. The transcription start site is indicated by an *arrow*. The putative transcription factor binding sites were identified with the MatInspector software (Quandt *et al.*, 1995).

As an alternative approach to perform an initial characterization of the proximal MCP-4 promoter, its CpG methylation status was assessed by genomic bisulfite sequencing. In mammalian somatic cells, 60-90% cytosine residues in cytidine-guanidine (CpG) sequences in genomic DNA are methylated at the 5' position (Singal and Ginder, 1999). Methylation of promoter regions of genes has been found to correlate with repression of the respective genes, probably by histone deacetylation and incorporation into heterochromatin (Razin, 1998). Cytosine methylation can be determined by bisulfite-induced reductive deamination, effectively leading to its conversion of cytosine to uracil. With 5'-methylated cytosine, the deamination step is kinetically inhibited by the methyl group, leading to an estimated half-life of 34.6 hr vs. 5.5 min of the unmethylated residue under the same conditions (Oakeley, 1999). The procedure effectively leads to conversion of all unmethylated genomic cytosines to uracil, i.e. thymidine after the strand-specific PCR amplification step and only previously methylated cytosines (in CpG motifs) are preserved in the sequence. Consequently, sequencing of the PCR product provides information on the CpG methylation status of the amplified genomic fragment before conversion.

Applying bisulfite sequencing to the monocyte/macrophage/DC system, all CpGs of the proximal promoter were found to be methylated in monocytes and macrophages, while in DCs, the two CpGs adjacent to the transcription start site (one single and two CpGs in tandem, located at -80 bp and -20 bp, respectively) become demethylated during differentiation. In MCP-4 non-expressing T- and B-lymphocytes as well as HL-60 with or without calcium ionophore treatment, THP-1 and U937 cells, the CpG motifs of the MCP-4 promoter were found to be methylated (data not shown).

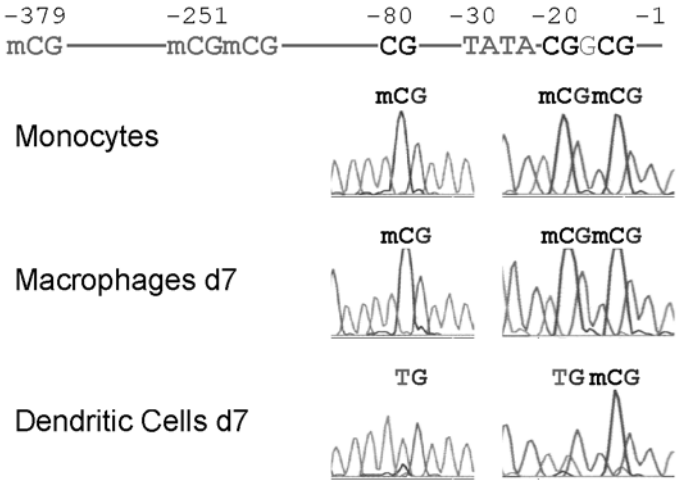


Figure 4.13 DC-specific demethylation of the MCP-4 promoter.
Sequencing pattern of the MCP-4 promoter at the -80 bp and the -20 bp CpG motifs after bisulfite conversion of unmethylated cytosine residues to thymidine (after PCR). Methylated cytosines are shown as mC.

The first intron of *MCP-4* also contains a number of CpG motifs which were found to remain methylated in curing monocyte to macrophage or DC differentiation.

The kinetics of CpG demethylation was assessed by bisulfite sequencing of DNA extracted from IL-4 and GM-CSF-treated monocytes after 0, 1, 2 and 4 days. As visible in Figure 4.14, demethylation of the –80 CpG was essentially completed after 24 h of culture, while maximal demethylation of the TATA-proximal CpGs at –20 bp took until day 4 and was incomplete.

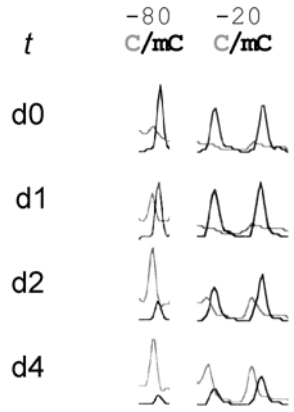


Figure 4.14 Kinetics of demethylation of the MCP-4 promoter CpG motifs in DCs.
Bisulfite sequencing of the MCP-4 promoter in monocytes cultured with 500 U/ml IL-4 and GM-CSF each in 10% FCS/RPMI for the indicated time periods. To circumvent automatic normalization performed by the sequencing software, raw data of the G (black) and A (gray) sequencing reactions of the antisense PCR product are shown, corresponding to methylated and demethylated cytosine residues of the genomic DNA, respectively.

4.3.6 Dendritic cell-specific demethylation of the MCP-4 promoter allows binding of a nuclear factor

In order to characterize the effects of monocyte-derived DC-specific CpG demethylation on nuclear protein binding to the MCP-4 promoter, electrophoretic mobility shift assays with nuclear extracts from macrophages and DCs were performed using probes that encompass either the single (-80bp) CpG motif upstream of the TATA box or the TATA box and the two CpG (-20 bp) motifs downstream (sequences are given in Table 4.2). While the latter oligonucleotide bound nuclear proteins of DCs and macrophages either in the methylated or unmethylated state (Figure 4.15A), the single (-80 bp) CpG motif-containing oligonucleotide only complexed nuclear protein when unmethylated, again from both cellular sources (Figure 4.15B). Competitor analysis revealed that unmethylated probe was displaced exclusively by unmethylated oligonucleotide but not by its methylated counterpart, indicating methylation-sensitive binding of an unknown factor to the (-80 bp) CpG motif.

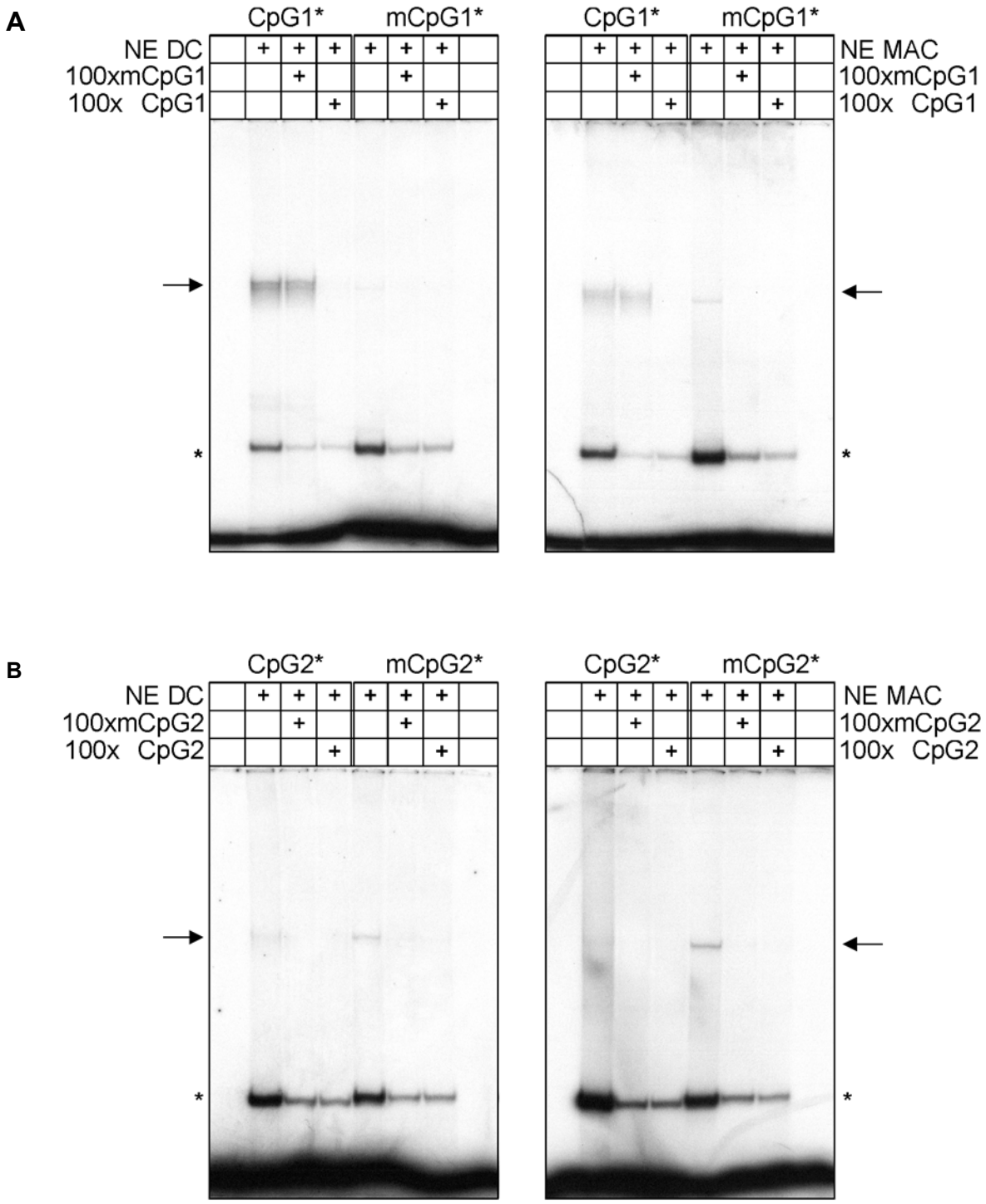


Figure 4.15 Methylation-sensitive binding of a nuclear factor at the –80 bp but not the –20 bp CpG motif.

A, Exclusive protein binding at the unmethylated –80 bp CpG motif which could only be competed for by unmethylated competitor oligonucleotide. EMSA using radioactively labeled unmethylated (**CpG1***) or methylated (**mCpG1***) oligonucleotide and nuclear extracts of monocyte-derived macrophage- (**MAC**) or dendritic cell (**DC**). For competition analysis, unlabeled oligonucleotides were added at 100-fold excess where indicated (**+**) above the respective lanes. **B,** Binding of nuclear proteins to the –20 bp CpG motifs is methylation-independent. EMSA using unmethylated (**CpG2***) or methylated (**mCpG2***) oligonucleotide as in A. Specific bands are marked with *arrows*, non-specific bands are marked with *asterisks*.

Table 4.2 Sequences of oligonucleotides used in gel shift analysis and site-directed mutagenesis.

Oligonucleotide	Sequence	Mutant construct
CpG1 (-80 bp)	5'-CATCATGACTTGGTCAACGCCCTGCTCA-3'	
M1	5'-CATCATGACTTGGTCAA**GC**CCCTGCTCA-3'	MCP-4pP1
M2	5'-CATCATGACTTGGTCAA**GAG**CCTGCTCA-3'	MCP-4pP2
CpG2 (-20 bp)	5'-CTCCCTATAAAAGGCCGGCGGAACAGCCAG-3'	

CpG motifs are *underlined*, introduced mutations are *bold faced*.

A query of the TRANSFAC database using the MatInspector tool (Quandt *et al.*, 1995) identified a putative core consensus binding site for the transcription factor winged-helix nude (whn) at the CpG motif at -80 bp (see Figure 4.12). Reversing the CpG (ACGC core to AGCC) or entirely eliminating the motif (ACGC to AGAG) almost completely abolished the ability of the mutant oligonucleotides to compete with wild type oligonucleotide for protein binding (Figure 4.16).

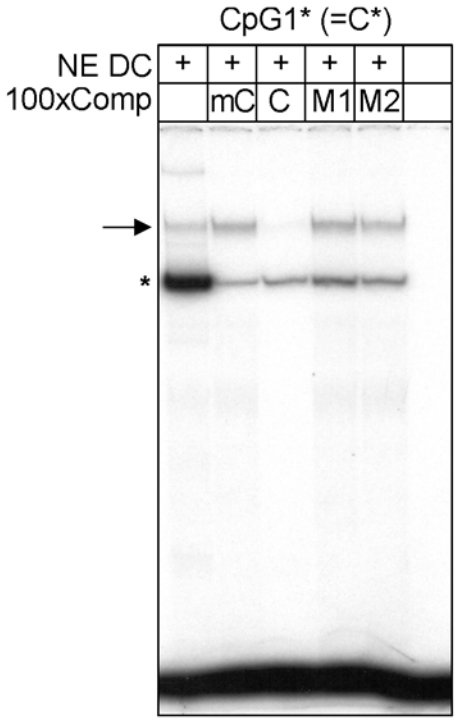


Figure 4.16 Mutation of the –80 bp CpG motif mimics CpG methylation in EMSA. Binding of nuclear extract protein from DCs to labeled, unmethylated CpG1* oligonucleotide (abbreviated as **C***) was only competed for by a 100-fold excess of unlabeled unmethylated CpG1 (**C**) but not methylated (**mC**), or mutated (**M1**, **M2**) CpG1 oligonucleotide. Specific bands are marked with arrows, non-specific bands are marked with asterisks.

4.3.7 Nuclear factor binding at the -80 bp CpG is necessary for maximal MCP-4 promoter activity in reporter assays in transiently transfected THP-1 cells

To date, there are no human DC lines which could be used as model systems for the analysis of gene regulation in DCs. However, EMSAs with nuclear extracts from the monocytic cell line THP-1 using the -80 bp CpG-containing oligonucleotide as probe displayed a protein binding pattern identical to that observed for monocyte-derived DCs and monocyte-derived macrophages, indicating that the factor binding at the –80 bp CpG motif is also present in these cells. (Figure 4.17).

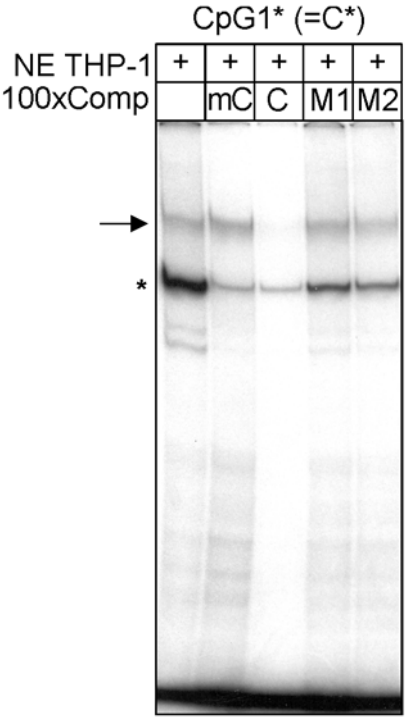


Figure 4.17 The CpG methylation-sensitive factor is also present in monocytic THP-1 cells.
Binding of nuclear extract protein from DCs to labeled, unmethylated CpG1* oligonucleotide (abbreviated as **C***) was only competed for by a 100-fold excess of unlabeled unmethylated CpG1 (**C**) but not methylated (**mC**), or mutated (**M1**, **M2**) CpG1 oligonucleotide. Specific bands are marked with arrows, non-specific bands are marked with asterisks.

To assess the effects exerted by inhibiting binding of the –80 CpG-binding factor on MCP-4 promoter activity, luciferase reporter assays were performed in THP-1 cells. As a first step, a luciferase reporter construct was prepared with the 5'-proximal MCP-4 promoter, ranging from -380 bp to -1 bp upstream of the transcription start, driving expression of firefly (*Photinus pyralis*) luciferase. Since the wild-type construct (MCP-4pP*) showed significant reporter activity in these cells, two plasmids with a mutated –80 bp CpG motif were constructed by site-directed mutagenesis employing the oligonucleotides listed in Table 4.2. As shown in Figure 4.18, the ACGC to AGCC

mutant (MCP-4pP1) exhibited 60% promoter activity compared to the wild type promoter while the ACGC to AGAG mutant (MCP-4pP2) yielded only 47% of the wild type promoter activity.

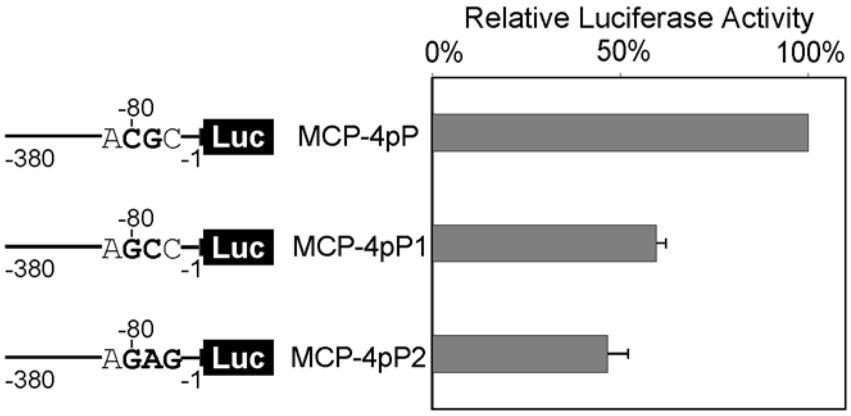


Figure 4.18 Mutation of the -80 bp CpG motif reduces the activity of the proximal MCP-4 promoter in THP-1 cells.
Mutations of the -80 CpG motif were introduced in a MCP-4 reporter construct by site-directed mutagenesis using the oligonucleotides listed in Table 4.2. The mutations are indicated in the schematic representations of the reporter constructs in *bold face*. Each construct was transiently transfected in duplicates into the myeloid cell line THP-1 and luciferase activity was determined after two days and normalized for total protein concentration of the samples. Luciferase activity is given relative to wild type MCP-4 promoter (100%) and values are the means + S.D. obtained for four independent experiments.

4.3.8 CpG demethylation of the MCP-4 promoter during monocyte to dendritic cell differentiation is differentiation stage-dependent

Recent reports suggested that only activated DCs and macrophages represent stable phenotypes and that non-activated macrophages can be converted to immature DCs and *vice versa* by addition or withdrawal of IL-4 and GM-CSF, respectively (Palucka *et al.*, 1998). To test whether this conversion is reflected by the CpG methylation status of the proximal MCP-4 promoter, seven day-old and immature DCs and macrophages were cultured for an additional seven days in 2% AB serum without or in 10% FCS with IL-4 and GM-CSF, respectively. MCP-4 mRNA expression was assessed by Northern blot analysis and the CpG methylation status of the proximal MCP-4 promoter was analyzed by bisulfite genomic sequencing. The conversion of the macrophage/DC phenotypes was confirmed by flow cytometric analysis of CD14, CD83 and HLA-DR expression (data not shown). As expected, differentiation of monocytes into DCs was accompanied by demethylation of the CpG motifs at –80 and –20 bp, while no demethylation took place during macrophage (Figure 4.19).

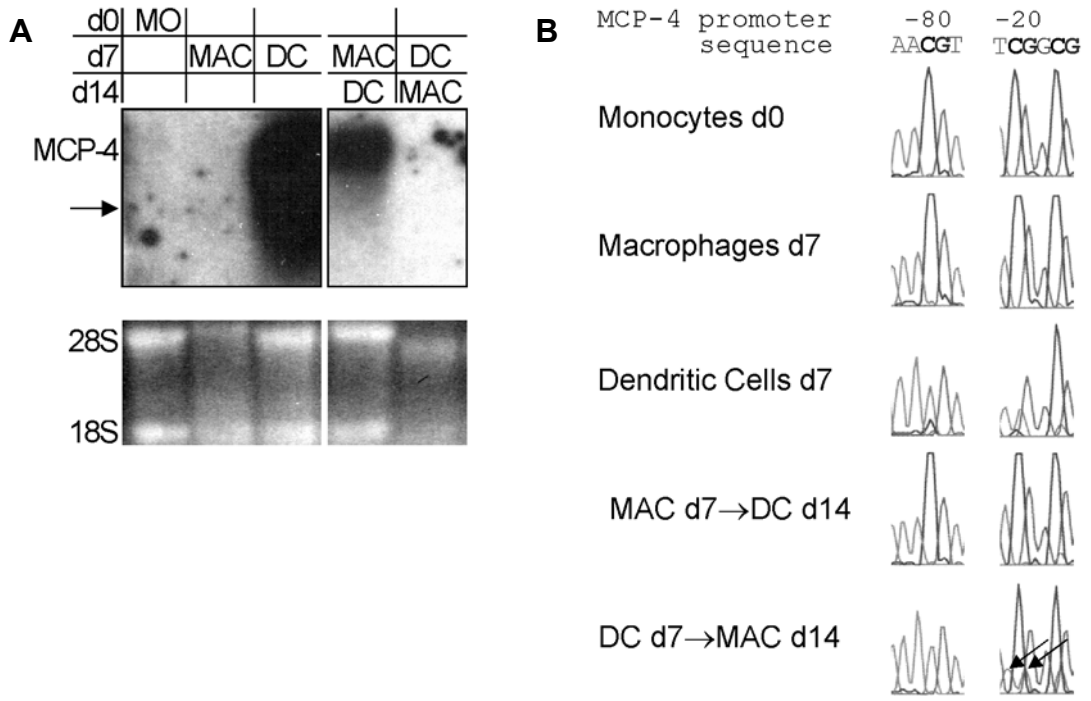


Figure 4.19 Analysis of MCP-4 mRNA expression and promoter CpG methylation after DC to macrophage conversion and *vice versa*.
A, Northern blot hybridization with an MCP-4-specific cDNA probe. An arrow marks a shorter MCP-4 sequence variant. The lower inset shows the EtBr-stained gel before blotting, 28S and 18S bands are indicated. **B,** genomic bisulfite sequencing. Freshly isolated monocytes (**MO**) were differentiated to macrophages(**MAC d7**) (2% human pooled AB serum/RPMI in teflon bags) or **DC**s **d7** (500 U/ml each of IL-4 and GM-CSF/10% FCS/RPMI in cell culture flasks) for seven days. Half of each cell preparation harvested and stained with antibodies against CD14, CD83 or HLA-DR or genomic DNA or total RNA extracted. The other half was washed twice with PBS and DCs were re-seeded in 2% human pooled AB serum/RPMI in teflon bags (**DC d14-MAC d7**), macrophages were re-seeded in 500 U/ml each of IL-4 and GM-CSF/10% FCS/RPMI in cell culture flasks (**MAC d7-DC d14**), for seven additional days. Two arrows indicate two thymidine peaks of equal amplitude.

Intriguingly, exposure of macrophages to IL-4 and GM-CSF in 10% FCS/RPMI also failed to induce demethylation. The lack of demethylation correlated with a reduced induction of MCP-4 mRNA compared to DCs. In DCs, removal of cytokines for seven days led to complete elimination of MCP-4 mRNA expression but did not alter CpG methylation. (The observed peak at the –20 bp cytosine in the last sequence in Figure 4.19 is probably an artifact of the automatic signal normalization algorithm of the sequencer. Comparison with the raw data as well as the conversion of the cytidine residues to thymidine which is apparent as a peak of similar amplitude as the neighboring thymidine peak confirmed the demethylation of the shown CpG residue). Similar results were obtained by using M-CSF as a DC de-differentiation agent instead of cytokine withdrawal. Maturation of DCs with LPS or TNF yielded identical results concerning CpG demethylation in both mature DC and mature DC after cytokine withdrawal (data not shown).

4.4 Hep27

Hep27 (HUGO nomenclature: dehydrogenase/reductase (SDR family) member 2, DHRS2) is a member of the short-chain alcohol dehydrogenase family with an as yet unidentified substrate and initially was cloned in HepG2 cells (Gabrielli *et al.*, 1995).

4.4.1 Predominant expression of Hep27 in monocyte-derived dendritic cells

Hep27 expression was further analyzed Northern blot hybridizations. As shown in Figure 4.4E, Hep27 mRNA expression was up-regulated in monocyte-derived DCs while freshly isolated monocytes or monocyte-derived macrophages at different time points expressed only low to undetectable levels of Hep27 mRNA (see also). Up-regulation of Hep27 mRNA expression was detectable after a minimum period of two days of culture and required the presence of both GM-CSF and IL-4.

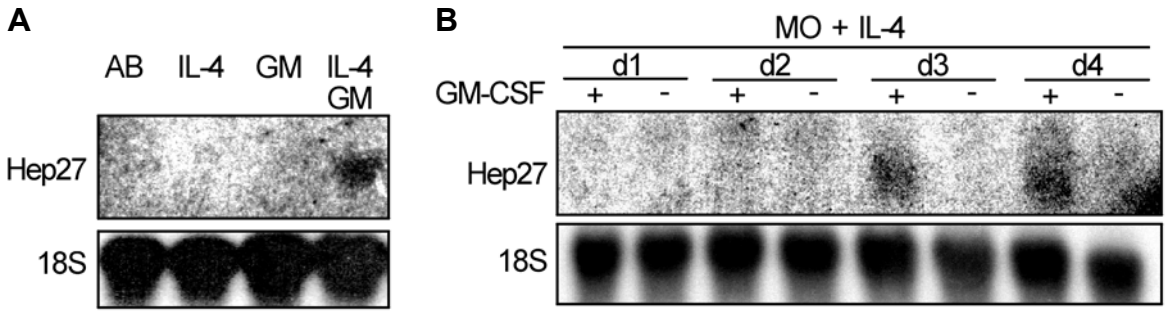


Figure 4.20 Both IL-4 and GM-CSF are necessary to induce Hep27 in monocytes.
Northern blots of total RNA of monocytes. **A**, Monocytes were cultured for seven days in 2%pooled human **AB**-positive serum/RPMI or 10%FCS/RPMI supplemented with 500 U/ml **IL-4**, **GM-CSF** or both. **B**, Monocytes were cultured in 10% FCS/RPMI with IL-4 (500 U/ml) with (**+**) or without (**-**) GM-CSF (500 U/ml) for the stated time periods.

Maturation stimuli like LPS or TNF led to down-regulation of Hep27 expression in monocyte-derived DCs Figure 4.4E and Figure 4.21).

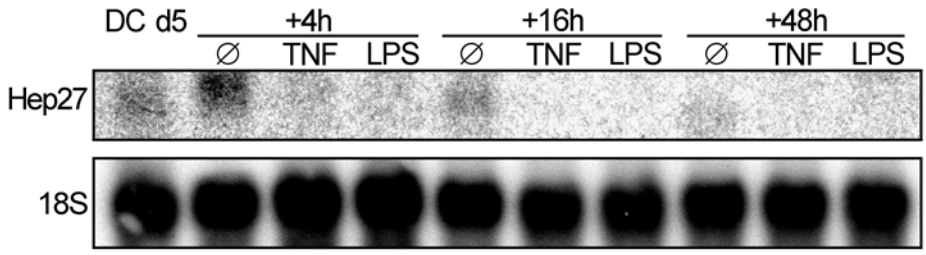


Figure 4.21 Hep27 mRNA expression by DCs is down-regulated upon terminal maturation induced by TNF or LPS.
On day 5 of culture, half of the medium was substituted for fresh medium (∅), or fresh medium supplemented with 20 ng/ml TNF or 200 ng/ml LPS and culture was continued for the indicated time periods. Blotting and hybridization was carried out as described before.

As shown in Figure 4.22, Hep27 protein could be detected in cytoplasmic extracts from monocyte-derived DCs by Western blotting, using a Hep27-specific antibody which was kindly provided by Dr. F. Gabrielli, University of Pisa, Italy.

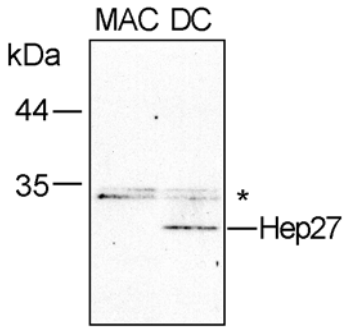


Figure 4.22 Western blot of cytoplasmic extracts from monocyte-derived macrophages and monocyte-derived dendritic cells.
Cytoplasmic extracts from macrophages and DCs were separated by SDS-PAGE, blotted on nitrocellulose membrane, probed with a mouse polyclonal anti-Hep27 serum and developed with an HRP-conjugated goat anti-mouse antibody and ECL. An unspecific band of 33 kDa is marked with an asterisk.

Cytoplasmic extracts from monocyte-derived macrophages did not contain detectable amounts of Hep27. A double band at 33 kDa was present in extracts from both monocyte-derived macrophages and monocyte-derived DCs. Since in macrophages no Hep27 mRNA could be detected, the signal might represent a cross reactivity of the antibody and possibly corresponds to 11β-hydroxysteroid dehydrogenase type 1 which is 28% identical to Hep27 on the protein level and has been reported to be up-regulated during monocyte to macrophage differentiation as well as in monocytes after stimulation with IL-4 (Thieringer *et al.*, 2001).

4.4.2 Tissue expression of Hep27 mRNA

To test whether Hep27 was expressed in cells other than monocyte-derived DCs and HepG2 cells, RNA samples from various cell types including granulocytes, B- and T lymphocyte-enriched elutriation fractions, HUVEC, dermal fibroblasts, the monocytic cell line THP-1, the promyeloblastic HL-60 cell line and the colon carcinoma cell lines HT-29 and CaCo-2 we analyzed for Hep27 mRNA by Northern blot hybridization.

As shown in Figure 4.23, apart from monocyte-derived DCs and HepG2 cells, only HT-29 and CaCo-2 cells produced appreciable amounts of Hep27 mRNA. The single transcript detected in monocyte-derived DCs was approximately 1.5 kb in length as opposed to HepG2 cells, where a 1.9 kb transcript was prominent as the main product and a shorter 1.3 kb sequence variant as well as a long transcript of about 4 kb length could be detected. Overexposure for 8 days revealed extremely weak bands of 1.5 and 1.3 kb in monocytes, monocyte-derived macrophages, HUVEC,

fibroblasts, THP-1 cells and HL-60, while in granulocytes or lymphocytes Hep27 mRNA was not detectable (data not shown).

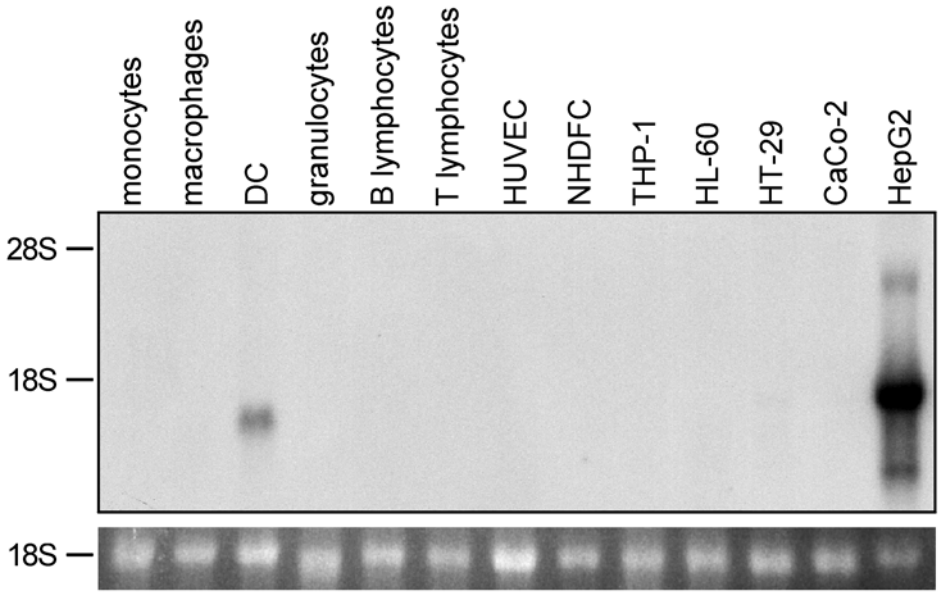


Figure 4.23 Northern blot analysis of Hep27 mRNA expression in various cell types.
10 µg of total RNA were loaded for each lane. The probe used spanned exons III through VIII and was labeled using an antisense primer (8as)**.** The positions of the 28S (5025 bp) and 18S (1868bp) rRNA bands are indicated. The bottom inset shows the EtBr-stained 18S rRNA bands after blotting.

Northern blot analysis of the tissues expression pattern of Hep27 mRNA revealed that the 1.3 and 1.5 kb transcripts described above were present in all tissues (Figure 4.24).

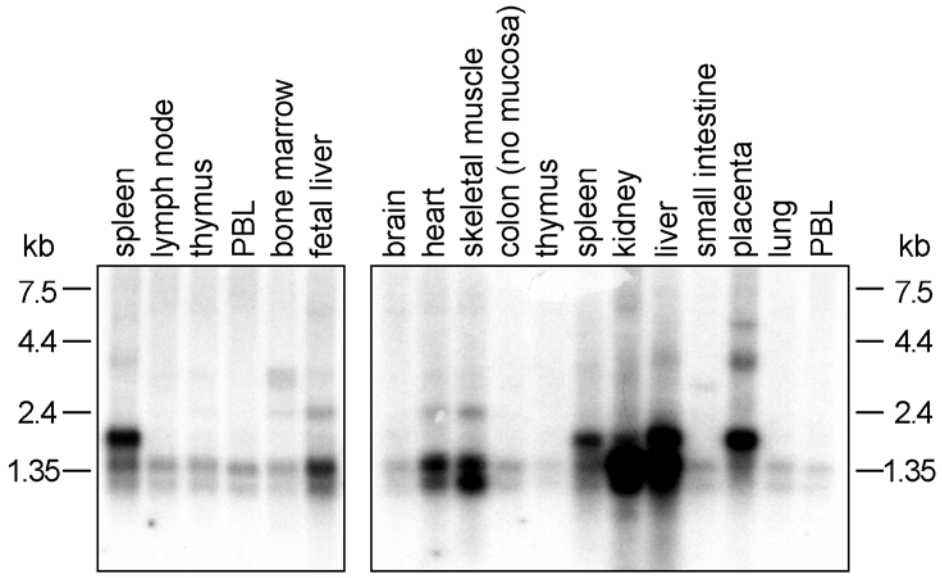


Figure 4.24 Hep27 mRNA expression pattern in human tissues.
Hep27 probe was hybridized to commercially available tissue Northern blots (Clonetech, Heidelberg, Germany) containing 2 µg polyA$^+$ RNA per lane.

Expression of the 1.3 kb and 1.5 kb transcripts was weakest in thymus and PBL, followed by lung, brain, colon, small intestine and spleen. In heart and skeletal muscle, the 1.3 and 1.5 kb variants exhibited stronger expression, accompanied by a longer transcript of approximately 2.5 kb. A very similar transcript pattern was detected in fetal liver. In liver and kidney, the two short variants were expressed the strongest accompanied by expression of the 1.9 kb transcript observed in HepG2 cells. The latter was the main transcript in spleen and placenta. Additionally, a 4 kb transcript was detectable in spleen, liver and placenta.

4.4.3 DC and HepG2 cells utilize different Hep27 promoters

The different transcript lengths of Hep27 in HepG2 cells and monocyte-derived DCs suggested different splicing and/or promoter activity in both cell types. In order to define the 5'-end and putative promoter sequences, 5'-RACE with primers for Hep27 was performed. Fig. 2A shows the resulting products of 860 bp from HepG2 and two products of 680 and 550 bp length from monocyte-derived DCs. Shotgun cloning and sequencing of 10 randomly picked clones for each cell type confirmed the previously published main transcription start site of Hep27 in

HepG2 cells, which lies in an LTR (Gabrielli *et al.*, 1995). In monocyte-derived DCs, however, most of the resulting clones contained a new 5'-end lacking the LTR portion which pointed to a new upstream exon and promoter employed by monocyte-derived DCs.

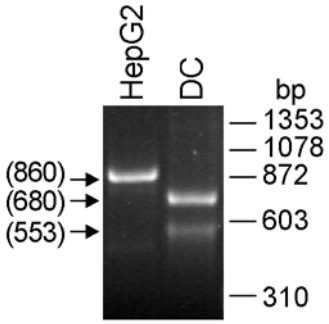


Figure 4.25 Monocyte-derived dendritic cells utilize a different Hep27 promoter than HepG2 cells.
EtBr-stained products of 5-RACE for Hep27 in HepG2 cells and monocyte-derived dendritic cells separated on an agarose/TAE gel. Products lengths (minus universal primer) in bp are noted in brackets.

To clone putative promoter sequences upstream of the newly identified Hep27 5'-end, promoter walking with primers derived from the newly discovered 5'-mRNA sequence of Hep27 was performed and the resulting PCR products were sequenced. As shown in Figure 4.26, the genomic sequence 5' of the Hep27 transcription start in DC contains a TATA box at -29 bp. Analysis of the sequence with the MatInspector program (Quandt *et al.*, 1995) identified a number of putative binding sites for

transcription factors including oct-1 and ets factors (core motifs at -131 bp and -101 bp, respectively), as well as an NF-AT/AP-1 module at around -210 bp.

DC

```
-350 TCTGTATTCCAAACCCTTTCTTTGAAAACTCCTACGTTCCCTCCACGAATTGAAGAGTGGAATTGCTTTC
                                                                    AP-1
-280 TACTCTTCCCCTGCTAGCATGGATAATAAAGACATCTTGCTCCCTCTTATCATAACTATTATTATTTTGA
      _____NF-AT_
-210 CGTCTTTCCACAAGCAGCAAGCAGCTGGACTCTTTTGCTGGTTATGCTTGTTAAGTGGTATGTGCAGCAT
       ____oct-1_____              _____ets_____ ets
-140 TCTGCAGAATATTACTCAGACAACAAGTCCTGCACTTCCCCTAGGTTTCCTCCCAAGTTGTGGGTGAGGC
                                              TATA box                  -1
 -70 CTTTTCTGAAACCCTCCTCCAACCTTTCTGAATTCTGGGGCTATAAATTCCAGGAATCTTTGTGTTGTTC
     ┌→Exon I
  +1 AGATAGAATCTGCAGTGCCT
```

HepG2

```
                CCAAT box                CCAAT box
-100 CTGTAAAATGGACCAATCAGCTCTCCATAAAATGGACCAATCAGCAGGATGTGGGTGTGGCCAGTTAAGG
      TATA-like motif          -1┌→Exon II
 -30 GAATAAAAGCAGGCTGCCTGAGCCGGCAGCAGCAACCTGCTCT
```

Figure 4.26 Genomic sequences upstream of the transcription start sites of Hep27 in DCs and HepG2 cells.
Sequences were determined by genome walking or sequence comparison using BLAST, respectively. Putative transcription factor binding sites are *overlined*, CCAAT, TATA and core recognition motifs are in *boldface*. Repetitive sequences are *underlined*, transcription start sites are marked by an *arrow*.

4.4.4 Genomic organization of *Hep27*

Comparison of the published Hep27 mRNA sequence (GenBank accession number U31875) and the newly defined 5'-end in DCs with a recently published BAC clone which contains the corresponding genomic sequence on chromosome 14q11.2 (accession number AL135999.2) yielded the intron/exon structure of *Hep27* as depicted in Figure 4.27.

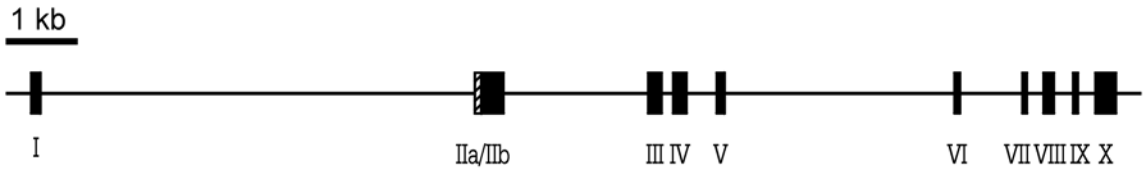


Figure 4.27 Genomic organization of *Hep27*.
Schematic representation of the intron/exon structure of *Hep27* as determined by BLAST. Exons are depicted as filled boxes and numbered with roman numerals, introns are represented by the connecting lines. Exon IIa is depicted as a hatched box. The drawing is up to scale.

Exon I represents the putatively DC-specific 5'-UTR. Together with the 350 bp proximal alternative promoter it is located at the beginning of a short 723 bp island of non-repetitive DNA following a repeat-only region of about 6 kb. Exon II lies in a

repeat-rich region and separates into a 5'-UTR upstream of the internal splice acceptor (exon IIa) and exon IIb downstream of the 3'-splice acceptor. Table 4.3 lists the sizes of introns and exons as well as the nucleotide and amino acid sequences at the exon/intron junctions. The genomic organization of *Hep27* has been deposited in GenBank (GenBank accession numbers AF244132 and AY036899).

Table 4.3 Positions of splice donor and acceptor sequences in the Hep27 gene.

Exon	Size (bp)	Position	Sequence at exon/intron junction: 5'-splice donor	Intron (bp)	3'-splice acceptor	Amino acid interrupted
I	121	1-121	**GCACAGCCAG**GTAAGCCTGG-	6231	-CACCTTTAAG**AGCTGTAACA**	untranslated
II	305	6353-6657	**TCGCCAAGTG**GTGAGTACCA-	2054	-TTTCCCCCAG**GCCTGATTCA**	untranslated
III*	178	8712-8889	**CCACCAGTGG**GTGAGTGCTG-	174	-CTGCATTCAG**GATCGGCTTT**	Ser-Gl/y-Ile
IV*	178	9064-9241	**GGTGGCCAAG**GTGAGGGGGC-	437	-CTCTCCGCAG**GCCCTGGAGC**	Lys-/-Ala
V	102	9679-9780	**CTGGGACAAG**GTGAGAGGCC-	3256	-CTGATTTCAG**ATCCTAAGTG**	Lys-/-Ile
VI*	68	13037-13104	**TGGAGAACAG**GTATGGCAGG-	891	-CTTGTCTCAG**GAGGGGTGCT**	Asn-Ar/g-Arg
VII	52	13996-14047	**TCCAGTAGTG**GTAAGTGCTT-	246	-CCCTTCCCAG**GCGCTGGGTG**	Val-/-Ala
VIII	135	14294-14428	**CAGCAAAGTG**GTGAGGATTG-	283	-CTTTGCCCAG**TTTCATGGGA**	Val-/-Phe
IX	56	14712-14767	**AGCTGCAGAG**GCAAGTGGGG-	259	-TTCCATCCAG**GATTGGGGAG**	Gln-Ar/g-Ile
X		15027->15306				

The main transcription starts site in HepG2 cells lies at 6249 bp. Start codon-containing exons are marked with an *asterisk*, exon sequence portions are in *boldface*.

4.4.5 Exclusive utilization of the upstream Hep27 promoter by monocyte-derived dendritic cells and alternative splicing

RT-PCR using sense primers derived from the first exon (1s) or exon IIa (a2s) and an antisense primer specific for exon V (5as), VIII (8as) or X (10as) indicated that exon I was exclusively transcribed in monocyte-derived DCs but not HepG2 cells, confirming the 5'-RACE results (Figure 4.28). Additionally, none of the weakly Hep27-expressing cell lines THP-1, HL-60, HT-29, CaCo-2 and HaCat were found to express the first exon (data not shown). Instead, Hep27 transcripts in these cells contained exon IIa which is spliced out in monocyte-derived DCs and is only present in the mature transcript if transcription is initiated by the LTR promoter.

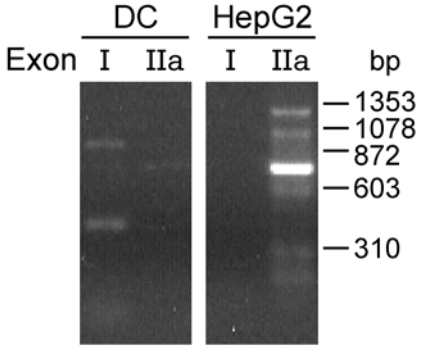


Figure 4.28 The upstream Hep27 promoter is not utilized in HepG2 cells. Shown are the products of RT-PCR for Hep27 using sense primers derived from exon I (1s) or exon IIa (a2s) with 1 µg total RNA from monocyte-derived dendritic cells or HepG2 cells. The antisense primer was derived from exon X (10as).

Cloning and sequencing of the products from both 5'-RACE and RT-PCR revealed at least three different splice variants in monocyte-derived DCs, comprising exons I and III through X, exons I, II and IV through X and exon I and VI through X. In HepG2 cells, the two predominant splice variants were sequenced, the first containing exons II through X, the second comprising exons II and VI through X. Figure 4.29 shows the predominant splice variants identified. Accordingly, the two bands of 955 bp and 370 bp observed for DCs in Figure 4.28 correspond to the first and third transcript depicted schematically in Figure 4.29, the longest (1142 bp) and the main PCR product (684 bp) from HepG2 cells accord with the full length transcript and the shorter splice variant shown in Figure 4.29, respectively. Exons III, IV and VI contain putative start codons. Consequently, the shorter splice variants code for the putative catalytic triad Ser172, Tyr185, Lys189 (Benach-Andreu, 1999) which is located in exons VII (Ser) and VIII (Tyr, Lys) but lack the putative coenzyme-binding region which is encoded by exons III through V.

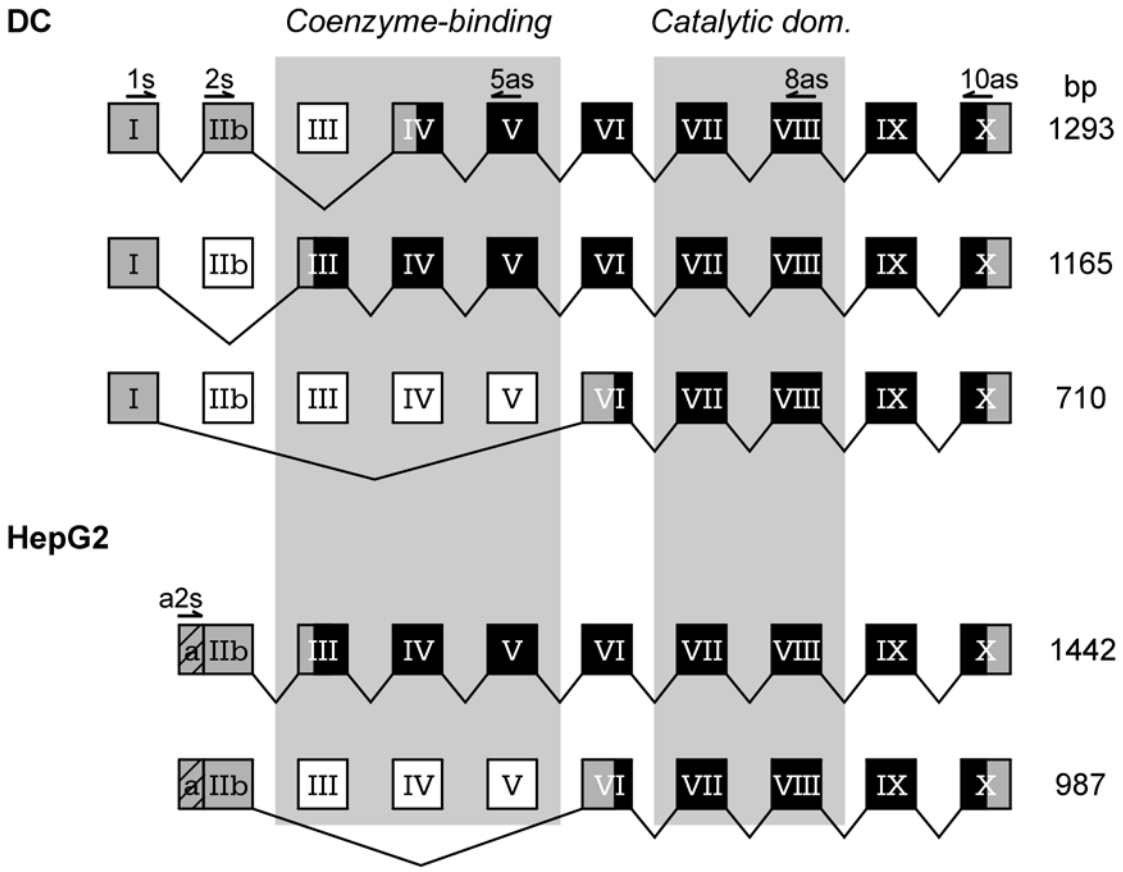


Figure 4.29 Hep27 mRNA splice variants in DCs and HepG2 cells.
Exons are represented by boxes. Spliced-out exons are empty, expressed exons are linked, untranslated regions are gray, ORFs are black. Exon IIa is hatched. The numbers behind each mature transcript represent the sums of the lengths of the included exons in bp. The orientation and approximate positions of the exon-specific RT-PCR primers are indicated by arrows. The coenzyme-binding domain- and catalytic domain-containing exons are underlaid in gray.

4.4.6 Butyrate treatment activates the downstream Hep27 promoter

Sequence analysis of the promoter utilized in HepG2 cells with the RepeatMasker program (Smit and Green, 2001) showed that Hep27 transcription is initiated at the already defined transcription start site of a solitary LTR of endogenous retroviral origin which is highly homologous to the LTR of the human endogenous provirus ERV9 (La Mantia *et al.*, 1992). Transposable elements are normally silenced in somatic cells, possibly by methylation of CpG residues and concomitant chromatin remodeling and translocation of the sequences to inactive heterochromatin (Yoder *et al.*, 1997; Razin, 1998; Razin, 1998). Silencing of retroviral sequences can be overcome by histone deacetylase inhibitors like sodium butyrate or trichostatin A which alleviate the chromatin-associated transcriptional repression (Barka, 1998; Chen and Townes, 2000; Chen and Townes, 2000).

As shown in Figure 4.30, treatment with sodium butyrate induced Hep27 expression in various cell lines. The Hep27 transcripts were of equal length as the main transcript in HepG2 cells which pointed to transcription being initiated at the LTR. Long-time exposure revealed that butyrate treatment also induced the other splice variants found in HepG2 cells in all cell types examined.

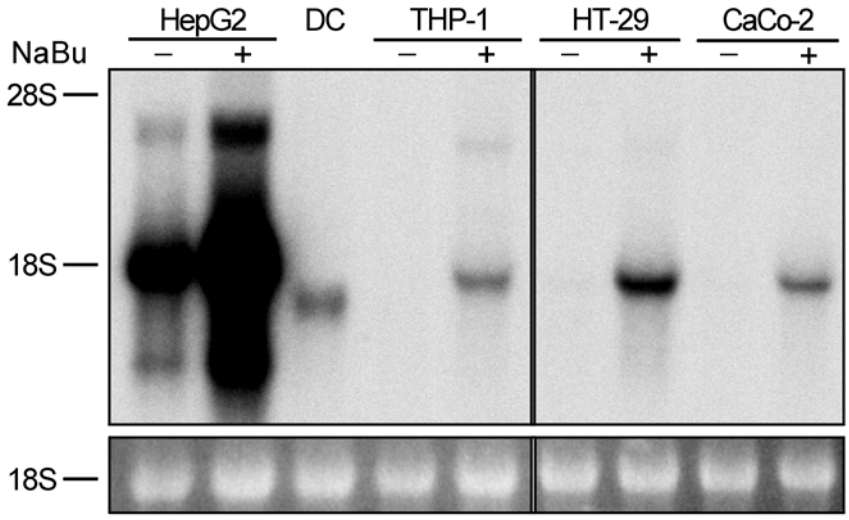


Figure 4.30 Effect of butyrate treatment on Hep27 mRNA expression in different cell lines.
Northern blot analysis of Hep27 mRNA expression in monocyte-derived dendritic cells and the cell lines HepG2, THP-1 HT-29 and CaCo-2. Culture conditions were 30 h and 48 h in the presence or absence of 10 mM sodium butyrate (HepG2 and THP-1, respectively) or 22 h and 30 h in the presence or absence of 5 mM sodium butyrate (HT-29 and CaCo-2, respectively). 10 µg of total RNA were in each lane were hybridized with a random-primed probe encompassing exons II-VIII. The bottom inset shows the EtBr-stained 18S rRNA bands immediately after blotting.

Parallel RT-PCR experiments which were performed as described for Figure 4.28 confirmed that the Hep27 transcripts induced by sodium butyrate contained exon IIa but not exon I, and thus had to be initiated at the LTR promoter as opposed to the upstream promoter which seems to be utilized exclusively by DCs (Figure 4.31). In separate experiments, butyrate-treatment also induced exon IIa-containing Hep27 transcripts in macrophages and DCs as assessed by RT-PCR (data not shown).

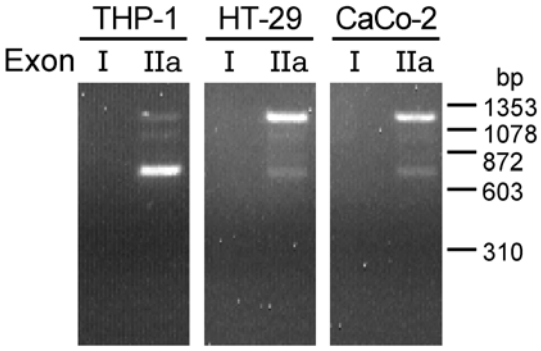


Figure 4.31 Sodium butyrate-treatment activates the downstream Hep27 promoter.
RT-PCR for *Hep27* was performed as described for Figure 4.28 with 1 µg each of the total RNA samples from butyrate-treated THP-1, HT-29 and CaCo-2 cells used in the Northern blot analysis shown in Figure 4.30.

5 Discussion

5.1 Identification of genes with DC-specific expression

The aim of this work was the identification of differentiation-associated genes with specific expression in DCs. RDA was employed as a screening method to subtract monocyte and macrophage-derived cDNA from DCs-derived cDNA. The resulting fragments were cloned and checked for DC specificity by reverse dot blot. Clones with exclusive expression in DCs were sequenced and the obtained sequences compared to published sequences in GenBank. This approach yielded nine differentially expressed fragments which mapped to six different genes. Three of the genes had previously been described to either be expressed by DCs (DC-CK-1 and complement C1q C-chain (Adema *et al.*, 1997; Schwaeble *et al.*, 1995)) or to be up-regulated in monocytes by IL-4 (15-LOX) (Conrad *et al.*, 1992). DC-specific expression of the remaining genes, MCP-4, Hep27 and FR-β had not been described before to be expressed by DCs. Further efforts focussed on the characterization of MCP-4 and Hep27 expression and the analysis of their regulation during DC differentiation.

5.2 MCP-4

Analysis of MCP-4 mRNA expression in monocytes, macrophages and DCs by Northern blot analysis confirmed the initial RDA data and revealed that MCP-4 expression was restricted to immature DCs. Although MCP-4 expression could be induced by adherence alone, this stimulus only led to short-term MCP-4 mRNA expression, with mRNA levels becoming undetectable after 16 h. Long-term MCP-4 expression required the presence of IL-4 which delayed the onset of MCP-4 mRNA expression. GM-CSF was found to further enhance IL-4-induced MCP-4 expression but was not sufficient to induce MCP-4 by itself.

Maturation of DCs can be induced by a number of „danger“ signals (Gallucci and Matzinger, 2001), TNF, LPS and CD40 ligation being most commonly used *in vitro*. Culture of DCs with either TNF or LPS led to down-regulation of MCP-4 expression, LPS exhibiting a more rapid effect than TNF.

Assessment of MCP-4 protein in DC supernatants showed that DCs secrete large amounts of MCP-4 into the supernatant, exceeding 3 ng/(24 h x 10^6 cells). The decrease in transcription induced by DC maturation also led to diminished to non-detectable protein levels in the supernatants.

Taken together, it could be shown that DCs express MCP-4 while in an immature state and that MCP-4 is down-regulated during DC maturation.

MCP-4 is a member of the chemokine family of proteins which to date comprises more than 50 members. Chemokines (derived from "chemotactic cytokines") elicit chemotactic responses (directional migration) of responsive leukocytes along chemokine gradients through interactions with a subset of seven transmembrane domain, G protein-coupled receptors. Chemokines are small (~8-14 kDa), mostly basic, structurally related proteins with four conserved cysteines close to the N-terminus which form two essential disulfide bonds (Cys1-Cys3 and Cys2-Cys4). Their tertiary structure is characterized by a typical chemokine fold containing a free amino terminus followed by the first cysteine residue, three antiparallel β-sheets and a carboxy-terminal α-helix. The chemokine fold seems to be critical to the activity of the free amino-terminal domain (Crump *et al.*, 1997), which contains the epitopes responsible for the specific interactions with the cognate chemokine receptors after the first contact of an exposed loop of the β-sheet between the second and third cysteine residue with the receptor has occurred (Clark-Lewis *et al.*, 1995).

Based on the arrangement and the presence of the two N-terminal cysteine residues, chemokines have been divided into four subfamilies. The two large CC-(β-) and CXC-(α-) families which include all but two of the known chemokines are characterized by the two cysteine residues being either adjacent (CC) or separated by one amino acid (CXC). The other two subfamilies only comprise one chemokine each, the transmembrane protein fractalkine/neurotactin forming the CX3C- or γ-class, the chemokine fold being attached to a transmembrane domain by a mucin-like stalk and the two cysteine residues separated by three amino acids in between, and lymphotactin, which only contains two instead of four cysteine residues, constituting the C- or δ-class (Baggiolini, 1998).

MCP-4 is a member of the MCP-subfamily of CC-chemokines which - in humans - comprises MCP-1 through –4 and eotaxin. The majority of the genes encoding CXC- and CC-chemokines are located in gene clusters on chromosomes 4q13 and 17q11.2-12, respectively, and share a common exon-intron structure (CXC, 4 exons; CC, 3 exons) (Baggiolini *et al.*, 1997). Accordingly, the MCP-4 gene contains three exons which encode for a 98 aa residue precursor protein with a 23 aa residue hydrophobic signal peptide that is cleaved to yield a 8 kDa, 75 aa mature MCP-4. Two longer MCP-4 variants of 77 and 82 residues termed LA-MCP-4 and FNPQGLA-MCP-4 have been described in the literature, the shorter of which was found to be 30-fold less potent than the main, 75 aa variant (Uguccioni *et al.*, 1996).

Mature MCP-4 lacks potential N-glycosylation sites and, like the other human MCPs, contains an N-terminal pyroglutamate proline motif. In its mature form, human MCP-4 shares 56-62% aa sequence identity with the other members of the MCP family, being most homologous to MCP-1 (60%),-3 (60%) and eotaxin (62%).

MCP-4 acts through the promiscuous chemokine receptors CCR2B, CCR3, CCR9 (cloned as D6 and CCR10) and CCR11 (Garcia-Zepeda *et al.*, 1996; Godiska *et al.*, 1997; Berkhout *et al.*, 1997; Stellato *et al.*, 1997; Nibbs *et al.*, 1997; Ruffing *et al.*, 1998; Schweickart *et al.*, 2000) and has been shown to be chemotactic for monocytes, T lymphocytes, eosinophils, basophils and immature monocyte-derived DCs (Uguccioni *et al.*, 1996; Sozzani *et al.*, 1997). MCP-4 mRNA is expressed strongly at sites with exposure to exogenous antigens like lung, small intestine and colon and displays consecutively weaker expression in other tissues such as heart, thymus, placenta, uterus, skeletal muscle, pancreas, liver, kidney, prostate, testis and peripheral blood leukocytes and is not expressed in brain and spleen (Godiska *et al.*, 1997; Stellato *et al.*, 1997; Berkhout *et al.*, 1997; Garcia-Zepeda *et al.*, 1996). Weak basal MCP-4 expression has been reported in airway endothelial cells and submucosal airway mononuclear cells (Chakravorty *et al.*, 2001). Elevated MCP-4 protein expression has been described in endothelial and epithelial cells in several inflammatory settings comprising atherosclerosis (Berkhout *et al.*, 1997), asthma (Lamkhioued *et al.*, 2000), acute renal inflammation (Chakravorty *et al.*, 2001) and basal epithelial cells in the crypts of inflamed tonsils as well as blood vessel-contacting basal keratinocytes in psoriatic skin lesions (Vanbervliet *et al.*, 2002).

MCP-4 expression by cells of the mononuclear infiltrate in interstitium or submucosa was always observed in $CD68^+$ areas.

In vitro, MCP-4 mRNA has been detected in primary endothelial and epithelial cells and cell lines (Garcia-Zepeda *et al.*, 1996), dermal and lung fibroblasts (Petering *et al.*, 1998; Teran *et al.*, 1999), the promonocytic U937 cell line and monocyte-derived macrophages (Godiska *et al.*, 1997).

During the course of this work, MCP-4 expression was also identified in monocyte-derived DCs by SAGE but only confirmed by RT-PCR (Hashimoto *et al.*, 1999). In the work presented here, monocyte-derived DCs could be shown to produce large amounts of MCP-4 mRNA as well as protein. MCP-4 mRNA was detectable as early as one day after the onset of culture, expression reaching its maximum after two days. While GM-CSF alone did not induce MCP-4 by itself, it had a synergistic effect on IL-4-induced MCP-4 production.

Proinflammatory stimuli like TNF, LPS or CD40-ligation lead to terminal differentiation of DCs, which was found to be accompanied by down-regulation of MCP-4 in monocyte-derived DCs, indicating that MCP-4 production may be characteristic of DCs at an immature stage *in vivo*.

Indeed, an *in vivo* DC subpopulation, mixed lineage-negative, CD11c- or CD123-positive blood DCs (BDCs) tested weakly positive for MCP-4 mRNA in RT-PCR. However, maturation did not alter MCP-4 expression in these cells which might be explained by their pertinence to a DC subset different from interstitial DCs and the monocyte-derived DC model (Dzionek *et al.*, 2000). Further experiments will be necessary to identify the source of MCP-4 in BDC preparations.

In contrast to the findings of Godiska et al. (Godiska *et al.*, 1997), MCP-4 could not be detected in monocyte-derived macrophages either by Northern blot hybridization and PCR or in Western blots. However, monocytes were found to express intermediate levels of MCP-4 mRNA when treated with LPS, while macrophages were less responsive (data not shown), suggesting that the macrophage preparation used in their experiments might have been activated by contaminating LPS.

Apart from epithelial cells and endothelium, subepithelial or subendothelial expression of MCP-4 in inflamed tissues has largely been attributed to macrophages,

either based on the morphology (Garcia-Zepeda *et al.*, 1996; Lamkhioued *et al.*, 2000) or CD68-positivity of the infiltrated area (Berkhout *et al.*, 1997; Ying *et al.*, 1999; Chakravorty *et al.*, 2001). Since both macrophages and DCs have been described to express CD68 *in vivo* (Betjes *et al.*, 1991), the findings that *in vitro*-derived immature DCs but not *in vitro*-derived macrophages produce copious amounts of MCP-4, suggest that the mononuclear MCP-4-expressing cells *in vivo* might be $CD68^+$ immature DCs.

The recently published promoter sequence of MCP-4 contains several putative GAS, IREγ and NF-κB consensus sites. These sites have been implied in the observed MCP-4 induction by IFN-γ and TNF in dermal fibroblasts (Hein *et al.*, 1999). In monocytes, however, IFN-γ did not induce MCP-4 message. On the contrary, IFN-γ abolished the induction of MCP-4 mRNA in monocytes if added prior to stimulation with IL-4. Analogous patterns of gene regulation in monocytes and DC have been described for 15-lipoxygenase (Dickensheets and Donnelly, 1997) and the chemokines DC-CK1/AMAC-1 (Adema *et al.*, 1997; Kodelja *et al.*, 1998) and MDC (Bonecchi *et al.*, 1998). This phenomenon has been suggested as a general regulation pattern for IL-4-inducible genes in monocytes (Levings and Schrader, 1999) and has been shown to be caused by suppression of IL-4-induced STAT6 tyrosine phosphorylation by interferons, probably through the induction of *de novo* expression of the JAK/STAT inhibitory gene SOCS-1 (suppressor of cytokine signaling) (Dickensheets *et al.*, 1999).

Additionally, comparison of MCP-4 mRNA expression by DCs and fibroblasts stimulated with various agents indicated that fibroblasts produce only negligible amounts of MCP-4, probably making their contribution to the overall MCP-4 production *in vivo* insignificant.

The trafficking of DCs in the body is guided by gradients of specific chemokines emanating from cells in the different tissues. Inflammation-induced chemokines cause directional migration of DCs towards the chemokine source and thus sites of inflammation and possible antigen uptake. Later on, chemokines are essential for DC homing to secondary lymphatic organs where they effectively support the induction of specific immune responses. DC-expressed chemokine receptors include CCR1,

CCR2, and CCR5 (Sozzani *et al.*, 1997), CCR3 (Delgado *et al.*, 1998), and CXCR1, CXCR2, and CXCR4 (Sozzani *et al.*, 1997), some of which have been suggested to mediate tissue-specific DC migration, such as CCR6 on Langerhans cells as the targeting receptor for the epidermis (Charbonnier *et al.*, 1999) or CCR7 as lymphoid organ-homing receptor (Dieu *et al.*, 1998).

DC function requires the interaction with other immune cells. Consequently, DCs have been found to express a number of chemokines in a differentiation-associated fashion, which direct effector cells like T lymphocytes to sites of antigen presentation and activation. Examples of chemokines produced by DC are DC-CK1, which attracts naive B and T lymphocytes (Adema *et al.*, 1997; Lindhout *et al.*, 2001), the activated and memory T cell-attracting chemokines TARC (Imai *et al.*, 1999), MDC (Tang and Cyster, 1999) and MIP-3β/ELC (Ngo *et al.*, 1998), as well as BLC (Vissers *et al.*, 2001), which acts on B cells and activated T and has been shown to be required for the development of most lymph nodes and Peyer's patches in mice (Ansel *et al.*, 2000).

At this point, one can only speculate on the putative role of MCP-4 expression by immature DCs. The receptors for MCP-4 are expressed mainly on monocytes, eosinophils, T lymphocytes and DCs themselves. One hypothesis that might explain the basal MCP-4 expression found in different tissues involves the fact that immature DCs form tight networks in these tissues, e.g. airway epithelium (Holt *et al.*, 1989), extracting antigen from each other and surrounding cells (Harshyne *et al.*, 2001) and exhibit constitutive turnover (Fossum, 1989; Holt *et al.*, 1994). Immature DCs express receptors for MCP-4, namely CCR2, CCR3 and CCR5 (Delgado *et al.*, 1998; Sallusto *et al.*, 1998) and have been reported to be attracted by MCP-4 (Sozzani *et al.*, 1997). Therefore, its expression by DCs as well as epithelial and endothelial cells (Berkhout *et al.*, 1997; Lamkhioued *et al.*, 2000; Vanbervliet *et al.*, 2002) might help to establish and keep a network of MCP-4-expressing, antigen-exchanging immature DCs in close association with the cells lining sites of possible antigen entry. Indeed, $CD11c^+$ DC were shown to localize to MCP-4-expressing blood vessel-contacting inflamed basal epithelial cells in tonsil crypts, which themselves are rich sources of MCP-4 (Vanbervliet *et al.*, 2002). The turnover of DCs in these tissues requires their maturation after a period of antigen sampling, including up-regulation of the homing receptor CCR7 (Dieu *et al.*, 1998) and concomitant down-regulation of MCP-4 and its

receptors (Delgado *et al.*, 1998; Sallusto *et al.*, 1998). On the one hand this would lead to their departure from the DC network in response to SLC, produced by secondary lymphoid tissue endothelial cells and on the other hand keep them from attracting the remaining, still immature DCs, leaving the network intact. The described increased DC turnover in inflammation (Holt *et al.*, 1994) and rapid recruitment of MCP-4-expressing immature DCs to the tissues (McWilliam *et al.*, 1994) would account for the elevated MCP-4 expression associated with CCR3- and CCR5-, CD68-positive infiltrates in inflamed tissues (Chakravorty *et al.*, 2001) and add to the observed attraction of $CCR3^+$ cells such as eosinophils, monocytes and T lymphocytes.

The hypothesis developed here is further complicated by the fact that *in vivo*, chemokines mostly do not work as isolated reagents that bring about one unique effect but rather act in concert, making it impossible to trace back a complex event like e.g. antigen-induced eosinophil influx to one single cellular signal. Clearly, further experiments are needed to elucidate the roles of MCP-4 expression *in vivo*.

Due to the high expression levels of MCP-4 and its exclusive presence in DCs, the MCP-4 promoter was characterized as a model for a DC-specific promoter.

The sequence of the MCP-4 promoter contains several putative binding sites for transcription factors, some of which have been implied in the regulation of DC-specific genes. Currently, no human DC lines are available which would allow analysis of MCP-4 promoter by transient reporter assays. However, the proximal promoter also contains a number of CpG motifs. Methylation of CpG motifs in mammals has been described as a repression mechanism active in X-chromosome inactivation, genomic imprinting and silencing of mobile elements (Siegfried and Cedar, 1997) and acts either directly via inhibition of binding of methylation-sensitive transcription factors to their CpG-containing DNA recognition sequence or indirectly, by recruiting methylcytosine-binding proteins which have been shown to both actively repress transcription through interaction with RNA polymerase II and to recruit histone deacetylases. These in turn deacetylate the surrounding histones which eventually leads to inaccessibility of the promoter to transcription factors and silencing of the gene (Razin, 1998). Due to its repressive effects, CpG methylation

has been implied in tissue-specific repression of genes with low CpG density (Razin and Cedar, 1991), CpG demethylation taking place as a differentiation-associated event that accompanies and enables gene activation.

Therefore, as an initial characterization, the methylation status of the MCP-4 promoter in monocytes, monocyte-derived macrophages and monocyte-derived DCs was analyzed. Using bisulfite sequencing, two CpG motifs of the proximal MCP-4 promoter were found to be demethylated during monocyte to DC differentiation and to remain methylated during monocytes to macrophage differentiation.

Two mechanisms of demethylation are discussed in the literature: passive demethylation, which is coupled to DNA replication, originating from suppressed maintenance methylation of the newly synthesized DNA strand, and active demethylation, which can take place in the absence of DNA replication. Up to now, active demethylation *in vivo* has only been demonstrated in early embryonic cells, where the paternal genome undergoes genome-wide demethylation before the onset of DNA replication (Mayer *et al.*, 2000). Demethylation in somatic cells after the embryonic phase so far has been assumed to be a passive event. Although there is evidence that active, i.e. DNA replication-independent, demethylation might exist in somatic cells (Lucarelli *et al.*, 2001), somatic active DNA demethylation has not yet been demonstrated conclusively.

Differentiation of human monocytes to DCs has been shown to proceed in the absence of DNA replication (Cavanagh *et al.*, 1998; Ardeshna *et al.*, 2000), effectively ruling out a DNA replication-based mechanism to explain the demethylation observed for the MCP-4 promoter. Thus, this finding probably represents the first report of active CpG demethylation in non-proliferating somatic cells.

Demethylation of one of the CpG motifs of the MCP-4 promoter facilitated binding of a nuclear factor that was present in both monocyte-derived macrophages and monocyte-derived DCs. Binding was sequence-specific and alterations of the CpG motif mimicked the effect of CpG methylation. In transient transfections of THP-1 cells, which express the CpG methylation-sensitive DNA-binding factor as well, mutation of the CpG motif reduced promoter activity by approximately 50%, indicating a possible role of this element in regulation of MCP-4 expression in DCs.

A query of the TRANSFAC database showed that the respective CpG lies in a core recognition motif of the murine winged-helix nude (whn) transcription factor, a member of the forkhead family of transcription factors. Several facts like the highly conserved core consensus binding sequence and the sensitivity of murine whn binding to DNA methylation (Lee *et al.*, 1999) point to human whn as the nuclear protein in question. However, its exclusive expression in skin and thymic epithelial cells makes it a less likely candidate. As a side note, in a recent paper by Thomassin *et al.* (Thomassin *et al.*, 2001), methylation-sensitive binding of a nuclear factor to an oligonucleotide containing a sequence motif identical (ACGC) to the whn core consensus binding site encompassing the –80 bp CpG of the human MCP-4 promoter is reported. The factor binds to a region within an enhancer of the rat liver-specific *Tat* gene after rapid chromatin remodeling which is followed by targeted CpG demethylation. Although the actual binding motif of the factor was not identified, it can be inferred from the hyperreactivity of the guanosine residue complementary to the second cytosine in the ACGC motif in the *in vivo* footprints that the factor binds to or in the immediate neighborhood of this motif. Together with the sensitivity of the binding to CpG methylation this suggests identity of the factor with the factor binding to the corresponding site of the MCP-4 promoter. Since HeLa cells also express the –80 bp CpG-binding factor, the protein itself is probably ubiquitously expressed. Identification of the factor after purification from nuclear protein extracts by affinity chromatography with the -80 CpG EMSA oligonucleotide will likely provide more insight into its possible properties as a target of CpG-induced silencing and its putative role in enhancing or enabling transcription.

Nearly 30 years after the predictions of Riggs (Riggs, 1975) and Holliday and Pugh (Holliday and Pugh, 1975) that tissue-specific genes would be found to be methylated within regulatory regions before the onset of transcription, and that programmed demethylation would allow transcription in the appropriate cell type, the role of CpG methylation in the tissue-specific regulation of gene expression is still controversial.

This is mainly due to the large body of correlative data of promoter demethylation coinciding with up-regulation of tissue-specific genes which has accumulated since then, without ever supplying evidence for demethylation as the actual cause for

upregulation of gene expression (Walsh and Bestor, 1999). Similarly, in DNA methyltransferase-deficient mice which exhibit genome-wide hypomethylation, DNA methylation could be shown to play central roles in genomic imprinting (Li *et al.*, 1993) (hypomethylation leads to biallelic expression of imprinted genes), X chromosome inactivation (Panning and Jaenisch, 1996) (hypomethylation leads to ectopic Xist gene expression and X chromosome inactivation) and silencing of mobile genomic elements (Walsh *et al.*, 1998), but there has been no indication of ectopic expression of tissue-specific genes in these mice.

Given the ambiguous data, Walsh and Bestor (Walsh and Bestor, 1999) have suggested that the observed correlations between demethylation and transcription might be due to demethylation being a consequence of transcriptional activation rather than the cause. In agreement with their analysis, demethylation of the MCP-4 promoter in DCs occurs after the onset of MCP-4 transcription and is not necessary to allow MCP-4 transcription, as can be seen in IL-4 and GM-CSF-treated macrophages which transcribe MCP-4 mRNA from a completely methylated promoter. Additionally, CpG demethylation alone is not sufficient to sustain MCP-4 expression in DCs after withdrawal of IL-4 and GM-CSF. However, since mutation of the –80 bp CpG decreases the activity of the MCP-4 promoter in THP-1 cells, CpG demethylation might be necessary to attain maximal MCP-4 expression in DCs which approximately coincides with completion of demethylation after two days of culture. In comparison, the weaker expression of MCP-4 mRNA in IL-4/GM-CSF-treated macrophages might be caused by the methylated –80 bp CpG in the MCP-4 promoter of these cells presumably by preventing transcription factor binding to the site. This would be in line with the recent finding that T_H2 differentiation-associated demethylation of the IL-4 locus is correlated with high-level IL-4 expression (Lee *et al.*, 2002). In order to verify the hypothesis that demethylation is necessary for maximal MCP-4 transcription, identification of the CpG-binding factor will enable to assess its role in realizing maximal MCP-4 promoter activity by analyzing its impact on the activity of methylated vs. unmethylated MCP-4 reporter constructs in cotransfection/ overexpression experiments.

A recent report (Palucka *et al.*, 1998) suggested that in their immature states, macrophages and DC retain plasticity in their differentiation potential, allowing them to convert from the DC to the macrophage phenotype and *vice versa*. Although this

might be true concerning the phenotypic aspects examined in this paper, the results presented here indicate that for the demethylation events in the MCP-4 promoter there is only a short time slot during which differentiation-coupled demethylation can take place during DC development. After completion of this period, the methylation status seems to become refractory to change and thus appears to conserve the differentiation history of the MCP-4 gene in each particular cell and to define its capacity to react to new stimulatory events, be it in macrophages or DCs. Hence the different states of CpG methylation of the MCP-4 promoter in macrophages and DCs seem to be characteristic of the distinct monocytic differentiation pathways.

5.3 Hep27

Hep27 was originally cloned from HepG2 cells where it is constitutively expressed and is up-regulated following butyrate-treatment (Donadel *et al.*, 1991; Gabrielli *et al.*, 1995). Hep27 protein has a molecular mass of approximately 27 kDa. Its amino acid sequence classifies Hep27 as a member of the short chain dehydrogenase/reductase (SDR) family of enzymes. The substrate for Hep27 has not yet been identified.

Northern blot analysis and RT-PCR confirmed that expression of Hep27 was restricted to DCs and HepG2 cells. Hep27 mRNA was absent from other hematopoietic cells such as granulocytes or lymphocytes as well as HUVEC, dermal fibroblasts and two colon epithelial cell lines. During differentiation of monocyte-derived DCs, Hep27 mRNA was detectable as early as two days after the onset of culture. Hep27 expression was restricted to monocyte-derived DCs at an immature state, since Hep27 was down-regulated by DC maturation stimuli like LPS or TNF.

Hep27 was initially described as a nuclear protein. However, using a polyclonal antiserum in Western blots, Hep27 protein was detectable only in cytoplasmic DC extracts but not in nuclear extracts nor the remaining nuclear envelopes. These findings are in line with more recent immunohistochemical data which indicates that Hep27 is mainly located in the cytosol and only a small fraction of the Hep27 protein pool can be found in the nucleus (Pellegrini *et al.*, 2002).

To understand the molecular basis for the highly selective expression of Hep27, the transcription start site in DCs and HepG2 cells was identified by 5’-RACE and the upstream genomic sequences were analyzed. The 5'-end of the HepG2-expressed

transcript is related to the LTR consensus of the human endogenous ERV9 provirus, pointing to a solitary ERV9-derived LTR driving Hep27 expression in these cells (La Mantia *et al.*, 1991). Alignment with the publicly available genomic sequence and analysis of the upstream sequence confirmed this hypothesis and showed that the start site of Hep27 transcription in HepG2 cells coincides with the previously described ERV9 transcription start site (La Mantia *et al.*, 1992). In DCs, 5'-RACE and subsequent promoter walking revealed a different promoter to be active. In contrast to the TATA-less retroviral HEP27 LTR promoter utilized by HepG2 cells, the DC-specific promoter, which lies approximately 6.2 kb upstream, does not contain repetitive sequences and features a regular TATA box 29 bp upstream of the transcription start as well as a number of putative binding sites for several transcription factors. Analysis of the surrounding genomic region with the RepeatMasker program (Smit and Green, 2001) showed that the alternative promoter and the first exon lie in a repeat-less island which is positioned in a repeat-rich stretch containing multiple remnants of retroviral integration.

Analyzing the Hep27 transcripts in HepG2 cells and monocyte-derived DCs a number of splice variants were identified, which in conjunction with the different transcription start sites were cell type-specific and could be distinguished by Northern blot analysis based on their different lengths. The main transcripts in HepG2 cells and monocyte-derived DCs were 1.9 kb and 1.5 kb in length, respectively.

In Northern blots of tissue mRNA, transcript variants similar to the ones observed for the different cell types could be detected. The short variants found in monocyte-derived DCs were present in almost all tissues, which is in agreement with the almost ubiquitous presence of DCs *in vivo*. A longer Hep27 transcript corresponding to the main transcript in HepG2 was strongly expressed in liver. It is also the main transcript in placenta and spleen, as well as in kidney. The ERV9 LTR promoter has been suggested to induce tissue-specific gene expression in erythroid cells (Long *et al.*, 1998). Hence the liver-specific expression of Hep27 might also be a trait of the LTR promoter. On the other hand, using the histone deacetylase inhibitor sodium butyrate, Hep27 transcription driven by the LTR promoter could be initiated in several non-hepatocytic cell lines as well as monocyte-derived DCs. Butyrate-induced histone hyperacetylation leads to an open chromatin conformation which in turn makes the DNA more accessible to the transcription machinery. Using Trichostatin A,

Plant *et al.* (Plant *et al.*, 2001) recently described similar findings for ERV9 LTR-initiated intergenic transcription in the human β-globin gene cluster which is normally erythrocyte-specific. The authors suggest that in non-transcribing cell types the block to transcription may lie at the level of chromatin structure rather than at the level of LTR-specific transcription factors, i.e. the lack thereof. As butyrate concentrations of portal vein blood can reach concentrations of up to 40 µM (Peters *et al.*, 1992), expression of the LTR-initiated Hep27 transcript in liver might be the result of LTR activation caused by the high butyrate concentrations. In fetal liver, where butyrate should not be present due to the absence of bacterial inhabitation of the fetal intestine, only the short, presumably exon I-containing Hep27 fragment is expressed, but not the longer, LTR-initiated transcript found in adult liver, pointing to a potential role of bacterially produced butyrate in Hep27 induction in adult liver. However, this reasoning does not account for the observation of the long transcript in spleen, kidney and placenta, where butyrate concentrations are extremely low (Peters *et al.*, 1992). One possible explanation, at least for the placental expression, might be the fact that placental DNA remains hypomethylated after blastocyst formation (Ehrlich *et al.*, 1982). Since CpG methylation has been suggested as one mechanism to suppress expression of endogenous retroviral sequences (Yoder *et al.*, 1997), placental DNA hypomethylation might induce Hep27 expression from its LTR promoter.

Expression of the Hep27 variant produced in immature monocyte-derived DCs is most prominent in non-lymphoid tissues like liver, kidney, heart and skeletal muscle where DCs are known to reside in an immature state (Hart , 1997). In spleen, where mature DCs are also present (McIlroy *et al.*, 2001), weaker expression of the shorter Hep27 mRNA variant was observed. Clearly, immunohistochemical studies with suitable antibodies or isolation and purification of DCs ex vivo followed by RT-PCR are necessary to confirm that DCs are sources of Hep27 *in vivo*.

5.4 Complement C1q C-chain

In line with the previously identified sources of C1q (Schwaeble *et al.*, 1995), Northern blot analysis for C1q C-chain expression confirmed macrophages and DCs as producers of C1q C-chain mRNA *in vitro*. Interestingly, macrophages, which are commonly viewed as the main source of C1q, only transiently expressed readily

detectable levels after four days of culture, i.e. at an intermediate stage of differentiation, and exhibited weaker C1q C-chain expression on day one and seven. Conversely, immature DCs produced far greater levels of C1q C-chain mRNA which was down-regulated by the maturation stimuli LPS and TNF. Similar results have been published recently for the mRNA expression of all three C1q subchains in a spleen-derived murine DC cell line (Granucci *et al.*, 2001). The data obtained for macrophages contradict recently published results for C1q C-chain expression in macrophages which show a continual increase of C-chain expression during monocyte to macrophage differentiation (Kaul and Loos, 2001). The differing results might arise from the different culture conditions (2% vs. 10% pooled human AB serum).

Complement C1q C-chain is a subcomponent of the C1q component of C1, the first component of the classical complement activation pathway. Together with the A- and B-chains, the C1q C-chain forms triple helices between their N-terminal collagen-like sequences. Six of these collagen-like “stalks” then self-assemble symmetrically to form the fibril-like central core of the umbrella-shaped C1q hexamer with the globular heads at their C-termini projecting on radial arms. Association of C1q with a $C1r_2C1s_2$ tetramer of the serine esterases C1r and C1s yields C1. Upon binding of antigen-bound to the globular heads, a conformational change activates C1r which in turn cleaves C1s. The activated C1s then hydrolyzes other complement components, thus initiating a protease cascade which leads to opsonization of antigen-bearing cells, release of chemotactic and pro-inflammatory degradation products (anaphylatoxins) and eventually to osmotic lysis of antigen-bearing cells through assembly of the Membrane Attack Complex.

Apart from its activating role in the classical complement pathway, C1q has been shown to bind to a variety of cell types as well as apoptotic cells (Korb and Ahearn, 1997; Navratil *et al.*, 2001) and to interact with a number of cognate cell surface receptors (reviewed in (Eggleton *et al.*, 2000)). The elicited effects depend on the triggered receptor and range from activation of respiratory burst in neutrophils and macrophages and inhibition of proliferation of activated T lymphocytes (Chen *et al.*, 1994) to enhancement of phagocytosis (Guan *et al.*, 1994), the initiation of macropinocytosis and uptake of apoptotic cells (Navratil *et al.*, 2001). The latter has recently been shown to proceed through binding of apoptotic cell-bound C1q to

calreticulin on the macrophage cell surface, which in turn is bound by CD91 (Ogden *et al.*, 2001).

In a recent report, Steinberger *et al.* (Steinberger *et al.*, 2002) showed one of the previously identified receptors for C1q, the phagocytosis-enhancing C1qRp (Nepomuceno *et al.*, 1997) to be identical to CD93 which had previously been shown to be expressed by monocytes, granulocytes and endothelial cells (Menetrier-Caux *et al.*, 1998) as well as immature DCs (Pickl *et al.*, 1996). Additionally, they found CD93 expression to be down-regulated upon DC maturation. (This is in contrast to another report on C1qRp (Fonseca *et al.*, 2001) which described C1qRp to be absent from both immature as well as mature DC.)

Functionally, the expression of C1q as well as its phagocytosis-enhancing receptors by DCs might contribute to the avid endocytic activity of immature DCs. Their maturation-induced down-regulation consequently would add to the general shut-down of endocytosis in maturing DCs. Additionally, the described antiproliferative effect of C1q on activated T cells (Chen *et al.*, 1994) might contribute to the tolerizing effect of immature DCs. Interesting questions to answer in further experiments therefore would be whether C1q is able to induce endocytosis levels in mature DCs comparable to immature DCs and whether addition of C1q to MLCs with mature DCs *vs.* immature DCs as stimulators alters the T cell proliferation rate.

5.5 15-Lipoxygenase

In agreement with previously published data showing induction of the lipid-peroxidating 15-lipoxygenase type 1 (EC 1.13.11.33) (15-LOX) in monocytes by IL-4 in the presence or absence of GM-CSF (Conrad *et al.*, 1992), 15-LOX was found to be strongly expressed in immature DCs and to be absent from macrophages and freshly isolated monocytes. DC maturation with LPS or TNF led to down-regulation of 15-LOX.

15-LOX has been implicated in a number of cellular processes, ranging from metabolic and structural changes to cell signaling and transcriptional control. 15-LOX is a non-heme iron-containing fatty acid-peroxidating enzyme and catalyses the stereospecific oxygenation of arachidonic acid (5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid) and linoleic acid (9*Z*,12*Z*-octadecadienoic acid) to yield mainly 15-HPETE (15*S*-

hydroperoxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid) (as well as minor amounts of 12-HPETE (12*S*-hydroperoxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid)) and 13-HPODE (13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid), respectively. The products are then rapidly reduced to the stable hydroxylated forms.

Unlike the other known lipoxygenases, 15-LOX is able to oxygenate its substrates either as free acid or esterified to lipid components or cholesterol in lipoproteins or biological membranes.

Since 15-LOX co-localizes with oxidized low density lipoprotein (oxLDL) in macrophage-rich regions of atherosclerotic lesions (Yla-Herttuala *et al.*, 1990) and has been shown to oxidize LDL to pro-atherogenic oxLDL *in vitro*, the enzyme has been implicated in the oxidation of LDL in vivo, a key event in the initiation of atherosclerosis. Due to contradicting evidence for a pro-atherogenic effect of 15-LOX in mice *vs*. anti-atherogenic effects in 15-LOX in rabbits, by gene knockout and 15-LOX overexpression, respectively, its actual role in atherogenesis is still a point of discussion (Funk and Cyrus, 2001).

In the functional context of DCs, several recent findings point to a possible involvement of 15-LOX in endocytic uptake of apoptotic cells, cross-presentation and the tolerogenic effects of immature DCs: Membrane translocation and activation of 15-LOX in eosinophils has been shown to be calcium-dependent (Brinckmann *et al.*, 1998). In a recent report, 15-LOX was shown to translocate to the cell membrane and to enhance actin-polymerization in IL-4-treated murine macrophages phagocytosing apoptotic cells, which could be inhibited using the specific 15-LOX inhibitor PD146176 (Miller *et al.*, 2001). Since phagocytosis of apoptotic cells by DCs has been shown to be calcium-dependent (Rubartelli *et al.*, 1997), 15-LOX might play a role in enhancing DC phagocytosis.

DCs have been shown to cross-present exogenous antigen to MHC class I-restricted $CD8^+$ T cells (Albert *et al.*, 1998b), owing to their unique ability to transport endocytosed antigen to the cytosol (Rodriguez *et al.*, 1999). DCs are avidly macropinocytic at an immature stage and macropinocytotic vesicles have been suggested to have a fatty acid composition similar to the ruffled membrane rafts they may be derived from (Nichols and Lippincott-Schwartz, 2001), containing elevated levels of arachidonic acid (Pike *et al.*, 2002). Since the membrane lipid-peroxidating

properties of 15-LOX have been shown to promote degradation of organelle membranes in reticulocytes (van Leyen *et al.*, 1998) and 15-LOX is expressed in immature DCs, 15-LOX might be involved in mediating permeabilization of macropinocytic vesicles and in generating the observed strong capacity of DCs to cross-present exogenous antigen. Additionally, the 15-LOX product 13-HODE is a potent ligand for the nuclear receptor PPAR-γ (Nagy *et al.*, 1998). Activation of PPAR-γ by binding of 13-HODE produced by IL-4-activated monocytes has recently been shown to suppress production of the key autocrine growth factor IL-2 by T lymphocytes (Yang *et al.*, 2002). Since the IL-4-induced expression of 15-LOX is downregulated by danger signals such as LPS or TNF, the production of 13-HODE by DCs or IL-4-activated monocytes might provide another feedback loop to prevent aberrant T cell activation and inflammation.

15-LOX has also been demonstrated recently in stem cell-derived DCs derived in the presence of IL-4, but as a caveat it has to be pointed out that although all the findings and conclusions for 15-LOX cited above do apply to DCs generated from monocytes *in vitro*, until now 15-LOX expression in DCs has not yet been demonstrated *in vivo*.

5.6 Folate Receptor β

Similar to most other genes identified in this study, folate receptor β (FR-β) or type 2 folate binding protein, was identified in immature DCs and its expression was downregulated upon maturation.

FR-β was originally cloned from placenta by Ratnam *et al*. (Ratnam *et al.*, 1989). Expression of FR-β has previously been demonstrated in spleen, thymus and neutrophils (Ross *et al.*, 1994; Ross *et al.*, 1999)as well as in synovial macrophages in rheumatoid arthritis (Nakashima-Matsushita *et al.*, 1999). Like the closely related FR-α, FR-β is a GPI-anchored membrane molecule and mediates uptake of folates by endocytosis. In contrast to FR-α, which binds the physiologically relevant 6S diastereomers of the reduced folates such as 5-methyltetrahydrofolate and 5-formyltetrahydrofolate, the FR-β has been reported to greatly favour binding of the unphysiologic 6R forms over the 6S diastereomers (Wang *et al.*, 1992).

Folic acid plays an important role as a carrier of one-carbon groups in several intracellular metabolic pathways. It is especially important for purine and thymidylate

synthesis, both of which are required for DNA synthesis. One could hypothesize that upregulation of FR-β on DCs might lead to depletion of folic acid around immature DCs which would halt proliferation of activated T cells in the vicinity of the non-activated DCs, similar to the inhibitory effect of tryptophane depletion by DC-produced indoleamine 2,3-dioxygenase (Hwu *et al.*, 2000). A proinflammatory signal would then relieve the metabolic T cell proliferation block by induction of DC maturation and downregulation of FR-β and thus validate the T cell-activating stimulus.

Further work is necessary to determine the relevance of these findings by assessing whether DCs express FR-β *in vivo* as well as to elucidate the function of FR-β expression in non-malignant adult cells.

6 Summary

During the course of this work, the genes DC-CK1, 15-LOX, MCP-4, Hep27, complement C1q C-chain and FR-β could be identified as being transcribed in monocyte-derived DCs but not in monocyte-derived macrophages or the precursor monocytes. Expression of all genes except DC-CK1 was restricted to immature DCs and was down-regulated upon DC maturation initiated by TNF or LPS.

MCP-4 expression in response to various stimuli could be shown to be different in DCs, macrophages, differentiating monocytes and human dermal fibroblasts on the mRNA level, immature DCs expressing the highest levels of MCP-4 mRNA as well as protein. Additionally, weak MCP-4 expression was observed in sorted blood DCs by RT-PCR.

DC specificity of expression of the SDR family member Hep27 (HUGO nomenclature: DHRS2) was confirmed in Western blots from cytosolic extracts from DCs and macrophages. Additionally, Hep27 mRNA could not be detected in a number of hematopoietic cells. Low level Hep27 mRNA expression could be demonstrated in all tissues examined; high level expression was found in heart and skeletal muscle, liver, kidney and placenta as well as spleen and fetal liver.

Several splice variants of Hep27 were identified and analyzed in DCs and HepG2 cells, which differ in their 5'-ends and overall length and seem to reflect the mature transcripts of distinct length detected in tissue Northern blots.

Its high-level expression in immature DCs made MCP-4 an interesting target for analysis of the mechanisms underlying its induction in DCs. Owing to the lack of DC cell line models suitable for classical reporter analysis to define a minimal promoter, the CpG methylation status of the MCP-4 promoter in monocytes, monocyte-derived macrophages and monocyte-derived DCs was analyzed by bisulfite sequencing. Exclusive CpG demethylation of two CpG sites at –80 bp and –20 bp, respectively, was detected in DCs.

Using electrophoretic mobility shift assays (EMSAs), binding of a nuclear factor to the demethylated but not the methylated or mutated –80 bp CpG motif could be demonstrated in macrophages, DCs, the monocytic cell line THP-1 and the human cervix carcinoma cell line HeLa.

Mutation of the –80 bp CpG abolished binding of the nuclear factor in EMSAs similar to CpG methylation and decreased activity of the proximal MCP-4 promoter by 50% in transient transfections of reporter constructs in THP-1 cells.

De-differentiation of immature DCs to macrophage-like cells and *vice versa* revealed the differentiation pathway dependency of the observed demethylation reaction, which apparently only takes place during the initial monocyte-DC transition and can not be induced at a later time point.

To elucidate the reasons for the DC-restricted Hep27 expression, the genomic structure of Hep27 was characterized. An alternative promoter could be defined, which is used exclusively in DCs, making it an interesting model for further analysis of DC-specific gene regulation.

7 References

Adema,G.J., Hartgers,F., Verstraten,R., de Vries,E., Marland,G., Menon,S., Foster,J., Xu,Y., Nooyen,P., McClanahan,T., Bacon,K.B. and Figdor,C.G. (1997). A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells. *Nature* **387**, 713-717.

Aderem,A. and Underhill,D.M. (1999). Mechanisms of phagocytosis in macrophages. *Annu. Rev. Immunol.* **17**, 593-623.

Albert,M.L., Pearce,S.F., Francisco,L.M., Sauter,B., Roy,P., Silverstein,R.L. and Bhardwaj,N. (1998a). Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J. Exp. Med.* **188**, 1359-1368.

Albert,M.L., Sauter,B. and Bhardwaj,N. (1998b). Dendritic cells acquire antigen from apoptotic cells and induce class I- restricted CTLs. *Nature* **392**, 86-89.

Andreesen,R., Picht,J. and Lohr,G.W. (1983). Primary cultures of human blood-born macrophages grown on hydrophobic teflon membranes. *J Immunol Methods* **56**, 295-304.

Ansel,K.M., Ngo,V.N., Hyman,P.L., Luther,S.A., Forster,R., Sedgwick,J.D., Browning,J.L., Lipp,M. and Cyster,J.G. (2000). A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* **406**, 309-314.

Ardeshna,K.M., Pizzey,A.R., Thomas,N.S., Orr,S., Linch,D.C. and Devereux,S. (2000). Monocyte-derived dendritic cells do not proliferate and are not susceptible to retroviral transduction. *Br. J. Haematol.* **108**, 817-824.

Baggiolini,M. (1998). Chemokines and leukocyte traffic. *Nature* **392**, 565-568.

Baggiolini,M., Dewald,B. and Moser,B. (1997). Human chemokines: an update. *Annu. Rev. Immunol.* **15**, 675-705.

Bain,B., Vas,M.R. and Lowenstein,L. (1964). The development of large immature mononuclear cells in mixed leukocyte cultures. *Blood* **23**, 108-116.

Barka,T. (1998). Effect of sodium butyrate on the expression of genes transduced by retroviral vectors. *J. Cell Biochem.* **69**, 201-210.

Benach-Andreu, J. X-ray Structure Analysis of Short-Chain Dehydrogenases/Reductases. 1999. Karolinska Institute, Center for Structural Biochemistry.
Ref Type: Thesis/Dissertation

Bennett,S.R., Carbone,F.R., Karamalis,F., Flavell,R.A., Miller,J.F. and Heath,W.R. (1998). Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* **393**, 478-480.

Berkhout,T.A., Sarau,H.M., Moores,K., White,J.R., Elshourbagy,N., Appelbaum,E., Reape,R.J., Brawner,M., Makwana,J., Foley,J.J., Schmidt,D.B., Imburgia,C., McNulty,D., Matthews,J., O'Donnell,K., O'Shannessy,D., Scott,M., Groot,P.E. and Macphee,C. (1997). Cloning, in vitro expression, and functional characterization of a novel human CC chemokine of the monocyte chemotactic protein (MCP) family (MCP-4) that binds and signals through the CC chemokine receptor 2B. *J Biol Chem* **272**, 16404-16413.

Betjes,M.G., Haks,M.C., Tuk,C.W. and Beelen,R.H. (1991). Monoclonal antibody EBM11 (anti-CD68) discriminates between dendritic cells and macrophages after short-term culture. *Immunobiology* **183**, 79-87.

Bonecchi,R., Sozzani,S., Stine,J.T., Luini,W., D'Amico,G., Allavena,P., Chantry,D. and Mantovani,A. (1998). Divergent effects of interleukin-4 and interferon-gamma on macrophage- derived chemokine production: an amplification circuit of polarized T helper 2 responses. *Blood* **92**, 2668-2671.

Borkowski,T.A., Letterio,J.J., Farr,A.G. and Udey,M.C. (1996). A role for endogenous transforming growth factor beta 1 in Langerhans cell biology: the skin of transforming growth factor beta 1 null mice is devoid of epidermal Langerhans cells. *J. Exp. Med.* **184**, 2417-2422.

Brinckmann,R., Schnurr,K., Heydeck,D., Rosenbach,T., Kolde,G. and Kuhn,H. (1998). Membrane translocation of 15-lipoxygenase in hematopoietic cells is calcium-dependent and activates the oxygenase activity of the enzyme. *Blood* **91**, 64-74.

Caux,C., Dezutter-Dambuyant,C., Schmitt,D. and Banchereau,J. (1992). GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. *Nature* **360**, 258-261.

Cavanagh,L.L., Saal,R.J., Grimmett,K.L. and Thomas,R. (1998). Proliferation in monocyte-derived dendritic cell cultures is caused by progenitor cells capable of myeloid differentiation. *Blood* **92** , 1598-1607.

Cella,M., Engering,A., Pinet,V., Pieters,J. and Lanzavecchia,A. (1997). Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells [see comments]. *Nature* **388**, 782-787.

Cella,M., Salio,M., Sakakibara,Y., Langen,H., Julkunen,I. and Lanzavecchia,A. (1999). Maturation, activation, and protection of dendritic cells induced by double-stranded RNA [In Process Citation]. *J Exp Med* **189**, 821-829.

Chakravorty,S.J., Howie,A.J., Girdlestone,J., Gentle,D. and Savage,C.O. (2001). Potential role for monocyte chemotactic protein-4 (MCP-4) in monocyte/macrophage recruitment in acute renal inflammation. *J. Pathol.* **194**, 239-246.

Charbonnier,A.S., Kohrgruber,N., Kriehuber,E., Stingl,G., Rot,A. and Maurer,D. (1999). Macrophage inflammatory protein 3alpha is involved in the constitutive trafficking of epidermal langerhans cells. *J. Exp. Med.* **190**, 1755-1768.

Chen,A., Gaddipati,S., Hong,Y., Volkman,D.J., Peerschke,E.I. and Ghebrehiwet,B. (1994). Human T cells express specific binding sites for C1q. Role in T cell activation and proliferation. *J. Immunol.* **153**, 1430-1440.

Chen,W.Y. and Townes,T.M. (2000). Molecular mechanism for silencing virally transduced genes involves histone deacetylation and chromatin condensation. *Proc. Natl. Acad. Sci. U. S. A* **97**, 377-382.

Chu,A., Eisinger,M., Lee,J.S., Takezaki,S., Kung,P.C. and Edelson,R.L. (1982). Immunoelectron microscopic identification of Langerhans cells using a new antigenic marker. *J. Invest Dermatol.* **78**, 177-180.

Clark-Lewis,I., Kim,K.S., Rajarathnam,K., Gong,J.H., Dewald,B., Moser,B., Baggiolini,M. and Sykes,B.D. (1995). Structure-activity relationships of chemokines. *J. Leukoc. Biol.* **57**, 703-711.

Conrad,D.J., Kuhn,H., Mulkins,M., Highland,E. and Sigal,E. (1992). Specific inflammatory cytokines regulate the expression of human monocyte 15-lipoxygenase. *Proc. Natl. Acad. Sci. U. S. A* **89**, 217-221.

Crump,M.P., Gong,J.H., Loetscher,P., Rajarathnam,K., Amara,A., Arenzana-Seisdedos,F., Virelizier,J.L., Baggiolini,M., Sykes,B.D. and Clark-Lewis,I. (1997). Solution structure and basis for functional activity of stromal cell- derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1. *EMBO J.* **16**, 6996-7007.

de Saint-Vis,B., Vincent,J., Vandenabeele,S., Vanbervliet,B., Pin,J.J., Ait-Yahia,S., Patel,S., Mattei,M.G., Banchereau,J., Zurawski,S., Davoust,J., Caux,C. and Lebecque,S. (1998). A novel lysosome-associated membrane glycoprotein, DC-LAMP, induced upon DC maturation, is transiently expressed in MHC class II compartment. *Immunity* **9**, 325-336.

Delgado,E., Finkel,V., Baggiolini,M., Mackay,C.R., Steinman,R.M. and Granelli-Piperno,A. (1998). Mature dendritic cells respond to SDF-1, but not to several beta- chemokines. *Immunobiology* **198**, 490-500.

Dhodapkar,M.V., Steinman,R.M., Krasovsky,J., Munz,C. and Bhardwaj,N. (2001). Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J. Exp. Med.* **193**, 233-238.

Dickensheets,H.L. and Donnelly,R.P. (1997). IFN-gamma and IL-10 inhibit induction of IL-1 receptor type I and type II gene expression by IL-4 and IL-13 in human monocytes. *J. Immunol.* **159**, 6226-6233.

Dickensheets,H.L., Venkataraman,C., Schindler,U. and Donnelly,R.P. (1999). Interferons inhibit activation of STAT6 by interleukin 4 in human monocytes by inducing SOCS-1 gene expression. *Proc. Natl. Acad. Sci. U. S. A* **96**, 10800-10805.

Dieu,M.C., Vanbervliet,B., Vicari,A., Bridon,J.M., Oldham,E., Ait-Yahia,S., Briere,F., Zlotnik,A., Lebecque,S. and Caux,C. (1998). Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J Exp Med* **188**, 373-386.

Donadel,G., Garzelli,C., Frank,R. and Gabrielli,F. (1991). Identification of a novel nuclear protein synthesized in growth- arrested human hepatoblastoma HepG2 cells. *Eur. J. Biochem.* **195**, 723-729.

Dubois,B., Massacrier,C., Vanbervliet,B., Fayette,J., Briere,F., Banchereau,J. and Caux,C. (1998). Critical role of IL-12 in dendritic cell-induced differentiation of naive B lymphocytes. *J Immunol* **161**, 2223-2231.

Dubois,B., Vanbervliet,B., Fayette,J., Massacrier,C., Van Kooten,C., Briere,F., Banchereau,J. and Caux,C. (1997). Dendritic cells enhance growth and differentiation of CD40-activated B lymphocytes [see comments]. *J Exp Med* **185**, 941-951.

Dzionek,A., Fuchs,A., Schmidt,P., Cremer,S., Zysk,M., Miltenyi,S., Buck,D.W. and Schmitz,J. (2000). BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. *J. Immunol.* **165**, 6037-6046.

Eggleton,P., Tenner,A.J. and Reid,K.B. (2000). C1q receptors. *Clin. Exp. Immunol.* **120**, 406-412.

Ehrlich,M., Gama-Sosa,M.A., Huang,L.H., Midgett,R.M., Kuo,K.C., McCune,R.A. and Gehrke,C. (1982). Amount and distribution of 5-methylcytosine in human DNA from different types of tissues of cells. *Nucleic Acids Res.* **10**, 2709-2721.

Fayette,J., Dubois,B., Vandenabeele,S., Bridon,J.M., Vanbervliet,B., Durand,I., Banchereau,J., Caux,C. and Briere,F. (1997). Human dendritic cells skew isotype switching of CD40-activated naive B cells towards IgA1 and IgA2. *J Exp Med* **185**, 1909-1918.

Fonseca,M.I., Carpenter,P.M., Park,M., Palmarini,G., Nelson,E.L. and Tenner,A.J. (2001). C1qR(P), a myeloid cell receptor in blood, is predominantly expressed on endothelial cells in human tissue. *J. Leukoc. Biol.* **70**, 793-800.

Fossum,S. (1989). Dendritic leukocytes: features of their in vivo physiology. *Res. Immunol.* **140**, 883-891.

Fritsche,J., Moser,M., Faust,S., Peuker,A., Buttner,R., Andreesen,R. and Kreutz,M. (2000). Molecular cloning and characterization of a human metalloprotease disintegrin--a novel marker for dendritic cell differentiation. *Blood* **96**, 732-739.

Frommer,M., McDonald,L.E., Millar,D.S., Collis,C.M., Watt,F., Grigg,G.W., Molloy,P.L. and Paul,C.L. (1992). A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci U S A* **89**, 1827-1831.

Funk,C.D. and Cyrus,T. (2001). 12/15-lipoxygenase, oxidative modification of LDL and atherogenesis. *Trends Cardiovasc. Med.* **11**, 116-124.

Gabrielli,F., Donadel,G., Bensi,G., Heguy,A. and Melli,M. (1995). A nuclear protein, synthesized in growth-arrested human hepatoblastoma cells, is a novel member of the short-chain alcohol dehydrogenase family. *Eur. J. Biochem.* **232**, 473-477.

Gallucci,S. and Matzinger,P. (2001). Danger signals: SOS to the immune system. *Curr. Opin. Immunol.* **13**, 114-119.

Garcia-Zepeda,E.A., Combadiere,C., Rothenberg,M.E., Sarafi,M.N., Lavigne,F., Hamid,Q., Murphy,P.M. and Luster,A.D. (1996). Human monocyte chemoattractant protein (MCP)-4 is a novel CC chemokine with activities on monocytes, eosinophils, and basophils induced in allergic and nonallergic inflammation that signals through the CC chemokine receptors (CCR)-2 and -3. *J Immunol* **157**, 5613-5626.

Geijtenbeek,T.B., Torensma,R., van Vliet,S.J., van Duijnhoven,G.C., Adema,G.J., van Kooyk,Y. and Figdor,C.G. (2000). Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* **100**, 575-585.

Geissmann,F., Launay,P., Pasquier,B., Lepelletier,Y., Leborgne,M., Lehuen,A., Brousse,N. and Monteiro,R.C. (2001). A subset of human dendritic cells expresses IgA Fc receptor (CD89), which mediates internalization and activation upon cross-linking by IgA complexes. *J. Immunol.* **166**, 346-352.

Gerloni,M., Lo,D. and Zanetti,M. (1998). DNA immunization in relB-deficient mice discloses a role for dendritic cells in IgM-->IgG1 switch in vivo. *Eur. J. Immunol.* **28**, 516-524.

Gilliet,M. and Liu,Y.J. (2002). Generation of human CD8 T regulatory cells by CD40 ligand-activated plasmacytoid dendritic cells. *J. Exp. Med.* **195**, 695-704.

Godiska,R., Chantry,D., Raport,C.J., Schweickart,V.L., Trong,H.L. and Gray,P.W. (1997). Monocyte chemotactic protein-4: tissue-specific expression and signaling through CC chemokine receptor-2. *J Leukoc Biol* **61**, 353-360.

Granucci,F., Vizzardelli,C., Pavelka,N., Feau,S., Persico,M., Virzi,E., Rescigno,M., Moro,G. and Ricciardi-Castagnoli,P. (2001). Inducible IL-2 production by dendritic cells revealed by global gene expression analysis. *Nat. Immunol.* **2**, 882-888.

Graw,R.G., Jr., Herzig,G.P., Eisel,R.J. and Perry,S. (1971). Leukocyte and platelet collection from normal donors with the continuous flow blood cell separator. *Transfusion* **11**, 94-101.

Grouard,G., Rissoan,M.C., Filgueira,L., Durand,I., Banchereau,J. and Liu,Y.J. (1997). The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *J. Exp. Med.* **185**, 1101-1111.

Guan,E., Robinson,S.L., Goodman,E.B. and Tenner,A.J. (1994). Cell-surface protein identified on phagocytic cells modulates the C1q- mediated enhancement of phagocytosis. *J. Immunol.* **152**, 4005-4016.

Guermonprez,P., Valladeau,J., Zitvogel,L., Thery,C. and Amigorena,S. (2002). Antigen presentation and T cell stimulation by dendritic cells. *Annu. Rev. Immunol.* **20**, 621-667.

Harshyne,L.A., Watkins,S.C., Gambotto,A. and Barratt-Boyes,S.M. (2001). Dendritic cells acquire antigens from live cells for cross-presentation to CTL. *J. Immunol.* **166**, 3717-3723.

Hart ,D.N. (1997). Dendritic cells: unique leukocyte populations which control the pimary immune response. *Blood* **90**, 3245-3287.

Hashimoto,S., Suzuki,T., Dong,H.Y., Nagai,S., Yamazaki,N. and Matsushima,K. (1999). Serial analysis of gene expression in human monocyte-derived dendritic cells. *Blood* **94**, 845-852.

Hein,H., Schluter,C., Kulke,R., Christophers,E., Schroder,J.M. and Bartels,J. (1999). Genomic organization, sequence analysis and transcriptional regulation of the human MCP-4 chemokine gene (SCYA13) in dermal fibroblasts: a comparison to other eosinophilic beta-chemokines. *Biochem Biophys Res Commun* **255**, 470-476.

Holliday,R. and Pugh,J.E. (1975). DNA modification mechanisms and gene activity during development. *Science* **187**, 226-232.

Holt,P.G., Haining,S., Nelson,D.J. and Sedgwick,J.D. (1994). Origin and steady-state turnover of class II MHC-bearing dendritic cells in the epithelium of the conducting airways. *J Immunol* **153**, 256-261.

Holt,P.G., Schon-Hegrad,M.A., Phillips,M.J. and McMenamin,P.G. (1989). Ia-positive dendritic cells form a tightly meshed network within the human airway epithelium. *Clin. Exp. Allergy* **19**, 597-601.

Huang,F.P., Platt,N., Wykes,M., Major,J.R., Powell,T.J., Jenkins,C.D. and MacPherson,G.G. (2000). A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. *J. Exp. Med.* **191**, 435-444.

Hubank,M. and Schatz,D.G. (1994). Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic Acids Res* **22**, 5640-5648.

Hwu,P., Du,M.X., Lapointe,R., Do,M., Taylor,M.W. and Young,H.A. (2000). Indoleamine 2,3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. *J. Immunol.* **164**, 3596-3599.

Imai,T., Nagira,M., Takagi,S., Kakizaki,M., Nishimura,M., Wang,J., Gray,P.W., Matsushima,K. and Yoshie,O. (1999). Selective recruitment of CCR4-bearing Th2 cells toward antigen- presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int Immunol* **11**, 81-88.

Ingulli,E., Mondino,A., Khoruts,A. and Jenkins,M.K. (1997). In vivo detection of dendritic cell antigen presentation to CD4(+) T cells. *J. Exp. Med.* **185**, 2133-2141.

Johnson,W.D., Jr., Mei,B. and Cohn,Z.A. (1977). The separation, long-term cultivation, and maturation of the human monocyte. *J. Exp. Med.* **146**, 1613-1626.

Jonuleit,H., Schmitt,E., Steinbrink,K. and Enk,A.H. (2001). Dendritic cells as a tool to induce anergic and regulatory T cells. *Trends Immunol.* **22**, 394-400.

Kadowaki,N., Antonenko,S., Ho,S., Rissoan,M.C., Soumelis,V., Porcelli,S.A., Lanier,L.L. and Liu,Y.J. (2001). Distinct cytokine profiles of neonatal natural killer T cells after expansion with subsets of dendritic cells. *J. Exp. Med.* **193**, 1221-1226.

Kamath,A.T., Pooley,J., O'Keeffe,M.A., Vremec,D., Zhan,Y., Lew,A.M., D'Amico,A., Wu,L., Tough,D.F. and Shortman,K. (2000). The development, maturation, and turnover rate of mouse spleen dendritic cell populations. *J. Immunol.* **165**, 6762-6770.

Kaul,M. and Loos,M. (2001). Expression of membrane C1q in human monocyte-derived macrophages is developmentally regulated and enhanced by interferon-gamma. *FEBS Lett.* **500**, 91-98.

Kitamura,H., Iwakabe,K., Yahata,T., Nishimura,S., Ohta,A., Ohmi,Y., Sato,M., Takeda,K., Okumura,K., Van Kaer,L., Kawano,T., Taniguchi,M. and Nishimura,T. (1999). The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)- 12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J. Exp. Med.* **189**, 1121-1128.

Kodelja,V., Muller,C., Politz,O., Hakij,N., Orfanos,C.E. and Goerdt,S. (1998). Alternative macrophage activation-associated CC-chemokine-1, a novel structural homologue of macrophage inflammatory protein-1 alpha with a Th2-associated expression pattern. *J Immunol* **160**, 1411-1418.

Korb,L.C. and Ahearn,J.M. (1997). C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. *J. Immunol.* **158**, 4525-4528.

Koski,G.K., Schwartz,G.N., Weng,D.E., Gress,R.E., Engels,F.H., Tsokos,M., Czerniecki,B.J. and Cohen,P.A. (1999). Calcium ionophore-treated myeloid cells acquire many dendritic cell characteristics independent of prior differentiation state, transformation status, or sensitivity to biologic agents. *Blood* **94**, 1359-1371.

Kurts,C., Cannarile,M., Klebba,I. and Brocker,T. (2001). Dendritic cells are sufficient to cross-present self-antigens to CD8 T cells in vivo. *J. Immunol.* **166**, 1439-1442.

La Mantia,G., Maglione,D., Pengue,G., Di Cristofano,A., Simeone,A., Lanfrancone,L. and Lania,L. (1991). Identification and characterization of novel human endogenous retroviral sequences prefentially expressed in undifferentiated embryonal carcinoma cells. *Nucleic Acids Res.* **19**, 1513-1520.

La Mantia,G., Majello,B., Di Cristofano,A., Strazzullo,M., Minchiotti,G. and Lania,L. (1992). Identification of regulatory elements within the minimal promoter region of the human endogenous ERV9 proviruses: accurate transcription initiation is controlled by an Inr-like element. *Nucleic Acids Res.* **20**, 4129-4136.

Laemmli,U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lamar,E.E. and Palmer,E. (1984). Y-encoded, species-specific DNA in mice: evidence that the Y chromosome exists in two polymorphic forms in inbred strains. *Cell* **37**, 171-177.

Lamkhioued,B., Garcia-Zepeda,E.A., Abi-Younes,S., Nakamura,H., Jedrzkiewicz,S., Wagner,L., Renzi,P.M., Allakhverdi,Z., Lilly,C., Hamid,Q. and Luster,A.D. (2000). Monocyte chemoattractant protein (MCP)-4 expression in the airways of patients with asthma. Induction in epithelial cells and mononuclear cells by proinflammatory cytokines. *Am. J. Respir. Crit Care Med.* **162**, 723-732.

Lee,D., Prowse,D.M. and Brissette,J.L. (1999). Association between mouse nude gene expression and the initiation of epithelial terminal differentiation. *Dev. Biol.* **208**, 362-374.

Lee,D.U., Agarwal,S. and Rao,A. (2002). Th2 Lineage Commitment and Efficient IL-4 Production Involves Extended Demethylation of the IL-4 Gene. *Immunity.* **16**, 649-660.

Levings,M.K. and Schrader,J.W. (1999). IL-4 inhibits the production of TNF-alpha and IL-12 by STAT6-dependent and -independent mechanisms. *J Immunol* **162**, 5224-5229.

Li,E., Beard,C., Forster,A.C., Bestor,T.H. and Jaenisch,R. (1993). DNA methylation, genomic imprinting, and mammalian development. *Cold Spring Harb. Symp. Quant. Biol.* **58**, 297-305.

Lindhout,E., Vissers,J.L., Hartgers,F.C., Huijbens,R.J., Scharenborg,N.M., Figdor,C.G. and Adema,G.J. (2001). The dendritic cell-specific CC-chemokine DC-CK1 is expressed by germinal center dendritic cells and attracts CD38-negative mantle zone B lymphocytes. *J. Immunol.* **166**, 3284-3289.

Lisitsyn,N. and Wigler,M. (1993). Cloning the differences between two complex genomes. *Science* **259**, 946-951.

Liu,Y.J., Kanzler,H., Soumelis,V. and Gilliet,M. (2001). Dendritic cell lineage, plasticity and cross-regulation. *Nat. Immunol.* **2**, 585-589.

Long,Q., Bengra,C., Li,C., Kutlar,F. and Tuan,D. (1998). A long terminal repeat of the human endogenous retrovirus ERV-9 is located in the 5' boundary area of the human beta-globin locus control region. *Genomics* **54**, 542-555.

Lucarelli,M., Fuso,A., Strom,R. and Scarpa,S. (2001). The dynamics of myogenin site-specific demethylation is strongly correlated with its expression and with muscle differentiation. *J. Biol. Chem.* **276**, 7500-7506.

Macatonia,S.E., Hosken,N.A., Litton,M., Vieira,P., Hsieh,C.S., Culpepper,J.A., Wysocka,M., Trinchieri,G., Murphy,K.M. and O'Garra,A. (1995). Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. *J. Immunol.* **154**, 5071-5079.

Manz,M.G., Traver,D., Miyamoto,T., Weissman,I.L. and Akashi,K. (2001). Dendritic cell potentials of early lymphoid and myeloid progenitors. *Blood* **97**, 3333-3341.

Mayer,W., Niveleau,A., Walter,J., Fundele,R. and Haaf,T. (2000). Demethylation of the zygotic paternal genome. *Nature* **403**, 501-502.

McIlroy,D., Troadec,C., Grassi,F., Samri,A., Barrou,B., Autran,B., Debre,P., Feuillard,J. and Hosmalin,A. (2001). Investigation of human spleen dendritic cell phenotype and distribution reveals evidence of in vivo activation in a subset of organ donors. *Blood* **97**, 3470-3477.

McWilliam,A.S., Nelson,D., Thomas,J.A. and Holt,P.G. (1994). Rapid dendritic cell recruitment is a hallmark of the acute inflammatory response at mucosal surfaces. *J Exp Med* **179**, 1331-1336.

Meierhoff,G., Krause,S.W. and Andreesen,R. (1998). Comparative analysis of dendritic cells derived from blood monocytes or CD34+ hematopoietic progenitor cells. *Immunobiology* **198**, 501-513.

Menetrier-Caux,C., Montmain,G., Dieu,M.C., Bain,C., Favrot,M.C., Caux,C. and Blay,J.Y. (1998). Inhibition of the differentiation of dendritic cells from CD34(+) progenitors by tumor cells: role of interleukin-6 and macrophage colony- stimulating factor. *Blood* **92**, 4778-4791.

Micallef,M.J., Ohtsuki,T., Kohno,K., Tanabe,F., Ushio,S., Namba,M., Tanimoto,T., Torigoe,K., Fujii,M., Ikeda,M., Fukuda,S. and Kurimoto,M. (1996). Interferon-gamma-inducing factor enhances T helper 1 cytokine production by stimulated human T cells: synergism with interleukin-12 for interferon-gamma production. *Eur. J. Immunol.* **26**, 1647-1651.

Miller,Y.I., Chang,M.K., Funk,C.D., Feramisco,J.R. and Witztum,J.L. (2001). 12/15-lipoxygenase translocation enhances site-specific actin polymerization in macrophages phagocytosing apoptotic cells. *J. Biol. Chem.* **276**, 19431-19439.

Mosialos,G., Birkenbach,M., Ayehunie,S., Matsumura,F., Pinkus,G.S., Kieff,E. and Langhoff,E. (1996). Circulating human dendritic cells differentially express high levels of a 55-kd actin-bundling protein. *Am J Pathol.* **148**, 593-600.

Muller,W., Hanauske-Abel,H. and Loos,M. (1978). Biosynthesis of the first component of complement by human and guinea pig peritoneal macrophages: evidence for an independent production of the C1 subunits. *J. Immunol.* **121**, 1578-1584.

Mullis,K., Faloona,F., Scharf,S., Saiki,R., Horn,G. and Erlich,H. (1986). Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harb. Symp. Quant. Biol.* **51 Pt 1**, 263-273.

Nagy,L., Tontonoz,P., Alvarez,J.G., Chen,H. and Evans,R.M. (1998). Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. *Cell* **93**, 229-240.

Nakashima-Matsushita,N., Homma,T., Yu,S., Matsuda,T., Sunahara,N., Nakamura,T., Tsukano,M., Ratnam,M. and Matsuyama,T. (1999). Selective expression of folate receptor beta and its possible role in methotrexate transport in synovial macrophages from patients with rheumatoid arthritis. *Arthritis Rheum.* **42**, 1609-1616.

Navratil,J.S., Watkins,S.C., Wisnieski,J.J. and Ahearn,J.M. (2001). The globular heads of C1q specifically recognize surface blebs of apoptotic vascular endothelial cells. *J. Immunol.* **166**, 3231-3239.

Nepomuceno,R.R., Henschen-Edman,A.H., Burgess,W.H. and Tenner,A.J. (1997). cDNA cloning and primary structure analysis of C1qR(P), the human C1q/MBL/SPA receptor that mediates enhanced phagocytosis in vitro. *Immunity.* **6**, 119-129.

Ngo,V.N., Tang,H.L. and Cyster,J.G. (1998). Epstein-Barr virus-induced molecule 1 ligand chemokine is expressed by dendritic cells in lymphoid tissues and strongly attracts naive T cells and activated B cells. *J Exp Med* **188**, 181-191.

Nibbs,R.J., Wylie,S.M., Yang,J., Landau,N.R. and Graham,G.J. (1997). Cloning and characterization of a novel promiscuous human beta- chemokine receptor D6. *J. Biol. Chem.* **272**, 32078-32083.

Nichols,B.J. and Lippincott-Schwartz,J. (2001). Endocytosis without clathrin coats. *Trends Cell Biol.* **11**, 406-412.

Oakeley,E.J. (1999). DNA methylation analysis: a review of current methodologies. *Pharmacol. Ther.* **84**, 389-400.

Ogden,C.A., deCathelineau,A., Hoffmann,P.R., Bratton,D., Ghebrehiwet,B., Fadok,V.A. and Henson,P.M. (2001). C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. *J. Exp. Med.* **194**, 781-795.

Ohshima,Y., Yang,L.P., Uchiyama,T., Tanaka,Y., Baum,P., Sergerie,M., Hermann,P. and Delespesse,G. (1998). OX40 costimulation enhances interleukin-4 (IL-4) expression at priming and promotes the differentiation of naive human CD4(+) T cells into high IL-4-producing effectors. *Blood* **92**, 3338-3345.

Osborn,L., Kunkel,S. and Nabel,G.J. (1989). Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. *Proc Natl Acad Sci U S A* **86**, 2336-2340.

Palucka,K.A., Taquet,N., Sanchez-Chapuis,F. and Gluckman,J.C. (1998). Dendritic cells as the terminal stage of monocyte differentiation. *J. Immunol.* **160**, 4587-4595.

Panning,B. and Jaenisch,R. (1996). DNA hypomethylation can activate Xist expression and silence X-linked genes. *Genes Dev.* **10**, 1991-2002.

Parmentier,H.K., van der Linden,J.A., Krijnen,J., van Wichen,D.F., Rademakers,L.H., Bloem,A.C. and Schuurman,H.J. (1991). Human follicular dendritic cells: isolation and characteristics in situ and in suspension. *Scand. J. Immunol.* **33**, 441-452.

Pavli,P., Hume,D.A., Van De Pol,E. and Doe,W.F. (1993). Dendritic cells, the major antigen-presenting cells of the human colonic lamina propria. *Immunology* **78**, 132-141.

Pellegrini,S., Censini,S., Guidotti,S., Iacopetti,P., Rocchi,M., Bianchi,M., Covacci,A. and Gabrielli,F. (2002). A human short-chain dehydrogenase/reductase gene: structure, chromosomal localization, tissue expression and subcellular localization of its product. *Biochim. Biophys. Acta* **1574**, 215-222.

Petering,H., Hochstetter,R., Kimmig,D., Smolarski,R., Kapp,A. and Elsner,J. (1998). Detection of MCP-4 in dermal fibroblasts and its activation of the respiratory burst in human eosinophils. *J Immunol* **160**, 555-558.

Peters,J.H., Xu,H., Ruppert,J., Ostermeier,D., Friedrichs,D. and Gieseler,R.K. (1993). Signals required for differentiating dendritic cells from human monocytes in vitro. *Adv. Exp. Med. Biol.* **329**, 275-280.

Peters,S.G., Pomare,E.W. and Fisher,C.A. (1992). Portal and peripheral blood short chain fatty acid concentrations after caecal lactulose instillation at surgery. *Gut* **33**, 1249-1252.

Pickl,W.F., Majdic,O., Kohl,P., Stockl,J., Riedl,E., Scheinecker,C., Bello-Fernandez,C. and Knapp,W. (1996). Molecular and functional characteristics of dendritic cells generated from highly purified CD14+ peripheral blood monocytes. *J. Immunol.* **157**, 3850-3859.

Pierre,P. and Mellman,I. (1998). Developmental regulation of invariant chain proteolysis controls MHC class II trafficking in mouse dendritic cells. *Cell* **93**, 1135-1145.

Pike,L.J., Han,X., Chung,K.N. and Gross,R.W. (2002). Lipid rafts are enriched in arachidonic acid and plasmenylethanolamine and their composition is independent of caveolin-1 expression: a quantitative electrospray ionization/mass spectrometric analysis. *Biochemistry* **41**, 2075-2088.

Plant,K.E., Routledge,S.J. and Proudfoot,N.J. (2001). Intergenic transcription in the human beta-globin gene cluster. *Mol. Cell Biol.* **21**, 6507-6514.

Quandt,K., Frech,K., Karas,H., Wingender,E. and Werner,T. (1995). MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res.* **23**, 4878-4884.

Randolph,G.J., Beaulieu,S., Lebecque,S., Steinman,R.M. and Muller,W.A. (1998). Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking [see comments]. *Science* **282**, 480-483.

Randolph,G.J., Inaba,K., Robbiani,D.F., Steinman,R.M. and Muller,W.A. (1999). Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. *Immunity.* **11**, 753-761.

Ratnam,M., Marquardt,H., Duhring,J.L. and Freisheim,J.H. (1989). Homologous membrane folate binding proteins in human placenta: cloning and sequence of a cDNA. *Biochemistry* **28**, 8249-8254.

Razin,A. (1998). CpG methylation, chromatin structure and gene silencing-a three-way connection. *EMBO J* **17**, 4905-4908.

Razin,A. and Cedar,H. (1991). DNA methylation and gene expression. *Microbiol. Rev.* **55**, 451-458.

Ridge,J.P., Di Rosa,F. and Matzinger,P. (1998). A conditioned dendritic cell can be a temporal bridge between a CD4+ T- helper and a T-killer cell. *Nature* **393**, 474-478.

Riggs,A.D. (1975). X inactivation, differentiation, and DNA methylation. *Cytogenet. Cell Genet.* **14**, 9-25.

Rissoan,M.C., Soumelis,V., Kadowaki,N., Grouard,G., Briere,F., de Waal,M.R. and Liu,Y.J. (1999). Reciprocal control of T helper cell and dendritic cell differentiation. *Science* **283**, 1183-1186.

Rodriguez,A., Regnault,A., Kleijmeer,M., Ricciardi-Castagnoli,P. and Amigorena,S. (1999). Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells. *Nat. Cell Biol.* **1**, 362-368.

Roncarolo,M.G., Levings,M.K. and Traversari,C. (2001). Differentiation of T regulatory cells by immature dendritic cells. *J. Exp. Med.* **193**, F5-F9.

Ronchese,F. and Hausmann,B. (1993). B lymphocytes in vivo fail to prime naive T cells but can stimulate antigen-experienced T lymphocytes. *J. Exp. Med.* **177**, 679-690.

Ross,J.F., Chaudhuri,P.K. and Ratnam,M. (1994). Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines. Physiologic and clinical implications. *Cancer* **73**, 2432-2443.

Ross,J.F., Wang,H., Behm,F.G., Mathew,P., Wu,M., Booth,R. and Ratnam,M. (1999). Folate receptor type beta is a neutrophilic lineage marker and is differentially expressed in myeloid leukemia. *Cancer* **85**, 348-357.

Rubartelli,A., Poggi,A. and Zocchi,M.R. (1997). The selective engulfment of apoptotic bodies by dendritic cells is mediated by the alpha(v)beta3 integrin and requires intracellular and extracellular calcium. *Eur. J. Immunol.* **27**, 1893-1900.

Ruffing,N., Sullivan,N., Sharmeen,L., Sodroski,J. and Wu,L. (1998). CCR5 has an expanded ligand-binding repertoire and is the primary receptor used by MCP-2 on activated T cells. *Cell Immunol* **189**, 160-168.

Saeki,H., Moore,A.M., Brown,M.J. and Hwang,S.T. (1999). Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. *J Immunol* **162**, 2472-2475.

Sallusto,F., Cella,M., Danieli,C. and Lanzavecchia,A. (1995). Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products [see comments]. *J Exp Med* **182**, 389-400.

Sallusto,F. and Lanzavecchia,A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* **179**, 1109-1118.

Sallusto,F., Schaerli,P., Loetscher,P., Schaniel,C., Lenig,D., Mackay,C.R., Qin,S. and Lanzavecchia,A. (1998). Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur J Immunol* **28**, 2760-2769.

Sambrook, J, Fritsch, E. F., and Maniatis, T (1989). "Molecular Cloning, a laboratory manual.," Cold Spring Harbor Laboratory Press.

Sanderson,R.J., Shepperdson,R.T., Vatter,A.E. and Talmage,D.W. (1977). Isolation and enumeration of peripheral blood monocytes. *J. Immunol.* **118**, 1409-1414.

Sanger,F., Nicklen,S. and Coulson,A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U. S. A* **74**, 5463-5467.

Sareneva,T., Matikainen,S., Kurimoto,M. and Julkunen,I. (1998). Influenza A virus-induced IFN-alpha/beta and IL-18 synergistically enhance IFN-gamma gene expression in human T cells. *J. Immunol.* **160**, 6032-6038.

Sauter,B., Albert,M.L., Francisco,L., Larsson,M., Somersan,S. and Bhardwaj,N. (2000). Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J. Exp. Med.* **191** , 423-434.

Schoenberger,S.P., Toes,R.E., van der Voort,E.I., Offringa,R. and Melief,C.J. (1998). T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* **393**, 480-483.

Schon-Hegrad,M.A., Oliver,J., McMenamin,P.G. and Holt,P.G. (1991). Studies on the density, distribution, and surface phenotype of intraepithelial class II major histocompatibility complex antigen (Ia)- bearing dendritic cells (DC) in the conducting airways. *J Exp Med* **173**, 1345-1356.

Schuurhuis,D.H., Ioan-Facsinay,A., Nagelkerken,B., van Schip,J.J., Sedlik,C., Melief,C.J., Verbeek,J.S. and Ossendorp,F. (2002). Antigen-antibody immune complexes empower dendritic cells to efficiently prime specific CD8+ CTL responses in vivo. *J. Immunol.* **168**, 2240-2246.

Schwaeble,W., Schafer,M.K., Petry,F., Fink,T., Knebel,D., Weihe,E. and Loos,M. (1995). Follicular dendritic cells, interdigitating cells, and cells of the monocyte-macrophage lineage are the C1q-producing sources in the spleen. Identification of specific cell types by in situ hybridization and immunohistochemical analysis. *J. Immunol.* **155**, 4971-4978.

Schweickart,V.L., Epp,A., Raport,C.J. and Gray,P.W. (2000). CCR11 is a functional receptor for the monocyte chemoattractant protein family of chemokines. *J. Biol. Chem.* **275**, 9550-9556.

Shapiro,A.L., Vinuela,E. and Maizel,J.V., Jr. (1967). Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochem. Biophys. Res. Commun.* **28**, 815-820.

Shortman,K. and Liu,Y.J. (2002). Mouse and human dendritic cell subtypes. *Nature Rev. Immunol.* **2**, 151-161.

Siegal,F.P., Kadowaki,N., Shodell,M., Fitzgerald-Bocarsly,P.A., Shah,K., Ho,S., Antonenko,S. and Liu,Y.J. (1999). The nature of the principal type 1 interferon-producing cells in human blood. *Science* **284**, 1835-1837.

Siegfried,Z. and Cedar,H. (1997). DNA methylation: a molecular lock. *Curr. Biol.* **7**, R305-R307.

Singal,R. and Ginder,G.D. (1999). DNA methylation. *Blood* **93**, 4059-4070.

Singh-Jasuja,H., Scherer,H.U., Hilf,N., Arnold-Schild,D., Rammensee,H.G., Toes,R.E. and Schild,H. (2000). The heat shock protein gp96 induces maturation of dendritic cells and down-regulation of its receptor. *Eur. J. Immunol.* **30**, 2211-2215.

Smit, A. F. A. and Green, P. unpublished data; URL: http://repeatmasker.genome.washington.edu. 2001.
Ref Type: Computer Program

Sornasse,T., Flamand,V., De Becker,G., Bazin,H., Tielemans,F., Thielemans,K., Urbain,J., Leo,O. and Moser,M. (1992). Antigen-pulsed dendritic cells can efficiently induce an antibody response in vivo. *J. Exp. Med.* **175**, 15-21.

Sozzani,S., Luini,W., Borsatti,A., Polentarutti,N., Zhou,D., Piemonti,L., D'Amico,G., Power,C.A., Wells,T.N., Gobbi,M., Allavena,P. and Mantovani,A. (1997). Receptor expression and responsiveness of human dendritic cells to a defined set of CC and CXC chemokines. *J Immunol* **159**, 1993-2000.

St Louis,D.C., Woodcock,J.B., Fransozo,G., Blair,P.J., Carlson,L.M., Murillo,M., Wells,M.R., Williams,A.J., Smoot,D.S., Kaushal,S., Grimes,J.L., Harlan,D.M., Chute,J.P., June,C.H., Siebenlist,U. and Lee,K.P. (1999). Evidence for distinct intracellular signaling pathways in CD34+ progenitor to dendritic cell differentiation from a human cell line model. *J. Immunol.* **162**, 3237-3248.

Steinberger,P., Szekeres,A., Wille,S., Stockl,J., Selenko,N., Prager,E., Staffler,G., Madic,O., Stockinger,H. and Knapp,W. (2002). Identification of human CD93 as the phagocytic C1q receptor (C1qRp) by expression cloning. *J. Leukoc. Biol.* **71**, 133-140.

Steinman,R.M. (1991). The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* **9**, 271-296.

Steinman,R.M. and Cohn,Z.A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J. Exp. Med.* **137**, 1142-1162.

Steinman,R.M., Turley,S., Mellman,I. and Inaba,K. (2000). The induction of tolerance by dendritic cells that have captured apoptotic cells. *J. Exp. Med.* **191**, 411-416.

Stellato,C., Collins,P., Ponath,P.D., Soler,D., Newman,W., La Rosa,G., Li,H., White,J., Schwiebert,L.M., Bickel,C., Liu,M., Bochner,B.S., Williams,T. and Schleimer,R.P. (1997). Production of the novel C-C chemokine MCP-4 by airway cells and comparison of its biological activity to other C-C chemokines. *J Clin Invest* **99**, 926-936.

Strunk,D., Egger,C., Leitner,G., Hanau,D. and Stingl,G. (1997). A skin homing molecule defines the langerhans cell progenitor in human peripheral blood. *J. Exp. Med.* **185**, 1131-1136.

Takahashi,K., Yamaguchi,H., Ishizeki,J., Nakajima,T. and Nakazato,Y. (1981). Immunohistochemical and immunoelectron microscopic localization of S- 100 protein in the interdigitating reticulum cells of the human lymph node. *Virchows Arch. B Cell Pathol. Incl. Mol. Pathol.* **37**, 125-135.

Tang,H.L. and Cyster,J.G. (1999). Chemokine Up-regulation and activated T cell attraction by maturing dendritic cells. *Science* **284**, 819-822.

Teran,L.M., Mochizuki,M., Bartels,J., Valencia,E.L., Nakajima,T., Hirai,K. and Schroder,J.M. (1999). Th1- and Th2-type cytokines regulate the expression and production of eotaxin and RANTES by human lung fibroblasts. *Am J Respir. Cell Mol Biol* **20**, 777-786.

Thieringer,R., Le Grand,C.B., Carbin,L., Cai,T.Q., Wong,B., Wright,S.D. and Hermanowski-Vosatka,A. (2001). 11 Beta-hydroxysteroid dehydrogenase type 1 is induced in human monocytes upon differentiation to macrophages. *J. Immunol.* **167**, 30-35.

Thomassin,H., Flavin,M., Espinas,M.L. and Grange,T. (2001). Glucocorticoid-induced DNA demethylation and gene memory during development. *EMBO J.* **20**, 1974-1983.

Towbin,H., Staehelin,T. and Gordon,J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U. S. A* **76**, 4350-4354.

Trinchieri,G. (1995). Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu. Rev. Immunol.* **13**, 251-276.

Tseng,S.Y., Otsuji,M., Gorski,K., Huang,X., Slansky,J.E., Pai,S.I., Shalabi,A., Shin,T., Pardoll,D.M. and Tsuchiya,H. (2001). B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. *J. Exp. Med.* **193**, 839-846.

Uguccioni,M., Loetscher,P., Forssmann,U., Dewald,B., Li,H., Lima,S.H., Li,Y., Kreider,B., Garotta,G., Thelen,M. and Baggiolini,M. (1996). Monocyte chemotactic protein 4 (MCP-4), a novel structural and functional analogue of MCP-3 and eotaxin. *J Exp Med* **183**, 2379-2384.

Urban,B.C., Willcox,N. and Roberts,D.J. (2001). A role for CD36 in the regulation of dendritic cell function. *Proc. Natl. Acad. Sci. U. S. A* **98**, 8750-8755.

Valladeau,J., Duvert-Frances,V., Pin,J.J., Dezutter-Dambuyant,C., Vincent,C., Massacrier,C., Vincent,J., Yoneda,K., Banchereau,J., Caux,C., Davoust,J. and Saeland,S. (1999). The monoclonal antibody DCGM4 recognizes Langerin, a protein specific of Langerhans cells, and is rapidly internalized from the cell surface. *Eur. J. Immunol.* **29**, 2695-2704.

Van den Eynde,B.J. and Morel,S. (2001). Differential processing of class-I-restricted epitopes by the standard proteasome and the immunoproteasome. *Curr. Opin. Immunol.* **13**, 147-153.

van Leyen,K., Duvoisin,R.M., Engelhardt,H. and Wiedmann,M. (1998). A function for lipoxygenase in programmed organelle degradation. *Nature* **395**, 392-395.

Vanbervliet,B., Homey,B., Durand,I., Massacrier,C., Ait-Yahia,S., de Bouteiller,O., Vicari,A. and Caux,C. (2002). Sequential involvement of CCR2 and CCR6 ligands for immature dendritic cell recruitment: possible role at inflamed epithelial surfaces. *Eur. J. Immunol.* **32**, 231-242.

Villadangos,J.A., Cardoso,M., Steptoe,R.J., van Berkel,D., Pooley,J., Carbone,F.R. and Shortman,K. (2001). MHC class II expression is regulated in dendritic cells independently of invariant chain degradation. *Immunity.* **14**, 739-749.

Vissers,J.L., Hartgers,F.C., Lindhout,E., Figdor,C.G. and Adema,G.J. (2001). BLC (CXCL13) is expressed by different dendritic cell subsets in vitro and in vivo. *Eur. J. Immunol.* **31**, 1544-1549.

Walsh,C.P. and Bestor,T.H. (1999). Cytosine methylation and mammalian development. *Genes Dev.* **13**, 26-34.

Walsh,C.P., Chaillet,J.R. and Bestor,T.H. (1998). Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. *Nat. Genet.* **20**, 116-117.

Wang,X., Shen,F., Freisheim,J.H., Gentry,L.E. and Ratnam,M. (1992). Differential stereospecificities and affinities of folate receptor isoforms for folate compounds and antifolates. *Biochem. Pharmacol.* **44**, 1898-1901.

Wu,L., Vandenabeele,S. and Georgopoulos,K. (2001). Derivation of dendritic cells from myeloid and lymphoid precursors. *Int. Rev. Immunol.* **20**, 117-135.

Wykes,M., Pombo,A., Jenkins,C. and MacPherson,G.G. (1998). Dendritic cells interact directly with naive B lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response. *J. Immunol.* **161**, 1313-1319.

Yang,X.Y., Wang,L.H., Mihalic,K., Xiao,W., Chen,T., Li,P., Wahl,L.M. and Farrar,W.L. (2002). Interleukin (IL)-4 indirectly suppresses IL-2 production by human T lymphocytes via peroxisome proliferator-activated receptor gamma activated by macrophage-derived 12/15-lipoxygenase ligands. *J. Biol. Chem.* **277**, 3973-3978.

Ying,S., Meng,Q., Zeibecoglou,K., Robinson,D.S., Macfarlane,A., Humbert,M. and Kay,A.B. (1999). Eosinophil chemotactic chemokines (eotaxin, eotaxin-2, RANTES, monocyte chemoattractant protein-3 (MCP-3), and MCP-4), and C-C chemokine receptor 3 expression in bronchial biopsies from atopic and nonatopic (Intrinsic) asthmatics. *J. Immunol.* **163**, 6321-6329.

Yla-Herttuala,S., Rosenfeld,M.E., Parthasarathy,S., Glass,C.K., Sigal,E., Witztum,J.L. and Steinberg,D. (1990). Colocalization of 15-lipoxygenase mRNA and protein with epitopes of oxidized low density lipoprotein in macrophage-rich areas of atherosclerotic lesions. *Proc. Natl. Acad. Sci. U. S. A* **87**, 6959-6963.

Yoder,J.A., Walsh,C.P. and Bestor,T.H. (1997). Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet.* **13**, 335-340.

Zhou,L.J., Schwarting,R., Smith,H.M. and Tedder,T.F. (1992). A novel cell-surface molecule expressed by human interdigitating reticulum cells, Langerhans cells, and activated lymphocytes is a new member of the Ig superfamily. *J Immunol* **149**, 735-742.

Zhou,L.J. and Tedder,T.F. (1996). CD14+ blood monocytes can differentiate into functionally mature CD83+ dendritic cells. *Proc Natl Acad Sci U S A* **93**, 2588-2592.

I would like to thank Prof. Dr. Reinhard Andreesen for taking me back as a Ph.D. student, his interest in my work and his continuous support during all those years.

I also wish to thank Prof. Dr. Buschauer for always going the extra mile for external Ph.D. students and for supervising and reviewing my thesis.

To PD Dr. Stefan Krause, I am very grateful to him for designing the project, for his enthusiasm and his guidance and all his support even in hard times.

I thank Dr. Harald Wagner for his early efforts.

I also would like to especially thank Dr. Michael Rehli for always being there for me, for our many discussions, for endless support and his faith in me and for pointing my nose in the right direction when I most desperately needed it.

I would like to thank everybody in the lab for the good atmosphere and fruitful discussions, for the occasional chats on arbitrary topics over a coffee, splendid breakfasts once in a while, a sporadic treat of cake and holiday-induced chocolate overloads and last but not least, the fun we all had together:

Ute Ackermann, Achim Ehrnsperger, Dr. Jana Fritsche, Dr. Eva Gottfried, Viola Hähnel, Silvia Kirchner, Dr. Marina Kreutz, Sabine Langmann, Dr. Krishna Mondal, Alexandra Müller, Sabine Pape, Alice Peuker, Dr. Ute Schulz, Lucia Schwarzfischer-Pfeilschifter and Sabine Sulzbacher.

Additionally, I would like to express special thanks to:

Dr. Christoph Ammon for sharing his experience with EMSA, for keeping up the good spirit and for the never ending offer to help printing out *_The Thesis_* (think rumbling thunder here!) ;-)

Lucia Schwarzfischer-Pfeilschifter, who introduced me to RDA, as well as to most of the other molecular biological techniques used in the lab.

Prof. Dr. Franco Gabrielli, for providing me with anti-Hep27 antibody and for fruitful and fun e-mail discussions on Hep27 biology.

Dr. Abdo Konur for all the time and discussions he invested in the immunohistochemical detection of MCP-4 and for recommending Slimfast™!

Dr. Guido Meierhoff for introducing me to MLCs.

Sandra Vogl for her diligent help with chemotaxis assays and RNA isolation.

Silvia Haffner for taking the gummi-bears in exchange for HUVECs.

Monika Laumer and PD Dr. Stefan Krause for performing the multi-color FACS analyses for BDC purity determination.

Dr. Carola Seitz from the Pathology Department for getting me going with fluorescence microscopy.

Dr. Stefan Faust for teaching me how to sort blood DCs with MACS.

Last but not least, I wish to thank my mother, Ingrid Heinz, for her love and support during all these years, enabling me to study and giving me the time to grow up, and my aunt Dr. Rosalinde Fuggersberger who turned me on to science a long time ago. (Parafilm™ did the trick.)

Finally, I have to thank my daughter Emily for ensuring that my home life was never boring.