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Donor-dependent effects of *para*-Phenylendiamine on human monocyte-derived dendritic cells

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To my children

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Abbreviations

ACD	Allergic contact dermatitis
AAB	4-aminoazobenzene
APC	Antigen presented cells
BB	Bandrowski Base
B7	B-lymphocyte activation antigen B7-1
CC chemokine	CC Motive chemokine
CCR2	CC chemokine receptor 2
CHS	Hapten-induced allergic contact hypersensitivity
DC	Dendritic cell
DNCB	2, 4-Dinitrochlorobenzene
ELISA	Enzyme-linked Immunoabsorbent Assay
FACS	Fluorescence Activated Cell Sorter
GM–CSF	Granulocyte-macrophage colony–stimulating factor
IFN-γ	Interferon-gamma
IL-1β	Interleukin 1 beta
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-8	Interleukin 8
IL-12	Interleukin 12
IP-10	Interferon gamma- inducible protein 10
LC	Langerhans cell
LPS	Lipopolysaccaride
MCP-1	Monocyte chemotactic protein 1
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MoDC	Monocyte-derived dendritic cells
NK	Natural killer cells
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PHA	Phytohemagglutinin
PMA	Phorbol-12-myristate-13-acetate
PPD	Para-Phenylendiamine
PTD	2-methyl-1, 4-phenylenediamine

RANTES	Regulated upon activation normally T- expressed and presumably secreted
Th1	T helper 1
Th2	T helper 2
TNF-α	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor
TNCB	Trinitrochlorobenzene

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1 Introduction

Allergic contact dermatitis (ACD) is one of the most frequent allergic skin diseases and often results from work-related skin contact with chemicals. The medical treatment for ACD is limited and the only means of healing are that the subjects concerned must change professions, which can have severe socio-medical and socio-economic impacts (Belsito 2000). To improve healing chances it is important to understand the underlying molecular mechanism responsible for the induction of ACD. Therefore, one key step is to study the impact of chemicals on dendritic cells because these cells are known to play a pivotal role in the induction of ACD.

1.1 Dendritic cells

Dendritic cells (DC) are professional antigen presenting cells. They play a key role in inducing and maintaining immune responses. DC link the innate and adaptive immune system and play a crucial part in initiating, amplifying and controlling the immune response especially to pathogenic microorganisms. In 1868 DC were first visualized as Langerhans cells (LC) in the skin as cells which exhibit dramatic dendritic morphology. Only later were they characterized in more detail by Steinman and his colleagues (1972). Dendritic cells represent a minor cell population in all tissues (Banchereau et al. 2000). Dendritic cells exhibit a unique morphological shape when alive, viewed by phase-contrast microscopy, DC extend large delicate processes or veils in many directions from the cell body. Langerhans cells (LC) exemplify one specific dendritic cell population of the skin and comprise between 1 and 2% of total epidermal cells (Hart 1997; Strunk et al. 1997). In 1992, in vitro culture systems were finally introduced, allowing for generation of larger numbers of mouse and human DC from blood. Since DC represent only a small fraction (ca. 0.3%) of the mononuclear cells in the blood, these culture systems considerably accelerated the studies of DC. One important finding is that DC change their morphology during their life time. The heterogeneous expression of a range of cell surface molecules called cluster of differentiation (CD) reflects the differences in their maturation states. Immature dendritic cells localized in peripheral tissues are specialized to capture and process invading pathogens to generate antigens. The traditional view is that antigen uptake by DC is associated with their maturation including migration into lymphoid organs, and antigen presentation in order to activate T cells (Banchereau and Steinman 1998). In addition to these interactions with T cells in the lymphoid organs it is well established that DC also interact with other cell types, including B cells and natural killer (NK) cells (Shortman and Liu 2002). Under these circumstances they remain in peripheral tissues and secrete inflammatory intermediatory

signals rather than migrating to lymph nodes (LNs). The numerous and often opposing roles now ascribed to DC are not carried out at once by the very same cell, so there should be different sets of DC that perform different functions (Shortman and Liu 2002). In this context one of the most important findings is that DC are not a single cell type but a heterogeneous collection of cells that have arisen from distinct bone marrow derived hematopoietic lineages (Rossi and Young 2005).

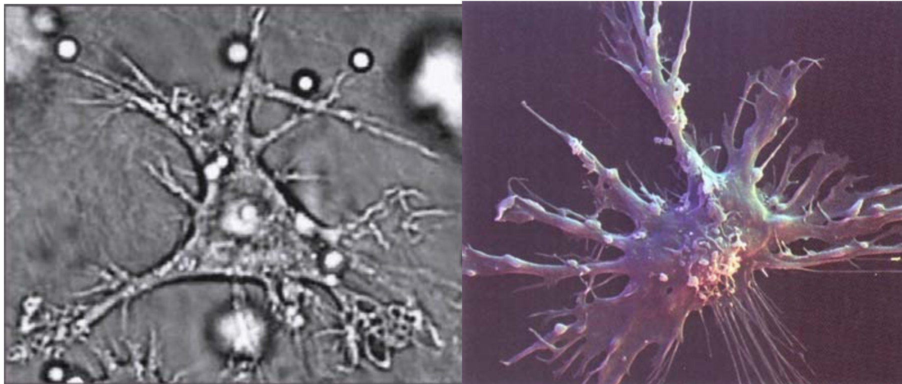

Figure 1: The shape of dendritic cells.
Left: dendritic cell view with phase contrast microscope (taken from Judith Bensen, 2007). Right: 3D view of dendritic cell with electron microscope (taken from Brian Khane, Immune Workshop 2004).

1.2 Dendritic cell subsets

Dendritic cells derive similar to other blood components from the same pluripotent hematopoietic stem cells which are located in the bone marrow. Hematopoietic stem cells mature and undergo changes in gene expression (the levels of genes change) that limit the cell types that it can become and also move it closer to a specific cell type (Sieburg et al. 2006). Within the first differentiation step the myeloid and lymphoid lineages are generated. Dendritic cells derive either from myeloid lineage and termed into interstitial DC or Langerhans cells or from lymphoid lineage and differentiated into plamacytoid DC (Kadowaki et al. 2001). Relevant insights into human DC subsets and their developmental origin came from studies of their development in culture from immature or precursor dendritic cells. These studies have led to the concept of distinct pathways of human DC development.

1.2.1 Lymphoid related dendritic cell pathway

The term lymphoid DC is meant to describe a distinct DC subtype that is closely linked to the lymphocyte lineage. Lymphoid DC may arise from progenitors that also have the potential to mature into T and natural killer (NK) cells (Galy et al. 1995; Grouard et al. 1997; Shortman

and Caux 1997; Bykovskaja et al. 1998; Marquez et al. 1998; Bykovskaia et al. 1999). Such progenitors are distributed in the thymus and in the T cell areas of secondary lymphoid tissues. A variety of functions has been attributed to lymphoid DC. They promote negative selection in the thymus and are co-stimulatory for different types of T cells (Austyn 1987; Shortman et al. 1997; Austyn 1998). It was found that they preferentially activate type 2 T-cell immune responses (TH2) which thereupon activate eosinophiles and help B cells to make the appropriate antibodies (Banchereau and Steinman 1998; Byrum et al. 1999). Because of their capacity to induce apoptosis and their role in eliminating potentially self-reactive T cells, it has been suggested that lymphoid DC primarily mediate regulatory rather than stimulatory immune effector functions (Shortman et al. 1997 ; Austyn 1998 ; Rissoan et al. 1999). Lymphoid DC in T cell areas have the capacity to regulate self-reactive T cells, for example by the production of interleukin (IL)-10 or other cytokines to delete them, for example by the induction of apoptosis via a member of the tumor necrosis factor (TNF) family like fadd associated domaine ligand (fasL) (Suss and Shortman 1996) or (CD30)L (Amakawa et al. 1996).

1.2.2 Myeloid related dendritic cell pathway: cluster domain 34+ (CD34+) haemopoietic progenitors

The earliest myeloid precursor is the CD34+ fraction, isolated from bone marrow or umbilical cord blood. Culture with granulocyte macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor alpha (TNF-α) leads to two types of intermediate precursor and to two apparently separate pathways of DC development. One pathway leads to DC resembling Langerhans cells, in that they have Birbeck granules and express Langerhans-cell associated antigen (Lag), langerin and E-Cadherin. The intermediates along this pathway express the cutaneous lymphocyte-associated antigen (CLA, a skin homing receptor) (Strunk et al. 1997), Cd11c and CD1a. This pathway depends on the presence of transforming growth factor ß (TGF-ß)(Strobl et al. 1996). Langerhans cells are skin cells and form a semi-contiguous network within epidermis (Strunk et al. 1997). The second pathway of development from CD34+ precursors leads to DC resembling interstitial DC, lacking Birbeck granules and Lag but expressing CD9, CD68 and coagulation factor XIIIa. The intermediates along this pathway lack CLA and CD1a but express the glycosylphosphatidylinositol (GPI) linked myeloid differentiation antigen CD14 and resemble blood monocytes in many respects (Shortman and Liu 2002). Interstitial DC can be found in most organs such as heart, liver, kidney, intestine and skin (Hart and Fabre 1981).

1.2.2.1 Myeloid DC pathway: peripheral blood mononuclear cells and CD14+ cells

Blood monocytes are the most commonly used precursor cells for generating human DC in vitro. In the presence of macrophage colony-stimulating factor (M-CF), they will generate macrophages but, in the presence of the cytokines GM-CSF and IL-4, dendritic cells are produced after six days (Sallusto and Lanzavecchia 1994; Bender et al. 1996; Romani et al. 1996) and little or no proliferative expansion is involved. Final maturation into CD14- CD38+CD86+ surface major histocompatibility complex II (MHC-II) ^{hi} DC is achieved by further stimulation with pro-inflammatory cytokines namely TNF-α or microbial products such as lipopolysaccharide (LPS). Helper CD4+ T cells, signaling by means of CD154, can also mature and activate these DC.

1.3 Dendritic cell activation for induction of immune reactions

Functional maturation of dendritic cells is mandatory in order to initiate T-cell negotiated immune reactions. The activation of dendritic cells and their maturation can occur by different mechanisms. As mentioned above, dendritic cells devise different functions dependent on their maturation status. While immature dendritic cells are specialized to capture antigens and can be viewed as players of the innate immune system, mature dendritic cells preferentially modulate T-cell responses either for tolerance induction or activation. There is an emerging consensus, pioneered by Janeway that evolutionary ancient innate immune system is essential to the initiation of adaptive immunity and that cells of the innate immune system are not constitutively active but are activated through pattern recognition receptors that recognize evolutionary distant pathogens. This self-non-self discrimination model assumes that the immune system protects the organism against everything which is foreign. Thus immune responses are directed towards external entities which are non-self antigens (Janeway 1992). Matzinger et al revised this concept, because it does not explain tolerance to specific antigens. They predicted in their 'danger model' that the immune system detects and then responds to anything dangerous and not necessarily foreign, and assumed that the main determinant leading to the initiation of an immune response is the presence of an antigen in the context of tissue destruction. If there is no damage and cells are unharmed or they die by apoptosis, no immune response ensues. However, if cells are injured, stressed or die by necrosis, an immune response is induced (Matzinger 1994; Matzinger 1998). Since an absolute majority of body cells presents only signal one which is crucial for specific antigen recognition, the default reaction of a responding T cell should be tolerance. This kind of behaviour is very beneficial to sustain the tolerant state and to avoid auto-aggression against self tissues. However, danger signals released by adjacent damaged tissue are predominantly cytokines like Interleukin 1 beta (IL-1ß), TNF-α, IL-6 which stimulate

maturation through signaling of specific receptors. In addition, the first signal which may result through invading pathogens releases microbial products, as for example the microbial cell wall component LPS, which activates dendritic cells via specific pattern recognition receptors such as toll like receptors (TLRs) (Gallucci et al. 1999). In addition to activation of specific cell surface receptors, antigens which were taken up by endocytose and phagocytose were processed and subsequently presented on the cell surface. Whether the ingested antigen is also able to activate immune responses depends on its potential to induce danger signals. The mechanism involved in antigen uptake depends on its location and size, biochemical composition as well as hydrophobicity of the antigen.

1.3.1 Antigen uptake

Immature DC have several features for antigen uptake. Beside phagocytosis, pincocytosis also interaction with specific receptors may be involved in capture of microbial pathogens, dead or dying cells, immune complexes, and other antigens. Phagocytosis is the cellular process of engulfing solid particles by the cell membrane to form an internal phagosome. The latter is usally delivered to the lysosome, an organelle involved in the breakdown of cellular components, which fuses with the phagosome. The contents are subsequently degraded, digested and either released extracellularly (exocytosis), or phagocytosed antigens are further processed for presentation. Capture of antigens with specific surface receptors such as membrane bound immunglobuline (mIg), mannose receptor, fragment crystallisable receptor (FcR) allows also delivery of antigens into lysosomal compartments. Antigens that fail to bind to surface receptors can still be taken up by pinocytosis. Pinocytosis is primarily used for the absorption of extracellular fluids, it generates very small vesicles which fuse with lysosomes and are then presented by APCs, but efficiency is much lower than in the case of receptor-mediated antigen uptake. Fluid phase uptake occurs via distinct mechanisms: a) micropinocytosis, i.e. uptake of small vesicles (0.1µm) via clathrin coated pits; and b) macropinoctosis, i.e. uptake of large vesicles (0.5-3µm) mediated by membrane ruffling driven by actin cytoskeleton (Sallusto et al. 1995)

1.3.2 Antigen processing

Processing is associated with antigen splicing and subsequent binding to presentation molecules such as major histocompatibility complexes (MHC). Dendritic cells are specialized to present the ingested antigens –professional antigen presenting cells-, while other immune cells like macrophages are predominantly equipped to digest them (Mellman and Steinman 2001).

1.3.2.1 Major histocompatibility complex II (MHCII) pathway

The MHC II pathway is mandatory to present extracellular soluble antigens uptaken by macropinocytose or receptor mediated endocytose. For MHC II presentation, ingested membrane coated antigens fuse with specific endosomes calling major histocompatibilitiy II compartiments (MIICs). MIICs continiously migrate inside the cell which is accompanied with the reduction of the MIICs specific pH value. The reduced pH value activates MIICs specific proteases to splice the antigens in shorter fragments. Other acid active proteases like catepsin S prepare the MHC II molecules for antigen binding by degradation of a MHC II binding specific placeholder namely clip fragment. At least MIIC specific enzymes like HLA-DM replace the clip fragment against the antigen peptid (Banchereau and Steinman 1998). During maturation of DC, MIICs convert to non-lysosomal vesicles that discharge their MHC-peptide complexes to the cell surface and presented the loaded antigen to CD4 T cells (Nijman et al. 1995).

1.3.2.2 Major histocompatibility complex I (MHCI) pathway

For presentation of endogenous antigens resulting most often from viral infections, proteins are degraded in the cytosol into peptides by a protein degrading enzyme complex (proteosome). A dedicated peptide transporter then translocates these peptides from the cytosol to the endoplasmatic reticulum, where they are connected to class I molecules. The peptide-loaded MHC class I complex then travels to the cell surface where it displays the antigen to CD8 T cells (Rodriguez et al. 1999).

1.3.2.3 DC Migration into lymph nodes

In peripheral tissue main responsibilities of DC appear to be the recognition, capture and processing of exogenous antigens. In contrast, antigen presentation takes place in the lymphoid tissues such as lymph nodes and spleen. The ability to present antigen is acquired by DC during the culture in the presence of appropriate cytokines (Jonuleit et al. 1997) and in vivo during their migration from periphery to draining lymphoid tissues. As a result phenotypical and functional properties change to enable DC to free themselves from the surrounding tissue matrix as a first step in the process of migration (Kimber et al. 2000). More precisely integrins and adhesions molecules as intercellular adhesion molecule-1 (ICAM-1), CD44 (Gabrilovich et al. 1994) and α6 integrins (Price et al. 1997) display altered expression. Metalloproteinases as matrix metalloproteinase 9 (MMP9) and MMP3 enable the journey through tissue matrix (Uchi et al. 1998) and several lectins for example dendritic cell specific ICAM-3 grabbing nonintegrin (DC-SIGN) function as rolling receptors that allow

leukocyte transendothelial migration for passing lymphoid endothelium (Steinman 2000). One main precondition for directed migration from peripheral sides into lymphoid organs is the expression of specific chemokines by inflammatory tissue, endothelial venules or lymphoid organs which direct DC to their destinations (Springer 1994). In response to specific chemokines, DC express corresponding receptors and, depending on their maturation, status expression profile of chemokine receptor changes. DC precursors typically express CC chemokine receptor 2 (CCR2) and CXC motive chemokine receptor 4 (CXCR4), immature dendritic cells express inflammatory chemokine receptors CCR1, CCR2, CCR5, CCR6, CXCR1, CXCR2 and CXCR4. When immature dendritic cells are recruited into lymphoid organs, expression of inflammatory chemokine decline and and lymphoid chemokine receptor CCR7 is rapidly induced (Ohl et al. 2004). Mature dendritic cells express only a single lymphoid chemokine receptor CCR7 (Sozzani et al. 1999). The CCR7 ligands CCL19 and CCL21 direct migration to the nodes and are required for the localization of DC within T cell zones (Scandella et al. 2004). Independent from the above described situation it should be mentioned that there are some situations in which the generic view of DC migration outlined above does not apply. First, in lymphoid organ resident DC antigen negotiated maturation and migration occur entirely within the lymphoid organ (Derbinski and Kyewski 2005). Second, it has been recognized that immature DC bearing self-antigens continuously migrate from peripheral sites to T cell zones, probably in order to maintain self tolerance (Hawiger et al. 2001; Steinman et al. 2003). This constitutive DC migration is possibly independent of inflammatory stimuli and DC maturation (Ohl et al. 2004), or the specific signals that stimulate this migration are unknown (Worbs and Forster 2007).

1.3.3 T-cell interactions for induction of immune responses.

Successful DC-T-cell interaction can be seen in lymphoid organs for all major classes of T-cell ligands but how the initial contact between DC and resting T cells, necessary for T cell activation, is established and regulated is largely unknown. Earlier studies indicated that DC and T cells form clusters in an antigen-independent manner (Banchereau et al. 2000). In general, both adhesion molecules and co-stimulatory molecules with their corresponding ligands such as lymphocyte function-associated antigen-3 (LFA-3) LFA-3/CD2 and LFA-1/ICAM -1, -2, or -3 are potential candidates for the cell-cell contact. More recently, it became evident that also DC-SIGN mediates transient adhesion between DC and T cells (Geijtenbeek et al. 2000). In addition to a stable connection between DC and T cells, two further signals are required in order to fully activate T cells. The first signal is antigen specific, provided through the specific T-cell receptor interaction with the antigen bearing MHC molecule on the surface of DC. A second non-specific signal, the co-stimulatory signal, is provided by the interaction between co-stimulatory molecules (Greenwald et al. 2005).

1.3.3.1 Co-stimulation

Co-stimulatory molecules provide the second signal determining the fate of naïve T cells after encounter with a MHC:peptid complex. Multiple co-stimulatory molecules with potent co-stimulatory activities both in vitro and in vivo have been found on antigen presenting cells. Among them are proteins such as CD24 (Liu et al. 1992), CD48 (Gonzalez-Cabrero et al. 1999), 4-1 BBL (DeBenedette et al. 1995), ICAM-1, LIGHT (Tamada et al. 2000), MHC class II invariant chain (Naujokas et al. 1993); but the far best characterized molecules are CD80 (B.7.1) and CD86 (B.7.2) expressed by DC. These two proteins are essential for a complete and effective T-cell activation as well as IL-2 production. The signaling is mediated by binding of these co-stimulatory molecules to CD28 on the T cell (CD28 ligation). If signaling fails at the moment of antigen recognition by T-cell receptor (TCR), an alternative T lymphocyte function may result, namely induction of anergy (lack of reaction). Another CD80/86 ligand, cytotoxic T-Lymphocyte antigen 4 (CTLA-4) is also induced on activated T lymphocytes and this protein may contribute to negative regulatory signaling. Several studies indicate that CD28 ligation also plays an important role in the qualitative outcome of immune responses by modulating the TH cell differentiation through selective regulation of Th1 and Th2 induction (Sansom DM 2003). In this context it could demonstrated that both strength of the TCR signal and the CD28/CD80/86 interaction are important factors influencing the priming for Th2 cells (Tao et al. 1997). Beside the nature of co-stimulatory molecules and duration of T-cell receptor engagement, further factors that contribute to the Th1-Th2 balance are dose of antigen and strength of antigenic stimulation promoted by specific cytokines such as IL-12 and IL-4 (Langenkamp et al. 2000).

1.3.3.2 Dendritic cell mediated T-cell polarization

Th1 and Th2 cells represent terminally differentiated effector cells characterized by different cytokine production and homing capacity. The generation of either type of response can confer protection against pathogens or lead to immunpathology. Th1 cells produce high levels interferon-γ (IFN-γ) and tumor necrosis factor beta (TNF-ß), cytokines that are instrumental in the induction of cell-mediated immunity against intracellular pathogens such as viruses, certain types of (myco) bacteria and protozoa. Effector Th2 cells produce high levels of IL-4, IL-5 and IL-13, cytokines that are predominantly known for their involvement in defense against helminth. A third set of Th cells are regulatory T cells that play a major role in the maintaince of tolerance against self- or harmless foreign proteins by downregulating effector T cell responses (De Jong 2005). The outcome of immune response is determined by the balanced development of the different types of effector and/or regulatory T cells, and is orchestrated by dendritic cells. Thus DC can also be distinguished between DC1 and DC2

according to different cytokine productions which mediate Th1 and Th2 responses. One study indicated that although myeloid cells give rise to DC1, plasmacytoide monocytes generates DC2 after in vitro culture with CD40L. However it is also clear that myeloid DCs can give rise either DC1 or DC2, depending on the nature of maturation stimulus influencing IL-12 production (Trinchieri et al. 2003).

DC1 induced Th 1 responses:

The critical Th1 polarizing cytokine is IL-12. It is produced by DC after stimulation with LPS, poly(I)oly(C) or CD40L (Macatonia et al. 1995; Cella et al. 1996; Cella et al. 1999) but it is not produced after stimulation with TNF-α, IL-1, fungal hyphae or nematode products (d'Ostiani et al. 2000; Whelan et al. 2000). IL-12 production can also inhibited in DC treated with vitamin D3 (D'Ambrosio et al. 1998; Na et al. 1999) or by agents that increase cyclic adenosine monophosphat (cAMP) such as prostaglandin E_2 (PGE2) or cholera toxin (Kalinski et al. 1997; Panina-Bordignon et al. 1997; Gagliardi et al. 2000). Besides of IL-12 also other family members such IL-23 and IL-27 and IL-18 and type I IFN cytokines also induce Th1 polarization. Furthermore, kinetics of DC activation influence the capacity of DC to prime T cells, not only towards effector Th1 or Th2, but also towards nonpolarized “central memory” T cells. Mature DC in draining lymph nodes have a reduced susceptibility to microbial products and predominantly respond to CD40 ligation by rapidly inducing their ligand (CD40L) on naïve T cells. Whereas the intrinsic capacity of mature effector DC to produce high IL-12 levels is imprinted by previous contact with selected pathogens during immature phase, the CD40-CD40L interaction drives mature DC to produce IL-12 and consequently Th1 responses (De Jong 2005).

DC2 induced Th2 responses:

Overall, Th2 responses are the result of a stimulation in the absence of IL-12 or IL-12 family members as polarizing factors. In contrast to the knowledge about Th1 inducing factors little is known about the promotion of Th2 cells by DC (Jankovic et al. 2000). Up to now its well established that IL-4, monocyte chemoattractant protein-1 (MCP-1) and OX40L are potent Th2 inducers (De Jong 2005).

Generation of regulatory T cells (Treg):

A growing body of evidence suggests that DC play a central role in the induction of peripheral tolerance via inducing development of adaptive regulatory T cells (Kubach et al. 2005). Immature dendritic cells are known to induce T cell tolerance after repetitive stimulation, probably due to the lack of co-stimulatory molecules. Such an immature state can be maintained after activation of immature DC (iDC) by certain anti-inflammatory cytokines, such as through the transforming growth factor beta (TGF-ß), IL-10 or by compounds that inhibit activation of nuclear factor kappa B (NF-kB), as for example corticosteroids, vitamin D3 and the N-acetyl-L-cysteine derivate nacystelyn (Steinbrink et al.

1997; de Jong et al. 1999; Gregori et al. 2001; Sato et al. 2003; Vosters et al. 2003). Immature DC lack the expression of functional chemokine receptors, which normally guide them to draining lymph nodes. Instead immature DC are prone to induce apoptosis or anergy in T cells and in addition may impose regulatory properties on the small number of remaining T cells. Specific pathogens are known to induce development of adaptive regulatory T cells (van der Kleij et al. 2002) and IL-10 is one factor that is responsible or coresponsible for induction of regulatory T-cell development. TGF-ß, several members of the B7 family (B7-H1, B7-DC, B7-H3, B7-H4), (Latchman et al. 2001; Selenko-Gebauer et al. 2003; Prasad et al. 2003 ; Sica et al. 2003 ; Suh et al. 2003) and proteins engaged in the Notch signaling pathway (Yvon et al. 2003) may also be candidates that induce negative signals to downregulate T cell responses.

1.4 Dendritic cells and their role in allergic contact dermatitis to low molecular weight chemicals

1.4.1 Influence of low weight chemicals on monocyte derived dendritric cells

Immune reactions to simple chemicals often provoke so-called allergic contact hypersensitivity reactions and establish the cutaneous disease “allergic contact dermatitis” (ACD). ACD is a cell mediated immune response to small molecular weight chemicals that contact and penetrate the skin. In 1963 Combs and Gel classified allergic contact dermatitis into the type IV hypersensitivity reactions which typically exert over a period of time inflammatory skin eruptions mediated by antigen specific T cells. ACD is restricted to industrialized countries and has an enormous sociomedical and socioeconomic impact (Belsito 2000). About 4000 compounds have the ability to induce ACD (De Groot et al. 1994). Medical treatment of the symptoms of ACD is rather unspecific and limited, because currently underlying cellular mechanisms responsible for the pathogenesis of ACD are only partially understood.

1.4.2 Basic cellular and biologic events in allergic contact dermatis

The mechanisms that are required for the acquisition of skin sensitization and for subsequent elicitation of allergic reactions in the skin are complex and dependent on highly orchestrated molecular and cellular interactions. The process occurs in two distinct phases. The first phase is called the induction or sensitization phase. Skin sensitization is induced when a susceptible subject is exposed topically to the inducing chemical allergen. Exposure must be sufficient to provoke a cutaneous immune response of vigour necessary for the development of sensitization which evokes that naïve antigen specific T cells are activated by antigen

presenting Langerhans cells. Once sensitization has been acquired in the second phase, called elicitation phase, the subject will respond to subsequent contact with the inducing allergen by mounting an accelerated and more aggressive secondary immune response that in turn will precipitate a cutaneous inflammatory reaction at the side of exposure (Saint-Mezard et al. 2004). The central event during induction of skin sensitization is the activation and clonal expansion of allergen specific T-lymphocytes in lymph nodes draining the site of exposure. The stimulation of T-lymphocytes responses requires that the antigenic stimulus is delivered from the skin in an appropriate form to draining lymph nodes. In case of chemicals, that may be an intricacy process (Kimber et al. 2003).

1.4.3 Dendritic cell mediated conversion of low weight chemicals into antigenic stimulus

In general, only chemicals which are small enough (<500 daltons) and lipid soluble are proper to transit the stratum corneum to reach the epidermis. Whereas most small and lipophilic chemicals do not cause ACD, a minority may bind to different cell-associated or cell-free proteins to form protein conjugates that are potentially antigenic. These protein conjugate formation leading to ACD are often referred as “haptens”. Depending on their chemical structure and chemical reaction mechanism, they can further be classify into “pro” or “pre” haptens. Approximately two-thirds of all tested sensitizing chemicals are electrophiles thereby directly protein reactive and called haptens. The other third of sensitizers is not directly protein reactive, because they are not electrophiles, and they must be activated by some means on or in the skin either via abiotic conversion called prehaptens or via metabolic (enzymatic) conversion called prohaptens (Aptula et al. 2007). Depending on the specific hapten, each one can be processed and presented in a different manner.

1.4.4 Antigen presentation of small weight chemicals

Low weight chemicals can be taken up by Langerhans cells (in case of ACD) and processed intracellulary into hapten-peptide conjugates. Subsequent critical events include association of processed peptides with MHCII and their presentation on the surface of DC. A second pathway involves direct conjugation with protein moieties already expressed on the DC surface, either the MHC molecules themselves (class I or class II) or peptides bound to these molecules, thereby circumventing endocytosis and intracellular processing altogether (Cavani et al. 1998; Cavani et al. 2001).

1.4.5 Research on the role of dendritic cells in ACD

During the past 25 years there have been tremendous advancements in our understanding of the underlying mechanisms of ACD. Animal models gave at least strong mechanistic foundations. Guinea pigs were the original species of choice for the identification of skin sensitising chemicals. Among the models developed, those most widely used were the guinea pig maximisation test and the occluded patch test introduced by Buehler. More recently alternative test methods, such as the murine local lymph node assay (LLNA) and the measurement of proliferative responses of local lymph node cells (LLC) following topical exposure to test chemicals, were established (Gerberick et al. 2000 ; Basketter et al. 2002). Over the past 10 years, scientists from academica and industry have been working to apply this understanding to the development of alternative, non-animal test methods for assessing the skin sensitization potential of a chemical. It is an enormuos challenge to reproduce in vitro the complexities of the immune system considering the fact that numerous cell types are involved (e.g. various T cells, Langerhans cells, keratinocytes) and a plethora of inflammatory mediators and chemokines in the initiation and development of ACD. However, in consideration of the important roles of LC and DC during initiation and regulation of skin sensitization, alternative approaches to hazard identification based upon chemical induced changes in the phenotype or functions of these cells were developed.

1.4.6 Role of DC in sensitization to chemical allergens as promising tool for in vitro testings

Langerhans cells, the principle antigen-presenting cell in the skin, constitute only 1-3% of all epidermal cells, but they play a key role in the development of ACD. Many isolation techniques have been developed to obtain purified populations of LC from human and murine sources but the numbers of cells obtained are relatively low and the availability of sufficient numbers of these cells has been a limiting factor in the development of LC-based in vitro methods. Furthermore, it became clear that isolated LC are subject to rapid changes in phenotype. Despite these obstacles, several investigators have focused on events that occur following exposure to chemical haptens (Herouet et al. 1999; Verrier et al. 1999). However, now there are methods available for their generation from progenitor cells in blood, cord blood, or bone marrow. Overall data about the impact of chemical allergens on DC were generated by using DC derived from human peripheral blood mononuclear cell (PBMC) derived DC namely monocyte derived DC (MoDC). These cells express surface proteins consistent with bone marrow derived DC, and they are capable of eliciting a primary allogenic mixed lymphocyte response (Sallusto and Lanzavecchia 1994; Bender et al. 1996).

1.4.7 Low weight chemicals induce functional changes and maturation of MoDC

For developing an in vitro model for contact sensitization, several investigators examined phenotypical alterations of MoDC under the influence of subtoxic concentrations of different chemicals and contact sensitizers. Depending on the type of chemical, enhanced or modified surface marker expression of HLA-DR; CD80; CD86; CD40; CD54; CD83 and CCR7 can be detected. These phenotypical changes are accompanied by cytokine production such as IL-6, TNF-α, IL-8 and IL-1ß (Aiba et al. 1997; Coutant et al. 1999; Aiba et al. 2000; Tuschl and Kovac 2001). All these results show that contact sensitizers are able to induce activation of human DC in spite of the lack of the skin environment, thus suggesting that DC act as crucial sensor of danger signals, including low molecular weight compounds. It has been speculated that simple chemicals may use similar signaling pathways as were found for infectious agents, e.g., LPS. Due to this fact several groups investigated if different haptens acquire the mature phenotype via the same signal transduction pathways. The precise mechanism for the survival and maturation of MoDC stimulated by LPS has been investigated furthermore it could be demonstrate that both mitogen activated protein kinase (MAPK) and NF-κB are responsible for maturation whereas phosphatidylinositol 3-kinase is important to maintain their survival (Ardeshna et al. 2000). As expected, several investigators could demonstrate that chemicals induce the same signaling pathway for surface marker or cytokine expression but dependent on the type of chemical either p38, JNK, ERK pathway or NF-κB or different combinations of these (Arrighi JF. 2001; Yoshimura et al. 2001; Aiba et al. 2003; Boisleve et al. 2005). It may be possible that other signal transduction pathways are activated through chemical sensitizers because it could be shown that surface markers, such as DC-SIGN, CD40 or CCR7, are inducted through different signalling, like JAK/STAT or PI3K Pathway in MoDC (Puig-Kroger et al. 2004; Sanchez-Sanchez et al. 2004; Chen et al. 2006).

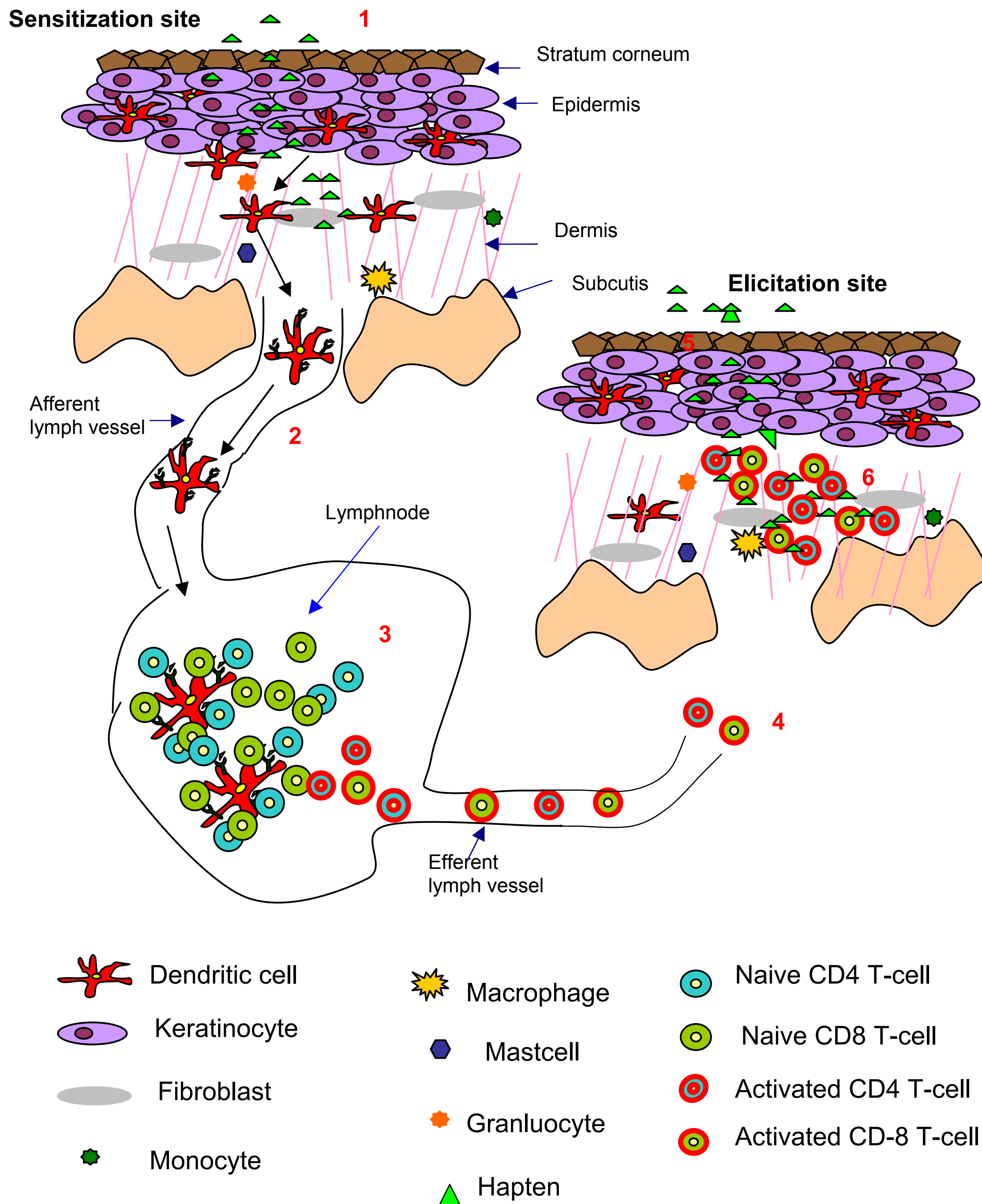

Figure 2: Pathophysiology of allergic contact dermatitis
Haptens penetrate the stratum corneum and are ingested by Langerhans cells and dermal dendritic cells. (step 1). Hapten uptake induces activation and migration of DC through the afferent lymphatic vessels to the draining lymph nodes. During the migration process the maturation process is induced by expression of MHC, co-stimulatory and adhesion molecules which graduate the dendritic cells to professional cells.(step 2). Migrating DC are located in the para-cortical area of the draining LN and present haptenated peptides on MHC class I and II molecules to specific CD8+and CD4+ T cell precursors which thereupon clonally expanded and differentiated into effector cells (step 3).Specific T effector cells exert the lymphnode through efferent lymphatic vessels and diffuse into the blood stream and subsequently re-circulate between lymph organs and skin. In response to skin-specific homing antigens (CLA and CCR4) they are capable to diffuse into the skin and become memory T cells (step 4). Renewed skin contact with the hapten induce hapten uptake of Langerhans cells and other skin resisting antigen presenting cells and subsequently presentation of the haptenated peptides with MHC to migrating specific T cells. (step 5) .Antigen presentation initiate activation and proliferation of specific T cells and induction of inflammation reactions responsible for subsequent keratinocyte

apoptosis and cytokine/chemokine production. Enhanced cytokine and chemokine expression at side of inflammation enable the recruitment of leukocytes from the blood to the skin leading to the development of skin lesions and after several days to a downregulation of the immunological mechanisms by the arrival of regulatory T cells. (step 6)

1.5 Aims of the study

The development of markers for the in vitro characterization of sensitization potentials of small molecular weight chemicals is still limited. Up to now, predominantly metals such as nickel have been studied. In contrast, the knowledge of other small molecular weight molecules is very limited. One example may illustrate *para*-phenylenediamine (PPD), a strong contact allergen which most often ranks on position 10-14 of the common allergens (Marks et al. 1998; Marks et al. 2000). As dye intermediate, the main industrial application for PPD is in dye formulations. Thus, skin is a primary target organ. Although PPD is a common cause for occupation-related ACD and many allergic persons may need to change their profession, up to now there is no successful therapy for this disease due to the fact that the mechanism of how small weight compounds induce ACD is only incompletely understood. This study will contribute to elucidating the immune modulatory and immunogenic potential of PPD because, up to date, it has not been clearly demonstrated whether PPD is itself a sensitizing agent. First, a characterisation of the metabolic competence of MoDC for this compound will be investigated. Afterwards, the effects of PPD on the functional maturation of MoDC with respect to changes in expression of the MHC molecule HLA-DR, the co-stimulatory molecules CD80, CD86, the maturation and late migration marker CCR7, and the DC-T cell interaction molecules DC-SIGN as well as CD11c will be investigated. Furthermore, estimation of effects in different persons and mediators induced by PPD will provide information about its potential to induce danger signals and give hints about the quantity and qualitiy of the immune response. Lastly, the analysis of PPD on pathogen-induced danger signals in MoDC elucidates not only the impact of PPD on inflamed or activated dendritic cells but also gives hints about the underlying signal transduction pathways involved in PPD-induced effects.

2 Materials and Methods

2.1 Material

2.1.1 Chemicals and reagents

Chemical	Supplier
- *Para*- Phenylenediamine (PPD)	Sigma Aldrich Co, Germany
-Mono-acetyl-PPD (MAPPD)	Sigma Aldrich Co, Germany
-4-Aminobenzoic acid (PABA)	Sigma Aldrich Co, Germany
-Dithiothreitol (DTT)	Sigma Aldrich Co, Germany
-4-Dimethylaminobenzaldehyd (DMAB)	Sigma Aldrich Co, Germany
-Acetyl coenzyme A sodium salt (AcCo-A)	Sigma Aldrich Co, Germany
-Acetonitrile (HPLC grade)	Sigma Aldrich Co, Germany
- Bradford reagent	Sigma Aldrich Co, Germany
- Bandrowski's Base (BB)	ICN Biomedicals, USA
- Phytohemagglutinin-M	CALBIOCHEM, Darmstadt, Germany
- Nickelsulfat ($NiSO_4$)	Sigma Aldrich Co, Germany
- Dinitrochlorobenzene (DNCB)	Sigma Aldrich Co, Germany
- Lipopolysaccharide (LPS)	Sigma-Aldrich, Steinheim, Germany

The antigens were freshly prepared, dissolved in PBS, and used to a final concentration as indicated.

2.1.2 Cells

2.1.2.1 Buffy coat

Fresh Buffy coats were provided from the blood bank of the University Hospital (Aachen, Germany).

2.1.2.2 Keratinocytes

Primary human foreskin keratinocytes were obtained from Fa Clonetics (Walkerville, MD).

2.1.3 Medium and solutions

2.1.3.1 Reagents for cell culture

Material:	Supplier:
- Keratinocyte Basal Medium	Clonetics, U.S.A.
- KGM-2 Bulletkin	Clonetics, U.S.A.

- RPMI 1640 Medium with L-Glutamine | PAA, Austria
- Phosphate Buffered Saline (PBS, pH=7.4) | Sigma, Deisendorf, Germany
- Fetal Calf Serum (FCS) | PAA, Austria
- Penicillin (100 units; ml) | Gibco BRL, Scottland
- Streptomycin (1µg/mL) | Gibco BRL, Scottland
- Amphotecerin B (25µg/ml) | Gibco BRL, Scottland
- Trypan blue | PAA, Austria
- Ficoll 400 | Amersham Biosciences, Germany
- EDTA | Sigma, Deisendorf, Germany
- Monocyte negative isolation kit | Dynal Biotech, Norway
- Trypan blue | PAA, Austria
- Propidium iodide | Sigma, Deisendorf, Germany

2.1.3.2 Reagents for Agarose gel electrophoresis

Material:	Supplier
- Agarose (SeaKem®Leeagarose)	FMC, Biozym, Heidelberg, Germany
- DNA Marker 50-1000 bp	FMC, Biozym, Heidelberg, Germany
- Loading Buffer:	Sigma, Deisenhofen, Germany
-.25 % Orange G	Sigma, Deisenhofen, Germany
- 0.25 % Ficoll 400	Amersham Biosciences, USA
- TAE Buffer:	Sigma, Deisendorf, Germany
- Ethidium bromide	Merck, Darmstadt, Germany

2.1.4 Antibodies

antibody	labelling	clone	Company
CD14	FITC	M5E2	Becton-Dickison (Heidelberg, G)
CD80	FITC	L307,4	Becton-Dickison (Heidelberg, G)
CD54	PE	HA58	Becton-Dickison (Heidelberg, G)
CD86	PE	FUN-1	Becton-Dickison (Heidelberg, G)
CD1a	FITC	HI149	Becton-Dickison (Heidelberg, G)
HLA-DR	FITC	G46-6	Becton-Dickison (Heidelberg, G)
CD83	PE	HB15e	Becton-Dickison (Heidelberg, G)
CD45/14	FITC/PE	2D1	Becton-Dickison (Heidelberg, G)
CD40	FITC	5C3	Becton-Dickison (Heidelberg, G)
CCR7	PE	3D12	Becton-Dickison (Heidelberg, G)
DC-SIGN	FITC	DCN46	Becton-Dickison (Heidelberg, G)
CD11c	PE	Be-ly6	Becton-Dickison (Heidelberg, G)

IFN-y	PE	4S.B3	Becton-Dickison (Heidelberg, G)
Anti-Caspase-3	Biotin	C9605	Becton-Dickison (Heidelberg, G)
IgG-1κ	PE	MOPC21	Becton-Dickison (Heidelberg, G)
IgG-1κ	FITC	MOPC21	Becton-Dickison (Heidelberg, G)
IgG-2a k	FITC	G155-178	Becton-Dickison (Heidelberg, G)
IgG-2a k	PE	G155-178	Becton-Dickison (Heidelberg, G)

2.1.5 Kits

1) Negative Monocyte Isolation Kit: Dynal Biotech (Oslo, Norway)
2) Cytofix Cytoperm: BD Biosciences (Heidelberg, Germany)
3) CBA Kit: BD Biosciences (Heidelberg, Germany)
4) High Pure RNA Isolation Kit from Bochringer (Mannheim, Germany)
5) Reverse Transcription: Perkin Elmer (Massachusets, USA)
6) PCR: Perkin Elmer (Massachusets, USA)

2.1.6 Equipment

CO_2-Inkubator	Sanyo (Tokio, Japan)
Microscope (Axiovert)	Zeiss (Jena, Germany)
Centrifuge (Rotixa, AP)	Hettich (Tuttlingen, Germany)
Eppifuge (Centrifuge 5417R)	Eppendorf (Hamburg, Germany)
Eppifuge (Centrifuge 5415C)	Eppendorf (Hamburg, Germany)
Rotator (RS-PL 28-10)	Heto (Holten, Denmark)
Laminar flow (LaminAir HB 2472)	Hereus (Hannau, Germany)
Precision scale	Sartorius (Göttingen, Germany)
MTP photometer (Spectra Max 250)	MWG (München, Germany)
Spectralphotometer (DU70)	Beckmann (München, Germany)
Spectral photometer (Synergy HT)	Bio-Tek (USA)
Flow cytometer (FACS Calibur)	Becton Dickinson (Heidelberg, D)

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Culture of human keratinocytes

Human foreskin keratinocytes were obtained from Fa Clonetics (Walkerville, MD) and cultured according to the instruction guidelines. Cell culture medium for keratinocytes were freshly prepared by addition of 5ml KGM-2 BulletKit (containing 2 ml beef pituitary extract, 0.5ml human recombinant epidermal growth factor, 0.5ml insulin, 0.5ml hydrocortison, 0.5ml transferrin, 0.5ml epinepherin and 0.5ml gentamicin, amphotericin-b) to 500ml keratinocyte basal medium. For culturing, human epidermal keratinocytes were thawed and transferred into preheated keratinocyte culture medium (87500 cells/5ml medium) and cultivated in a $25cm^2$ culture flask at 37°C in a humidified atmosphere of 5% CO_2. The culture medium were changed completely every day and cells were cultured until 60% confluence was reached. For passaging, cells were washed with 3ml Hepes- buffer and detached with 3ml Trypsin/EDTA solution. After 6 to 8 min, 3ml trypsin neutralization solution was added and cells were washed with 220g for 5min. For further cultivation cells were counted by trypan blue exclusion (3500cells/cm^2), seeded in freshly cell culture flask and cultivated at 37°C in a humidified atmosphere of 5% CO_2

2.2.1.2 Generation of immature dendritic cells

PBMC were isolated from buffy coats of healthy blood donors obtained from the local blood bank. The cell blood concentrate was diluted (1:2 with sterile PBS, pH 7.4), layered carefully onto equal volumes of Ficoll-Paque and centrifuged at 435 g without brake for 40 minutes at room temperature. After centrifugation time, the lymphocytes platelets (PBMC ring) appeared as a cloudy ring at the PBS/Ficoll interface. These cells were carefully harvested, diluted with PBS (1:2), and centrifuged again at 245×g for 10 minutes at room temperature. The resulting supernatant was discarded. The cell pellet was washed twice by gently re-suspending the cells in 50 ml PBS subsequent centrifugation at 245×g for 10 minutes at room temperature. Viable cells were counted using trypan blue exclusion. The PBMC were suspended in the RPMI 1640 with L-Glutamine supplemented with 10% heat-inactivated FBS.

Monocytes were isolated from other PBMC by the use of adherence method. Isolated PBMC were plated on petri-dishes (d=10cm) at a concentration of $6x10^6$ cells/ml and incubated at 5% CO_2 and 37°C for 60min. To deplete residual inherent thymocytes, supernatants enriched with B cells; T cells; natural killer cells and granulocytes were removed and adherent monocytes were incubated with 0.5mM EDTA in PBS at 37°C and 5% CO_2 for 30 min. Afterwards monocytes were detached by plating the petri-dishes on ice for 5 to 10 min. and

subsequent scraping from the plastic surface. To block the residual EDTA action, cells were diluted in medium containing 10% heat-inactivated FBS and counted for a further purification with the negative monocyte isolation kit.

Cells were centrifuged at 245 x g for 10 min and the pellet was resolved in PBS /0.1% BSA to a cell density of $5x10^7$ cells/ml. For further depletion of residual B-cells, T cells, natural killer cells and granulocytes, the adherence purified cells were treated with mouse IgG antibodies for CD2, CD7, CD16, CD19, CD56 and CD235a and specific blocking reagents at 2-8°C for 20min. Subsequently, to delete unbound antibodies and residual blocking reagents, cells were washed with PBS/0.1% BSA and centrifuged at 300 x g for 8 min. The cell pellet was re-suspended in PBS/0.1% BSA to a cells concentration of $1x10^7$cells/ml and added to 1ml depletion beats per 1ml cells. The mixture was incubated at 4°C (to avoid ingestion of the beads by monocytes) for 40 minutes on an apparatus that provides both gentle tilting and rotation. Afterwards, the lymphocytes were depleted using the Dynal Magnetic Particle Concentrator. The monocytes were then collected as unbound cells. The isolated monocytes were washed with PBS and centrifuged at 245×g for 10 minutes (2 times). The resulting monocytes were re-suspended in the RPMI 1640 with L-Glutamine supplemented with 10 % heat-inactivated FBS.

Freshly isolated monocytes were subsequently cultured in six-well plates ($3x10^6$ cells/well) in RPMI 1640 medium containing L-Glutamin supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100µg/ml streptomycin, 25µg/ml amphotericin B, 800U/ml GM-CSF and 1000U/ml IL-4. 1ml each of the culture medium including cytokines was replaced on day 2, 4, 6 and on day 8, only 10% of culture medium was replaced with cytokine containing medium and the appropriate stimulus.

2.2.2 Chemical treatment of cultured immature DC

On day six, the cultured cells were treated with 1µg or 0.1µg/ml LPS, 4mM $NiSO_4$, 5µM DNCB, various concentrations PPD (10µM, 50µM, 100µM, 200µM, 500µM and 1000µM), and various concentrations of PPD in combination with LPS (1µg/ml) for 24h and 96h.

2.2.3 Gene expression analysis

2.2.3.1 RNA isolation

Total RNA from PBMC, monocyte derived dendritic cells (MoDC), keratinocytes and human hepatoma cell line cells (HepG2) were isolated using the High Pure RNA Isolation Kit from Boehringer (Mannheim, Germany) according to the instruction guidelines. After thawing, cells were embedded in lysis buffer for some minutes and afterwards RNA was applied on the columns. Isolated complete RNA (50µl) was aliquoted and immediately stored at –80°C to avoid RNA degradation. For quantification, 5µl of the isolated RNA was mixed with 500µl DEPC-water and measured in a spectrophotometer (DU 70, Beckmann, Germany). The ratio between the absorbance values at 260 and 280 nm gives an estimate of nucleic acid purity. Since OD_{260nm} of 1 comply 40µg of RNA, the RNA concentration calculated as follows:
Concentration of RNA (µg/ml) = 40 × $OD_{260\ nm}$ × dilution factor.

2.2.3.2 Reverse transcription of RNA

Reverse transcription (RT) was performed using RT-PCR-Kit. For one reverse transcription procedure we normally use 1µg RNA which is mixed with other reagents to a final volume of 10µl as follows:

2µl	$MgCl_2$ 25mM
1µl	10x buffer without $MgCl_2$
1µl	dNTP 10mM (dATP, dGTP, dTTP, dCTP)
0.5µl	reverse transciptase (RT)
0.5µl	random-hexamers
0.5µl	RNase inhibitor

cDNA was synthesized in a thermal cycler at the following temperatures: 22°C for 10 min, 42°C for 15 min, 99°C for 5 min, 5°C for 5 min. Corresponding reactions without the addition of reverse transcriptase (-RT) were set up to check for DNA contamination prior to every NAT amplification.

2.2.3.3 Polymerase chain reaction (PCR)

PCR was performed for NAT-1 and NAT-2 cDNA using primer located in the coding sequence. Forward primers were 5`-gga aca aat tgg act tgg aaa c-3` for NAT-1 and 5'-gat gac aaa tag aca aga tt-3' for NAT-2 and the common reverse primer was 5'-gag agg ata tct gat agc cac ata-3' yielding the product sizes of 861 bp for NAT-1 and 906 bp for NAT-2 (Kloth et al. 1994). PCR reactions were carried out in:

2.5µl	PCR buffer without MgCl2
2.5 µl	cDNA,
1.5 µl	$MgCl_2$ 25mM
0.5µl	dNTPs 10mM (dATP, dGTP, dTTP, dCTP)
0.25µl	forward primer (100pmol/µl)
0.25µl	reverse primer (100pmol/µl)
0.15µl	Ampli-TaqGold Polymerase
17.35µl	DEPC-water

Samples were amplified using the following conditions:

Denaturation	95°C	9min
(35cycles)		
Denaturation	95°C	1min
Annealing	51°C	1min
Elongation	72°C	1min
Elongation	72°C	10min

The resulting PCR products were separated with agarose gel electrophoresis (2.2%), stained with ethidium bromide and visualized under UV light. PCR was performed using cDNA from immature MoDC, cultured primary keratinocytes and HepG2 cells, which served as controls. Amplification of genomic DNA served as positive control.

2.2.3.4 Agarose gel formation

2 g agarose (2%) was dissolved in 100 ml 1× TAE electrophoresis buffer, heated in a microwave oven until completely melted. After cooling the solution to about 60°C, 1µL ethidium bromide (10 mg/ml) was added. Agarose was poured into a casting tray containing a sample comb and store until needed. Samples containing DNA, DNA marker were mixed with 2 µL loading buffer, and then loaded into the sample wells. The gel was run at 300-500 V for approximately 20 minutes, and bands were analyzed using a transilluminator.

2.2.4 N-Acetyltransferase 1 activity assay

N-Acetyltransferase 1 (NAT-1) activity was estimated by a modification of published protocols for arylamine determination (Sinclair et al. 1998; Kawakubo et al. 2000).
In brief, harvested cells were washed twice with PBS and re-suspended in cold lysis buffer (50 mM Tris-HCl buffer pH 7.5) containing 1mM Dithiothreitol (DTT) and 1 tablet protease inhibitor per 10 ml (complete mini, EDTA-free, Roche Diagnostics, Mannheim, Germany). Cell lysates were prepared by sonication (UP50H, Dr. Hielscher GmbH, Stuttgart, Germany, 4x8 pulses) on ice, centrifuged for 10 min at 20,000 x g at 4°C, and the resulting supernatants were used for the NAT activity assays. The protein concentration was determined by the method of Bradford (1976).
For the acetylation, a reaction mixture (100 µl final volume) containing cell lysate (a volume appropriate to 50 µg protein) and 10 µl substrate solution or 10 µl PBS were prepared on ice. PABA (1mM in PBS, pH 7.4) was used as the NAT-1 specific arylamine substrate. The reaction was started by the addition of 1mM AcCo-A. After incubation at 37°C for 30 min, the reaction was stopped by the addition of 100 µl ice cold acetonitrile. The mixture was centrifuged 10 min at 20,000 x g to remove precipitated proteins.
The supernatant was mixed 1:4 with 4-dimethylaminobenzaldehyd [DMAB, 5% w/v, in HCl-acidic acetonitrile/water (9:1) solution] and the absorbance at 420 nm was measured by spectral photometer (Synergy HT, BIO-TEK) in a 96-well microplate for quantification of the remaining acetylated arylamine. The amount of residual arylamine was determined from a standard curve, which was linear over the range of concentrations. To determine the substrate specific enzyme activity, the measured quantity of remaining arylamine was subtracted from the added amount, in order to achieve the quantity of acetylated NAT substrate. This was related to total protein and reaction time. The resulting enzyme activity is given in [nmol/mg/min].

2.2.5 Determination of acetylated substrates in cell culture supernatants

Supernatants of non-treated and PPD-treated cells were extracted with ethyl acetate, evaporated under nitrogen gas flow and re-dissolved in 50% acetone nitrile. These solutions were analyzed for the known acetylated PPD-derivatives (mono- and di-acetyl-PPD) by HPLC [Shimadzu HPLC system equipped with LC10 AD gradient pump, security guard column, Nucleosil C18 column (5 µm, 4.1 x 250 mm), a SPD M10A DA detector and ClassVP chromatography software]. The flow rate of the mobile phase was 1.0 ml/min and its composition varied over time as follows: 0 min B = 0 %; 7 min B = 0 %; 31 min B 60 %; 36 min B = 60 %; 38 min B =100 %; 41 min B = 100 %; 44 min B = 0 %; 49 min B = 0 %. Eluent A was 92% 25mM ammoniumacetate and 8% acetone nitrile; eluent B was 100% acetonitril. The detection was performed at 255 nm and retention times were 3.4 min for *para*-

phenylenediamine (PPD), 5.5 min for monoacetyl-PPD (MAPPD) and 13.0 min for diacetyl-PPD (DAPPD). Peaks were identified and quantified by comparison with standard curves for PPD, MAPPD and DAPPD, which were linear over the range of concentrations. Measured concentrations were corrected by the appropriate extraction recovery factors. In order to determine these factors, samples with known amounts of PPD, MAPPD and DAPPD were carried along with every extraction process and analyzed as described above.

2.2.6 Flow cytometric analysis

2.2.6.1 Dendritic cell characterisation by flow cytometric analysis

Cells were analyzed on day zero, 2, 4, and 6 as well as 24h and 96h after chemical treatment. Cells (1-2 x 10^5 cells/ml) were washed, re-suspended in PBS and incubated with fluorescent-labelled Abs (CD45-FITC combined with CD14-PE, CD80-FITC, CD86-PE, CD1a-FITC, CD83-PE, CD40-FITC, HLA-DR-FITC, CCR7-PE or isotype matched controls (BD Biosciences, Heidelberg, Germany)). Stained cells were washed to remove excess Abs, re-suspended in 300µl PBS and were subsequently analyzed in the FACS Calibur cell analyzer using Cell Quest Pro software (Becton Dickinson). Cellular debris was eliminated from the analysis using a gate on forward and side scatter.

2.2.6.2 Popidium iodide staining

Immature MoDC were treated with 1µg LPS, 4mM $NiSO_4$, 5µM DNCB, various concentrations of PPD (10µM, 50µM, 100µM, 200µM, 500µM and 1000µM), or various concentrations of PPD in combination with LPS (1µg/ml, Steinheim, Deutschland) for 24h and 96h. After stimulation MoDC were centrifugated at 245 x g for 10 min and the pellet was resolved in PBS to a cell densitiy of 1x10^6 cells/ml. 2µl of propidium iodide solution (c(PJ)= 0.1mg/ml) was added to 200µl cell suspension and cells were subsequentely measured by FACS analysis. Incubation duration of propidium iodide should not exceed 5min.

2.2.6.3 Caspase-3 activation

Caspase activation was measured 24h and 96h after stimulation with different concentrations of PPD; PPD in combination with LPS; LPS alone or 4mM NiSO4 as positive control. Cells (1 x 10^6cells/ml) were washed with PBS, fixed and permeabilized using the Cytofix/Cytoperm Kit (Becton Dickinson, Heidelberg) for 20min at room temperature (RT), centrifuged with 500xg for 5min at RT and washed with the orginal kit containing Perm/WashTM buffer. Pellets of each sample were solved in 5µl Perm/WashTM buffer and for staining; cells were

incubated with 20µl biotinylated rabbit anti-active caspse-3 mAb (BD, Heidelberg) for 60min at RT in the dark. Following incubation with the primary Ab, cells were washed with Perm/WashTM buffer, resuspended in 5µl Perm/WashTM buffer and incubated after addition of 5ng Avidin-FITC for 30min at RT in the dark. Following incubation, cells were washed once with Perm/ Wash buffer TM and analyzed by flow cytometry using Cell Quest Pro software (FACS Calibur, BD, Heidelberg).

2.2.6.4 Cytometric beat array (CBA): Determination of cytokines

With the cytometric beat array we could measure the PPD induced secretion of 6 different cytokines in supernatants of PPD stimulated MoDC. To establish a kinetic, we separated supernatants from MoDC of four different donors stimulated with different concentrations of PPD (10µM, 50µM, 100µM and 500µM) or LPS (0.1µg/ml)as positive control for 1h, 2h, 4h, 8h and 24h. For practical application we use the human inflammation kit from BD Biosciences. The principle of the test is as follows:
Six bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for IL-8, IL-1β, IL-6, IL-10, TNF-α and IL-12p70 proteins. The six bead populations are mixed together to form the BD™ CBA which is resolved in the FL3 channel of a flow cytometer such as the BD FACScan™ or BD FACSCalibur™ flow cytometer. The capture beads, PE-conjugated detection antibodies, and recombinant standards or test samples are incubated together to form sandwich complexes. Following acquisition of sample data using the flow cytometer, the sample results are generated in graphical and tabular format using the BD™ CBA Analysis Software.

2.2.7 Determination of TNF-α using Enzyme-linked Immunosorbant assay (ELISA)

After incubating MoDC cultures with the relevant chemicals, culture supernatants were separated and TNF-α determined by human ELISA Kits obtained from (R&D, Systems) according to the instructions of the producer. This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TNF-α has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TNF-α present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TNF-α is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, an enhanced luminol/peroxide substrate solution is added to the wells and light is produced in proportion to the amount of

TNF-α bound in the initial step. A microplate luminometer is used to measure the intensity of the light emitted.

3 Results

3.1 In vitro generation of dendritic cells

For most experiments, we generated monocyte derived dendritic cells as described in the materials and methods section. To ensure a high quality and adequate quantity of immature dendritic cells, we measured on day 2, 4 and 6 the maturation progress of freshly isolated peripheral blood monocytes. Thus, we measured the downregulation of the monocyte marker, namely cluster domain 14 (CD14) and the concomitant upregulation of CD45. Only those cells which downregulated CD14 by more than 50% between day 2 and 4, were considered as responder cells and used for further experiments. As shown in Figure 3, we measured the marker expressions of CD14 and CD45 on day of isolation (day 0) and on day 2, 4 and 6.

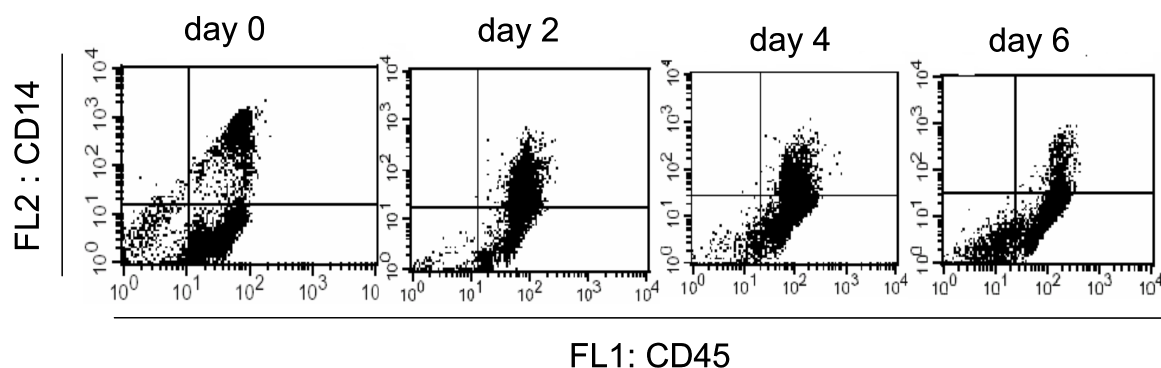

Figure 3: Differentiation of monocytes to immature dendritic cells.
In vitro generated CD14+ peripheral blood monocytes were differentiated to immature dendritic cells by culturing cells in cytokine enriched medium. Cells were stained with CD45FITC/CD14PE conjugated anitbodies and analyzed by flow cytometry using FACScalibur (Becton Dickinson, Heidelberg, Germany) on culturing day 2, 4 and 6.

Addition of IL-4 and GM-CSF into the culture medium of freshly isolated monocytes delivers the differentiation process into immature dendritic cells. Figure 4 represents an optimal maturation progress in which monocytes differentiate into immature dendritic cells. CD14/CD45 double-stained cells degrade time dependently while the simple CD45 stained cell population is concomitantly enhanced. On day 6 these results were found: more than 95% simple CD45 positive cells and almost no CD14/CD45 double stained or CD14 simple stained cells.

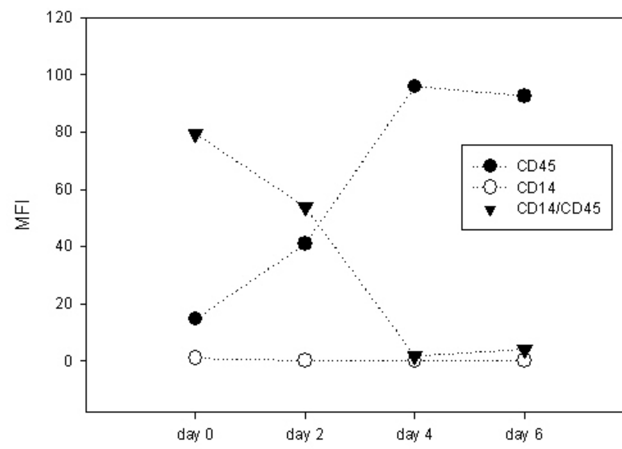

Figure 4: In vitro generation of immature dendritic cells
Qualitiy of MoDC is characterized by downregulation of CD14 with concomitant upregulation of CD45.

3.2 Characterization of N-Acetyltransferase (NAT) in MoDC

3.2.1 Presence of NAT-1 and NAT-2 mRNA in monocyte-derived dendritic cells

For characterization of the metabolic capacity of MoDC in comparison with hepatocytes (HepG2) and keratinocytes, we determined the m-RNA expression of N-acetyltransferase 1 and 2 (NAT-1, NAT-2) mRNA with RT-PCR. In contrast to HepG2 cells, for MoDC and for keratinocytes, only NAT-1 mRNA expression was detected (Figure 5).

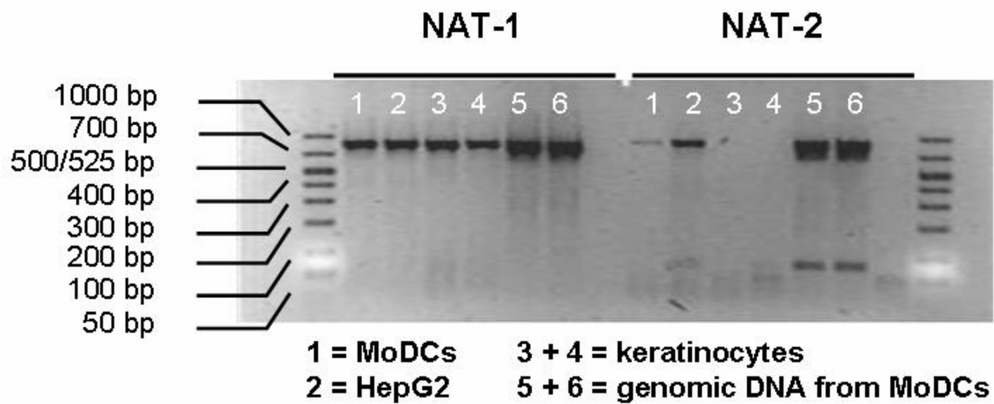

Figure 5: Presence of NAT-1 and NAT-2 mRNA in monocyte-derived dendritic cells
MoDC (1), HepG2 cells (2) and primary cultured keratinocytes (3, 4) were analyzed for NAT-1 (861 bp) and NAT-2 (906 bp) mRNA levels. PCR products were separated by electrophoresis on a 2.2% (w/v) agarose gel stained with ethidium bromide.

3.2.2 NAT-1 activity in MoDC

To examine the metabolic activities in these in vitro generated DC, N-acetyltransferase 1 activity assays (as described above) were performed for MoDC, as well as for PBMC and the cell line HepG2, which served as controls. As shown in Figure 6, MoDC cell lysates of all investigated donors (DC 1-3) showed NAT-1 activities between 23.4 and 26.6 nmol/mg/min. PBMC NAT-1 activities of three different donors (PBMC A, B, C) varied between 8.0 and 23.5 nmol/mg/min. HepG2 cell lysates exhibited NAT-1 acitvities of 10.5 ± 0.3 nmol/mg/min.

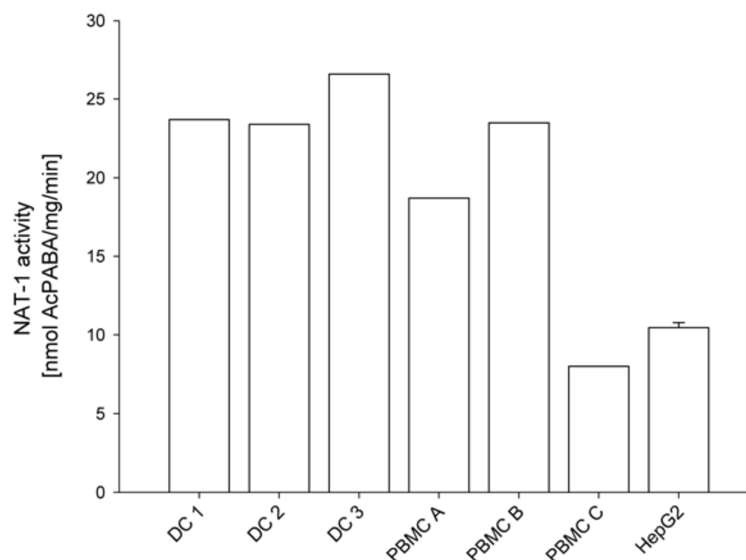

Figure 6: NAT-1 activity of MoDC
NAT-1 enzyme activity of MoDC was determined by performing AcCoA-dependent acetylation of 1 mM PABA by cell lysates as described. NAT-1 activity is given in quantity of acetylated PABA per total protein and reaction time [nmol/mg/min]. All samples were measured in duplicates. Results of individual donors (PBMCs and MoDC) and in case of HepG2 a mean of three independent experiments ±S.E. are depicted.

3.2.3 Determination of acetylated PPD derivatives in MoDC cell culture supernatants

In order to validate our findings we examined the acetylation capacity of cultured, intact MoDC. Therefore, we determined the acetylated derivatives of para-phenylenediamine (PPD) in cell culture supernatants. MoDC were incubated with PPD (0, 10, 100, 500 µM) for 24 h, and afterwards supernatants were collected and extracted with ethylacetate. Amounts of mono- and diacetyl-PPD (MAPPD and DAPPD) were quantified by HPLC analysis. MAPPD and/or DAPPD were found in cell culture supernatants of MoDC from 4 of 6 donors. Measured concentrations varied between 0.9 µM and 23.9 µM for MAPPD and 1.8 µM and 12.1 µM for DAPPD depending on the donor and the added PPD concentrations. (Figure 7).

	Donor-and PPD-concentration dependent formation of acetylated PPD derivatives (MoDC)	
Added PPD [µM] (studied subjects)	**MAPPD formation**[a]	**DAPPD formation**[b]
0 (n=6)	0	0
10 (n=5)	1	2
100 (n=4)	1	2
500 (n=5)	4	3

[a]amount of donors forming MAPPD
[b]amount of donors forming DAPPD

Figure 7: Donor- and PPD- dependent acetylation of MoDC
Donor-dependent secretion (n=6) of monoacetyl-PPD and diacetyl-PPD (MAPPD and DAPPD) by monocyte-derived dendritic cells (MoDC) after addition of different PPD concentrations (24 h, HPLC analysis).

3.3 Effect of PPD on phenotypical and functional properties of MoDC

3.3.1 Effects of different low concentrations of PPD on the maturation of MoDC

Maturing of immature antigen ingesting and processing MoDC are associated with differentiation into mature antigen presentig MoDC and are accompanied by phenotypical changes. Immature dendritic cells were characterized as CD14 negative, CD45 positive (53%), CD80 positive (10%), and CD86 positive (15.4%), all of which displayed a low expression of CD83 (3%). The histograms of cells incubated for 24h and 96h with 10μM, 50μM PPD and 5μM DNCB (dissolved in 0.1% DMSO), a known chemical sensitizer, and also 1 μg/ml LPS are shown in Figure 8. PPD induces no significant changes in the expression of CD80, CD86 and HLA-DR expression in contrast to the included controls. This was found for all donors studied (n=9).

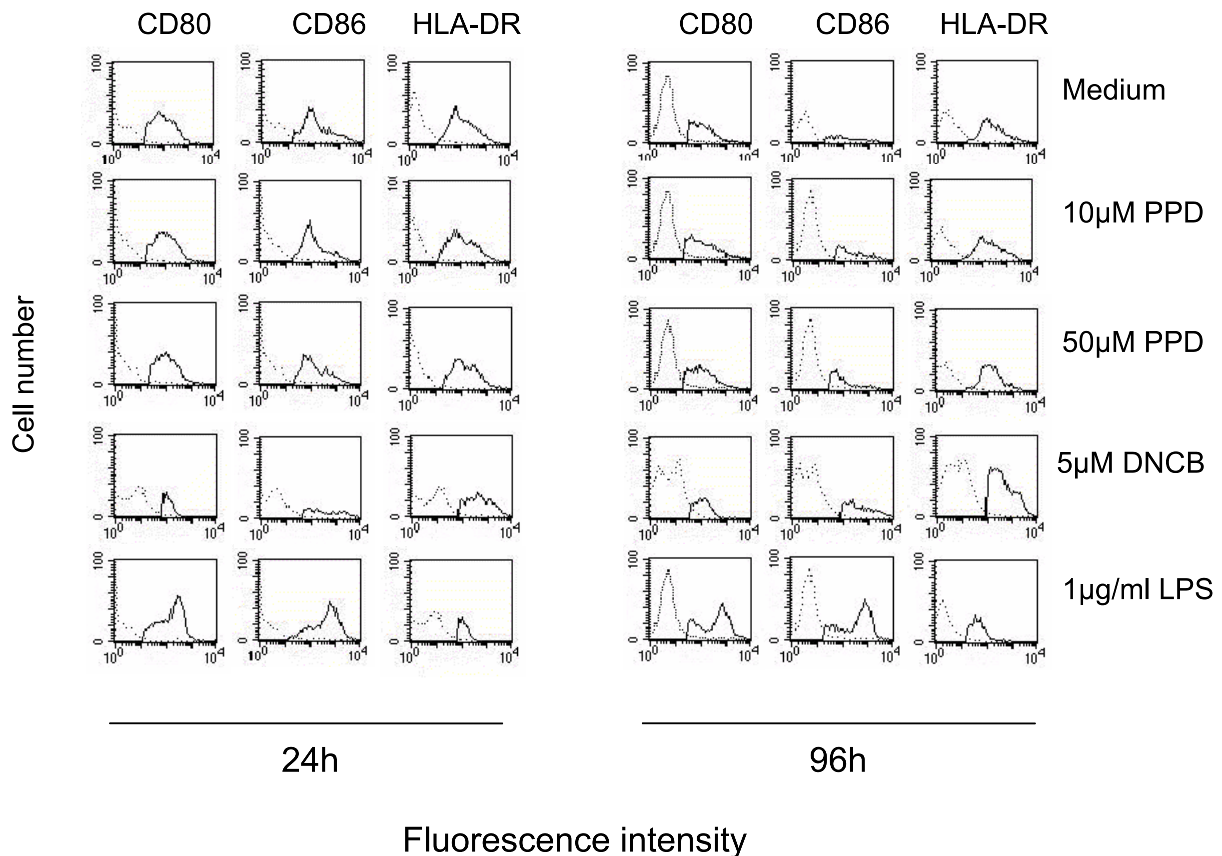

Figure 8: Representative flow cytometry of CD86, CD80, and HLA-DR expression on MoDC treated for 24h and 96h with various concentrations of PPD compared to LPS and DNCB
Freshly isolated peripheral blood monocytes (three independent donors were studied) were cultured in the presence of GM-CSF and IL-4 as described, and resulting MoDC were stimulated on day 6 with 10 µM and 50 µM PPD, respectively. LPS (1µg/ml) and 5 µM DNCB (dissolved in 0.1% DMSO) served as positive controls. Cells were stained with FITC- or PE- conjugated mABs against CD80, CD86, and HLA-DR molecules. Cell numbers of positive cells for CD86, CD80, and HLA-DR were measured 24h and 96h after stimulation. In order to assess unspecific bindings, cells were stained with specific isotype controls (open histogram). Staining was analyzed using FACScalibur (Becton Dickinson). Data are expressed as histogram overlay depicting the staining of positive cells (solid areas) and the isotype control (open, dotted line).

The results are summarized in Figure 9. The MFI values for 1µg/ml LPS increased significantly after 24h and varied between 189 and 219 for CD80; 321 and 390 for CD86 and between 304 and 424 for HLA-DR (n=3 donors) compared to medium. The results for 10 µM and 50 µM PPD did not change after an incubation time of 96h. Levels varied for CD80 between 129 and 145, for CD86 between 126 and 145, and for HLA-DR between 102 and 195. In contrast, CD86 and HLA-DR levels of DNCB increased considerably more at this point. CD86 varied donor dependently between 1347 and 1899, and HLA-DR between 349 and 1655. LPS clearly increased MFIs of all three co-stimulatory molecules.

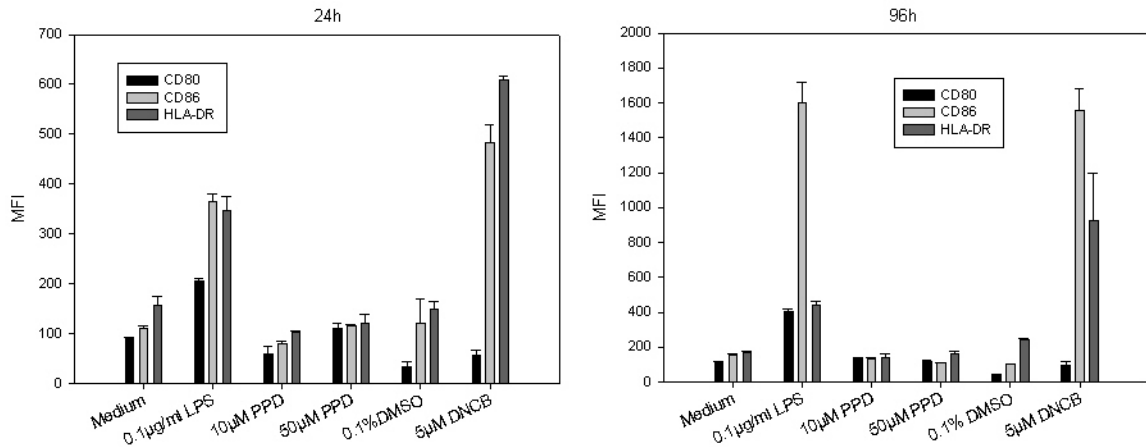

Figure 9: Summarization of effects of 10µM and 50µM PPD on MoDC maturation
MoDC were cultured with medium, PPD (10 µM, 50 µM), LPS (1 µg/ml) and DNCB (5 µM, dissolved in 0.1% DMSO) for 24h (panel a) and 96h (panel b). Cells were stained with FITC- or PE- conjugated mABs against CD80, CD86, and HLA-DR molecules. MFI values are shown for the positive cells. The data are expressed as mean values (+/- SEM) of three individual donors with the exception of DNCB (24h, n=2 donors) and DMSO (24h and 96h, n= 2 donors).

3.3.2 Effects of different high concentrations of PPD on the maturation of MoDC

To be certain that the results as limited to the concentrations used, further experiments were performed. They included higher PPD concentrations, namely of 0.1 mM, 0.2 mM, 0.5 mM, and 1 mM.

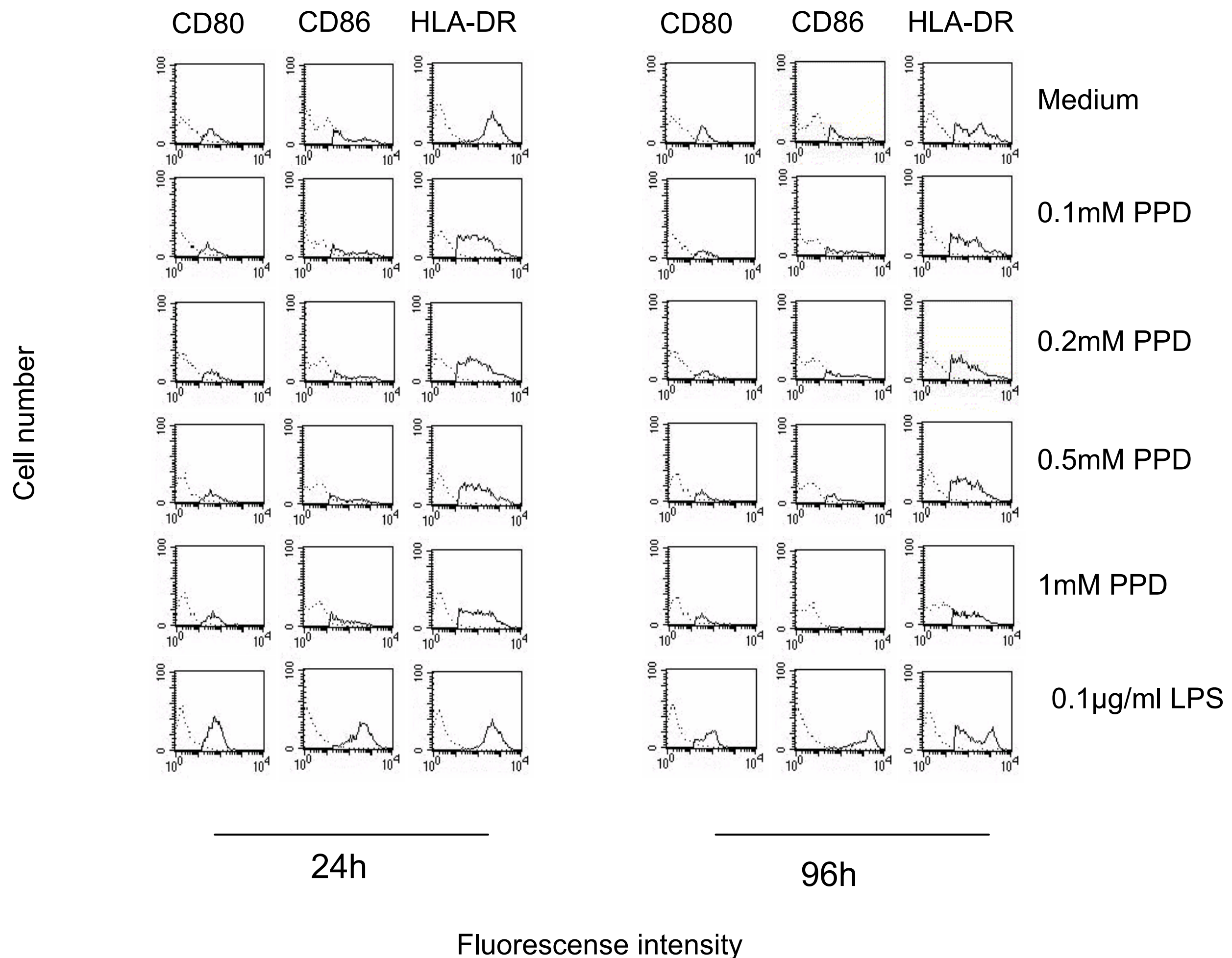

Figure 10: MFI values of CD80, CD86, and HLA-DR of MoDC after treatment with high concentrations of PPD, LPS, and DNCB.
MoDC were cultured with medium, PPD (0.1mM, 0.2mM, 0.5mM and 1mM), LPS (1 µg/ml) for 24h and 96h. Cells were stained with FITC- or PE- conjugated mABs against CD80, CD86, and HLA-DR molecules. In order to assess unspecific bindings, cells were stained with specific isotype controls (open histogram). Staining was analyzed using FACScalibur (Becton Dickinson). Data are expressed as histogram overlay depicting the staining of positive cells (solid areas) and the isotype control (open, dotted line). At least three independent experiments have been performed.

The results are summarized in Figure 11. Again, PPD induced no significant upregulation of CD80, CD86 and HLA-DR surface expression after 24h, while LPS (1µg/ml) enhanced MFI for CD80 (41-55), CD86 (218-347), HLA-DR (167-561) clearly (n=3). After 96h incubation with these concentrations, we found that 0.5 mM and 1.0 mM PPD reduced the MFI values for CD80, CD86, and HLA-DR compared to the controls. For instance, a concentration of 0.5 mM PPD reduced CD80 MFIs from 39 to 26, and the presence of 1mM PPD reduced values from 39 down to 22 (56%) in. MFIs for CD86 decreased from 67 to 46 (0.5 mM, 68%) and dropped to 29 (43%) after incubation with 1mM PPD. Values for HLA-DR dropped from 239 to 112 (47%) and for 1mM PPD to 106 (44%).

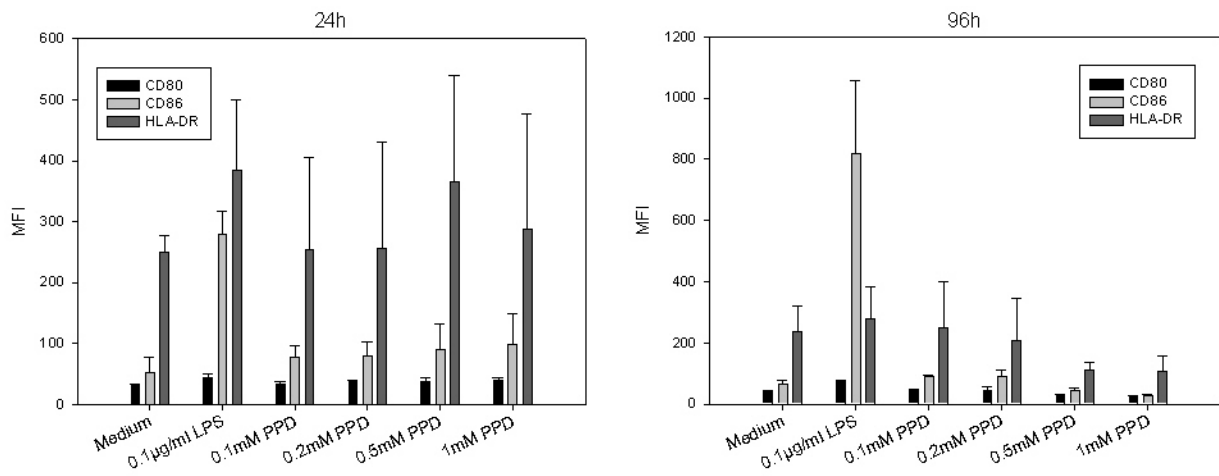

Figure 11: Summarisation: effects of 0.1mM, 0.2mM and 0.5 mM ppd on dendritic cell maturation.
MoDC from 3 subjects were treated with 1mM, 0.5mM, 0.2mM or 0.1mM PPD, and 1 µg/ml LPS for 24h (panel a) and 72h (panel b). Alteration of MFI from CD80, CD86, and HLA-DR was estimated by flow cytometry. Data are depicted as mean ± SEM for three independent experiments.

3.3.3 Effect of PPD on TNF-α secretion of MoDC

The effect of haptens on the MoDC phenotype is often accompanied by the production of TNF-α. It could be shown that bacterial components and some haptens trigger phenotypic maturation probably via the production of TNF-α (Arrighi et al. 2000; Aiba et al.1997). Coutant (et al. 1999) could demonstrate that strong contact allergens like $NiSO_4$ also show a higher potential to induce TNF-α in MoDC and that in contrast to LPS the hapten induced TNF-α production is generally rather weak. Because PPD was not able to enhance co-stimulatory molecules, it was expected that PPD might not enhance the basal TNF-α production in MoDC either.

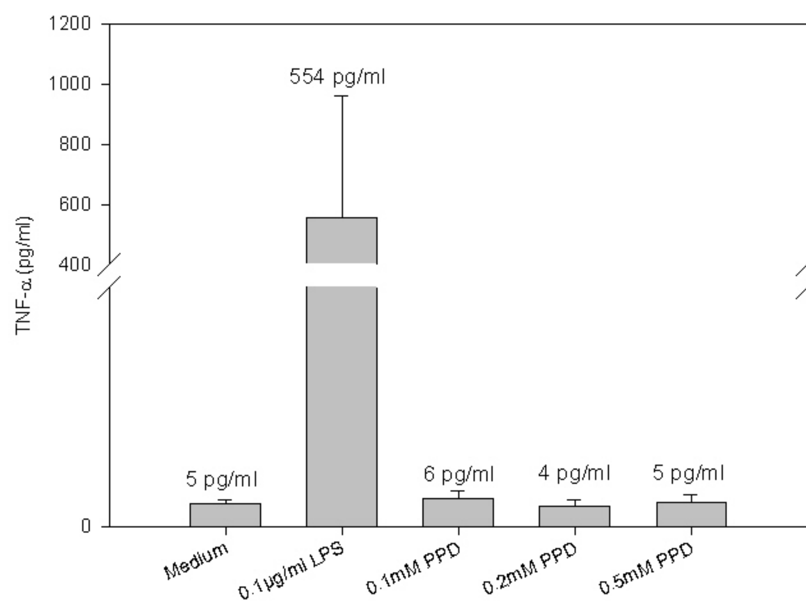

Figure 12: Effect of PPD on TNF-α production in MoDC
DC´s (1x10^6 cells/ml) were stimulated with PPD (0.5mM, 0.2mM or 0.1mM) or LPS (0.1µg/ml) as positive control. After 24h of incubation the concentration of TNF-α was determined by cytometric beat array (CBA). Mean values derived from 4 different donors and are presented on the top of each sample bar.

As illustrated in Figure 12, our assumption could be confirmed by these data, namely that no PPD concentration induced TNF-α production in MoDC whereas, in supernatants of LPS stimulated cells, we measured high TNF-α concentrations.

3.3.4 The impact of PPD on CCR7 expression in dendritic cells

The CCR7 receptor, also called the homing receptor, enables cells after encountering with the corresponding ligands SLC or MIP-3ß to enter into local lymphnodes (Sozanni et al. 1999). Immature DC, upon induction of maturation after stimulation by bacterial products or inflammatory cytokines, upregulate CCR7, a chemokine receptor that drives the migration to the lymphatics. The migration of DC through CCR7 has also been observed after hapten stimulation (Aiba et al. 2000). We measured the expression of CCR7 in MoDC in response to LPS and PPD stimulation after 24h and 96h. We detected enhanced CCR7 expression of LPS stimulated MoDC only after 96h. In contrast to LPS, for PPD we could always detect an enhanced CCR7 expression after 24h (Figure 13).

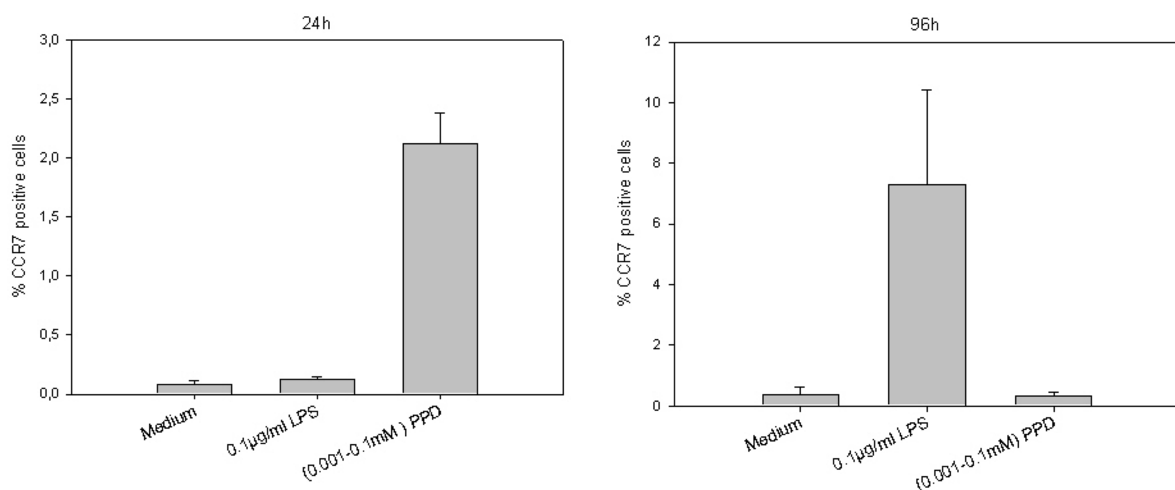

Figure 13: Effects of PPD on the CCR7 expression of MoDC.
MoDC (1x106 cells/ml) were cultured on day 6 in culture medium alone (control) with LPS, or in the presence of different concentrations of Paraphenylenediamine (PPD) for 24h h. Surface expression CCR7 was analyzed by flow cytometry. Each value represents the mean ± SEM from 5 independent donors.

Successful migration from peripheral tissue to 2nd lymphnodes requires LC not only to express CCR7, but numerous modification need to occur. One main prerequisite for successful migration is a rapid change of adhesion molecules on the cell surface of dendritic cells. We therefore measured how PPD regulated the expression of two dendritic cell characteristic adhesion molecules, DC-SIGN and CD11c.

3.3.5 Impact of PPD on expression of adhesion molecules DC-SIGN and CD11c

DC-SIGN is a DC specific adhesion molecule crucial to the specific migratory process of DCs. In detail, DC-SIGN shows a high affinity to ICAM-2 and thereby mediates DC rolling and transendothelial migration into 2nd lymphnodes. Moreover DC-SIGN enables transient DC-T cell interaction by its high affinity interaction with ICAM-3 (Geijtenbeek et al. 2000). Another well known DC-specific marker is CD11c. Purified CD11c supports the endothelial cell adhesion and interacts with multiple ligands of endothelial cells including ICAM-1, ICAM-2, and VCAM-1. Meunier et al have demonstrated that CD11c is involved in antigen presentation by DC; furthermore (Sadhu C 2007) reported that CD11c play a significant role in delayed type hypersensitivity. Measurement of DC-SIGN and CD11c after PPD stimulation could produce necessary insights into the impact of PPD on the regulation of DC specific adhesion molecules.

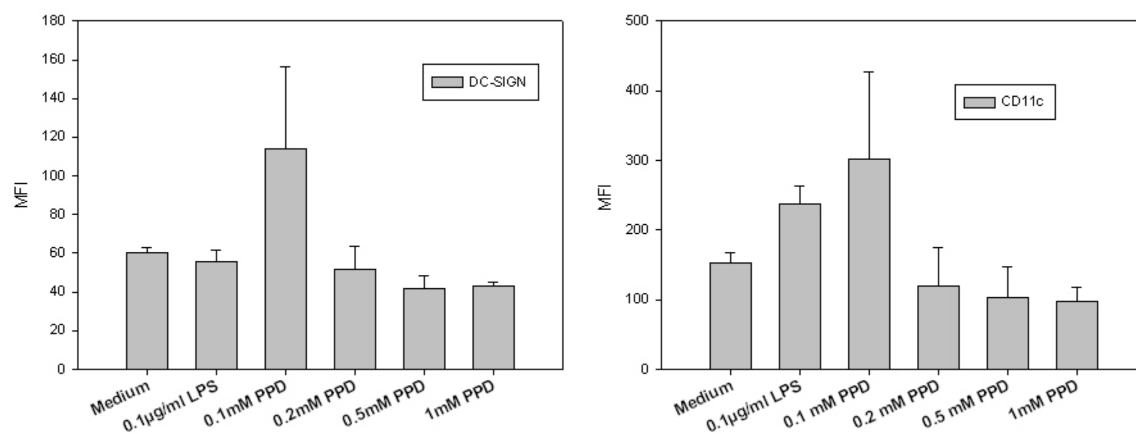

Figure 14: Effects of PPD on CD11c and DC-SIGN expression of MoDC.
MoDC (1x106 cells/ml) were cultured on day 6 in culture medium alone (control) with LPS, or in the presence of different concentrations of Paraphenylenediamine (PPD) for 24h. Surface expression of left DC-SIGN and right CD11c was analyzed by flow cytometry. Each value represents the mean ± SEM from 3 independent donors.

Figure 14 depicts the finding in one of three donors investigated: that 0.1mM PPD enhances the expression of CD11c and DC-SIGN after 24h. Higher PPD concentrations, namely 0.2mM, 0.5mM and 1mM, showed no effects on CD11c and DC-SIGN expression in MoDC.

3.4 Influence of PPD on mediator secretion in MoDC

DC can respond to chemicals by changing their expression of co-stimulatory molecules or HLA-DR antigen and their production of pro-inflammatory cytokines in different fashions, which subsequently may induce a difference in the final immune response. Cytokine expression patterns and the cytokine secretion time points are different for each chemical. Therefore, we investigated if different concentrations of PPD are able to augment the secretion of different cytokines in MoDC, and collected the cell culture supernatants after administration of 0.1mM, 0.2mM, 0.5mM or 1mM PPD for 1h, 2h, 4h, 8h and 24h. In addition, we included LPS (0.1µg/ml) as positive control.

3.4.1 Interleukin 1 beta (IL-1ß) expression of MoDC

IL-1ß is upregulated by contact allergens and is necessary for successful skin sensitization. In in vitro testings, Reutter et al 1997 could show that chemicals are able to increase mRNA for IL-1ß in LC-like cells. Pichowski et al. 2001 could confirm these findings for MoDC but remark that the chemical induced changes in mRNA expression are modest and show strong inter-individual differences. In addition they could demonstrate that PPD elicits comparatively robust increases in IL-1ß mRNA expression as strong contact sensitizer like $NiSO_4$ and DNCB. Furthermore, others (Aiba et al 1997) measured enhanced IL-1ß protein expression in MoDC after DNCB or $NiCl_2$ stimulation.

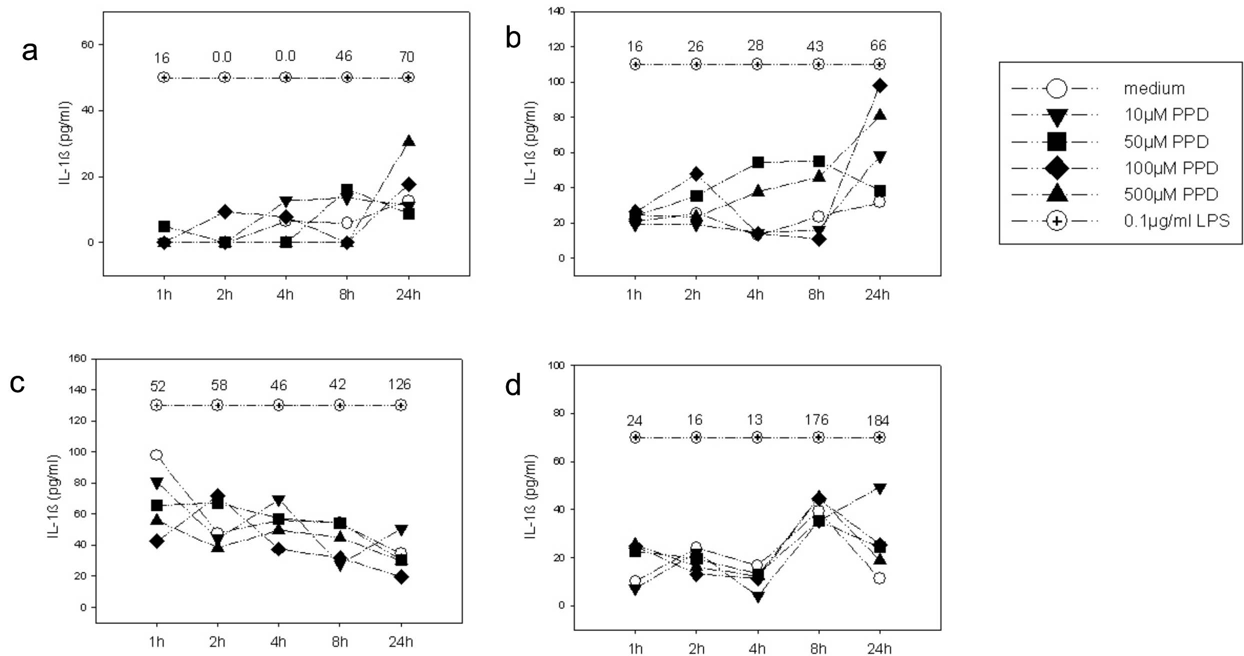

Figure 15: IL-1ß expression
Immature MoDC were unstimulated or stimulated with either different concentrations of PPD or 0.1µg/ml LPS as positive control. After 1h, 2h, 4h, 8h or 24h IL-1ß concentrations were determined in cell culture supernatants with Cytometric Bead Array (CBA). The assay sensitivity for IL-1ß is 7.2 pg/ml.

The positive control LPS induces in all 4 donors, a clearly enhanced IL-1ß secretion after 24h. By comparison of the IL-1ß expression patterns of 4 different donors it is noticeable that 3 of 4 donors show, at least with one PPD concentration an enhanced IL-ß expression after 24h. Various concentrations of PPD induce in donor b a strong IL-1ß secretion already after 4h of incubation (Figure 15).

3.4.2 TNF-α expression of MoDC

TNF-α modulates the functional activity of LC and DC, in part due to regulation of the expression of certain co-stimulatory and adhesions molecules and other membrane determinants (Kimber et al. 2000). LPS is a powerful stimulator of TNF- α synthesis in MoDC and triggers phenotypic maturation (Arrighi JF. 2001). Coutant et al could demonstrate that the effects of haptens are accompanied by the production of TNF-α. Because we could not measure enhanced TNF-α concentrations in 24h old supernatants of PPD treated cells, we were surprised to detect TNF-α after shorter incubation durations.

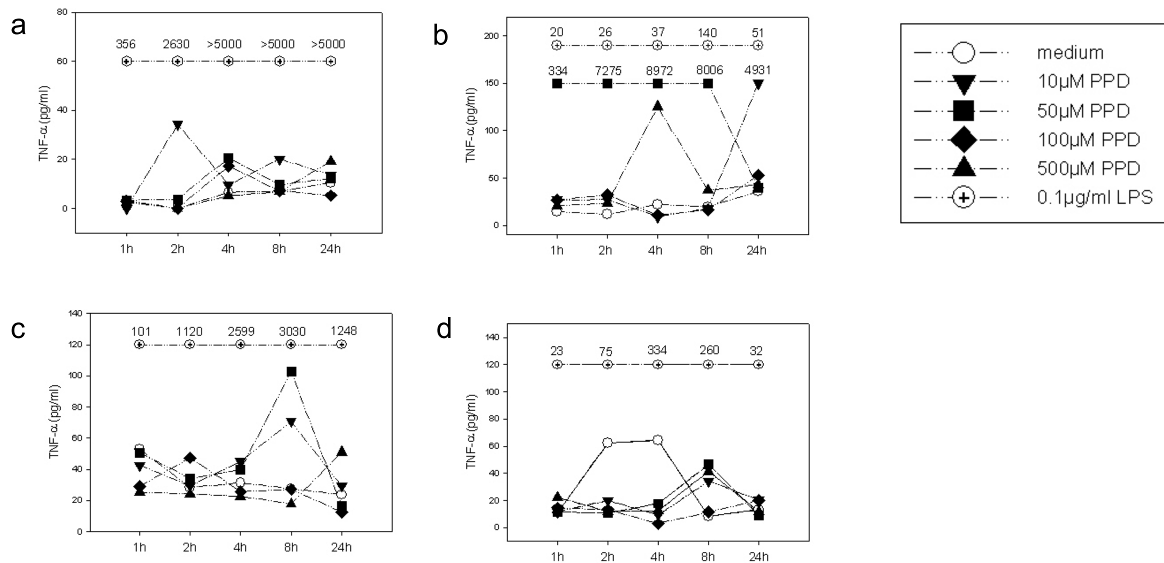

Figure 16: TNF-α expression
Immature MoDC were unstimulated or stimulated with either different concentrations of PPD or 0.1µg/ml LPS as positive control. After 1h, 2h, 4h, 8h or 24h TNF-α concentration were determined in cell culture supernatants with Cytometric Bead Array (CBA). The assay sensitivity for TNF-α is 3.7pg/ml.

LPS successfully function as positive control because a significantly enhanced TNF-α secretion could be measured in all 4 donors. Different PPD concentrations induced in donor a, b and c an increased TNF-α secretion for a short time. 50µM PPD induced in donor b an extremely high TNF-α distribution already after 1h, 2h 4h and 8h of incubation, whereas 10µM PPD strongly raised the TNF-α secretion only after 24h. The secreted

TNF-α values of donor b are significantly higher after treatment with 50µM PPD than after LPS stimulation for every time point (Figure 16).

3.4.3. IL-6 expression

IL-6 displays multiple biologic activities including in association usually with IL-1ß, co-stimulation of T lymphocyte activation. The primary sources of IL-6 in allergen activated draining lymphnodes are dendritic cells (Hope et al. 1994; 1995). In vitro testings, Verheyen et al. 2005 could detect enhanced IL-6 mRNA expression in CD34+ derived DC after stimulation with contact allergens. LPS induces in MoDC after 3-4h of incubation strong IL-6 secretion (Langenkamp et al. 2000).

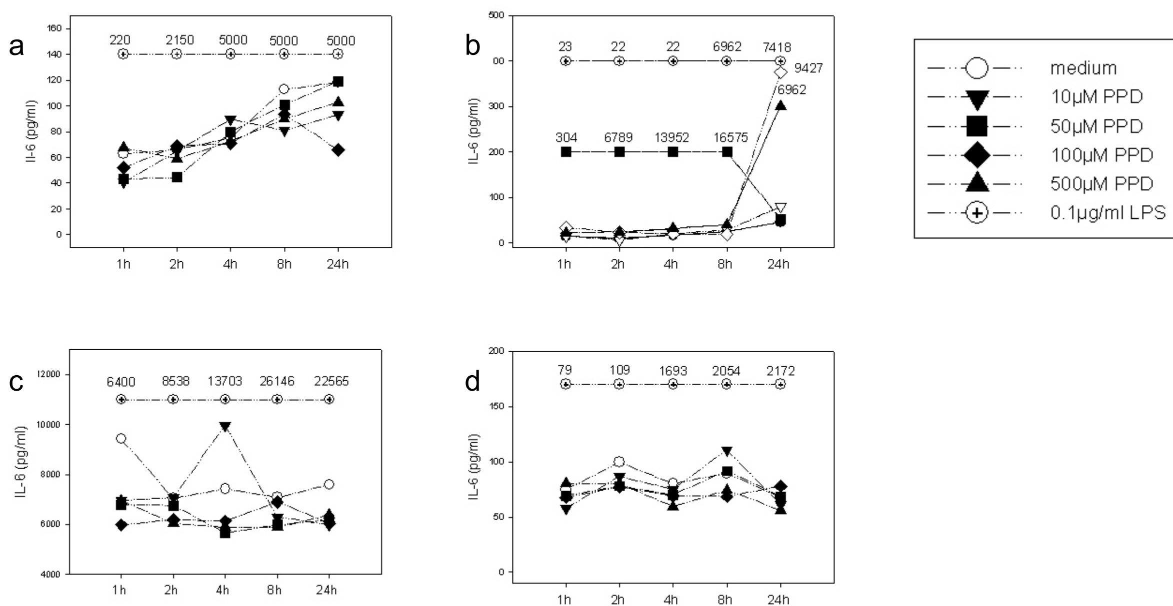

Figure 17: IL-6 expression
Immature MoDC were unstimulated or stimulated with either different concentrations of PPD or 0.1µg/ml LPS as positive control. After 1h, 2h, 4h, 8h or 24h, IL-6 concentrations were determined in cell culture supernatants with Cytometric Bead Array (CBA). The assay sensitivity for IL-6 is 2.5 pg/ml.

The positive control LPS induced in all 4 donors a clearly enhanced IL-6 secretion. In contrast, PPD induced only in donor b significant IL-6 secretion, but 50µM PPD especially induce immediately high IL-6 production (Figure 16).

3.4.3 Interleukin (IL-8) expression of MoDC

IL-8 is a chemokine and known to be expressed in dendritic cells immediately after encounter with any inflammation stimuli. Maturing DC´s have been shown to produce large amounts of inflammatory chemokines like IL-8 after exposure to LPS (Sallusto et al, 1999; Nagerson et al. 2004). The earliest sign of LPS activation in MoDC is the enhanced production of IL-8; it is already detectable after 2h of incubation (Hellmann P, 2007). Toebak et al. 2006 observed an increased expression of IL-8 protein in MoDC exposed to sensitizers and suggested that IL-8 might be involved in the induction and elicitation phase of allergic contact dermatitis. Toebak´s observation suggests an enquiry into wether or not PPD also induces IL-8 expression in dendritic cells.

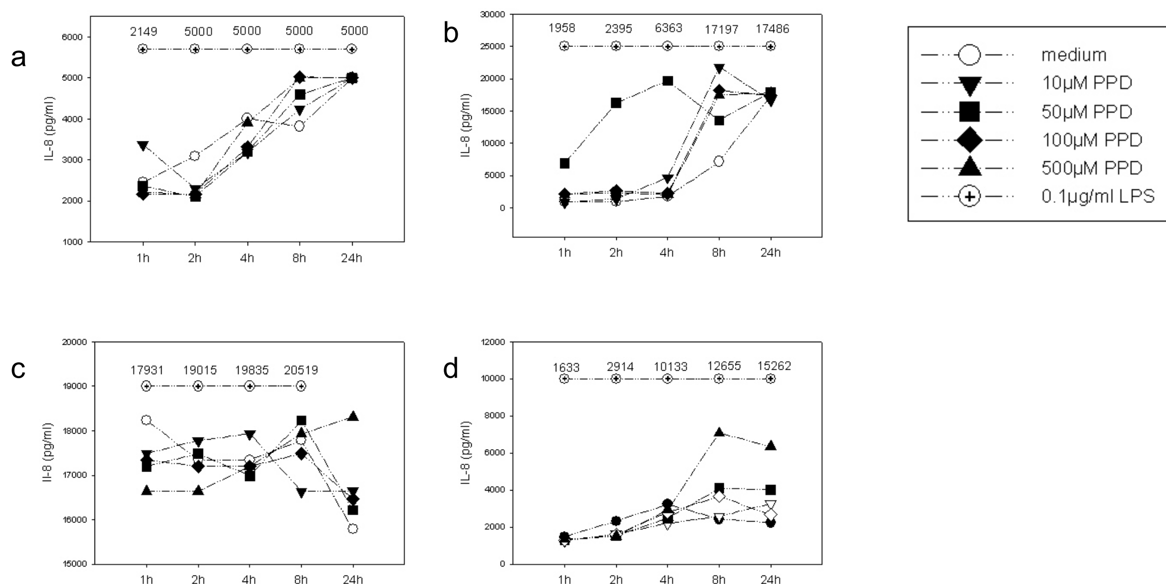

Figure 18: IL-8 expression
Immature MoDC were unstimulated or stimulated with either different concentrations of PPD or 0.1µg/ml LPS as positive control. After 1h, 2h, 4h, 8h or 24h, IL-8 concentrations were determined in cell culture supenatants with Cytometric Bead Array (CBA). The assay sensitivity for IL-8 is 3.6 pg/ml.

After 8h of incubation, LPS induced in 3 of 4 subjects increased IL-8 secretion and in all subjects after 24h. PPD enhanced in all donors the basal IL-8 production. The IL-8 secretion follows donor dependently after different points in time and through different PPD concentrations. Again donor b distinguished himself from other donors in his remarkable IL-8 production (Figure 18).

3.4.4 IL-12P70 expression of MoDC

Dendritic cells, matured through infectious agents or inflammatory products, produce high amounts of IL-12P70 and turn CD4 T Helper cells into IFN-γ producing Th1 cells (Banchereau and Steinman 1998). Langenkamp et al 2000 could demonstrate that LPS induces temporary a strong IL-12P70 secretion in MoDC after 8h of incubation. We also detected enhanced IL-12P70 secretion after LPS stimulation in all donors. The capacity to produce IL-12P70 in response to LPS is highly donor dependent, because values considerably varied between 32pg/ml and 1398pg/ml. 2 of 4 donors PPD notably increase the IL-12P70 secretion in MoDC. Donor b showed an exceptionally high IL-12p70 secretion following stimulation with 50µM or 10µM PPD after 8h or 24h (Figure 19).

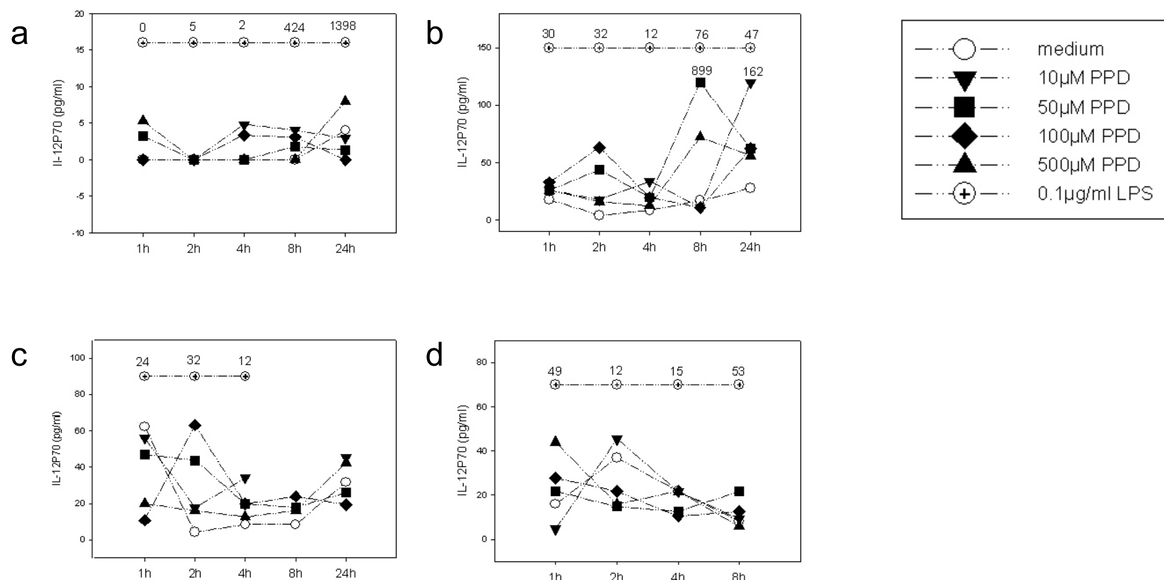

Figure 19: IL-12P70 expression
Immature MoDC were unstimulated or stimulated with either different concentrations of PPD or 0.1µg/ml LPS as positive control. After 1h, 2h, 4h, 8h or 24h, IL-8 concentrations were determined in cell culture supenatants with Cytometric Bead Array (CBA). The assay sensitivity for IL-12P70 is 1.9 pg/ml.

3.4.5 IL-10 expression of MoDC

IL-10 is a cytokine which was originally described as a Th 2 cell product that inhibited Th1 cell cytokine production (Fiorentino et al. 1989; Cumberbatch et al. 2005). IL-10 has been shown to be involved in modulating cutaneous immune responses, including contact hypersensitivity (Ferguson et al.1994; Wang B et al. 1999).

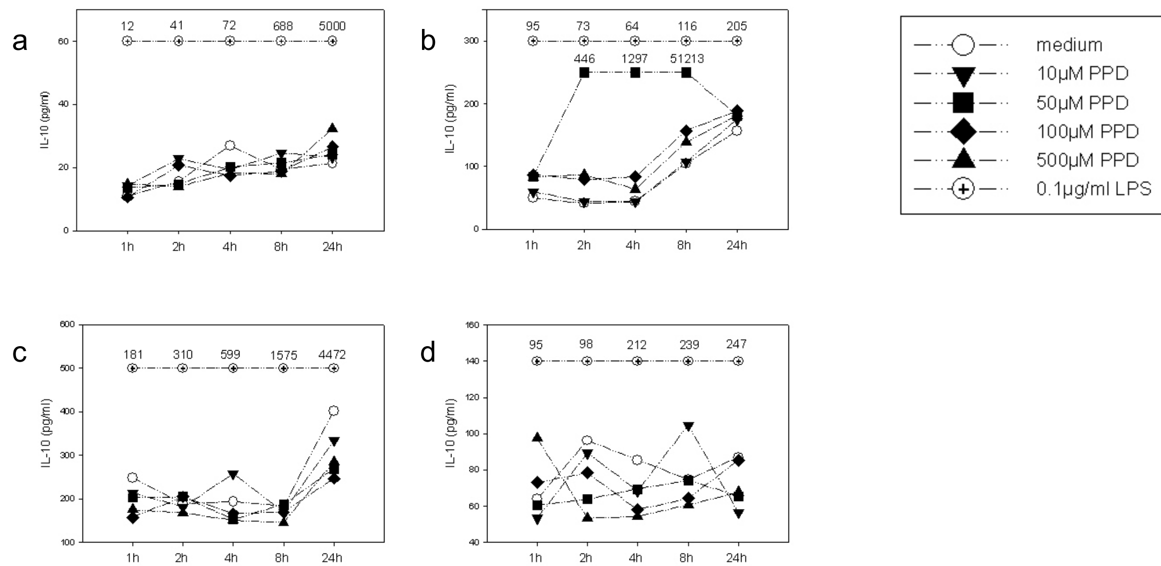

Figure 20: IL-10 expression
Immature MoDC were unstimulated or stimulated with either different concentrations of PPD or 0.1µg/ml LPS as positive control. After 1h, 2h, 4h, 8h or 24h IL-10 concentrations were determined in cell culture supernatants with CytometricBead Array (CBA). The assay sensitivity for IL-10 is 3.3 pg/ml.

LPS induced in MoDC a strong IL-10 secretion after 6h (Langenkamp et al. 2000). LPS induced in all 4 subjects an enhanced IL-10 secretion and for PPD only 2 of 4 subjects show an increased IL-10 secretion (Figure 20).

3.4.6 Summarization of effects of PPD on cytokine expression profile from MoDC of 4 different donors

This frequency table was generated to acquire the point of time when each cytokine is predominantly expressed. The length of the columns corresponds to the number of donor which responds to any PPD concentration. We only validate measured cytokines if following prerequisitions were given.

Mean for medium has to exceed the doubled sensitivity limit of each cytokine.

Mean for positive control LPS has to double the mean of medium.

Mean for LPS has to be valid for the measured point in time, as referred by the literature.

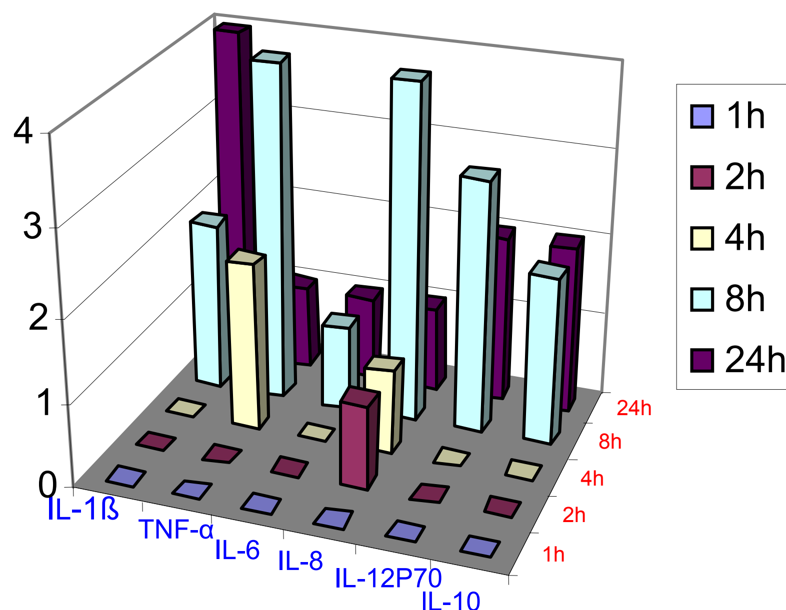

Figure 21: Concentration independent summarization of the impact of PPD on cytokine expression profile of MoDC from 4 different donors.
The frequency table summarizes the effects of PPD on the 4 different donors. Length of columns corresponds to the number of donors who respond to any PPD concentration with cytokine production. IL-1ß was produced by each donor after 24h and by two donors after 8h. TNF-α was secreted after 4h from 2 donors, after 8h from 4 donors and after 24h from one donor. IL-6 was produced after 8h and 24h from one donor namely donor b. IL-8 was produced from each donor after 8h and additional from donor b after 2h, 4h, and 24h. IL12P70 was secreted from 3 donors after 8h and from 2 donors after 24h. IL-10 was produced from 2 donors after 8h and 24h.

With these severe guidelines, we had to delete many measured values for PPD, especially those which were expressed at early points in time. However, with the frequency table it can be summarized that the cytokines IL-1ß, TNF-α, IL-8 were most dominantly expressed, followed by IL-12P70 and IL-10. IL-6 was expressed only from one donor (Figure 21).

3.4.7 Quantitative expression of different cytokines in response to PPD

Table 2 gives an overview about the individual maximal cytokine expression rate for each donor. In comparison to the positive control LPS, donor a, c and d expressed rather small amounts of each cytokine in response to PPD, values were all in pg area, and none of these donors could secrete detectable amounts of IL-6. In contrast, donor b expressed high amounts of each cytokine including IL-6 and values were all remarkably higher than values gained by LPS (Figure 22).

	TNF-α	IL-1ß	IL-12P70	IL-10	IL-8	IL-6
donor a	14 pg/ml	18 pg/ml	8pg	11pg/ml	1,2 ng/ml	n.d.
donor c	74 pg/ml	16 pg/ml		30 pg/ml	0,4 ng/ml	n.d.
donor d	39 pg/ml	38 pg/ml	21,7 pg/ml		4,6 ng/ml	n.d.
donor b	*8,9 ng/ml*	*66 pg/ml*	*870 pg/ml*	*5 ng/ml*	*18 ng/ml*	*20 ng/ml*

Figure 22: Time independent summarization of the impact of PPD on cytokine expression profile of MoDC from 4 different donors.
This table represents the quantitative and qualitative cytokine responses of each donor upon PPD stimulation. For each donor the highest expressed value is registered time independentely.

3.5 Impact of PPD on LPS treated MoDC

Because allergic contact dermatitis to PPD is often accompanied with an infection, we investigated the impact of PPD on LPS treated MoDC.

3.5.1 Viability analysis with propidium iodide

To avoid unspecific results from toxic effects of LPS and PPD, we first measured the toxic effects of 1µg/ml LPS and 1µg/ml LPS combined with (0.1mM; 0.2mM; 0.5mM and 1mM) PPD on MoDC. As described in material and methods the viability was determined with propidium iodide staining. When MoDC were treated with 1µg/ml LPS, cell viability fell to 1% and addition of PPD further decreased the viability for 0.1mM to 2%, for 0.2mM to 4%, for 0.5 mM to 7.4% and for 1mM to 9.7% after 24h of incubation. After 96h, LPS decreased the viability to 2%. Addition of 0.1 and 0.2mM PPD did not reinforce LPS cytotoxity any more, while 0.5mM decreased the viability to 3.6% and 1mM to 7.2% (Figure 23).

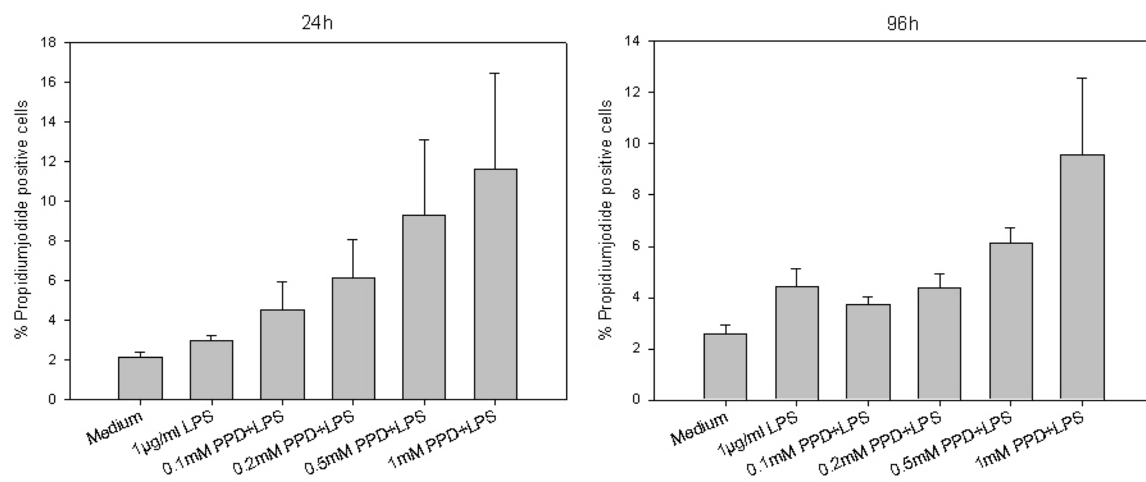

Figure 23: Cell viability determined with propidium iodide staining by flow cytometry. MoDC (1x106 cells/ml) were cultured on day 6 in culture medium alone (control), with LPS, or with LPS in the presence of increasing concentrations of PPD for 24h or 96h. Cell viability was determined with propidium iodide staining. Results are given as mean MFI of five independent donors, error bars indicate SEM.

3.5.2 Influence of PPD on LPS induced surface marker expression

To test the influence of PPD on LPS matured dendritic cells, we co-stimulated MoDC with LPS and different concentrations of PPD. In detail, we measured if 0.1mM, 0.2mM, 0.5mM or 1mM PPD modifies the CD80, CD86 and HLA-DR expression of LPS stimulated MoDC after 24h.

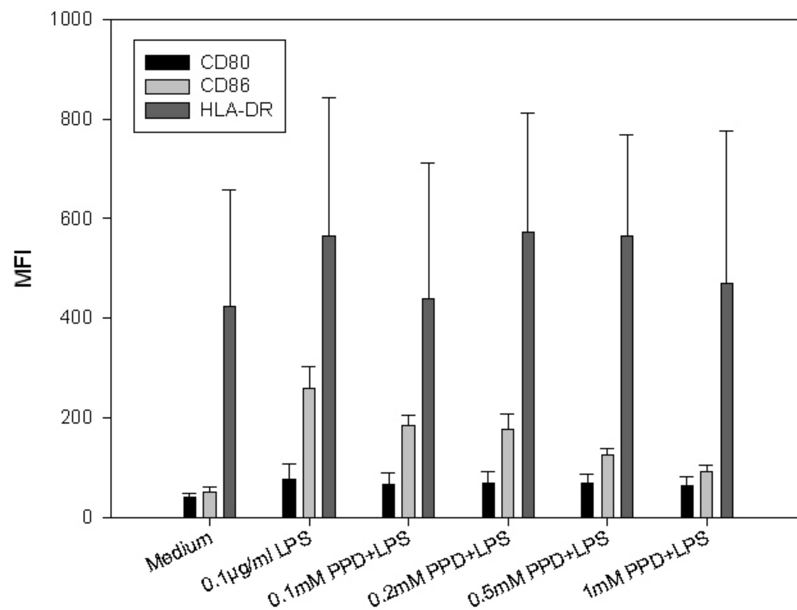

Figure 24: Paraphenylenediamine inhibit LPS induced CD86 expression after 24h of incubation.
MoDC ($1x10^6$ cells/ml) were cultured on day 6 in culture medium alone (control), with LPS, or with LPS in the presence of increasing concentrations of PPD for 24h. Surface expression of CD80, CD86 and HLA-DR was measured using flow cytometry. Results are given as mean MFI of three independent donors, error bars indicate SEM.

As shown in Figure 24, pretreatment with increasing concentrations of PPD marginally decreased the LPS induced upregulation of CD86, while CD80 or HLA-DR expression were not modified. In detail, LPS increased the MFI of CD86 significantly compared to medium, MFI varied between 347 and 214 and 0.1µM PPD decreased the MFI between 226 to 158, 0.2µM PPD pretreated cells gained MFI values between 137 to 236, 0,5µM PPD between 109 and 151 and 1mM PPD between 80 to 160.

3.5.3 Late effects of PPD on LPS treated MoDC

In regard to LPS, which is known to exert its effects on MoDC at a later time point, we also measured the impact of different concentrations of the LPS induced maturation after 96h.

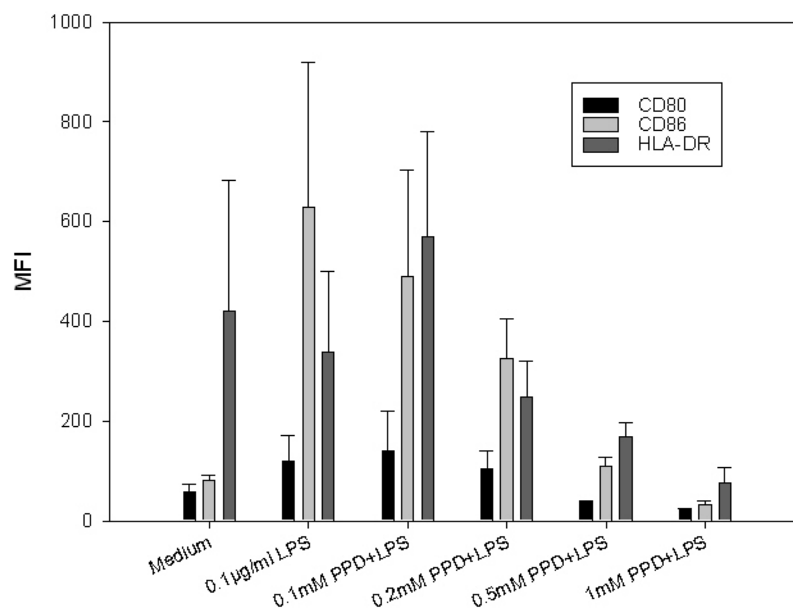

Figure 25: Paraphenylenediamine inhibits LPS induced CD86 expression after 96h of incubation.
MoDC ($1x10^6$ cells/ml) were cultured on day 6 in culture medium alone (control), with LPS, or with LPS in the presence of increasing concentrations of PPD for 96h. Surface expressions of CD80, CD86 and HLA-DR were measured using flow cytometry. Results are given as mean MFI of three independent donors, error bars indicate SEM.

In contrast to the short incubation duration, we found that PPD not only inhibited the CD86 expression but also the expression of CD80 and HLA-DR. Furthermore the PPD induced downregulation of CD86 was considerably more effective after 96h. Accurately, the mean MFI for CD86 (3 donors) induced by LPS was 629. Addition of 0.1µM PPD decreased the CD86 expression to 22%, 0.2µM PPD to 48%, 0.5µM PPD to 83% and 1mM PPD to 95%. The MFI for CD80 induced by LPS varied between 67 and 222. In contrast to 0.1µM PPD which showed no inhibitory effect on LPS induced CD80 expression, the addition of 0.2µM PPD reduced the CD80 expression to 13%, 0.5µM to 77% and 1mM PPD to 81%. In contrast to higher PPD concentrations, 0.1µM PPD clearly enhanced the LPS induced HLA-DR expression. The mean MFI for HLA-DR of LPS stimulated MoDC ranked

between 171 and 662, whereas 0.1µM PPD enhanced the mean value by about 40%, 0.2µM PPD decreased the mean value by 26%, 0.5µM PPD by 50% and 1mM by 77%. (Figure 25)

3.5.4 Impact of PPD on the LPS induced TNF-α secretion

As elucidated above, TNF-α is one of the key cytokines of dendritic cells which induce migration, maturation, communication with other cells and apoptosis. We therefore also analyzed the potential of PPD to modulate the LPS induced TNF-α secretion in MoDC. We again stimulated MoDC either with 1µg/ml LPS combined with 0.1mM, 0.2mM and 0.5mM PPD or with 0.1mM, 0.2mM or 0.5mM PPD alone, again LPS was used as positive control. We measured the TNF-α secretion with CBA and ELISA after 24h and 96h. (Figure 26, 27)

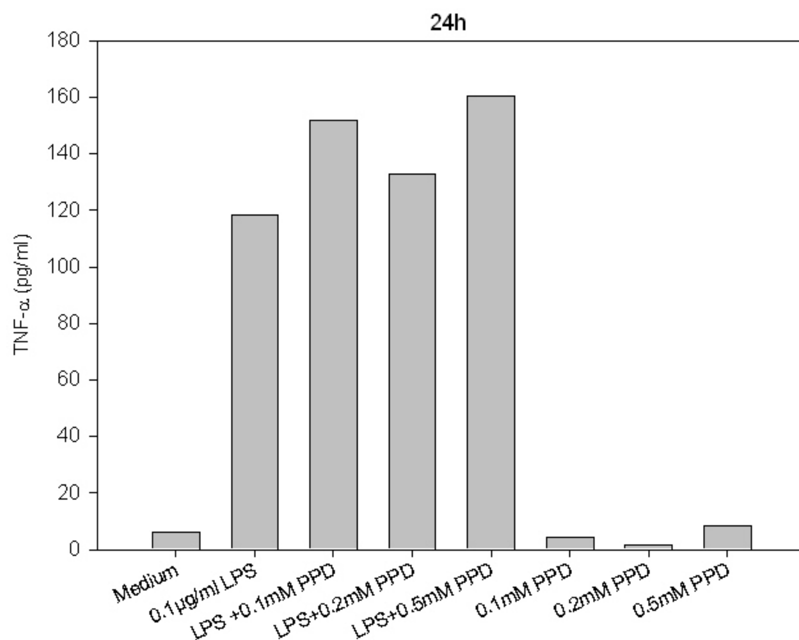

Figure 26: Impact of PPD on LPS induced TNF-α production after 24h of incubation, determined by flow cytometry with cytometric beat array (CBA).
Immature MoDC were cultured with LPS (0.1µg/ml, positive control) or either with increasing concentrations of PPD (0.1µM, 0.2µM, 0.5µM) or the same concentration of PPD combined with LPS for 24h. Supernatants were analyzed for TNF-α determination by CBA. The figure shows one representative example out of 2 different LPS responders.

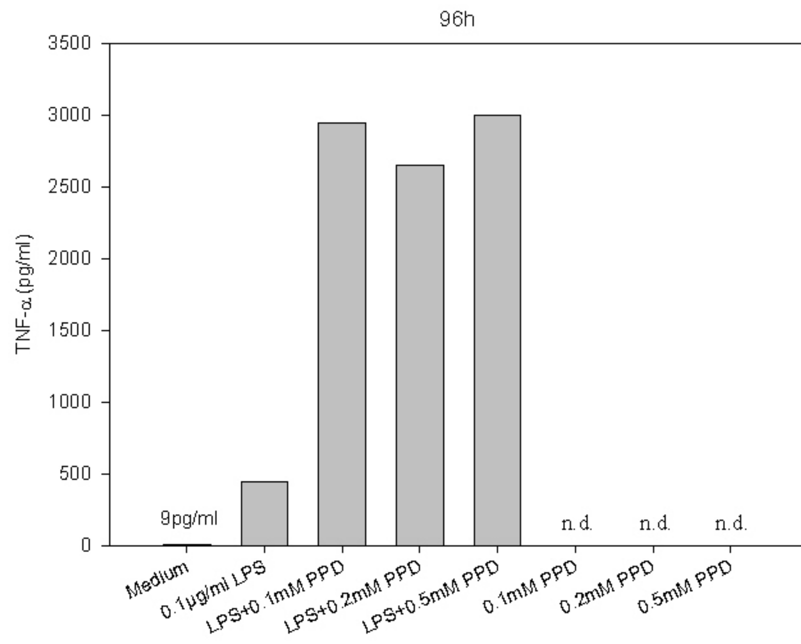

Figure 27: PPD enhances the LPS-induced TNF-α production.
Immature MoDC were cultured with LPS (0.1µg/ml, positive control) or either with increasing concentrations of PPD (0.1µM, 0.2µM, 0.5µM) or the same concentration of PPD combined with LPS for 96h. Supernatants were analyzed for TNF-α determination by ELISA. The figure shows one representative example out of 3 different LPS responders.

Figures 26 and 27 show that PPD alone fails to induce TNF- α production in MoDC; TNF-α concentrations in supernatants of PPD treated MoDC are even lower than control samples. Surprisingly, PPD in combination with LPS significantly enhances the TNF- α production. This effect could be shown with ELISA as well as with CBA testings, as summarized: 5 out of 6 LPS responders show this effect.

3.5.5 Effects of PPD on native apoptosis rate in MoDC

It is commonly established that LPS induces apoptosis in MoDC via ROS formation (Chiang E, et al 2006). An investigation into wether or not PPD alone induces apoptosis in MoDC because until now, this has not been determined. High concentrations of $NiSO_4$ are known to initiate apoptosis in MoDC, and that would suggest that 4mM $NiSO_4$ might be used as a positive control.

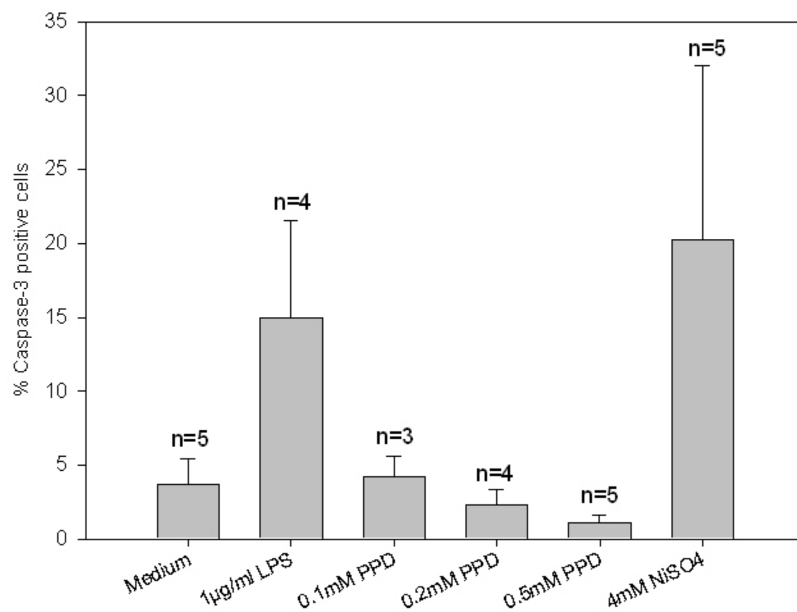

Figure 28: Impact of Paraphenylenediamine (PPD) on caspase-3 induction in MoDC.
MoDC ($1x10^6$ cells/ml) were incubated in culture medium alone (negative control), LPS (positive control), $NiSO_4$ (positive control) or increasing concentrations of PPD for 24h-96h. After incubation, cells were intracellularly stained with biotinylated anti- caspase-3 by flow cytometry.

As demonstrated in Figure 28, PPD alone reduces the native apoptosis rate in MoDC whereas 1µg/ml LPS and 4mM NiSO4 significantly induce apoptosis.

3.5.6 Effects of PPD on LPS induced apoptosis in MoDC

To ascertain the impact of PPD to modify the LPS-induced caspase-3 formation, we co-stimulated MoDC with LPS and 0.1mM, 0.2mM and 0.5mM PPD. For positive control, we again used NiSO4 and LPS.

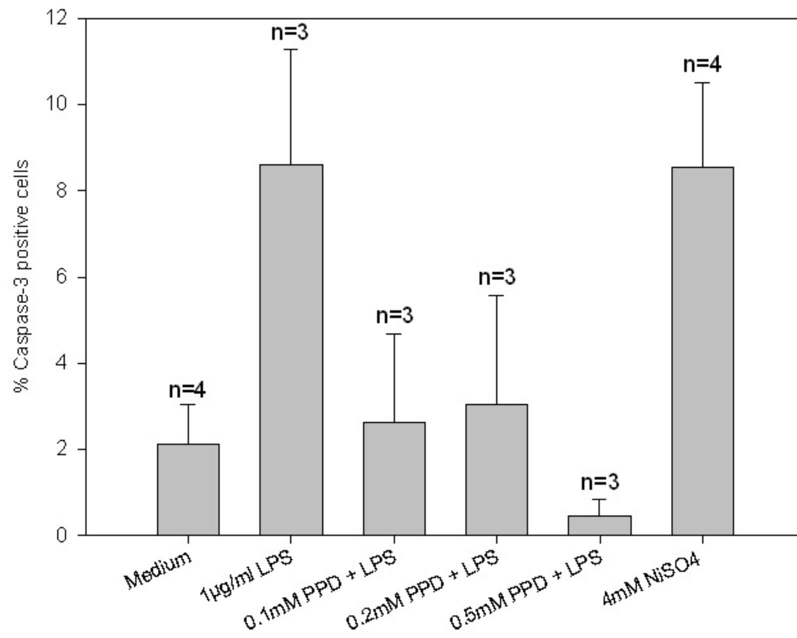

Figure 29: Impact of Paraphenylenediamine (PPD) on LPS mediated caspase-3 induction. MoDC (1x10^6 cells/ml) were incubated in culture medium alone (negative control), LPS (positive control), $NiSO_4$ (positive control) or LPS in the presence of increasing concentrations of PPD for 24h-96h. After incubation, cells were intracellularly stained with biotinylated anti- caspase-3 by flow cytometry.

As expected, PPD clearly decreased the LPS inducted apoptosis rate in MoDC. Strong standard deviations show donor-dependent susceptibility against pro-apoptotic substances. (Figure 29)

4 Discussion

4.1 PPD modification and classification

The aim of the current study was to investigate wether or not PPD is able to stimulate an adaptive immune response by activating naïve T cells. For sensitizing, PPD has to activate professional antigen presenting cells in order to induce stimulation of naive T cells. For establishment of an appropriate in vitro test system, one of the main questions is the assortment of antigen. Until now it is not clear how PPD does sensitize, meaning that the immunogens in PPD reactions are unknown. It is well accepted that PPD is a non-protein reactive sensitizer and has to modify into an electrophil in order to bind to intracellular proteins and evolve sensitizing potential. PPD can be classified as prohapten and also as prehapten, because it can be converted metabolically and abiotically into protein reactive derivates.

4.1.1 Metabolic conversion of PPD in MoDC.

a) Oxidation

4.1.2. Modification of PPD in MoDC

Enzymatic oxidation of PPD attracts much attention because of the N-oxidation leading to the formation of an N-hydroxylarylamin which is regarded as a critical step in the conversion of the parent arylamine to proximate carcinogenic metabolite that ultimately leads to the development of bladder cancer. Recently, we demonstrated the expression of cytochrome P450 (Cyps) gene families 1A1, 1A2, 1B1, 2A6, 2B6, 2C6, 2C19, 2D6, and 3A15 in monocyte-derived DCs (Sieben et al. 1999) and in a former mRNA study we found that MoDC enhance the mRNA synthesis of P450 A1 and 1B1 after stimulation with PPD (Hamidi et al. in preparation) but until now we have not investigated the biologic function of these Cyps and their potential oxidation of PPD.

b) Acetylation

To gain further understanding of potential metabolism of PPD within this study we investigated the N-acetylation capacities of MoDC. We could show that MoDC express metabolically active N-acetyltransferase and that PPD was donor-dependent more or less mono- and diacetylated 24h after PPD exposure.

4.1.2 Abiotic conversion

Oxidation by athmospheric oxygen

PPD is an unstable chemical and it is well established that PPD auto-oxidizes by exposure to light into benzochinondiimin (BQ) which is converted, in addition with PPD, into the trimer Bandrowski Base (BB). Among others, (Coulter et al. 2006) measured the auto-oxidation products of PPD in medium using on-line radiometric HPLC and mass spectronomy, and could thereby demonstrate that PPD is not only converted into BB but also into one other unidentified product.

Taken together, until now it could be demonstrated that PPD is converted in MoDC per enzymatic activation into MAPPD, DAPPD and per abiotic conversion into BB, BQ and into unidentified products. To select which of these antigens are suitable for our in vitro testing, we studied the literature as follows. For MAPPD and DAPPD, (Aeby et al in preparation) shows that both acetylated derivates of PPD are not able to activate MoDC. In previous studies it was proposed that BB was the main allergen in patients reacting to PPD (Krasteva et al. 1993). These observations could be disproved by actual findings from others (White et al. 2006) who found that only a few PPD positive patch-test subjects also reacted positive to BB. Furthermore, (Coulter et al. 2006) demonstrate in an in vitro study that BB does not cause significant functional maturation of MoDC. Both results strongly indicate that BB cannot be an important haptenic species involved in the development of sensitization to the prohapten PPD. (Dupuis and Benezra 1982) promoted the concept that PPD become reactive via oxidative metabolism to BQ but analogically to BB newer data substantiate that only the minority of PPD allergic subjects react to BQ (White et al. 2006). In contrast to BQ and BB, most of PPD allergic subjects respond to PPD. Thus, we only used PPD as single antigen in our in vitro test system. We preferred to conduct our experiments with in vitro generated human monocyte derived dendritic cells, because previous in vivo investigations with animals like guinea pigs or mice have been shown to be conferrable on the human system only to a limited degree (White et al. 2006).

Taking into account that PPD must not only provide the chemical signal but also have to activate and thereby convert dendritic cells from functionally immature antigen recognition cells into mature and potent antigen presenting cells, we investigated the impact of PPD on the functional maturation of in vitro generated MoDC.

4.2 Impact of PPD on the functional maturation of MoDC

Up to now the impact of PPD on MoDC was only fragmentarily regarded. Most often it was tested in screening investigations in which only one PPD concentration was applied or one marker was measured (Aeby et al. 2004) or only one time point was analyzed (Hulette et al. 2005). In contrast we measured the influence of PPD on the functional maturation extensively and therefore used different PPD concentrations to analyze changes of co-stimulation, DC-TC interactions and cytokine expression at different time points.

4.2.1 Impact on co-stimulatory molecules

Co-stimulatory interactions are essential for T cells to undergo complete activation, and they occur when the constitutively expressed T cell costimulatory ligand CD28 binds the co-stimulatory molecules on APCs, CD80 (B.7.1.), or CD86 (B.7.2). In the absence of co-stimulatory molecules, T cells enter a state of anergy or apoptosis; hence, CD28 activation is essential for T cell activation (Jonuleit and Schmitt 2003). However, in addition to the interaction of CD80 and CD86 molecules with CD28, CTLA-4 which is upregulated after T cell activation, reaches its maximal cell surface expression after 2-3 days after stimulation, and acts thereby as inhibitory ligand. CTLA-4 has a stronger affinity for both CD80 and CD86 compared to CD28 and is responsible for downregulation of TCR signaling. CD80 and CD86 are primary expressed on APCs with different kinetics. CD86 is constitutively expressed on resting APCs, albeit at low levels, while CD80 is not detectable. When studied individually, rather than on the same APCs, CD80 is a more potent co-stimulator of T cell activation compared with CD86 (Thomas et al. 2007).
Investigations using organ-cultured mice skin demonstrated that contact allergens induce CD80 and CD86 mRNA and surface marker expression (Katayama et al. 1997; Matsunaga et al. 1998). Furthermore, in vivo experiments with mice analyzing the function of these molecules during contact sensitivity reactions induced by the haptens showed that CD86 but not CD80 is involved in the hapten induced contact sensitivity (Reiser H 1996). Others reported about distinct roles for CD80 and CD86 to induce epicutanous antigen or hapten induced allergic contact hypersensitivity (CHS) responses (Rauschmayr-Kopp et al. 1998). Although it is well known that PPD induces positive reactions in animal-skin sensitization models and also that it causes allergic contact dermatitis in humans, it is up to now not clear if PPD induces the upregulation of co- stimulatory molecules CD80 or CD86. In vitro investigations with human MoDC show that contact allergens predominantly augment the expression of CD86 but not CD80 (Aiba et al. 1997), and moreover CD86 is generally considered a suitable marker for developing an in vitro test system for contact sensitizers

(Aiba et al. 1997; Pichowski et al. 2000; Pichowski et al. 2001; Tuschl and Kovac 2001). In contrast, by flow cytometric protein analysis we could reveal that PPD was neither able to induce the expression of CD80 nor CD86 in MoDC using a wide range of concentrations (10µM-1mM) and two time points, whereas clear differences in surface-marker expression were consistently found when using LPS and DNCB, respectively. By comparing our results with data from the literature, we found no data about the impact of PPD on CD80 expression. CD86 was measured of different groups, and the results reported are controversial. In detail (Aeby et al. 2004) demonstrated, using pooled immature DCs from different donors, that 0.048-2.5mM PPD enhance the expression of CD86 in a concentration dependent manner. (Hulette et al. 2005) also detected enhanced CD86 expression in MoDC but only with concentrations above 2.5mM PPD and speculated that this effect is due to the concomitant cytotoxity as a consequence of a danger signal first described by (Matzinger 1994; Matzinger 2002). In contrast, (Coulter et al. 2006) could confirm our data and could not detect any CD86 upregulation in response to PPD using MoDC. As a result of these data we can not approve that CD86 is a suitable marker for developing an in vitro test system for contact sensitizers. Assumed that PPD is the immunogenic compound in the PPD allergy, the co-stimulation has to be provided by other proteins. In this context (Coulter et al. 2006) reported that PPD may be able to initiate CD40 expression in MoDC and induces T-cell proliferation in allogenic mixed lymphocyte reaction. They predicted that CD40 mediated signaling is related to susceptibility to PPD sensitization because CD40 receptor ligand binding is known to stimulate an important signaling pathway involving NF-κB that results ultimately in further dendritic cell activation (Quezada et al. 2004). We could not confirm CD40 upregulation in every donor because we found only in rare cases (2 of 11) enhanced CD40 expression using a comparable in vitro system. It is generally premature to assume that expression of any co-stimulatory molecule allows sensitization without any additional data. To conclude, CD40 may be responsible for sensitization; to verify this, further investigations for instance T-cell sensitization assay or CD40 knock out mice or application of CD40 antibodies are clearly necessary.

4.2.2 Impact of PPD on dendritic cell / T-cell interactions

However, not only the co-stimulation but also the maturation status and the T-cell interaction capacity determine wether or not PPD activated MoDC are able to prime T cells. We therefore measured the impact of PPD on phenotypical changes which enable the antigen presentation, migration, and T cell adhesion.

4.2.2.1 Influence of PPD on HLA-DR expression

Human leucocyte antigen (HLA)-DR is a class of the major histocompatibility complex (MHC) and among HLA-DQ, HLA-DP encoded by the HLA complex. Small numbers of HLA-DR, HLA-DQ and HLA-DP molecules are constitutively expressed by antigen presenting cells like dendritic cells, macrophages, or B-cells (Banchereau and Steinman 1998). During the processing phase foreign antigens, e.g., proteins from pathogens or modified self-proteins resulting from chemicals, were digested, loaded onto the surface of these molecules, and transported to the cell surface. Consequently, upregulation of these molecules is a hallmark of relevant exposure. The primary function of MHC is presentation of antigens to T-cells for the purpose of eliciting or suppressing T-(helper) cell responses. Investigations using mice demonstrated that, 24h after application of haptens, a significant percentage of Langerhans cells (LC) in the epidermis increase surface expression of these molecules. These LC, immediately after harvesting, exhibit enhanced syngeneic and allogeneic T-cell stimulatory function when compared to control LC (Aiba and Katz 1990).
In humans, presentation of antigens from pathogens is mainly performed by HLA-DR. Contact allergens such as nickel or trinitrophenol are predominantely restricted to HLA-DR or HLA-DQ (Sinigaglia et al. 1985; Vollmer et al. 1999), and previous findings from our group could demonstrate that both HLA-DR and HLA-DP are involved in the stimulation of PPD specific T-cell clones recovered from allergic subjects (Sieben et al. 2002). These previous findings at least initiated us to ascertain if PPD has nonetheless the potential to activate professional dendritic cells by inducing the expression of HLA-DR.
For PPD, we can demonstrate in this study that low concentrations of PPD are not able to enhance the basal HLA-DR expression in MoDC. PPD is obviously different from other chemical contact allergens. Former in vitro testings using MoDC, others reported that haptens like $NiSO_4$, DNCB, aminophenol, and chlorpromazine cause significant increase in the surface expression of HLA-DR after 24h and 48h (Coutant et al. 1999). For PPD we found only in rare cases and only after 24h an enhanced HLA-DR expression for higher concentrations as 0.1mM, 0.2mM, 0.5mM and 1mM which is demonstrated in Figure 8 by the high standard error. (Coulter et al. 2006) detected in MoDC an enhanced HLA-DR expression in response to 10µM PPD after 16h. Apart from the shorter incubation durations, it is difficult to compare these with our own data because we miss any instructions about the underlying significance of their observed HLA-DR expression. Independently, other results from this study are in line with our previous findings although be studied the elicitation phase. One remarkable finding was that recognition of PPD by memory T-cell clones does not require processing, which originally has been considered as a prerequisite to stimulate T cells. Considering these observations and publications on mechanisms of drug

hypersensitivity reactions (Pichler 2005; Pichler et al. 2006), we postulated that PPD is capable of binding extracellulary to a single molecule or to different molecules that function as classical antigen-presenting molecules and interact with the T-cell receptor (TCR) of helper T-cells. Furthermore, although clearly not shown for the sensitization phase of allergic contact dermatitis, recent studies support the possibility that haptens do not enter cells but bind non- or covalently to MHC molecules present on antigen presenting cells.

4.2.2.2 Impact of PPD on migration and adhesion of MoDC

CCR7 is actually a chemokine receptor but is also known as a maturation marker, because if dendritic cells are induced to mature, they change their chemokine receptor expression profile: The expression of inflammatory receptors is gradually lost while expression of a single chemokine receptor, CCR7, is rapidly induced. Expression of CCR7 is particulary important, as CCR7 deficient mice show an impaired migration of activated LC into draining lymph nodes after skin painting with FITC and consequently lack any contact hypersensitivity (Forster et al. 1999). In humans, in vitro generated MoDC express CCR7 mRNA. Using CD34+ derived dendritic cells, (Boisleve et al. 2004) could show in vitro that both $NiSO_4$ and DNCB are able to induce CCR7 in combination with expression of the maturation marker CD83. The impact of PPD on CCR7 expression has to be reviewed in detail. Although we could not detect any CD83 upregulation, we did measure CCR7 expression in PPD stimulated MoDC and found that PPD significantly induces the expression of CCR7 after just 24h. In contrast to PPD, we found that LPS stimulated MoDC only express CCR7 after 96h. Early CCR7 upregulations have also been documented for other contact sensitizers like DNCB, $NiSO_4$, or ascaridol. Some investigators measured the expression of CCR7 mRNA after 7h, and others the protein expression after 24h. The observed late CCR7 surface marker expression after LPS stimulation was also confirmed by another group (Macagno et al. 2006) while they detected other maturation markers after 20h, CCR7 was primally analyzed after 44h.

Different expression times may be due to different pathways inducing CCR7 expression. However, the fact that low doses of PPD such as 1µM induce strong CCR7 expression indicates that the upregulation is not due to any unspecific danger signal resulting from cytotoxicity of PPD, but rather through a substance specific effect of PPD. A prerequisite for CCR7 negotiated migration is the presence of the corresponding chemokine gradient with CCL19 or CCL21 expressed by mature dendritic cells or endothelial cells. The next step concerning PPD mediated CCR7 induction is to prove the bioactivity of CCR7 by establishing in vitro migration assays using corresponding chemokines. Another condition for successful migration but also for a proper DC-T-cell binding is the reorganization of adhesion molecules.

In this context, we additionally measured if PPD also influences the expression of DC-SIGN and CD11c.

DC-SIGN plays an important role in establishing the initial contact between DC and resting T lymphocytes through recognition of ICAM-3, and it also mediates DC trafficking through interactions with endothelial ICAM-2 (Geijtenbeek et al. 2000). Therefore DC-SIGN appears to be a critical mediator of the migratory and T-cell-interacting capabilities exhibited by MoDC. The impact of contact allergens on DC-SIGN expression in dendritic cells is of some interest, considering its influence on immune responses. Up to now we could not find literature about this, neither in animal models nor in human systems.

In our study we could not detect a significant upregulation for DC-SIGN in every donor, but for one of three tested donors, we could measure enhanced DC-SIGN expression after stimulation with 0.1mM PPD. Without any comparable literature we can only speculate that PPD induces DC-SIGN in rare cases and only for certain concentrations, and this may have a pro-sensitizing impact. Since we measured the DC specific marker DC-SIGN in combination with CD11c which is also a specific marker for dendritic cells, we could exclude the fact, that PPD differentiating MoDC into another cell type. Among other cell types CD11c is expressed by dendritic cells, and as a result of its high expression level, it is considered as a specification marker of these dedicated APCs (Meunier et al. 1994 ; Brocker et al. 1997 kein abstract). Although involvement of CD11c in diverse functions of DC is relatively unexplored, Meunier et al have demonstrated a complete inhibition of allogenic T cell proliferation mediated by cutaneous Langerhans cells in the presence of CD11c specific antibody. This new observation definitely implies a role of CD11c in antigen presentation by DCs. In mice, Sadhu C et al. 2007 found that CD11c binds to ICAM-2 and VCAM-1 and plays a significant role in delayed type hypersensitivity reactions. Up to now, literature about the impact of contact allergens on CD11c expression in humans is lacking. In most papers CD11c was frequently used to define the specific gate adjustment and to analyze at least expression of other dendritic cell markers. This study measured if PPD modulates the CD11c expression and found in one of three blood donors enhanced CD11c. We have to take into account that the donor with enhanced CD11c expression is the same who also shows an enhanced DC-SIGN expression in response to 0.1mM PPD. Taken together, the CD11c/DC-SIGN study demonstrated that certain PPD concentrations may potentially deliver the reorganisation of adhesion molecules in MoDC of some donors. In the future, investigations using a broader spectrum of PPD concentrations are needed to affirm these speculations.

4.2.3 Validation of the PPD mediated functional maturation

Taking all the data, we can predict that PPD is not able to induce CD80 or CD86 expression; only in rare cases can induce HLA-DR, CD11c and DC-SIGN expression but induces CCR7 expression. In contrast to others studying PPD or other contact allergens, this study cannot confirm that CD40 is significantly expressed in response to PPD; instead we measured a significant CCR7 expression using very low concentrations (1µM PPD). With the assumption that a few PPD presenting immature dendritic cells are competent to migrate into 2nd lymph nodes, the question arises: how could they affect an immunological response without any co-stimulation? As reported by Kuhbach 2005 and others, it is well established that, upon presentation of antigen to T cells in the lymphoid organ, the maturation state of DC controls the outcome of the immune response. Antigens taken up by immature DCs in the steady state are presented in a tolerogenic manner. Based on our in vitro results for PPD that would mean that PPD induces tolerance. This hypothesis speaks against results from studies demonstrating data of PPD specific T cell clones from PPD allergic subjects. However, the capacity of PPD to initiate CCR7 expression suggests that PPD induces processing in MoDC and thereby actively influences their cell signaling. Following this hypothesis, effects of PPD on the secretion of different mediators was measured, because the cytokine profiling is also considered being a practical application for the characterization and identification of immunogenic chemicals.

4.2.4 Impact of PPD on cytokine expression of MoDC

The capability of chemicals to induce activation of in vitro generated DC can further be evaluated through determining the production of various cytokines or chemokines for example TNF-α, IL-1ß, IL-6, IL-8, IL-10, and IL-12 (Lore et al. 1998 ; Aiba and Tagami 1999)(Lore et al 1998, Aiba 1999). It is generally agreed that application of different chemicals induces in cells a typical cytokine fingerprint which denotes that, for each chemical releases, a different composition of mediators is secreted at different times. In conjunction with allergic contact dermatitis, some pro-inflammatory cytokines, like TNF-α or IL-1ß, are known to serve as danger signals which in turn may induce further maturation and migration of dendritic cells. IL-6 is not only an important inflammation marker but also has co-stimulatory functions during antigen presentation. IL-8 is an early chemokine which is predominantly important to recruit cells of the innate immune system, and IL-12 and IL-10 are particularly dedicated to direct the mode of an immune response. To get more insights about the effect of PPD on MoDC activation concerning the type of immune response and about the time frame when signaling starts, we analyzed wether or not different PPD concentrations induce the secretion

of the major cytokines IL-1ß, TNF-α, IL-10, IL-12p70, IL-8, and IL-6 after different incubation points in time.

To evaluate the underlying data, we also needed the inset of specific positive controls for each time point, but this could not be realized because it goes beyond the scope of technical application. Because we have reliable data from literature, we take only LPS as the sole positive control which induces all measured cytokine expression in MoDC. The disadvantage is that LPS primally induces some cytokines after later incubation duration. Due to this and in order to have a valid positive control for all cytokines, we probably lost some PPD signals because we could not evaluate all the data obtained.

IL-1ß is the first cytokine known to be rapidly induced after topical application of contact sensitizers onto mouse skin (Enk and Katz 1992) and exclusively produced by LC (Heufler et al. 1992; Matsue et al. 1992). In this instance, this cytokine is involved in LC migration after treatment with chemical allergens (Cumberbatch et al. 1997; Cumberbatch et al. 1999) and absence or neutralization is associated with impaired or inhibited contact sensitization (Enk et al. 1993; Shornick et al. 1996). Unfortunately, treatment of human MoDC with chemical contact allergens results in no or modest increase of IL-1ß (Pichowski et al. 2000). For this study the measuring of IL-1ß has been of especial interest because (Pichowski et al. 2001) could measure that PPD elicits increases in IL-1ß mRNA in responders. On the basis of these data they assumed that PPD itself may have some intrinsic allergenic potential. This study found that different concentrations of PPD at least doubled the amounts of IL-1ß in 3 of 4 donors after 24h. Even a very strong response being at almost a threefold increase was observed in one subject. In contrast with others (Aiba et al. 1997; Pichowski et al. 2001), large donor-to-donor variations or high degree of non-responding subjects (more than 50%) could not be approved in this study. In sum, PPD, like other strong contact allergens, is able to augment the production of IL-1ß.

TNF-α is one of the most influential cytokines when considering DC maturation. In response to high concentrations of TNF-α, dendritc cells increase their surface expression of MHC subtype II, ICAM I, LFA-3, CD40, and B7 by two or threefold, whereas expression of Ii and FcRII decreases (Sallusto and Lanzavecchia 1994). (Kimber and Cumberbatch 1992) showed that in mice, contact sensitization induced TNF-α production by keratinocytes and that this cytokine acts in a paracrine fashion with local LC to provide one signal for their mobilization and migration. (Aiba et al. 1997; Manome et al. 1999) found that human MoDC are able to produce in vitro TNF-α in response to $NiCl_2$ or $MnCl_2$ treatment. (Coutant et al. 1999) confirmed this finding and demonstrated further that TNF-α was enhanced in a dose-dependent manner by several chemicals, namely Dinitrochlorobenzene (DNCB), Aminophenol (4-Ap), Chlorpromazine (Clpz), and Nickelsulfate ($NiSO_4$). An early and brief response (3-4h) was observed for bacterial lipopolysaccharide (LPS) (Langenkamp et al.

2000). High amounts of TNF-α were found after PPD treatment of cells of each donor but a dose dependency was not noted. Quantitatively, results were comparable to those reported by Coutant and co-workers. They showed that the basal production of TNF-α was upgraded between 10pg/ml and 20pg/ml by 4-AP, DNCB, and Clzp treatment for 48h, whereas NiSO4 elicited a stronger release of approximately 80pg/ml.

For three donors (A, C and D in figure 16), PPD increased TNF-α levels between 14 and 74 pg/ml already after 8h. With this study, we can demonstrate for the first time that PPD itself is or early generated intermediates are indeed able to induce TNF-α production. At this point, it should be mentioned that TNF-α levels peaked after 8h and reached control levels after 24h or 96h.

IL-8 is a low weight chemotactic chemokine and responsible for chemotaxis induction, which directs migration of cells to a site of inflammation. Monocytes and macrophages represent the principal cellular source but also other nucleated cells including dendritic cells secrete IL-8 in response to multiple stimuli as lipopolysaccharide or early proinflammatory cytokines. One of the most important annotations is that in rats and mice IL-8 does not exist. These mammals do have chemokines, which fulfill the same biological functions as IL-8, but they do not have IL-8 (Remick DG 2005).

Following exposure of CD34+ -progenitor-derived DC to allergens or irritants, mRNA expression for IL-8 was examined by real-time PC (Verheyen et al. 2005). As documented for other cytokines, significant interindividual variations were observed. Lately, (Toebak et al. 2006) investigated the chemokine secretion by DC after allergen or irritant treatment and they postulate that measurement of chemokine secretion, in particular IL-8 secretion, may provide a novel tool to identify potential contact allergens in vitro. This study confirms this for PPD by finding that cells of each donor were able to enhance IL-8 production after 8h whereas LPS increased basal IL-8 production for all measured time points between 2h and 24h. Overall, levels were comparable to data by (Toebak et al. 2006) for DNCB.

Interleukin-6 (IL-6) production, a multifunctional cytokine that regulates various immune and inflammatory responses (Van Snick 1990 kein abstract), was produced by draining lymph node cells following primary contact sensitization in mice (Hope et al. 1994). Next, it was demonstrated that murine epidermal LC constitutively express IL-6 protein and that the expression can be augmented following application of a skin sensitizing chemical. For humans, (Verheyen et al. 2005) reported that mRNA for IL-6 is among IL-1ß and IL-8 upregulated after exposure of CD34+ progenitor derived DC to allergens. Therefore, we also determined the IL-6 secretion after PPD stimulation using for representative donors. Just one donor reacted with an aggressive IL-6 production which manifold exceeded the response of the positive control (LPS), demonstrating that IL-6 secretion is not a robust marker in this context.

Last but not least, IL-12 and IL-10 have a major impact on generation of an immune response. The balance of early produced IL-10 and IL-12 by DCs after contact with various pathogens (which is, generally speaking, the microenvironment present at the time of T cell activation) is believed to be critical for the development of a polarized immune response in vivo. They influence the differential development of Th cells into functionally distinct memory TH cells, such as Th1 and Th2 cells. Overall this process should be tightly regulated, since an inappropriate Th1 development can be extremely harmful to the host such as found in autoimmune diseases (Snijders et al. 1998). A very powerful factor here is IL-12, which is the only known cytokine directing the development of Th1 cells and is responsible for their high production of INF-γ. (Hsieh et al. 1993; Trinchieri et al. 2003). Memory T cells induce, via CD40 engagement, IL-12 production in dendritic cells, but during sensitization is CD40 engagement by naïve T cells insufficient for IL-12 induction due to the fact that naïve T cells require a second signal. The latter can be provided by exogenous factors which evoke Th1 responses like LPS. Once IL-12 is secreted by dendritic cells, naïve T cells will develop into Th1 cells which respond with IFN-γ production and induce, in combination with CD40 engagement, an elevated IL-12 production in dendritic cells (positive feedback).

Some chemical allergens known to induce contact sensitization induce activation of Th-1 cell subsets in mice. Secondly, draining lymph node cells excised from mice treated topically with DNCB expressed high levels of IFN-γ and IL-12 but relatively low levels of type 2 cytokines IL-4, IL-5 IL-10 and IL-13 (Dearman and Kimber 2002 kein abstract). Up to now there is no published research showing that contact allergens are able to induce IL-12 expression in MoDC, whereas an early IL-12 production in response to LPS is well established. After this very limited time span, ceases the production, and DCs fail to respond to further stimulation with IL-12 (Langenkamp et al. 2000). Surprisingly, we detected some IL-12 (subtype IL12P70) in 3 of 4 donors after PPD stimulation. In support of observations reported by others (de Saint-Vis et al. 1998), we found substantial levels of IL-12P70 in MoDC of all measured donors after LPS stimulation. In contrast to LPS, supernatants of cells stimulated with PPD (3 of 4) comprised enhanced IL-12P70 concentrations predominantly after short incubation times, whereas for the fourth donor showed significant levels even after 8h and 24h.

One action of IL-10 may be to prevent immune activation or to cause the delivery of a tolerogenic, rather than immunogenic signal (Steinbrink et al. 1997). Another possibility is that, under conditions where the influence of IL-10 predominates, the development of DCs may be directed in such a way that the DCs into which they mature preferentially stimulate type 2 immune responses. Contact allergens induce epidermal cells to produce not only pro-inflammatory cytokines but also anti-inflammatory cytokines, including IL-10. (Wang et al. 1999) could demonstrate with IL-10 -/- knock-out mice that IL-10 inhibit the emigration of DC

through downregulation of TNF-α. It has been demonstrated that IL-10 is also secreted by monocytes/macrophages, B cells, mast cells, eosinophiles, APC, and keratinocytes. IL-10 inhibits Th1 cytokine production such as IL-1, TNF-α and IL-12, downregulates molecules such as B7 and suppresses the APC function of LCs. Investigations with IL-10 knock-out mice demonstrated that the response to the contact sensitizer oxalzone is increased in both magnitude and duration (Wang et al. 1999). The immunosuppressive effects are partially assigned to the migratory inhibiting effect of IL-10, because IL-10 downregulates IL-1 and TNF-α which are known to be the mandatory signals for contact allergen induced migration. Prolonged exposure of mice to DNCB provokes IL-1ß expression but not IL-10 expression, allowing rapid migration of LC and the development of a type 1 response (Cumberbatch et al. 2005). To date, enhanced IL-10 amounts in supernatants of MoDC stimulated in vitro for 48h with contact allergen have not been published (Coutant et al. 1999). This study found only a marginal increase in IL-10 secretion in 1 of 4 donors, whereas another donor expressed extremely high levels of IL-10 after 8h of stimulation with 50µM PPD. Since we have no comparative data concerning IL-10 or IL-12P70 expression of MoDC stimulated with other contact allergens, we cannot classify the acquired data for PPD and subsequently we cannot speculate wether or not a Th1 or a Th2 induction would dominate.

4.2.5 Validation of PPD mediated cytokine expression profile in MoDC

With this kinetic study, we could show that PPD induces donor dependently quite different cytokine fingerprints. Cytokines were donor dependently expressed at different points of time and in response to different PPD concentrations so that we only formulate a qualitative prediction: PPD is able to induce temporarily the expression IL-1ß, TNF-α, and IL-8, thereby showing an intrinsic potential to activate MoDC. Donor dependently also IL-12 and IL-10 expression could be temporarily observed, but this allows no prediction if PPD induces Th1 rather than Th2 activation. It is remarkable that the qualitative cytokine profiles of 3 donors are similar, and the values of expressed mediators are comparable by means of other contact allergens. In contrast, MoDC of 1 donor (1 of 4) show not only quantitatively but also qualitatively a different cytokine profile: it is obvious that cells repond to small PPD (10µM and 50µM) amounts with high levels of cytokines, and expression is frequently not limited to only one or two hours. These two concentrations are also mentioned in studies of Coulter et al as the most potent ones, because 10µM and 50µM are most effective for CD40 and HLA-DR expression in MoDC.

Overall, most peculiar is the distinct cytokine expression profile of 1 donor compared to the others. One explanation may be that MoDC of that donor (named b in figure 15,16,17,18,19 and 20) have different intracellular preconditions possibly due to any infections which may

modulate sensitizing potential of PPD. This assumption that PPD may interfere with concomitant inflammation speaks for preliminary data of a case report from a PPD-sensitized 55 year old woman which show, compared to this measured cytokine profile of donor b, striking symptoms. She had recurrent dermatitis on the scalp, ears, neck, generalised itch and recurrent staphylococcal infection for 3 years. In addition, after dying her hair, she always got itchy and exudative eruptions on her ears and scalp. Repeated long-time antibiotic treatments did not solve her problems, and patch testing with chemical hair dyes found strong reactions to various dyes and PPD. Her problems resolved only after cessation of hair dying.

Another striking aspect is that, although we detected that PPD enhances the TNF-α expression in all donors after 8h, we could not measure in any of many investigated donors that these PPD concentrations induce the surface expression of co-stimulatory molecules. One explanation may be that the time frame in which TNF-α is expressed is too short and is unable to accumulate, because of results from additional ELISA or CBA analyzes originating from supernatants of MoDC treated with PPD for 24h or 96h. Another explanation may be that PPD actively inhibits co-stimulation in MoDC. With the prospect of getting answers for both incomprehensible aspects, we were encouraged to ascertain wether or not PPD itself can interfere with responses to LPS.

4.3 Impact of PPD on LPS treated MoDC

To investigate the impact of PPD on LPS treated cells, we co-stimulated MoDC with LPS and different concentrations of PPD and ascertained changes in cytotoxicity, maturation, TNF-α expression, and apoptosis. As reported by others, we confirmed that, in response to LPS, mature MoDC show a strong enhancement of cytokine production as well as an increased surface marker expression of HLA-DR and co-stimulatory molecules (Cella et al. 1997; Verhasselt et al. 1997). We found that, 24h after stimulation with PPD, the LPS induced CD86 expression was slightly inhibited, whereas the CD80 and HLA-DR expression remained unaffected. In contrast, after 96h PPD clearly inhibited the LPS induced expression of CD80, CD86, and HLA-DR. Exclusion of these effects may be caused by any cytotoxic impact of high concentrations of PPD and LPS; we excluded this possibility via measurement of cell viability and found that was not the reason for our observations. In percentage, 1µg/ml LPS maximally reduced the viability of MoDC by 2%, and addition of the highest PPD concentration further reduced the viability by 9.7%. Since others (Ade et al. 2007) had defined the optimal test concentration for DC activation at about 75% cell viability, the

conclusion is that the observed downregulations of CD86, CD80, and HLA-DR are not associated with cytotoxic effects of PPD and LPS.

Independently, inhibition of co-stimulation has been reported for some drugs such as corticosteroids (Matasic et al. 1999; Piemonti et al. 1999), cyclosporine (Lee et al. 1999), or 1α,25-dihydroxyvitamin D3 (Berer A 2000). Similarly to our results for PPD, others also found that 1α,25-digidroxyvitamin D3 (Piemonti et al. 2000) or curcumin and lycopene (Kim et al. 2005) strongly inhibit the LPS mediated DC maturation by downregulation of MHCI, MHCII, CD80, CD86, CD40, and CD83 expression. From this point of view, we can speculate that PPD can repress adaptive cell mediated immune responses under certain conditions while concomitant the non-specific immune defence is enhanced. To further judge if the PPD induced downregulation of LPS initiated surface marker expression may be due to apoptosis which inhibit the general protein production and expression, we measured the effects of PPD on LPS induced caspase-3 activation and thus excluded all apoptotic effects from the results. It is well established that LPS activates mitogen-activated protein kinases (MAPKs) signaling namely extracellular regulated kinase (ERK), jun kinases (JNK) and p38 MAPK as well as NF-kB. For LPS-treated MoDC, it has been demonstrated that p38 MAPK is critically involved in upregulation of CD80, CD83, and partially for CD86 but not for CD1a, CD40, and HLA-DR (Arrighi JF. 2001). Furthermore, NF-κB is believed to be essential for upregulation of HLA-DR, CD86, CD83 and CD40 expression (Yoshimura et al. 2001). Based on our findings presented here, we speculate that PPD may infer with these pathways. An unexpected finding in this context was that PPD significantly enhanced LPS-induced TNF-α release after 24h and 96h. In contrast to PPD, other substances which also impair the LPS induced co-stimulation exert additional immunosuppressive effects through inhibition of pro-inflammatory cytokines including TNF-α (Piemonti et al. 2000). For some of the substances, it could even be demonstrated that the inhibition was due to the blocking of MAPK and NF-kB activation. However, TNF-α is not only produced via MAPK (JNK, ERK, p38) or NF-kB activation but also through other pathways and possibly via additional unidentified pathways. It should be mentioned that the enhancement of the TNF-α secretion and the reduction of the CD86 expression may result from epigenetic effects of PPD. In this context, El-Gazzar reported that TNF-α superinduction can be associated with simultaneous silencing of NF-kB dependent genes coding for inflammatory proteins such as pro-inflammatory cytokines and thereby also CD86 surface expression (El Gazzar et al. 2007). Which signaling pathways are involved in PPD mediated inhibition of LPS maturation remains to be investigated in more detail. Based on our findings that PPD can induce CCR7 expression and enhance the LPS induced TNF-α expression, we propose that PPD not only suppresses but also activates some signaling pathways.

4.4 Concluding remarks

Taken together, one of the salient results of this study was that PPD significantly enhances the CCR7 expression. While expression of other classical maturation markers such as HLA-DR and CD40 or CD80, and CD86 were very insignificantly or not enhanced at all, the considerable CCR7 expression hinted that PPD has the potential to activate MoDC. Analysis of mediators further confirmed this because the majority of tested donors responded with enhanced TNF-α, IL-1ß, and IL-8 levels for a short time period, and the concentrations are comparable with those of strong contact sensitizers. The question of the extent to which these temporarily expressed signals in combination with enhanced CCR7 expression together are sufficient to empower MoDC to sensitize naïve T cells remains unpredictable and demands further investigation.

In contrast to the general cytokine profile represented from 3 donors, the profile of a subject which may have a higher susceptibility to PPD sensitization by virtue of the extremely high cytokine expression was presented. There may be different causes for these aberrant responses to PPD. One cause may be that this donor has a concomitant bacterial or viral infection, and PPD is due to a inflammatory milieu more oxidized than in healthy subjects. This hypothesis suggests that PPD is able to enhance the LPS mediated TNF-α expression. For example, oxidized PPD is likely able to activate yet not well-defined different signaling pathways via ROS production and oxidative stress. Which pathways are involved in our findings remains to be investigated in the future but, based on the current knowledge; we propose that PPD may suppress the NF-kB and p38 MAPK signaling. Furthermore, observation of occasionally enhanced DC-SIGN and CD40 expression gives hints that JAK-STAT signaling might be involved. In addition, the explicitly enhanced expression of CCR7 indicates that PPD also activates the PI3K signaling, but in that case, PPD should also be able to induce CCL19 expression in MoDC. Detailed investigations about the involved pathways are important to find medical attendance for the PPD induced allergic contact dermatitis. However independent of the underlying mechanisms we searched for other explanations for elevated susceptibility to PPD. One important cause may certainly be genetic factors, which are known to influence the sensitization and reaction process. In a clinical study, Blömeke B et al 2007 (in press) demonstrated that a TNF-α polymorphism is associated with an increased risk for PPD sensitization. Alternatively, the described genetic polymorphisms in the NAT1 gene (Vatsis and Weber 1993; Grant et al. 1997) which modify the individual acetylation status may also be responsible for the individual susceptibility to PPD. We further cannot exclude the possibility that PPD induces epigenetic changes in MoDC which may influence at least the expression of crucial proteins. Finally it seems that all potential risks which may lead to sensitization to PPD occur rather infrequently. In detail we

opine either the coincidence that PPD induces in one subject coevally the upregulation of CD40, HLA-DR and CCR7 or that anoter subject expresses a specific cytokine profile or exhibits an appropriate genetic profile. This observation equals the in vivo situation because only the minority of subjects (1-4% of adults) in Germany are sensitized to PPD. The risk of establishing an allergic contact dermatitis as a result of sensitization to PPD is enhanced through environmental circumstances like hair dyeing. This observation is supported by the fact that hair dressers often suffer from allergic contact dermatitis to PPD (Gottlober et al. 2001). In combination with additional oxidative products, as in the case of hair coloring, the risk that more oxidative PPD derivates with sensitizing potential are developed arises (Regenery et al. in process). It is remarkable that, even though the frequency of PPD allergy is rather small, the allergic reactions of sensitized subjects are very strong. This might be because PPD sensitized persons usually react to other arylamines, para-amino compounds and some azo dyes as reported in patch-test studies and supported by another study in our team (Skazik and Grannemann, in revision), but this is a topic of the elicitation phase of allergic contact which will have to discussed in another context.

5 Summary

Allergic contact dermatitis (ACD), a delayed hypersensitivity reaction to small molecular weight compounds, is a common skin reaction causing personal and occupational problems. Chemicals, such as PPD (Paraphenylenediamine) and other related para-aminobenzenes, frequently found in dyes, are raising increasing concerns about their strong allergenicity. Up to now, the knowledge about cellular immune responses initiated by PPD is limited. In the current study, we investigated the immune modulatory potential of PPD to find answers to the still open question of whether or not PPD itself is able to sensitize naïve T cells or wether PPD is solely an allergen. Taking into account that dendritic cells play a pivotal role in the sensitization process for ACD, we analyzed the impact of PPD to induce and modulate functional maturation in monocyte derived dendritic cells (MoDC). To initially estimate the amounts of actual PPD available after stimulation, we validated the metabolic compentence of MoDC to acetylate PPD. Thereby, we characterized the *N-acetyltransferase 1* and *2* (*NAT-1*- and *2*) mRNA expression and further determined the metabolic activity of NAT1 enzyme. We found that MoDC express NAT-1 enzyme in 9 of 10 donors and detected mono- as well as di-acetylated PPD in extracts of cell culture supernatants in 4 of 6 donors confirming that in vitro-generated MoDC express metabolic active N-acetyltransferase-1 (NAT-1). In consideration that acetylated PPD is unlikely to be able to activate dendritic cells we can assume that initially added PPD cannot be completely available for dendritic cell activation. In order to elucidate the sensitizing potential of PPD, we measured the expression of surface molecules crucial for presentation, co-stimulation, late migration, and dendritic cell/ t-cell interaction after stimulation with different PPD concentrations and different incubation durations with FACS analysis. In summary, we found that PPD significantly enhances the chemokine receptor 7 (CCR7) expression and insignificantly affects HLA-DR, DC-SIGN, and CD11c expression. The enhanced CCR7 expression strongly indicates that PPD is, despite the fact that it cannot induce the traditional co-stimulatory molecules CD80 and CD86, able to activate dendritic cells. Augmentative cytokine kinetic measurements did substantiate this hypothesis. With cytometric beat array analysis, we were able to measure coevally mediators such as interleukin 1 beta (IL1ß), tumor necrosis factor-alpha (TNF-α), IL-8, IL-6, IL-12P70, and IL-10 and therewith to demonstrate that PPD induces danger signals in all tested donors. In addition we could show strong inter-individual differences in the PPD-induced cytokine production in terms of magnitude and secretion times. Surprisingly, the qualitative composition was then comparable. While in 3 of 4 donors enhanced cytokine expression was rather weak and of short duration, we measured, in one donor, amazing cytokine responses, particularly after incubation with 10 and 50μM PPD. This donor might represent a person who is more susceptible for sensitizing to PPD than others. Independently, the fact that PPD

is able to induce danger signals in all subjects but no significant upregulation of traditional maturation markers (with the exception of CCR7) we further searched if PPD is able to actively modulate dendritic cell responses. We therefore measured the influence of PPD on activated dendritic cells and co-stimulated the MoDC with PPD and lipopolysaccharide (LPS). The third indication that PPD does indeed have immunogenic impact (the first: enhanced CCR7 expression, and the second: induction of danger signals) comes from test results, showing that PPD enhances the LPS induced TNF-α expression and also reduces the LPS induced expression of CD80 and CD86.

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7 Acknowledgement

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8 Lebenslauf

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9 Summary in German

Allergische Kontaktdermatitis ist eine zellvermittelte verzögerte Überempfindlichkeitsreaktion auf niedermolekulare Substanzen, die häufig Hautreaktionen hervorrufen und dadurch sowohl persönliche als auch berufliche Probleme verursachen. Chemikalien wie *para*-Phenylendiamin (PPD) und andere verwandte *para*-Aminobenzole werden häufig in Farben eingesetzt und erlangen aufgrund ihrer starken Allergenität eine immer größere Aufmerksamkeit. Bis jetzt ist das Wisen über zelluläre Immunantworten auf PPD nur begrenzt verstanden. In dieser Studie haben wir das immunmodulatorische Potential von PPD untersucht, vor allem hinsichtlich der Fragestellung, ob PPD selber naive T-Zellen sensibilisieren kann oder ob PPD letztendlich nur ein Allergen ist. In Anbetracht der Tatsache das dendritische Zellen (DZ) eine vorherrschende Rolle bei der allergischen Kontaktdermatitis spielen, haben wir untersucht, in wie weit PPD in der Lage ist, funktionelle Reifung zu modulieren. Um abschätzen zu können, welcher Anteil an PPD nach der Stimulation zur potentiellen Aktivierung tatsächlich zur Verfügung steht, haben wir die metabolische Kompetenz von „monocyte derived dendritic cells" (MoDC) untersucht, PPD zu acetylieren. Dazu haben wir die N-Acetyltransferase 1 (NAT-1) und N-Acetyltransferase 2 (NAT-2) mRNA Expression charakterisiert und weitergehend die metabolische Aktivität von NAT-1 Enzym bestimmt. Mit diesen Versuchen konnten wir zeigen, dass MoDC von 9 aus 10 Spendern das NAT-1 Enzym exprimieren und konnten in 4 von 6 Spendern acetyliertes PPD in Extrakten aus Zellkulturüberständen detektieren. In Anbetracht der Tatsache, dass acetyliertes PPD nicht in der Lage ist, MoDC zu aktivieren, können wir davon ausgehen, dass der Anteil an acetyliertem PPD zur DC Aktivierung nicht zur Verfügung steht. Um das sensibilisierende Potential von PPD herauszufinden, haben wir nach Stimulation mit verschiedenen PPD Konzentrationen die Expression von Oberflächenmolekülen, die für die Antigenpräsentation, Co-Stimulation, späte Migration und dendritische Zell-/T-Zell-Wechselwirkung entscheidend sind, zu verschiedenen Zeitpunkten mittels Durchflusszytometrie Messungen (FACS) gemessen. Zusammengefasst konnten wir zeigen, dass PPD signifikant die „chemokine receptor 7" (CCR7) Expression und nicht signifikant die

„human leukocyte antigen – DR“ HLA-DR, „DC-specific C-type lectin intercellular adhesion molecule-3-grabbing nonintegrin“ (DC-SIGN) und „cluster domain“ 11c (CD11c) Expression erhöht. Die erhöhte CCR7 Expression ermutigte uns, weiter nachzuforschen, ob PPD trotz der Tatsache, dass es keine traditionelle CD80 und CD86 Expression induzieren kann, fähig ist, DCs zu aktivieren. Ausgiebige Zytokinkinetikmessungen konnten diese Hypothese bestätigen. Mit Cytometric Beat Array (CBA) Messungen konnten wir gleichzeitig Mediatoren wie „Interleukin -1ß“ (IL-1ß), „tumor necrosis factor-alpha“ (TNF-α), IL-8, IL-6, IL-12P70 und IL-10 messen und damit demonstrieren, dass PPD in der Lage ist, bei allen Spendern „Danger Signals“ zu induzieren. Zudem konnten wir zeigen, dass die PPD induzierte Zytokinausschüttung interindividuell schwankte und zu unterschiedlichen Zeitpunkten erfolgte. Überraschenderweise war die qualitative Zusammensetzung der Zytokine vergleichbar. Während in 3 von 4 Spendern die Zytokinexpression eher schwach erhöht war und von kurzer Dauer, haben wir in einem Spender vor allem mit den Konzentrationen von 10µM und 50µM PPD verblüffend hohe Zytokinausschüttungen gemessen. Dieser Spender scheint eine Person zu repräsetieren, die empfänglicher für eine PPD Sensibilisierung ist als andere. Unabhängig davon, hat uns die Tatsache, dass PPD in allen Spendern „Danger Signals“, aber keine gesteigerte Expressionsrate von traditionellen Reifungsmarkern (mit Ausnahme von CCR7) induzierte, veranlasst zu untersuchen, ob PPD anderweitig fähig ist, in MoDC Immunantworten zu modulieren. Daraufhin haben wir den Einfluss von PPD auf aktivierte DCs gemessen und die Ergebnisse bestätigten die Annahme. Weiterhin konnten wir zeigen, dass PPD immunogenes Potential aufweist. Aufgrund der Tatsache, dass die LPS induzierten Signaltransduktionswege in MoDC gut charakterisiert sind, konnten wir mit dieser Studie zusätzlich Hinweise über die PPD induzierte Signalgebung liefern.