

**Generation of danger signals by small molecular weight
environmental compounds**

-Impact of *para*-phenylenediamine on monocytes-

Vom Fachbereich VI
Geographie/Geowissenschaften
der Universität Trier
zur Verleihung des akademischen Grades
'Doktor der Naturwissenschaften' (Dr. rer. nat.)
genehmigte Dissertation

vorgelegt von
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Tag der wissenschaftlichen Aussprache: 31. August 2006
Erscheinungsort und -jahr: Trier, 2006

To my parents

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Abbreviations

ACD	Allergic contact dermatitis
AAB	4-aminoazobenzene
APC	Antigen presented cells
BB	Bandrowski's Base
B7	B-lymphocyte activation antigen B7-2
CC chemokine	CC Motive chemokine
CCR2	CC chemokine receptor 2
CCR4	CC chemokine receptor 4
CCR5	CC chemokine receptor 5
CCL2	CC chemokin ligand 2
CCL7	CC chemokin ligand 7
CCL8	CC chemokin ligand 8
CCL13	CC chemokin ligand 13
CHS	Hapten-induced allergic contact hypersensitivity
CTACK	Cutaneous T cell-attracting chemokine
CX3C chemokine	CX3C Motive chemokine
CXC chemokine	CXC Motive chemokine
DC	Dendritic cells
DNCB	2, 4-Dinitrochlorobenzene
ELISA	Enzyme-linked Immunoabsorbent Assay
FACS	Fluorescence Activated Cell Sorter
GM-CSF	Granulocyte-macrophage colony –stimulateding factor
IFN- γ	Interferon-gamma
IL-1 β	Interleukin 1 beta
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-8	Interleukin 8
IL-12	Interleukin 12

Abbreviations

IP-10	Interferon gamma- inducible protein 10
I-TAC	Interferon inducible T cell alpha hemoattractant
LC	Langerhans cells
LPS	Lipopolysaccharide
MCAF	Monocyte chemotactic and activating factor
MCD	Mucular corneal dystrophy
MCP-1	Monocyte chemotactic protein 1
MIG	Monokine induced by interferon gamma
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MoDC	Monocyte-derived dendritic cells
MPD	Meta-Phenylenediamine
NK	Natural killer cells
OPD	Ortho-Phenylenediamine
PARC	Pulmonary and activation-regulated chemokine
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
SDF1	Stromal cell-derived factor 1
SMC-CF	Smooth muscle cell-derived chemotactic factor
TARC	Thymus and activation-regulated chemokine
Th1	T helper 1
TCDD	2, 3, 7, 8-tetrachlorodibenzo-p-dioxin
Th2	T helper 2
TNF- α	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor
TNCB	Trinitrochlorobenzene

1. Introduction

1.1. Allergic contact dermatitis to small molecular compounds

Contact hypersensitivity (CHS) is a frequent cause for occupational hapten-induced allergic contact dermatitis (ACD). ACD results from a T cell response to harmless, low-molecular weight chemicals (haptens) applied to the skin. ACD is especially common in industrialized countries with an enormous sociomedical and socioeconomic impact [1].

1.1.1. Sensitization phase

Following primary application to the skin, epidermal Langerhans cells (LCs) take up the chemical, process it, and migrate towards the regional lymph nodes, where the antigen is presented to naive T cells. During this process, LCs convert from a resting into an activated functional state. This activation of LCs is initiated by keratinocytes and most likely by other skin related cells such monocytes and macrophages that secrete inflammatory cytokines as a result of the hapten application, and is possibly also due to direct effects of the haptens on LCs themselves [2]. This inflammatory reaction, the so-called danger signal, seems to be essential for the whole process.

After activation LC secrete then various cytokines such as interleukins (IL) such as IL-1 β , IL-6, and IL-12. They enhance their expression of certain cell-surface molecules [major histocompatibility complex (MHC) class I and II, adhesion molecules, costimulatory molecules], alter their antigen (Ag) uptake, processing, and finally Ag presenting capacities [3]. The induction of migration towards the lymph node appears to depend on the capacity of haptens to induce IL-1 β in LCs, which is a specific and almost immediate effect after epicutaneous hapten application. This secretion seems be specific, and is not observed after contact with irritants or tolerogens [4-7]. In addition, chemokines, tumor necrosis factor alpha (TNF- α), and granulocyte-colony stimulation factor (GM-CSF) may also contribute to LC activation and migration [8-10]. Thus, haptens themselves by virtue of their capacity to induce a specific cytokine pattern are the primary stimulus that activate LCs and induce sensitization. However, besides LCs other antigen presenting cells (APCs) such as dermal dendritic cells may also contribute to priming of naive T cells [11].

In this whole process are also chemokines crucial involved both in the induction and the effector phase of ACD. The kinetics and temporal pattern of chemokine expression during ACD indicate an early involvement of interleukin 8 (IL-8) followed by monocyte chemoattractant proteins (MCP) 1, and a protein called RANTES (regulated upon activation normally T- expressed and presumably secreted). This is followed by a robust burst of CXC3 receptor agonists [12].

1.1.2. Effector phase

After priming and clonal expansion of specific T-cell precursors [13], ACD can develop only after a second encounter with the hapten. At that stage (effector phase), the clinical features of ACD, characterized by rapid recruitment and activation of specific T cells at the site of hapten challenge, can be viewed as the result of activation of two distinct T cell subsets endowed with opposite functions: effector T cells and down-regulatory T (Tr) cells [14]. The expression of both murine CH and human ACD correlates with the activity of hapten-specific CD8+ T cells, which exert their effector function through direct cytotoxic activities on skin cells as well as the release of cytokines [15-20]. The inflammatory infiltrate in ACD is composed mainly of CD4+ and CD8+ T cells, monocytes and DCs, with an early and transient presence of neutrophils [21]. CD4+ T cells play a more complex role with Th1 and Th2 subsets contributing to disease expression, and Tr cells are primarily involved in modulation and/or termination of the reaction [21]. The latter emphasizes the self-limiting character of the reaction, suggesting that regulatory mechanism is actively involved in the termination of ACD.

More recently, it had been shown that the expressed chemokine profile influences clearly the pattern of T cell subsets recruited during inflammation. This supports the concept already earlier announced by D'Ambrosio and colleagues [22] who hypothesize that different thresholds for Th1 and Th2 cells exist that differently localize Th cell subsets in inflammation. Goebeler et al. [23] analyzed the expression pattern of chemokines in the skin at different times after hapten challenge and suggested that MCP-1 seems to be the dominant monocyte/macrophage attractants expressed during the elicitation phase of CHS [23]. During the later phase of CHS, strong expression of the interferon- γ -inducible chemokines interferon gamma inducible protein 10 (IP-10) and monokine induced by

gamma interferon (MIG), and a moderate expression of macrophage inflammatory protein (MIP)-1 α/β may activate and attract preferentially Th1 cells that express CXC receptor (CXCR) 3 and CC receptor (CCR) 5, respectively [24]. In contrast, other T-cell-attracting chemokines detected show less preferences for Th1 subtypes and are rather specific for Th2 subtypes such as thymus and activation-regulated chemokine (TARC) and mucular corneal dystrophy (MDC) [25]. Expression of more promiscuous T cell attractants such as MCP-1 and RANTES may explain the presence of bystander cells that are detected in CHS [26].

1.1.3. Recognition of small molecular weight chemicals by antigen presenting cells

Small molecular weight compounds are not directly recognized by the cells of the immune system. These compounds need to be presented by subsets of antigen presenting cells to the immune system. In this regard, epidermal Langerhans cells (LC) and the cells into which they mature (dendritic cells) are believed to play a pivotal role in the sensitization process of the allergic contact dermatitis. They are so-called professional antigen presenting cells. LC bind the haptens, internalize them, and present them in association with their major histocompatibility complex (MHC) to T cells (see Figure 1). Several mediators are released by these cells after the contact with haptens. One of the first proteins released into the environment is interleukin (IL)-1 β . Immediately after the antigen enters the cells and IL-1 β mRNA levels are up regulated and followed by the secretion of mature IL-1 β . Later one other cytokines such as IL-6, IL-12 and IL-18 are secreted as well. The second major cytokine involved in the early events is tumor necrosis factor-alpha (TNF- α). This protein is released by LC but even more by the keratinocytes. These two cytokines - IL-1 β , TNF- α - provide major signals for the initiation of the LC migration [27]. In addition, various chemokines are released to support the migration of LC from the skin to the draining lymph nodes via the afferent lymphatic system. In the lymph nodes, chemokines also direct the LC to the areas where the naïve T cells reside.

During the migration of LC they undergo morphological and phenotypical changes, and mature into dendritic cells and they up regulate the co-stimulatory molecules such as CD54, CD80, CD86 and CD40. With the exception of CD86, these modifications are also

influenced by TNF- α . In the regional lymph nodes, the dendritic cells present the antigens to the responsive naïve T cells, and generate thereby the effector cells [27, 28].

During the effector phase of ACD haptens can be presented not only by professional antigen presenting cells. Under these circumstances even keratinocytes and cells of the monocyte/macrophages lineages are able to present allergens efficiently to effector cells. The impact of chemicals on professional antigen presenting cells is rather limited. Most studies focused on mice, and only few studies used human cells. Aiba *et al* found that NiCl₂ elicited a strong expression of co-stimulatory molecules. These changes were accompanied by a remarkable release of IL-1 β and IL-6 [29], [30]. But 2, 4-Dinitrochlorobenzene (DNCB) induced only a slight increase of IL-1 β secretion. This type of investigations has been hampered in the past due to the limited availability of LCs especially in humans. They are a minor cell population within epidermal cells, and therefore is it difficult to isolate sufficient numbers of LCs. Furthermore, the isolation procedures itself often lead to unspecific activation of LCs. Progress in this field arose from new methods allowing to generation of large numbers of immature DCs resembling LCs from human blood precursors. DCs.

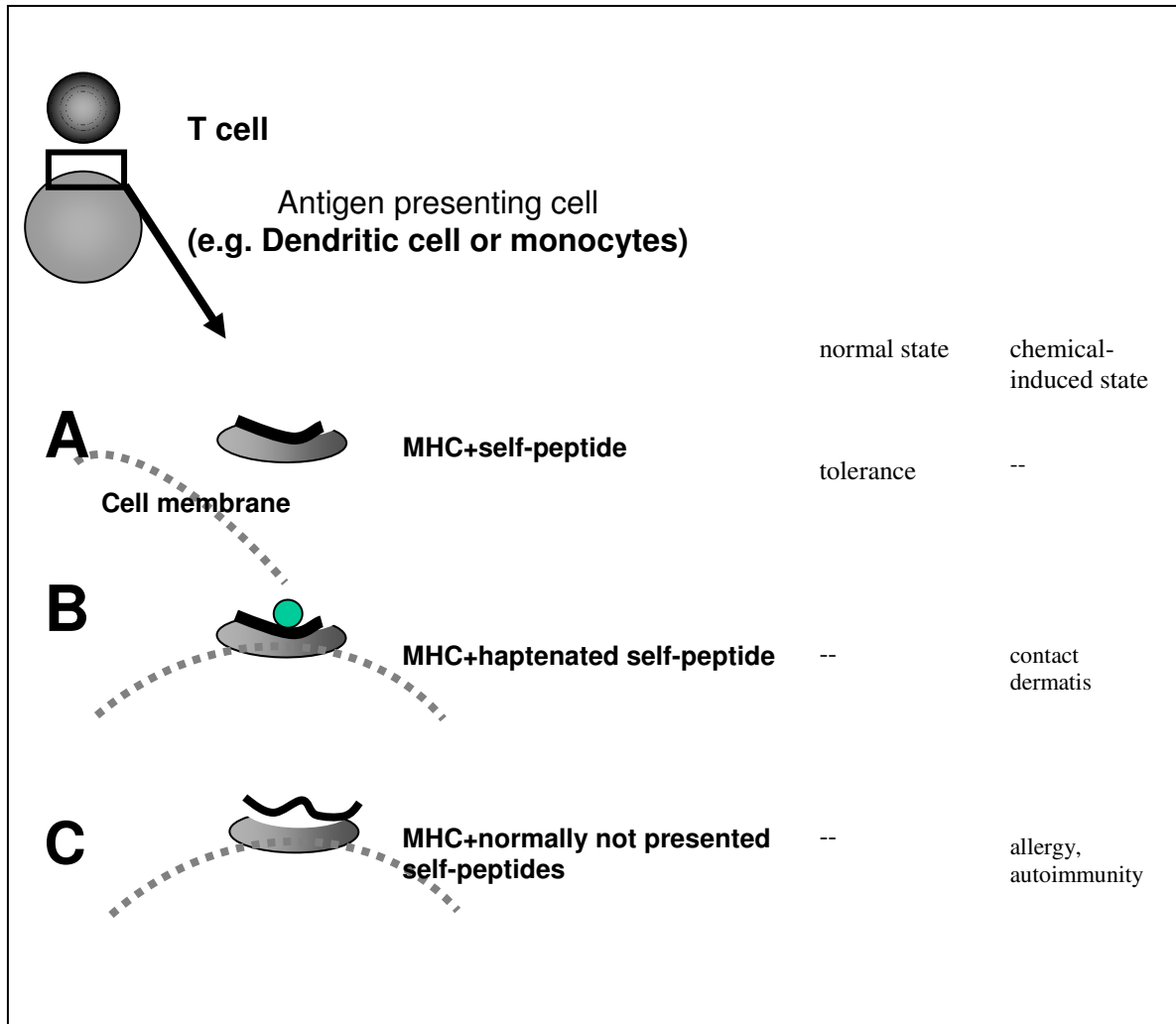


Figure 1. Possible outcomes of the interaction between T cells and antigen presenting cells. Hapten form protein-adducts with self-peptides. T cells recognize only peptides lying in a groove of MHC. T cells with receptors which would recognize self-peptides are eliminated in the thymus (A) or kept in an inactive state in the healthy situation. (B) Low molecular weight chemicals which can bind to peptides, covalently or not, change the form of the peptide, which no longer „looks“ like a self-antigen, and T cells can be activated. Binding of the hapten to the peptide can occur inside or outside of the cells. This depends on the chemical (C). Low molecular weight chemicals can change either the protein degradation or the loading of peptides in the cell. This leads to a changed pattern of presentation namely presentation of normally not presented peptides. Thus, even if presented peptides are „self“ peptides, T cells will exist with receptors specific for them because the selection processes in the thymus will have used the normal set of self-peptides, and not the cryptic ones. It should be noted that binding of the T cell receptor to the peptide inside the MHC groove is necessary but not sufficient for T cell activation. A second signal, i.e. yet another contact of surface molecules by T cells and APC, must be provided. This is discussed in the context of the danger hypothesis in the text (scheme modified after Esser 2005).

1.2. The role of mediators in ACD

1.2.1. Cytokines

As mentioned in chapter 1.2 many mediators are involved in the induction and effector phase of ACD. This knowledge comes mostly from animal studies of contact dermatitis including knock-out models but detailed knowledge about the effects of chemical-related contact dermatitis is very limited with the exception of metals. It is commonly accepted that the major donors for the needed interleukin IL-1 β [31, 32] GM-CSF [33] and TNF- α [9] after antigen encounter comes initially from skin cells such as keratinocytes, monocytes and macrophages. These cytokines provide the danger signals needed for the activation of Langerhans cell (LC), which then produces in a positive feedback, loop various cytokines themselves. TNF- α and IL-1 β are also major stimuli for the increased expression of the major histocompatibility complex (MHC) class I and II, co-stimulatory molecules such as intercellular adhesion molecule-1 (ICAM-1), B7-1, B7-2, CD40, and for LC migration [9, 34]. But still, interleukin-12 (IL-12) is the essential cytokine for successful contact sensitization known so far, that is produced by LCs themselves [4, 35].

Various groups investigated the kinetics of these cytokines on human skin and found that application of various haptens to the skin led indeed to an rapid up regulation of IL-1 β and TNF- α mRNA and protein levels in the skin [36, 37]. Besides the keratinocytes are the monocyte/macrophage cell populations discussed as a potential dermal source of IL-1 β [38] in humans as well as in mice. Further, TNF- α and IL-1 β have been described to play a crucial role in the elicitation phase of allergic contact dermatitis upon exposure to haptens. Especially well-known are the pleiotropic functions of TNF- α . TNF- α activates the immune responses through further induction of IL-1, IL-8 and transforming growth factor type β (TGF- β) [39]. In addition, it also involved in tissue remodelling, cell motility, cell cycle, and apoptosis. By regulating a characteristic large set of chemokines, including newly identified TNF- α targets, it attracts neutrophils, macrophages, and skin-specific memory T-cells. Therefore, TNF- α initiates not only ACD but is involved also in inflammation and responses to injury, repair [40], and elicitation phase of ACD. TNF- α mediates their effects via two distinct cell-surface receptors TNFR1 and TNFR2 are known for TNF- α [41].

1.2.2. Chemokines

As mentioned above, chemokines are involved the induction and expression of ACD. They can influence the danger signal generation, migration, homing of T cells in the local lymph nodes as well as their recruitment back into the skin.

In general, they cause directed cells migration (chemotaxis) and are expressed by diverse cell types. They mediate the recruitment of leukocytes to inflammatory sites, the constitutive trafficking of cells through the secondary lymphoid organs, modulation of T cell proliferation, and cytokine production [42-46]. Furthermore, chemokine-induced activation of several integrands is important for temporal adhesion of migrating cells [47, 48]. The chemokines (IL-8, MIP3A and cutaneous T cell-attracting chemokine; CTACK) were increased after contact allergens (nickel sulfate and potassium dichromate) [49].

In total, forty-five human chemokines and eighteen chemokine receptors have been described up to date [50]. The biological effects of chemokines are mediated by a large family of G-protein coupled, seven transmembrane receptors [51-53]. The selective expression of chemokine receptors on target cells determines to a significant degree the specificity. The ligand specificities of the receptors can overlap as some chemokines bind to multiple receptors, and some receptors can bind multiple chemokines, yielding a complex and highly redundant regulatory network [54]. The chemokine superfamily is divided into four subfamilies characterised in part by the position of four conserved cysteine residues in their primary amino acid sequence:

1. CXC chemokines or alpha-chemokines (CXC Motive chemokine), with one amino acid separating the first two cysteine residues, act as chemoattractants for neutrophil granulocytes and T lymphocytes. Stromal cell-derived factor 1 (SDF-1) and interleukin 8 (IL-8) are members of this family.

2. CC chemokines (CC-motive chemokine) such as RANTES and monocyte chemotactic protein 1 (MCP-1), the first two cysteines are immediately adjacent to one and other. They are generally chemoattractants for monocytes and T lymphocytes [55] but they are also active on granulocytes, B lymphocytes, natural killer cells (NK cells), and dendritic cells (DC) [56-59].

3. C chemokine family consisting of only lymphotactin- α and - β . They lack the second cysteine residue but share significant homology at their 3' end with members of the CC subfamily. They are expressed by natural killer cells (NK) cells, and attract themselves as well as T-lymphocytes [60, 61].

4. CX3C (CX3C- motive chemokine) family with three amino acids separating the two N terminal cysteine residues. Fractalkine, the only known member of this group, exists as a soluble as well as a membrane bound form. This chemokines again attracts T-lymphocytes and monocytes [62, 63].

1.2.2.1. The CC chemokines

Among the known CC chemokines, MCP-1, MCP-2, MCP-3, MCP-4 (CCL-2, CCL-8, CCL-7, and CCL-13, respectively), MCP-5 [42-45, 64], MIP-1 α , MIP-1 β , I309, all have monocyte chemoattractant activity in vitro. Monocytes express at least three CC chemokine receptors, namely CCR1, CCR2, and CCR5. CCR2 binds only MCP-1 with high affinity but binds also MCP-3 and MCP-5 [65-67]. MCP-1, MCP-2, MCP-3, and MCP-4 target besides the already mentioned leukocyte subsets further basophiles, and eosinophils [47, 68]. The lifespan of these chemokines is limited via proteolysis. Indeed, production of prototypically processed chemokines has been reported in cell cultures [69-72]. Migration and cell recruitment are multistep processes (rolling-adhesion-transmigration). This is supported by specific adhesion molecules, which coordinate the interactions between migrating cells and endothelial cells [73]. The needed well coordinated tissue and extracellular matrix degradation is mainly performed by the action of various matrix metalloproteinase's (MMPs). It is gradually becoming clear that these proteases even degrade CC chemokines [71, 72].

1.2.2.2. Monocyte chemoattractant protein-1 and its receptor

Dependent of ACD several in vivo studies suggest that MCP-1 recruits monocytes to sites of inflammation in a variety of other pathological conditions, including atherosclerosis [74-76], rheumatoid arthritis [77], and pulmonary fibrosis and granulomatous lung disease [78]. Therefore, it can be concluded that MCP-1 is a potent chemoattractant both in vitro

[79-81] and in vivo [82, 83], and additionally its expression is markedly induced by inflammatory stimuli [84, 85].

The genes for MCP-1 and its receptors have been cloned. The murine gene of MCP-1 was discovered in 1983 and was named JE [86]. The homologous human gene was described in 1989 [87] and named MCP-1 also called monocyte chemotactic and activating factor (MCAF) [79, 88] and smooth muscle cell derived chemotactic factor (SMC-CF) [89, 90] because of its chemoattractive effects on monocytes [81]. Their receptors were cloned in 1994 and 1996, respectively [91, 92]. The receptors share sequence homologies of 80 %. Already 1997, CCR2 defective mice were generated [93, 94]. MCP-1/JE, are clustered on chromosome 17q11.2-12 [95-97] or mouse chromosome 11 [98, 99]. Both natural and recombinant JE/MCP-1 proteins are potent chemoattractants in vitro [81, 88]. However, the ability of MCP-1 to cause monocyte accumulation in vivo still remains contradictory, with both positive [83, 100-102] and negative [80, 103] reports.

The receptor expression varies between mice and humans. Boring et al. first indicated that CCR2 is the primary receptor for MCP-1 in the mouse, and pointed to its important role in monocyte recruitment as well as in the production of inflammatory cytokines [93]. In humans, there are two well-characterized receptors for MCP-1, CCR2A, and CCR2B, which are splice variants of a single gene [104]. In the mouse, it was detected only a single MCP-1 receptor [91].

Various cytokines influence the expression of MCP-1 and CCR2. The MCP-1 expression is promoted by LPS as well as chemicals [105]. The latter is mediated by external stimuli such as interleukin 1 (IL-1), TNF- α or interleukin 4 (IL-4) [106]. On the other side, monocytes stimulated with MCP-1 secrete IL-1, interleukin 6 (IL-6) but no TNF- α [107].

In addition, interferon- γ (IFN- γ) [31, 35] is a strong inducer of MCP-1. The mechanism behind the MCP-1 induction is mediated by increased gene expression [108]. This has been proven in a number of cell types, and typically IFN- γ rapidly and potently induces MCP-1 mRNA accumulation and MCP-1 protein production [109-112].

In contrast, IL-1 β and TNF- α down regulate CCR2 mRNA and protein expression [113, 114], and the MCP-1-mediated transmigration of monocytes, whereas IFN- γ did not affect CCR2 expression in monocytes [113, 115]. In contrast, IL-2 induced CCR2 expression on monocyte [113]. The mechanism of this regulation has been studied in detail in the monocytic cell line THP-1 (human acute monocytic leukemia cells) [116]. Here, it has been found that the down regulation may be not transcriptionally regulated. The presence of TNF- α as well as IL-1 induced in THP-1 a rapid down regulation of human CCR2 expression and loss of receptor protein [114] which is consistent with an impairment on CCR2 mRNA stability rather than reduced transcription [113]. This is likely a general mechanism because similar results were found for LPS. In contrast, the impact of small molecular weight compounds on MCP-1/CCR2 system has not been studied.

1.3. The influence of MCP-1 on T cell polarization

The impact of MCP-1 on the polarization of naive T cells has been studied only recently. This is presumably mediated by its impact on interleukin 12 (IL-12). This cytokine is known for its major impact on naive T cells and expression of the Th1 phenotype [117]. In the presence of high IL-12 level, naive T cells are polarized into Th1 cells while in the absence of IL-12, and most likely presence of an yet unknown Th2 promoting mediator and IL-4 [117], cells develop after antigen-T-cell receptor interactions into Th2 cells. It has been found, that MCP-1 also can infer with the expression of these cytokines and regulate the balance between proinflammatory Th1 and anti-inflammatory Th2 cytokines on several levels [118, 119]. MCP-1 influences directly activated macrophages by decreasing their IL-12 expression [78]. Furthermore, even newer results expanded this effect to activated monocytes showing that MCP-3, MCP-2 and MCP-4 all are able to inhibit IL-12 production [120]. Furthermore, MCP-1 seems also able to stimulate directly IL-4 production by CD4⁺ T cells [46]. These results could explain its possible influence on T cell polarization and suggest that MCP-1 may polarize T cells into Th2 cells but detailed studies are still missing especially in humans.

Different and conflicting results were gained using knock-out models. Lymph node cells from immunized MCP-1^{-/-} mice synthesized extremely low levels of IL-4, and were unable to mount Th2 responses or have at least defects in Th2 responses [119, 121, 122].

In agreement with these results are studies demonstrating the resistance of MCP-1^{-/-} mice to the effect of *Mycobacterium tuberculosis* [122] which is indicative for a fully functional Th1 response. Nevertheless, there are reports on Th1 defects in CCR2^{-/-} mice [93, 123]. This discrepancy might not be surprising given the fact that at least two additional high affinity ligands are known in the mouse (MCP-3 and MCP-5), which might stimulate Th1 polarization via other receptors. Further, there is evidence out for another MCP-1 receptor [124], known as D6, may not have a functioning signal cascade [125].

Overall support these data that MCP-1's may influence the development of polarized immune responses in vivo. Despite the effects of MCP-1 on monocytes and macrophages, which may be indicative for a role in Th1-polarization, other data indicate a more likely an effect on the development of Th2 responses.

1.4. Para-Phenylenediamine (PPD)

Up to now, we know more than six million chemicals and approximately 2,800 are known to have contact-sensitizing and/or allergenic properties [126]. The most common contact allergens include nickel, fragrances, certain antibiotics, rubber components, preservatives as well as *Para*-phenylenediamine (1, 4-diaminobenzene, PPD, see Figure 1), and some dyes.

PPD, an aryl amine, can be found as an ingredient in dye formulations [127]. As a dye intermediate it most often ranks on position 10-14 of the most common allergens [128, 129]. In Europe, PPD and other diaminobenzenes are allowed at a maximum concentration of 6% in hair dye products [130]. In addition, it may be found in textiles[131], antioxidants, temporary tattoos [132], photographic developer and lithography plates, photocopying and printing inks; black rubber, accelerators for rubber vulcanization, oils, greases, and gasoline [133]. PPD itself is a colourless substance that requires oxidation to form coloured complexes. Among others, PPD allergic persons often show cross-reactions to para-aminobenzoic acid used as UV screens, or drugs like sulfonamides [134], and drugs such as benzocain or procain [135].

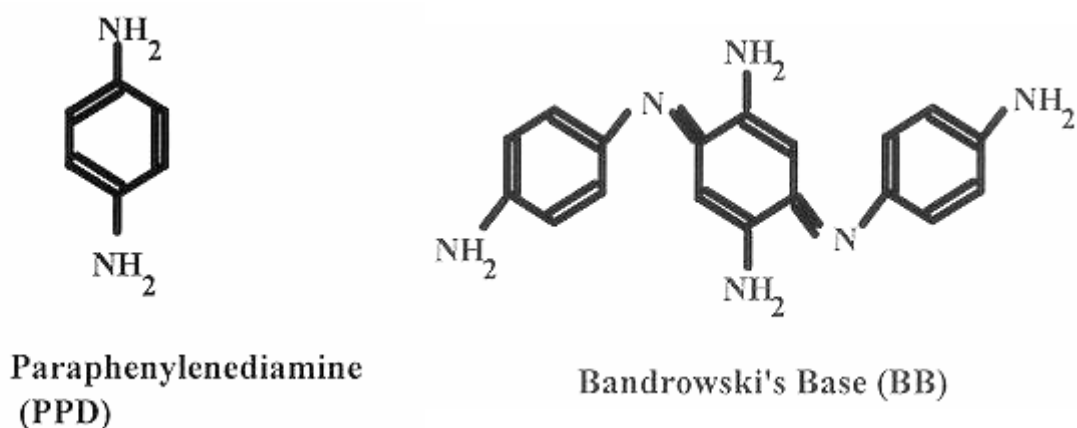


Figure 2. Structure of para Phenylenediamine and Bandrowski's Base

The skin is one of the main targets for PPD exposure [136]. Usually, allergic reactions are mild and found only at very sensitive body sites such as eyelids or the rims of the ears. In more susceptible persons, there may be marked reddening and swelling of the scalp and the face. Up know, it is not clear whether PPD itself or oxidized compounds cause the sensitization. In contrast, more is known for the elucidation phase and its effects in human keratinocytes. PPD may be oxidized to benzoquinone diimine, which, in turn may form the trinuclear dye N, N-bis (4-aminophenyl)-2, 5-diamino-1, 4-quinone-diimine called Bandrowski's base (BB), which is clearly involved in the allergy as shown in our and other groups [137, 138]. While initial investigations using peripheral blood mononuclear cells (PBMC) from PPD-allergic persons found only T-cell reactivity to BB and not to PPD [137, 139] our laboratory demonstrated that specific T-cell clones (TCC) responded indeed also to PPD [138]. A detailed mechanistic analysis of the requirements for the antigen presentation revealed that BB needed processing and possibly metabolism for T cell stimulation. The mechanism underlying reactions to PPD was quiet different and contradictory to the common expectations. PPD itself was recognized by T cells through a processing-independent pathway. These results forced us to re-examine the effects of PPD especially on antigen presenting cells. In this study we focussed on monocytes while an independent thesis investigate the impact of PPD on maturation of monocyte derived dendritic cells (MoDC).

1.5. Aims of the study

PPD is a common cause for occupational related allergic contact dermatitis [140-143] but the mechanism of small weight compounds induced allergic contact dermatitis is not well defined. Furthermore, investigations are warranted for several other aspects: Up to now there is no successful therapy for this disease. These circumstances explain why many allergic persons have to change their profession (a social-economic aspect). Secondly, PPD allergic persons show often cross-reactions to further chemicals including dyes, and even more importantly certain medications. In addition, up to date, it has not been clearly demonstrated if PPD itself is a sensitizing agent, and can provide the signals needed for this process. Thus, this study aimed on the potential of PPD to provide the danger signals for sensitization using monocytes by studying IL-1 β , TNF- α , and MCP-1.

In detail, human monocytes, peripheral blood mononuclear cells (PBMC) from healthy volunteers were chosen first to study the in vivo relevance. These cells are well-known for their high capacities to induce these signals even ex vivo. In addition, they allow for the identification of possible individual differences. In addition, two human two monocyte cell lines namely U937 (human histiocytic lymphoma cells [144]) and THP-1 we chosen to study kinetic and mechanistic aspects in more detail.

2. Materials and Methods

2.1. Reagents:

- *Para*- Phenylenediamine (PPD)
- *Mono*- Phenylenediamine (MPD)
- *Ortho*- Phenylenediamine (OPD)
- Bandrowski's Base (BB)
- Phytohemagglutinin-M
- NiSO₄
- Dinitrochlorobenzene (DNCB)
- Lipopolysaccharide (LPS)

Supplier

- Sigma, Deisendorf, Germany
- Sigma, Deisendorf, Germany
- Sigma, Deisendorf, Germany
- ICN Biomedicals, USA
- CALBIOCHEM, Darmstadt, Germany
- Sigma, Deisendorf, Germany
- Sigma, Deisendorf, Germany
- Sigma-Aldrich, Steinheim, Germany

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

2.2. Buffy coats

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

2.3. Cell culture of PBMC, human monocyte, and cell lines

Material

- RPMI 1640 Medium with L-Glutamine
- Phosphate Buffered Saline (PBS, pH=7.4)
- Fetal Calf Serum (FCS)
- Penicillin (10000 units; g/mL)
- Streptomycin Sulfat (10 mg/mL)
- Trypan blue
- Fetal Calf Serum (FCS)
- Human AB serum
- Ficoll 400
- Dynalbeads M-450 CD19 (Pan B)
- Dynalbeads M-450 CD2 (Pan T)

Supplier

- PAA, Austria
- Sigma, Deisendorf, Germany
- PAA, Austria
- PAA, Austria
- PAA, Austria
- PAA, Austria
- PAA, Austria
- PAA, Austria
- Sigma, Deisendorf, Germany
- Amersham Biosciences, USA
- Dynal Biotech, Hamburg, Germany
- Dynal Biotech, Hamburg, Germany

2.3.1. Cell lines

- Human Caucasian histiocytic lymphoma (U-937) DSMZ, Hannover, Germany
- Human monocytic leukaemia (Human THP-1) DSMZ, Hannover, Germany

The Human Caucasian histiocytic lymphoma (U-937) was derived from malignant cells of a pleural effusion of 37 year old Caucasian male with diffuse histiocytic lymphoma. The human monocytic leukaemia (Human THP-1) was derived from the peripheral blood of a 1 year old male with acute monocytic leukaemia. The cell lines were obtained from German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ). Cell lines were cultured in RPMI 1640 with L-Glutamine supplemented with 10 % heat-inactivated (at 56°C for 25 minutes) FCS (for cell lines) or human AB serum for PBMC and monocytes, 1% Penicillin and Streptomycin Sulfat cells were seeded at a density of 1×10^6 , and incubated with the antigen at 37°C in a humidified atmosphere of 5% CO₂. After incubation with the compounds, cells were washed for 5 minutes 245×g at room temperature, and counted by trypan blue exclusion.

2.3.2. Isolation of peripheral blood mononuclear cells (PBMC)

PBMC were isolated from Buffy coat of healthy blood donors obtained from the local blood bank. The cell 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 the 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 resuspended the cells in 50 ml PBS and 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 FCS or human A/B serum.

2.3.3. Negative isolation of monocytes

Monocytes were separated from PBMC by depletion of B cells, T cells, natural killer cells, and thymocytes using magnetic anti-CD19 and anti-CD2 beads (Dynal, Hamburg, Germany). Prior usage beads were washed 4 times with PBS/0.1% FCS. Subsequently, they were added to PBMC at a ratio of 1:1.1:1 (PBMC, CD2, and CD19) per 10^7 /mL PBMC. 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 are then collected as unbound cells. The isolated monocytes were washed with PBS and centrifugation at $245\times g$ for 10 minutes (2 times). The resulting monocytes were resuspended in the RPMI 1640 with L-Glutamine supplemented with 10 % heat-inactivated FCS or human AB serum.

2.4 Freezing and Thawing of cells

2.4.1 Freezing of cells

To freeze the cells they should be growing well or known to be in the log phase. The cells were counted, resuspended (5.0×10^6 cells/mL) in freezing media (90% fetal calf serum and 10% DMSO) and transferred into 1.5 mL cryovials. Freezing is performed slowly in a cryocontainer filled with isopropanol (this ensured cooling at a rate of 1°C per minute). After 2 hours the container is first transferred to -20°C for another 2 hours, and then to -80°C freezer for 24 hours. Afterwards, vials were transferred to liquid nitrogen for long-term storage.

2.4.2. Thawing of cells

The cells were quickly thawed in water at 37°C. To quickly remove DMSO, the cell pellet was transferred in 10 ml warm RPMI 1640 supplemented with 10 % FCS, and centrifuged at $245\times g$ for 5 minutes. Supernatant was aspirated and the cell pellet was diluted in 5 mL of prewarmed RPMI 1640 supplemented with 10 % FCS. The resuspended cells were

transferred in the T-25 flask, incubated at 37°C and 5 % CO₂, and then expanded into a T-75 flask after two days.

2.5. The MTT viability test

2.5.1. Assay principle

The assay is based on the cleavage of the yellow tetrazolium salt MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] to purple formazan crystals by metabolic active cells. This cellular reduction involves the pyrimidine nucleotide cofactors NADH and NADPH. The formazan crystals formed are solubilized, and the resulting colored solution is quantified using absorbance at 550-690 nm by a spectrophotometer (Synergy HT, BIO-TEK®, USA).

2.5.2. Assay procedure

U-937 cells (1×10^6 /ml) were grown in microtiter plates (tissue culture grade, 96 wells, flat bottom) in a final volume of 300 µl culture medium per well. Desired concentration of antigens was added to the cells. After the incubation time, 30 µl of the MTT (Roche Biochemicals, Mannheim, Germany) labelling reagent (final concentration 0.5 mg/ml) were added to the cells, and incubated for additional 4 hours (time may vary depending on cell type and experimental design) at 37°C and 5 % CO₂. The cells were centrifuged in the microtiter plate at 400xg for 10 minutes, and the supernatant discarded. The resulting cell pellets were resuspended in 200 µl PBS and 100 µl solubilization solution (provided with the MTT kit) were added into each well. After an overnight incubation at 37°C and 5 % CO₂ the absorbance of each sample was measured at wavelength between 550 and 690 nm.

2.6. Flow Cytometric analysis

The CCR2 protein expression on the cell surface of THP-1 cells was determined after treatment with various antigens by flow cytometry. For staining, cells (1×10^5) were washed with 1 mL PBS, and concentrated by centrifugation at 10000xg for 15 seconds. The cells

were resuspended in 150 μ L PBS, and stained with 3 μ l human anti-CCR2-PE antibodies (R&D Systems, Heidelberg, Germany). Cells were incubated for 20 minutes at room temperature in the dark. Afterwards, excess antibodies were removed by washing with 1 mL PBS and centrifugation (10 minutes at $301 \times g$). The stained cells were then resuspended in approximately 350 μ L PBS for the flow cytometric analysis using 488nm wavelength laser excitation (FACScalibur, cellquest software, B&D Heidelberg, Germany).

2.7. Quantitative Reverse Transcriptase PCR (RT-PCR)

2.7.1 RNA isolation

Total RNA from PBMC, human monocyte and U-937 cells were isolated using the High Pure RNA Isolation Kit (Roche Biochemicals, Mannheim, Germany). The isolated RNA was stored at -70°C . For quantification, the isolated RNA was diluted (1:100) in RNase free water, and the concentration was determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer (Synergy HT, BIO-TEK®, USA). An absorbance of 1 unit at 260 nm corresponds to 40 μ g of RNA per ml ($A_{260}=1.40 \mu\text{g/mL}$). The ratio between the absorbance values at 260 and 280 nm gives an estimate of nucleic acid purity. Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1. Calculation of RNA concentration was calculated as follows: Concentration of RNA ($\mu\text{g/mL}$) = $40 \times A_{260 \text{ nm}} \times \text{dilution factor}$.

2.7.2 Reverse transcription of total RNA (cDNA synthesis)

Materials:

- GenAmp® RNA PCR Applied Biosystems, Weiterstadt, Germany
- dNTPs (100 mM) Roche Biochemicals, Mannheim, Germany

RNA was reverse transcribed into cDNA by a reverse transcriptase (RT) reaction. Frozen RNA was denatured by heating at 95°C for 3 minutes and direct cooling on ice. In a PCR tube, $1 \times$ PCR-buffer, 25 mM dNTPs, 5 mM MgCl_2 , 2.5 μM random hexamers, 1 U Rnase inhibitor, and 2.5 U Mul V reverse transcriptase were mixed with 0.5-1 μ g total RNA in a final volume of 10 μ L. The reaction mixture was incubated with the following

temperatures using an thermal cycler (9700 Perkin Elmer, Weiterstadt, Germany): 10 minutes at 22°C, 15 minutes at 42°C, 5 minutes at 99°C, 5 minutes at 5°C, and stored then at -20°C for longer storage or used immediately.

2.8. Quantification of mRNA using cDNA (RT-PCR)

Quantification of the different mRNAs (TNF- α , IL-1 β , MCP-1, and CCR2) from PBMC, monocytes, and U-937 cells was performed by quantitative reverse transcriptase reaction (QRT-PCR). Therefore, methods were established for each target in order to perform rapid PCR, and simultaneously quantify, and analyze the PCR product by monitoring fluorescence during amplification using the LightCycler™ Instrument (Roche Diagnostic, D-Mannheim, Germany). This is a rapid thermal cycler combined with a micro-volume fluorimeter. In comparison to conventional PCR machines, heating and cooling are controlled by ambient air as the medium for temperature transfer. This rapid thermal cycling technique uses glass capillaries as reaction vessels. Because of the high ratio of surface area to volume of these capillaries, they are highly efficient at transferring heat, thus permitting rapid cycling conditions allowing for 30-40 PCR cycles in 20-30 minutes. During thermal cycling, reactions are monitored on-line as high-precision stepper motors position the samples above the fluorimeter optics for fluorescence measurements (LED=light emitting diode) with peak emission at 470 nm, 530 nm, 640 nm, and 710 nm offering multi-color detection. Furthermore, the glass capillary serves as an optical element for signal collection, piping the light and concentrating the signal at the tip of the capillary.

By using SYBR Green II dye (intercalator) added to the amplification reaction, double stranded (ds) DNA is stained, and the fluorescent signal (measured at 530 nm) increases proportionally with the increasing amount of dsDNA during PCR.

The specificity can be monitored by melting of the product. Every DNA fragment melts at a characteristic temperature, called the melting temperature (T_m), defined as the temperature where 50% of the DNA is single stranded. The LightCycler Instrument is able to monitor the fluorescence continuously, while raising the temperature gradually. When the temperature in the capillary reaches the T_m of the fragment under study, this results in a sharp decrease in fluorescence, because SYBR Green II dye is released from the

amplicon. Product differentiation, smaller products like primer-dimers have lower T_m than specific fragments.

PCR-condition for amplification of TNF- α , IL-1 β , MCP-1 and CCR 2 by the Light Cycler technology

For the reaction were prepared 10 μ l of following components as described below:

Materials:	Supplier
- Forward Primer	TibMolBiol, Berlin, Germany
- Reverse Primer	TibMolBiol, Berlin, Germany
- FASTSYBR Green	Roche Biochemicals, Mannheim, Germany
- MgCl ₂	Roche Biochemicals, Mannheim, Germany

2.8.1. Primers

Tab. 1. Primer sequences used for quantification of the mRNA by RT-PCR

Gene symbol	Sequence 5'→3' forward primer	Sequence 5'→3' reverse primer
CCR2	TCTCACTGCCCTATGCCTCT	GGATTGAACAAGGACGCATT
IL-1 β	GACACATGGGATAACGAGGC	ACGCAGGACAGGTACAGATT
IFN γ	GCAGGCAGGACAACCATTAC	GCATCCAAAAGAGTGTGGAG
MCP-1	CAATAGGAAGATCTC AGTGC	GTGTTCAAGTCTTCGGAGTT
TNF- α	CCCAGGGACCTCTCTAATC	ATGGGCTACAGGCTTGCTACT

2.8.2. Procedure:

	INFγ	TNFα	IL-1β	MCP-1	CCR2
Sterile deionized H ₂ O	1.8 μ L	2.1 μ L	2.3 μ L	2.6 μ L	3 μ L
MgCl ₂ (25 mM)	1.2 μ L	1.2 μ L	0.9 μ L	1.2 μ L	0.6 μ L
Forward Primer (100 pmol/ μ l)	0.5 μ L	0.35 μ L	0.4 μ L	0.1 μ L	0.2 μ L
Reverse Primer (100 pmol/ μ l)	0.5 μ L	0.35 μ L	0.4 μ L	0.1 μ L	0.2 μ L
Fast SYBR Green I	1 μ L	1 μ L	1 μ L	1 μ L	1 μ L *
cDNA	5 μ L	5 μ L	5 μ L	5 μ L	5 μ L
<hr/>					
Final volume	10 μ L	10 μ L	10 μ L	10 μ L	10 μ L

Accordingly, mixture was pipetted into precooled LightCycler Capillary, centrifuged at $100 \times g$ for 5 seconds (1000 rpm in a standard bench top microcentrifuge) and placed into the LightCycler Instrument for gene-specific amplification.

2.8.3. Light Cycler programs

Light Cycler program for TNF α

	Target Temperature	time sec	slope °C/sec	Acquisition Mode	Cycles
<u>Preincubation</u>	95	600	20	None	1
<u>Amplification</u>					40
Denaturation	95	5	20	None	
Annealing	55	5	20	None	
Elongation	72	14	20	Single	
<u>Melting Curve</u>					1
Denaturation	95	0	20	None	
Annealing	58	10	20	None	
Melting	95	0	0.1	Continuous	
<u>Cooling</u>	40	30	20	None	1

Light Cycler program for IL-1 β

	Target Temperature	time sec	slope °C/sec	Acquisition Mode	Cycles
<u>Preincubation</u>	95	600	20	None	1
<u>Amplification</u>					50
Denaturation	95	10	20	None	
Annealing	62	10	20	None	
Elongation	72	10	20	Single	

Melting Curve 1

Denaturation	95	10	20	None
Annealing	45	10	20	None
Melting	95	0	0.2	Continuous

Cooling 1

	40	30	20	None
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Light Cycler program for IFN γ

	Target	time	slope	Acquisition	Cycles
	Temperature	sec	°C/sec	Mode	
<u>Preincubation</u>					1
	95	600	20	None	
<u>Amplification</u>					40
Denaturation	95	5	20	None	
Annealing	55	5	20	None	
Elongation	72	14	20	Single	
<u>Melting Curve</u>					1
Denaturation	95	0	20	None	
Annealing	58	10	20	None	
Melting	95	0	0.1	Continuous	
<u>Cooling</u>					1
	40	30	20	None	

Light Cycler program for MCP-1

	Target	time	slope	Acquisition	Cycles
	Temperature	sec	°C/sec	Mode	
<u>Preincubation</u>					1
	95	600	20	None	
<u>Amplification</u>					50
Denaturation	95	0	20	None	
Annealing	60	5	20	None	
Elongation	72	8	20	Single	
Measuring	80	8	20	Single	

<u>Melting Curve</u>					1
Denaturation	95	0	20	None	
Annealing	65	10	20	None	
Melting	95	0	0.2	Continuous	
<u>Cooling</u>					1
	40	30	20	None	

Light Cycler program for CCR2

	Target	time	slope	Acquisition	Cycles
	Temperature	sec	°C/sec	Mode	
<u>Preincubation</u>					1
	95	600	20	None	
<u>Amplification</u>					40
Denaturation		95	10	20 None	
Annealing	64	10	20	None	
Elongation	72	30	20	Single	
Measuring	80	8	20	Single	
<u>Melting Curve</u>					1
Denaturation	95	0	20	None	
Annealing	60	10	20	None	
Melting	95	0	0.1	Continuous	
<u>Cooling</u>					1
	40	30	20	None	

2.9. Traditional Polymerase Chain Reaction (PCR)

Materials:

- Forward primer
- Reverse primer
- Taq DNA polymerase
- 10 × PCR-Puffer TaqGold
- dNTPs

Supplier

- TibMolBiol, Berlin, Germany
- TibMolBiol, Berlin, Germany
- Applied Biosystems, Weiterstadt, Germany
- Applied Biosystems, Weiterstadt, Germany
- Applied Biosystems, Weiterstadt, Germany

5 μ l of cDNA (1:5 diluted) was added to 10-50 pmol/ μ L forward and reverse primer (see Tab. 1), 5 units Taq DNA polymerase, 200 μ M of dNTP, in 1 \times PCR-Puffer TaqGold (contains 15 mM MgCl₂) to a total volume of 100 μ L. The PCR was performed by the following PCR program. After an initial denaturation for 10 minutes at 95°C, DNA fragments were amplified 1 minute at 95°C, 1 minute at the annealing temperature for that gene, and 2 minutes at 72°C.

40 cycles were performed. A final extending step was added for 2 minutes at 72°C. PCR product was purified from primers using MicroSpin™ S300 HR Columns (Amersham Biosciences, New Jersey, USA). Subsequently, DNA concentration was determined in a spectrophotometer. To verify the DNA purity, 10 μ L of DNA-Fragment were loaded on a 3 % agarose gel, and separated for 20 min at 500V.

2.10. Agarose gel electrophoresis

Material:

- Agarose (SeaKem®Leeagarose)
- DNA Marker 50-1000 bp
- Loading Buffer:
 - .25 % Orange G
 - 0.25 % Ficoll 400
- TAE Buffer:
 - 40 mM Tris
 - 20 mM acid acetic
 - 1 mM EDTA, pH 7.8
- Ethidium bromide

Supplier

- FMC, Biozym, Heidelberg, Germany
- FMC, Biozym, Heidelberg, Germany
- Sigma, Deisenhofen, Germany
- Sigma, Deisenhofen, Germany
- Amersham Biosciences, USA
- Sigma, Deisendorf, Germany

- Merck, Darmstadt, Germany

Procedure

2 g agarose (2%) was dissolved in 100 mL 1 \times TAE electrophoresis buffer, heated in a microwave oven until completely melted. After cooling the solution to about 60°C, 1 μ L 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 μ L 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.

2.11. Determination of cytokines and chemokines using Enzyme-linked Immunosorbant assay (ELISA)

The measurement for TNF α , IL-1 β , MCP-1, and MCP-3 were performed on PBMC, human monocytes and U-937 using TNF α , TNF α hypersensitivity, IL-1 β , IL-1 β hypersensitivity, MCP-1, and MCP-3 ELISA kits obtained from R&D Systems (Heidelberg, Germany).

This technique is based on trapping or capture of the protein by one antibody and the detection by another antibody according to the manufacturers' instructions.

Cell culture supernatant was separated from cell pellets after centrifugation for 10 minutes at 301 \times g and absorbance was measured (Synergy HT, BIO-TEK®, Badreichenhall, Germany).

3. Results

Cytokines and chemokines are both crucial danger signals for the induction phase of ACD. IL-1 β , TNF- α and MCP-1 are such early induced mediators. In addition is TNF- α involved in the regulation of the MCP-1 expression, while IL-1 β , one of the earliest induced cytokines during contact sensitization, stimulates TNF- α production. Therefore, we investigated the effects of PPD on the expression of these molecules.

3.1. Cytotoxicity of PPD using MTT in U937

In order to control for unspecific effects of the test compounds such as cellular proliferation, viability or activation in response to them, we conducted first the so called MTT (3-[4, 5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromid) test in U-937 cells. The MTT [145] is recommended as cytotoxicity assay in viable cells. The assay is based on cleavage of the yellow tetrazolium salt, MTT, to purple formazan crystals by the active mitochondrial enzymes in metabolic active cells.

First, we studied whether PPD reacts with the MTT and other reagents itself. We also included BB, an autoxidation product of PPD called Bandrowski's base, and LPS a positive control. Figure 3 shows that only PPD reacted in the absence of cells with the detection dye. We concluded from these preliminary results, that we had to wash the cells carefully after treatment to avoid interactions of PPD with the detection reagent. The summary of the viability tests are shown in Figure 4. The assays were performed in the presence and absence of serum. The results indicated that the viability of U-937 cells is not inhibited by the indicated concentrations of PPD and BB neither in the presence or absence of serum. Only a partial but not significant reduction of cell viability was observed for PPD in absence of serum. Altogether, PPD had no toxic effect on U-937 cells.

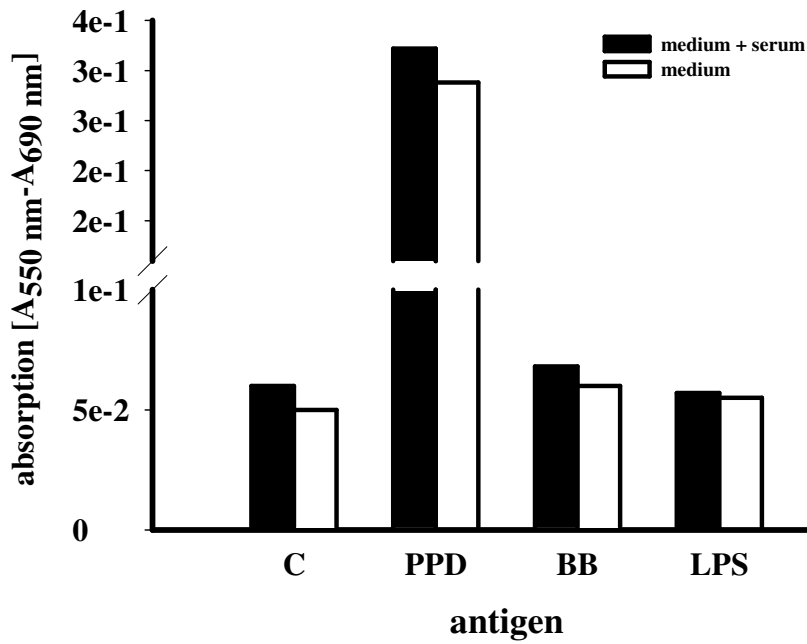


Figure 3. Absorbance of culture media (RPMI 1640) and serum in the presence of antigens and absence of cells. RPMI + serum (10 % FCS) +MTT and RPMI + MTT were incubated with PPD (50 μ M), BB (50 μ M), LPS (50 ng/mL) for 2h. Absorption was quantified at 550 nm and 690 nm.

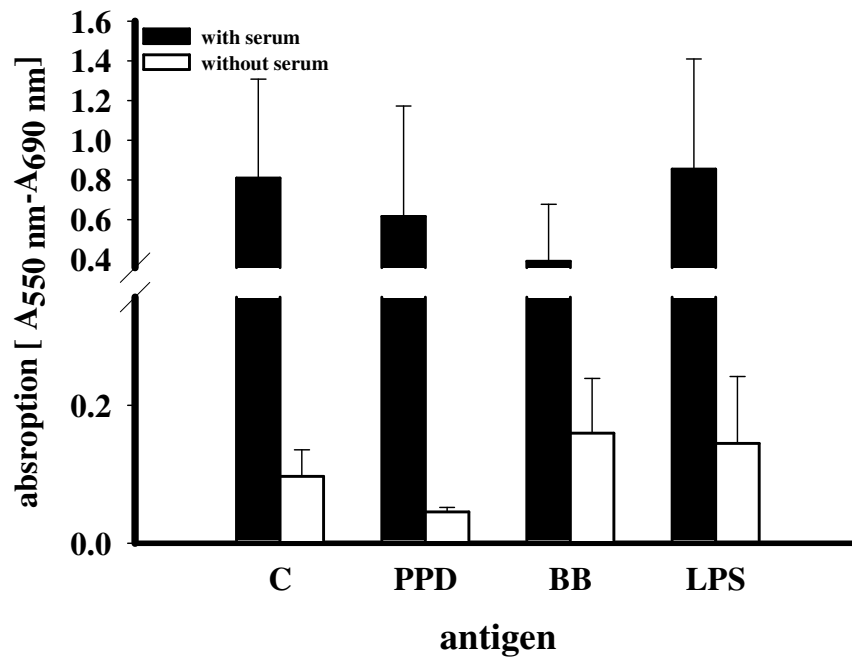


Figure 4: U-937 cells (1×10^6 /mL) were incubated with solvent (medium, control, (C)), PPD (50 μ M), BB (50 μ M) and LPS (50 ng/mL) for 24 h in culture medium (RPMI) with 10 % FCS and without serum (RPMI). Absorption was quantified by 550 nm and 690 nm. Results are presented as mean and SE (n=5).

3.2. The influence of PPD on cytokines and chemokines in antigen presenting cells

The production of pro-inflammatory cytokines such as IL-1 and TNF- α after antigen encounter is an hallmark for the induction of ACD [4, 5, 146]. These danger signals are first expressed by various cells at the contact site. Then, TNF- α induces MCP-1 and other chemokines, more cytokines, growth factors, and cell-surface receptors that allow for the immigration of further cells to the hapten-contact area. Such cells include haematopoietic cells, neutrophils, memory T cells, monocytes, and macrophages. They all contribute to further enhancement of the danger signals, inflammation [147], and migration of the LCs. In addition, both IL-1 β and TNF- α as well as IL-4 and IFN- γ have been shown to regulate the expression of MCP-1 [84, 148, 149]. The knowledge in humans is more limited especially because generated DCs from human monocytes as model for LCs are not able or show only a modest IL-1 β mRNA increase in vitro after incubation with contact allergens [150, 151].

Thus, we studied the effects of PPD on IL-1 β , TNF- α and MCP-1 in PBMC and isolated human monocytes from healthy individuals, U-937 and THP-1 cells. BB was also included. We found in previous studies optimal proliferations for PPD around 5.0 $\mu\text{g/mL}$ with a narrow range without toxic side effects [138]. Therefore, we used in this study predominantly that concentration.

3.2.1. IL-1 β expression in PBMC and human monocytes

The IL-1 β kinetics is best studied in mice where it is the earliest epidermal cytokine to be up regulated following 30 minutes after DNCB application to the skin. Based on these data, we studied the effects of the antigens in PBMCs from healthy individual donors after 45 minutes and up to 24 hours. Surprisingly, we found a time dependent decrease of IL-1 β mRNA expression by PPD, measured in several independent experiments (n=3). As an example, shows figure 5 the results for all antigens. PPD clearly decreased the IL-1 β mRNA already significantly after 2 hours in PBMC. In contrast was BB not able to decline the mRNA of IL-1 β . A significant up regulation was found after 2 and 24 hours for BB and LPS.

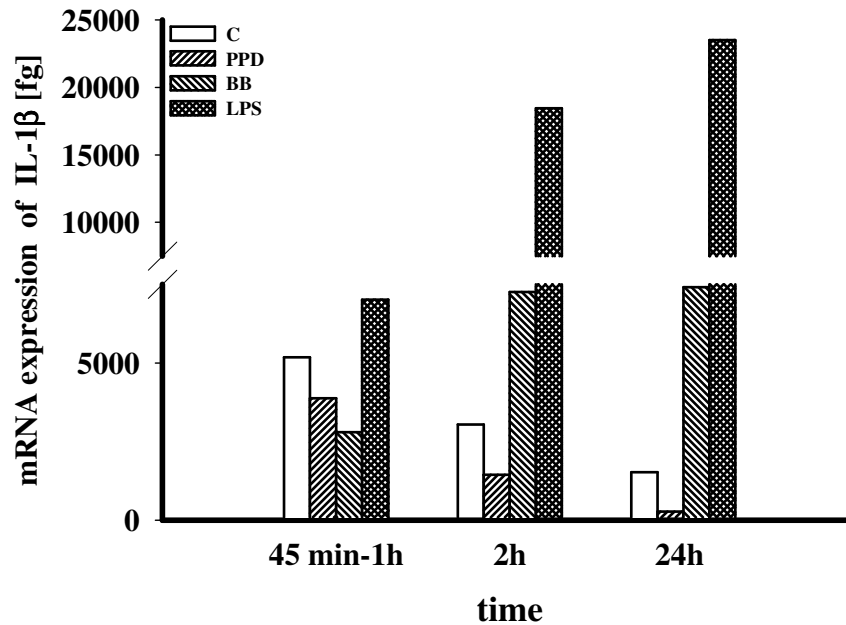


Figure 5. IL-1 β mRNA expression of PBMC estimated by quantitative real-time RT-PCR. PBMC (1×10^6 /mL) were cultured with RPMI + 10 % human AB serum (C), treated with PPD (50 μ M), BB (50 μ M), LPS (50 ng/mL) and incubated for 45 min-1h, 2 h, and 24h.

3.2.2. IL-1 β protein secretion of human PBMC and human monocytes

We were wondering whether PPD also decreased the protein level of IL-1 β and investigated this aspect in PBMC after hapten contact. In this series of experiments we included a time course (2, 4, 8, and 24h).

Due to donor differences varied the results widely (by 10 fold, $n=5$), and representative results are shown. In agreement with the mRNA results a decrease of the secreted IL-1 β protein levels were found by analyses of cell culture supernatants by ELISA. PPD clearly decreased the IL-1 β protein levels starting at 4 hours and lasting up to 24 hours, while LPS increased IL-1 β protein levels under these conditions. In contrast, the IL-1 β protein levels were not significantly changed after treatment with BB (figure 6A). To gain more insights into the specificity of the observed effects we additionally studied the effects of meta-Phenylenediamine (MPD) and ortho-Phenylenediamine (OPD) over a time period of 24 hours. Figure 6B shows the results for OPD and MPD. These results clearly indicate that only PPD was able to decrease the IL-1 β mRNA and protein release significantly. This observed highly specific effect of PPD was then further investigated in purified human

Results

monocytes from PBMC. These cells are major antigen presenting cells and precursor for DC/LCs. In addition, the monocyte/macrophage cell population has been suggested as a potential dermal source of IL-1 β [38].

For these reasons, the influences of PPD on IL-1 β protein were studied in monocytes derived from healthy donors. Incubation times varied between 2 and 24 hours. Again, PPD-treated monocytes significantly decreased IL-1 β protein levels already after 30 minutes (figure 7A) and continued up to 24 hours (figure 7B). IL-1 β protein were not changed significantly above control levels by BB whereas treatment of the monocytes with LPS and Phytohemagglutinin (PHA)-M, a mitogen and a second positive control, caused a significant increase in IL-1 β levels.

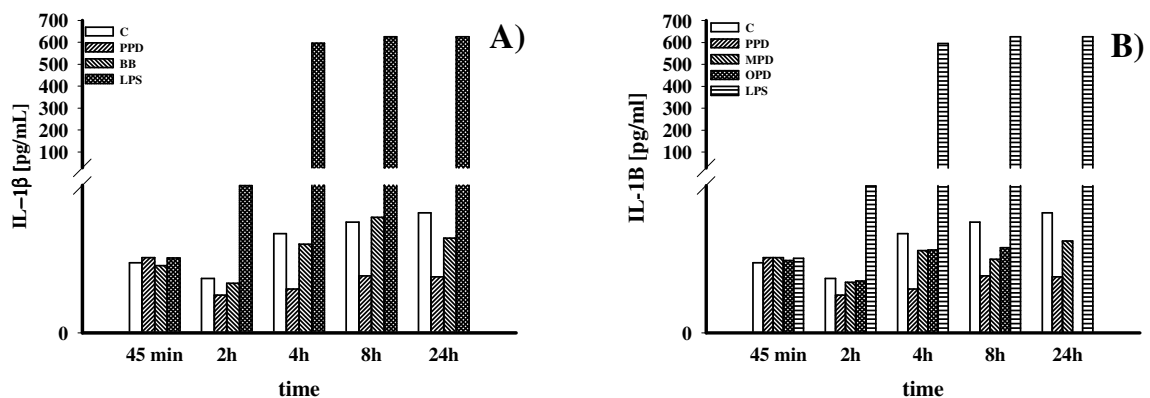


Figure 6. Kinetics of IL-1 β protein release by PBMC. PBMC (1×10^6 mL) were cultured with RPMI + 10 % human AB serum (C), treated with PPD (50 μ M), BB (50 μ M) and LPS (50 ng/mL) and incubated for 45 min-1h, 2 h, 4h, 8h and 24h (panel A). Panel B shows the results for MPD (50 μ M) and OPD (50 μ M) in comparison to PPD and LPS. The IL-1 β sensitivity of is typically 4 pg/ml

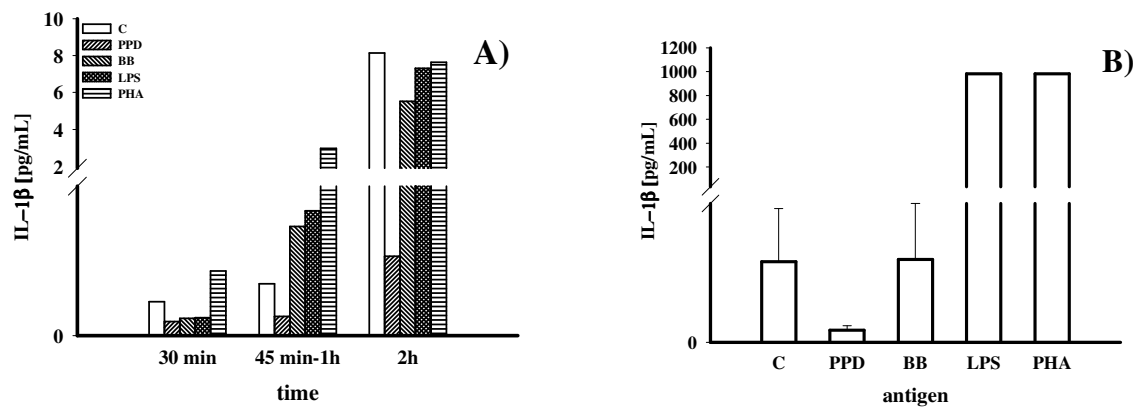


Figure 7. IL-1 β release in human monocytes . Human monocytes (1×10^6 / mL) were cultured with RPMI + 10 % human A/B serum (C), treated with PPD (50 μ M), BB (50 μ M), LPS (100 ng/mL) and PHA (25 μ g/mL) for 30 min, 45 min, and 2 h (panel A). The results for 24 h are depicted in panel B (mean and SE for 3 experiments).

3.2.3. TNF- α expression of PBMC and monocytes

To verify that PPD modifies as likely consequence of the observed reduced IL-1 β expression levels also TNF- α we tested that in our model. First we investigated the mRNA expression in PBMC within 24 hours after incubation with PPD, BB, and LPS. The results are shown in figure 8. Interestingly but expected, PPD decreased TNF- α mRNA levels. This occurred at later time points. The effect was moderate after 45 minutes but a significant reduction was observed in PBMCs after 24 hours. The latter was not observed for BB or LPS. For LPS, an increase was detected already after 1 hour, the highest levels were found after 2 hours, and levels decreased transiently after 24 hours. An mRNA increase was found for BB only after 24 hours.

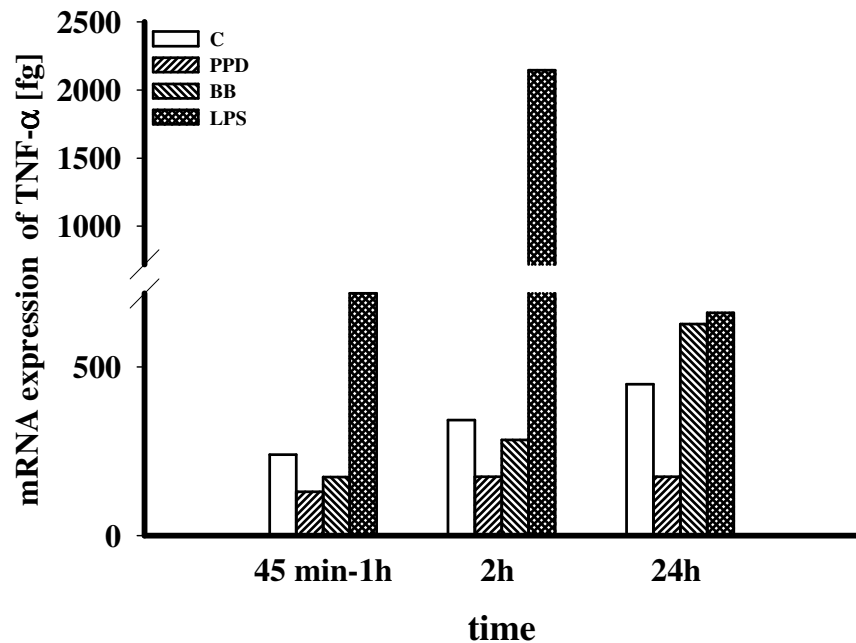


Figure 8. Time course of TNF- α mRNA expression of PBMC estimated by quantitative real-time RT-PCR. PBMC (1×10^6 /mL) were cultured with RPMI + 10 % human A/B serum alone (C) and treated with PPD (50 μ M), BB (50 μ M), LPS (50 ng/mL) for 45 min-1h, 2h, and 24h.

3.2.4. TNF- α secretion of PBMC and human monocytes

To confirm the results we determined the TNF- α protein release in cell culture supernatants of PBMC and monocytes after incubation with the antigens. The aim was to determine whether PPD is able to inhibit TNF- α also on the protein level in PBMC and monocytes similar to the TNF- α mRNA levels. ODP and MPD were also included in this series of experiments using PBMCs as well as monocytes. The results are depicted in figure 9 and 10. As expected from the previous set of experiments only PPD showed a significant reduction of the TNF- α protein levels detected already after 2 hours. Neither BB nor MPD or OPD were able to decrease the TNF- α of protein levels in these cells.

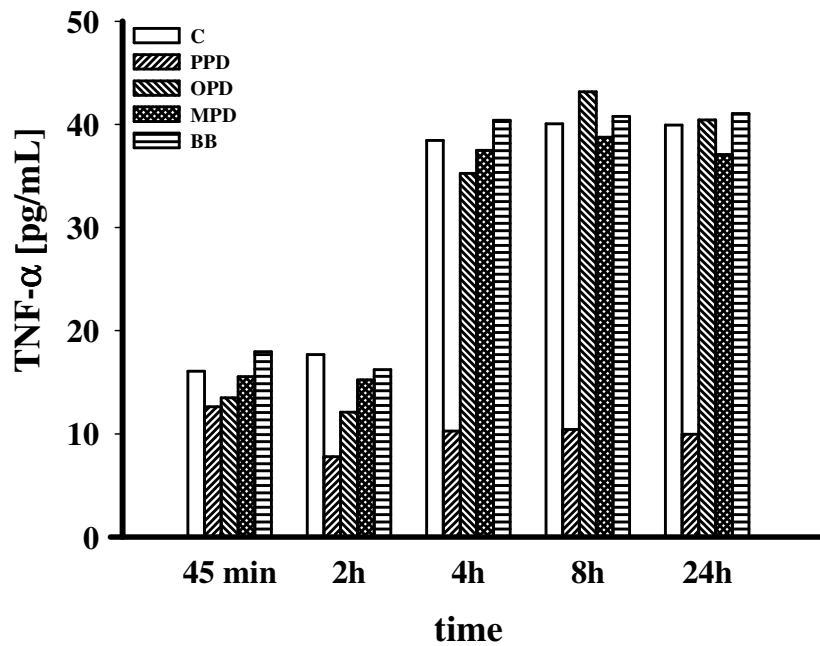


Figure 9. Time dependent TNF- α secretion by PPD, MPD, OPD, and BB in cell culture supernatants of PBMC. PBMC (1×10^6 /mL) were cultured with RPMI + 10 % human AB serum alone (C) and treated with PPD (50 μ M), MPD (50 μ M), OPD (50 μ M), and BB (50 μ M) for 45 min, 2h, 4h, 8h, and 24h. The sensitivity of TNF- α assay was 0.06-0.32 pg/mL.

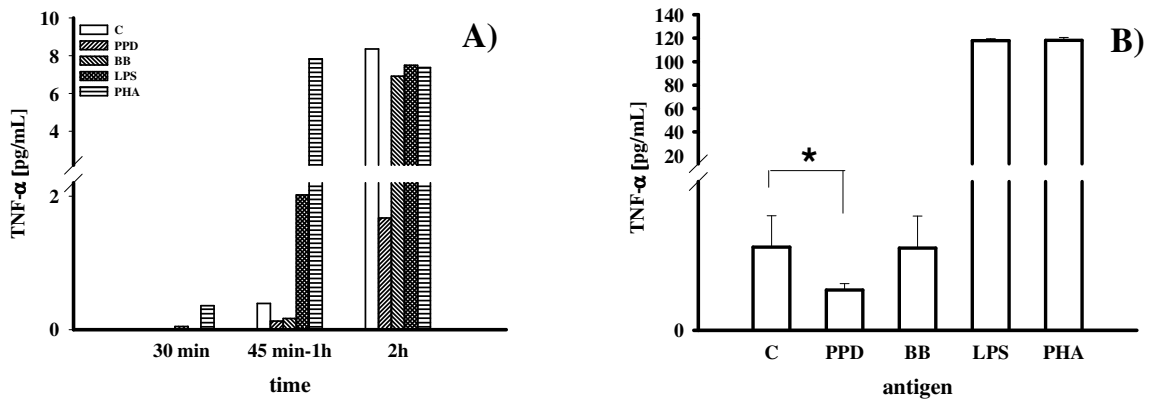


Figure 10. Time dependent TNF- α levels in cell culture supernatants of human monocytes. Human monocytes (1×10^6 /mL) were cultured with RPMI + 10 % human AB serum alone (C), treated with PPD (50 μ M), BB (50 μ M), LPS (100 ng/mL) and PHA (25 μ g/mL) and incubated for 30 and 45 min, 2h (Panel A), and 24h (panel B).

3.2.5. The impact on MCP-1 expression in PBMC

As above mentioned, both IL-1 β and TNF- α have been shown to regulate the expression of MCP-1. Furthermore, beside MCP-1, RANTES and MDC are also frequently detected chemokines in hapten-induced allergic contact dermatitis [56, 152]. This may point to the predominance of mononuclear cells, even lymphocytes and macrophages the areas of the antigen-exposed skin. In addition, regardless of the applied allergen MCP-1 was the first chemokine that was found up-regulated after the elicitation phase of ACD. We therefore investigated the regulation of MCP-1.

The results are shown in Figure 11. Again LPS [105, 110] and PHA [105, 153, 154] were used as two positive controls for MCP-1. In addition, IFN γ was included as a strong inducer for MCP-1 [110, 153]. We demonstrated again that only PPD inhibited MCP-1 mRNA expression after 45 minutes up to 24 hours in PBMC while IFN γ induced the expression of MCP-1 mRNA clearly.

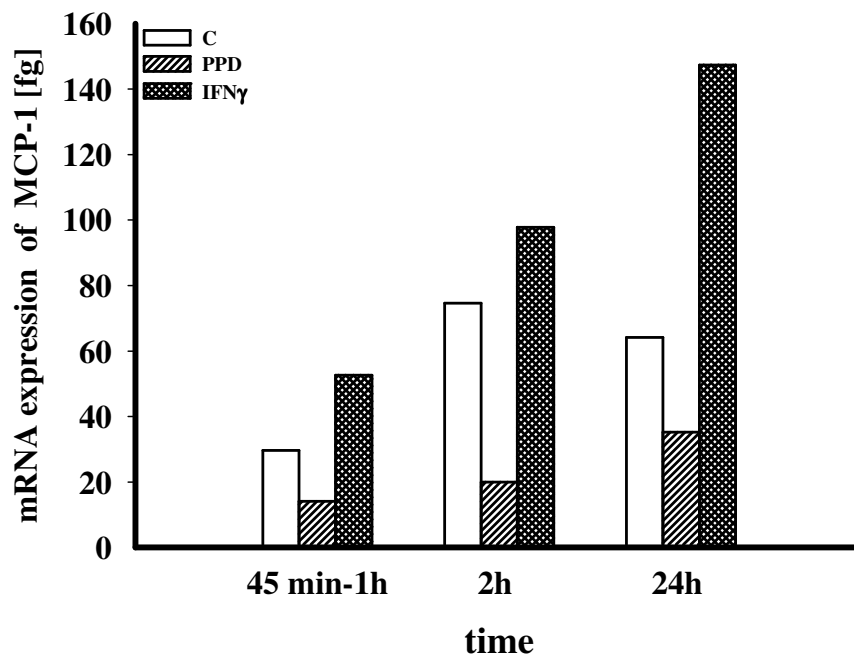


Figure 11. Time dependent MCP-1 mRNA expression in PBMC estimated by quantitative real-time PCR. PBMC (1×10^6 mL) were cultured with RPMI + 10 % human AB serum alone (C) treated with PPD (50 μ M), IFN γ (50 ng/mL) incubated for 45 min-1h, 2h and 24h.

3.2.6. Protein levels of MCP-1 and MCP-3

Infiltrating monocytes, resident keratinocytes as well as DCs themselves are sources for increased MCP proteins that are essential for successive boosts of lymphocyte arrival at the contact area of the allergen. To further clarify that the influence on the mRNA levels are indeed comparable to protein levels, we quantified them in the cell culture supernatants of PBMC and monocytes. This was studied for PPD, MPD, OPD as well as BB. MCP-3 was also included because it also is expressed by monocytes and binds to CCR2 with high affinity.

Figure 12 summarizes the results for MCP-1. Interestingly, we reproduced our previous findings, and verified our results regarding the specificity for PPD.

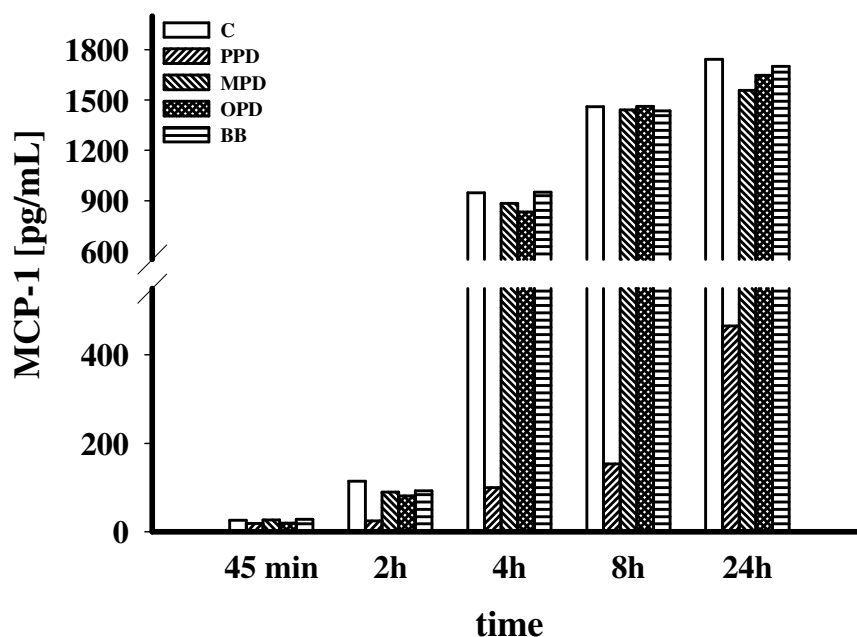


Figure 12. Antigen-specific MCP-1 release in PBMC. PBMC (1×10^6 mL) were cultured with RPMI + 10 % human AB serum alone (C), treated with PPD (50 μ M), MPD (50 μ M), OPD (50 μ M), BB (50 μ M), and IFN γ for 45 min, 2h, 4h, 8h and 24h. The sensitivity of MCP-1 assay is typically less than 5.0 pg/mL.

Figure 13 (A, B) shows the comparison of the gained MCP-1 and MCP-3 levels in monocytes. Unexpectedly, PPD caused a stronger down regulation of MCP-3 (9.5 fold) compared to MCP-1 (4.87 fold). Both LPS and PHA were not effective as positive controls for MCP-3. It was possible that LPS and PHA did not increase the MCP levels in the

presence of human AB serum. This was tested by using fetal calf serum (FCS) instead of human AB serum during cell culture and antigen incubation. Indeed, the protein levels were enhanced in the presence of FCS using monocytes from the same blood donor (figure 13C). After further investigations it became clear that culture media with only 5 % human AB serum gave comparable results for MCP-1 and MCP-3 (data is not shown). Similar results were reported from other groups. In this respect mentioned Minty and co-workers that they also believe that LPS is not a consistent good inducer of MCP-1 and MCP-3 mRNA in vitro [153]. The results of them and our results clearly demonstrate that the MCP-1 release in vitro varies dependent on the culture conditions. On the other side provide the experiments with different culture conditions further evidences that the observed strong effects of PPD are indeed substance-specific, and independent from different culture media.

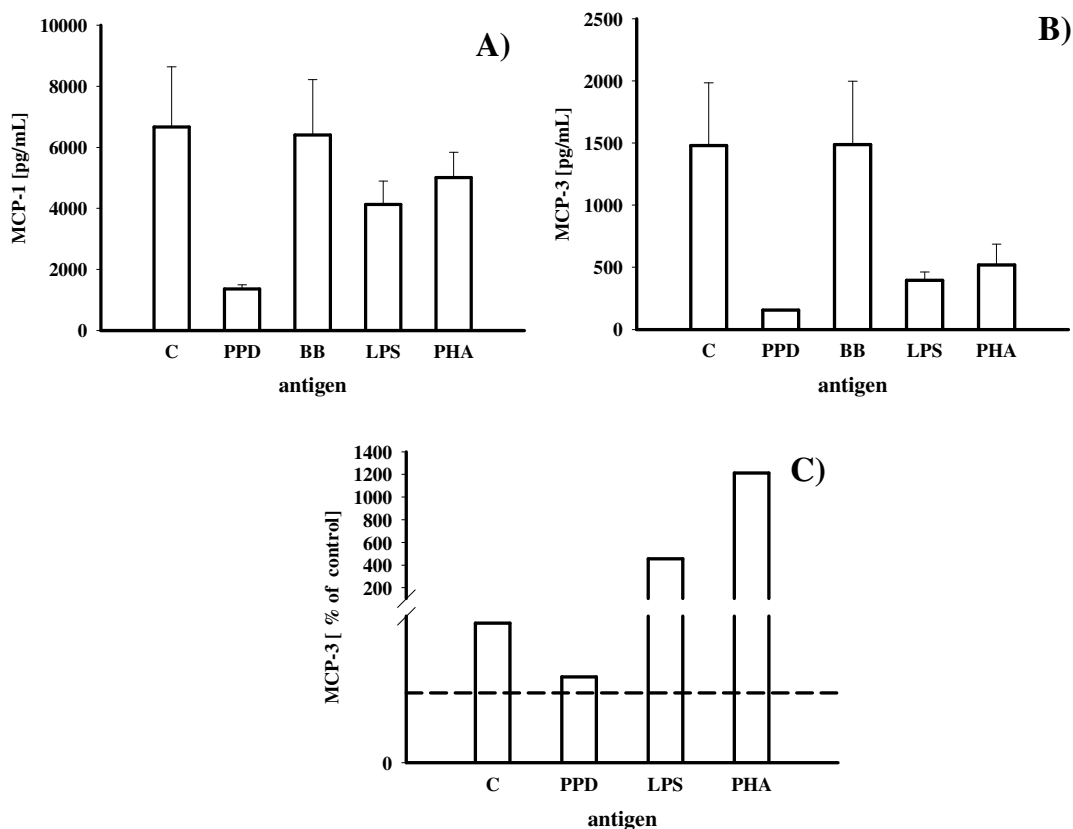


Figure 13. MCP-1 (A), MCP-3 (B, C) release in human monocytes. Human monocytes (1×10^6 mL) were cultured with RPMI + 10 % human AB serum (A, B) or with RPMI + 10 % FCS (C). Cells were treated with medium only (C), PPD (50 μ M), BB (50 μ M), LPS (100 ng/mL), and PHA (25 μ g/mL) for 24 h. Results are presented as mean and SE. The sensitivity of MCP-3 ranged from 0.29-8.52 pg/mL.

3.2.7. CCR2 expression in PBMC

The impact of PPD on MCP-1 and its receptor CCR2 was first investigated in primary cells using PBMCs by quantification of their mRNAs. We included several informative controls into this set of experiments. LPS was used as an active down regulator of CCR2 [113, 155], and PHA was included to see the impact of a mitogen-induced proliferation on CCR2 mRNA levels. Here, we found a significant increase of the CCR2 mRNA in the presence of PPD (figure 14). In contrast, LPS decreased rapidly the CCR2 mRNA confirming other reports. As expected, the mitogen PHA induced the CCR2 expression significantly only after 2 hours. These results for PPD were clear and reproducible. They were either based on an active CCR2 up regulation or the consequence of a fact auto regulation pathway induced by the decreased MCP-1 levels. In order to gain better insights to the underlying mechanisms, the effects of PPD were then studied in two different cell lines. The usage of the monocytic cell line U937 allowed for investigations in the absence of CCR2 because they do not express the CCR2 protein at their surface (data not shown). In contrast, THP-1 cells could be used for the CCR2 receptor regulation.

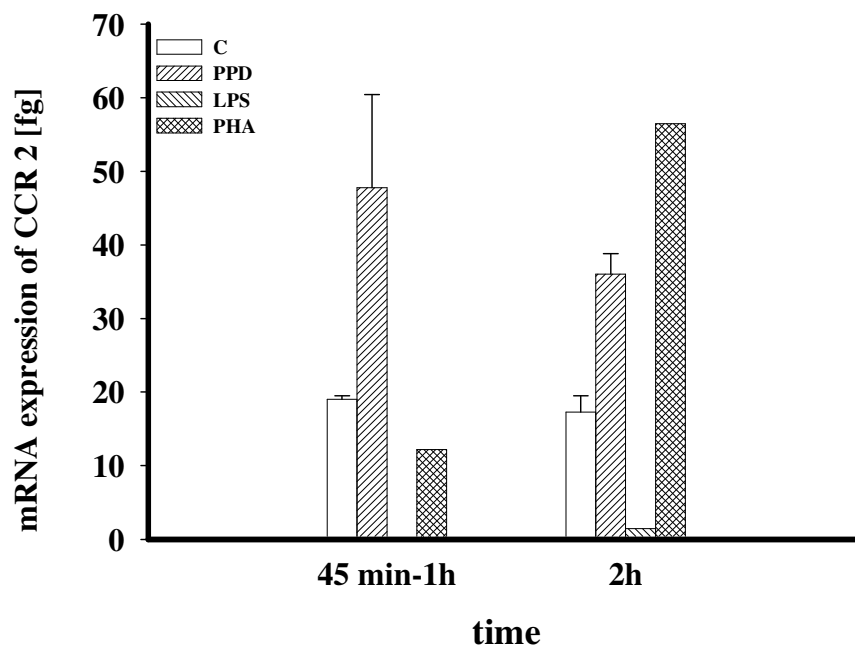


Figure 14. Time dependent CCR2 mRNA expression of PBMC estimated by quantitative real-time PCR. PBMC (1×10^6 /mL) were cultured with RPMI + 10 % human AB serum alone (C), treated with PPD (50 μ M), LPS (50 ng/mL), and PHA (25 μ g/mL) for 45 min-1h, and 2h.

3.2.8. The influence of PPD on the MCP-1 expression in U-937

In addition to the above mentioned mechanistic aspects, the two cell lines helped validating the findings independently from blood donor differences. These cells are fast growing cells and the resulting large quantities are helpful when studying dose response effects. In addition to this technical aspect, the question whether PPD can indeed modify the MCP-1 and CCR2 in persistently growing cells could be answered.

Therefore, U-937 cells were incubated for 4 hours with increasing concentrations of PPD (1 μ M-200 μ M). 2,4-dinitrochlorobenzene (DNCB) was used as an independent chemical sensitizer [156], IL-4 as positive control for CCR2, IFN γ as positive control for MCP-1.

Figure 15 showed that PPD was again able to inhibit MCP-1 mRNA expression in a dose dependent manner in the absence of CCR2 signalling. This was confirmed on the protein level both in the absence and presence of serum after 24 hours. The absolute levels were low in these cells, therefore earlier time points did not allow for the quantification of released MCP-1 (data not shown).

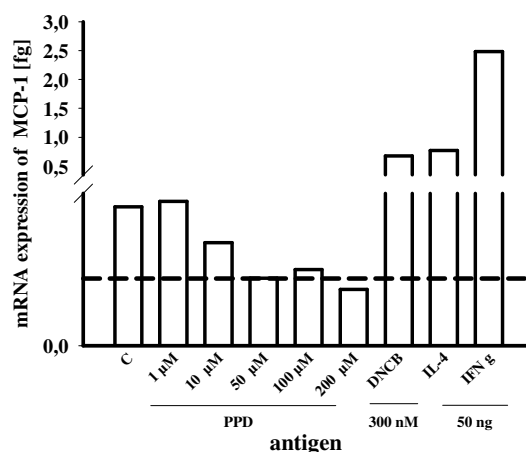


Figure 15. Dose-response effects of compounds on the MCP-1 mRNA in U-937 estimated by quantitative real-time RT-PCR. U-937 (1×10^6 mL) were cultured in RPMI alone (C) and treated with PPD (1, 10, 50, 100 and 200 μ M), DNCB (300 nM), IL-4 50 (ng/ mL) and IFN γ (50 ng/ mL) for 4h.

3.3. The influence of PPD on CCR2 surface protein expression in THP-1

Despite some obvious differences as being an immortalized cell line, the responses of THP-1 are normally very similar to those reported from human monocytes [114]. They

express functional CCR2 on their surface in detectable amounts (about 5000 per cell), and the impact of PPD on the surface expression of CCR2 was studied by flow cytometry. In preliminary experiments we established the best conditions for reproducible detection of this sensitive receptor. Based on published data THP-1 cells were incubated in RPMI medium with 0.5% FCS [157]. Figure 16 showed that cells needed a period of 4 hours after washing and plating them into the test chambers prior to the addition of compounds. In these series PPD increased the CCR2 expression significantly after 4 hours.

In the following series of experiments the impacts of PPD, BB, and a well-known sensitizer NiSO₄ [158] was studied in more detail.

Figure 17 shows the histograms for the PPD- and NiSO₄- induced surface expression of PPD. PPD up regulated CCR2 expression in THP-1 in a time- and dose dependent manner. In the presence of 50 μ M PPD 5% positive cells were detected after 6 hours and 10.6% after 12 hours. This response was increased further in the presence of 200 μ M PPD (8.1% and 13.7 % respectively). No significant time differences were found in the presence of NiSO₄.

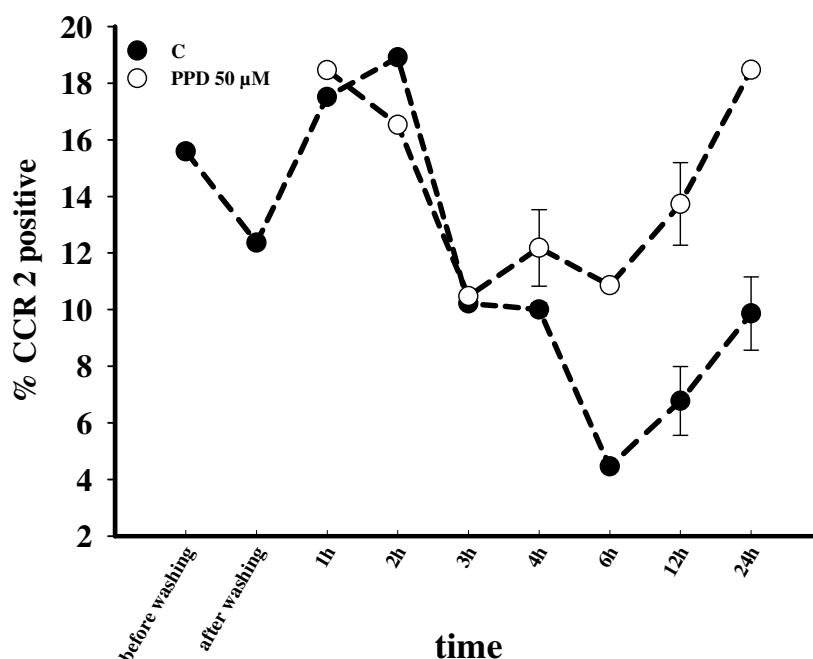


Figure 16. Time- and treatment dependent CCR2 expression of THP-1 cells (percentage of positive cells) THP-1 (1×10^6 / mL) were cultured in RPMI + 0.5 % FCS alone (C) and with PPD (50 μ M) for the indicated time points. Cells (1×10^5) were stained with PE-conjugated anti-CCR2 antibodies. To detect unspecific bindings, cells were stained with specific isotype controls, and analysed by flow cytometry using FACScalibur (Becton Dickinson, Heidelberg, Germany). The bars show the mean and SE, $p < 0.05$.

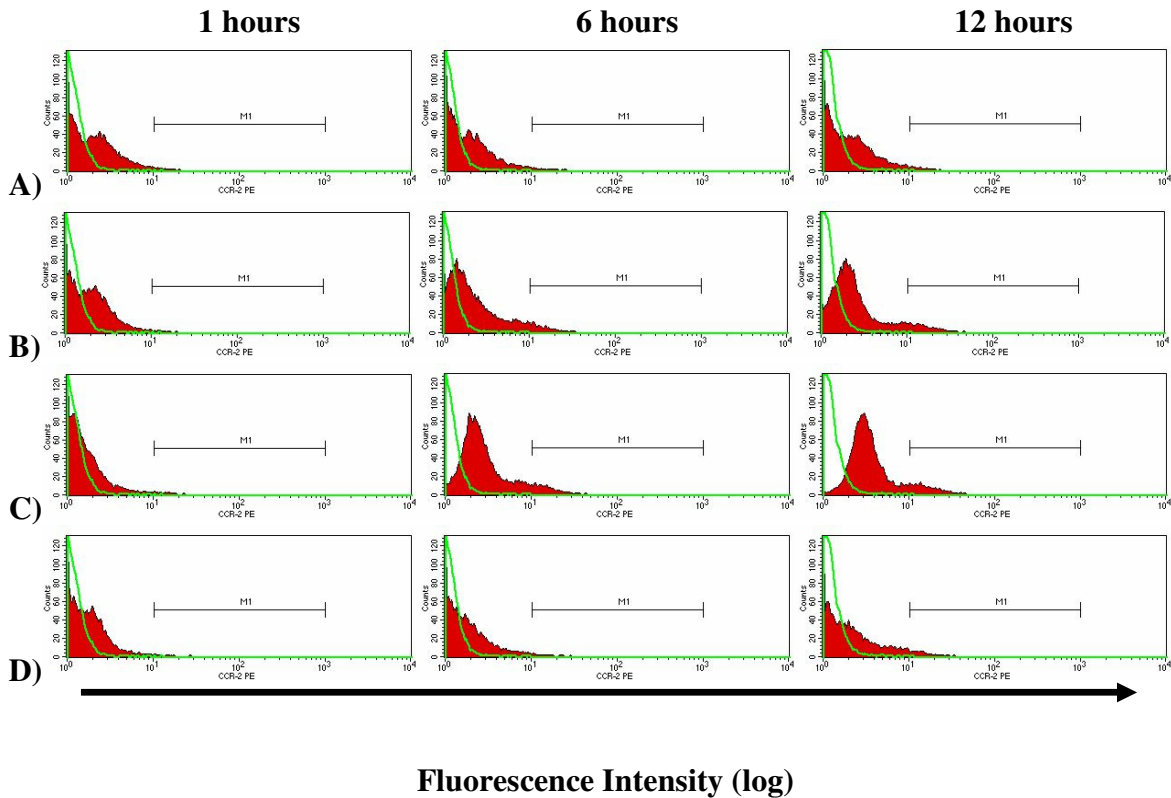


Figure 17. Time-dependent CCR2 expression of THP-1 cells (histogram) THP-1 ($1 \times 10^6/\text{mL}$) were cultured in RPMI + 0.5 % FCS, treated with PPD and NiSO₄ for 1h, 6 h, and 12 h. Cells (1×10^5) were stained with PE-conjugated anti-CCR2 antibodies. To detect unspecific bindings, cells were stained with specific isotype controls (green), and analysed by flow cytometry using FACScalibur (Becton Dickinson, Heidelberg, Germany). A) Untreated cells, B) 50 μM PPD, C) 200 μM PPD, D) 500 μM NiSO₄.

Figure 18 shows the time course for PPD, BB, and NiSO₄. Panel A shows that PPD selectively caused a significant time-dependent increase in CCR2 surface protein expression after 6 hours. No such up regulation was found for BB (figure 18B), and DNCB (data not shown). An unexpected, but highly reproducible, finding was the behaviour of the cells after 2 hours.

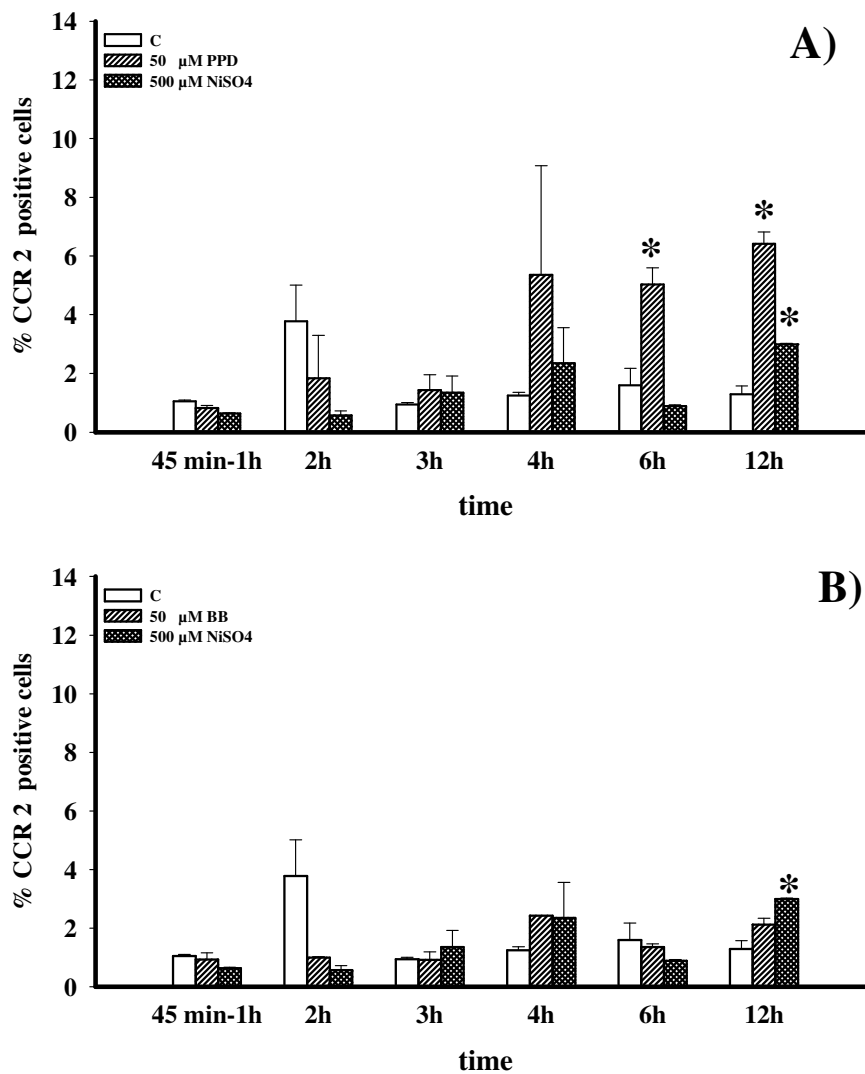


Figure 18. Time-dependent percentages of CCR 2 positive THP-1 cells determined by flow cytometry. THP-1 (1×10^6 /mL) were cultured in RPMI + 0.5 % FCS alone (C), treated with PPD (A), BB (B), and NiSO4 for 1h, 2 h, 3h, 4h, 6h and 12h. The bars show the mean (n=3) and SE, *, p< 0.05.

In order to study the impact of MCP-1 on the CCR2 up regulation, experiments were performed in presence of externally added MCP-1. These results demonstrated that pre-treatment of THP-1 cells with MCP-1 increased CCR2 receptor protein 2-3 fold less than in absence of external MCP-1 (data not shown).

3.3.1. Dose and time dependency

The up regulation of CCR2 surface protein was already detectable in a series of Dose- and time- dependent experiments. Figure 19 shows the dose response curves. In summary, the results support the hypothesis that PPD clearly can modulate the MCP-1/CCR2 expression in primary cells and cell lines.

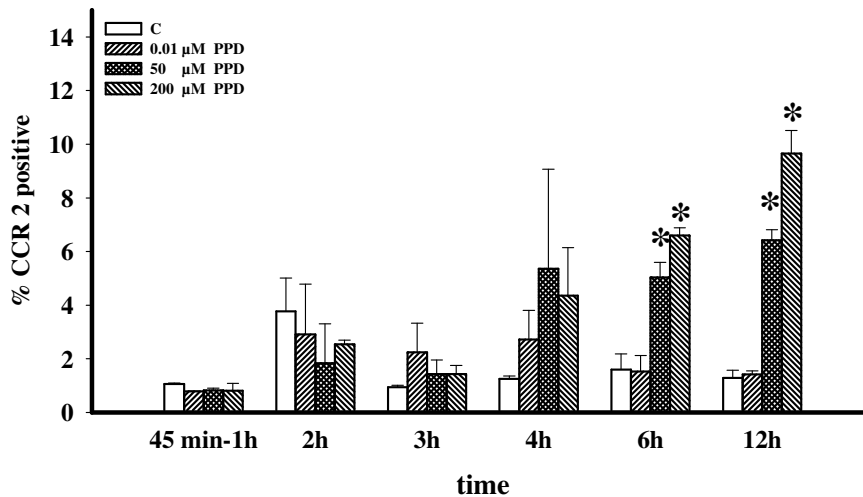


Figure 19. Dose-dependent percentages of CCR 2 positive THP-1 cells determined by flow cytometry. THP-1 (1×10^6 / mL) were cultured in RPMI + 0.5 % FCS alone (C) and indicated concentration of PPD for 1h, 2h, 3h, 4h, 6h and 12h. Results are presented as mean and SE, *, $p < 0.05$.

4. Discussion

4.1. Allergic contact dermatitis to small molecular weight compounds such as *para*-phenylenediamine

The chemical-induced allergic contact dermatitis (ACD) is a frequent, often occupationally related human disorder of the skin. ACD is a cell-mediated (type IV) allergic reaction. Different cells are involved in this reaction. This includes T cells, B cells, natural killer cells, and antigen presenting cells such as Langerhans cells, keratinocytes, and monocytes or macrophages.

The distribution of ACD is restricted to industrialized countries. Numbers are still increasing. Up to now, there is no successful treatment available, thus ACD has an enormous sociomedical impact [1]. It has been estimated that there are more than six million chemicals in the environment, and some authors speculated that about 2,800 of these compounds have allergic or to a lesser degree also contact-sensitizing or immunogenic properties causing ACD [159]. In general, immunogenic and/or allergic chemicals are rather small, with molecular weights varying between 100 and 5,000. Due to the assumption that small molecules are not directly recognized by the immune system, it is believed that they need binding to proteins before they can interact with the immune system. Most chemicals are *per se* non-reactive (prohaptens), they need metabolic activation in order to become immunogenic (see also Figure 1). Only few chemicals are known that can bind directly to proteins (*per se* reactive). Well-known examples are here certain antibiotics [160, 161]

A chemical that was originally believed to need metabolic activation in order to become allergenic is the aryl amine *para*-phenylene diamine (PPD). PPD is one of the common contact allergens [162, 163]. It is used as an antioxidant, an ingredient of hair dyes, intermediate of dyestuff, and is found in chemicals used for photographic processing [136]. More recently, also allergic skin reactions to temporary black henna tattoos, which often contain some PPD, are increasingly reported [140, 164]. Recent data from our laboratory indicated that PPD may directly interact with effector T cells. Therefore, we asked in this

investigation whether PPD is even able to stimulate the innate immune system, and create a danger signal for a sensitization process.

4.2. The impact of PPD on the generation of danger signals in monocytes

Several environmental compounds and some drugs can interact with the immune system. This may lead to either immunosuppression or immunostimulation of relevant cells. For instance, the environmental compound 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) can inhibit the necessary cell proliferation of T cell precursor cells [165, 166]. In contrast, the allergic properties of *para*-phenylenediamine (PPD) have been studied initially by means of the proliferative responses of lymphocytes. In this context it should be mentioned that investigators first found only responses to a derivative of PPD. Several investigations demonstrated that PBMC from persons with positive skin reactions to PPD exhibited significant T-cell proliferations solely triggered by its autooxidation product Bandrowski's base (BB), and not by PPD itself [137, 139]. But newer results from our laboratory demonstrated that PBMC as well as PPD specific T-cell clone responded to both PPD and BB [138, 167]. Therefore, this work focused now on the question whether PPD itself is indeed able to provide danger signals in antigen presenting cells such as monocytes.

The theory of a danger signal [168] claims that T cells will react only if antigen is presented in the context of a so-called danger signal. Danger signals are thought to act on dendritic cells and stimulating them to mature so that they can present foreign antigens in order to stimulate T lymphocytes.

Some classical endogenous danger signals are heat-shock proteins, nucleotides, reactive oxygen intermediates, extra cellular-matrix breakdown products, neuromediators, and cytokines like the IFNs [168-174]. The chemical can provide the danger signal itself, either by inducing cell damage (irritation), or by direct up-regulation of co-stimulatory molecules or cytokines [175] like IL-1 β and TNF- α [4, 176, 177].

4.2.1. The impact of PPD on interleukin-1 β and tumor necrosis factor - α and on related cytokines

Interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) are both pro-inflammatory cytokines that are believed to provide the signals needed for the activation of Langerhans cells, and for non-professional antigen presenting cells. To the latter group belong the monocytes which have been used in this study. Thus, IL-1 β and TNF- α play a major role in sensitization and elicitation phase of contact dermatitis [4, 5, 146]. The latter has been clearly demonstrated in animal models [35, 178] while studies with in vitro generated monocyte derived dendritic cells were not so informative up to now. Other studies also indicated that IL-1 β is induced first. This cytokine induces then TNF- α which then stimulates its own production via a positive feedback loop [179]. In our laboratory, the cytokine pattern of PPD and/or BB (Bandrowski's base, a derivative of PPD) specific monoclonal T-cell clones was determined previously. Surprisingly, PPD-/BB specific T-cell clones exhibited a Th2 like cytokine pattern. These T-cell clones released high amounts of IL-4 and IL-13 but no IL-10.

The data presented in this investigation demonstrating that PPD decreased the expression and release of IL-1 β and TNF- α support clearly our initial findings in T cells, and extend them to antigen presenting cells such as monocytes. These cells were chosen in this study instead of monocyte-derived dendritic cells due to their already mentioned limited capacities to release the investigated cytokines in vitro.

The impact of PPD on these cytokines was studied in different lineages of monocytes. The received results clearly indicated that PPD inhibited both IL-1 β and TNF- α in a highly specific, time- and dose-dependent manner. This was found in primary human monocytes as well as in a cell lines generated from monocytes (U-937). These effects were evaluated on the protein and mRNA level. So far, no comparable investigations have been performed for aryl amines such as PPD. In contrast, several reports revealed that the application of haptens such as nickel, or DNCB on human skin led to an upregulation of IL-1 β mRNA levels in various antigen presenting cells [37],[150]. Similar results were found in Langerhans cells derived from mice. The latter was found in mice as early as 15 minutes after skin painting with allergens [4, 178]. Furthermore, increased IL-1 β mRNA levels were observed in the epidermis, dermis, hair follicles about 90 minutes following topical

allergen exposure [180]. The results found for PPD are as well controversial to prior studies in mice indicating that TNF- α mRNA levels increased within 45 min after hapten painting [178].

Therefore, the intensive specificity studies were performed to validate the results for PPD. These included studies using other chemicals. These findings were confirmed through additional investigations including *meta*-phenylenediamine (MPD) and *ortho*-phenylenediamine (OPD). The down regulation of the cytokines was clear again, and highly reproducible. Included time course experiments demonstrated significant reductions already 2 hours after administration of PPD to the cells. Based on these results we conclude that PPD is not able to mount a cascade for the induction of danger signals in monocytes. Following that line, the results point to the open question whether PPD itself is indeed able to stimulate and mature professional antigen presenting cells such as LC or in vitro immature dendritic cells. Nevertheless, these results support our previous findings in T cells demonstrating that PPD can indeed stimulate effector T cells without processing [138], and mostly likely without danger signals.

Although the results for PPD may be different in professional antigen presenting cells such as LCs, the presented findings of PPD, a well-known contact allergen, allow for new discussions about the unique role of IL-1 β and TNF- α during the induction process of danger signals. On the other side, it has been taken into account that the stimulation of memory T cells, which is performed predominantly by monocytes in the effector phase, may not need danger signals for their stimulation.

Independently, inhibition of the investigated cytokines has been found for compounds such as retinoic acid [181], chloroquine (hydroxychloroquin) [182], and rooperol- tetraacetate. They all reduced the mRNAs levels in U937 cells for IL-1 β and TNF- α both in the presence and absence of “professional” inducers of danger signals such as lipopolysaccharides (LPS) [183]. Further investigations for these compounds that they exhibit immunosuppressive features or induced immunosuppressive effects. Following these arguments, it could be speculated that PPD itself may have such features but clearly more investigations are needed to support this finding. In that case, the allergic properties in the PPD-contact allergy should be mediated by other compounds generated from PPD

via autooxidative processes or through metabolites formed by the action of specific enzymes in the skin. This clearly points to facts that more studies are needed to better understand the modes of action of these small molecular weight environmental chemicals.

4.2.2. The impact of MCP

Chemokines are also crucial regulators of both the induction and expression of ACD. The kinetics and their temporal expression pattern during ACD resemble the wellknown course during wound healing. Interleukin-8 (IL-8) is expressed first followed by the monocyte chemotactic protein 1 (MCP-1), and a protein called RANTES (Regulated upon activation normally T- expressed and presumably secreted) [184]. MCP-1 seems to play an even more relevant role in ACD. Informative results were gained using mice models. A transgenic mice strain characterized by high MCP-1 protein expression levels in the basal keratinocytes showed enhanced contact hypersensitivity responses together with an increased number of infiltrating DCs after encounter with various chemicals [185]. The results found in this investigation using human primary cells and a monocytic cell line (U-937) revealed that PPD down regulated the expression of this protein in a time- and dose-dependent manner. These results show again the specificity of PPD or may point to species differences. The importance of MCP-1 for ACD has been studied only recently, and the results are not well understood right now.

Investigations using mice demonstrated that MCP-1 modifies the cytokine pattern released by memory T cells. In this study, MCP-1 was found to be able to stimulate IL-4 production in CD4+ T cells [46]. MCP-1 acts also on activated macrophages by influencing and decreasing their IL-12 expression drastically [78]. These findings using mice indicate already that MCP-1 may also influence the cytokines released by T cells and likely other cells in humans. Additional support comes from derivatives of MCP-1. Some investigators were able to show that not only MCP-1 but also MCP-2, MCP-3, and MCP-4 are able to inhibit IL-12 production in human monocytes and DCs [120]. These results are suggestive for strong direct influence of MCPs on the polarization of naïve T cells into Th2 cell. But still more data are needed to better define their functions. Interestingly, we found that PPD but not BB reduced MCP-1 secretion while these cells released high amounts of IL-4. The

latter results clearly point to differences between mice and humans, and show that more research is needed on this subject.

We extended our investigations of PPD to the regulation of MCP-1 and its receptor namely CCR2. This was studied in human peripheral blood mononuclear cells (PBMC) and monocytes. Here we found clear evidence for substance specificity but independent from the cell-type studied. We found that PPD significantly inhibited MCP-1 protein synthesis after 2 hours, while the mRNA levels reduced already after 45 minutes in PBMC. In contrast, we found at the same time an up regulation of the mRNA levels for the CCR2 receptor. Similar to results gained for the cytokines, we found a clear substance – specificity. Therefore, we can speculate that this effect was highly dependent on the *para*-substitution.

During studying the literature carefully, we found that our results differ from reports focusing on chemicals as well. In these studies MCP-1 was identified as the first chemokine expressed in the basal epidermal layer, but still increasing as early as 6 hours after allergen contact. This indicates that the levels are most likely released by infiltrating monocytes and lymphocytes [23].

Several explanations could account for the reduced MCP-1 protein levels and mRNA levels. It is for example known that sodium salicylate - an anti-inflammatory agent - suppressed the mRNA levels by reduced transcriptional activity [186]. In addition, some researchers observed that destabilization of the mature mRNA can be an explanation for reduced MCP-1 levels. Although we did not study the stability of the mRNA, postulate that PPD may destabilize the MCP-1 mRNA. The latter has been reported for hypoxia on human monocytic cells [187].

4.2.3. Impact of the PPD-induced increased CCR2 surface protein expression

The CCR2 receptor plays an important role in the regulation of T cell responses. The CCR2 is even more known for its effects on inflammatory sites [188]. In this regard, studies using CCR2^{-/-} animals had a defect in Th1 cytokine production and were hypersensitive to *Leishmania* [118]. The main importance for the CCR2 comes from

results demonstrating that CCR2 is critical for migration [189]. We also studied the specificity of the CCR2 up regulation, and found that beside PPD, other haptens such as nickel sulfate, or a terpenoid also induced an up regulation of this receptor. The biological consequences of these findings need further investigations. In contrast, exposure of another monocytic cell line (THP-1) with phorbol-12-myristate-13-acetate (PMA) reduced the expression of the CCR2 gene [114].

5. Summary

The allergic contact dermatitis (ACD) to small molecular weight compounds is a common inflammatory skin reaction. ACD is restricted to industrialized countries, has an enormous sociomedical and socioeconomic impact. About 2,800 compounds from the six million chemicals known in our environment are believed to have allergic, and to a lesser degree also contact-sensitizing or immunogenic properties causing allergic contact dermatitis. ACD results from T cell responses to harmless, low molecular weight chemicals (haptens) applied to the skin. Haptens are not directly recognized by the cells of the immune system. They need to be presented by subsets of antigen presenting cells to the cells of the immune system. In this regard, epidermal Langerhans cells (LC) and the cells into which they mature (dendritic cells) are believed to play a pivotal role in the sensitization process for ACD. LC are able to bind the haptens, internalize them, and present them to naive T cells and induce thereby the development of effector T cells. They are so-called professional antigen presenting cells. This process is initiated and maintained by the release of several mediators, which are released by various cells after their contact with the haptens. One of the first proteins secreted into the environment is interleukin (IL)-1 β . This cytokine is produced and secreted minutes after an antigen enters the cell. It is commonly believed that the large amounts of this protein and other cytokines such as granulocyte-colony stimulation factor (GM-CSF) and tumor necrosis factor alpha (TNF- α) needed for the initiation and activation of ACD are coming first from other cells residing in the skin, e.g., keratinocytes, monocytes and macrophages. These cytokines provide the danger signals needed for the activation of the Langerhans cell (LC), which then produce via a positive feedback loop various cytokines themselves. In addition, other proteins such as chemokines influence the generation of danger signals, migration, homing of T cells in the local lymph nodes as well as the recruitment of T cells into the skin. Thus, a small molecular compounds or hapten needs to be able to induce danger signals in order to become immunogenic.

In this study, we investigated whether *para*-phenylenediamine (PPD), an arylamine and common contact allergen, is able to induce danger signals and likely provide the signals needed for an initiation of an immune response[162, 163]. PPD is used as an antioxidant, an ingredient of hair dyes, intermediate of dyestuff, and PPD is found in chemicals used

for photographic processing. But up to date, it has not been clearly demonstrated if PPD itself is a sensitizing agent.

Thus, this study aimed on the potential of PPD to provide the danger signals by studying IL-1 β , TNF- α , and monocyte chemoattractant proteins (MCP-1) in human monocytes, peripheral blood mononuclear cells (PBMC) from healthy volunteers, and also in two human monocyte cell lines namely U937, and THP-1. This study found that PPD decreased dose- and time-dependently the expression and release of three relevant mediators involved in the generation of danger signals. Namely, PPD reduced the mRNA and protein levels for IL-1 β , TNF- α , and MCP-1 in primary human monocytes from various donors. These findings were extended and validated by investigations using the cell line U937. The data were highly specific for PPD, and no such results were gained for its known auto oxidation product called Bandrowski's base or for *meta*-phenylenediamine (MPD), and *ortho*-phenylenediamine (OPD). Therefore, we can speculate that this effect is likely to be dependent on the *para*-substitution. Based on these results we conclude that PPD itself is not able to mount a cascade for the induction of danger signals.

It should be mentioned that it is still possible that PPD induces danger signals for sensitization by other unknown processes. Therefore, more research is still needed focusing on this subject especially in professional antigen presenting cells in order to solve the still open question whether PPD itself sensitizes naive T cells or if PPD is solely an allergen.

Independently we found unexpectedly that PPD as well as other haptens such as 2, 4-Dinitrochlorobenzene, nickelsulfate, as well as some terpenoide increased clearly the expression of CC chemokine receptor 2 (CCR2), the receptor for the chemokine MCP-1. Up to date, the main importance for the CCR2 receptor comes from results demonstrating that CCR2 is critical for the migration of monocytes after encounter with bacterial lipopolysaccharides. Under these circumstances the receptor disappears from the cell surface and is down regulated. An up regulation of CCR2 has not been reported for haptens, and deserves further investigations.

Zusammenfassung

Die allergische Kontaktdermatitis (ACD) durch kleinmolekulare Umweltsubstanzen – sogenannte Haptene - ist sehr häufig in den westlichen Industrieländern. Man geht davon aus, dass von den ca. 8000 bekannten Umweltchemikalien annähernd 2800 Allergene oder eventuell sogar immunogene Wirkungen hervorrufen können. Trotz intensiver Forschungsaktivitäten während der letzten 20 Jahre ist für viele Umweltchemikalien noch unklar, welche Molekülbestandteile oder Seitengruppen die immunogenen und/oder allergenen Effekte hervorrufen. Dies trifft sowohl für die Sensibilisierungsphase als auch für die Auslösephase der ACD zu. Beide Phasen werden durch eine Anzahl von Mediatoren wie verschiedene Zytokine und Chemokine ausgelöst und unterstützt. So ist bekannt, dass die notwendigen Interleukine (IL) wie beispielsweise IL-1 β , der Granulozyten-Makrophagen koloniestimulierende Faktor (GM-CSF), Tumor-Nekrose-Faktor Alpha (TNF- α) und Chemokine wie das Monozyten-chemotaktische Protein 1 (MCP-1) nach einem Antigenkontakt von Hautzellen wie Keratinozyten sowie Monozyten und Makrophagen ausgeschüttet werden. Diese Zytokine bilden die notwendigen *danger signals*- für die Aktivierung der beteiligten Zellen. Für die weitaus meisten Chemikalien ist bisher nicht bekannt, ob und inwieweit sie die erforderlichen *danger signals*- selbst auslösen können.

Im Rahmen dieser Arbeit wurde dieser Frage anhand der Modellsubstanz *para*-Phenylendiamin (PPD), einem Arylamin, nachgegangen. PPD ist ein bekanntes und häufiges Kontaktallergen. Die Substanz wird vornehmlich als Koppler bei der Farbpigmentherstellung eingesetzt. Voruntersuchungen legten nahe, dass PPD die Signalwege für die Bildung von *danger signals* stimuliert und möglicherweise naive T-Zellen sensibilisieren kann.

Die Induktion der Genexpression und Sekretion von IL-1 β , TNF- α , und MCP-1 durch PPD wurde *in vitro* an primären humanen Monozyten verschiedener Spender untersucht. Die IL-1 β Sekretion wurde in Monozyten durch PPD bereits nach 30 Minuten gehemmt, während eine Hemmung der TNF- α Sekretion erst nach einer Stunde signifikant meßbar war. Des weiteren wurde eine signifikante Verringerung der IL-1 β und TNF- α mRNA

Spiegel und der Proteinmenge nach zweistündigem PPD-Kontakt in peripheren mononukleären Zellen des Blutes verschiedener Spender nachgewiesen. Die MCP-1 mRNA Spiegel zeigten nach 45 Minuten eine eindeutige Verringerung, während die verminderte MCP-1 Ausschüttung nach zwei Stunden nachgewiesen wurde. Neben MCP-1 verringerte sich auch die Sekretion von MCP-3. Das Ausmaß der PPD-induzierten Effekte variierte zwischen den verschiedenen Zellspendern, sie konnten jedoch bei jedem Zelldonor signifikant nachgewiesen werden.

Zur detaillierten Analyse der Wirkungen auf die Chemokine wurden Untersuchungen in zwei Monozytenzelllinien (U937, THP-1) durchgeführt. Zur Erfassung des Einflusses von PPD auf die Chemokin-induzierte Wanderung der Zellen wurde seine Wirkung auf den MCP-1 Rezeptor CCR2 (CC-Chemokinrezeptor 2) auf der Zelloberfläche ebenfalls analysiert.

PPD hemmte in U937 Zellen, die den CCR2 Rezeptor nicht an der Zelloberfläche exprimieren, die MCP-1 mRNA Spiegel und die Sekretion von MCP-1 in die Umgebung. Somit waren die Effekte unabhängig von der Signalkette des CCR2 Rezeptors. In THP-1 Zellen, die den CCR2 Rezeptor an der Zelloberfläche exprimieren, regulierte PPD die Rezeptorexpression auf der Zelloberfläche dosis- und zeitabhängig hoch. Diese Untersuchungen deuten auf eine aktive Hochregulation des Rezeptors durch PPD hin.

Zur Spezifitätsanalyse der gewonnenen Ergebnisse wurde neben PPD sein bekanntes Autooxidationsprodukt, die Bandrowski's Base, *meta*-Phenylendiamin und *ortho*-Phenylendiamin in die Untersuchungen einbezogen. Nur PPD konnte die bekannten Signalwege für die Induktion von *danger signals* hemmen. Aus diesen Analysen wurde ersichtlich, dass die *para*-Stellung der beiden NH₂-Gruppen für die Effekte essentiell ist. Aufgrund der Ergebnisse kann geschlossen werden, dass PPD die Signalwege für die Induktion der *danger signals* nicht eigenständig induzieren kann.

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Acknowledgements

The present study was started in the Laboratory of Molecular Epidemiology, Department of Dermatology, University Hospital Aachen (RWTH Aachen) and finished in the Department of Ecotoxicology/Toxicology (Fachbereich VI), University of Trier (Germany).

I wish to express my deepest gratitude to my supervisor, Professor Dr. Brunhilde Blömeke, for the opportunity to work in her laboratory. Her support, enthusiastic attitude and excellent guidance have made it possible to complete this thesis. Her optimism has kept my spirits up all these years. She is also acknowledged as Head of the Institute, providing the singular working environment placed at my disposal.

I would like to express my respectful thanks to Professor Dr. Dr. h.c. mult. Paul Müller for his support.

I am grateful to Professor Dr. Jobst Meyer for the critical reviewing of the thesis and for his valuable suggestions.

I extend my warmest thanks to all my past and present colleagues in Aachen and in Trier, in particular Dr. Sebastian Breuer, Dr. Xiaohua He, Beate Pfister, Martin Holschbach and Gabriele Schmidt.

Finally, I would like to thank my family and my friends. My mother, sister and brothers have always been encouraging and supportive. Without their help, especially that of my mother, this thesis could not have been completed.

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