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„Role of the Aryl Hydrocarbon Receptor while Langerhans Cell Differentiation and Maturation“

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Die Endlosigkeit des wissenschaftlichen Ringens sorgt unablässig dafür, dass dem forschenden Menschengeist seine beiden edelsten Antriebe erhalten bleiben und immer wieder von neuem angefacht werden:

Die Begeisterung und die Ehrfurcht.

Max Planck

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Abstract

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor which has been implicated in the regulation of immune responses. Toxic ligands have been reported to exert immune-suppressive effects via AhR activation. Apart from these negative toxic effects of AhR ligands the pharmaceutical drug VAF347 shows that AhR ligands could be of potential therapeutic use. The compound provokes an inhibition of allergic lung inflammation and is able to suppress pancreatic islet allograft rejection in mice. It has been shown that these immune-suppressive functions are likely mediated by altered dendritic cell function. Therefore we investigated AhR protein levels in DC subsets and found that Langerhans cells (LCs) and monocyte-derived Dendritic Cells (moDCs) express the highest protein levels followed by interstitial DCs (intDCs) and monocyte-derived LCs (moLCs). Investigations on VAF347 treated LCs have previously shown that activated AhR diminishes LC differentiation potential and dampens the maturation process. With respect to the varying levels of AhR in DC subsets we here ask the question whether moLCs are equally influenced by AhR ligands VAF347 and FICZ. We could show that moLC generation was increased in the presence of AHR ligands and that these cells expressed lower levels of E-cadherin. The maturation process was only slightly decreased upon LPS treatment but left unchanged upon PGN activation. Therefore we conclude that LCs and moLCs generation is differentially influenced by AhR ligands and that the maturation capacity is only slightly affected depending on the activation stimulus. We were able to stain AhR in human skin LCs thereby proofing the existence of AhR in-vivo in human skin. Our data show that independent of activation stimuli AhR is constantly present in the nucleus. Furthermore we focus on the molecular role of AhR in mediating $NiSO_4$-induced hypersensitivity reactions in LCs. Interestingly we could show that the activation of LCs and moLCs with the chemical sensitizer $NiSO_4$, but not chemical irritant SDS, led to the activation of AhR. The same pattern could be found for RelB which plays an important role in regulating DC maturation in general. With regard to existing reports suggesting an interaction between AhR and RelB signaling, we ask the question whether AhR and RelB interplay in the specific hypersensitivity reaction induced by chemical sensitizers in LCs. We made use of the retroviral gene transduction system and analyzed the AhR/RelB target IL8 to address this question. We found that AhR still translocates to the nucleus upon $NiSO_4$ activation although RelB was cytoplasmically sequestered by the p100ΔN construct. Interestingly we observed a tendency to an immediately increased IL8 production and secretion. Therefore we hypothesize that AhR and RelB indeed interplay in the regulation of hypersensitivity reaction in means of preventing overreaction by controlling gene expression in the nucleus.

Zusammenfassung

Der Aryl Hydrocarbon Rezeptor (AhR) ist ein Ligand-aktivierter Transkriptionsfaktor der im Immunsystem eine wichtige Rolle spielt. Toxische Liganden aktivieren AhR und führen dadurch zur Unterdrückung der Immunantwort. Die Immun-Suppression durch AhR hat aber auch potentiell therapeutische Anwendungsgebiete bei der Unterdrückung von Autoimmun-Erkrankungen und Transplantat-Abstoßungen. Es wurde gezeigt, dass die immun-suppressive Funktion von AhR größtenteils durch eine veränderte Funktion der dendritischen Zellen (DZs) zustande kommt. Aus diesem Grund haben wir uns gefragt wie die AhR Protein Levels in verschiedenen DZs sind und fanden heraus das Langerhans Zellen (LZs) und Monozyt-abgeleitete DZs (moDZs) die höchsten Protein Levels an AhR exprimieren, gefolgt von interstitiellen DZs (intDZs) und Monozyt-abgeleiteten LZs (moLZs). Untersuchungen von VAF347 generierten LZs haben gezeigt, dass die Aktivierung von AhR zu einer beeinträchtigten LZ-Differenzierung führen. Außerdem war das Reifungs-Potential von LZs verringert. Da unsere Ergebnisse bereits unterschiedliche AhR Levels in LZs und moLZs zeigten, haben wir uns die Frage gestellt, ob moLZs im gleichen Ausmaß durch AhR Liganden beeinflussbar sind. Wir konnten zeigen, dass AhR-Ligand-generierte moLZs ein höheres Potential zur Differenzierung haben und dass diese Zellen eine geringere E-cadherin Expression aufweisen. Außerdem ist der Reifungsprozess der Zellen nur bei LPS Stimulation geringfügig verringert während PGN Stimulation keinen signifikanten Einfluss hat. Aus diesem Grund können wir schlussfolgern, dass die Differenzierung von LZs und moLZs unterschiedlich stark von AhR Liganden beeinflusst wird. Außerdem ist die Reifung der Zellen nur geringfügig beeinflusst wobei der Effekt vom Aktivierungsstimulus abhängt. Wir konnten AhR in LZs von humanen Hautschnitten färben und so zeigen, dass AhR, unabhängig vom Aktivierungsstatus konstitutiv im Kern lokalisiert ist. Interessanterweise wurde gezeigt, dass menschliche Haut mit endogenen AhR Liganden gesättigt ist, was die andauernde Aktivierungsstimulus-unabhängige AhR Aktivierung erklären könnte. Weiters haben wir untersucht ob in der Hypersensitivitätsreaktion auf $NiSO_4$ in LZs ein potenzielles AhR/RelB Zusammenspiel stattfindet, da wir interessante Übereinstimmungen in AhR und RelB Aktivierung nach $NiSO_4$ Stimulation festgestellt haben. Wir verwendeten ein retrovirales Infektionssystem um Veränderungen in der IL8 Produktion und Sekretion zu untersuchen. Dieses Gen wird in der Literatur bereits als potentielles Ziel von AhR/RelB Interaktionen beschrieben. Wir konnten zeigen, dass AhR unabhängig von RelB in den Kern transloziert und dass RelB Inhibierung zu einer verstärkten IL8 Produktion und Sekretion nach nur drei Stunden führt. Zusammenfassend können wir sagen, dass AhR und RelB tatsächlich zusammen wirken um die Hypersensitivitäts-Reaktion auf $NiSO_4$ zu regulieren. Weiters konnten wir feststellen, dass dieser Mechanismus erst auf Ebene der Genregulation im Zellkern stattfindet.

Introduction

1. Hematopoiesis

Hematopoiesis defines the process of the sequential differentiation steps from hematopoietic stem cells towards multiple cell lineages which altogether build up the unique hematopoietic and immunologic system. During this stepwise differentiation process the initially multipotent hematopoietic stem cells gradually lose their differentiation potential and become restricted towards a lineage. Reya et al (Reya et al. 2001) established the bi-lineal model of the hematopoiesis which claims that the hematopoietic stem cell (HSC) (Morrison et al. 1995) first differentiates either into a common myeloid precursor (CMP) (Akashi et al. 2000) or into a common lymphoid precursor (CLP) (Kondo et al. 1997). After this first cell fate decision the CLPs can commit into three different cell lineages, namely B-cells, T-cells and NK-cells which form the lymphoid part of the immune system. The CMPs on the other hand lost their lymphoid potential and differentiate into either a megakaryocyte-erythro-progenitor (MEP) or a granulocyte-macrophage-progenitor (GMP). The terminally committed cell lineages of eosinophils, basophils, megacaryocytes and erythrocytes differentiate from the MEPs. Myeloid cell types like granulocytes, monocytes, macrophages and dendritic cells develop from GMPs (Figure 1). Later on scientist further identified a common dendritic progenitor (CDP) cell type in the bone marrow which either gives rise to the pre-DC progenitor or plasmacytoid DCs (del Hoyo et al. 2002). Pre-DC progenitors leave the bone marrow and migrate through the blood stream to the lymphoid organs where they further differentiate into tissue-specialized DC subsets (Liu et al. 2009). However, in the last years there have been reports that besides the CLP and CMP, there exists a third multipotent progenitor, namely the lymphoid-primed multipotent progenitor (LMPP) (Adolfsson et al. 2005; Lai and Kondo 2006; Yoshida et al. 2006). In fact, this implies that the first decision in HSC differentiation is between megacaryocyte/erytho and myeloid/lymphoid cell fates and the granulocyte/macrophage lineage can arise from either LMPPs or GMPs (Luc et al. 2007). Most of the dendritic cells arise from the myeloid pathway, although CLP have recently been reported to have the potential to generate DCs (Akashi et al. 2000; Manz et al. 2001; Wu et al. 2001).

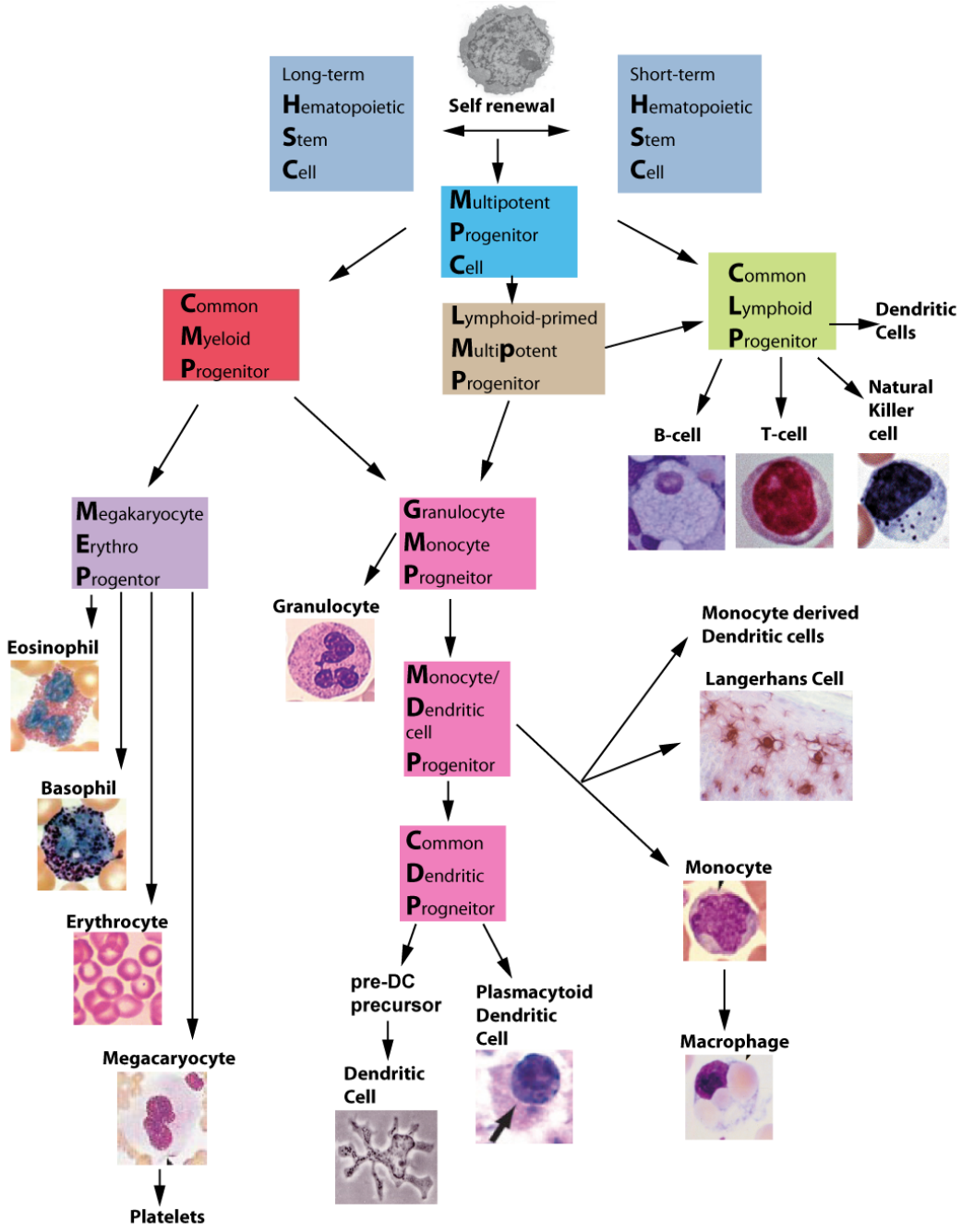


Figure 1: Hematopoietic differentiation
Adapted from https://daley.med.harvard.edu/assets/Willy/willy.htm, supplemented with pictures (see figure refrences)

Hematopoiesis is tightly regulated not only by intrinsic factors such as transcriptional programs but also by extrinsic signals from the microenvironment. Hematopoietic cells and immune cells mainly communicate via cytokines (interleukins, chemokines, and interferons) and are partly characterized by their production and responsiveness to these low molecular weight proteins which are part of a sophisticated network of cellular signaling. For example the DC progenitors are characterized by their ability to produce and respond to Fms-related tyrosine kinase 3 ligand (FLT3L) (Schmid et al. 2010).

2. Immune system:

The immune system is constituted by specialized cells of the hematopoietic system and is able to protect the organism from invading microbes, viruses, macromolecules, proteins, polysaccharides and small chemicals which are potentially harmful. The immune response itself is produced by two parts of the immune system, namely the innate immune system (early response) and the adaptive immune system (later responses). The innate immune response only recognizes structures shared by groups of related microbes, has no memory and always reacts in the same way. It is comprised of physical and biochemical barriers such as epithelia and antimicrobial substances. In addition to this the proteins of the peripheral blood complement system and phagocytic cells like macrophages or dendritic cells exert immediate protective functions within the first 12 hours of an infectious encounter thereby limiting the expansion of the infection and activating the adaptive immune system which targets the infection more specifically via antigen recognition. The main players of the adaptive immunity are the lymphocytes. On the one hand the humoral immunity mediated by B-cells which blocks infections by producing antibodies directed against specific extracellular antigens and subsequently leading to the elimination of the infection. On the other hand the cell-mediated immunity by T-cells which reacts to intracellular pathogens. CD8 positive T-cells exert the cytotoxic killing response whereas the CD4 positive T-cells can differentiate into 4 T helper subsets with divergent functions. Th1 cells act against intracellular pathogens and are involved in allergy induction. Th2 cells play a crucial role in defense against extracellular pathogens and induction of allergy and act as T effector-cells. Th17 cells are implicated in autoimmunity and antimicrobial mechanisms against extracellular parasites and fungi. T-regs control the induction of tolerance and immune responses and function as important lymphocyte homeostasis. For T-cells it is essential to interact with other antigen-presenting-cells (APCs) such as macrophages or dendritic cells which process and present the antigen on major histocompatibility complexes (MHC) class I and II on their cell surface. Activated T-cells then initiate the immune response by either directly killing infected cells (MHC class I presentation of cytosolic antigens to CD8+ T lymphocytes) or acting as T helper cells to induce other mechanisms of clearance or tolerance (MHC class II presentation of vesicular antigens to CD4+ T-lymphocytes). The defining characteristics of the later immune response are the specificity against antigens enabled by clonal selection of T-cell clones with the highest specificity for antigens. In addition to this T cells are unique in their immense diversity of antigen recognition which is possible because of the somatic recombination of receptor gene segments. Furthermore the adaptive immune system has the ability to memorize antigens and elicit more effective immune responses to the same antigen. (compare (Abbas et al. 2007)

2.1. Dendritic cell subsets:

Dendritic cells are distributed in multiple organs throughout the body and are professional antigen-presenting cells. Their function is crucial for the induction of the adaptive immune response. Research of the last decades showed that dendritic cells are composed of a heterogeneous pool of cells with different surface marker expression, function and origin. First of all DCs are divided into conventional DCs and non-conventional DCs (Shortman and Naik 2007). Conventional DCs differentiate from CDPs and pre-DC precursors and act as normal antigen capturing and processing cells. On the other hand we have the non-conventional class of DCs which either originate from a different source than CDP or pre-DC precursors or have different functions than the conventional DCs. All DC subsets which differentiate from monocytes in steady-state or inflammation are counted to the non-conventional class of DCs. They are found in peripheral tissues such as lung, liver, skin, intestine or kidneys where they function as classical antigen presenting cells (APCs) and subsequently migrate to the draining lymph nodes to initiate the adaptive immune response. Furthermore the plasmacytoid DCs (pDCs), found in lymphoid or non-lymphoid organs, are also classified as non-conventional subsets because their function differs markedly from common DCs. They secret high amounts of type I interferon upon viral infection (Guiducci et al. 2008), continuously present endogenous viral antigen even in the activated state (Young et al. 2008) and they play an important role in the maturation of activated B-cells towards antibody-secreting plasma cells (Jego et al. 2003). The conventional class of DCs is further divided into migratory and lymphoid-tissue resident DC subsets. The migratory DC subsets are located in multiple organs such as intestine, lung, liver, kidney or the skin where they monitor incoming pathogens and subsequently process them to present them in the draining lymph nodes. The lymphoid-tissue resident DCs on the contrary are found in lymphoid organs such as the thymus, spleen or lymph nodes and lack any migratory capacities. They are differentiated from pre-DCs and are further classified according to their surface marker expression of CD4 and CD8 (Naik et al. 2006). There are three types of lymphoid-resident DCs, namely the CD8+CD4-, CD8-CD4- and the CD8-CD4+ cells. CD8+CD4- DCs play a crucial role in viral immunity because they present viral antigen via MHC class I and secret high amounts of interferon-α in response to viral infections and intracellular pathogens which increases the cyto-toxicity of NK-cells and T-cells (Hochrein et al. 2001). CD8-CD4- double negative DCs secret high amounts of interferon-γ and initiate a CD8+ cyto-toxic T-cell response (McLellan et al. 2002). CD8-CD4+ DCs are predominately described as immune-tolerating and autoimmune-dampening cells which are potent inducers of CD4+ T-cell responses (Dudziak et al. 2007; Legge et al. 2002). Above mentioned classifications of DC subsets are also reviewed in (Kushwah and Hu 2011; Liu and Nussenzweig 2010; Merad and Manz 2009) and are illustrated in figure 2.

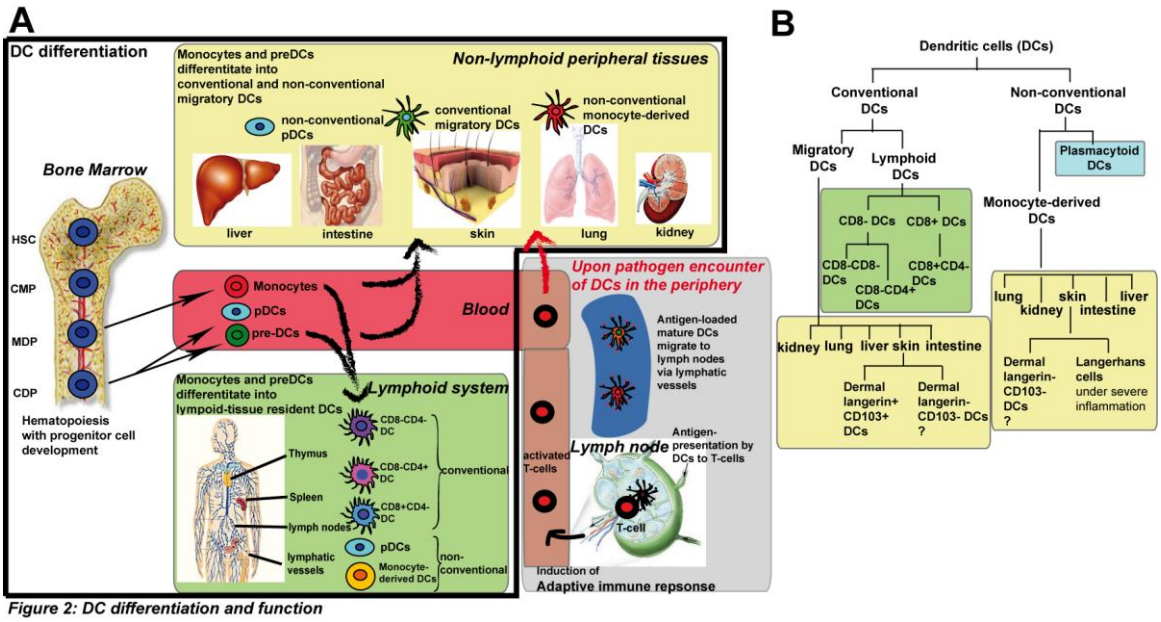


Figure 2: DC differentiation and function

(A) shows a schematic of the DC differentitaion starting with the hematopoiesis in the bone marrow. Hematopoietic stem cells develop into CMPs and further into MDPs and CDPs. MDPs give rise to monocytes and CDPs to pre-DC precursors and pDCs. These cell types are then transfered to the peripheral organs via the blood stream. In non-lymphoid tissues monocytes and pre-DCs differentiate into migratory DCs. In the lymphoid organs pre-DCs differentitate into lympoid-tissue resident DCs (CD8+ and CD8- DCs) and monocytes differentiate into migratory DC subsets. pDCs can either diffuse in the blood stream or migrate to peripheral organs. Picture refrences can be found in the figure reference section.
(B) shows a schematic of the DC subset classification. (adapted from Kushwah et al 2011). Dendritic cells are classified into conventional and non-conventional DCs according to their origin and function. Conventional DCs arise from CDP and pre-DCs whereas non-conventional DCs arise from monocytes, CDPs and CLPs. The DC subsets in the peripheral tissues such as kidney, lung, liver, skin and intestine are migratory DCs. CD8- and CD8+ DCs are termed lymphoid tissues resident DCs and harbour no migratory potential. There are also non-conventional monocyte-derived DC subsets in peripheral tissues, to which we count the Langerhans cells under inflammatory condition. Plasmacytoid DCs also count to the non-conventional class of DCs because of their origin and function.

2.2. Dendritic cell function

The basic characteristic of immature dendritic cells (DCs) is the very potent antigen processing function which allows them to take up and thus monitor incoming antigens for their pathogenic potential. Being the link between the innate and the adaptive immune system these specialized cells are able to decide whether to tolerate a certain antigen or induce a pro-inflammatory immune response. Upon the encounter of an antigen DCs undergo the so-called maturation process, in which they lose the antigen processing capacity and gradually gain antigen presenting and T-cell stimulatory functions. DCs are specialized in taking up and processing antigens to subsequently present antigen peptides on the cell surface via MHC class I and II molecules. Activated DCs initiate the expression of the chemokine receptor CCR7 and are thereafter capable of responding to chemokines which lead to the migration of the antigen-loaded cells to the adjacent draining lymph nodes (Saeki et al. 1999; Stoitzner et al. 2002). In these secondary lymphoid organs DCs migrate to the T-cell rich zone where they present the processed antigen to T-cells. For the proper interaction with T-cells in the lymph nodes DCs need to undergo further phenotypical changes while the maturation process. They gradually increase the expression of co-stimulatory molecules like CD86, CD80 and CD40 which are necessary for T-cell activation and increase the expression of accessory molecules like CD83 and antigen-presenting molecules like MHC class I and II (eg. HLADR). Furthermore, activated DCs initiate the production of specific,

antigen-dependent cytokines which then govern the differentiation of T-cells towards a particular phenotype (T helper 1, 2 and 17 and Treg) and thereby shape the upcoming immune response (de Jong et al. 2005). In addition to this DCs are also essential for the maintenance of self-tolerance. Upon the encounter of self-antigens DCs are not properly activated in means of a dampened up-regulation of co-stimulatory molecules and pro-inflammatory cytokines. These cells are able to induce apoptosis or anergy of T-cells or the differentiation of regulatory T-cells (Steinman et al. 2003).

2.3. Langerhans cells:

The skin is the biggest organ of the human body and is constantly exposed to environmental particles such as chemicals or microorganisms. Langerhans cells are the dendritic cells of the epidermis and form a regular network (Romani et al. 2003). As protective guards they reside in the skin for long time periods (Merad et al. 2002). Nowadays scientists mostly believe, that hematopoietic progenitors populate the skin during embryonic development which then give rise to LCs during life (Ginhoux and Merad 2010). New results from mice studies suggest that LCs, populating the healthy adult skin have a dual origin. The majority of LCs originates from fetal liver monocytes which differentiate from myeloid progenitors in the liver before they migrate to the dermis and further differentiate into LC precursors. Apart from this the authors also found that yolk sac primitive macrophages contribute to LCs by migrating through the blood stream to the developing skin of the embryo to give rise to myeloid precursors. Although it is speculative the authors hypothesize that LC precursors are present in the skin before the hematopoiesis starts in the bone marrow while embryogenesis (Hoeffel et al. 2012). It is believed that LCs can repopulate themselves by self-renewal under steady state conditions (Chorro et al. 2009). However, Ginhoux et al could show that inflammatory conditions enable peripheral blood monocytes to differentiate into LCs (Ginhoux et al. 2006). Recently there have been two groups indicating that CD14 positive cells either from the blood or dermal resident cells can differentiate into LCs and therefore constitute possible LC self-renewal mechanisms (Larregina et al. 2001; Schaerli et al. 2005). Langerhans cells are phenotypically characterized by the expression of the dendritic surface marker CD1a (Fithian et al. 1981) and the mannose-specific C-type lectin Langerin (CD207) (Valladeau et al. 2000). In addition to this they express E-cadherin to interact with the surrounding keratinocytes (Tang et al. 1993). It has been shown that this molecule inhibits uncontrolled maturation of LCs (Riedl et al. 2000). However, E-cadherin is subsequently down-regulated upon maturation stimuli to ensure DC migration towards the adjacent draining lymph node and therefore the initiation of an immune response (Schwarzenberger and Udey 1996). Once LCs arrive in the draining lymph nodes they can either present the

antigen directly to T-cells or hand over the antigen to resident CD8+ lymphoid DCs which in turn cross-present it to CD4+ and CD8+ T-cells (Allan et al. 2006; Carbone et al. 2004; Stoitzner et al. 2006). Monocytic markers like CD11b and CD14 are not present on LCs. Furthermore LCs have special intracellular rod- or rocket like structures called Birbeck granules (Birbeck et al. 1961) which are characteristic for this cell type.

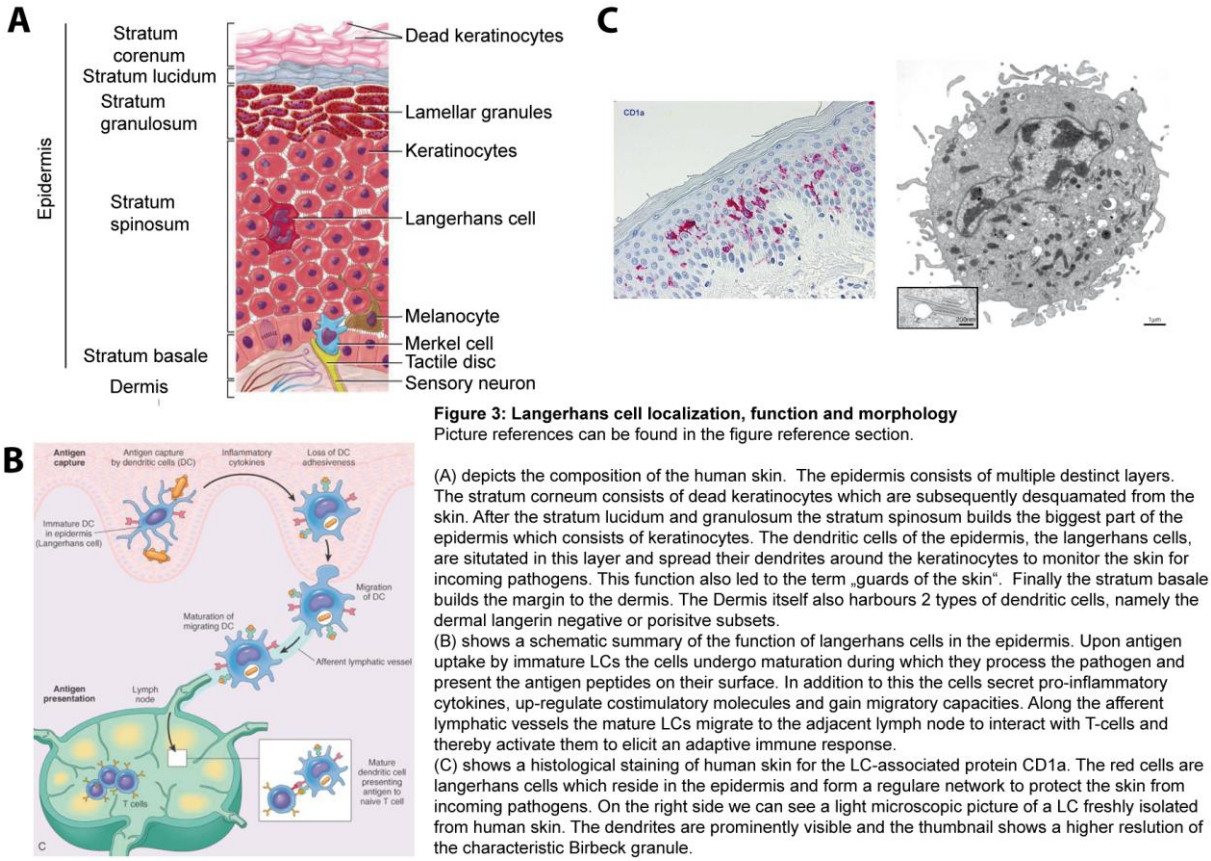


Figure 3: Langerhans cell localization, function and morphology
Picture references can be found in the figure reference section.

(A) depicts the composition of the human skin. The epidermis consists of multiple destinct layers. The stratum corneum consists of dead keratinocytes which are subsequently desquamated from the skin. After the stratum lucidum and granulosum the stratum spinosum builds the biggest part of the epidermis which consists of keratinocytes. The dendritic cells of the epidermis, the langerhans cells, are situtated in this layer and spread their dendrites around the keratinocytes to monitor the skin for incoming pathogens. This function also led to the term „guards of the skin". Finally the stratum basale builds the margin to the dermis. The Dermis itself also harbours 2 types of dendritic cells, namely the dermal langerin negative or porisitve subsets.
(B) shows a schematic summary of the function of langerhans cells in the epidermis. Upon antigen uptake by immature LCs the cells undergo maturation during which they process the pathogen and present the antigen peptides on their surface. In addition to this the cells secret pro-inflammatory cytokines, up-regulate costimulatory molecules and gain migratory capacities. Along the afferent lymphatic vessels the mature LCs migrate to the adjacent lymph node to interact with T-cells and thereby activate them to elicit an adaptive immune response.
(C) shows a histological staining of human skin for the LC-associated protein CD1a. The red cells are langerhans cells which reside in the epidermis and form a regulare network to protect the skin from incoming pathogens. On the right side we can see a light microscopic picture of a LC freshly isolated from human skin. The dendrites are prominently visible and the thumbnail shows a higher reslution of the characteristic Birbeck granule.

2.4. Cytokines required for DC differentiation:

In the process of identifying a common precursor for monocytes and dendritic cells, research on peripheral blood mononuclear cells was pioneering. Kasinrerk et al. (Kasinrerk et al. 1993) found that the cytokine granulocyte-macrophage colony stimulating factor (GM-CSF) is a substantial requirement for the induction of the dendritic cell marker CD1 expression. Today we use GM-CSF and additional IL4 for the efficient in-vitro generation of DCs from peripheral blood monocytes, because it was shown, that IL4 addition leads to a decrease of monocytic marker CD14 and enhances the accessory functions of DCs (Romani et al. 1994; Ruppert et al. 1993; Sallusto and Lanzavecchia 1994). Interestingly in 2003 Mallah et al. reported of a GM-CSF independent generation model of LC like cells from CD34 positive cells (Mollah et al. 2003). However, with ongoing research progress it was then possible to isolate CD34 positive hematopoietic stem cells (HSC) from the cord blood. Researches then started to elucidate the culture conditions to generate committed cell lines. Caux et al.

cultured CD34 positive HSCs in serum supplemented medium and found that the cells gain DC characteristics when differentiated in the presence of GM-CSF and tumor necrosis factor α (TNFα) (Caux et al. 1992). About 20% of the CD1a positive cells were Birbeck granule positive LCs. The addition of IL4 to this cytokine mix increased the CD1a positive cell fraction (Rosenzwajg et al. 1996; Strunk et al. 1996) and the supplementation of stem cell factor (SCF) further enhanced the DC yield (Szabolcs et al. 1995; Young et al. 1995). However, the breakthrough in elucidating the cytokine requirements for proper LC in-vitro generation came with the identification of TGF-β1 as a crucial factor for LC differentiation (Strobl et al. 1996). This research group tried to establish a serum-free LC generation model to make future medical application more applicable. They differentiated the cells in serum-free medium with GM-CSF, TNFα, SCF and TGF-β1 and observed a significant increase in CD1a positive cells compared to the cells without TGF-β1 treatment. In addition to this cells formed the typical DC clusters and around 20% of the TGF-β1 cultured cells showed birbeck granules and a positive staining for Langerin. It was further shown, that the promoting effect of TGF-β1 in the in-vitro generation of dendritic cells is achieved by inhibiting apoptosis of progenitor cells (Riedl et al. 1997). The results of this group are in line with the observation that TGF-β1 knock-out mice selectively lack Langerhans cells in the skin while still generating other dendritic cell types and monocytes in the peripheral blood (Borkowski et al. 1996). Ten years later it has been discovered that the TGF-β1, produced by LCs itself, is crucial for LC development and survival in an autocrine/paracrine way (Kaplan et al. 2007). Not only LCs produce their own TGF-β1 but also epidermal keratinocytes secret this cytokine. The serum-free LC generation model with GM-CSF, TNFα, SCF and TGF-β1 offered an ideal experimental set up to find out that the cytokine fms-related tyrosin kinase 3 ligand (Flt3L), which was already known to act as growth factor for hematopoietic progenitors (Lyman et al. 1994; Maraskovsky et al. 1996), specifically promoted the expansion of a common progenitor of monocytes and LCs, because the percentage of CD1a positive cells with molecular features of LCs was significantly increased while the ratio between monocytic and dendritic cells in the culture system was unchanged (Strobl et al. 1997). Above mentioned cytokine findings are also reviewed in (Strobl and Knapp 1999; Strobl et al. 1998).

It is quite interesting that TGF-β1 inhibits the maturation of immature LCs in murine bone marrow derived dendritic cells (Yamaguchi et al. 1997). One can speculate that this indeed is a clever move of nature concerning the fact that epidermal LCs are constantly exposed to environmental particles and need to have a sophisticated mechanisms to establish tolerance and control immune reactions. Apart from TGF-β1-maturation suppressing function, ongoing research further sheds light on the tolerance establishment in DCs. One interesting candidate is the β-catenin/Wnt signaling pathway which is activated via E-cadherin disruption. Upon the release of E-cadherin-sequestered β-catenin due to cell-cell junction

disruption or E-cadherin downregulation upon antigen encounter, β-catenin translocates to the nucleus where it binds to T cell factor/lymphoid enhancer factor (TCF/LEF) and turns on the expression of immune tolerance inducing genes (IL10, TGF-β1) (Fu and Jiang 2010; Manicassamy et al. 2010; Van den Bossche et al. 2012). This leads to the hypothesis that LCs continuously induce tolerance by TGF-β1 and β-catenin signaling and are only able to induce immune responses when pathogen recognition receptor signaling efficiently overwrites tolerogenic signals with inflammatory danger signals.

2.5. Transcription factors directing DC differentiation:

As mentioned above, GM-CSF is an essential cytokine in DC differentiation by acting as growth factor for common precursor cells. GM-CSF signaling on the one hand activates signal transducer and activator of transcription (STAT) 5, which represses the transcription factor interferon regulatory factor (IRF) 8 important for the differentiation of plasmacytoid dendritic cells. On the other hand GM-CSF signaling activates STAT3, which induces the important DC differentiation factor IRF4 (Schiavoni et al. 2002). FLT3L cytokine is essential for DC differentiation and it mediates its effects, amongst others, through the hematopoietic master transcription factor PU.1. This transcription factor is expressed in all DC subsets and CDP progenitors and is required for myeloid and lymphoid DC differentiation in an instructive and concentration-dependent manner (Anderson et al. 2000; Carotta et al. 2010; Guerriero et al. 2000). Another important transcription factor in DC differentiation is RelB, the NFκB family member of the non-canonical pathway. Platzer et al found that RelB over-expression promotes the generation of monocytic intermediates which are the precursors of CD11b+CD1a+ interstitial DCs while not affecting LC differentiation (Platzer et al. 2004). The transcription factor Ikaros is especially important for the differentiation of DC subsets from common lymphoid progenitors (Wu et al. 1997). The transcription factor growth factor independent 1 (Gfi1) in contrast seems to function as a transcriptional repressor because the Gfi knock-out mice show an increased number of LCs, whereas lymphoid resident DCs were reduced (Hock et al. 2003). Apart from this it was also shown that this transcription factor seems to be important in the DC versus macrophage decision (Rathinam et al. 2005). As TGF-β1 is a crucial cytokine in LC differentiation it is not astonishing that the main transcription factors in LC development are activated by its signaling. One interesting candidate is the TGF-β1 responsive transcription factor inhibitor of differentiation 2 (Id2). Id2 knockout mice lack LCs (Hacker et al. 2003) whereas the over-expression of Id2 in hematopoietic stem cells leads to an inhibition in plasmacytoid differentiation (Spits et al. 2000). Heinz et al. reported that TGF-β1 induces PU.1 and Id2 up-regulation in CD34 derived LCs and that PU.1 strongly promotes LC differentiation while Id2 suppresses monocyte

differentiation (Heinz et al. 2006). Runt related transcription factor 3 (Runx3) also counts to the TGF-β1 signaling inducible transcription factors involved in LC differentiation as Runx3 knock-out mice completely lack LCs (Fainaru et al. 2004). Vitamin D 3 receptor (VDR) has also been described to be induced by TGF-β1 and is specifically induced while LC differentiation. GATA1 on the other hand is induced by IL4 in monocyte-derived dendritic cell differentiation. Göbel et al showed that GATA1 functions as a repressor of VDR (Gobel et al. 2009). To sum up the downstream targets of TGF-β1 signaling: Id2, Runx3 and Pu.1 are strictly obligatory for LC differentiation. Above described transcription factors are in part reviewed in (Merad and Manz 2009; Zenke and Hieronymus 2006).

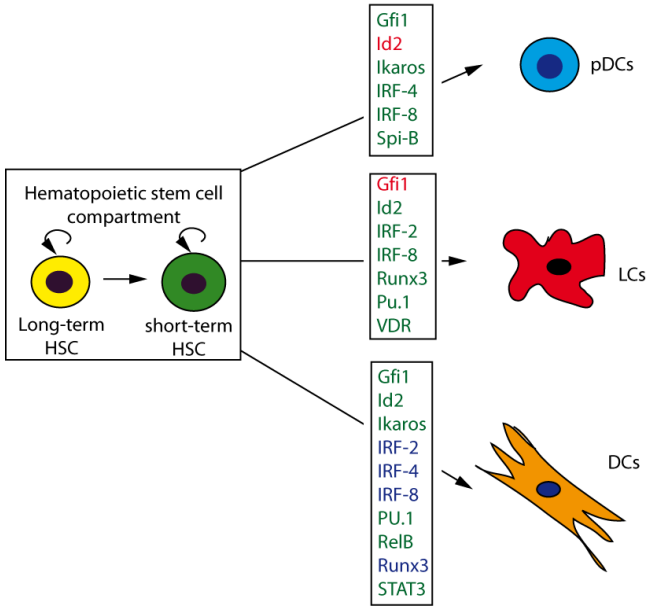


Figure 4: Transcription factors in DC differentitation
Adapted from Zenke et al.

2.6. Signal transduction pathways in DC maturation

DCs express a cell-type specific repertoire of pattern recognition receptors (PRRs) on their surface. The family of PRRs comprises four different classes, namely the nucleotide oligo dimerization domain (NOD)-like receptors, retinoid acid receptor inducible gene I (RIG-I)-like receptors, C-type lectin receptors (CLRs) and the toll-like receptors (TLRs) which enable them to recognize pathogen associated molecular patterns (PAMPs) of bacteria, viruses or fungi in their environment. Each DC subset expresses a unique repertoire of PRRs which leads to a specific and diverging response towards extracellular stimuli (Netea et al. 2005). Human dendritic cells express 10 different TLRs which can further be divided into extracellular TLRs (TLR1, 2, 4, 5, and 6) which recognize exterior compounds of bacteria and fungi such as cell wall components. Intracellular TLRs (TLR3, 7, 8 and 9) recognize nucleoside-containing structures like RNA or un-methylated DNA from bacteria and viruses.

Table 1:Toll-like receptor signaling in immune responses
Adapted from Kumar et al. 2009

receptor	location	ligand	adapter molecule	signaling
TLR1/2	PM	Triacyl lipopeptides (bacteria and mycobacteria)	MyD88 and TIRAP	NFκB
TLR2	PM	PGN (gram+ bacteria), LAM (Mycobacteria), hemagglutinin (measles virus), phospholipomannan (Candida), glycosylphosphophatidyl inositol mucin (Trypanosoma)	MyD88 and TIRAP	NFκB
TLR3	Endosome	ssRNA virus (WNV), dsRNA virus (Reovirus), RSV and MCMV	TRIF	NFκB and IRF3/7
TLR4	PM	LPS (gram- bacteria), mannan (candida), glycoinositolphospholipids (Trypanosoma), envelope proteins (RSV and MMTV)	MyD88, TIRAP, TRAM and TRIF	NFκB and IRF3/7
TLR5	PM	Flagellin (flagellated bacteria)	MyD88	NFκB
TLR6/2	PM	Diacyl lipopeptides (Mycoplasma), LTA (Streptococcus), Zymosan (Saccharomyces)	MyD88 and TIRAP	NFκB
TLR7	Endosome	ssRNA viruses (VSV, Influenza virus)	MyD88	NFκB and IRF7
TLR8	Endosome	ssRNA from RNA viruses	MyD88	NFκB and IRF7
TLR9	Endosome	dsDNA viruses (HSV, MCMV), CpG motifs from bacteria and viruses, hemozoin (Plasmodium)	MyD88	NFκB and IRF7

PM = Plasma membrane, LAM = Lipoarabinomannan, WNV = West Nile virus, RSV = Respiratory syncytial virus, MCMV = Murine cytomegalovirus, MMTV = Mouse mammary tumor virus, LTA = Lipoteichoic acid, VSV = Vesicular stomatitis virus, HSV = Herpes simplex virus, CpG = Cytidine-phosphate-guanosine.

In general all TLRs use the adapter molecule myeloid differentiation primary-response protein 88 (MyD88) with the exception of TLR3 and 4. TLR3 instead engages with the TIR-domain-containing adapter-inducing interferon-β (TRIF) molecule whereas TLR4 can use both adapter molecules (Akira and Takeda 2004). In general TLR ligand encounter leads to the dimerization of TLRs and subsequent recruitment of adaptor molecules. MyD88 then activates IRAK1/4 which further induces TRAF6. Together with TAK1, TAB1 and TAB2, TRAF6 then activates the NFκB and MAP kinase signaling pathways. TLRs predominantly exert their functions through the activation of NFκB and IRF signaling which ultimately leads to the induction of pro-inflammatory cytokines, type I interferons, induction of co-stimulatory molecules and the morphological changes towards a migratory phenotype. With the exceptions of TLR3 and TLR4 MyD88 is strictly required for DC maturation. TLR signaling of

DCs is also reviewed in (Akira and Takeda 2004; Kawai and Akira 2010; Kumar et al. 2009) LPS stimulation via the TLR4 is special because it needs a LPS-binding protein (MS2) which dimerizes with the co-receptor CD14 upon LPS binding. The activated TLR4 complex is then able to induce two different responses. The early response signals via MyD88 and induces NFκB whereas the late response is MyD88-independent but needs the TRIF/TRAM signaling cascade to activate NFκB and IRFs (Palsson-McDermott and O'Neill 2004).

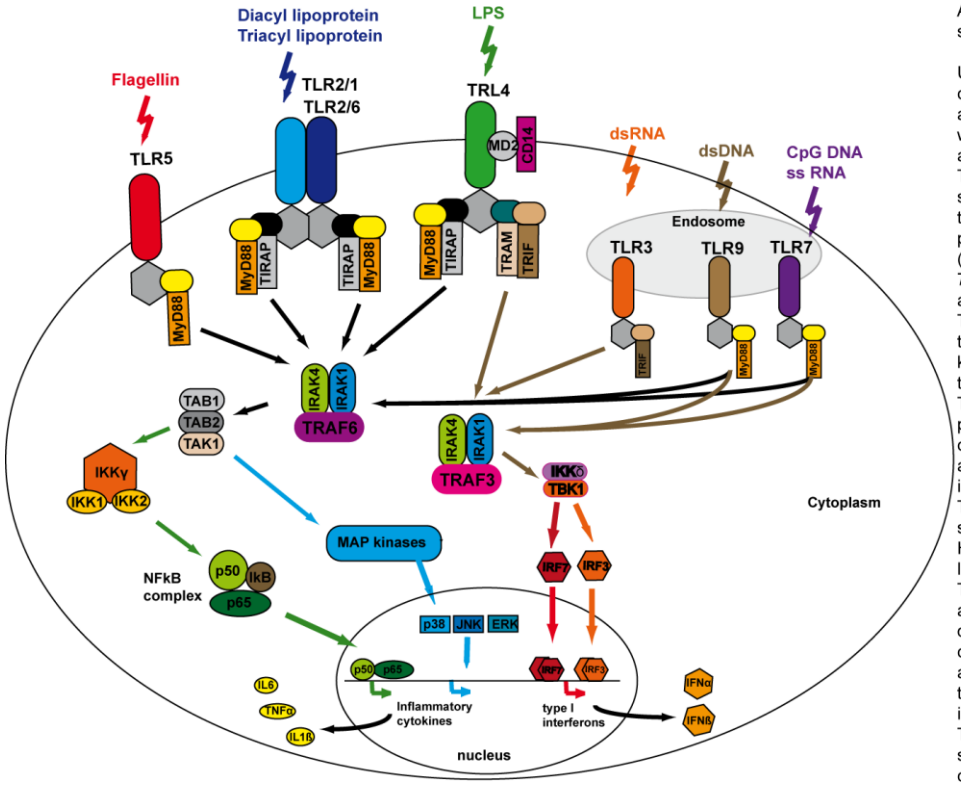


Figure 5: TLR signaling
Adapted from Martin et al. , supplemented with Kumar et al.

Upon a distinct stimulus the corresponding TLRs are activated and in turn dimerize with MyD88. TLR1, TLR2, TLR4 and TLR6 additionally need the TIRAP molecule for activation of signaling. IRAK1 and IRAK4 are then in turn activated and further propagate the signal to TRAF6 (TLR1-6) or TRAF 3 (TLR4, TLR 7 and TLR9). When TRAF6 is activated TAK1 together with TAB1 and 2 can either activate the NFkB signaling or the MAP kinases and turn on the production of inflammatory cytokines. TLR4 is special in means of the possibility of two signaling cascades, one via MyD88-TRAF6 and the other one MyD88-independently via the TRAF3-TBK1 axis. Also TLR3 is able to signal MyD88 independently. Here the activation of the signaling cascade either goes via TRAM and TRIF (TLR4) or TRIF alone (TLR3). TRAF3 subsequently activates the TBK1 molecule which further relaies its activation state to IRF3 or IRF7 to turn on transcription of type I interferons. The endogenous TLR7 and TLR9 are also able to signal via two independent cascades, namely the TRAF6-TAK1 or the TRAF3-TBK1 cascade.

2.7. MAP kinase and NFκB signaling in DC maturation:

A lot of immune functions are mediated via the NFκB signaling cascade, also because it is an ideal platform to interlink signals from innate and adaptive immunity. Knock-out mice studies of NFκB showed that the function of antigen-presenting cells and lymphocytes are highly regulated by this transcription factor family. NFκB transcription factors always bind to κB DNA sequences in promoters and enhancers of target genes as NFκB/Rel heterodimers or p50 and p52 homodimers. They can exert positive and negative effects on transcription and play a role in multiple cell types. The NFκB transcription factor family is comprised of 5 proteins which characteristically harbor an N-terminal Rel domain for rel protein interaction. RelA (p65), RelB and c-Rel additionally possess C-terminal transactivation domains (TADs) which render them capable of initiating transcription. NFκB1 (p50 from p105) and NFκB2

(p52 from p100) are kept in the cytoplasm in an inactive precursor form, namely p105 or p100 respectively. These NFκB1 and 2 proteins possess an inhibitory domain which keeps them in the cytoplasm. While the p105 precursor is processed spontaneously the p100 precursor only gets processed upon activation. However the inhibitory domains are phosphorylated, ubiquitinylated and processed in a proteosome-dependent way, resulting in the active p50 or p52 proteins displaying a nuclear localization sequence (NLS) necessary for nuclear translocation of the NFκB/Rel hetero- or homodimers. Moreover the NFκB signaling cascade consists of 5 different inhibitory proteins called inhibitor of κB (IκBs), namely IκBα, β, ε, γ and Bcl-3. The function of these proteins is to sequester the NFκB/Rel heterodimers or homodimers in the cytoplasm and inhibit signaling. Only upon activation signals the Inhibior of κB kinase complex (IKK) is activated. This in turn leads to the phosphorylation and ubiquinitylation of the inhibitory IκB inducing the proteosomal degradation. The transcriptionally active dimmers are now able to translocate into the nucleus. Generally the NFκB signaling pathway is divided into the cananonical (classical) pathway and the non-canonical (alternative) pathway. The canonical pathway, including RelA dimers with p50 or c-Rel, is activated by ligand-activated cytokine receptors such as TNFα- or IL-1 receptors, activated PRRs such as toll-like receptors or antigen-receptors such as the T-cell receptor (TCR). The RelA/c-Rel heterodimer forms a complex with IκBα whereas the C-terminus of the p105 precursor first has to be degraded to the p50 protein which then forms heterodimers with RelA. This p105-degradation process occurs spontaneously and the thereby produced p50 proteins can either heterodimerize with RelA or c-Rel or form homodimers. The p50 heterodimer are also inhibited from nuclear translocation by the association of an IκB inhibitory protein. Signal propagation occurs via IKKβ (IKK2) which forms a complex with IKKα (IKK1) and the regulatory protein IKKγ (NEMO). This activated IKK complex then phosphorylates the IκB protein which ultimately leads to its ubiquinitylation and proteosomal degradation. The active p50 or RelA/c-Rel heterodimers then translocated to the nucleus where they initiate transcriptional changes. The non-canonical or so-called alternative NFκB pathway on the other hand signals through RelB/p52 heterodimers. TNF cytokine family and ligands such as CD40L, BAFF and lymphotoxinβ induce the alternative signaling pathway by activating the NFκB-inducing kinase (NIK). NIK subsequently phosphorylates IKKα which is essential for the alternative pathway whereas NEMO is not needed in this process (Sun 2011). P100 precursor finally gets phosphorylated and ubiquitinylated leading to the proteosomal degradation of the C-terminus generating the p52 protein with a nuclear localization sequence. P52 exists in complex with RelB and forms the transcriptionally active heterodimer which performs transcriptional changes after nuclear translocation and DNA binding to κB sites. It has to be said that p50 and p52 homodimers can be formed and mostly exert repressive effects on DNA sequences by competing with

transcriptionally active heterodimers (Zhong et al. 2002). However, whether the homodimers repress or activate transcription is also determined by special IκB association. When considering the ability of IKKα and IKKβ to crosstalk with multiple signaling cascades (p53, MAP kinases and IRF pathways), it becomes obvious why the NFκB signal transduction cascade plays such an important role in integrating signals from innate and adaptive immunity to exert an adequate immune response. (reviewed in (Ghosh and Hayden 2008; Hayden and Ghosh 2012; Hayden et al. 2006)

It is important to mention that nuclear RelB expression in DCs serves as hallmark for maturation as immature DCs show no RelB in the nucleus whereas mature DCs in T-cell areas of lymph nodes and tonsils are highly positive for nuclear RelB (Clark et al. 1999; Pettit et al. 1997). Overall the classical NFκB signaling cascade is immediately turned on after danger signals and exerts pro-inflammatory responses. The alternative RelB pathway on the contrary serves as immune-regulatory pathway as LCs deficient in RelB signaling show hyper-maturation (Jorgl et al. 2007; Saccani et al. 2001). These results are in line with the hyper-inflammatory phenotype of RelB knock-out mice (Weih et al. 1995). Additionally in the last years there have been reports claiming that RelB and RelA NFκB members are also able to interact with signaling molecules beyond the NFκB family, namely the aryl hydrocarbon receptor (AhR) (Kim et al. 2000; Ruby et al. 2002; Vogel et al. 2007). This topic will be discussed in chapter 3.5. in more detail.

In our experiments dedicated to investigate the interaction between RelB and AhR we make use of a p100ΔN construct, which lacks C-terminal ankyrin repeats essentially needed for subsequent proteosomal degradation of the p100 precursor to the active p52 protein. It has been described that this truncated form of the p100 precursor inhibits nuclear translocation of the RelB/p52 heterodimer (Solan et al. 2002). Our lab has already worked with this construct and found that inhibiton of the alternative RelB signaling cascade inhibits intDC development by promoting a monocytic intermediate (Platzer et al. 2004). Furthermore Jörgl et al. showed that conditionally active LC maturation is counter regulated by RelB. RelB inhibition led to LC hyper maturation (Jorgl et al. 2007).

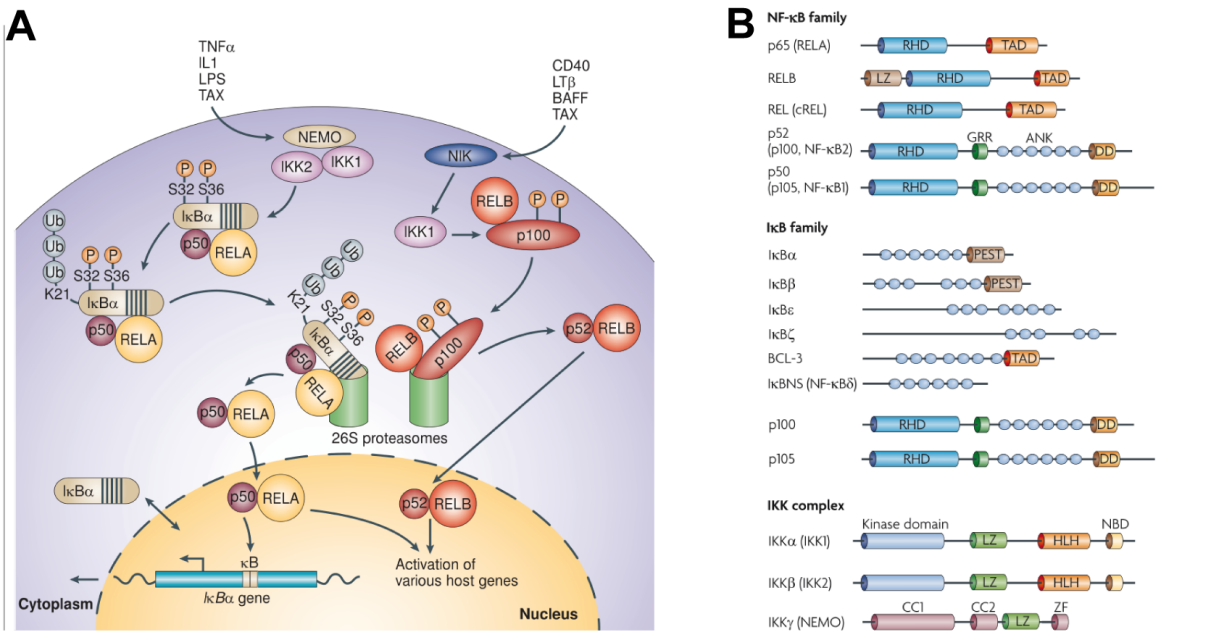


Figure 6: NFkB signaling
Picture A adapted from Chen and Greene et al. 2004, Picture B adapted from Ghosh and Hayden et al. 2008

Picture A shows a schematic of the 2 different NFkB signaling pathways. On the left side we see the canonical pathway with the p50/RelA heterodimer starting with cell activation through cytokines (TNFα, IL1) or antigenic stimuli like LPS. The IKK complex with NEMO gets activated and phosphrylates the inhibitory protein IkBα which further leads to its ubiquitinylation and subsequent degradation in the proteasome. The free p50/RelA heterodimer then translocates to the nucleus and turns on transcription of target genes among them the IkBα gene as negative feedback loop. On the right side wee see the non-canonical pathway with the p52/RelB heterodimer. This cascade is activated by CD40L, lymphotoxinß, BAFF and TAX proteins. NIK protein is stabilized and can activate itself by autophosphorylation. It further activates the IKK1 kinase which phosphorylates the p100 precursor protein in turn leading to proteosomal processing of p100 to p52. The p52/RelB heterodimer then translocates to the nucleus and turns on transcription.
Picture B shows an overview of the NFkB, IkB and IKK family members involved in the NFkB signaling. RHD (Rel homology domain), TAD (transactivation domain), LZ (leucine-zipper), GRR (glycine-rich region), ANK (ankyrin repeats), DD (death domain), PEST (proline, glutamic acid, serine and threonine rich), HLH (helix loop helix domain), NBD (NFkB essential modulator binding domain), CC1/2 (coiled-coil domain), ZF (zinc finger).

Mitogen activated protein kinase (MAPK) signaling cascades play an important role in the generation and regulation of immune responses. This class of signal transduction depends on sequential phosphorylation steps on and from kinases which ultimately phosphorylate transcription factors which in turn exert immunological responses via transcriptional alterations. As a first step extracellular signals are received via receptors or simply diffuse into the cell. The spectrum is wide and ranges from hormones, growth factors, antigenic stimuli, cytokines, interferons and oxidative stress to small chemicals. These priming signals activate MAP kinase kinase kinase (MAPKKK) proteins by phosphorylation. This activation signal is then transmitted to the next instance, namely the MAP kinase kinases (MAPKKs) These enzymes finally phosphorylate 3 classes of MAP kinases (MAPKs), namely the c-Jun N-terminal kinse (JNK), extracellular-signal regulated kinase (ERK) and p38 MAPK. Activated MAPKs then activate a wide range of transcription factors. These transcriptional program alterations finally enable the cell to adequately respond to the signals received from the environment. Candidates are signal transducer and activator of transcription (STATs), AP-1 (c-jun, c-Fos and activation of transcription (ATF), NFκB1, E twenty six like transcription factor (ELK1), T-cell factor (TCF) and cAMP responsive-element binding protein (CREB3). While the ERK pathway has distinct MAPKKKs and MAPKK which specifically activate the

ERK MAPK, p38 and JNK signaling cascades are not easily distinguishable. Already on the MAPKKK level they share some activation proteins such as TAK1, ASK1, MLK3 and MEKK1, 3 and 4 (Blank et al. 1996; Karandikar et al. 2000; Waetzig and Herdegen 2005). In addition it was shown that MAP kinase kinase enzyme MKK4 has the capacity to activate both MAPKs JNK and p38 while other MAPKKs like MKK7 (JNK) and MKK3/6 (p38) are specific for either cascade (Ganiatsas et al. 1998). The MAPK-ERK pathway is generally known as survival pathway mediating proliferation and differentiation whereas the MAPK JNK is implicated in cellular signaling, immune responses and apoptosis. The four isoforms of p38 (α, β, γ, δ) mostly respond to stress signals and induce inflammatory or apoptotic responses and are implicated in cell cycle regulation. (reviewed in (Cargnello and Roux 2011; Junttila et al. 2008; Puga et al. 2009). MAP kinase phosphatases (MKP) add another level of complexity to the MAPK signaling as they are able to inhibit kinase activity of more than one pathway (Junttila et al. 2008).

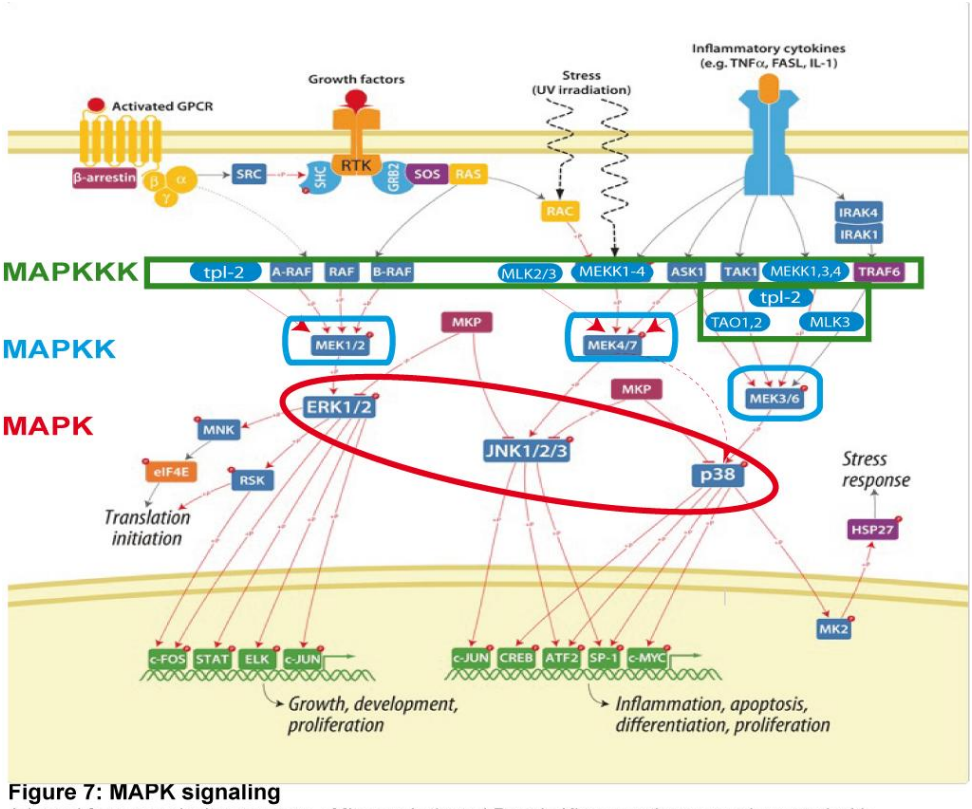


Figure 7: MAPK signaling
Adapted from www.invitrogen.com ->Mitogen Activated Protein Kinase pathway supplemented with www.quiagen.com -> MAPK signaling and Nakahara et al. 2006

Extracellular signals like hormons, cytokines, growth factors, UV radiation and oxidative stress or small chemicals activate the MAPKKKs. In the ERK cascade the MAPKKKs are RAF proteins and the c-Mos kinase tpl-2 whereas MEKK1-4, ASK1, TAK1 and MLK2/3 are implicated in the JNK pathway. TAK1, MEKK1,3,4, ASK1, tpl2, TAO1/2, MLK3 and TRAF6 are the MAPKKKs of the p38 pathway. The MAPKKs MEK1/2 activate the MAPK ERK1/2. MAPKs JNK1/2/3 are activated by MEK4 and MEK7 whereas p38 activation requires MEK3 and MEK6. MAPKKKs can be shared among the different cascades. Additionally also MEK4 kinase can either activate JNK or p38 signaling. MAP kinase phosphatases (MKPs) add additional complexity to this signaling system by suppressing kinase activity from multiple pathways and thereby interlinking the signaling cascades. The activated MAPK ERK, JNK and p38 ultimately phosphorylate multiple transcription factors important for the adequate elicitation of a cellular response to environmental conditions. The response ranges from growth, development and proliferation to differentitaion, inflammation and apoptosis.

For our experiments we are using special inhibitors to elucidate the role of MAPK and NFκB signaling in the activation of LCs and subsequent AhR nuclear translocation. SB203580 inhibitor specifically inhibits p38α, p38β and p38β MAPK catalytic activity by blocking the ATP binding pocket rather than inhibiting its phosphorylation (Cuenda et al. 1995; Kumar et al. 1999). Other MAPK pathways are unharmed by this inhibitor. SB216763 inhibits the GSK3α and β kinase activity. These enzymes usually phosphorylate β-catenin and target it for degradation thereby inhibiting active β-catenin/Wnt signling. Besides multiple other actions in various signaling pathways SB216763 inhibits GSK3β and turns on the β-catenin/Wnt signaling (Coghlan et al. 2000). SP600125 inhibitor specifically abrogates the phosphorylation of JNK MAPK therefore inhibiting this signaling cascade (Bennett et al. 2001). SN-50 inhibitor retains the NFκB family member p50 in the cytoplasm by sequestering the nuclear translocation sequence and anchoring it to the plasma membrane via a hydrophobic region (Lin et al. 1995).

2.8. Contact hypersensitivity:

Haptens are low molecular weight chemicals which cause a contact hypersensitivity reaction when applied to the skin. This biological response leads to contact dermatitis which can be classified into irritant and allergic dermatitis. Irritant contact dermatitis on the one hand is a classical inflammatory reaction caused by the toxic character of certain haptens. On the other hand the allergic contact hypersensitivity is an antigen-specific immune response of the adaptive immune system towards haptens. In our studies we use the chemical irritant sodium dodeyl sulfate (SDS) as inducer of irritant contact hypersensitivity reactions and the chemical sensitizer nickel sulphate ($NiSO_4$) as inducer of allergic hypersensitivity reactions. The allergic contact hypersensitivity is characterized by the sensitization and elicitation phase each comprised of 3 sequential steps. The sensitization phase is initiated by antigen invasion and activation of langerhans cells which subsequently start the maturation process and migrate to the draining lymph nodes where they finally present the processed antigen to CD4 and CD8 positive T-cells. The following elicitation phase starts with the migration of activated T-cells to the skin where LCs, T-cells and keratinocytes crosstalk via cytokines and interferons. Finally keratinocytes are targeted by inflammatory responses elicited by T-cells triggering apoptosis and thereby dermatitis symptoms. It is important to mention that the research team around Setsuya Aiba could show in multiple studies that only chemical sensitizers inducing allergic hypersensitivity lead to the maturation process of DC subsets in vivo and in vitro while chemical irritants function via different pathways (reviewed in (Sasaki and Aiba 2007). Concerning the investigation of signaling transduction leading to DC maturation upon hapten encounter there are multiple studies from various labs most of them

outlining the involvement of p38 MAPK among others. First it was shown that strong haptens induce phosphotyrosines in moDCs (Kuhn et al. 1998), later on it was shown that p38 MAPK activation is involved in the maturation process upon treatment with chemical sensitizers of in vitro moDCs (Aiba et al. 2003; Arrighi et al. 2001). Boislève et al. showed that p38 MAPK induces CCR7 expression upon $NiSO_4$ treatment which is essential for the induction of a migratory phenotype of mature DCs (Boisleve et al. 2004). Miyazama et al implicated p38 and ERK MAPK activation in the $NiSO_4$-treated THP-1 cell line. P38 inhibition led to a decrease in co-stimulatory molecule expression (Miyazawa et al. 2007). There are convincing studies linking the p38 MAPK activation to an imbalance in the redox system in the cell detected by a recued GSH/GSSG ratio (Sasaki and Aiba 2007). DCs activate IL-12 production upon $NiSO_4$ activation by a mechanism that involves STAT1 phosphorylation and further IRF1 induction by redox stress. This mechanism further proves the activation of MAPKs by intracellular redox imbalance caused by haptens (Antonios et al. 2010). However knockout mice selectively lacking LCs showed controversial results from unchanged to reduced and amplified contact hypersensitivity (Bennett et al. 2005; Kaplan et al. 2005; Kissenpfennig et al. 2005). These findings suggest that LCs are not the first responders to haptens in contact hypersensitivity. In fact it was proposed that CD14 positive dermal DCs play an important role. Kissenpfennig et al. made the important discovery that upon hapten skin painting of mice dermal DCs were the first cells present in the lymph node after only 24 hours whereas LCs arrived 2-4 days later. Additionally these two DC subsets migrated to distinct areas in the lymph node (Kissenpfennig et al. 2005). Above mentioned contact dermatitis findings were also partially reviewed in (Sasaki and Aiba 2007).

There has been an interesting paper by Jux et al. showing that LC maturation and contact hypersensitivity are impaired in Aryl hydrocarbon receptor-null mice. It has also been shown that $NiSO_4$ but not DNCB treatment of human DCs triggers RelA activation which in turn is important for the maturation process as the inhibition of nuclear RelA impaired this process. Interestingly RelA activation is independent of the p38 MAPK pathway which is also induced by $NiSO_4$ treatment (Ade et al. 2007). With regard to our interest in aryl hydrocarbon receptor (AhR) function in LCs we investigate AhR levels in human skin upon the application of contact irritant (SDS) and contact sensitizer ($NiSO_4$). We found interesting signaling analogy between AhR and RelB and further know that RelB plays an important role in limiting immune responses (Jorgl et al. 2007). Additionally Vogel et al. proposed a model where AhR and RelB synergistically act in immune responses (Vogel et al. 2007). Therefore we investigate the role of AhR and RelB interplay in LCs upon $NiSO_4$ stimulation.

3. Aryl hydrocarbon receptor

3.1. AhR signaling and structure:

The aryl hydrocarbon receptor (AhR) belongs to the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family of transcriptional regulators. AhR is induced by ligand-binding and was first identified about 30 years ago in the field of pharmacology as a major mediator of chemical toxicity (Burbach et al. 1992). Environmental pollutants such as polyaromatic hydrocarbons (PAHs) or dioxins were identified as ligands of this receptor. Their binding to the receptor ultimately leads to the activation of the so-called AhR-gene-battery consisting of phase I and II detoxifying enzymes such as cytochrome p450 family members Cyp1A1, Cyp1A2 and Cyp1B1 (Hankinson 1995; Nebert et al. 1993). These classes of enzymes ensure the biotransformation of low molecular weight chemicals and their excretion of the cell. Depending on chemical properties such as the receptor binding affinity, metabolizing capacity and the length of exposure it is possible that the biotransformation itself is blocked or metabolic intermediates accumulate and harm the cell by toxic effects. For example PAHs are known to be metabolized quickly after AhR induction by the actions of the detoxifying enzymes thereby rendering the chemicals water soluble and enabling excretion. Dioxins on the contrary have the highest binding affinities for AhR and are not properly metabolized which leads to prolonged AhR activity and accumulation of dioxins inside the cell where they trigger toxic effects. Dioxin toxicity in humans has gained great awareness because of multiple tragic incidents. While the Vietnam war the US military used the defoliant agent orange in a wide geographical scale as a military strategy to weaken guerilla fighters. For 10 years U.S. military planes atomized huge amounts of Agent Orange over wide landscapes. Only later it was discovered that the agent orange had been contaminated with the highly toxic AhR ligand 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD). According to estimates about half a million of people had been intoxicated or died immediately. Another half million of children had birth defects (York and Mick 2008). Another tragic incident happened in Seveso, Italy, where a chemical accident led to the release of a TCDD cloud which contaminated a wide area. Common symptoms of dioxin intoxication range from hepatocellular damage, thymic involution, immune suppression, chloracne, epithelial hyperplasia, teratogenesis to tumor promotion and birth defects in newborns (Sweeney and Mocarelli 2000).

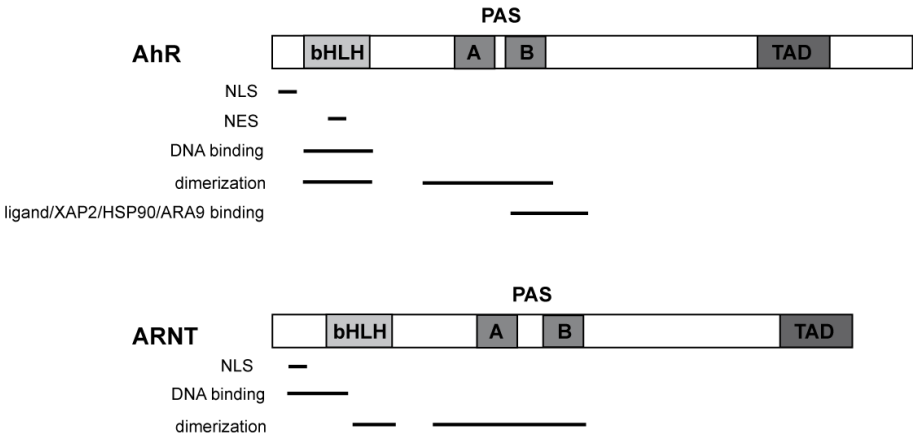


Figure 8: AhR and ARNT structure
Adapted from Stevens et al.

Structurally the Ah-receptor is comprised of an N-terminal bHLH domain for DNA binding and dimerization. This part also harbors the nuclear localization and export sequences (NLS and NES) which regulate receptor shuttling. In the center of the receptor is the PAS domain with two degenerate repeats (A and B) which not only serve signal sensor purposes by ligand binding but also mediate heterodimerization with ARNT, DNA recognition and chaperone interactions. The C-terminus has a transactivation domain for receptor transformation and potential co-activator recruitment (Stevens et al. 2009).

The inactive AhR protein is located in the cytoplasm and forms a multi-protein complex with 2 chaperone molecules heat shock protein 90 (Hsp90) (Heid et al. 2000), one X-associated protein 2 (XAP2) (Meyer et al. 1998), one co-chaperone molecule p23 (Kazlauskas et al. 1999) and the aryl hydrocarbon associated 9 (Ara9) protein (Carver et al. 1998). Exogenous AhR-ligands diffuse into the cell and bind to AhR which subsequently undergoes a conformational change leading to the exposure of a nuclear localization sequence (NLS) in the N-terminal part of the protein (Ikuta et al. 1998). The multiprotein complex is then translocated to the nucleus where it dissociates from the chaperone molecules and binds to AhR nuclear translocator (ARNT) protein (Reyes et al. 1992). This AhR/ARNT heterodimer harbors high DNA binding affinity and targets xenobiotic response elements (XREs) in the promoter and enhancer regions of particular genes and turns on their expression (Swanson et al. 1995). After transcription induction the receptor is exported to the cytoplasm where it gets ubiquitinylated and finally degraded in the proteosome (Davarinos and Pollenz 1999). The receptor regulates itself via a negative feedback loop by inducing the expression of the aryl hydrocarbon receptor repressor (AHRR) which competes with AhR for ARNT dimerization (Mimura et al. 1999; Watanabe et al. 2001).

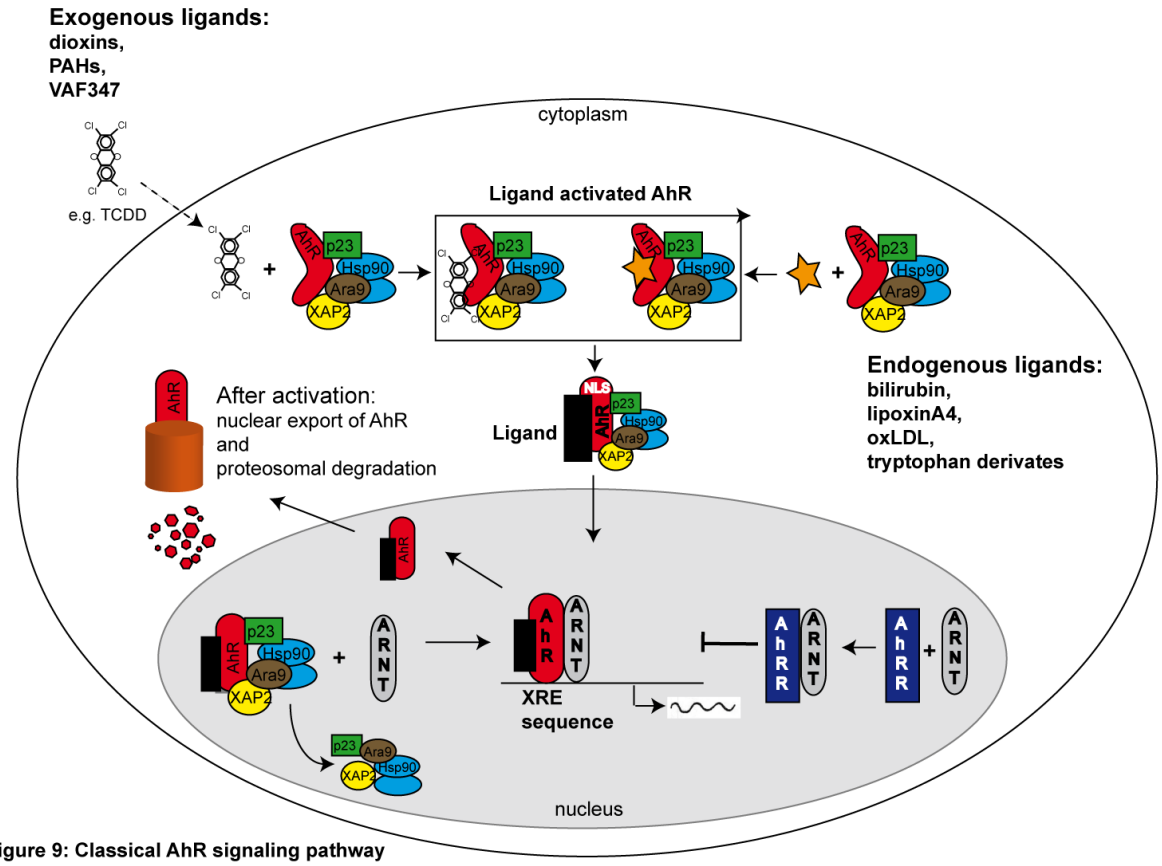


Figure 9: Classical AhR signaling pathway

Ahr exists in a multiprotein complex together with chaperone molecules that help to hold AhR in an inactive state in the cytoplasm able to bind its ligands. Exogenous ligands, mostly environmental pollutants or pharmacological drugs enter the cell via diffusion. Endogenous ligands are physiological molecules that are produced in metabolic processes. However upon ligand encounter AhR is activated and undergoes a conformational change that exposes a nuclear localization sequence. The activated multiprotein complex is now able to translocate to the nucleus where it subsequently dissociates from its chaperon proteins and dimerizes with the ARNT protein. This heterodimer is then capable of binding to xenobiotic response elements (XREs) in the promoter and enhancer regions of target genes thereby inducing transcription. AhR repressor (AhRR) competes with AhR for ARNT dimerization and constitutes a negative feedback loop for AhR driven transcription. After activation AhR is exported from the nucleus and degraded in the proteosome.

However in the last decade the receptor began to attract interest of immunologists as lots of the above mentioned physiologic consequences of dioxin intoxication led to immune suppressive disabilities. Apart from this, genomic screening analysis revealed that lots of genes involved in innate immune responses harbor a considerable amount of XREs in their promoters (Sun et al. 2004). Furthermore it is intriguing that many cell types involved in immune responses express AhR receptor protein (also reviewed in (Kerkvliet 2009).

Table 2: XREs of genes involved in innate immune responses
Adapted from Kerkvliet et al. 2009 as summarized from Sun et al. 2004

Innate response genes	XREs	Receptor genes	DREs	Associated molecules	DREs
Tlr1	5				
Tlr 2	2				
Tlr 3	3				
Tlr 4	5				
Tlr 5	9				
Tlr 6	3				
Tlr 7	5				
Tlr 8	2				
Tlr 9	3				
Il1		Il1r1	5	Irak1	5
		Il1r2	7	Irak1bp	7
				Irak4	4
				Il1rap	12
Il6	3				
Il18	10	Il18r1	3	Il18bp	1
				Il18rap	1
Tnfα	1				

3.2. AhR ligands and their implication in immune responses:

First of all it has to be said that there are some determining criteria in the ligand characteristics which are important for AhR activation and subsequent cellular effects. The AhR ligand spectrum is very wide. Ligands are characterized into endogenous and exogenous ligands (Denison and Nagy 2003). There are not only environmental pollutants binding to AhR and inducing toxic responses but also physiologic ligands play a crucial role in normal processes such as immune control and regulation. In the last decades a multitude of distinct ligands and alternative signaling pathways contributes to the complexity of the signalosome of AhR. Generally it is believed that AhR triggering leads to an overall immune suppression as lots of studies with the highly toxic AhR agonist TCDD have revealed. However newly discovered endogenous physiologic AhR ligands such as indigorubins, bilirubin, lipoxin A4 or oxidized low density lipoprotein (oxLDL) are implicated in modulating rather than initiating or suppressing immune responses. Apart from this, dietary compounds such as flavonoids, bisphenyls, indigorubins or indols were also identified to bind to AhR and induce anti-inflammatory and immune-regulatory effects (Benson and Shepherd 2011). (also reviewed in (Kerkvliet 2009; Nguyen and Bradfield 2008)

Moreover tryptophan derivates are another important class of endogenous ligands. Indoleamine 2,3-dioxygenase (IDO) is an immune-suppressing enzyme of the tryptophan metabolism and is present in multiple organs in the organism. Vogel et al. showed that AhR activation leads to the induction of IDO (Vogel et al. 2008). It produces kynurenin from tryptophan and thereby generates a potent AhR ligand. Kynurenin has been implicated in the progression of cancer as a patho-physiological factor that suppresses antitumor immune responses and promotes tumor progression (Opitz et al. 2011). In addition to this it has also been shown that kynurenin produced in DCs and the subsequent activation of AhR leads to a negative regulatory effect on the balance of Treg versus Th17 T-cell differentiation thereby significantly influencing the outcome of the immune response (Nguyen et al. 2010). On the other hand the tryptophan derivate 6-formylindolo[3,2-b]carbazole (FICZ) is a high affinity AhR ligand which is produced in the skin upon UVB irradiation and is quickly metabolized after AhR induction (Fritsche et al. 2007). Interestingly, this ligand is reported to rather induce than suppress immune reactions by favoring Th17 T-cell differentiation and IL-22 production leading to worsened autoimmune disease in an EAE mouse model (Veldhoen et al. 2008). Quintana et al. pose a model in which AhR activity controls and regulates differentiation of T-cells towards a Th17 or Treg phenotype thereby significantly influencing the outcome of an immune response (Quintana et al. 2008). In our studies we use FICZ as representative endogenous ligand with a concentration of 100nm. VAF347 on the other hand is a pharmacological drug designed by Novartis. It has been characterized as tryptophan derivate and potent exogenous AhR agonist (Lawrence et al. 2008). Studies revealed anti-inflammatory and autoimmune-suppressive effects triggered by diminishing the up-regulation of maturation markers such as CD86 and HLADR and a decrease in IL6 production (Ettmayer et al. 2006). It was further shown that VAF347 administration prolonged graft survival in a pancreatic transplantation model. Additionally mice treated with VAF347 yield a higher count of regulatory Treg cells (Hauben et al. 2008). For our experiments we use VAF347 as representative exogenous AhR ligand with a concentration of 100nm.

Other exogenous ligands are predominantly environmental pollutants. The most famous ligand is TCDD, a tetra-chlorinated dioxin which is not metabolizable and binds AhR with very high affinity. It induces immune-suppressive effects in a multitude of models. The immune suppressive effects in mice models treated with TCDD range from suppression of antibody-producing B-cells and CTL response to prolonging graft-survival and severe impairment of virus infection clearance (reviewed in (Kerkvliet 2009). A tremendous amount of studies exists for this AhR agonist, although it might not be the right ligand to study the physiological role of AhR in the immune system as TCDD leads to a prolonged and persistent AhR activation. This mechanism could mask subtle differences in AhR signaling by simply inducing toxic-related symptoms. Nonetheless TCDD was the most important AhR

ligand for elucidating molecular mechanisms of AhR activation. Another class of exogenous ligands are the non-halogenated polycyclic aromatic hydrocarbons (PAHs) which affect the cell rather by toxic than by immune-suppressive effects. PAHs such as benzo(a)pyrene diffuse into the cell and bind to AhR, thereby activating the transcriptionally active receptor. The main difference between dioxins and PAHs is their susceptibility to cellular metabolism. Whereas dioxins are not degradable and accumulate in the cell, PAHs are rapidly metabolized by AhR-induced detoxifying enzymes such as the Cyp family of enzymes. During this process toxic intermediates are generated which harm the cell by multiple ways, including diol-epoxide- mediated DNA damage (Davila et al. 1995; Mann et al. 1999; Ward et al. 1985).

Apart from these AhR functions in terminally differentiated cells there have been reports of a physiologic role of AhR in the regulation of HSCs and other stem/progenitor cell populations. AhR expression is essential for quiescence maintenance in HSCs whereas down regulation of AhR enables cells to proliferate pointing towards a negative regulatory function of AhR in hematopoiesis (reviewed in (Singh et al. 2009). Additionally Boitano et al. recently published a report about an AhR antagonist, StemRegenin 1(SR1), which promotes the expansion of human CD34 positive hematopoietic stem cells (Boitano et al. 2010). In our laboratory we could show that AhR triggering via VAF347 leads to an inhibition of LC differentiation from monocytic precursors which also underlines the negative regulatory function in hematopoietic differentiation processes (Platzer et al. 2009).

3.3. AhR signaling cascades and the alternative non-genomic signaling pathway:

For a long time the dogma in AhR biology was that the receptor can only signal through the classical genomic pathway of ligand binding and subsequent ARNT heterodimerization. It was believed that only this complex is in turn able to initiate transcription. This basic model of AhR action was mostly defined by studies which used TCDD as AhR ligand. Indeed there are lots of reports showing inflammatory responses in a TCDD-AhR signaling cascade-dependent manner. Vogel et al. reported that TCDD treatment of wild-type mice led to an induction of keratinocyte chemoattractant (KC) and monocyte chemoattractant protein 1 (MCP-1) in various organs in an AhR-dependent manner. Furthermore these chemoattractant molecules led to an infiltration of macrophages into these organs (Vogel et al. 2007). However it has always been a problem to solemnly explain all elicited inflammatory effects of TCDD by the oversimplified model of TCDD induced AHR/ARNT mediated gene transcription. For instance Sun et al. made a genome wide screening analysis of human, mouse and rat DNA for XRE sequences. He identified 2437 genes of which 48 were conserved throughout all species. Astonishingly only 19 genes were positionally conserved

of which only 7 were responsive to TCDD. In the end only 6 genes induces TCDD dependent intracellular changes and all of these genes harbor detoxifying functions (Sun et al. 2004). TCDD intoxication also leads to pronounced immune suppression which was so far not explainable with regard to the classical genomic pathway and the XRE-mediated TCDD responsiveness of genes (reviewed in (Matsumura 2009)). In the last years there has been a multitude of reports showing alternative AhR signaling pathways where ARNT is not involved as critical factor. The group of Fumio Matsumura was the first to show that kinase activity was involved in early TCDD administration to rats. In more detail they implicated the cAMP-dependent protein kinase (PKA), protein kinase C (PKC), tyrosine kinases and Src kinase $pp60^{c\text{-}src}$ in the process of TCDD activation and that these signaling pathways lead to the activation of EGF receptor which in turn plays a crucial role in the wasting syndrome of dioxin intoxication (Bombick et al. 1985; Bombick and Matsumura 1987). Also other groups reported of an immediate and rapid increase of free Ca^{2+}-concentration in TCDD treated Hepa1 cells (Puga et al. 1992). Nebert et al. even directly connected the TCDD induced Ca^{2+} rise to signal transduction activity of AhR leading to an activation of transcriptionally active proteins such as AP-1 (Nebert et al. 1993). The Matsumura group further emphasized that TCDD treatment of the MCF10a cell line led to an immediate increase of arachidonic acid (AA) in the culture medium. AA is released by the cytosolic phospholipase (cPLA2) which is activated by elevated Ca^{2+} levels. These immediate effects were dependent on AhR but independent of ARNT and Src kinase. Therefore they conclude that TCDD activated AhR induces an intracellular Ca^{2+} increase triggering the activity of cPLA2. Next the activated cPLA2 activates the Src kinase which then induces the expression of the pro-inflammatory gene Cox-2 via AP-1 transcription factor (Dong and Matsumura 2008). Park et al. could further show that TCDD administration to the MCF10a cell line led to the suppression of growth and differentiation via Src kinase induced ERK MAPK signaling (Park et al. 2004). Interestingly Scullio et al. investigated the same TCDD mediated early signaling mechanisms but found different results in the U937 cell line differentiated macrophages which more likely resemble immunologic cells than the epithelial cell line MCF10a. TCDD activated macrophages respond with the production of pro-inflammatory cytokine TNFα thereby clearly underlining the immunogenic capacity of the toxic compound TCDD. However the striking difference was that the Ca^{2+} level increase and subsequent cPLA2 activation led to Src kinase-independent Cox-2 gene expression (Sciullo et al. 2008). Apart from this nongenomic AhR signaling pathway there are other implications of alternative signaling cascades in AhR function. In more detail, Ikuta et al. reported that AhR intracellular localization is regulated by cell density and cell-cell contact signals. They identified Ser-68 in the nuclear export sequence as phosphorylation target upon accumulation of activated AhR in the nucleus. The

Ser-68 phosphorylation happened in a p38 MAPK, okadaic acid and Ca^{2+} concentration dependent way (Ikuta et al. 2004).

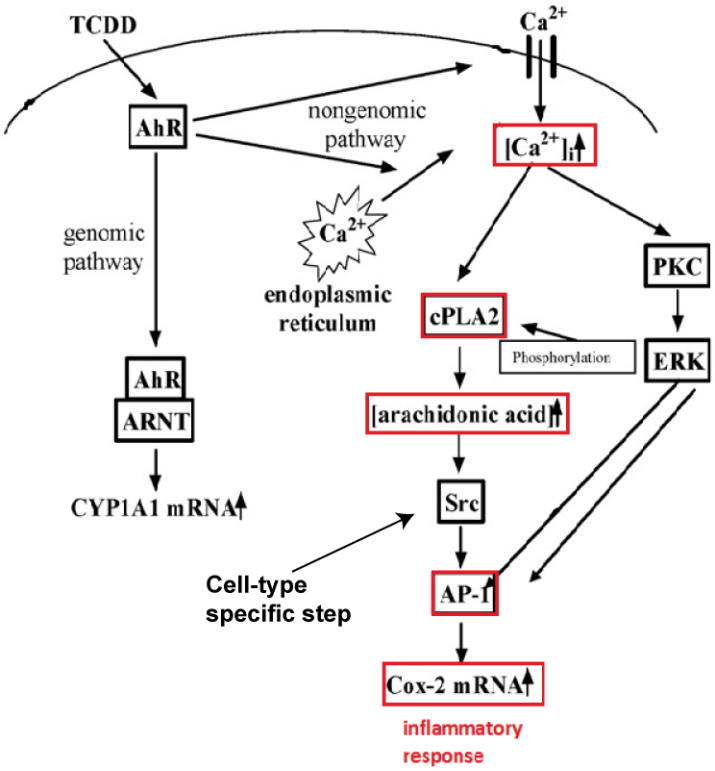


Figure 10: Non-genomic AhR signaling pathway
Adapted from Matsumura et al. 2009

TCDD exposure activates AhR. Besides the classical genomic pathway (AhR/ARNT dimerization), Dong et al propose a new alternative nongenomic signaling pathway mainly acting through a rapid elevation in free Ca2+ ions (Dong et al. 2008). This in turn leads to the activation of cytoplasmic phospholipase 2 and subsequent arachidonic acid release. Thereafter Src kinase is activated although this step is not essential as only MCF10a cell line used this kinase step whereas U937 macrophages did not activate Src kinase (Sciullo et al. 2008). However in any case the transcription factor AP-1 is induced and turns on the transcription of inflammatory response genes such as Cox-2. Additionally the rise in intracellular free Ca2+ ions leads to the activation of PKC signaling which in turn activates ERK MAPK. This step provides supplemental activation of AP-1. Red highlighted steps are essential in the nongenomic-cPLA2-dependent pathway.

Nowadays the involvement of MAPK signaling in AhR function is accepted although clear mechanisms of action are lacking due to an immense diversity depending on the cell type, environmental surrounding and the ligand used for AhR activation. However MAP kinase signaling pathways are implicated in receptor shuttling, immune response modulation by enhancing, repressing or termination of AhR signaling (reviewed in (Henklova et al. 2008)). P38 MAPK was implicated in TCDD-mediated gene transcription in mouse and human hepatoma cell lines, as inhibition of p38 signaling by a chemical inhibitor suppressed transcription of AhR target genes (Shibazaki et al. 2004). Tan et al on the other hand reported the opposite, namely the induction of ERK and JNK signaling but not p38 MAPK upon TCDD activation in an AhR-independent way (Tan et al. 2002). Another level of complexity was reached when Weiss et al. reported that c-jun transcription factor (JNK MAPK), is activated in an AhR-dependent p38 MAPK signaling cascade (Weiss et al. 2005) (reviewed in (Puga et al. 2009). In our experiments we focus on the question which MAPK signaling cascade triggers the nuclear translocation of AHR in in-vitro PGN-activated LCs. With this approach we want to shed light on the situation in activated DCs by other stimuli than exogenous AhR ligands with respect to an endogenous physiologic role of AhR in DC maturation. For this purpose we make use of special MAPK inhibitors discussed in chapter 2.7..

3.4. Role of AhR in DCs:

As it became obvious that AhR plays an important role in immune responses, researches started to investigate various cell types of the immune system for potential effects of AhR activity. As described above DCs have a pivotal role in linking the innate to the adaptive immune response. DCs are therefore the lynchpin in eliciting proper immune responses. In addition to this our lab and others showed that DCs express high levels of AhR implicating a physiologic cell-specific role for this receptor. Jux et al. reported that LCs of AhR-deficient mice show a reduced potential to up-regulate co-stimulatory molecules and down-regulate phagocytic capacity upon contact hypersensitivity pointing towards a role of AhR in LC maturation (Jux et al. 2009). The results of Bankoti et al. are in agreement with this notion because they indicate an increased MHC class II, CD40 and CD86 expression and augmented pro-inflammatory cytokine production (IL-6, TNFα) upon TCDD-induced AhR signaling. Additionally they saw down-regulated RelA levels but an increase of RelB upon TCDD administration to BMDCs from mice. Interestingly these effects were strictly AhR-dependent but not exclusively XRE-mediated (Bankoti et al. 2010). Dalei Wu et al. were the first to pose a model of ligand-independent AhR signaling interaction with classical TLR and NFκB mediated transcriptional immune responses. They treated wild-type and AhR knock-out mice with LPS and found that LPS induced gene transcription was impaired in a tissue and time-dependent manner as were the binding affinities of classical transcription factors such as NFkB, AP-1 and C/EBP (Wu et al. 2011). Nguyen et al. further reported that BMDCs of AhR-deficient mice are unable to up-regulate IDO. This enzyme is in turn needed to generate Kynurenin from tryptophan. Kynurenin is a known AhR ligand and influences the T-cell balance between Th17 and Treg cells. This group implicates a negative immune-regulatory role for AhR in LPS or CpG induced immune reaction (Nguyen et al. 2010). There exists a large body of proof that AhR in DCs is necessary to further regulate AhR-dependent immune regulatory functions in other cell types such as T-cells (Quintana et al. 2008; Veldhoen et al. 2008). Benson et al. could proof that dietary non-toxic AhR ligands such as indole-3-carbinol (I3C) and indirubin-3'-oxime (IO) induce immune-suppressive and anti-inflammatory effects in BMDCs from mice. Maturation markers such as CD40 and CD54 were decreased but MHC class II and CD80 were increased. Pro-inflammatory cytokines such as TNFα, IL-6, IL-12 and IL-1β were decreased while immune suppressive cytokine IL-10 was increased. Immune regulatory enzymes such as IDO were up-regulated and T-cell stimulatory capacity was altered towards Treg induction. All these effects were partially AhR-mediated (Benson and Shepherd 2011). VAF347, a tryptophan derivate and anti-autoimmunity drug mediates its effects in DCs. In an allergic asthma mouse model researchers could show that AhR induction with VAF347 ligand decreases HLADR and CD86 maturation marker expression in DCs. In addition VAF347 treated DCs display a

diminished potential of proliferation and IL-2 production in T-cells (Ettmayer et al. 2006). VAG539 (pro-drug of VAF347 which gets converted in vivo) administration to mice and subsequent isolation and transfer of DCs to transplanted mice markedly prolongs graft survival. The same is true for VAF347 in-vitro differentiated BMDCs (Hauben et al. 2008).

3.5. AhR interplay with candidates other than ARNT and its implication:

So far we have learnt about the genomic and nongenomic signaling pathway of AhR. Further complexity was added by the various MAPK signaling cascades implicated upstream and downstream of AhR signaling in immunologic responses. In the last decade the controversial discussion about distinct AhR functions in immunologic processes even gained intricacy when researchers found that AhR is able to interact with NFκB family members (Tian et al. 2002). AhR was reported to physically interact with RelA and thereby inhibit typical RelA driven transcription (Tian et al. 1999). Ruby et al made an interesting finding when they treated a dendritic cell line with TCDD and subsequently activated the cells with TNFα or CD40. They observed that the translocation of RelA/p50 heterodimers to the nucleus was suppressed. At the same time AhR binding to RelA seemed increased while DNA binding was blocked. Interestingly inhibitory p50 homodimers were not affected by TCDD-activated AhR and could freely bind to DNA. The authors pose a model in which activated AhR alters the balance between RelA/p50 heterodimers and p50 homodimers which leads to the defects in DCs resulting in immune suppression (Ruby et al. 2002). Nevertheless there were also reports suggesting a positive transcriptional role of AhR/RelA cooperation. Namely Kim et al. observed an AhR/RelA heterodimer which binds to NFκB binding sites in the promoter of c-myc and transactivates its transcription in breast cancer cell lines (Kim et al. 2000). Apart from this, AhR also interacts with RelB although this interaction tends to augment transcription of immunologic genes such as cytokines and chemokines. Vogel et al. reported that TCDD-mediated stimulation of U937 macrophages resulted in the induction of chemokines in an ARNT-independent way but RelB was obligatory. They further proposed that AhR and RelB physically interact and bind to a novel AhR/NFκB binding site in the promoters of these genes (Vogel et al. 2007). Vogel et al. further refined their model and proposed that the chemokine induction is AhR and protein kinase A (PKA) dependent. Activated AhR and RelB physically interact and bind to novel sites in the DNA thereby linking two signaling pathways. Nonetheless the authors claim that classical NFκB sites normally targeted by RelB/p52 heterodimers can also serves as target for the novel AhR/RelB heterodimer even without stimuli indicating a normal physiological role (Vogel et al. 2007). Later the same group reported about the involvement of RelB and AhR in TCDD-stimulated breast cancer cell lines by forming an AhR/RelB-heterodimer which ultimately leads to a

pronounced IL-8 over-expression (Vogel et al. 2011). To sum up the findings by Vogel et al., the group proposes a model in which AhR/RelB heterodimers link the classical AhR/ARNT pathway and the alternative NFκB pathway (Vogel and Matsumura 2009).

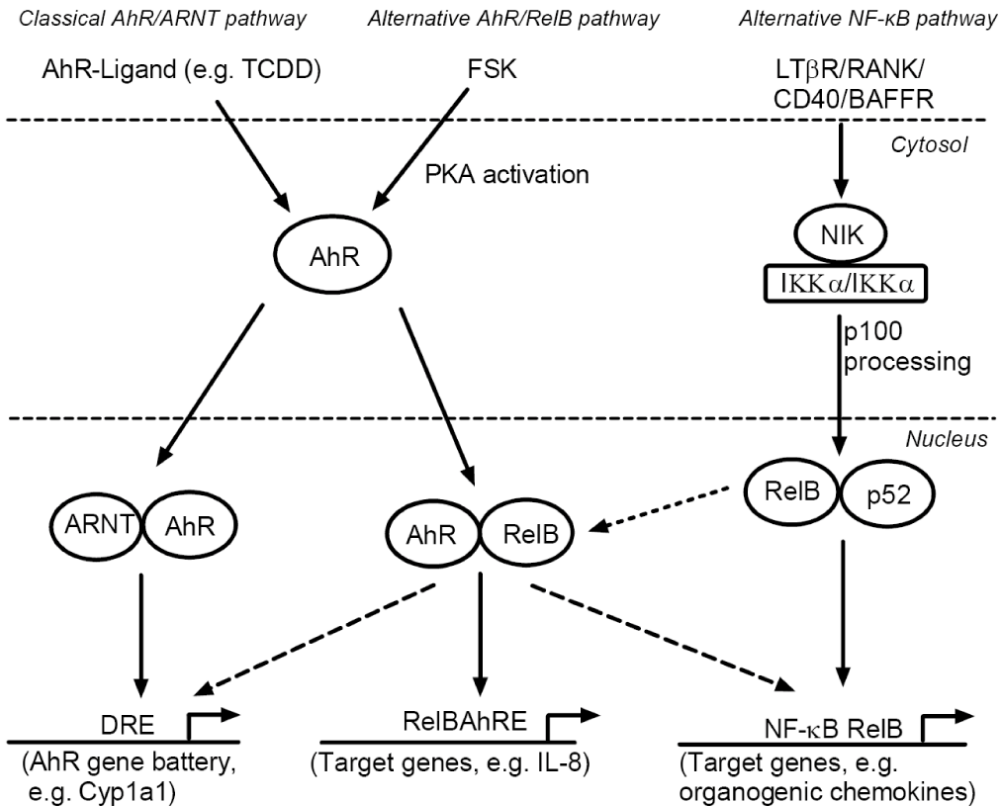


Figure 11: AhR/RelB heterodimer signaling
Adapted from Vogel and Matsumura et al. 2009

In our studies we focus on the question whether AhR and RelB interplay in CD34 derived in-vitro generated LCs upon $NiSO_4$ activation.

Baglole et al. also reported about an AhR-dependent regulation of RelB in lung fibroblasts. AhR-deficient fibroblasts showed a heightened tobacco-smoke induced Cox-2 and Prostaglandine production and a loss in RelB expression whereas the symptoms in AhR-deficient fibroblasts transfected with an AhR expressing plasmid, were attenuated by a mechanism that maintained RelB expression (Baglole et al. 2008). Another group investigated the mechanism of the synergistic induction of IL-6 by AhR and inflammatory signaling. They came to the conclusion that TCDD-activated AhR primes the IL-6 promoter by binding to upstream non-consensus XREs thereby facilitating IL1β-induced RelA/p50 or c-REL/p50 binding. Furthermore they could pose a mechanism because they subsequently observed the recruitment of histone deacetylase (HDAC) 1 and 3 to AhR primed promoter, reorganizing repressive complexes on DNA and priming the locus for transcription (DiNatale et al. 2010). Jensen et al. reported the opposite phenomenon in a bone-marrow derived cell line. They saw a TCDD-activated AhR-dependent inhibition of IL-6 transcription upon LPS

activation. At least for AhR agonist 7,12-Dimethylbenz[a]anthracen (DMBA) they report a decrease in RelA/p50 and c-Rel/p50 heterodimer binding at the IL-6 promoter (Jensen et al. 2003). Again it becomes obvious that there is no common signaling mechanism for AhR-mediated effects in the immune system. Every cell type investigated shows a distinct pattern. This further underlines the concept that AhR signaling receives multiple crosstalk from other signal transduction pathways and that this process is cell-type, micro-environment and cell-state specific.

Material and Methods

4. Cell culture:

4.1. Media, buffers and solutions:

Table 3: Media

Media		Supplementations	Company
X-Vivo	Serum-free hematopoietic cell medium	125U/ml penicillin 125U/ml streptomycin 2,5mM GlutaMAX	Lonza
Cell-Gro DC	Serum-free dendritic cell medium	125U/ml penicillin 125U/ml streptomycin 2,5mM GlutaMAX	CellGenix
RPMI-1640	(Roswell Park Memorial Institute) Cell line medium for suspension culture	10% FCS 125U/ml penicillin 125U/ml streptomycin 2mM L-gluatmine	Sigma Aldrich
DMEM	(Dulbecco´s modified eagle´s medium) Cell line medium for adherent culture	10% FCS 125U/ml penicillin 125U/ml streptomycin 2mM L-gluatmine	Sigma Aldrich
PBS	Phosphate-buffered saline	-	Sigma Aldrich

Table 4: Buffers and solutions

Buffers/solutions	Ingredients
10xPBS	400g NaCl (Roth) 10g KCl (Roth) 72g Na_2HPO_4 (Roth) 10g KH_2PO_4 (Roth) adjust to pH 7,4 in 5l dH_2O
PBST	1x PBS 0,05% Tween20 (Roth)
PBSTT	1xPBS 0,1% Tween20 (Roth) 0,05% TritonX-100 (Polysciences, Inc.)
1x PBS/BSA/Azide	1xPBS 20% BSA (Roth) 0,4% Na_3N (Roth)
Beriglobin	Beriglobin (CSL Behring) diluted 1/8 in 1xPBS
MACS buffer	250ml 10xPBS 20mM EDTA 5% BSA Dilute 1/10 with H_2O dest.
10x blotting buffer	30,3g Tris (Roth) 144g Glycin (Roth) in 1L dH_2O

10x SDS-PAGE buffer	30,2g Tris (Roth) 144g Glycin (Roth) 10g SDS (Roth) in 1L dH_2O
Stripping buffer	100ml 20%SDS (Roth) 2,5ml 1M Tris/ HCl pH 7,5 7,3ml β-mercaptoethanol (Sigma) 830,2ml dH_2O
8% separating gel	2134µl 30% Acrylamid/Bis (Biorad) 2000µl Tris/HCl pH 8,8 3736µl dH_2O (Fresenius) 40µl 20% SDS (Roth) 10µl TEMED (Biorad) 80µl 10% APS (Biorad)
4% stacking gel	396µl 30% Acrylamid/Bis (Biorad) 378µl Tris/HCl pH 6,8 2196µl dH_2O (Fresenius) 15µl 20% SDS (Roth) 5µl TEMED (Biorad) 20µl 10% APS (Biorad)
4x sample buffer	0,5M Tris/HCl pH 6,8 40% Glycerol (Roth) 4% SDS (Roth) 5µl/ml Bromphenolblue (Roth) 5% β-mercaptoethanol (Sigma)
2xHBS	8g NaCl (Roth) 6,5g HEPES (Sigma) 105mg NaH_2PO_4 (Roth) in 500ml dH_2O (Fresenius) adjust to pH 7,00
2M $CaCl_2$	14,70g $CaCl_2$ (Roth) in 50ml H_2O
dH_2O	Aqua bidest. “Fresenius” (Fresenius Kabi)
ACK lysis buffer	8,3g NH_4Cl (Roth) 1g $KHCO_3$ (Roth) 37,2mg Na_2-EDTA (Roth) in 1L dH_2O adjust to pH 7,2-7,4 sterilize with 0,2µm filter
Blocking solution	0,2g BSA (Roth) 100µl of a 10% Tween20 stock solution 5µl of a 20% Sodium azide stock solution Fill up with 1xPBS to 10ml

4.2. Reagents and cytokines:

Table 5: Reagents		
Reagents	**Company**	**Working concentration**
FCS (fetal calf serum)	Gibco/Invitrogen	Heat inactivated at 65°C water bath for 30 min prior to use
L-glutamine	Sigma Aldrich	2mM
GlutaMAX	Gibco/Invitrogen	2,5mM
Trypsin-EDTA	Sigma Aldrich	3ml/10cm dish
Penicillin	Sigma Aldrich	125U/ml

Streptomycin	Sigma Aldrich	125U/ml
Donkey-anti-human IgG	Jackson ImmunoResearch	10µg/ml
Delta-1 IgG	Kindly provided by Bernstein ID	1µg/ml
Chloroquin	Sigma Alrich	50mM
Retronectin	Takara Bio	30µg/ml

Table 6: Cytokines

Cytokines		Company	Working concentration
GM-CSF	Granulocyte-macrophage colony stimulating factor	Kindly provided by Novartis Institutes of Biomedical Research and PeproTech	100ng/ml
TPO	Thrombopoietin	PeproTech	50ng/ml
Flt3Ligand	Fms-related tyrosine kinase 3 ligand	PeproTech	50ng/ml
SCF	Human stem cell factor	PeproTech	20ng/ml
TNF-α	Tumor necrosis factor alpha	PeproTech	2,5ng/ml
TGF-β1	Transforming growth factor beta1	R&D Systems GmbH	0,5ng/ml for LC 10ng/ml for moLC
IL4	Interleukin 4	PeproTech	2,5ng/ml

4.3. AhR ligands and activation stimuli:

Table 7: AhR ligands

AhR ligands		Company	Working concentration
VAF347	4-(3-chlorophenyl)-N-[4-(trifluoromethyl)phenyl]pyrimidin-2-amine, 4-(3-Chlorophenyl)-N-(4-(trifluoromethyl)phenyl)pyrimidin-2-amine	Kindly provided by Novartis	100nm
FICZ	6-formylindolo[3,2-b]carbazole	Biomol International, Inc.	100nm

Table 8: Activation stimuli

Activation stimuli		Company	Working concentration
PGN	Peptidoglycan	Sigma Aldrich	10µg/ml
LPS	Lipopolysaccharide	Sigma Aldrich	2µg/ml
SDS	Sodium dodecyl sulfate	Roth	20µg/ml
$NiSO_4$	Nickel sulfate	Roth	500µM

4.4. Isolation of cord blood CD34 and peripheral blood CD14 positive cells:

Cord blood is obtained from full-term deliveries of healthy donors. The Medical University of Vienna review board approved these studies and informed consent is provided according to the Declaration of Helsinki. Human peripheral blood samples from healthy donors and cord blood samples are diluted 1:1 with 1xphosphate buffered saline (1xPBS). To separate the mononuclear cell fraction from serum, erythrocytes and granulocytes the diluted blood is covered over a density gradient of lymphoprep (Axis Shield PoC AS) and centrifuged without deceleration for 25 minutes with 1500 rpm. The mononuclear cell ring is carefully harvested and washed with 1xPBS. To get rid of contaminating erythrocytes the cells are incubated in 4°C cold ACK lysis buffer for 10 minutes on ice. After washing the cells for 10 minutes in PBS with 1200 rpm the isolation is carried out according to the manufacture's protocol. For CD34 positive hematopoietic stem cell isolation from cord blood we use the EasySep human CD34+ selection kit (StemCell Technologies Inc.). CD14 positive monocyte isolation from peripheral blood is achieved by labeling the mononuclear cell fraction with 20µl of CD14-PE labeled antibody (BioLegend Inc) for 15 minutes on ice. After washing with 1xPBS for 10 minutes with 1200rpm the cells are coupled to MACS-anti-PE magnetic beads (Miltenyi Biotech) for 15 minutes on ice. After another washing step with 1xPBS for 10 minutes with 1200rpm cells are dissolved in MACS buffer and isolated over LS MACS columns (Miltenyi Biotech) according to manufacturer´s protocol. Isolated cell purity is analyzed by FACS (~90%).

4.5. Cell lines:

The Phoenix-GalV packing cell line (PhGP) is cultured in DMEM complete medium (supplemented with 10%FCS, 2mM L-glutamine, 125U/ml of both Penicillin and Streptomycin) in Nunclon^{TM}ΔSurface 10cm culture dishes (Nunc) and split every 2nd day 1/7-1/9. For splitting adherent cells we first aspirate the culture supernatant and add 3 ml of Trypsin-EDTA (Sigma Aldrich). When cells start to detach, the reaction is stopped by adding 8ml of DMEM medium. After a washing step cells are split 1/7 and seeded in a new dish. This cell line is maintained at 37°C with 5% CO_2.

4.6. Freezing and thawing of cells:

~1*10^6 cells are pelleted by a 5 minute centrifugation step with 1200rpm. The pellet is dissolved in FCS + 10%Dimethyl Sulfoxide (DMSO; Sigma Aldrich) and placed in a Mr. Frosty freezing container (Sigma Aldrich) and stored at -80°C. For thawing the cells, we used 10ml of pre-warmed medium to dissolve the cells and wash them in a 5 minute centrifugation

step at 1200rpm. The cells were then plated in the according medium and maintained at 37°C and 5% CO_2.

4.7. Cell culture and differentiation of primary cells:

CD34+ hematopoietic stem cells isolated from cord blood are expanded for 3-7 days in serum-free X-vivo complete medium in the presence of 50ng/ml of each Flt3L, TPO and SCF. The expanded CD34+ cells are then used to in-vitro differentiate interstitial dendritic cells (IntDCs) or langerhans cells (LCs). The latter cell type is generated in serum-free Cell Gro DC complete medium in the presence of 100ng/ml GM-CSF, 20ng/ml SCF, 50ng/ml Flt3L, 2,5ng/ml TNFα and 2ng/ml TGF-β1. After 7 days of differentiation LCs form typical cell clusters which can be purified over PBS. The pipette tip is cut off to carefully resuspend the cells and transfer them on 8ml 1xPBS in a 15ml falcon tube. The clusters settle on the bottom of the tube after 2-5 minutes and the supernatant is discarded. IntDCs are generated with a 2-step protocol. First the expanded CD34+ cells are cultured in Cell Gro DC complete medium in the presence of 100ng/ml GM-CSF, 20ng/ml SCF, 50ng/ml Flt3L and 2,5ng/ml TNFα for 5 days. Afterwards the cells are harvested and centrifuged for 5 minutes with 1200rpm and seeded 1:2 in RPMI-1640 complete medium in the presence of 100ng/ml GM-CSF and 2,5ng/ml IL-4 for another 5 day differentiation period.

CD14 positive monocytes isolated from peripheral blood can either be differentiated into monocyte-derived DCs (moDCs) or monocyte-derived LCs (moLCs). To generate moDCs the CD14+ monocytes need to be cultured in RPMI-1640 complete in the presence of 100ng/ml GM-CSF and 2,5ng/ml IL-4 for 4 days. MoLC generation requires a special coating of the culture plates with Notch ligand Delta-1. 24-well non-tissue suspension culture dishes (Greiner) are incubated with 10µg/ml donkey-anti-human IgG in PBS for 1 hour at 37°C 5%CO_2. After 1 hour of RPMI complete blocking at 37°C supernatant is discarded and 1µg/ml Delta-1 in PBS is transferred onto the blocked wells for 3-4 hours at 37°C or overnight at 4°C. The CD14+ monocytes are then seeded onto the Delta-1 coated wells in RPMI complete medium in the presence of 100ng/ml GM-CSF and 10ng/ml TGF-β1 and incubated for 4 days.

All primary cells are maintained in an incubator with 37°C and 5% CO_2 saturation.

Table 9: Differentiation models of primary cells Adapted from (Haslwanter 2012)

Precursor	Cell type	Medium	Cytokines	Time [d]	Reference
CD34	LC	Cell Gro DC complete	GM-CSF, SCF, Flt3L, TNFα, TGF-β1	7	(Strobl et al. 1996)
CD34	IntDC	Cell Gro DC complete & RPMI complete	1st: GM-CSF, SCF, Flt3L and TNFα 2nd: GM-CSF, IL-4	10	(Caux et al. 1996)
CD14	moDC	RPMI complete	GM-CSF, IL-4	5	(Sallusto and Lanzavecchia 1994)
CD14	moLC	RPMI complete	GM-CSF, TGF-β1 Delta-1 coated wells	4	(Hoshino et al. 2005)

Maturation studies were done after the initial differentiation period. LCs are cluster purified whereas the other cell subsets are not purified. The cells are cultured in Cell Gro DC complete medium in the presence of 100ng/ml GM-CSF. 2µg/ml SDS, 500µM $NiSO_4$, 2µg/ml LPS or 10µg/ml PGN are incubated for 24 hours at 37°C with 5% CO_2 saturation.

4.8. Retroviral infection and transfection:

For the study of a potential RelB/AhR interplay in LC maturation upon $NiSO_4$ treatment, we infect CD34+ hematopoietic stem cells with the constitutively active retroviral vector pBMNp100ΔN-IRES-GFP (Solan et al. 2002). The amphotropic virus is generated in the 293T derived packaging cell line Phoenix-GP which is transformed with an E1a adenovirus and carries a temperature sensitive T antigen co-selected with neomycin. These cells encode two retroviral genes Gag and Pol which enable virus packaging and viral replication (Nolan). Phoenix-GP cells are split one day before transfection in the normal 1/7-1/9 ratio and seeded onto Nunclon™ΔSurface 6cm culture dishes (Nunc) in DMEM complete medium. On the next day the vector plasmid is transfected into the packing cell line by calcium-phosphate precipitation. The DNA precipitate is formed by pipetting together 12µg vector DNA, 8µg GalV plasmid DNA, 3µg gagpol plasmid DNA and 63µl 2M $CaCl_2$. After adding 430µl dH_2O and 500µl 2xHBS buffer pH 7.1, the mixture is immediately vortexed for 45 seconds and incubated at room temperature for 15 minutes. In the meantime 3µl of 50mM chloroquin (Sigma Aldrich) are applied drop wise to the Phoenix-GP culture. After 5 minutes of incubation the DNA precipitates are carefully pipetted drop wise onto the Phoenix-GP cells which are then incubated at 37°C 5% CO_2 for 6 hours. Transfection rates is checked by FACS, 90-100% transfection efficiency must be achieved for further infection. Then the medium is carefully aspirated and 2ml of DMEM complete are carefully added to the cells.

2days later the culture supernatant containing the virus particles is filtered through a 0,45µm syringe filter (Iwaki) and the cells itself were analyzed for transfection efficiency by FACS. The virus producing Phoenix-GP cells are again incubated with 2ml of DMEM complete over night. On the next day CD34+ cells are further infected with the viral supernatant. For this we coat 24-well non-tissue suspension culture dishes (Greiner bio-one GmbH) with 1mg/ml Retronectin (Takara Bio) in 1xPBS over night at 4°C. 700µl of filtered viral supernatant is transferred onto the Retronectin-coated wells and incubated for 4 hours at 37°C. 1 day expanded CD34+ hematopoietic stem cells isolated from cord blood are dissolved in X-vivo medium complete with a cell density of 2*10^4/700µl. 400µl of viral supernatant are carefully taken off and 700µl of cells are added onto the wells. CD34 expansion mix is prepared (100µl X-vivo complete medium with 50ng/ml of TPO, SCF and Flt3L) and 10µl are added per well. On the next day the second infection round is done by carefully exchanging 400µl culture supernatant with filtered viral supernatant. The CD34 cells are then again expanded in the presence of new expansion mix until the next day. After the infection procedure the infection rate is analyzed by FACS. When an infection rate of 60-90% is achieved cells are further differentiated into LCs (see differentiation of primary cells chapter 4.7).

5. Immunfluorescence stainings:

5.1. Fluorescent activated cell sorting (FACS analysis):

Surface marker expression is analyzed by FACS. Up to 5*10^5 cells are stained per micronic tube (Thermo Scientific). First the cells are washed with 1xPBS/BSA/Azide for 5 min at 300g centrifugation at 4°C. All further steps are carried out on ice and incubation times at 4°C in the fridge. The supernatant is discarded and the remaining 50µl of cell suspension is vortexted and incubated with 10µl beriglobin for 10 minutes at 4°C to block unspecific Fc-binding of the antibodies. Afterwards 10µl of each antibody dilution are added to the beriglobin blocked cell suspension and incubated for 20-30 minutes at 4°C. In case the primary antibody is biotinylated instead of directly fluorophore-coupled, cells are washed once with 1xPBS/BSA/Azide for 5 minutes at 300g. The secondary antibody (SA-PerCP) is incubated for 30 minutes at 4°C. Afterwards cells are washed twice with 1xPBS/BSA/Azide and either analyzed right away or fixed with 100µl of FIX medium (An der Grub) for 7 minutes in the dark and washed once with PBS/BSA/Azide for 5 minutes with 300g. The micronitc tube is filled up with PBS/BSA/Azide and stored in the fridge. Thereafter cells can be analyzed for up to one week. Prior to analysis cells need to be washed once in 1xPBS to get rid of the Azide and supernatant was aspirated. Cells are then vortexted in the remaining 50µl 1xPBS. All antibody dilutions are made with 1xPBS/BSA/Azide and prepared as mastermix before addition to the cells. For flow cytometric analysis of the cells we use the

BD LSRII cytometer and data analysis is done with the FlowJo software (BD Biosciences). For the list of antibodies used and the corresponding dilution see table 10.

Table 10: Antibodies for FACS analysis

Antibody	Company	Stock concentration
CD1a-Pacific Blue	BioLegends	1/10
Langerin-PE (CD207)	Immunotech	1/10
E-cadherin-APC	BioLegends	1/20
CD14-APC-Cy7	BD Bioscience	1/30
CD14-FITC	BD Bioscience	1/5
CD11b-PE-Cy7	BD Bioscience	1/50
CD40-PE	BD Bioscience	1/20
HLADR-biot	BD Bioscience	1/10
CD80- biotylated	BD Bioscience	1/5
CD83-APC	BD Bioscience	1/5
CD86-FITC	BD Bioscience	1/10
CD86-biotylated	BD Bioscience	1/5
Streptavidin(SA)-PerCP	BD Bioscience	1/100

5.2. AhR and RelB staining of moLCs and LCs:

Cells are harvested and washed once with 1xPBS for 5 minutes at 1200rpm.

AhR staining:

Then cells are resuspended in 1xPBS at a concentration of 2-8*10^4cells/50µl. 12-spot adhesion slides (Marienfeld) are then prepared by rinsing off the green protection film with water and 1xPBS. Afterwards 50µl of the cell suspension are pipetted onto each spot and incubated in a liquid chamber for 10-15 minutes at RT. For fixation of the cells the slides are incubated in -20°C methanol for 10 minutes and shortly washed in 1xPBS. Next, the cells are blocked for 10 minutes with 30µl beriglobin per spot. Primary antibody incubation is done over night at 4°C with 1/100 anti-AhR antibody or 1/100 goat IgG antibody in 2%BSA/PBS. On the next day slides are washed three times with 1xPBS for 3 minutes before blocking with 5% donkey serum in 1xPBS for 30 minutes. Secondary antibody donkey-anti-goat-AF488 coupled (or –AF546 coupled) is diluted 1/500 in 2%BSA/PBS and incubated for 1 1/2hours at RT in the dark. After three washing steps of 3 minutes in 1xPBS we do a specificity blocking step with 5% mouse serum in 1xPBS for 30 minutes before we counter-stained with CD1a-AF546 1/750(or –APC coupled 1/50) diluted in 2%BSA/PBS for 4 hours at RT in the dark. After another three 3 minute washes in 1xPBS the nuclei are stained with 1/100 dapi dilution in 1xPBS for 10 minutes in the dark. Then slides are washed two times 5 minutes in 1xPBS and mounted in fluoroshield (Sigma Aldrich) with a cover slip and the edges are sealed with nail polish. Microscopic pictures are made with 20x or 40x objectives with a Zeiss LSM700 confocal microscope at the imaging facility of the medical university of Vienna.

RelB staining:

Cells are resuspended in 50µl 1xPBS and 50µl FIX and PERM solution (ADG Bio Research GmbH) is incubated for 15 minutes at RT. After a washing step with 500µl PBS for 5 minutes at 1200rpm the cells are resuspended in 1xPBS at a concentration of 2-8*10^4. 12-spot adhesion slides (Marienfeld) are then prepared by rinsing off the green protection film with water and 1xPBS. Afterwards 50µl of the cell suspension are pipetted onto each spot and incubated in a liquid chamber for 20 minutes at RT. For fixation of the cells the slides were incubated in -20°C methanol for 3 minutes and 30 seconds in -20°C aceton. Permeabilization of the cells is achieved by incubation of the slides in 0,1%TritonX-100 in 1xPBS for 3 minutes at RT. Cells are then blocked for 30 minutes in a blocking solution (0,2gBSA, 100µl Tween20 (of a 10% stock solution) and 5µl sodium azide (of a 20% stock solution) dissolved in a total volume of 10ml 1xPBS). The primary antibody incubation is done over night at 4°C in a liquid chamber with 1/250 anti-RelB antibody or rabbit IgG antibody dilution in blocking solution. On the next day the slides are first washed for 7 minutes in blocking solution and then for 5 minutes in 1xPBS. Specificity blocking is done with a 5% goat serum in 1xPBS dilution for 30 minutes before the secondary antibody goat-anti-rabbit-AF488(or –AF546 coupled) 1/500 dilution in blocking solution is applied for 1 1/2hours in the dark. Thereafter slides are washed two times for 5 minutes in 1xPBS before we apply a 5% mouse serum in 1xPBS solution for specificity blocking for another 30 minutes. Afterwards we counter-stain with 1/750 anti-CD1a-AF546 (or –APC coupled 1/50) antibody dilution in blocking solution for 2 hours at RT in the dark. Then we wash the slides two times 5 minutes with 1xPBS and incubate the cells with a 1/100 dapi dilution in 1xPBS for 15 minutes before we wash another two times 5 minutes with 1xPBS. Finally, the slides are mounted in fluoroshield medium (Sigma Aldrich) with a cover slip and the borders were sealed with nail polish. Microscopic pictures were made with 20x or 40x objectives with a Zeiss LSM700 confocal microscope at the imaging facility of the medical university of Vienna.

Table 11: Antibodies for Immunfluoresence stainings on single cells

Antibody	Dilution	company
Ah Receptor (N-19): sc-8088	1/100	Santa Cruz Biotechnology, Inc
NFκB p50 (C-19): sc-226	1/250	Santa Cruz Biotechnology, Inc
Goat IgG	1/100	Santa Cruz Biotechnology, Inc
Rabbit IgG	1/250	Santa Cruz Biotechnology, Inc
Dapi	1/100	Sigma Aldrich
Langerin-PE	1/50	Immunotech
CD1a-APC (HI146)	1/50	BD Biosciences
CD1a-AF546	1/750	Kindly provided by Elbe-Bürger Lab.
Donkey-anti-goat-AF488 /–AF546 coupled	1/500	Invitrogen
Goat-anti-rabbit-AF488 /–AF546 coupled	1/500	Invitrogen

5.3. AhR staining of human skin sections:

Human skin samples from plastic surgery (abdominoplasty or mammoplasty) are first fumigated with Betaisodona (Multipharma Ges.m.b.H.) and subsequently freed from cloth and adipose tissue with a scalpel. The dermis and epidermis are then cut in small squares and incubated in 24-well plates in 1ml RPMI complete medium with 100ng/ml GM-CSF without stimulation and 1µl SDS or $NiSO_4$ stimulation overnight. On the next day the skin parts are cryo-frozen in Tissue-Tek O.C.T. compound embedding medium (Sakura) in liquid nitrogen and stored at -80°C. Skin sections are then cut with a cryostat and placed on microscopic glass slides. After fixing the skin sections with -20°C cold aceton for 10 minutes the O.C.T. compound rests are removed and the sections are encircled with a hydrophobic pen. Now the samples are blocked for 30 minutes with 2%BSA/PBS at room temperature (RT). Primary antibody anti-AhR and the goat-IgG isotype control antibody are dissolved 1/100 in 2%BSA/PBS and incubated over night at 4°C in a humid chamber. After three washing steps with 1xPBS for 5 minutes we block with 10% donkey serum in 1xPBS for 30 minutes at RT. Secondary antibody donkey-anti-goat-AF488 is dissolved 1/500 in 2%BSA/PBS and incubated 1 ½ hours at RT in the dark. Thereafter slides are again washed three times 5 minutes with 1xPBS and blocked with 10%mouse serum in 1xPBS for 30 minutes. We counter-stain with 1/50 Langerin-PE antibody dissolved in 2%BSA/PBS for 4 hours at RT. Slides are again washed three times 5 minutes in 1xPBS. In the end we incubate the slides with 1/100 Dapi dye in 1xPBS for 10 minutes. After another threes washing steps in 1xPBS for 5 minute the slides are mounted in fluoroshield (Sigma Aldrich) with a cover slip. Microscope pictures are taken with a Zeiss LSM700 confocal microscope with 20x or 40x objectives at the imaging core facility of the medical university of vienna.

Table 12: Antibodies for Immunfluoresence staining on skin cryosections

Antibody	Dilution	company
Ah Receptor (N-19): sc-8088	1/100	Santa Cruz Biotechnology, Inc
Donkey-anti-goat-AF488	1/500	Santa Cruz Biotechnology, Inc
Dapi	1/100	Sigma Aldrich
Langerin-PE	1/50	Immunotech

6. DNA and protein analysis:

6.1. qPCR

For mRNA level determination we use quantitative real-time PCR. We harvest cells, wash them once in 1xPBS and count them with the CasyCounter (Schärfe System GmbH). Cells are again washed in 1xPBS and the supernatant is discarded. For total RNA isolation we use the RNeasy® Mini Kit (Quiagen). All working steps are carried out on an RNAZip treated work bench and the pipette tips are filtered. Cells are disrupted by re-suspension in 150µl

RLT buffer with 1/100 β-mercaptoethanol by vortexing for 1 minute. At this step samples are stored at -20°C and RNA isolation is done later according to the manufacturers´ protocol. The thawed samples are mixed with 150µl 70% ethanol and immediately transferred to the RNeasy columns and centrifuged for 15 seconds with 8000g. The column is washed with 350µl RW1 buffer for 15 seconds at 8000g. DNA eradication is done by a 15 minute incubation at RT with 80µl of a DNase solution (10µl DNase I stock solution plus 70µl RDD buffer (Quiagen)). Finally the column is washed with 350µl RW1 buffer and 500µl RPE buffer for 15 seconds at 8000g each and with 500µl of RPE buffer for 2 minutes at 8000g. Before RNA elution from the column with RNase-free water (10µl) for 1 minute at 8000g we perform an additional centrifugation step for 1 minute at full-speed to get rid of excess liquid. For an increased RNA recovery from the column the elution step is repeated with another 10µl of RNase-free water. As a next step cDNA is produced from the isolated RNA. For this we use 16,25µl RNA and mix it with 1µl oligodT primers (25mM) in an eppendorf PCR tube. Primer annealing is achieved after 5 minutes of 70°C followed by 5 minute 4°C incubation. Afterwards we add 5µl 5x RevertAid [TM] H Minus M-MuLV RT buffer, 1.25µl dNTP mix (10mM) , 0,5µl RiboLock RNase Inhibitor (40u/µl) and 1µl of RevertAid [TM] H Minus M-MuLV RT (200u/µl) (all reagents were obtained from Fermentas). cDNA generation is accomplished by a 2-step cycle PCR program starting with 42°C for 1 hour followed by 70°C for 15 minutes and cooling down to 4°C. cDNA is stored at -20°C and has to be diluted 1/3 with RNase-free water before usage for quantitative real-time PCR. However, samples for real-time qPCR are pipetted in a 96-well 20µl capillary plate (BioRad). Detection is done with the CFX96 Real Time Detection System (BioRad). qPCR mix includes: 5µl Sybr Green mix (Life Technologies, Applied Biosystems®), 0,5µl of each forward and reverse primers (10µM; MWG Eurofins Operon), 3µl RNase-free water (Quiagen) and 1µl cDNA (1:3 diluted). For standard curve cDNA is used 1/3, 1/8, 1/16 and 1/32 diluted. Every condition is pipetted as duplets to get an average value. Amplification program is 95°C for 5 minutes followed by 40 cycles of 95°C (15 seconds) and 60°C (60 seconds) and ends with 4°C cooling. All primers are designed with the Primer3 software and ordered from MWG Eurofins Operon. Data analysis is done with the BioRad CFX Manager software. Gene of interest mRNA levels are normalized to GAPDH mRNA levels. Values represent the fold-change to un-stimulated cells. Following primer sequences are used:

GAPDH primers:

Forward: 5´-GAAATCCCATCACCATCTTCCAGG-3´

Reverse: 5´-CGCGGCCATCACGCCACAGTTTCC-3´

Cyp1a1 primers:

Forward: 5´-CAGCTGACTTCATCCCTATTC-3´

Reverse: 5´-AGCTGGACATTGGCGTTCTCA-3´

IL8 primers:

Forward: 5´-GTGTGAAGGTGCAGTTTTGC-3´

Reverse: 5´-AATTTCTGTGTTGGCGCAGT-3´

6.2. Western Blot

To determine protein levels in cells the method of choice is western blot analysis. Cells are harvested, washed once in 1xPBS for 5 minutes at 1200rpm and counted with the CasyCounter (Schärfe System GmbH). Then cells are transferred to an 1,5ml eppendorf tube and washed again in 1xPBS for 5 minutes at 1200rpm. The cell pellet is vortexed and resuspended in an appropriate amount of 4x SDS sample buffer to reach a concentration of 1*10^5 cells per 10µl 1xSDS sample buffer. To lyse the cells the samples are heated for 15 minutes at 95°C on a shaking heating block. Finally the western blot samples are stored at -20°C. Before loading the protein extract (~1/10^5 cells) onto the gel, samples are again heated up for 10 minutes at 95°C. We used 8% separating with 4% stacking denaturating sodium dodecylsulfate-polyacrylamide gels (SDS-PAGE) for protein separation. Electrophoretic protein separation on the gel is done in 1x SDS-PAGE buffer with 80V (start with 40V). When the running front of the protein extracts is running out of the gel the gel run is stopped and the proteins are transferred onto a polyvinylidenfluorid (PVDF) membrane (Immobilon-P, Millipore) with 250mA for 1 ½ hours inside a Biorad Criterion Blotter aperture (BioRad) with ice cold 1xblotting buffer with an ice cube to cool the system and an magnetic stirring ball to mix the liquid conduction buffer. After this step we make a Ponceau red staining (Sigma Aldrich) for 1 second to check proper protein transfer. The red staining is washed out with distilled H_2O and 1xPBS. Afterwards the membrane is blocked for 45 minutes in 5% low-fat milk powder in 1x/PBS-T at RT. Primary antibody dilutions are incubated over night at 4°C shaking. For the list of antibodies see table 13. On the next day the antibody dilution is recovered and the membrane washed in 1xPBS-T three times 10 minutes shaking. Next the secondary antibody is diluted in 3% low-fat milk/PBS-T and incubated for 1 ½ hours at RT shaking. After another three washing steps in PBS-T for 10 minutes and a last washing step in 1xPBS the membrane is analyzed. Chemiluminescent

substrate SuperSignal WestPico and WestDura Kit (Pierce Biotechnology) are mixed according to the manufacturers' protocol and applied onto the membrane. Pictures are taken with a Fujifilm Las-4000 developer machine. Actin is used as normalization. Repeated incubation with antibodies requires an antibody stripping step in between. Membranes are incubated with stripping buffer for 10 minutes at 57°C in a shaking water bath. After two times 10 minutes PBS-T washing at RT on a shaker the incubation starts again with 5% low-fat milk/PBS-T incubation for 45 minutes at RT and subsequent antibody incubations (see steps above). Western Blot quantifications are done with the ImageJ program, calculations are done in Microsoft Excel and statistical analysis is carried out with the Graphpad Prism4 software. Protein levels are normalized to the Actin levels.

Table 13: Antibodies for Western Blot analysis

Antibody	company	Working concentration
AhR	Santa Cruz Biotechnology, Inc.	1/500 (in 3% milk/PBS-T)
RelB	Santa Cruz Biotechnology, Inc.	1/500 (in 3% milk/PBS-T)
Actin	Sigma Aldrich	1/1000 (in 3% milk/PBS-T)
phospho-p38	Cell Signaling Technology, Inc.	1/500 (in 5%BSA/TBST)
VDR	Santa Cruz Biotechnology, Inc.	1/500 (in 3% milk/PBS-T)
p65	Santa Cruz Biotechnology, Inc.	1/500 (in 3% milk/PBS-T)
PU.1	Santa Cruz Biotechnology, Inc.	1/500 (in 3% milk/PBS-T)
Goat-anti-rabbit-HRP	Thermo Scientific (Pierce)	1/5000 (in 3% milk/PBS-T)
Donkey-anti-goat-HRP	R&D Systems GmbH	1/2500 (in 3% milk/PBS-T)

6.3. ELISA measurement of cytokines in the culture supernatant:

About 1*10^6 (moLCs) to 1*10^5 (LCs) cells are activated for 24 hours. Supernatant of each well is collected and cytokine measurements are done with the Luminex system (Austin, TX).

7. Statistical analysis

Statistical analyses are conducted with the Graphpad Prism4 program by using the paired, two-tailed student t-test. P-values less than 0,05 are considered as significant. *P<0,05, **P<0,01, ***P<0,001

Results

8. Aryl hydrocarbon receptor in DC subsets

The Aryl hydrocarbon receptor (AhR) has a longstanding research interest in our lab. Platzer and Richter et al. published data about the role of this molecule in Langerhans cell development from CD34+ hematopoietic progentitor cells. They found out that the activation of AhR by AhR-ligand VAF347 leads to an arrest of LC development in a monocytic stage due to an inhibition of up-regulation of the crucial LC transcription factor PU.1. They also investigated AhR levels in different myeloid cell subsets by western blot and found that LCs express highest levels of AhR followed by monocytes, whereas granulocytes and the progenitor cells itself express only very low to undetectable levels of AhR protein. (Platzer et al. 2009) The first part of this project is to further elucidate the function of this protein in different dendritic cell subsets and in vivo in human skin.

8.1. Generation of dendritic cell subsets: differentiation and purity

To generate Langerhans cells (LCs) and interstitial dendritic cells (intDCs) we isolate CD34 positive hematopoietic progenitor cells from cord blood with a positive selection kit from EasySep®. After an expansion period of 3-7 days in the presence of Flt3L, SCF and TPO the cells are set up in LC mix (GM-CSF; Flt3 ligand, SCF,

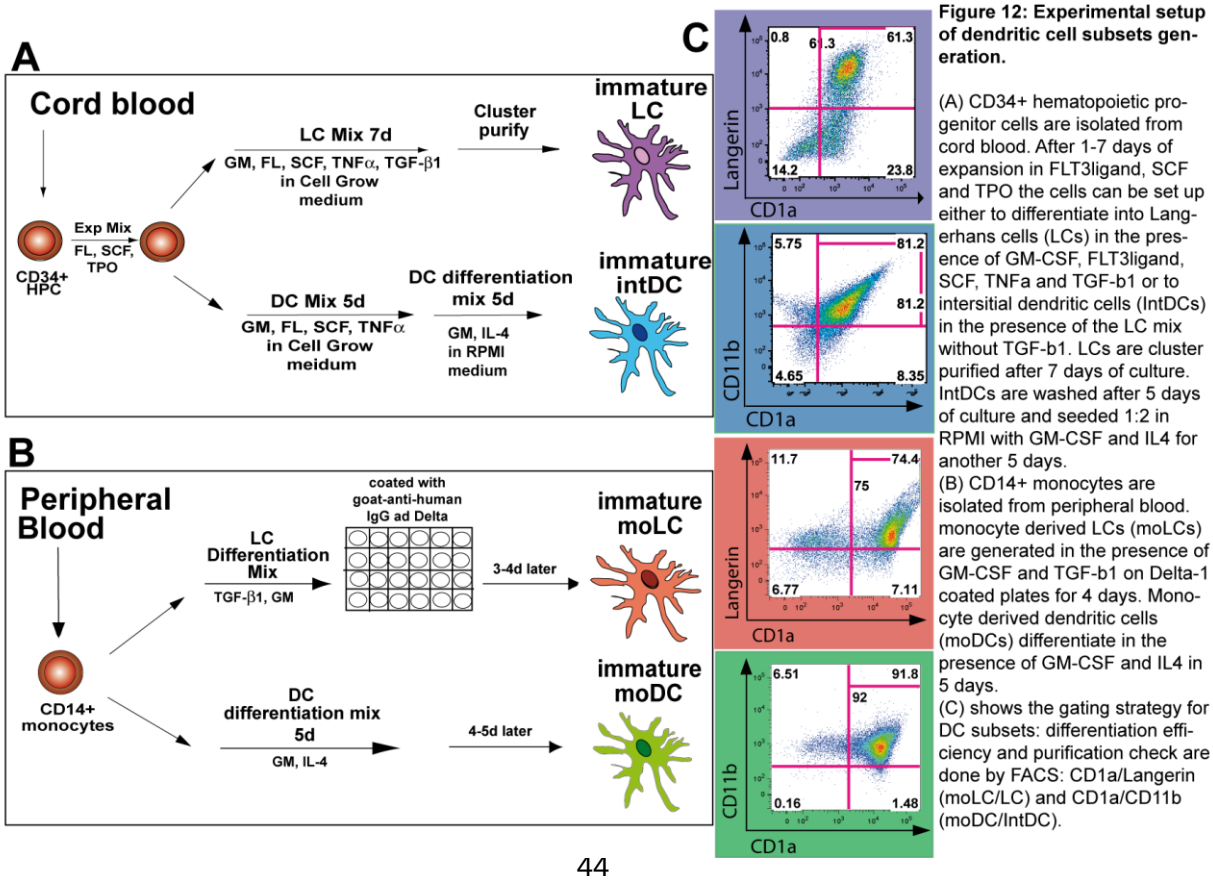


Figure 12: Experimental setup of dendritic cell subsets generation.

(A) CD34+ hematopoietic progenitor cells are isolated from cord blood. After 1-7 days of expansion in FLT3ligand, SCF and TPO the cells can be set up either to differentiate into Langerhans cells (LCs) in the presence of GM-CSF, FLT3ligand, SCF, TNFa and TGF-b1 or to intersitial dendritic cells (IntDCs) in the presence of the LC mix without TGF-b1. LCs are cluster purified after 7 days of culture. IntDCs are washed after 5 days of culture and seeded 1:2 in RPMI with GM-CSF and IL4 for another 5 days.
(B) CD14+ monocytes are isolated from peripheral blood. monocyte derived LCs (moLCs) are generated in the presence of GM-CSF and TGF-b1 on Delta-1 coated plates for 4 days. Monocyte derived dendritic cells (moDCs) differentiate in the presence of GM-CSF and IL4 in 5 days.
(C) shows the gating strategy for DC subsets: differentiation efficiency and purification check are done by FACS: CD1a/Langerin (moLC/LC) and CD1a/CD11b (moDC/IntDC).

TNFα and TGF-β1) for 7 days and cluster purified over a PBS gradient to enrich LCs. To set up intDCs, cells differentiate for 5 days in the LC mix without TGF-β1 and are then washed and transferred to RPMI medium with GM-CSF and IL4 for another 5 days. Monocyte-derived LCs (moLCs) and DCs (moDCs) are differentiated from CD14 positive monocytes isolated from peripheral blood with a positive selection kit from Miltenyi Biotec®. While moDCs need GM-CSF and IL4 for 4 days, moLCs are generated in the presence of GM-CSF and TGF-β1 on Delta-1 coated plates for 4 days. To ensure differentiation efficiency and purity of the dendritic cell subsets we perform a FACS staining for CD11b and CD1a (intDCs and moDCs) and CD1a and Langerin (LCs and moLCs). Figure 12A and B show an overview of the experimental set up of the differentiation methods. The representative FACS blots depicted in figure 12C show the gating strategy for the DC subsets used in this project. The average purity for moDCs and IntDCs is between 80-95%, for LCs around 60-80% and for moLCs around 50-70% under optimal conditions.

8.2. Aryl hydrocarbon receptor is most prominently expressed in LCs and moDCs followed by intDCs and moLCs

Dendritic cell subsets are generated according to the generation methods described in chapter 8.1. Western blot samples are prepared according to the cell number. Figure 13 shows a representative western blot of the dendritic cell subsets and figure

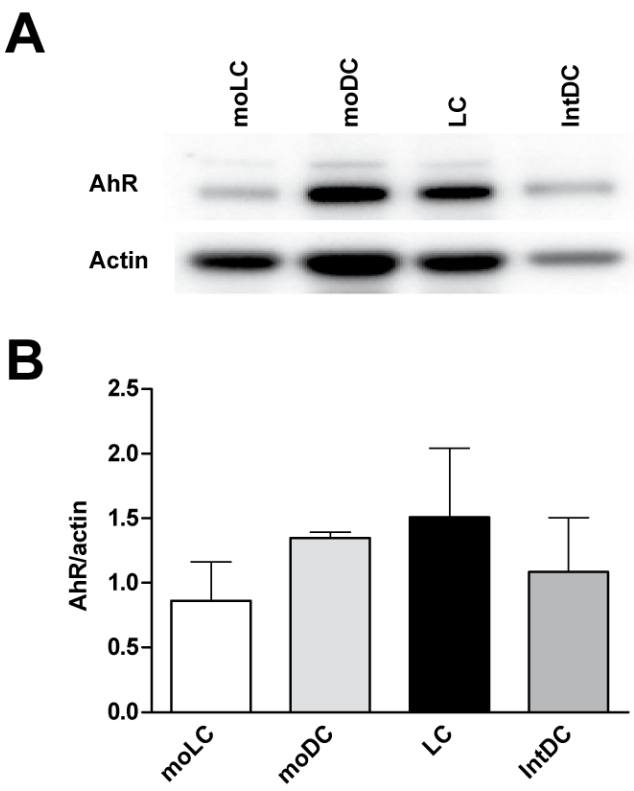


Figure 13: DC subsets show different levels of AhR protein: LCs and moDCs expressing the highest amounts follwed by intDCs and moLCs.

MoLCs and moDCs are generated from CD14+ monocytes isolated from peripheral blood, whereas IntDCs and LCs are differentiated from CD34+ progenitor cells isolated from cord blood. Differentiation efficiency and purity are checked by FACS staining for CD1a/Langerin double positive cells (moLCs and LCs) or CD1a/CD11b double positive cells (IntDCs and LCs) respectivley. Average purity of samples is:
moLCs: 50-70%
moDCs: 80-90%
IntDCs: 80-90%
LCs: 60-80%
Cells are counted and Western Blot samples are prepared.
(A) shows a representative Western Blot analyzed for AhR and Actin protein levels.
(B) shows statistical data after quanitfication of all Western Blots conducted. Bars show the mean +/- SEM of 3 independet donors. The results show that LCs and moDCs have the highest levels of AhR followed by IntDCs whereas moLCs have the lowest levels.

2B shows the statistical quantification of three independent donors. From these results it becomes obvious that LCs express the highest levels of AhR followed by and moDCs and intDCs whereas moLCs only express low levels of the protein.

8.3. Cytoplasmic localization of AhR and subsequent nuclear translocation upon $NiSO_4$ activation

As we have now shown, that AhR is present in DC subsets, we further want to investigate the cellular localization of the protein. In addition to this we want to analyze the behavior of the AhR upon cellular stimulation with contact hypersensitivity inducing agents such as the contact irritant (SDS) or contact sensitizer ($NiSO_4$) to see whether there are potential differences in the protein reaction in these two different stimuli. To address this question we generate moLCs and LCs according to the steps described in chapter 8.1. After the differentiation period the cells are activated with SDS and $NiSO_4$ for 24 hours. On the next day the cells are fixed and stained for AhR and CD1a with a nuclear counterstaining with dapi. Figure 14 shows representative immunfluoresence pictures of moLCs without stimulation (A), SDS (B) and $NiSO_4$ (C) treatment. These results depict, that about 75% of the cells show a cytoplasmic AhR staining in immature moLCs. Preliminary data suggest that upon SDS activation AhR translocates to the nucleus in about 20% of the cells whereas 60% of the cells have AhR in both, the cytoplasm and nucleus. $NiSO_4$ activation on the other hand leads to a strong nuclear translocation of AhR with about 90% of the cells showing a strong and exclusively nuclear staining for AhR as presented by the statistical analysis shown in figure 14 D. Figure 15 shows the results of the same experiment performed on LCs. The quantification of three independent donors is shown in part D of figure 15. LCs show a similar outcome, namely the predominantly cytoplasmic localization of the protein (~90% of the cells) in the immature state. Upon SDS activation of the cells AhR translocates to the nucleus in approximately 10% of the cells and 20% show a cellular distribution between the cytoplasm and the nucleus. However, $NiSO_4$ is a stronger inducer of the nuclear translocation of the protein, which is also reflected in the statistical analysis. The quantification shows that over 60% of the cells are strongly positive for nuclear AhR staining while the rest of the cells show AhR signal in both, the cytoplasm and the nucleus.

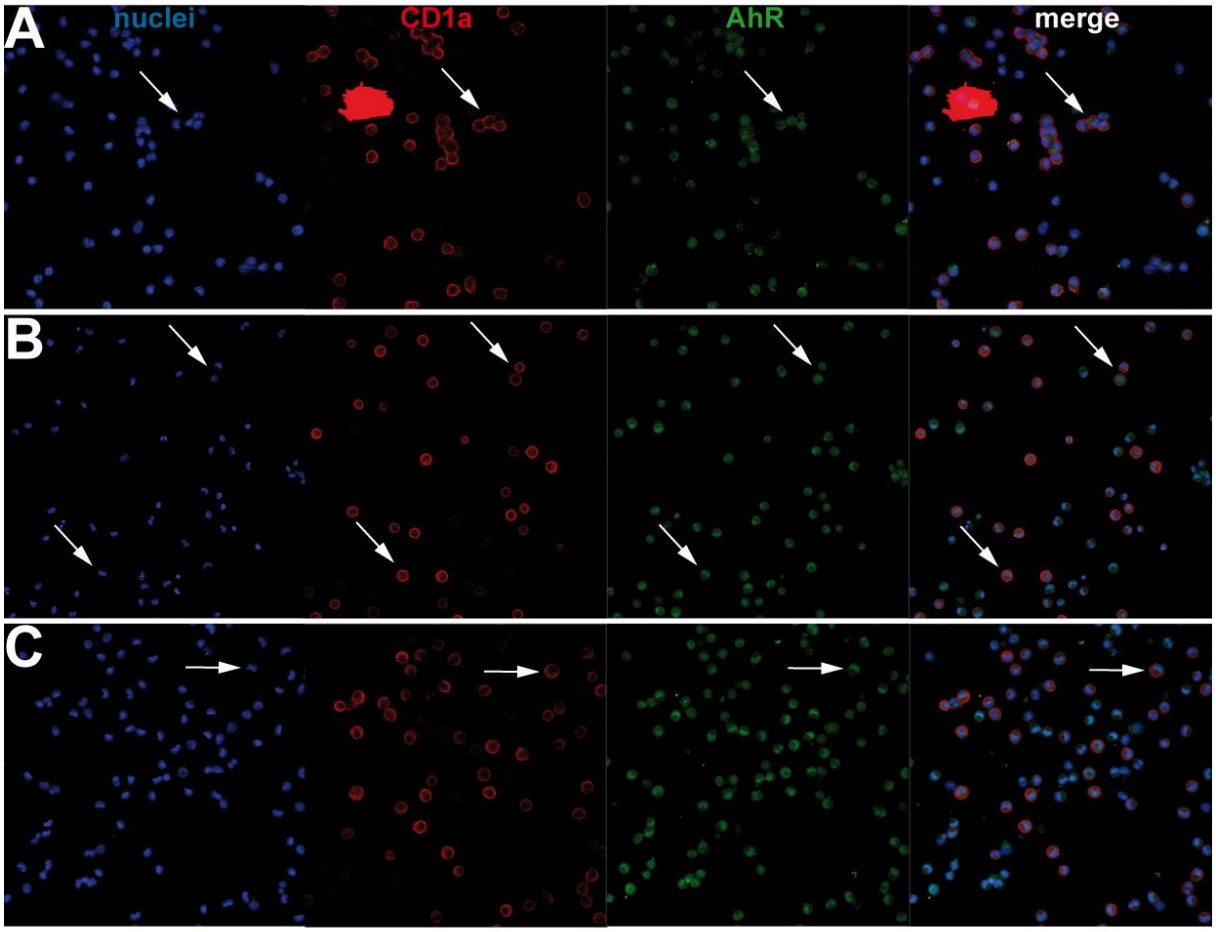
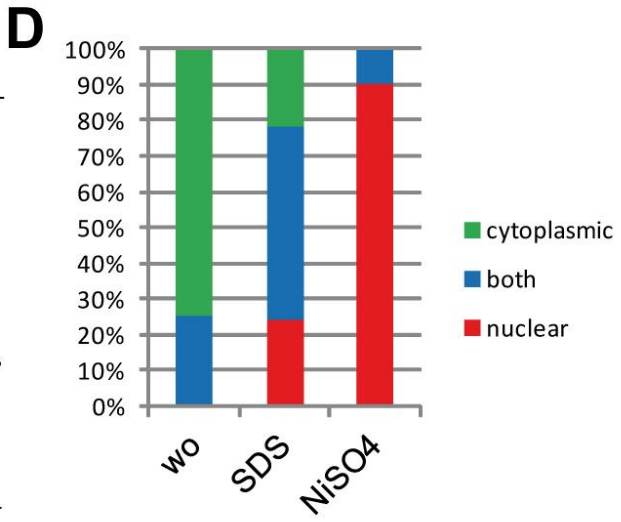


Figure 14: moLCs show a strong nuclear AhR staining upon NiSO4 activation.

CD14 positive monocytes are isolated from peripheral blood and differentiated into moLCs with GM-CSF and TGF-b1. After 4 days of culture the cells are stimulated with SDS or NiSO4 for 24 hours. On the next day cells are harvested, fixed and immunostained on an adhesion slide. Each column shows the signal of one single laser channel, whereas each row shows one condition (A wihout , B SDS and C NiSO4 activation). The last row shows the merger of all 3 channels. The arrows indicat monocyte-derived langerhans cells with the typical staining. What we can see is that without activation AhR shows a cytoplamic staining. Upon SDS we see a slight nuclear staining pattern for AhR and upon NiSO4 activation AhR strongly translocates to the nucleus.
(D) shows the quantification of one experiment from the wo, SDS and NiSO4 condition for nuclear, cytoplasmic or both cellulare locations of the AhR protein.



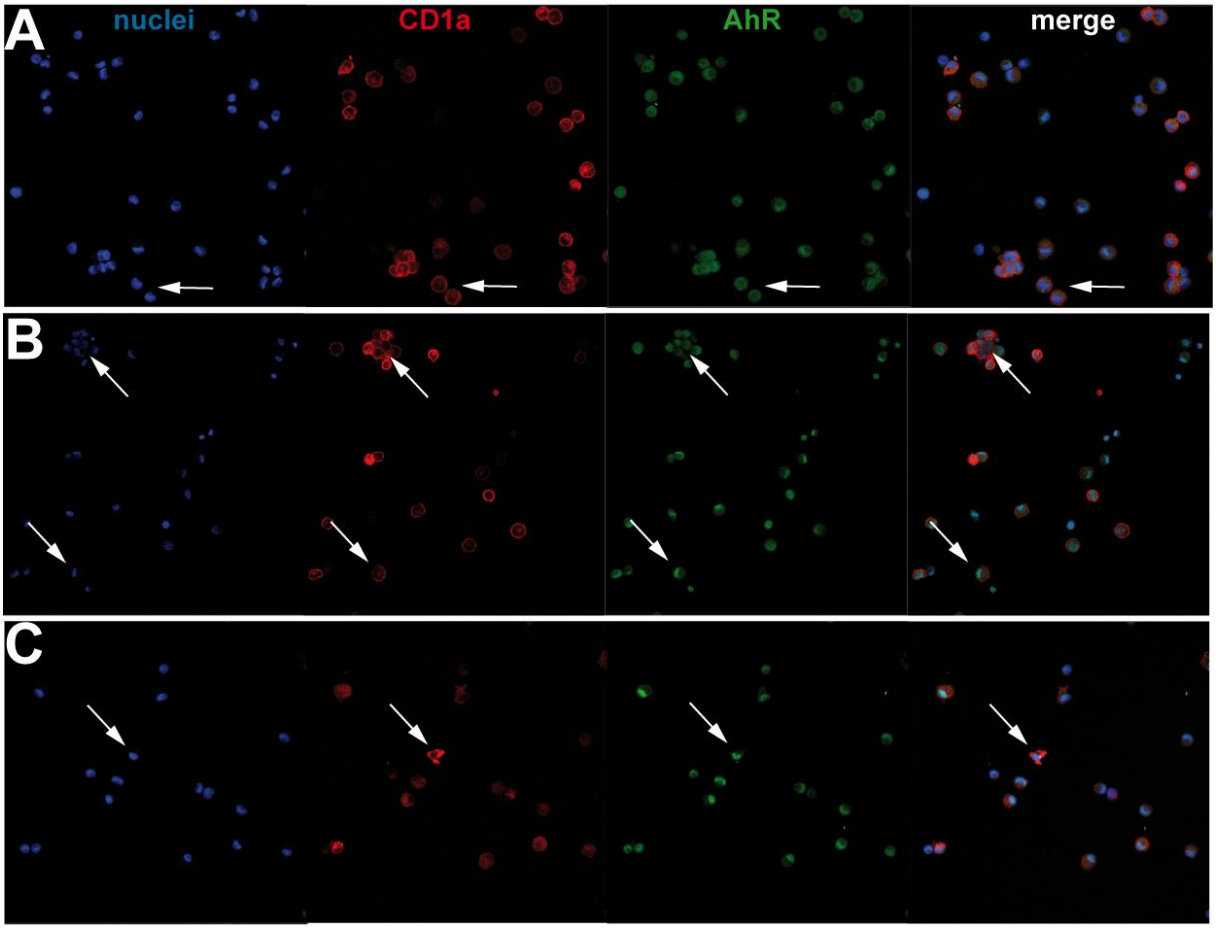
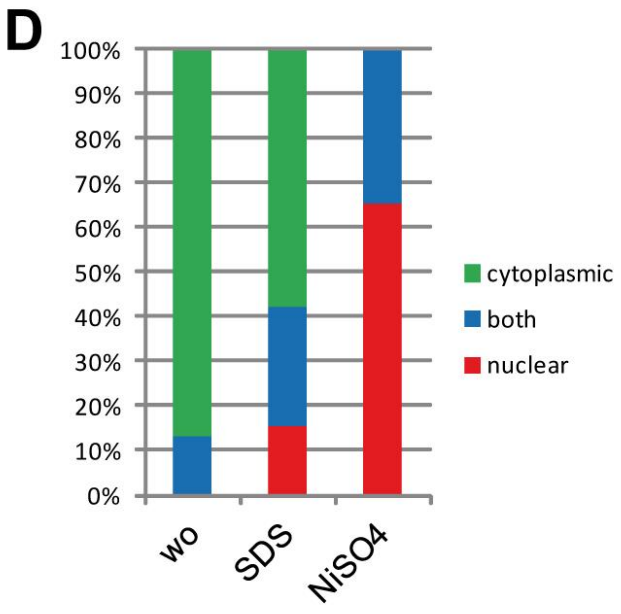


Figure 15: LCs show a strong nuclear AhR staining upon NiSO4 activation.

CD34 positive HSCs are isolated from cord blood and differentiated into LCs. After 7 days of culture the cells are cluster purified and stimulated with SDS or NiSO4 for 24 hours. On the next day cells are harvested, fixed and immunostained on an adhesion slide. Each column shows the signal of one single laser channel, whereas each row shows one condition (A wihout , B SDS and C NiSO4 activation). The last row shows the merger of all 3 channels. The arrows indicat langerhans cells with the typical staining. These results show, that AhR is cytoplasmically located without stimuli. SDS only leads to a weak nuclear translocation while NiSO4 activation induces a strong nuclear translocation of the protein.
(D) shows the statistical quantification of three independent donors. Cells from all three conditions are counted regarding to their cellular localization of the AhR protein.



8.4. AhR is present in the nucleus of in-vivo LCs from human skin

As we have confirmed the expression of AhR in in-vitro generated DC subsets and showed its nuclear translocation upon $NiSO_4$ activation we want to shed further light on the in-vivo situation of LCs in human skin. To accomplish this question we prepare cryofrozen sections of human skin grafts activated with SDS or NiSO4 over night or

without treatment as a control. After blocking the skin sections, we stain AhR shown in green and CD1a shown in red. In the end we counterstain with Dapi shown in blue to visualize the nuclei. Figure 16 shows representative immunfluoresence pictures. In (A) we see the untreated cells. The white arrow points towards a Langerhans cell in the epidermis, which is positive for AhR in the cytoplasm and the nucleus. Part (B) and (C) of figure 16 show representative pictures of the treated conditions, SDS and $NiSO_4$ respectively.

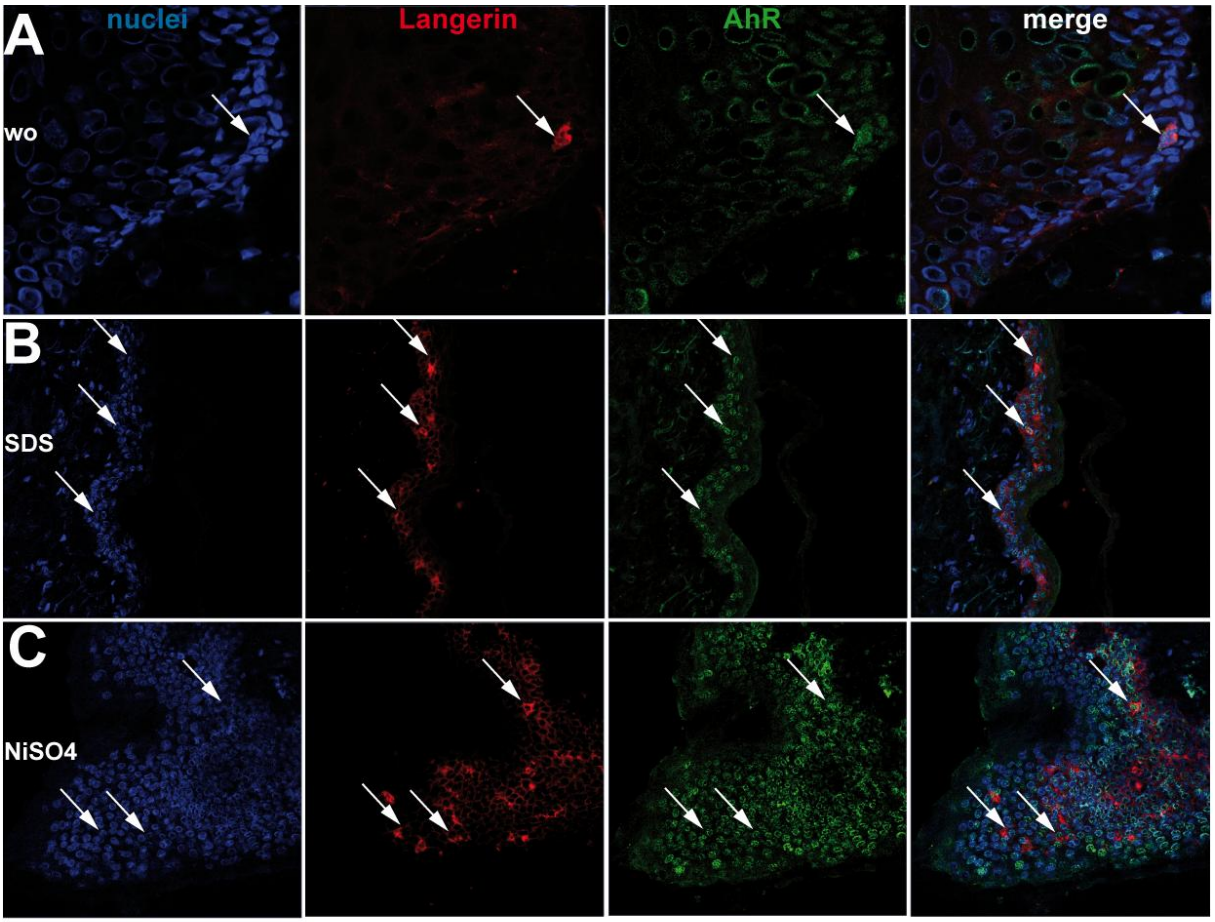


Figure 16: AhR is present in Langerhans cells of in human skin

Skin sections are prepared as desribed in the methods section and incubated over night in RPMI without, SDS or NiSO4 stimuli. On the next day the skin sections are cryofrozen and cut into sections and fixed on a glas slides with aceton. The sections are stained for AhR protein with a secondary antibody Alexa Fluor AF488 labeled (green). To visualize the langerhans cells, the skin sections are counterstained with Langerin antibody labeled with a PE fluorophore (red). The nuclei are stained with Dapi dye (blue). Pictures are taken with a Zeiss LSM700 confocal microscope with 20x and 40x objectives.
(A)-(C) show representative pictures of skin sections treated without, SDS and NiSO4 stimuli respectively. The first 3 lanes show the single channel signal, where the first lane shows the nucleic staining, the second lane the langerin and finally the third lane the AhR staining. The fourth lane shows the merger of the single channel signals. The white arrows indicate the langerhans cells in the corresponding channels. These results show that AhR ist present in vivo in human skin.

So far we conclude, that AhR is present in the nucleus of LCs in human skin no matter whether we activated the skin or not. Further experiments are needed to clarify whether there is a shift towards a more prominent nuclear staining upon activation with SDS or $NiSO_4$.

9. Monocyte-derived LCs treated with AhR ligands

Susanne Richter has already extensively investigated CD34 positive derived LCs, which were generated in the presence of AhR ligands FICZ and VAF347. To sum up her findings, what she saw was that LCs with an activated AhR show a diminished differentiation capacity and a decreased maturation potential. Upon FICZ- or VAF347-induced AhR activation while the differentiation period, LCs had an increased CD14 expression and lower basal levels of AhR expression. When these cells were subsequently activated with PGN, AhR upregulation was decreased and the typical maturation potential of LCs was down-regulated. In more detail the up-regulation of maturation markers was decreased, the secretion of cytokines declined and the induction of Th1+Th22 T helper cell induction was reduced (Richter 2010). As the literature regards these CD34 positive HSC derived LCs to resemble more the steady state LCs we also want to see how the inflammatory LCs behave in the same context. Therefore the second part of the project focuses on the generation of moLCs from peripheral blood monocytes in the presence of two different AhR ligands and takes a closer look on the differentiation capacity and the maturation potential of these cells.

9.1. MoLCs generated in the presence of AhR ligand FICZ show a tendency towards more Langerin/CD1a double positive cells

To address the question whether moLCs generated in the presence of AhR ligand FICZ show differences in differentiation and maturation, we differentiate moLCs from CD14 positive peripheral blood monocytes with or without the addition of AhR ligand FICZ. After 4 days of differentiation the cells are analyzed by FACS staining to investigate the surface marker expression profile. In addition to this cells are activated with lipopolysaccharide (LPS) and peptidoglycan (PGN) for 24 hours to investigate their maturation potential. On the next day the cellular supernatant is analyzed for cytokine levels and the cells are again analyzed by FACS staining. We also prepare western blot samples to see whether AhR translocates to the nucleus and RelB gets activated upon maturation of the cells.

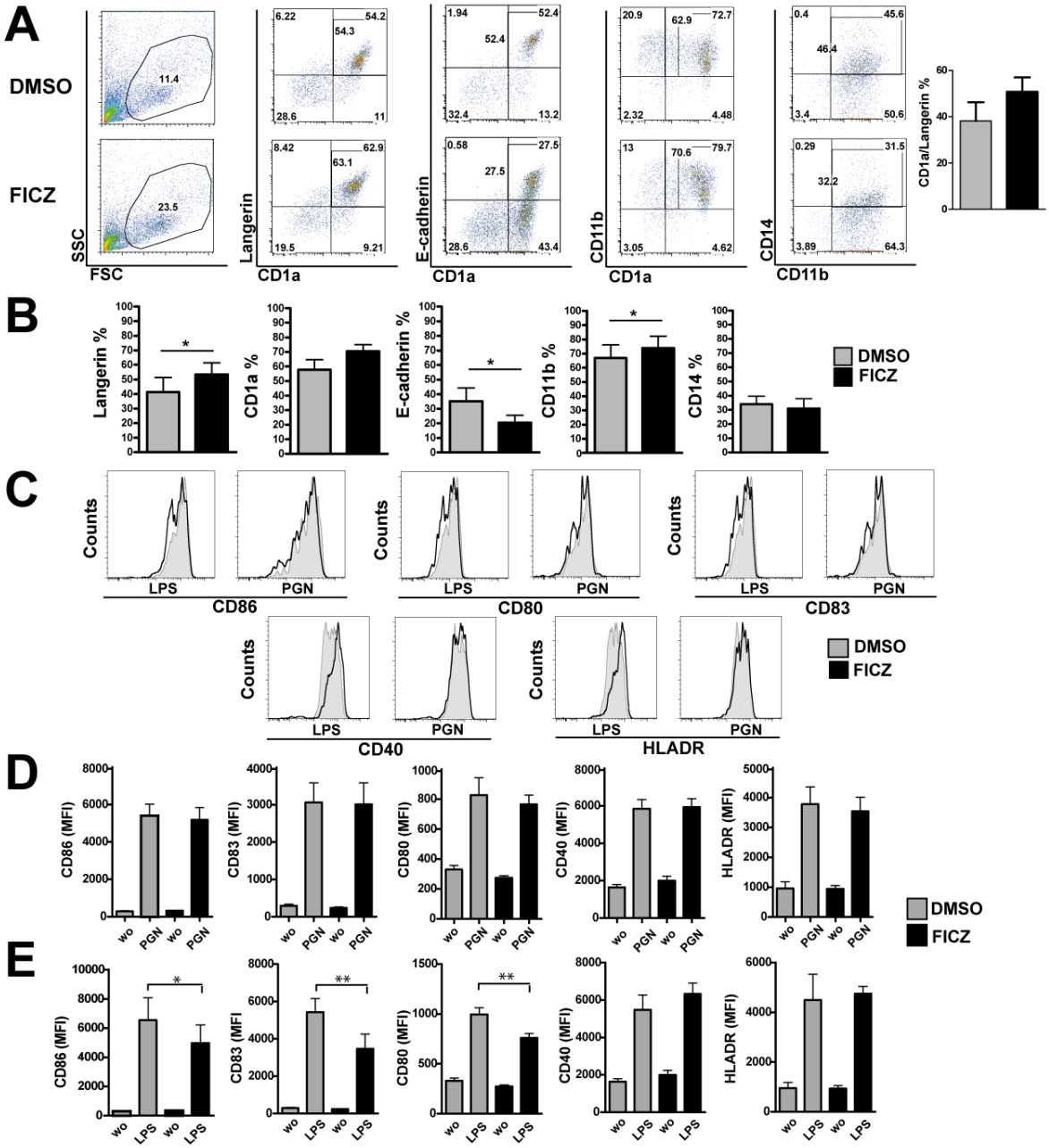


Figure 17: FICZ generated moLCs show a tendency towards inreased CD1a/Langerin double positive cells which only show a decreased maturation marker expression upon LPS stimulation
CD14+ monocytes are isolated from peripheral blood and differentiated into moLCs with GM-CSF and TGF-b1 in the presence of AhR ligand FICZ. After 4 days of culture we analyze the cell purity by FACS and activate the cells with LPS and PGN for 24 hours. On the next day the cells are again analyzed by FACS to investigate the maturation potential
(A) shows a representative FACS blot of differentiated moLCs generated in the presence of AhR ligand FICZ. FICZ treated cells show an increase in CD1a/Langerin positive cells and a decrease in CD1a/E-cadherin double positive cells. (B) shows the graphs of the statistical analysis from the quantified data of 6 independet donors. The bars represent the mean+/- SEM. (C) shows representative histogramms of gated langerhans cells for maturation markers. (D)-(E) show the graphs from the statistical analysis of the MFI of the activation markers. The bars represent the mean of 6 (LPS) or 3 independent donors (PGN) +/- SEM. FICZ generated langerhans cells show a tendency towards decreased LPS but not PGN activation which can be seen with CD86, CD80 and CD83, whereas CD40 and HLADR markers show no change in expression.

Figure 17 shows data acquired by these experiments. In Figure 17A we see a representative FACS blot of DMSO versus FICZ treated moLCs. What we can see is a tendency towards more CD1a/Langerin double positive cells in FICZ treated cells, which can also be seen in the diagram summing up the percentages of six

independent donors. In addition to this we can also see a decrease in E-cadherin expression and a slight increase in CD11b expression whereas CD14 gets slightly down-regulated. Figure 17B underlines the above described effects by the statistical quantification of six independent donors. The results show a significant increase in Langerin and CD11b positive cells. CD1a positive cells increase, whereas E-cadherin positive cells significantly decrease and CD14 positive cells slightly decrease in FICZ treated cells. Figure 17C, D and E show the results of the maturation FACS analysis. We focus on the well established maturation marker of dendritic cells, namely CD80, CD83, CD86, CD40 and HLADR. In the histogram overlays of DMSO versus FICZ treated cells of the mean fluorescent intensity from the maturation markers gated on moLCs we see no difference upon PGN activation but LPS activation leads to slight decrease of CD80, CD86 and CD83 whereas CD40 and HLADR show a slight drift towards higher expression. If we now focus on Figure 17D for PGN and 17E for LPS activated cells, we see that this trend is also reflected in the statistical analysis throughout all six independent donors. PGN activation shows no change in activation marker expression between DMSO and FICZ treated moLCs. However, LPS on the other hand shows a significant down-regulation of maturation marker expression of CD86, CD83 and CD80 and a tendency towards a slight increase of CD40 and HLADR expression. Figure 18 presents the data of the maturation analysis by western blot and cytokine measurements. Figure 18A shows a representative western blot which shows a decrease in AhR signal in the DMSO and FICZ treated cells in the unstimulated and stimulated conditions. AhR down-regulation in the unstimulated condition is most likely due to slight pre-activation of the cells because of physical handling. RelB gets up-regulated upon activation stimuli LPS and PGN in both generation models. Figure 18B shows the statistical quantification analysis from four independent donors. The AhR/actin ratio decreases in LPS and PGN stimulated conditions when compared to the 0h time point. The RelB/actin ratio increases upon stimulation. Figure 18C and D show the results of the cytokine measurements of LPS and PGN activated cells respectively. What we can conclude from these data is that LPS treatment of FICZ treated cells tends to lead to a higher cytokine secretion with the exception of IL10. PGN activation of FICZ treated cells on the other hand leads to a down-regulation of cytokines with the exception of IL6 and Il12p40.

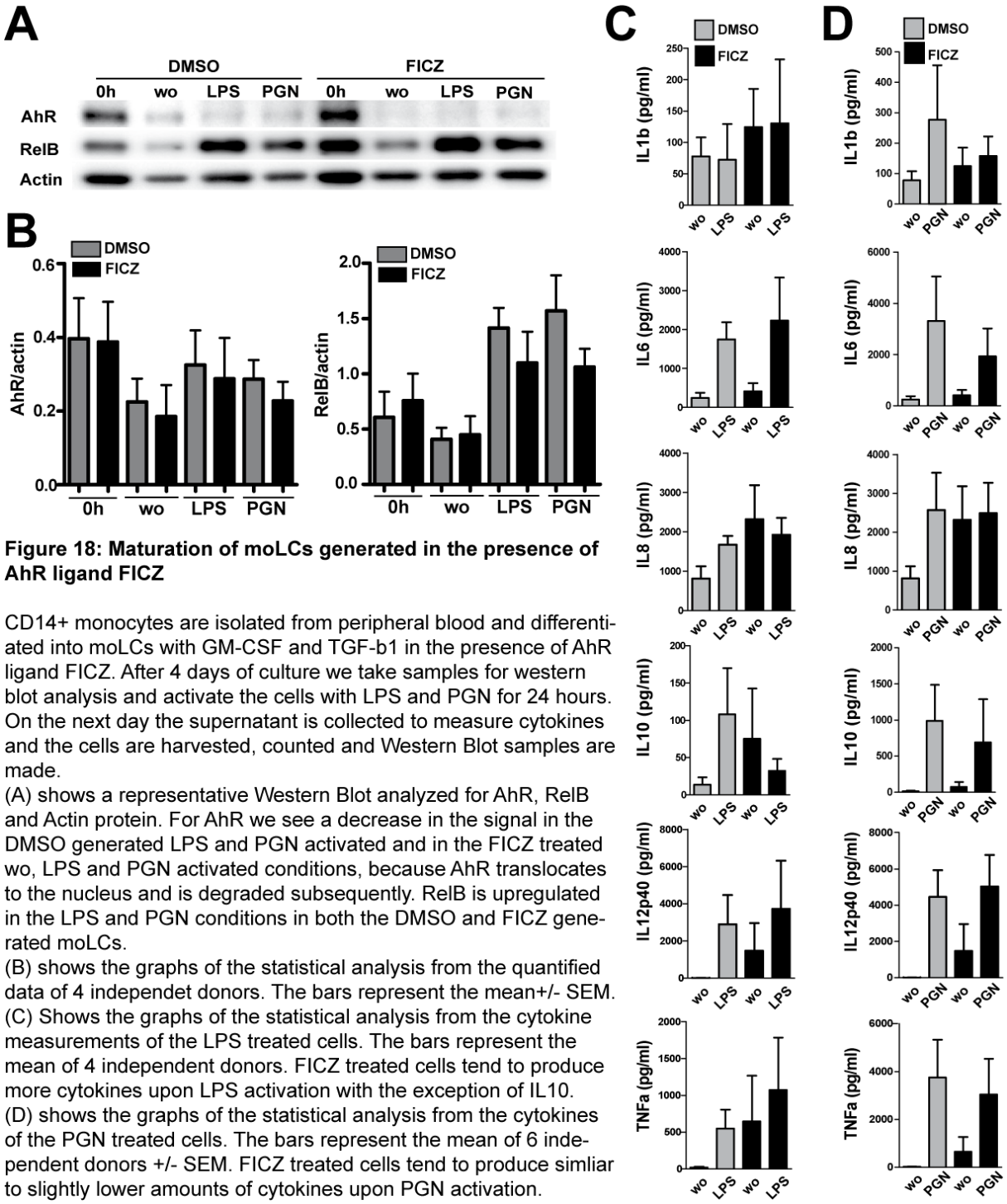


Figure 18: Maturation of moLCs generated in the presence of AhR ligand FICZ

CD14+ monocytes are isolated from peripheral blood and differentiated into moLCs with GM-CSF and TGF-b1 in the presence of AhR ligand FICZ. After 4 days of culture we take samples for western blot analysis and activate the cells with LPS and PGN for 24 hours. On the next day the supernatant is collected to measure cytokines and the cells are harvested, counted and Western Blot samples are made.
(A) shows a representative Western Blot analyzed for AhR, RelB and Actin protein. For AhR we see a decrease in the signal in the DMSO generated LPS and PGN activated and in the FICZ treated wo, LPS and PGN activated conditions, because AhR translocates to the nucleus and is degraded subsequently. RelB is upregulated in the LPS and PGN conditions in both the DMSO and FICZ generated moLCs.
(B) shows the graphs of the statistical analysis from the quantified data of 4 independet donors. The bars represent the mean+/- SEM.
(C) Shows the graphs of the statistical analysis from the cytokine measurements of the LPS treated cells. The bars represent the mean of 4 independent donors. FICZ treated cells tend to produce more cytokines upon LPS activation with the exception of IL10.
(D) shows the graphs of the statistical analysis from the cytokines of the PGN treated cells. The bars represent the mean of 6 independent donors +/- SEM. FICZ treated cells tend to produce simliar to slightly lower amounts of cytokines upon PGN activation.

To sum up the results we state that FICZ treated monocytes tend to generate more moLCs, which show lower levels of E-cadherin and upon LPS, but not PGN activation, show a decreased maturation potential. In addition to this we see a decrease in AhR protein levels after stimulation in both conditions which is evidence of a nuclear translocation upon activation. RelB also gets activated upon stimulation without any difference between FICZ treated and control cells.

9.2. MoLCs generated in the presence of AhR ligand VAF347 tend to generate more LCs but show no significant change in maturation potential

While FICZ is a physiologic, endogenous AhR ligand, VAF347 is an exogenous pharmaceutically engineered AhR ligand. As it becomes more and more obvious that endogenous and exogenous AhR ligands have different effects on cellular behavior, we next asked the question whether FICZ and VAF347 have similar effects on moLC differentiation and maturation. Therefore we generate moLCs in the presence of VAF347 and perform the same experimental analysis as with the moLCs generated in the presence of FICZ (see chapter 9.1.). Figure 19 shows the results of the FACS analysis of differentiation and maturation of moLCs. Part A of figure 19 shows a representative FACS blot of differentiated cells. Compared to control cells, VAF347-treated cells show a tendency towards more CD1a/Langerin double positive cells. VAF347 treated cells display a slightly reduced expression of E-cadherin and a slightly increased CD11b expression. The statistical analysis of three independent donors depicted in figure 19B also reflects this trend: CD1a/Langerin double positive cells increase in the presence of VAF347. Langerin, CD1a and CD11b mean fluorescent intensities (MFI) are slightly higher in VAF347-treated cells, whereas the E-cadherin and CD14 MFI tends to slightly decrease. Figure 19C shows representative histogram overlays of the maturation markers from gated moLCs of DMSO versus VAF347 treated cells. We see a tendency of higher MFI levels of CD86, CD80 and CD83 whereas CD40 and HLADR show a slightly lower expression. The statistical analysis of three independent donors shows that there is only a significant change for CD86. Regarding these results we conclude that there are only minor effects on the maturation potential of VAF347 treated moLCs. Figure 20A shows a representative western blot of PGN activation. We can see that AhR protein signal decreases in FICZ and VAF347 treated cells in both, without and PGN activated conditions. RelB on the other hand is activated upon PGN maturation in DMSO, FICZ and VAF347 treated cells. The statistical analysis of the quantification of four independent donors shown in figure 20B reflects the same trends. In figure 20C we see the diagrams of the cytokine secretion analysis of the culture supernatant after PGN activation. Preliminary data of two independent donors suggest that VAF347-generated cells tend to produce slightly more IL10, IL8 and IL12p40 whereas IL6 and TNFα show no change. IL1β tends to be secreted to a lesser extent.

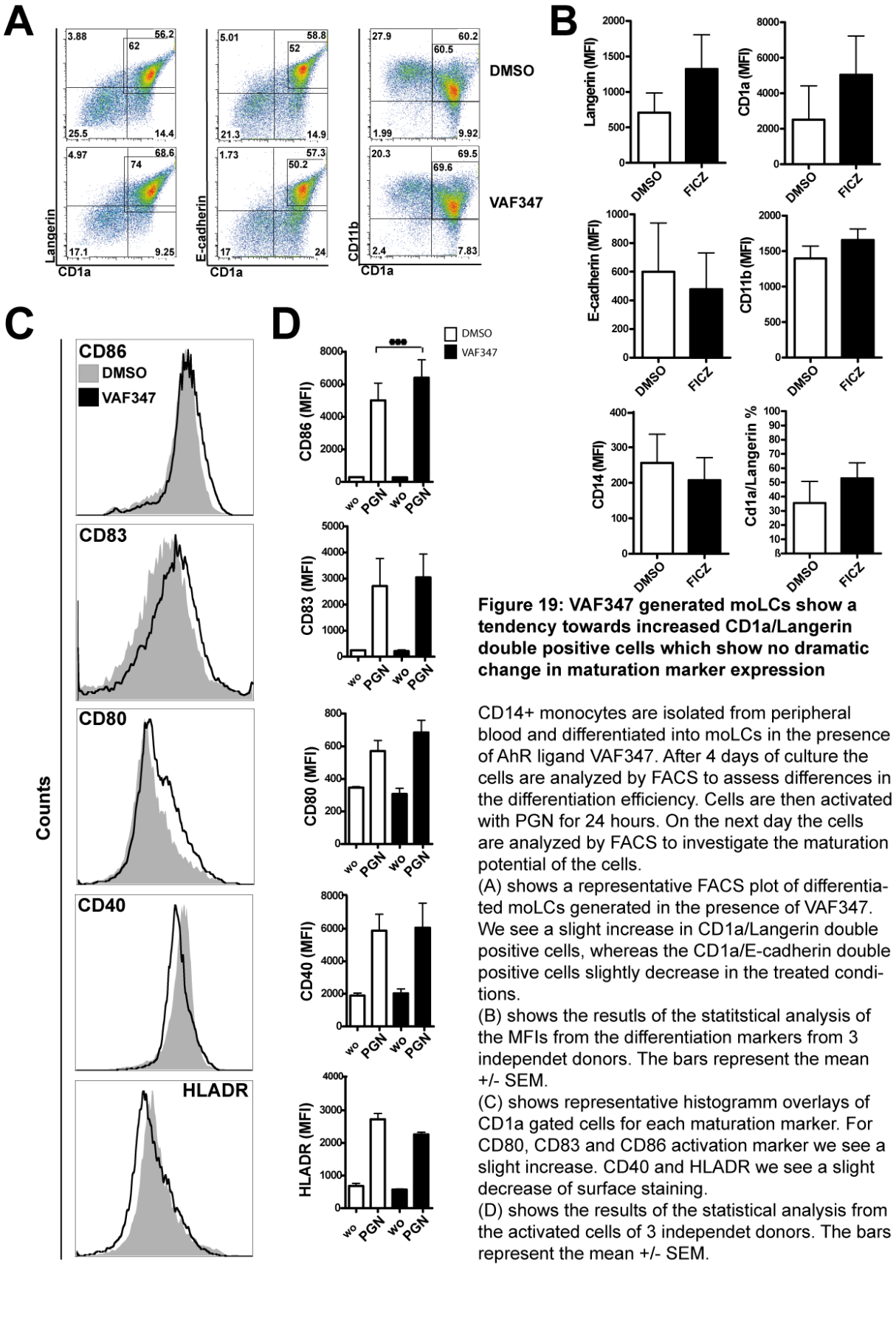


Figure 19: VAF347 generated moLCs show a tendency towards increased CD1a/Langerin double positive cells which show no dramatic change in maturation marker expression

CD14+ monocytes are isolated from peripheral blood and differentiated into moLCs in the presence of AhR ligand VAF347. After 4 days of culture the cells are analyzed by FACS to assess differences in the differentiation efficiency. Cells are then activated with PGN for 24 hours. On the next day the cells are analyzed by FACS to investigate the maturation potential of the cells.
(A) shows a representative FACS plot of differentiated moLCs generated in the presence of VAF347. We see a slight increase in CD1a/Langerin double positive cells, whereas the CD1a/E-cadherin double positive cells slightly decrease in the treated conditions.
(B) shows the resutls of the statitstical analysis of the MFIs from the differentiation markers from 3 independet donors. The bars represent the mean +/- SEM.
(C) shows representative histogramm overlays of CD1a gated cells for each maturation marker. For CD80, CD83 and CD86 activation marker we see a slight increase. CD40 and HLADR we see a slight decrease of surface staining.
(D) shows the results of the statistical analysis from the activated cells of 3 independet donors. The bars represent the mean +/- SEM.

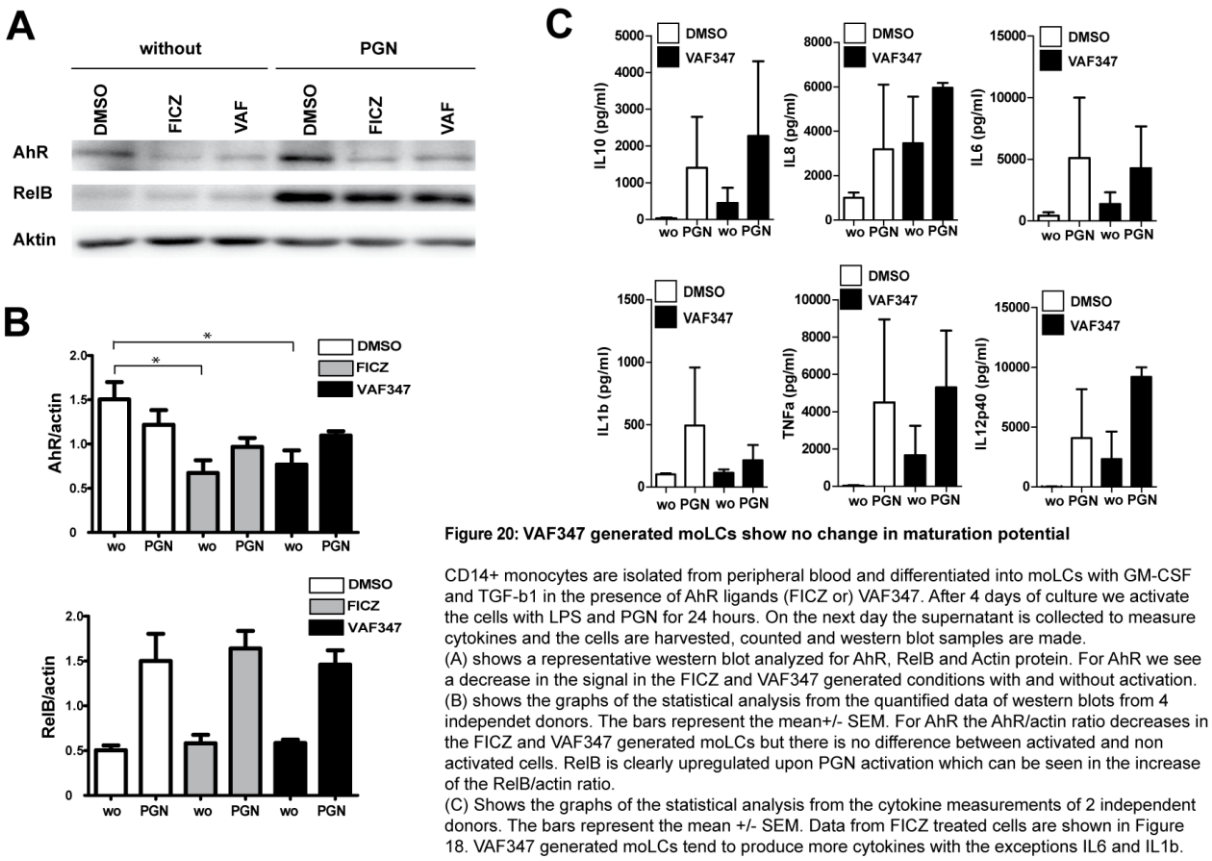


Figure 20: VAF347 generated moLCs show no change in maturation potential

CD14+ monocytes are isolated from peripheral blood and differentiated into moLCs with GM-CSF and TGF-b1 in the presence of AhR ligands (FICZ or) VAF347. After 4 days of culture we activate the cells with LPS and PGN for 24 hours. On the next day the supernatant is collected to measure cytokines and the cells are harvested, counted and western blot samples are made.
(A) shows a representative western blot analyzed for AhR, RelB and Actin protein. For AhR we see a decrease in the signal in the FICZ and VAF347 generated conditions with and without activation.
(B) shows the graphs of the statistical analysis from the quantified data of western blots from 4 independet donors. The bars represent the mean+/- SEM. For AhR the AhR/actin ratio decreases in the FICZ and VAF347 generated moLCs but there is no difference between activated and non activated cells. RelB is clearly upregulated upon PGN activation which can be seen in the increase of the RelB/actin ratio.
(C) Shows the graphs of the statistical analysis from the cytokine measurements of 2 independent donors. The bars represent the mean +/- SEM. Data from FICZ treated cells are shown in Figure 18. VAF347 generated moLCs tend to produce more cytokines with the exceptions IL6 and IL1b.

To sum up, VAF347 shows similar effects as FICZ during the differentiation process of moLCs. There is a tendency towards increased expression of LC markers like Langerin and CD1a whereas E-cadherin is decreased. Regarding the data of the maturation analysis we conclude that there is no significant change in the maturation of VAF347 treated moLCs compared to control cells.

9.3. MoLCs generated in the presence of FICZ and increasing concentrations of TGF-β1 show a decrease in E-cadherin expression

As we have seen an effect of AhR ligands on the generation of moLCs we ask the question whether this effect is dependent on TGF-β1, the crucial cytokine for LC differentiation. To examine this question we generate moLCs in the presence of FICZ with increasing concentrations of TGF-β1 (0, 1, 3,3, 5, 15 and 30ng). Figure 21 presents the data gained by these experiments. In figure 21A we see the statistical quantification of two independent donors which show that CD1a/E-cadherin double positive cells are dramatically decreased in FICZ treatedcells over the whole TGF-β1 concentration range. This can also be seen in the E-cadherin MFI gated on living cells. In figure 21B we see a representative FACS blot of CD1a versus Langerin of cells differentiated in the presence of DMSO or FICZ. The histogram shows the E-cadherin MFI of CD1a/Langerin double positive gated cells. We see a drastic

decrease in E-cadherin signal which is also reflected in the E-cadherin MFI statistics of two independent donors. Finally in part C of figure 21 we see representative light microscope pictures of cells differentiated with or without TGF-β1 in the presence of DMSO or FICZ. It becomes obvious that FICZ treated cells generate more clusters in comparison to DMSO treated cells but they are smaller in size and less dense. From these results we can conclude that TGF-β1 is required to generate moLCs and that the AhR ligand effect on the E-cadherin expression of moLCs is independent of TGF-β1 but strictly dependent on ligand activated AhR.

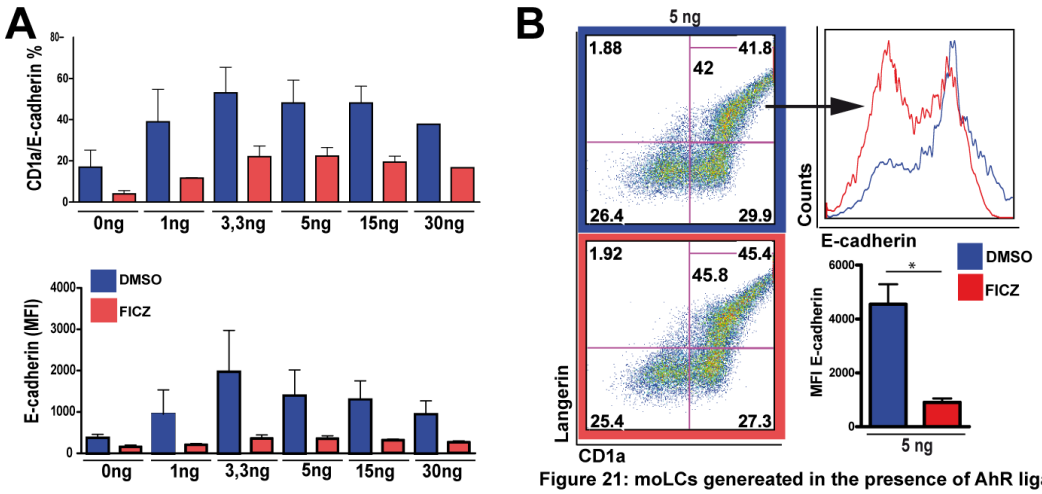


Figure 21: moLCs genereated in the presence of AhR ligand FICZ and increasing TGF-b1 concentrations show a decrease in E-cadherin expression.

CD14+ monocytes are isolated from peripheral blood and differentiated into moLCs in the presence of AhR ligand FICZ and increasing concentrations of TGF-beta1 (0; 1; 3,3; 5; 15 and 30ng/ml). After 4 days of culture the cells are analyzed by FACS and light microscopy.
(A) shows the graphs of the statistical analysis from the FACS staining of 2 independent donors.The bars show the mean +/- SEM. The percentage of CD1a/E-cadherin double positive cells decrease in FICZ treated cells as does the E-cadherin MFI.
(B) shows a representative FACS blot from the 5ng condition of CD1a against Langerin. The histogramm shows the E-cadherin intensity of CD1a/Langerin double positive cells and the diagramm shows the statistcal analysis of the E-cadherin MFI of 2 independet donors +/- SEM.
(C) shows representative light microscopy pictures of without and 30ng TGF-beta1 conditions from DMSO and FICZ treated cells in comparison What gets obvious from these results is that FICZ treated cells generate more clusters but they are smaller in size and less compact.

10. Effects of AhR on transcription profile and signaling in LCs

Researchers from our lab have investigated the effect of AhR ligands in LC differentiation on the transcriptional level and found that VAF347 treatment impairs LC differentiation by inhibiting PU.1 up-regulation in a monocytic precursor form at an early differentiation state. We want to further shed light on the transcription factor profile after 7 days of culture to see whether AhR ligands also influence the transcriptional program at a later time point. What becomes clear from the current state of literature is that AhR signaling varies from cell-type to cell-type and is dependent on a multitude of endogenous and exogenous factors. Some papers point towards an involvement of p38 MAPK whereas others object to this and implicate JNK and ERK MAPK or NFκB cooperation. We want to further shed light on the situation in LCs. Additionally we want to investigate RelB signaling in LCs and moLCs in response to $NiSO_4$ and SDS to unravel potential similarities because this molecule as well has been implicated in AhR signaling (Vogel et al. 2007).

10.1. Transcription factor profile of AhR ligand treated LCs shows no change in fully differentiated or activated LCs

To further investigate how AhR ligands influence the generation of LCs we have analyzed samples (kindly provided by Susanne Richter) of LCs which were generated in the presence of FICZ or VAF347 AhR ligands. After 7 days of differentiation the cells are FACS sorted for CD1a/Langerin double positive cells. Western blot samples normalized to the cell number are prepared and the rest of the cells are activated with PGN for 24 hours. On the next day cells are harvested, counted and western blot samples are prepared normalized to cell numbers. The results in figure 22A show two representative Western Blots of differentiated LCs analyzed for the most prominent LC transcription factors, namely AhR, RelB, VDR, PU.1 and p65 (RelA). There is no change in transcription factor expression in AhR ligand treated LCs. The same is true for transcription factor levels after PGN maturation, which can be seen in figure 22B. Only PU.1 and AhR tend to be reduced in AhR ligand-treated cells due to AhR-ligand-dependent blockage of PU.1 up-regulation (Platzer et al. 2009) and AhR activation through the ligands and the following protein degradation respectively.

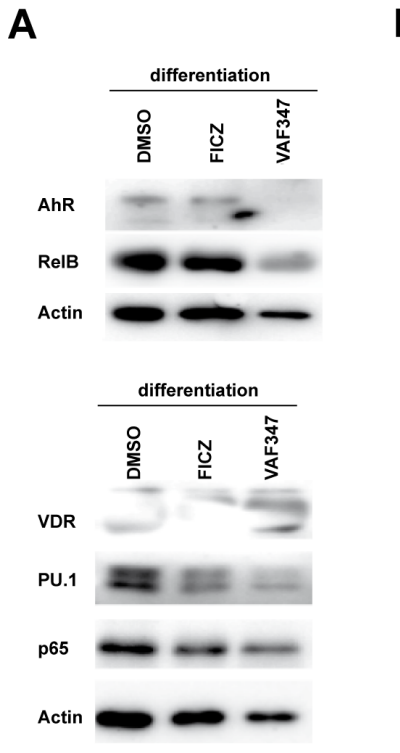


Figure 22: Sorted LCs generated in the presence of AhR ligands FICZ and VAF347 show no difference in the transcriptional profile neither after differentiation nor PGN-induced maturation.

CD34+ progenitor cells are differentiated in the presence of AhR ligands FICZ or VAF347 in LC mix. After 7days clusters are purified over PBS and FACS sorted for CD1a/Langerin double positive cells. Then the cells are activated with PGN for 24 hours, afterwards Western Blot extracts are made.
(A) shows two representative Western Blot of the samples from the differentiation analyzed for the most prominent LC differentiation transcription factors AhR, RelB, VDR, PU.1 and p65. Results show no change in transcription factor expression in FICZ and VAF347 generated LCs compared to DMSO control.
(B) shows two representative Western Blot of the samples from the maturation after PGN activation analyzed for the same transcriton factors. The data imply that there is no difference in the transcription factor profil upon maturation bewteen DMSO and AhR ligand generated LCs.

10.2. AhR nuclear translocation is disabled by a JNK inhibitor while NFkB inhibition enforces the nuclear translocation

While the functional steps of AhR activation have been unraveled, the signaling cascade which is triggered to translocate AhR into the nucleus is still unknown. In previous experiments (see chapter 8.3.) we have encountered strong nuclear AhR translocation upon $NiSO_4$ activation. In the literature there have been publications indicating that $NiSO_4$ leads to the phosphorylation of p38 MAP kinase (Arrighi et al. 2001; Boisleve et al. 2005; Miyazawa et al. 2007). Therefore we want to further investigate the importance of the p38 signaling cascade in LC maturation. We specifically focus on compounds which either inhibit p38 signaling (SB203580-> ERK inhibitor), β-catenin/Wnt siganling (SB216763 -> GSK3 inhibitor), JNK signaling (SP600125) or NFkB (RelA) signaling (SN-50). To accomplish this question we generate LCs from CD34+ hematopoietic progenitor cells. After 7 days of culture we purify the cell clusters and pre-incubate them with the corresponding inhibitors for 1 hour before we activate the cells over night with PGN. On the next day we prepare western blot samples to analyze AhR protein levels in inhibitor-treated, activated cells. Figure 23A shows the experimental set up. In figure 23B we see a representative western blot, which shows a decrease in AhR protein signal in the without (wo) and PGN-activated conditions, which is most likely due to AhR activation through physical handling. Nevertheless it becomes obvious that the SP inhibitor

disables the nuclear translocation of AhR upon PGN activation because the signal does not decrease in PGN activated cells. SN-50, an NFkB (RelA) inhibitor on the contrary significantly enhances nuclear translocation of AhR upon PGN activation. P38 MAPK inhibitor on the contrary does not interfere with nuclear translocation and for the β-catenin/Wnt inhibitor the results are varying but overall shows no consistent inhibition of the nuclear translocation of AhR as can be seen in the statistical analysis of three independent donors (figure 23C).

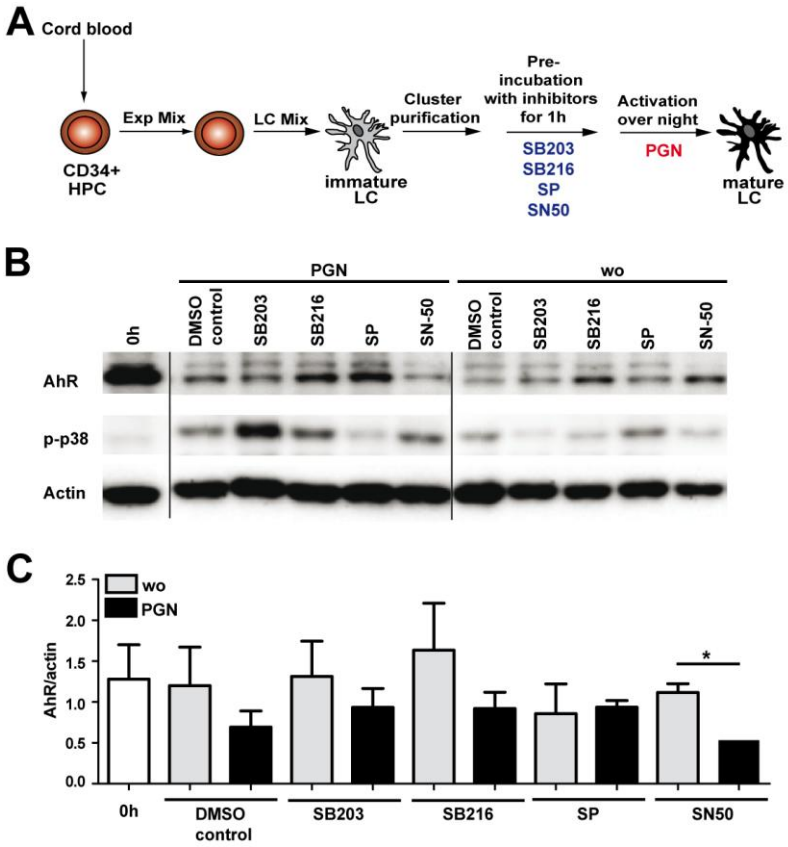


Figure 23: LCs treated with signaling inhibitors - JNK and RelA inhibition influences AhR nuclear translocation upon PGN-induced maturation

LCs are generated from CD34+ progenitor cells. After 7 days of culture the clusters are purified and preincubated with different siganling inhibitors for 1h.

SB203 (SB203580) -> p38 (ERK)
SB216 (SB216763) -> p38 (GSK3)
SP (SP600125) -> JNK
SN50 -> NFkB

Afterwards the clusters are activated with PGN overnight. On the next day cells are harvested, counted and Western Blot extracts are made.
(A) shows the experimental set up.
(B) shows a representative Western Blot analyzed for AhR, phosphorylated p38 and actin protein levels.
(C) presents the graph with statistical data of the quantification from 3 conducted experiments. The bars show the mean +/- SEM of 3 independet donors. SP inhibitor prevents the translocation of AhR after activation with PGN, whereas the other inhibitors show no inhibition of AhR translocation. SP inhibitor disables nuclear translocation of AhR upon PGN activation. However, SN50, a NFkB inhibitor enforces nuclear translocation.

10.3. RelB, an important factor in LC maturation, shows a similar activation pattern upon SDS and $NiSO_4$ treatment

Previous work from our lab has shown the importance of RelB during LC maturation as a regulator to prevent hyper-activation of LCs.(Jorgl et al. 2007) As this regulator is very important especially in LC maturation we want to investigate the RelB levels in different DC subsets. For this purpose we generate moLCs and moDCs from CD14+ peripheral blood monocytes and intDCs and LCs from CD34+ hematopoietic progenitor. After differentiation the cells are analyzed by FACS to ensure differentiation efficiency and purity of the cells. Western Blot samples are prepared normalized to the cell number. Figure 24A shows a representative western blot displaying the highest RelB levels in moDCs while moLCs, LCs and intDCs express

similarly lower levels of RelB. These results are also reflected in the statistical analysis of the quantification of 3 independent donors shown in figure 24B.

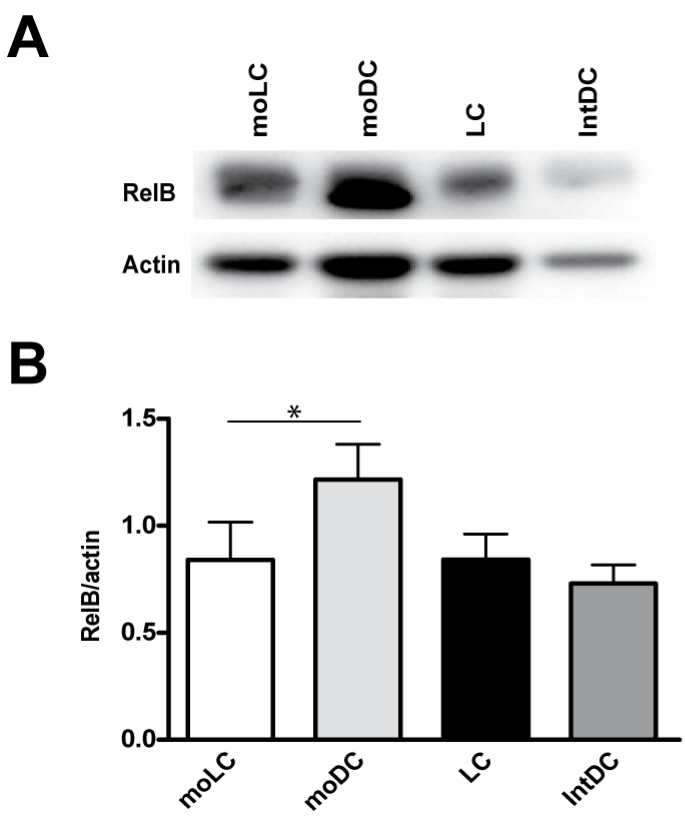


Figure 24: DC subsets show different levels of RelB protein.

moLCs and moDCs are generated from CD14+ monocytes isolated from peripheral blood, whereas IntDCs and LCs are differentiated from CD34+ progenitor cells isolated from cord blood. Differentiation efficiency and purity are checked by FACS staining for CD1a/Langerin double positive cells (moLCs and LCs) or CD1a/CD11b double positive cells (IntDCs and LCs) respectivley. Average purity of samples was:
moLCs: 50-70%
moDCs: 80-90%
IntDCs: 80-90%
LCs: 60-80%
Cells are counted and Western Blot samples are prepared.
(A) shows a representative Western Blot analyzed for RelB and Actin protein levels. (B) shows the statistical data after quanitfication of all Western Blots conducted. Bars show the mean +/- SEM of 3 independet donors.

10.4. Immature moLCs and LCs show a cytoplasmic cellular localization of RelB which translocates to the nucleus upon $NiSO_4$ activation

With our previous experiment we could show that RelB is expressed in all dendritic cell subsets. For AhR we could show that the stimulation with contact sensitizer ($NiSO_4$) or contact irritant (SDS) in moLCs and LCs leads to a differential nuclear translocation of the protein. As a next step we want to elucidate whether RelB reacts in a similar way. After generating moLCs and LCs, we activate them for 24 hours with SDS and $NiSO_4$. On the next day we fix the cells and stain them for RelB shown in green, CD1a shown in red and the nucleus shown in blue. Figure 25 shows representative pictures of the stainings performed in moLCs. The inactivated condition (wo) is presented in A, which shows a cytoplasmic distribution for RelB. Part B and C show the SDS and $NiSO_4$ activated conditions respectively. These results point out that only the $NiSO_4$ activation leads to a nuclear translocation of RelB, while SDS treatment has no impact on RelB activation.

Figure 26 presents representative data of the same experiments conducted in LCs (pictures kindly provided by Rene Köffel). Concerning the RelB activation and nuclear translocation, LCs show the same results like moLCs. Taken together, these results are quite interesting, also in relation to the similar behavior of AhR in the same context.

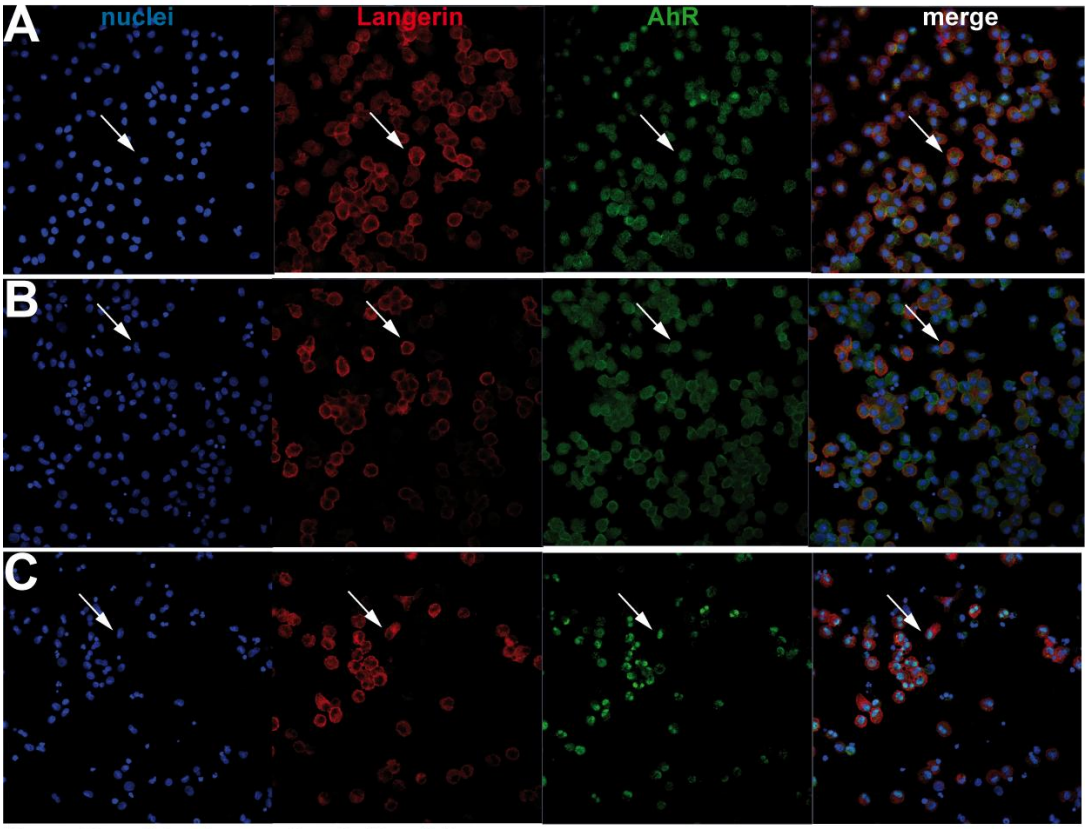
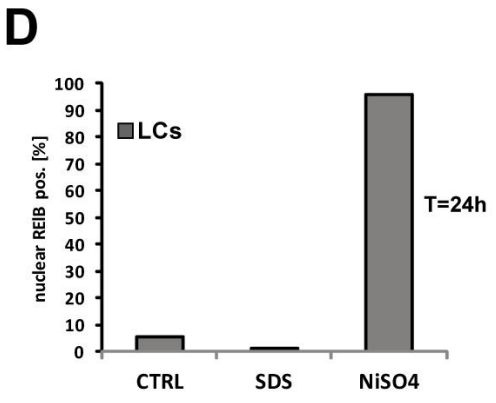


Figure 25: moLCs show nuclear RelB staining upon NiSO4 activation.

CD14+ monocytes are isolated from peripheral blood and differentiated into moLCs with GM-CSF and TGF-b1. After 4 days of culture the cells are stimulated with SDS or NiSO4 for 24 hours. On the next day cells are harvested and fixed and immunostained on an adhesion slide. Each column shows the signal of one single laser channel, whereas each row shows one condition (A wihout , B SDS and C NiSO4 activation). The last row shows the merger of all 3 channels. The arrows indicated monocyte deeived langerhans cells with the typical staining. What becomes obvious from these results is that RelB only translocates to the nucleus upon NiSO4 activation whereas the SDS and unactivated cells show no nuclear translocation of RelB. The quantification of nuclear RelB GFP positive cells in (D) of one donor underlines this effect.



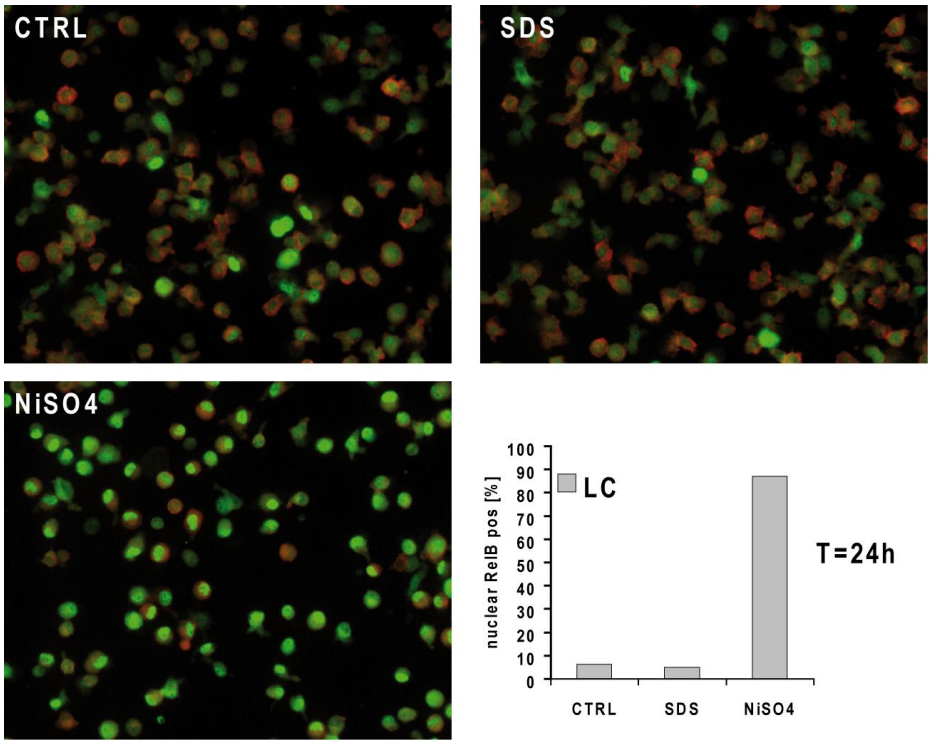


Figure 26: LCs show nucelar RelB staining upon NiSO4 treatment

Figure kindly provided by Rene Köffel

CD34+ cells are isolated from cord blood and LCs are generated. After 7 days of culture the LC clusters are purified and activated with NiSO4 and SDS for 24 hours. On the next day immunfluorescence stainings are made for RelB (green) and CD1a (red). We can see that the control and SDS condition show a cytoplasmic RelB localization while the NiSO4 treatment leads to pronounced nuclear translocation and induction of RelB. This effect can also be seen in the quantification of nuclear RelB positive cells from one donor as shown in the diagram: about 85% of the cells are positive in the NiSO4 condition while control and SDS treated cells are negative for nuclear RelB.

10.5. $NiSO_4$ activation of DC subsets leads to nuclear translocation of AhR and activation of RelB

So far we could show that AhR and RelB are expressed in all dendritic cell subsets investigated. In addition to this we could proof that in moLCs and LCs, $NiSO_4$ activation leads to a potent nuclear translocation of both proteins. With our next experiment we want to investigate whether this nuclear translocation is also detectable with the western blot method and whether this effect is equally pronounced in all DC subsets to further underline its importance. We generate moLCs, moDCs, intDCs and LCs. After the differentiation period the cells are activated with $NiSO_4$ for 24 hours and on the next day western blot samples are prepared according to the cell number. Preliminary results are depicted in figure 27, where A shows a representative western blot and B the corresponding quantification of the results. If we draw our attention to the western blot we can see that $NiSO_4$ activation leads to a dramatic decrease of AhR signal which is due to the nuclear translocation of the protein and its subsequent degradation in the cytoplasm. We can also see that the RelB signal slightly increases in the $NiSO_4$ treated conditions throughout all DC subsets, although LCs do not show such a pronounced effect, which could be due to a pre-activated state of the cells in the wo condition because of physical handling. The trends we see in the western blot are also underlined by the quantification of the two western blots from one donor shown in figure 27B. Priliminary data show that the AhR/actin ratio decreases upon $NiSO_4$ activation in all DC subsets, whereas the RelB/actin ratio

increases with activation with the exception of LCs due to the above mentioned pre-activated state of the cells.

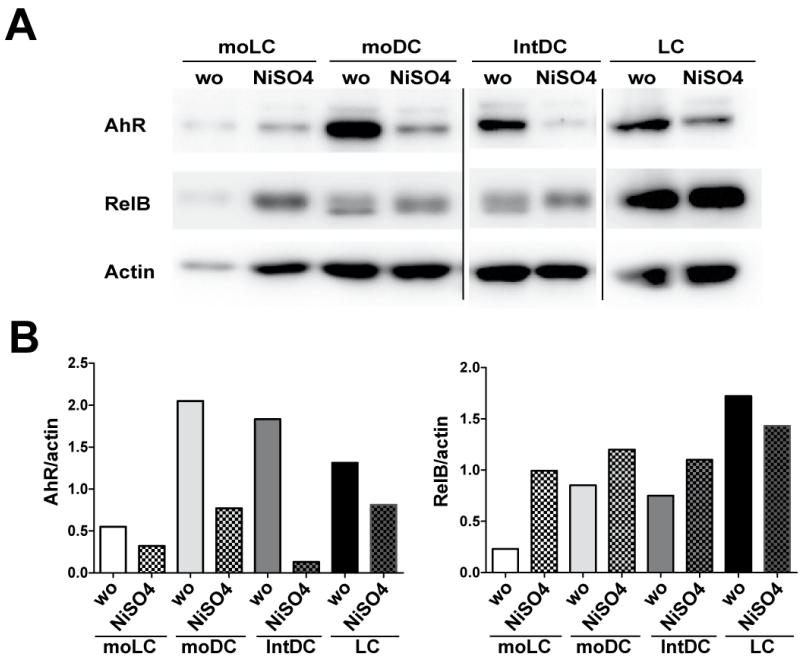


Figure 27: AhR nulear translocation upon NiSO4 activation in DC subsets.

DC subsets are generated as described before. After the differentiation period LC clusters are purified and then all DC subsets are checked for their purity rate with FACS. Cell purity is around 70-90% for all subsets. Cells are activated with NiSO4 for 24hours. On the next day the cells are harvested and counted and Western Blot samples are prepared.
(A) shows a representative blot analyzed for AhR, RelB and actin protein. NiSO4 activation leads to the nuclear translocation of AhR and adjacent degradation of the protein in the cytoplasm. Therefore we see a decrease of AhR protein level in the treated conditions in all dendritic cell subsets.
(B) shows the diagramms of the quantification. The bars show the mean of two blots from one donor. Whereas AhR/actin ratio is clearly decreased in the NiSO4 treated DC subsets, the RelB/actin ratio is increased in all subsets but LCs.

11. p100ΔN infected LCs underline potential interplay between AhR and RelB

As we have discovered an interesting similarity in the $NiSO_4$ maturation pattern of AhR and RelB protein in LCs we further ask the question to which extent these two proteins interact during this process. There are groups which have already reported about an AhR/RelB heterodimer (Vogel and Matsumura 2009; Vogel et al. 2007). This AhR/NFκB heterodimer was found to bind to newly discovered AhR/NFκB DNA recognition sites in promoters of genes associated with immune reactions such as chemokines (Vogel et al. 2011; Vogel et al. 2007). Our LC generation protocol poses an ideal experimental investigation system, as we could already show, that these cells express high levels of AhR and RelB. In addition to this $NiSO_4$ maturation causes the activation of both proteins and leads to nuclear translocation. Moreover we have seen that our LCs produce high amounts of IL8 upon maturation (Nighat Yasmin, unpublished data), which is indeed interesting with regard to a recent report implicating the IL8 promoter to harbor such newly described NFκB/AhR heterodimer binding sites (Vogel et al. 2011). Furthermore we have the system of retroviral infection well established in our lab and are therefore able to transduce these cells with a retroviral construct, namely the pBMNp100ΔN construct. Figure 28 B and C illustrates the construct in use. It consists of a p100 protein version which lacks ankyrin repeats. These repeats in turn are crucial for further proteosomal degradation of the p100 precursor into the p52 protein. Only the processed p52 form is able to undergo nuclear translocation. The p100ΔN construct

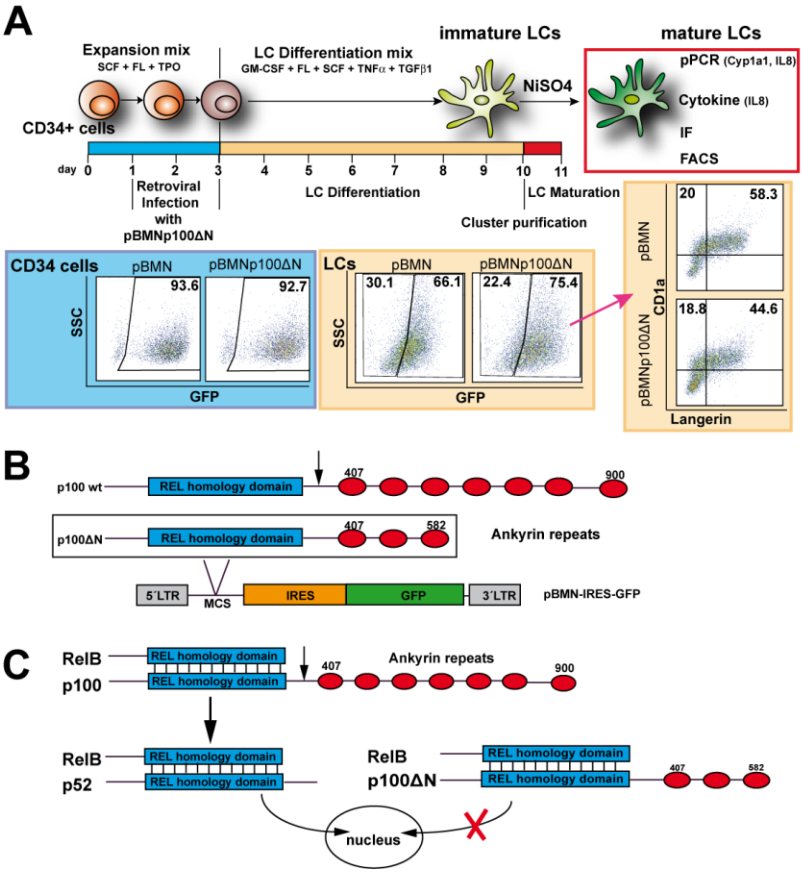


Figure 28: Experimental setup of infection experiments on CD34+ hematopoietic progenitor cells.

(A) CD34+ hematopoietic progentior cells are isolated from cord blood and expanded overnight. On the first and second day of culture cells are infected with the pBMNp100ΔN-IRES-GFP construct and the empty control vector pBMN-IRES-GFP. On the third day the infection efficiency of CD34 cells is checked by FACS. Infection works very good up to 90% of the cells are infected with constructs, which can be seen in the blued out rectangle. The cells are now set up for Langerhans cell differentiation for 7 days. After the differentitaion period cell clusters are purified over a PBS gradient and activated with NiSO4 at different time points over a period of 24 hours (wo, 3h, 6h, 24h). After 24 hours the supernatant is analyzed for cytokine secretion of IL8, total RNA is isolated to investigate the gene expression pattern of IL8 and Cyp1a1. In addition to this cells are immunostained to see the cellulare localization of AhR and RelB. The cells are also analzyed by FACS. The yellow rectangle shows a representative blot of the gating strategy for GFP positive and negative cells and the CD1a/Langerin double positive fraction gated on the GFP positive cells.
(B) shows a schematic of the p100 protein which dimerizes with RelB. The protein consits of a REL homolgy domain for RelB association and of ankyrin repeats, which are important for subsequent proteosomal processing of the protein complex to be translocated into the nucleus. The framed version of the protein lacks ankyrin repeats and is therefore named p100ΔN. It is cloned into the pBMN-IRES-GFP retroviral backbone vector. Figure adapted from Solan et al..
(C) illustrates the physiologic role of the p100ΔN construct. Upon proteosomal degradation of p100, p52/RelB dimer is able to translocate into the nucleus. p100ΔN on the other hand cannot be processed and is unable to undergo nuclear translocation. Therefore RelB is sequestered in the cytoplasm.

leads to a sequestration of RelB/p100 heterodimers in the cytoplasm. To investigate the question of a potential interplay between AhR and RelB in our LC system we infect CD34 positive hematopoietic progenitor cells with the pBMNp100ΔN construct. The experimental set up is outlined in figure 28A. The infected and expanded progenitor cells are then set up to differentiate into LCs. After the differentiation period the LC clusters are purified over a PBS gradient and set up for an activation time course with $NiSO_4$ at 3, 6 and 24 hours. The culture supernatant is analyzed for cytokine secretion and the cells are analyzed by FACS staining. Total cellular RNA is isolated to assess levels of IL8 and Cyp1a1 mRNA to investigate the induction of gene expression upon maturation. In addition to this cells are immunostained for RelB, to proof the effectiveness of the transduced construct, and for AhR to see whether nuclear translocation still takes place when RelB is kept in the cytoplasm.

11.1. p100ΔN transduced LCs show no change in differentiation and maturation

Figure 16 shows data from the FACS staining after LC maturation. In Figure 29A we see a representative FACS diagramm. The infection rate is around 50-70% for both the construct and the empty vector control. Cells are gated for GFP positive cells (marked red). In the control cells (first two lines), LC generation efficiency is around 50-60% and shows no significant change in the pBMNp100ΔN transduced cells. This can also be seen in the diagram depicting CD1a/Langerin double positive cells of

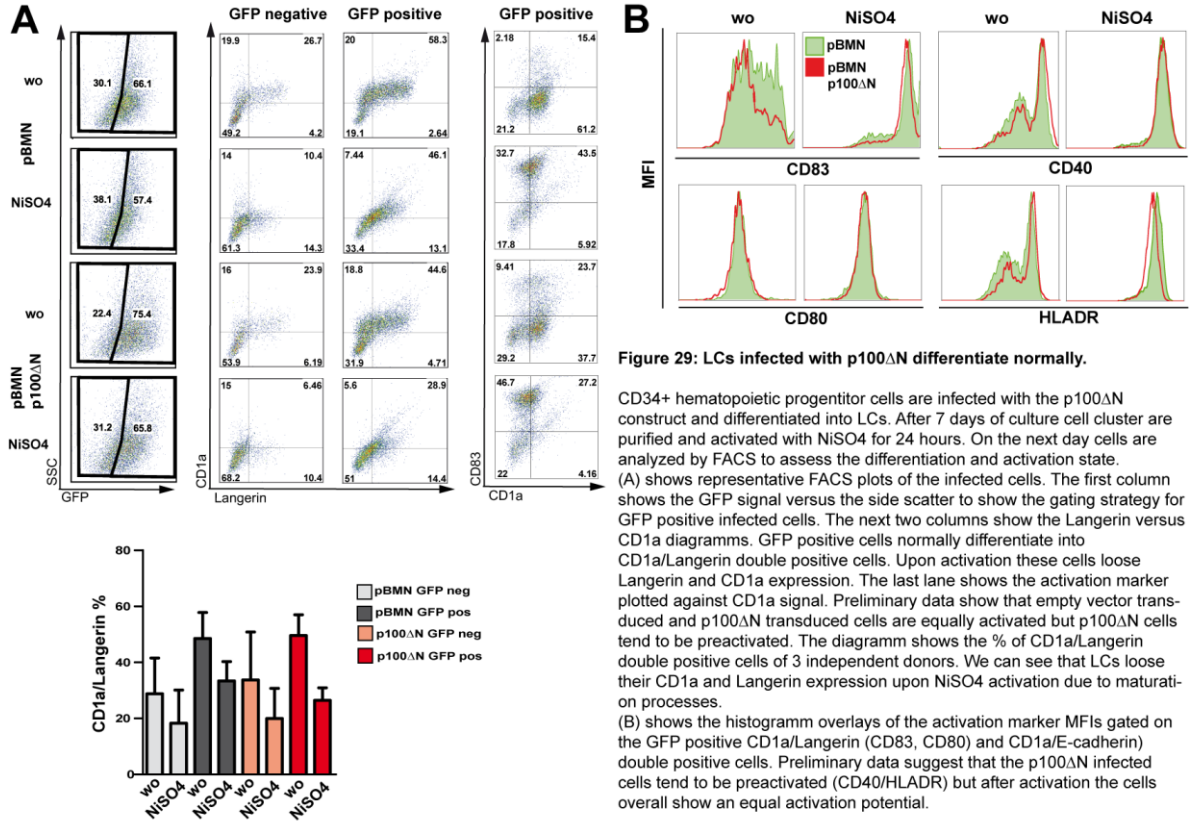


Figure 29: LCs infected with p100ΔN differentiate normally.

CD34+ hematopoietic progentitor cells are infected with the p100ΔN construct and differentiated into LCs. After 7 days of culture cell cluster are purified and activated with NiSO4 for 24 hours. On the next day cells are analyzed by FACS to assess the differentiation and activation state.
(A) shows representative FACS plots of the infected cells. The first column shows the GFP signal versus the side scatter to show the gating strategy for GFP positive infected cells. The next two columns show the Langerin versus CD1a diagramms. GFP positive cells normally differentiate into CD1a/Langerin double positive cells. Upon activation these cells loose Langerin and CD1a expression. The last lane shows the activation marker plotted against CD1a signal. Preliminary data show that empty vector transduced and p100ΔN transduced cells are equally activated but p100ΔN cells tend to be preactivated. The diagramm shows the % of CD1a/Langerin double positive cells of 3 independent donors. We can see that LCs loose their CD1a and Langerin expression upon NiSO4 activation due to maturation processes.
(B) shows the histogramm overlays of the activation marker MFIs gated on the GFP positive CD1a/Langerin (CD83, CD80) and CD1a/E-cadherin) double positive cells. Preliminary data suggest that the p100ΔN infected cells tend to be preactivated (CD40/HLADR) but after activation the cells overall show an equal activation potential.

three independent donors. Upon maturation with $NiSO_4$, the cells loose Langerin and CD1a expression and up-regulate CD83 (Figure 29A, last row), CD40, CD80 and HLADR (Figure 29B). Figure 29B shows representative histogram overlays of the maturation markers gated on LCs. While CD40 and CD80 show no difference in activation state CD83 and HLADR tend to be slightly less expressed compared to the empty control vector. CD40 and HLADR show slightly pre-activated cells in the inactivated condition, whereas CD80 and CD83 do not show this effect. Nevertheless these changes are not significant and taken together there seems to be no effect in maturation potential regarding the expression profile of activation markers.

11.2. AhR nuclear translocation upon maturation is not inhibited by RelB cytoplasmic sequestration

Figure 30 and 31 show representative pictures of the immunostainings for RelB and AhR respectively. Cells are fixed on adhesive slides and stained for RelB or AhR, shown in red. CD1a staining is done to identify LCs and is shown in white. Nuclei are counterstained with dapi and shown in blue. Infected cells can be distinguished from normal cells through the GFP signal, as the transduced vectors have a GFP epitope to track the constructs. In figure 30 we can see the RelB staining of construct or empty transduced cells from inactivated and $NiSO_4$ activated cells. As already previously shown inactivated cells show a cytoplasmic localization of the protein, indicated with white arrows in Figure 30A and C. What becomes obvious from the pictures in figure 30B and C is that RelB nuclear translocation upon maturation is only completely blocked in strongly pBMNp100ΔN transduced cells, indicated by the white arrows, whereas slightly transduced cells (indicated by the yellow arrows) and empty vector control transduced cells show a normal RelB nuclear translocation upon stimulation. The immunostaining of AhR of transduced LCs is shown in figure 31. Part A and B of figure 31 show the pictures of the empty vector transduced cells without (A) and with $NiSO_4$ activation (B). Upon maturation we see a nuclear signal for AhR. The same is true for the pBMNp100ΔN transduced cells. To sum up we can state that RelB sequestration in the cytoplasm does not inhibit AhR nuclear translocation upon $NiSO_4$ activation which in turn indicates that AhR and RelB proteins independently translocate to the nucleus.

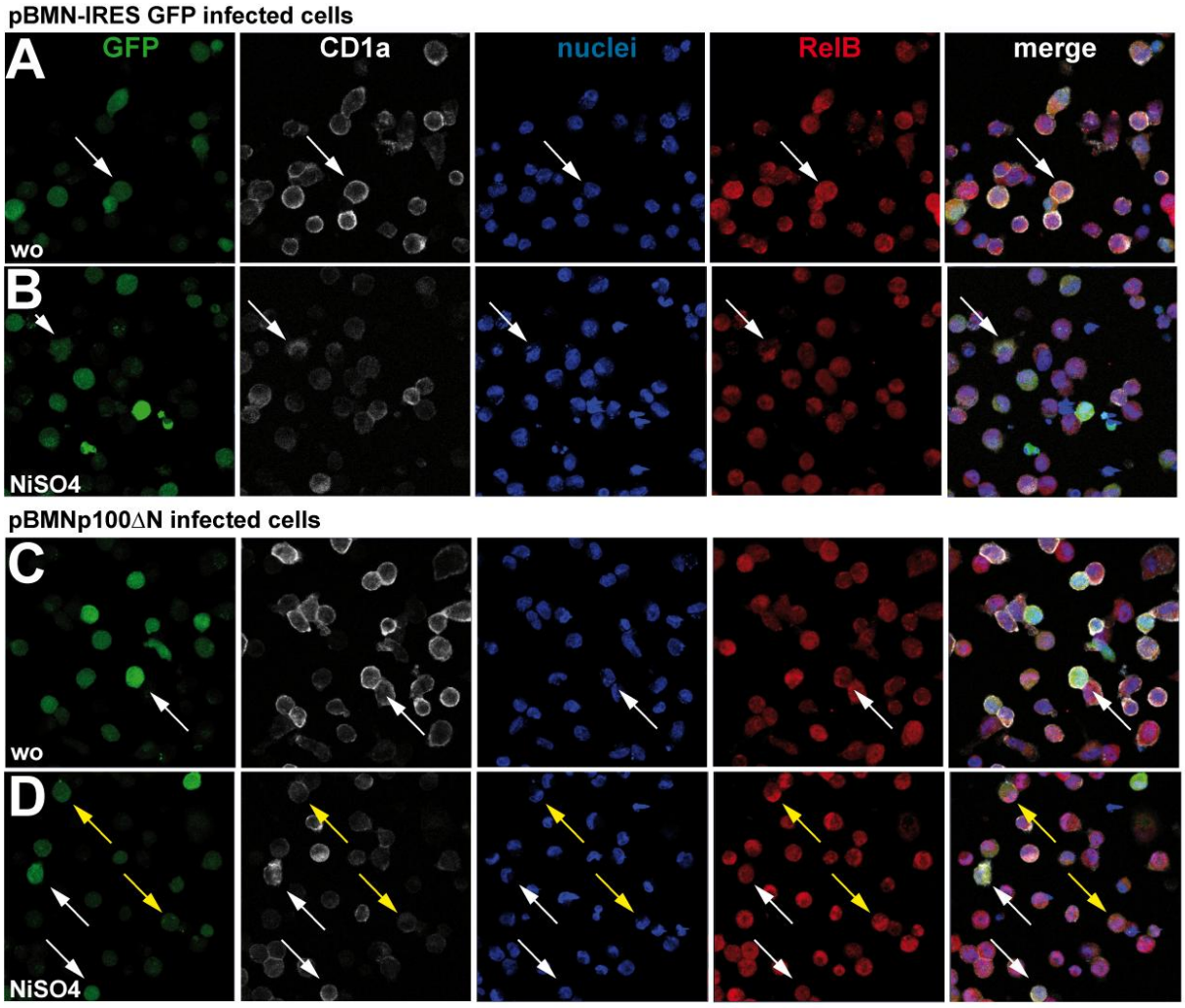


Figure 30: RelB nuclear translocation upon NiSO4 is not completely inhibited by the p100ΔN construct.

CD34+ hematopoietic progenitor cells are isolated from peropheral blood and infected with the p100ΔN construct. After LC generation the cells are activated with NiSO4 for 24 hours. The cells are then fixed and immunostained on an adhesion slide. The first column in green shows the GFP signal to visualize infected cells. The second column, in white, shows the CD1a staining to identify Langerhans cells. The nucleus is stained with dapi and can be seen in the third column in blue. AhR is stained in red and can be seen in the fourth column. The last column shows the merger of the single channel laser signals. The pictures are taken with a ZEISS LSM700 confocal microscope with a 40x objective. The arrows indicate infected langerhans cells with the typical staining.
(A) and (B) are representative pictures of LCs transfected with the empty vector backbone pBMN-IRES-GFP, with and without activation respectively. What we can see is that the cells loose Cd1a upon activation. In addition to this RelB is located all over the cell equally before activation. Upon NiSO4 activation RelB translocates to the nucleus.
(C) and (D) are representative pictures of LCs transfected with the p100ΔN construct, with and without activation respectively. From these results it becomes obvious that the p100ΔN infected LCs too loose CD1a upon activation. Apart from this RelB nuclear translocation upon NiSO4 activation is not completely inhibited by the p100ΔN construct.The white arrow indicates strongly transduced cells where RelB is kept in the cytopslam. The yellow arrows on the other hand point to infected cells where we can see a nuclear signal for RelB.

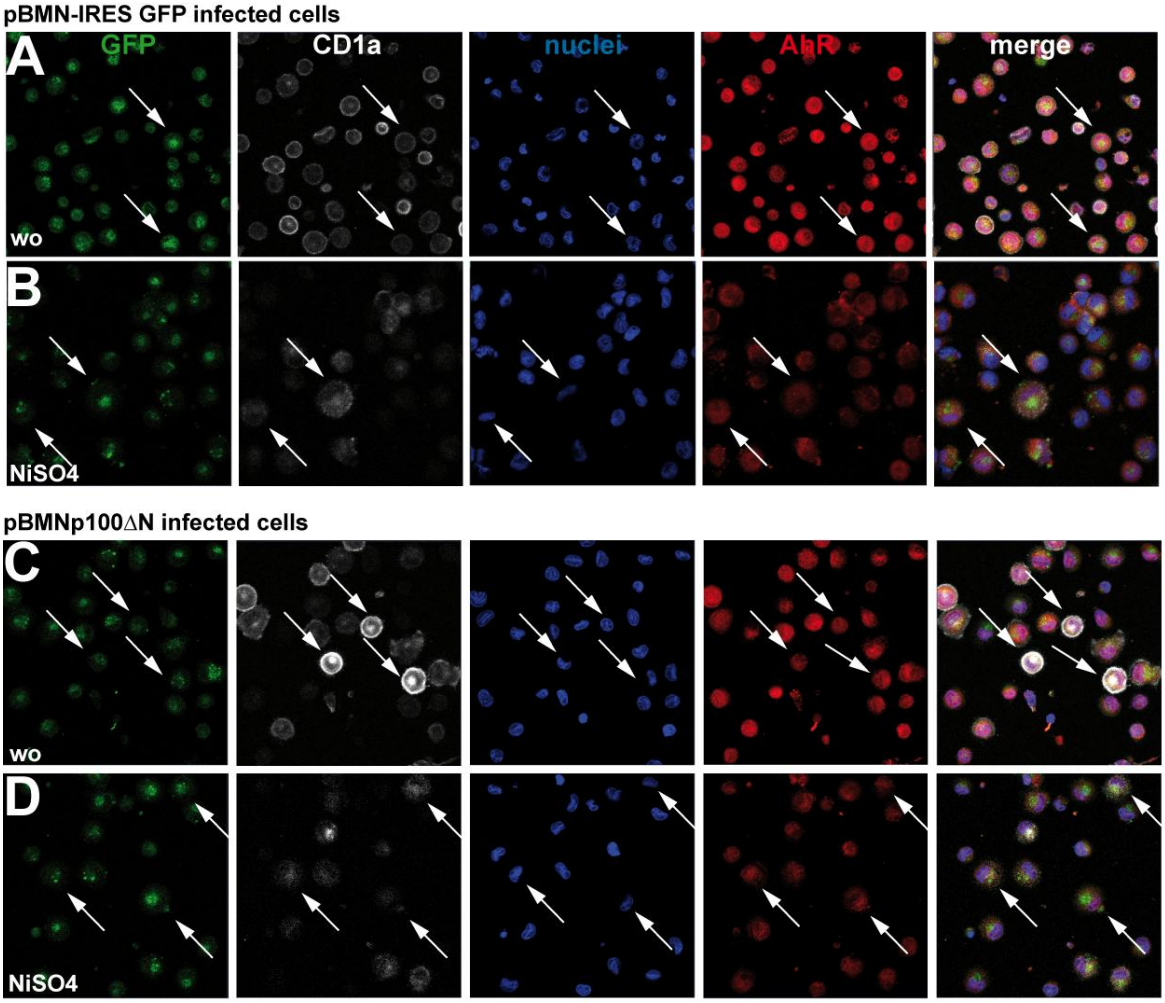


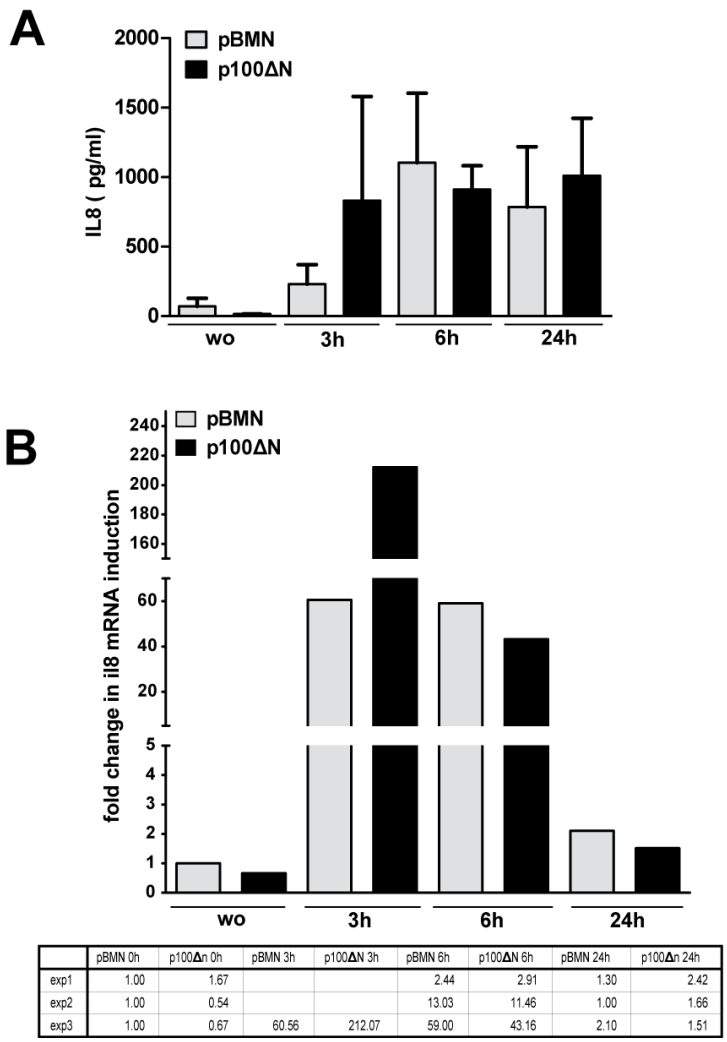
Figure 31: AhR still translocates to the nucleus in p100ΔN infected LCs

CD34+ hematopoietic progenitor cells are isolated from peripheral blood and infected with the p100ΔN construct. After LC generation the cells are activated with NiSO4 for 24 hours. The cells are then fixed and immunostained on an adhesion slide. The first column in green shows the GFP signal to visualize infected cells. The second column, in white, shows the CD1a staining to identify Langerhans cells. The nucleus is stained with dapi and can be seen in the third column in blue. AhR is stained in red and can be seen in the fourth column. The last column shows the merger of the single channel laser signals. The pictures are taken with a ZEISS LSM700 confocal microscope with a 40x objective. The arrows indicate infected langerhans cells with the typical staining.
(A) and (B) are representative pictures of LCs transfected with the empty vector backbone pBMN-IRES-GFP, with and without activation respectively. What we can see is that the cells loose Cd1a upon activation. In addition to this AhR already translocates to the nucleus to a certain extent in the wo condition probably due to physical handling. Upon activation the AhR signal decreases because the protein is tranlocated to the nucleus and gets degradeed subsequently.
(C) and (D) are representative pictures of LCs transfected with the p100ΔN construct, with and without activation respectively. From these results it becomes obvious that the p100ΔN infected LCs too loose CD1a upon activation. Apart from this AhR still translocates to the nucleus upon activation and the AhR signal gets less because the protein is degraded.

11.3. IL8 secretion and il8 mRNA production are increased in p100ΔN transduced LCs

Figure 32A shows the cytokine levels of after a time course of $NiSO_4$ activation. IL8 cytokine secretion tends to be increased in the pBMNp100ΔN transduced cells after 3 hours. This effect is leveling out after 6 to 24 hours. These data indicate that RelB inhibition has a very immediate effect on the IL8 secretion. Results from the pPCR

analysis for cyp1a1 are not shown here as we have not seen any detectable induction of the gene. This is a further indication that AhR, upon $NiSO_4$ activation indeed translocates to the nucleus but does not bind to commonly known XREs, including the Cyp proteins, but has other target sites to exert different signals. The data from the il8 gene qPCR are presented in figure32B. The diagram shows the representative data of one experiment of the x-fold induction change in il8 mRNA production normalized to the house keeping gene gapdh. Preliminary data point towards an increased il8 mRNA production after 3 hours of $NiSO_4$ induction.



	pBMN 0h	p100Δn 0h	pBMN 3h	p100ΔN 3h	pBMN 6h	p100ΔN 6h	pBMN 24h	p100Δn 24h
exp1	1.00	1.67			2.44	2.91	1.30	2.42
exp2	1.00	0.54			13.03	11.46	1.00	1.66
exp3	1.00	0.67	60.56	212.07	59.00	43.16	2.10	1.51

Figure 32: LCs infected with p100ΔN tend to secret higher levels of IL8 cytokine.

CD34+ hematopoietic progenitor cells isolated from cord blood are infected with the p100**Δ**N construct and differentiated into langerhans cells. After 7 days of culture the cell clusters are purified and activated with NiSO4 at different time points (3, 6 and 24hours). The culture supernatant is analyzed for cytokine levels and total RNA is isolated to perform real time PCR for IL8 and Cyp1a1.
(A) shows the graph from the statistical analysis of the cytokine measurements from 3 independent donors. The bars represent the mean +/- SEM. The p100**Δ**N infected cells show a tendency towards higher levels of secreted IL8 cytokine upon NiSO4 activation especially after 3hours.
(B) shows the diagramm of the IL8 qPCR of experiment 3. The table shows the results of all 3 experiments. Preliminary data suggest that the IL8 gene is highly upregulated after 3 hours of NiSO4 treatment.

Discussion

AhR and RelB levels in DC subsets: In our lab we showed before that the highest AhR expression in the myeloid cell lineages are found in LCs followed by monocytes whereas granulocytes and neutrophils show only basal to no AhR levels (Platzer et al. 2009). Therefore we further concluded that AhR is characteristically expressed in dendritic cells and might have an essential role in these cell types. In our studies we investigated various dendritic cell subsets for their basal AhR levels and found that CD34 derived LCs and monocyte derived DCs express the highest levels followed by interstitial DCs. Inflammatory monocyte derived LCs on the other hand only express low levels of AhR. Additionally we analyzed RelB levels in these DC subsets and could show that moDCs express the highest RelB levels followed by LCs, moLCs and intDCs. To sum up moDC are the DC subtype with the highest levels of AhR and RelB followed by LCs. Although we crossed out experiments with generation rates less than 50% purity, the obstacle of these experiments is that purity rates vary between the different donors and therefore protein levels have to be interpreted with caution. The best analysis method would be to sort the generated cells for cell lineage specific markers to be sure to analyze a homogenous population. Nevertheless the cellular stress from the cell sorting process could interfere with protein levels therefore we stick to the conventional method of FACS purity check and immediate western blot sample preparation to preserve natural protein levels from inactivated cells. Indeed it is interesting that LCs, which are situated in the epidermis and are continuously in contact with the environment, express the highest levels of AhR and RelB, which in turn are two proteins involved in immune-regulatory processes. One can speculate that DC subtypes which are constantly exposed to environmental stress need to have a mechanism to regulate immune responses to establish a certain tolerance to prevent immune overreaction. DC subsets which are not constantly exposed to the environment (e.g. intDCs) or arise under inflammatory conditions (moLCs) express lower levels of these immune regulatory proteins.

AhR and RelB localization after $NiSO_4$ activation in LCs: LC activation with the chemical sensitizer $NiSO_4$ or the chemical irritant SDS induces a divergent cellular localization pattern of AhR and RelB. By using immuno-histochemistry we were able to show that only $NiSO_4$ induces a strong nuclear translocation of AhR in LCs and moLCs. SDS in comparison only slightly induced nuclear translocation of AhR although moLCs tend to have more nuclear AhR upon SDS treatment compared to LCs. Our protein level analysis of AhR in DC subsets revealed that moLCs express very low levels of basal AhR, which could be the reason why

SDS is able to slightly translocate this AhR whereas the high basal levels of AhR in LCs seem to be less susceptible to SDS-induced nuclear translocation. Apart from this it has to be mentioned that also physical handling of the cells can lead to AhR activation and translocation into the nucleus although this activation is not as intense as the $NiSO_4$ activation.

We did the same experiments for RelB and could show that upon $NiSO_4$ but not SDS activation RelB translocates into the nucleus in LCs and moLCs. Therefore we speculate that AhR translocation upon activation could be an indicator which distinguishes reactions to contact sensitizers to those from contact irritants. Nevertheless further experiments are needed to confirm this hypothesis. It would be interesting to compare immune responses of AhR knockout mice to $NiSO_4$ and SDS in contact hypersensitivity reactions on the skin, to see whether AhR in epidermal LCs really plays a crucial role in the induction of an appropriate immune response to these chemicals.

Preliminary data from western blot analysis of different DC subsets for AhR and RelB upon $NiSO_4$ treatment showed that all DC subtypes harbor the same cellular mechanism in response to $NiSO_4$. AhR activation and therefore decrease in signal takes place in all DC subsets just as RelB gets up-regulated in response to $NiSO_4$ stimulation. Therefore we assume that all DC subtypes react in the same way to $NiSO_4$ treatment although the basal levels of the proteins determine the strength of the reaction. Additionally it is interesting that both, AhR and RelB, are strongly translocated to the nucleus of $NiSO_4$ stimulated LCs whereas SDS treatment does not lead to this strong nuclear co-localization as shown with immunostainings. This could imply a potential interplay between AhR and RelB in the cellular immune reaction towards chemical sensitizers.

<u>AhR in ex-vivo human skin:</u> So far we could show that in in-vitro generated LCs AhR is cytoplasmically located and only translocates into the nucleus upon activation signals. To elucidate the in-vivo situation we stained AhR in human skin explants. What we have observed so far does not reflect the findings on in-vitro LCs. AhR seems to be in the nucleus in epidermal LCs independently of the activation state of the cells. Preliminary data imply that activation stimuli lead to a more intense and exclusively nuclear signal of AhR. Nevertheless for this assumption more donors have to be analyzed. Aside from this the basic nuclear localization of AhR in epidermal LCs can furthermore be explained by the fact that FICZ, an AhR agonist, is constitutively present in the skin. Through UV irradiation tryptophan gets metabolized to FICZ and is saturated in the epidermis (Fritsche et al. 2007). Constant AhR

activation through this endogenous physiologic ligand could explain constant activation and nuclear AhR localization in human skin LCs. However the interpretation of this finding is demanding as FICZ has been reported to worsen autoimmune diseases in a mouse EAE model and having a rather immune-stimulatory than an immune-repressive function (Quintana et al. 2008; Veldhoen et al. 2008). One possible model could be that the constant but short-lived AhR activation in the skin renders LCs less responsive to other environmental AhR ligands, thereby constituting a higher activation threshold in this cell type. Another possibility is that ligand activated AhR needs other inflammatory signals to properly induce immune reactions. It is also possible that the continuous AhR activation by the endogenous physiologic ligand FICZ impairs LC maturation so that T-cells cannot be properly activated and are therefore cleared or the balance shifts towards T-reg differentiation (Quintana et al. 2008; Veldhoen et al. 2008).

A possible future experiment could be to determine which influence light has on the proper immune reaction to contact hyper sensitivity reagents such as $NiSO_4$. As a model one could use wild-type and AhR knock-out mice which are kept exclusively in the dark. First one would have to show that FICZ levels are depleted in the skin of mice which are kept in the dark. Second of all one would have to compare the immune reactions to $NiSO_4$ and SDS in wild-type and AhR knock-out mice and see whether a potential phenotype can be reversed by the application of light and the subsequent generation of AhR ligand FICZ.

moLCs treated with AhR ligands FICZ and VAF347: FICZ is a physiologic endogenous high affinity ligand of AhR. After 4 days of differentiation of moLCs in the presence of FICZ we observed more Langerin/CD1a double positive cells. In more detail Langerin, CD1a and CD11b marker expression was increased whereas E-cadherin expression was significantly decreased. CD14 on the other hand was not influenced by AhR ligand FICZ. When moLCs were generated in the presence of the exogenous ligand VAF347 we observed similar trends. The MFI values of Langerin, CD1a and CD11b were increased as was the percentage of Langerin/CD1a double positive cells. E-cadherin MFI was decreased and CD14 expression was not influenced to a great extent.

These findings are interesting with regard to the findings in LCs because AhR ligands seem to influence steady state LCs and inflammatory moLCs in a different way. In our lab it has been shown that LC differentiation in the presence of VAF347 is inhibited at a monocytic precursor state with elevated CD14 levels (Platzer et al. 2009; Richter 2010). Our findings show that AhR in these two LC subsets responds differentially to the same AhR ligands. A

possible explanation for this phenomenon could be the different basal levels of AhR in these two cell types and also makes sense with regard to their physiologic role. While steady-state LCs have to set up a complex tolerance system in the skin, inflammatory moLCs are only recruited in response to danger signals and ongoing inflammation with the purpose to clear the infection rather than induce tolerance. Monocytes recruited to the place of infection differentitate into moLCs. It could be that the presence of AhR ligands (e.g. FICZ) or potentially harmful environmental compounds (e.g. TCDD) increase the capacity of monocytes to differentiate into moLCs in an AhR-dependent manner to help clear the infection more rapidly.

Furthermore we have analyzed the maturation potential of moLCs generated in the presence of AhR ligand FICZ. We have activated the cells with two different activation stimuli, namely Lipopolysaccharide (LPS) and Peptidoglycan (PGN). LPS signals through the TLR4 receptor and needs additional proteins for proper signal transduction such as lipopolysaccharide binding protein (LBP), CD14 receptor and MD-2. TLR4 levels on our in-vitro LCs are known to be very low and therefore LPS is considered a weak stimulus. PGN on the other hand can be recognized by CD14 (Dziarski et al. 1998), TLR2 (controversial) (Dziarski and Gupta 2005; Travassos et al. 2004), nucleotide-binding oligodimerization domain-containing proteins (NODs) (Franchi et al. 2006) and PGN-recognition proteins (PGRPs) (Yoshida et al. 1996). We and others could show that TLR2 is expressed on in-vitro generated LCs and therefore PGN acts as strong activation stimulus (Flacher et al. 2006; Jurkin et al. 2010; Renn et al. 2006; Rozis et al. 2008).

Interestingly our data are not reflecting this typical characterization. We have observed that LPS stimulation of FICZ treated moLCs leads to a slightly reduced expression of co-stimulatory molecules. PGN treatment on the other hand did not influence this expression. Same was true for cytokines as LPS tends to increase levels of IL1β, Il6, IL8 and TNFα whereas IL10 is slightly decreased and IL12p40 is not influenced. PGN on the other hand lead to an overall reduction of cytokine levels with the exception of IL8 and IL12p40 which were not changed. Additionally basal levels of cytokines without stimuli seemed to be slightly induced in FICZ treated cells. It is possible that activated AhR somehow primes cytokine promoters for transcription and facilitates their transcriptional control as has been implicated before in other models (DiNatale et al. 2010).

We further analyzed the AhR activation in DMSO versus FICZ treated moLCs upon LPS and PGN stimulation. We could show that AhR is activated to the same extent in both conditions even in the absence of stimulus. This can be explained by physical handling of the cells leading to stress which can ultimately induce activation.

VAF347 treatment during moLC differentiation led to slightly different effects compared to FICZ treated moLCs. The strong stimulus PGN applied to VAF347 treated cells only caused a weak and partial up-regulation of co-stimulatory molecules. CD40 and CD83 showed no changes whereas CD80 and CD86 were slightly up-regulated and HLADR actually tended to be down-regulated. Basal cytokine levels were generally slightly induced, again underlining the concept of promoter priming of activated AhR. The cytokine profile slightly differed from the results we have obtained for FICZ treated cells. Preliminary data showed that IL10, IL8 and IL12p40 tend to be slightly induced whereas IL6 and TNFα were not affected and IL1β seemed to be slightly down-regulated. Activation studies showed that AhR in VAF347 treated cells is translocated to the nucleus in PGN activated and inactivated conditions to a similar extent. We would have expected that control LCs down-regulate AhR more extensively upon PGN activation compared to VAF347 treated cells. Furthermore the hypothesis would implicate that VAF347 treatment leads to a decreased AhR activation capacity due to continuous stimulation with AhR ligand. Nevertheless we could not see this trend as AhR levels in VAF347 treated cells were already very low before actual activation treatment. This shows that VAF347 is a strong agonistic AhR ligand. RelB on the other hand was nicely up-regulated in PGN treated conditions reflecting the proper activation of the cells.

In summary we found that AhR ligand treatment during moLC generation leads to an increased differentiation potential of moLCs. Upon activation of these cells the maturation process is only slightly decreased or left unchanged. Nevertheless it becomes clear that LPS and PGN stimulation have different effects on AhR ligand treated cells. For example PGN treatment of VAF347 cells leads to a slight tendency towards increased co-stimulatory molecule expression and slightly induced cytokine production. LPS treatment of VAF347 treated cells leaves the co-stimulatory molecule expression unchanged and only slightly reduces cytokine production. LPS and PGN activation of FICZ treated cells shows that the weaker LPS stimulation leads to a decreased co-stimulatory molecule expression profile but an increased cytokine production whereas PGN activation leaves maturation overall unchanged. These results confirm that AhR function in maturation depends on the stimulus present as has already been proposed by (Richter 2010). Nevertheless there are only small changes therefore we reason the effect of AhR ligands on maturation of moLCs is negligible and does not have an in-vivo effect.

TGF-β1 and FICZ-activated AhR interplay in moLCs: There have been studies investigating the signal interplay between AhR and TGF-β1. What becomes clear from the literature is that this interplay is highly tissue and cell-type specific. For example in a prostate epithelial cell

line it has been shown that TGF-β1 suppresses AhR-induced target gene expression via the inhibition of AhR expression and down-regulation of nuclear AhR in a SMAD4-dependent way. Interestingly, AhR signaling induced by TCDD does neither affect TGF-β1 regulated gene expression nor epithelial-to-mesenchymal transition (Starsichova et al. 2012). In another report Guo et al. showed that AhR represses TGF-β genes in an AhR-ligand-independent way (Guo et al. 2004). Chang et al. observed that AhR deficient mouse fibroblasts proliferated slower than wild type fibroblasts and that this happened in an AhR-ligand-independent way. In addition to this they reported that AhR-deficient fibroblast secret higher levels of TGF-β1 and have higher levels of activated SMAD4 and TGF-β1 mRNA (Chang et al. 2007). E-cadherin expression is a key hallmark of immature LCs. E-cadherin is responsible for the interaction with epithelial cells and keeps the epidermal dendritic LCs in a regular network in the skin. Strobl et al could show that TGF-β1 is essential for in-vitro generation of LCs from CD34 positive HSC. In culture these cells form clusters in a strictly TGF-β1 dependent way and express high levels of E-cadherin (Strobl et al. 1996). Upon activation Langerhans cells down-regulate E-cadherin and the clusters are loosened and broke down. This down-regulation of E-cadherin is essential for LC maturation because the cells need to leave the epidermis and migrate to the draining lymph node. Riedl et al could show that ligation of E-cadherin on immature LCs prevents them from acquiring a mature state. They further concluded that this constitutive E-cadherin-mediated suppression of LC maturation in the epidermis could prevent uncontrolled LC activation (Riedl et al. 2000). In our experiments we asked the question what effects FICZ-activated AhR might have on TGF-β1-dependent moLC generation. We found that FICZ treated moLCs, independent of TGF-β1 concentration, express lower levels of E-cadherin, while other TGF-β1-dependent LC typical markers such as CD1a or Langerin were not affected. Furthermore we have observed that typical LC cluster formation is disrupted in FICZ treated cells in a way that cells form more but smaller and less compact clusters. E-cadherin is a TGF-β1 target gene in LC differentiation and FICZ is an AhR ligand that is constitutively present due to UVB-irradiation-dependent processing of tryphtophan to FICZ (Fritsche et al. 2007). LCs need to have mechanisms for potent tolerance induction because this cell type is constantly exposed to environmental influences. It has even been reported that LCs travel to the draining lymph node at a basal rate in steady-state conditions in the absence of danger signals to present self-antigen and induce tolerance (Steinman and Nussenzweig 2002). Therefore we speculate that LCs in FICZ-saturated epidermis are constitutively expressing lower levels of E-cadherin in an AhR-dependent way which renders them more motile. This effect could facilitate the semi-maturation of LCs in the absence of danger signals and therefore enables the generation of semi-activated LCs. This hypothesis needs further testing by FACS analysis of the maturation state and comparison between various AhR ligands. Interestingly

there are two groups linking E-cadherin to induction of tolerance. In more detail E-cadherin down-regulation leads to the activation of β-catenin/Wnt signaling which is implicated in tolerance induction (Fu and Jiang 2010; Manicassamy et al. 2010). It would be interesting to test in-vivo E-cadherin levels of LCs compared to other epithelial DC cell types. In addition it would also be interesting to compare E-cadherin levels of LCs from wild-type and AhR knock-out mice. Furthermore it would be interesting to investigate β-catenin/Wnt signaling in this process to further unravel a potential tolerance inducing mechanism in the skin immune system.

Transcription factor profile in AhR ligand treated LCs: Platzer and Richter et al. investigated CD34 derived LCs for AhR ligand effects during early differentiation. They could show that VAF347 impairs LC differentiation from CD34+ HSC. Activated AhR inhibited PU.1 up-regulation which is necessary for the differentiation of monocytes towards LCs (Platzer et al. 2009). In our studies we further analyze the effect of AhR ligands on LCs regarding their transcription factor profile before and during PGN maturation. LCs which were generated in the presence of AhR ligands and analyzed after 7 days of differentiation showed no remarkable difference in transcription factor profiles of PU.1, p65, VDR, AhR or RelB. Only PU.1 and AhR were reduced in AhR ligand treated cells due the blockage of PU.1 up-regulation (Platzer et al. 2009) and AhR activation through the ligands and the following protein degradation. Additionally PGN induced maturation did not lead to a change in transcription factor expression. These results are in line with the findings of Platzer and Richter et al because they further underline the early effect of AhR on transcription factors in progenitor cell differentiation rather than a late function in differentiated LCs or maturation process.

AhR signaling in LCs: As described in the introduction there have been many - partly controversial - reports about the signaling cascades involved in AhR activation and subsequent nuclear translocation. P38 MAPK has been implicated in AhR activation by multiple groups. On the one hand it has been proposed that p38 MAPK phosphorylates the NES sequence of AhR protein at Ser-68 thereby inhibiting AhR nuclear export which leads to prolonged AhR signaling (Ikuta et al. 2009). However there have been further studies investigating the p38 MAPK cascade in this context. One report poses a role for p38 MAPK in TCDD-activated AhR-mediated transcription of target genes (Shibazaki et al. 2004). On the other hand Tan et al reported that not p38 but rather ERK and JNK MAP kinases are

important for AhR activation (Tan et al. 2002). These controversial results suggest a specialized AhR function in each cell type also depending on the basic expression level of involved signaling proteins and susceptibility to signaling cascades.

In addition to this we show that $NiSO_4$ activation of LCs leads to the nuclear translocation of AhR. $NiSO_4$ signaling is known to activate p38 MAPK (Miyazawa et al. 2008) therefore we speculate that p38 MAPK might play an important role in AhR activation. In our studies we analyzed the signaling events in PGN induced LC maturation by investigating AhR activation. We applied multiple signaling cascade inhibitors targeting p38 MAPK signaling (SB203580), β-catenin/Wnt signaling (SB216763), NFκB (RelA) signaling (SN-50) and JNK signaling (SP600125). We have observed that p38 MAPK inhibition does not completely inhibit AhR activation although SB203580 inhibitor tends to partially suppress it. One possible reason for this phenomenon could be that p38MAPK has four different isoforms namely α, β, γ and δ. The SB203580 inhibitor only inhibits the α and β subunit. It could be that the other two isoforms are involved in AhR activation. Dong et al have already implicated c-Src kinase $pp60^{Src}$ in AhR signaling (Dong et al. 2011; Dong and Matsumura 2008). It is known that this kinase activates the p38δ MAPK isoform.

A possible way of elucidating the question which p38 MAPK isoform is implicated in AhR activation would be to infect LCs with retroviral constructs expressing the various isoforms and repeat the experiment to monitor changes in AhR activation. Interestingly the JNK inhibitor showed the strongest inhibition of AhR nuclear translocation. It is important to mention that there are controversial reports in the literature suggesting the SP inhibitor to be an AhR ligand, either with agonistic (Dvorak et al. 2008) or antagonistic effects (Joiakim et al. 2003). Our results would imply an antagonistic function. Nevertheless further experiments are needed to clarify the question whether AhR inhibition comes from an antagonistic function or really from JNK inhibition. As the SP600125 inhibitor only prevents the upstream MAPKK to phosphorylate JNK, the inhibition is not quite specific. So far there are no inhibitors on the market which directly affect JNK MAPK. In addition to this it has to be said that p38 and JNK signaling are only hardly distinguishable because they are interlinked at many steps. The only possibility to specifically investigate JNK function in AhR signaling would be JNK knockout mice. Nevertheless only single knock-out mice are viable but not the JNK1/2 double knockout mice.

Apart from JNK, NFκB subunit RelA seems to have the opposite effect. RelA inhibition enhances AhR activation. There are no reports in the literature suggesting an AhR agonistic role for the NFκB SN-50 inhibitor. Therefore it is reasonable to hypothesize that RelA plays a regulatory role in the process of AhR activation induced by PGN. However, further

experiments are needed to elucidate the role of signaling cascades in AhR activation. Considering the fact that lots of inhibitors share chemical structures of AhR ligands the method of choice for further investigations on AhR signaling are knock-out mice of the respective signaling cascades.

AhR interplay with the NFκB subunit RelB in $NiSO_4$-induced LC maturation: Infection experiments with the pBMNp100ΔN construct in CD34+ derived HSC achieved good transduction rates. We could reach an infection rate of 80-90%. After 7 days of LC differentiation GFP positive cells made up about 40-60% of the culture. As we did not sort the cells to avoid maturation due to physical handling we have to keep in mind that the results we have obtained come from a mixed cell population of GFP negative and GFP positive cells. However, differentiation does not change to a great extent in the GFP positive cells as we have analyzed with FACS staining. Upon $NiSO_4$ activation GFP positive and GFP negative LCs loose CD1a/Langerin double positive expression due to maturation processes. Although Jörgl et al. showed that constitutive RelB cytoplasmic sequestration induces a significant hyper-maturation of LCs we did not see this pronounced phenotype (Jorgl et al. 2007). Maturation markers of LCs in our experiments tended to be only slightly up-regulated or were left unchanged. However primary data suggest that inactivated cells tend to be slightly pre-activated before the actual $NiSO_4$ stimulation, although further donors are needed to ensure this phenotype. Furthermore we performed immunostaining to investigate AhR and RelB localization. We could show that only strongly transduced LCs with the pBMNp100ΔN construct really show a potent inhibition of nuclear RelB staining, whereas slightly or intermediate positive GFP cells still show nuclear RelB staining although the intensity was reduced in large measures.

One main question was whether AhR still translocates to the nucleus when RelB is held in the cytoplasm. With the help of immunostainings for AhR of transduced LCs we could show that AhR still translocates to the nucleus independently of the cellular localization of RelB. Therefore we can rule out that AhR and RelB form a physically attached heterodimer in the cytoplasm which only then translocates to the nucleus. NFκB signaling works in a manner where subunits form hetero- or homodimers in the cytoplasm which then are transferred to the nucleus (Hayden and Ghosh 2012). It is interesting that the potential AhR/RelB signaling interference does not make use of this cytoplasmic dimer formation mechanism. Furthermore this implies that AhR and RelB are regulated and modulated separately and independently of each other and that their signals are integrated at a later signaling stage.

Another interesting point of our experiment was to investigate whether RelB inhibition has any effect on IL8 cytokine production and secretion because Vogel et al has already reported about a role of AhR/RelB heterodimers in IL8 production in breast cancer cell lines. They showed that the inhibition of either AhR or RelB led to a decrease of IL8 production (Vogel et al. 2011). We observed that RelB inhibition in our system has a very immediate positive effect on IL8 cytokine secretion after 3 hours of $NiSO_4$ activation. At later time points this effect dampens. It would be interesting to investigate even earlier time points of $NiSO_4$ activation to get a better understanding of time kinetic characteristics of this reaction. These results are inconsistent to the findings of Vogel et al because they showed that TCDD activation of cells led to an AhR- and RelB-dependent induction of IL8 which was in turn decreased after AhR or RelB silencing (Vogel et al. 2011).

We further analyzed the il8 mRNA production by quantitative realtime PCR. Primary data support the model of an immediate (3h) positive effect on the IL8 promoter which levels out at later time points. When we integrate the data of this experiment we can speculate that $NiSO_4$ activated AhR translocates to the nucleus and primes the IL-8 promoter which leads to subsequent and immediate transcription of the gene. RelB seems to have a regulatory role in this process because its inhibition leads to a pronounced increase in IL8 production and secretion. Vogel et al introduced a new AhR/NFκB DNA recognition element which is present in the promoter of IL8. In line with this we could not detect any induction of the typical AhR XRE-mediated target Cyp1a1 gene (data not shown). But at the same time AhR/NFκB-target IL8 was strongly induced. Nevertheless we could not observe the strictly AhR and RelB dependent IL8 induction as the cells with inhibited RelB in fact showed an up-regulation of the IL8 production. Therefore we conclude that activated AhR alone is able to induce IL8 production and that nuclear RelB binding to AhR modulates the transcription of IL8 by yet unknown mechanisms.

Further investigation of the IL8 promoter in our $NiSO_4$-activated and p100ΔN infected LC system is needed to elucidate mechanisms taking place at DNA level in the regulation of IL8 production. This hypothesis is quite astonishing because AhR and RelB signaling integration at the level of DNA poses a regulatory and fast-responding platform for fine-tuning of gene expression as transcriptional response to various danger signals. CHIP analysis and mobility shift analysis in combination with time kinetic studies are suitable for this purpose. We suggest that the recruitment of histon modification enzymes such as HCATs happens in an AhR-dependent way and is regulated by the interplay of RelB signaling.

In addition to this it would be interesting to repeat the same experimental set up with an AhR inhibitor to see whether the ablation of IL8 production can be achieved. Investigation of IL8 promoter regulation of AhR or RelB knockout mice bone marrow derived DCs would be another interesting experiment although we have to keep in mind that at this stage of complex transcriptional regulation multiple aspects shape the outcome and cannot easily be applied to other cell types or even other species as demonstrated above with the different results from our lab compared to the findings of Vogel et al in breast cancer cell lines.

In conclusion we found that AhR translocates to the nucleus independently of RelB upon the stimulation of Langerhans cells with the chemical sensitizer $NiSO_4$. Nevertheless the interplay between AhR and RelB is needed on the level of gene expression regulation on DNA to fine-tune the immediate transcriptional response to $NiSO_4$.

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Figure references:

Figure 1	B-cell	http://theboxmove.weebly.com/1/post/2011/02/canaries-and-panthers.html
	T-cell	http://immunotrends.blogspot.co.at/2010/04/t-lymphocyte-family-at-glance.html
	NK-cell	http://www.pathnet.medsch.ucla.edu/educ/lecture/pathrev/casestudy/case9/case9q.htm
	Erythrocyte	http://www.cord.edu/faculty/todt/336/lab/blood/BloodSmear/erythrocytes.htm
	Macrophage	http://www.polconsultant.com/conteduc/immunology/images/6-q358-Macrophage-900.jpg
	Langerhans cell	(Modlin and Bloom 2001)
	Granulocyte	http://www.svt.ac-versailles.fr/IMG/jpg/polynuc1.jpg
	Eosinophil	http://student.nu.ac.th/wuth_web/pic.htm
	Basophil	
	Monocyte	
	Megakaryocyte	
	Dendritic cell	http://www.laskerfoundation.org/awards/2007_b_description.htm
	Plasmacytoid dendritic cell	http://ajcp.ascpjournals.org/content/131/2/174/F1.large.jpg
	Hematopoietic stem cell (HSC)	(Wojakowski et al. 2011)
Figure 2	Bone marrow	http://www.ilo.at/text.php?M_ID=32
	Lymph node	http://o.elobot.de/artikel/aids-im-zusammenhang-lymphombehandlung-pdq
	Lymph system	http://www.gesundheitspraxis-widmer.ch/index.php?option=com_content&view=article&id=88&Itemid=75
	Intestine	http://www.mayoclinic.com/health/medical/IM01780
	liver	http://www.virtual-liver.de/index_de.html
	Lung	http://www.yourlunghealth.org/lung_disease/
	skin	http://barefacedtruth.com/skin-101/
	kidney	http://kandh.org/
Figure 3	LC function	http://pencildown.files.wordpress.com/2012/07/blog-07182012_3.png
	LCs in skin	http://www.dermpath.de/cd1a.jpg
	Skin	http://www.imperial.edu/~thomas.morrell/cha_5_tortora_integument.htm
	Birbeck granule	(Girolomoni et al. 2002)

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Curriculum vitae

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2000 - 2005	Secondary schools with a particular emphasis on music , Feldkirch
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2005 - 2006	Medical university of Vienna, Human Medicine
Since 2006	University of Vienna, Molecular Biology with main topics:
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Lab experience

Cell biology internship	2 months. Lab of Prof. Gerhard Wiche, University of Vienna. Research: cytoskeleton, cytolinkers – plectin. Model: knock-out mice. Technicques: cell culture (neurons, melanocytes, fibroblasts, keratinocytes), immunostainings and WB
Biochemistry internship	2 months. Lab of Prof. Gustav Ammerer, University of Vienna. Research: MAPK osmotic stress signaling pathway. Model: Saccharomyces cerevisiae. Techniquea: WB and cloning

Immunology internship	2 months. Lab of Prof. Daniel Legler, Biotechnology Institute Kreuzlingen, University of Konstanz. Research: PGE_2-mediated migration of DCs. Techniques: primary cell culture and cell lines, migration and protein interaction assays, WB and immunostainings
Immunology diploma thesis	12 months. Lab of Prof. Herbert Strobl, Institute of Immunology, Medical university Vienna. Research: Role of AhR in Langerhans Cell Differentitaion and Maturation. Techniques: primary cell culture and cell lines, immunostainings on cells and skin sections, WB, retroviral infections, qPCR, cloning, FACS

Techniques

Western Blot, FACS, biochemical protein interaction assays, immunfluorescent analysis (cells and tissue) with confocal or normal microscopic analysis and life imaging, real-time PCR, cloning, cell culture of primary cells and cell lines, cell isolations from mouse and human samples, retroviral gene transduction.

Language and computer skills

Computer knowledge	Windows and Mac operating system, Microsoft Office, FlowJo, Endnote, Graphpad Prism4, Adobe Illustrator and Photoshop, ImageJ, Zeiss ZEN
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