The role of the lipid oxidizing enzyme
12/15-lipoxygenase as regulator of
dendritic cell maturation and function

Untersuchung der Rolle der 12/15-Lipoxygenase während des
Reifungsprozesses Dendritischer Zellen und in der Ätiologie
von Autoimmunprozessen

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Abstract

Dendritic cells (DCs) form a heterogeneous population of immune cells that can bridge innate and adaptive immunity by presenting antigens to T cells. They play a key role in the initiation of immune responses and the maintenance of self-tolerance. A critical hallmark of DC biology is their capacity to rapidly change into a maturated state, which subsequently allows them to initiate and orchestrate T cell–driven immune responses. DC maturation must be tightly controlled in order to avoid random T cell activation and development of autoimmunity.


The work presented in this thesis reveal a role of the lipid-oxidizing enzyme 12/15-LO during the regulation of DC maturation and the modulation of the adaptive immune response. The key findings of the work presented here have previously been published in the Journal of Clinical Investigations (Rothe, Gruber et al. 2015).
Zusammenfassung


Die hier vorgestellten Ergebnisse weisen somit auf eine wichtige Rolle der 12/15-LO in der Regulierung des DZ Reifungsprozesses und zusätzlich der Steuerung der adaptiven Immunantwort hin.
1 Introduction

1.1 Dendritic Cells

The human body is permanently challenged by a myriad of potentially pathogenic microorganisms. To maintain health a properly functioning innate and adaptive immune system is vital. In the late 1970s Ralph Steinman and Zanvil Cohen discovered the dendritic cell (DC), a cell that has the unique feature to bridge both these arms of immunity. DCs are specialized sentinel cells distributed throughout the body that recognize and present antigens to T cells, without directly engaging in effector activities such as pathogen killing. Upon exposure to pathogen-associated molecular patterns and endogenous danger signals, DCs undergo a maturation process, which is a prerequisite for initiating specific T-cell responses. DC maturation involves a change in the pattern of secreted cytokines as well as the upregulation of co-stimulatory molecules. Furthermore, DC-derived signals guide the immune response by orchestrating the differentiation of naïve T-cells into specific effector T-cell subsets. Since DCs do not only ingest pathogens, but also acquire self-antigens during the steady state, their maturation has to be tightly controlled to avoid priming of auto-reactive T-cells and autoimmunity.

However, since their discovery by Steinman, knowledge about DC biology has increased substantially in the recent decades, revealing DC as a heterogeneous group of antigen-presenting cells that considerably differ in ontogeny, localization, cytokine pattern and immunological function.

1.1.1 Diversity of dendritic cells

Probably due to the existence of a variety of pathogens, routes of infection and immune escape mechanisms, multiple subsets of DCs have evolved. Besides the fact that they all arise from proliferating progenitors in the bone marrow, DCs from all subsets, however, do share some common features, e.g., they all constitutively express the integrin CD11c and efficiently ingest and process antigens for
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presentation to naïve T cells. Additionally, upon maturation, they all upregulate major histocompatibility complex II molecules (MHCII) on their surfaces and most of them exhibit a characteristic dendritic morphology.

The complexity of the DC family was accompanied with numerous classification systems distinguishing between, immature and mature DCs, resident and migratory DCs, or lymphoid tissue and non-lymphoid tissue DCs. Recently, Guilliams and colleagues have proposed a simplified and unifying model of human and mouse DC subsets, based on functional similarities of the DC subsets found in both lymphoid and non-lymphoid tissues (Figure 1). According to this model, there are at least five types of DCs: the classical (also known as conventional) DCs (cDCs), a category which itself comprises many subsets, and the nonclassical DCs, comprising the plasmacytoid DCs (pDCs), monocyte-derived or inflammatory DCs (moDCs), and the Langerhans Cells (LCs). For reason of clarity, this work will describe only mouse but not human DC subsets in the following sections.

![Figure 1: Unifying model of mouse DC subsets.](image)

Mouse DC subsets can be subdivided into five broad groups irrespective of their primary anatomical location. These five subsets correspond to: (i) Langerhans Cells (LC) (green); (ii) CD11b+ cDCs (blue); (iii) CD8+ and CD103+ cDCs (violet); (iv) plasmacytoid DCs (pDCs) (brown) and monocyte-derived inf-DCs (infDCs) (orange). The phenotype used to identify these subsets is specified for each group and their proposed functional specialization is indicated above. ROI, radical oxygen intermediates; NOI, nitric oxygen intermediates. Modified from Guilliams et al., 2010.
1.1.2 Classical DCs

Classical DCs can be found in lymphoid tissue, including spleen, lymph nodes (LN), and bone marrow (BM), as well as most nonlymphoid tissue. They can be further divided into at least two main subsets characterized by either CD8α and CD103 or CD11b expression.

1.1.2.1 CD8α+ and CD103+ cDCs

CD8α+ DCs represent 20-40% of spleen and LN cDCs. An equivalent population also exists in nonlymphoid tissues, whereas these cells are identified by the expression of the integrin CD103 but do not express CD8α (Bursch, Wang et al. 2007; del Rio, Rodriguez-Barbosa et al. 2007). The relationship between CD8α+ and CD103+ cDCs was revealed by comparing the corresponding transcriptional profiles. These transcriptome profiling showed that, regardless of the tissue environment in which they reside, CD8α+ and CD103+ cDCs are distinct from the rest of the DCs, including pDCs, and CD11b+ cDCs in the same tissue (Miller, Brown et al. 2012). For instance, these studies have found that CD8α+ and CD103+ cDCs express a similar Toll-like receptors (TLRs), C-type lectin receptor, and chemokine receptor profile. In particular, both are the only cDCs that express the Toll-like receptors 3 and 11, and the scavenger receptor CD36.

CD103+ cDCs are primarily distributed to connective tissues in close contact with the environment and efficiently migrate charged with tissue antigens to the T cell zone of the draining LN (Helft, Ginhoux et al. 2010). CD8α+ cDCs are localized at strategic sites within the spleen and the LN, where they filter blood or afferent lymphatics, respectively. From these areas, CD8α+ cDCs migrate to the T cell zone to present antigens from tissue or the circulation to T lymphocytes. Splenic CD8α+ cDCs are functionally specialized in directly presenting endogenous self or viral antigens on MHC I molecules to CD8+ T cells. Moreover, CD8α+ cDCs have the superior ability to present exogenous antigens via MHC I, a process named cross-presentation (den Haan, Lehar et al. 2000; Belz, Shortman et al. 2005). Cross-presentation activity has also been shown for skin-derived CD103+ cDCs (Bedoui, Whitney et al. 2009).

Notably, CD8α+ and CD103+ cDCs are the main source of IL-12 and IL-15, two
cytokines involved in the differentiation of cytotoxic CD8+ T cells (Mattei, Schiavoni et al. 2001; Mashayekhi, Sandau et al. 2011).

1.1.2.2 CD11b+ cDCs

In contrast to CD8α+ and CD103+ DCs, the population currently defined as CD11b+ cDCs is heterogeneous and remains less well characterized, so do some CD11b+ cDCs, for instance, additionally express the surface marker CD4. CD11b+ cDCs are most abundant cDCs in lymphoid organs except for the thymus and can also be found in nonlymphoid tissues. Like all cDCs, CD11b+ cDCs can sense pathogens and migrate from nonlymphoid tissues to regional LNs bearing self and foreign antigens, whereas in the spleen they are preferentially located in the red pulp and the marginal zone. Compared with CD8α+ and CD103+ cDCs, CD11b+ cDCs are inefficient in cross-presenting antigens to CD8+ T cells but superior in the induction of CD4+ T cell immunity, due to their prominent expression of MHC II molecules. CD11b+ cDCs express distinct pattern recognition receptors PRRs compared to CD8α+ and CD103+ cDC and can further be characterized by their production of cytokines, such as IL-6 and IL-23 (Persson, Uronen-Hansson et al. 2013; Schlitzer, McGovern et al. 2013). Furthermore, splenic CD11b+ cDCs were shown to be potent producers of proinflammatory chemokines after TLR ligand exposure, such as chemokine (C-C motif) ligand 3 (CCL3), CCL4, and CCL5 (Proietto, O'Keeffe et al. 2004).
1.1.3 Nonclassical DCs

1.1.3.1 Monocyte-Derived DCs

Upon inflammation or infection, monocytes can infiltrate lymphoid and nonlymphoid organs that give rise to DCs called “monocyte-derived DCs” (moDCs) or “inflammatory DCs” (iDCs). These are transiently formed DCs that disappear once the inflammation resolves, performing a crucial role in T cell activation. Most inflammatory DCs are characterized by the expression of CD11b, MHC II, Ly6C, and intermediate CD11c (Dominguez and Ardavin 2010). However, since Ly6C is downregulated upon tissue entry, it is difficult to distinguish moDCs from tissue-resident CD11b+ cDCs. Recent studies have shown, that in response to lipopolysaccharide (LPS) injection moDC accumulate in the LNs which are positive for the lectin DC-SIGN (CD209a) and the mannose receptor CD206 (Cheong, Matos et al. 2010). Furthermore moDCs express CD64 (also termed FcγRI), indicating their monocytic origin (Tamoutounour, Henri et al. 2012).

In addition another subset of monocyte-derived inflammatory DCs was identified upon infection of animals with Listeria monocytogenes. Due to their capacity to produce high amounts of TNFα and iNOS (inducible nitric oxide synthase) they were named TNFα/iNOS-producing DCs (TipDCs), playing a role in immune defense against bacterial infection (Serbina, Salazar-Mather et al. 2003).

1.1.3.2 Plasmacytoid DCs

Plasmacytoid dendritic cells (pDCs) are best characterized by their unique functional property to rapidly secrete massive amounts of type I interferon (IFNα/β) in response to foreign nucleic acids (Siegal, Kadowaki et al. 1999; Nakano, Yanagita et al. 2001). However, they possess overlapping characteristics with both lymphocytes and cCDs, creating confusion over their exact ontogeny. Although they exhibit an exclusive cell morphology within the DC family, pDCs and cDCs are closely related (Soumelis and Liu 2006; Bar-On, Birnberg et al. 2010). This way they derive from a common DC progenitor and share key transcription factors, such as interferon regulatory factor 8 (IRF8). pDCs develop in the bone marrow and reside
primarily in the lymphoid organs in the steady state, entering the lymph nodes from
the blood. Pathogen detection is mediated by pDC expression of endosomal nucleic
acid-sensing TLR7 and TLR9, which bind to single-stranded RNA or unmethylated
CpG-containing DNA (CpG), rendering them good responders to viral antigens. TLR-
induced pDC activation includes also secretion of IL-12 and the upregulation of
MHCII and costimulatory molecules on the surface of the pDCs, allowing them to
efficiently present and cross-present antigens (Reizis, Bunin et al. 2011). As a
consequence of all that, pDCs contribute to the recruitment and activation of several
immune cell types, e.g. natural killer (NK) cells to destroy virus-infected cells.

Development and homeostasis of pDCs are regulated by the helix-loop-helix
transcription factor E2-2 that provides pDC development by directly suppressing
expression Id2, a critical transcription factor for cDC development (Ghosh, Cisse et
al. 2010). Deletion of E2-2 in mature pDCs causes spontaneous differentiation into
cDC-like cells, suggesting that pDCs have a close relationship to cDCs (Cisse, Caton et
al. 2008).

1.1.3.3 Langerhans Cells

Long before Steinman and Cohen defined the classical DC, it was Paul Langerhans
who originally discovered the first DCs in 1868, a cell restricted to the epidermal
layer of the human skin. Subsequently these cells were named after him; however,
he assumed that they were nerve cells. Later it was recognized that Langerhans cells
(LCs) represent the DC population of the epidermis, representing the first
immunological barrier against pathogens that breach body surfaces. Upon
activation, LC increase their expression of MHCII and costimulatory molecules and
migrate to the T cell areas of regional lymph nodes where they initiate a systemic
immune response by presenting antigens to T cells, a process often called
Langerhans paradigm. In fact, the study of LCs led to the realization of distinct
“immature” and “mature” DC stages, and their functional consequences in
stimulating T cell proliferation (Schuler and Steinman, 1985). One characteristic
feature of LCs is their Birbeck granules (BG), which can be found via electron
micrography. The function of BG is still not completely understood, but they seem to
be implicated in receptor-mediated endocytosis, since they are induced by langerin,
a C-type lectin receptor that is expressed at high levels in LCs (Kissenpfennig, Ait-
Yahia et al. 2005). Moreover, LCs differ from cDCs in many aspects. The LC compartment is established before birth from fetal liver-derived monocytes (Hoeffel, Wang et al. 2012), and is maintained by self-renewal or by local precursors in the absence of inflammation (Merad, Manz et al. 2002). Under inflammatory conditions LCs are replaced by circulating monocytes (Ginhoux, Tacke et al. 2006). In addition and in contrast to cDCs, LC development is independent of FMS-like tyrosine kinase 3 ligand (Flt3L), but requires Csf1r receptor engagement and its alternative ligand IL-34 rather than Csf1 (Wang, Szretter et al. 2012). Moreover, gene expression alignments suggest that LCs are closer related to tissue-resident macrophages than to cDCs (Miller, Brown et al. 2012).

1.1.4 Dendritic cell ontogeny

Except for Langerhans cells, cDCs are generally short-lived hematopoietic cells constantly replenished from bone marrow precursors in a Flt3L-dependent manner. While plasmacytoid DCs complete development in the bone marrow, most DCs complete development in lymphoid and peripheral tissue (Liu and Nussenzweig 2010). The differentiation and expansion of the specific lineages are mainly controlled by the interplay of specific cytokines and transcription factors (Satpathy, Murphy et al. 2011; Belz and Nutt 2012). The classical model of DC development starts from bone marrow-derived hematopoietic stem cells. Lymphoid and myeloid lineages diverge early in hematopoiesis into common myeloid precursors (CMPs and CLPs), both with the potential to give rise to cDCs (Manz, Traver et al. 2001). CMPs differentiate into granulocytes/macrophage progenitors (GMP), which give rise to the bipotent macrophage and DC precursor (MDP). MDPs have the capacity to differentiate into common DC precursors (CDP) or monocytes. These CDPs are restricted to generate cDCs and pDCs. While pDCs leave the bone marrow terminally differentiated, so called pre-cDCs exit the bone marrow and migrate through the blood to lymphoid and non-lymphoid organs, where they terminally differentiate into cDCs, including the CD8+ /CD103+ and CD11b+ subsets (Naik, Metcalf et al. 2006; Liu, Victora et al. 2009). Analogous to the CDP, a common monocyte progenitor (cMoP) has recently been identified that is downstream of MDPs and give rise to monocytes but not DCs (Hettinger, Richards et al. 2013). A small proportion
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of DCs may also be derived from CLPs in the bone marrow and from early T cell progenitors in the thymus (summarized in Figure 2).

![Diagram of DC origin and differentiation](image)

Figure 2: DC origin and differentiation.

The developmental pathways from myeloid and lymphoid progenitors to precursor dendritic cells (pre-DCs) in the bone marrow and the peripheral diversification of DC subsets are shown. In addition, the key transcription factor as well as the key growth factors required for the development of the specific DC subsets are indicated. HSC, hematopoietic stem cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; GMP, granulocyte-macrophage progenitor; MDP, macrophage and DC progenitor; CDP, common DC progenitor. Modified from Belz and Nutt, 2012.

Several cytokines and transcription factors are critical for DC development. Besides Flt3L, some of the major cytokines involved in DC generation are macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Flt3 is expressed very early in hematopoiesis and maintained on DC precursors, whereas expression is lost as progenitors become committed to non-DC lineages. M-CSF signaling, in turn, favors the differentiation of MDP towards monocytes instead of DC direction, as M-CSF receptor expression is reduced in pre-cDCs and lost on CD8⁺ and CD103⁺ cDCs. GM-CSF itself is a hematopoietic growth
factor, and its receptor (GM-CSFR) is expressed on several precursors during DC development, although mice lacking GM-CSF or its receptor display only minor impairment in the development of spleen and LN cDCs (Vremec, Lieschke et al. 1997). The different effects of various cytokines have also been used in vitro to generate several kinds of DCs in large numbers out of bone marrow cultures. Flt3 drives the differentiation of bone marrow progenitors into pDCs and cDCs, while the use of GM-CSF produces DCs resembling moDCs (Xu, Zhan et al. 2007).

In addition to growth factor, the expression of distinct transcription factors controls the development of the DC lineages. For instance, the differentiation of both DCs and monocytes depends on high concentrations of PU.1, which regulates the expression of the cytokine receptors Flt3R, M-CSFR, and GM-CSFR. The development of CD8α+ and CD103+ cDCs relies on the stepwise activity of interferon-regulatory factor 8 (IRF8), inhibitor of DNA binding 2 (ID2), E4 promoter-binding protein 4 (E4BP4) and basic leucine zipper transcription factor, ATF-like 3 (BATF3), as well as on Flt3 signaling. CD11b+ cDCs depend on a unique set of transcription factors, including RELB, IRF2, IRF4 and Ikaros, while pDC lineage requires IRF8, a low level of PU.1 and the absence of ID2.
1.1.5 Maturation of dendritic cell and T-cell activation

One of the key features of DC biology is their ability to undergo the process of maturation, which enables them to initiate T-cell-driven immune responses. DCs are constantly sampling their environment via phagocytosis, micropinocytosis and receptor-mediated endocytosis in an immature state. Therefore they are equipped with a variety of sensors like pattern-recognition receptors (PRRs), such as langerin and DC-SIGN, and TLRs. TLRs are a major group of receptors recognizing pathogen-associated molecular patterns (PAMPs), which comprise lipids, lipoproteins, proteins and nucleic acids, derived from various pathogens including bacteria, viruses, parasites and fungi (Kawai and Akira 2010). Upon receptor binding, DCs start to migrate to the draining lymph nodes in a CCR7-dependent manner to get in contact to T-cells, although DC maturation need not be coupled to migration. During their migration DC undergo multiple phenotypical as well as functional changes. This maturation involves the upregulating of several surface markers, most importantly MHC II and the co-stimulatory molecules and activation markers CD40, CD25, CD86, CD83 and CD80 (Reis e Sousa 2006). While activated DCs stabilize peptide-loaded MHC II molecules to enhance their antigen presentation, they lose their capacity to phagocytose antigens (van Niel, Wubbolts et al. 2008). In addition, DC activation also leads to increased survival and thus to prolonged antigen presentation in the spleen or LNs, mediated by differential expression of apoptosis signaling molecules (Chen, Huang et al. 2007). Moreover, during their maturation process, DCs start to express a specific cytokine profile, which in turn polarizes naïve T-cells towards T helper (Th) 1, Th2, Th17 or regulatory T-cell (Treg) differentiation (Figure 3) (O'Shea and Paul 2010).
In fact DCs deliver 3 signals to determine the fate of naïve T-cells. Mature DCs deliver the first signal through the T-cell receptor (TCR) via engagement with an appropriate peptide-MHC complex and the second signal through CD28 when binding to CD80 and/or CD86, referred to as “co-stimulation”. These two signals in combination were thought to induce “immunity”, while “Signal 1” alone leads to peripheral tolerance in the steady state or effector immunity in the injured state.

Figure 3: DC controllers of adaptive immunity.

This illustration summarizes key DC functions, highlighting their importance as regulators of adaptive immune functions in lymphoid and nonlymphoid tissues. DC subsets that populate peripheral tissues capture commensals, food antigens, or exogenous antigens and migrate in a CCR7-dependent manner to the draining lymph node, where they present tissue-derived antigens to CD8+ T cells (cross-presentation) and CD4+ T cells (direct presentation), thereby inducing peripheral tolerance in the steady state or effector immunity in the injured state. The concerted interplay of co-stimulatory molecules and secreted cytokines mediates the polarization into the different T-cell subsets, either inducing effector immunity or tolerance. From Merad et al., 2013.
“tolerance”. However, it was shown that tolerogenic DCs can also be phenotypically mature (high levels of MHCII and costimulatory molecules). Therefore it requires a third signal to determine differentiation of T-cells into effector cells. This is mediated through cytokines produced by DCs eventually polarizing T-cells into their different subsets (reviewed in Reis e Sousa, 2006).

For example, IL-12, IL-18 and IFNα promote TH1 differentiation, cells that mainly secrete the pro-inflammatory cytokine IFNγ and are responsible for killing intracellular pathogens and for perpetuating autoimmune responses. TH2 differentiation is driven by IL-4, resulting in a T-cell subset producing IL-4, IL-5 and IL-13, with an important role in coordinating the immune response to extracellular pathogens and allergies. The concurrent presence of IL-6, IL-23 and TGF-β1 leads to the differentiation of TH17 cells, a third group of T cells that are potent inflammatory mediators and contribute to several autoimmune disorders, such as rheumatoid arthritis, multiple sclerosis and asthma. They are also important for immune responses against fungi and extracellular bacteria. TH17 cells are characterized by the transcription factor RORγ and the secretion of the cytokines IL-17A and IL-17F, which activate and recruit neutrophils, IL-21, which activates B cells and TH17 cells in an autologous manner, and IL-22, which has pro-inflammatory and tissue-protective functions (Hemdan, Birkenmeier et al. 2010). However, TGF-β1 alone induces Treg differentiation, a subset that can suppress the development and progression of immune reactions. They also play an important role in the balance between tolerance and autoimmunity.

1.1.6 Dendritic cells and immune tolerance

Besides the role of DCs in activating T-cells to resist foreign antigens, they can also make the immune system tolerate harmless self-antigens, from tissue, cells and protein. This process has to be fine balanced since DC that fail to maintain tolerance lead to autoimmune diseases. On the other hand, if DCs are too tolerant, this can create a permissive environment for chronic infections or tumors. There are two kinds of immune tolerance the DC system appears to play a pivotal role in: central and peripheral tolerance.
1.1.6.1 Central tolerance

The induction of central tolerance takes place in the primary lymphoid organs. B cells are selected in the bone marrow and T cells in the thymus. In the thymus, endogenous CD8+ cDCs and blood-derived CD11b+ cDCs contribute to central tolerance through induction of T\textsubscript{reg} cells or the negative selection of developing thymocytes that bear “self-reactive antigens”.

1.1.6.2 Peripheral tolerance

Although negative selection in the thymus is a highly effective mechanism, some autoreactive T cells still escape to the periphery. It is therefore important that peripheral tolerance mechanisms exist that can inactivate these T cells. DCs play an important role in maintaining peripheral tolerance, as they are capable of inducing deletion, anergy and the induction of Treg cells. In the steady state, DC pick up dead cells, process them and present them in the context of MHC to T cells, which are thereby inactivated (Steinman, Turley et al. 2000). It has been shown that a DC-specific deficiency in uptake of apoptotic material inhibits cross-presentation \textit{in vivo} leading to an accumulation of self-reactive T cells in the periphery (Luckashenak, Schroeder et al. 2008).
1.2 Lipoxygenases as lipid oxidizing enzymes

During inflammatory processes several bioactive lipid mediators act as key regulators, orchestrating the course of the immune response from the onset of the inflammation to its resolution. The lipoxygenase family is one of the members of enzymes that are responsible for metabolizing these lipid mediators.

Lipoxygenases form a family of non-heme iron containing enzymes, which dioxygenate polyunsaturated fatty acids (PUFA) at specific positions to produce a series of hydroperoxy fatty acids. A variety of free fatty acids, membrane phospholipids and lipoproteins can be used as substrates for peroxidation by lipoxygenases. This family of enzymes is widely distributed in the animal and plant kingdom (Mack, Peterman et al. 1987; Grechkin 1998; Brash 1999; Kuhn and Thiele 1999). Lipoxygenases have also been discovered in lower organisms, such as corals (Brash, Boeglin et al. 1996), fungi (Bisakowski, Perraud et al. 1997; Su and Oliw 1998) and even bacteria (Porta and Rocha-Sosa 2001). The nomenclature of the enzyme is based upon the number of the carbon atom which is preferentially oxygenated in arachidonic acid (AA). The positional nomenclature is further specified by mentioning the tissue source of the lipoxygenase e.g. leukocyte or platelet, and the stereospecificity by adding S and R to the name.

1.2.1 12/15-Lipoxygenase

Among mammalian enzymes with lipoxygenase activity a broad diversity within the family exists, involving difficulties to classify them properly. In general the mammalian lipoxygenases can be subdivided into 4 phylogenetically related subtypes, sharing similarities in their structures, sequences and activities: 12/15-lipoxygenases (12/15-LO), 5-lipoxygenases (5-LO), platelet-type 12-lipoxygenases and the epidermis-type lipoxygenases (Kuhn and Thiele 1999). The 12/15-LO family consists of murine 12/15-LO, which is often referred to as “leukocyte-type” 12-lipoxygenase and its orthologues in other species such as human 15-lipoxygenase (15-LO-1) and rabbit 15-lipoxygenase (Noguchi, Yamashita et al. 2002; Kuhn and O’Donnell 2006). These phylogenetic closely related enzymes are highly, but not
exclusively, expressed in distinct myeloid cells such as monocytes, macrophages, eosinophils or dendritic cells (MacMillan, Hill et al. 1994; Huo, Zhao et al. 2004; Rothe, Gruber et al. 2015). Although immature red blood cells, polymorphonuclear leukocytes or vascular cell display detectable mRNA and protein levels of 12/15-LO, there is barely evidence for a significant enzymatic activity. In contrast, certain cells of the myeloid lineage such as resident murine peritoneal macrophages constitutively express high levels of the enzyme and were shown to exhibit strong 12/15-LO activity. Despite inflammatory Ly6chs monocytes, newly recruited inflammatory macrophages, and in vitro-generated bone marrow-derived macrophages are reported to express only minor levels of this enzyme, expression of 12/15-LO in these cells can be easily induced by exposure to the Th2 cytokines IL-4 and IL-13 (Sun and Funk 1996; Kuhn and O’Donnell 2006; Uderhardt, Herrmann et al. 2012).

The oxygenation of PUFAs like AA by 12/15-LOs naturally results in the formation of products such as 12- and 15-hydroxyeicosatetraenoic acid (12-HETE and 15-HETE) as well as 13-hydroxyoctadecadieionic acid (13-HODE). As this enzyme exhibit dual positional specificity with AA, the ratio of 12-HETE to 15-HETE products differs between the lipoxygenases from different species. Thus murine 12/15-LO predominantly generates 12-HETE while the human orthologous 15-LO-1 mainly produces 15-HETE. These HETEs in turn serve as precursors for families of more complex molecules. For instance, the coaction of 12/15-LO and 5-LO leads to the generation of lipid metabolites, including lipoxins, which exert various anti-inflammatory effect and thereby contributing to the resolution of inflammation (Yamamoto, Ueda et al. 1988; Levy, Clish et al. 2001; Lawrence, Willoughby et al. 2002; Serhan, Chiang et al. 2008).

In addition, 12/15-LO-derived 15-HETE and 13-HODE are implicated in modulating the activity of the anti-inflammatory peroxisomal proliferator-activating receptor-γ (PPARγ). PPARs, in turn, are a family of ligand-dependent nuclear receptors that have been implicated in regulation of fat and glucose metabolism as well as macrophage function. Depending on the concentration and on the positional isomeric form of the 12/15-LO products, the modulating effects of PPARs, however, can be both activating and inhibiting. For instance, 12/15-LO activity was shown to

Furthermore, 12/15-LO was shown to play an important role during the clearance of necrotic and apoptotic cells (ACs). In the mouse model of peritonitis ACs were exclusively taken up by 12/15-LO-expressing resident macrophages rather than by non-resident 12/15-LO-negative, inflammatory monocytes. Since ACs are a major sources of autoantigens this non-immunogenic disposal by resident macrophages maintains self-tolerance and prevents development of autoimmune disease (Munoz, Janko et al. 2010; Uderhardt, Herrmann et al. 2012).

Nevertheless, 12/15-LO activity has been also implicated in the pathogenesis of various diseases, such as atherosclerosis, diabetes, bronchial diseases, carcinogenesis, allergy, hypertension, and myeloproliferative disorders (Kuhn and Thiele 1999; Kuhn and O'Donnell 2006; McDuffie, Maybee et al. 2008; Zarbock, Distasi et al. 2009; Dobrian, Lieb et al. 2011). Especially the contribution of 12/15-LO in the pathogenesis of atherosclerosis has been addressed intensively. In this disease oxidation of low-density lipoproteins (LDL) particles occurs within the arterial walls, leading to foam-cell formation and vascular inflammation. In this process oxidized LDL (oxLDL) acts as chemoattractant for monocytes, activates endothelial cells, macrophages and smooth muscle cells further promoting infiltration of pro-inflammatory cells and thereby formation of atherosclerotic plaques (Lusis 2000). In addition, macrophages within these plaques are capable of both initiate the oxidation of LDL and take up oxLDL in an unregulated manner, leading to foam-cell formation (Cushing, Berliner et al. 1990). This oxidation of LDL seems to be mediated by 12/15-LO, as mice deficient for this enzyme display decreased lipid peroxidation, which was paralleled by a reduction in the size of atherosclerotic lesions (Cyrus, Pratico et al. 2001; Funk and Cyrus 2001; Huo, Zhao et al. 2004). In addition, 15-LO has been shown to oxidize LDL in vitro (Kuhn, Belkner et al. 1994b). However, several recent reports also provide evidence for an anti-atherogenic role of 12/15-LO. Thus, a study in transgenic rabbits expressing human 15-LO revealed a significant reduction of atherosclerotic lesions when fed western type diet (Shen, Herderick et al. 1996). These results, however, maybe
Introduction

linked to differences in the preferential oxidation product of the murine and human lipoxygenases.

These findings demonstrate that the role of 12/15-LO during inflammation is critically dependent on the availability of specific substrates, and expression has to be tightly controlled under physiological conditions.

1.2.2 Oxidized phospholipids

While a major substrate of LOs are unesterified PUFAs, 12/15-LO is also capable of oxidizing esterified fatty acids in the form of phospholipids or cholesterol esters. Phospholipids (PLs) are the major components of eukaryotic cell membranes and play an important role in the biochemistry of all cells. They provide a permeable barrier as well as serving as substrates for the generation of essential signaling mediator such as eicosanoids. PLs comprise a glycerol backbone connected to two nonpolar fatty acids at sn1 (typically saturated) and sn2 (typically unsaturated) the major part where oxidation will occur. The sn3 position generally contains a phosphate-containing polar head-group. Among the different PL species, such as phosphatidylethanolamine (PE) or -serine (PS), phosphatidylcholine (PC) is the most abundant PL in eukaryotic cell membranes (40-50%). Oxidation of PLs can form hundreds of diverse molecules, with a huge range of biological activities (Spickett and Dever 2005; Bochkov, Oskolkova et al. 2010; Salomon 2012; Aldrovandi and O’Donnell 2013). They can mediate both pro-inflammatory and anti-inflammatory actions, including activation of platelet aggregation and monocyte adhesion, and suppression of cytokine generation and neutrophil superoxide release. Most reports suggest strong pro-inflammatory effects of OxPLs, due to their ability to interact with PRR of the innate immune system. Nevertheless, different molecular species of OxPLs are able to inhibit effects of LPS in vitro and in vivo. Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (OxPAPC) and other classes of oxidized PLs (oxPLs) were shown to bind to lipopolysaccharide binding protein (LBP), soluble and membrane-bound CD14, and MD-2, and thereby inhibits interactions of these proteins with LPS, which are critically important for activation of TLR4 (Bochkov, Kadl et al. 2002).
1.2.3 Oxidative stress response

Oxidative stress plays an important role in immune regulation and fundamentally affects the pattern of immune responses (Nathan and Cunningham-Bussel 2013). Oxidative stress occurs when oxidants overwhelm antioxidant defenses. More specifically, to compete against microorganisms cell start to produce reactive oxygen species (ROS), including superoxide, hydrogen peroxide, singlet oxygen and organic peroxides (Nathan and Ding, 2010). Excessive intracellular production of ROS, however, can lead to oxidative damage within the cells. To counteract this oxidative stress, cells have developed an elaborate defense mechanism to maintain redox homeostasis, by inducing an antioxidative response program (Nguyen, Sherratt et al. 2003). Thereby oxidative and electrophilic stresses lead to enhanced expression of a multitude of antioxidant and phase II enzymes that restore redox homeostasis (Kobayashi, Li et al. 2009). The nuclear factor erythroid 2-related factor (NRF2) is a transcription factor that functions as the key controller of this redox homeostatic gene regulatory network. Under basal conditions, NRF2-dependent transcription is repressed by its negative regulator Keap1, which functions as an adapter for CUL3-based E3 ligase leading to proteasomal degradation of NRF2 (Nguyen, Sherratt et al. 2003; Kensler, Wakabayashi et al. 2007). During oxidative stress, Nrf2 is activated and detaches from its cytosolic inhibitor to translocate to the nucleus where it binds to antioxidant responsive elements (AREs) in the promoter region of target genes, leading to their transcriptional induction (Kensler, Wakabayashi et al. 2007). NRF2 targets comprise genes such as hemeoxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase-1 (NQO1), and both modifier and catalytic subunits of glutamate-cysteine ligase (GCLM and GCLC, respectively) (Ishikawa, Navab et al. 1997; Gargalovic, Imura et al. 2006).

Notably, oxidized phospholipids seem to represent a major source of intra- and extracellular oxidative stress (Kansanen, Jyrkkänen et al. 2012). This holds true both for phospholipid oxidation products that derive from ROS-dependent oxidation (formed e.g. during bacterial inflammation) and for enzymatically-generated (e.g. 12-15-LO-derived) oxidized phospholipids. Several antioxidant genes were identified to be regulated by OxPLs. It was recently shown that the oxidation of PAPC by UVA-1 induces an antioxidant stress response in skin cells (Gruber, Mayer
et al. 2010). Furthermore, oxidized phospholipid-treated murine macrophages were reported to induce an antioxidative response program via NRF2 (Kadl, Meher et al. 2010). The same induction of NRF2 could be demonstrated in dendritic cells by the prooxidant activities of ragweed extract (Rangasamy, Williams et al. 2010). Moreover, it was shown that this NRF2-mediated oxidative stress response significantly effects dendritic cell maturation and function, as deficiency in NRF2 leads to the dysregulation of pathways that control expression of co-stimulatory receptors in dendritic cells (Aw Yeang, Hamdam et al. 2012; Al-Huseini, Aw Yeang et al. 2013).
2 Material and Methods

2.1 Material

2.1.1 Media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMDC medium (R10)</td>
<td>Roswell Park Memorial Institute medium (RPMI 1640, Invitrogen) was supplemented with 10 % heat-inactivated fetal calf serum (FCS, PAA Laboratories, Pasching, Austria), 1%L-Glutamine and 1 % penicillin-streptomycin (10 000 U/ml, Gibco).</td>
</tr>
<tr>
<td>IMDM medium (T cell skewing)</td>
<td>Iscove’s Modified Dulbecco’s Medium; 10% FCS (heat-inactivated); 2mM L-Glutamine; 1% Penicillin/Streptomycin (10 000 U/ml); 50 μm β-mercaptoethanol.</td>
</tr>
<tr>
<td>Human moDCs</td>
<td>RPMI 1640 (Lonza) supplemented with 1% (vol/vol) of each of the following: heat-inactivated human serum type AB (Lonza), Penicillin/Streptomycin/L-Glutamine (PAA Laboratories), and 10 mM HEPES (Lonza) as well as 800 IU/ml (day 0) or 400 IU/ml (day 3) recombinant human GM-CSF and 250 IU/ml (day 0 and 3) recombinant IL-4 (both Cell Genix)</td>
</tr>
<tr>
<td>Ag8653 myeloma cells medium</td>
<td>Roswell Park Memorial Institute 1640 Medium; 10% FCS (heat-inactivated), 2mM L-Glutamine, 1% Penicillin/Streptomycin and 50 μM b-mercaptoethanol.</td>
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</tbody>
</table>
2.1.2 Cell culture

<table>
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<th>Supplier</th>
</tr>
</thead>
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<td>β-mercaptoethanol</td>
<td>GIBCO® by life technologies (#31350010)</td>
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<tr>
<td>FCS</td>
<td>PAA Laboratories (#A15-043)</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>GIBCO® by life technologies (#15140-122)</td>
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<tr>
<td>RPMI 1640</td>
<td>GIBCO® by life technologies (#11875-093)</td>
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<tr>
<td>L-Glutamine</td>
<td>GIBCO® by life technologies (#25030-081)</td>
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<tr>
<td>PBS</td>
<td>GIBCO® by life technologies (#14200-067)</td>
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<tr>
<td>IMDM with HEPES</td>
<td>Lonza (#12-726F)</td>
</tr>
<tr>
<td>Lymphoprep™</td>
<td>Axis- Shield PoC AS (#1114544)</td>
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<tr>
<td>Human Serum, AB</td>
<td>Lonza (# 14-490E)</td>
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<tr>
<td>HEPES</td>
<td>Gibco (#15630080)</td>
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2.1.3 Buffers

<table>
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<tr>
<th>Buffer</th>
<th>Composition</th>
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</thead>
<tbody>
<tr>
<td>FACS buffer</td>
<td>4% FCS in PBS</td>
</tr>
<tr>
<td>ELISA wash buffer</td>
<td>0,05% Tween 20 in PBS</td>
</tr>
</tbody>
</table>
| Tris-buffered saline (TBS)     | 50mM Tris  
150 mM NaCl;  
pH 7.6                                                                 |
| RBC lysis buffer               | 4g NH4Cl  
2,35g HEPES  
50µl 1M EDTA (pH 7,8)  
add 500ml;  
the pH was adjusted to 8,0 by adding ammonium |
Material and Methods

### 2.1.4 Enzymes and proteins

<table>
<thead>
<tr>
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<th>Supplier</th>
</tr>
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<tbody>
<tr>
<td>DNAse I</td>
<td>Thermo Scientific Fermentas (#EN0521)</td>
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<td>DNAse I</td>
<td>Sigma-Aldrich (#DN25)</td>
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<tr>
<td>Proteinase K</td>
<td>AppliChem (#A3830)</td>
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<td>Collagenase IV</td>
<td>Sigma-Aldrich (#C5138)</td>
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<tr>
<td>BSA</td>
<td>Sigma-Aldrich (#A7030)</td>
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<tr>
<td>Pertussis Toxin, Islet-Activating Protein</td>
<td>List/Quadratech Diagnostics (#180)</td>
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<tr>
<td>MOG peptide 35–55</td>
<td>(Charité Berlin)</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis (H37Ra)</td>
<td>Difco/BD PharMingen (#231141)</td>
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### 2.1.5 Chemicals and reagents

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<td>2-Propanol</td>
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<td>Agarose low EEO</td>
<td>AppliChem (#A2114)</td>
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<td>Ammonium chloride</td>
<td>Sigma-Aldrich (#254134)</td>
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<tr>
<td>Ammonium persulfate (APS)</td>
<td>Sigma-Aldrich (#A3678)</td>
</tr>
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<td>Material/Reagent</td>
<td>Supplier/Part Number</td>
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<td>-----------------------------------</td>
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<td>Brefeldin A</td>
<td>Biolegend (#420901)</td>
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<tr>
<td>DMSO</td>
<td>AppliChem (#A3608,001)</td>
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<tr>
<td>ECL Western Blotting Substrate</td>
<td>Thermo Scientific (#32106)</td>
</tr>
<tr>
<td>ELISA stop solution</td>
<td>eBioscience (#BMS409)</td>
</tr>
<tr>
<td>ELISA substrate I and II</td>
<td>eBioscience (#BMS402/3)</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid (EDTA)</td>
<td>Sigma-Aldrich (#E9884)</td>
</tr>
<tr>
<td>Freund’s Adjuvant, Incomplete</td>
<td>Sigma-Aldrich (#F5506)</td>
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<tr>
<td>GenLadder 100bp + 1,5kp</td>
<td>Genaxxon</td>
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<tr>
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<td>Merck (#1.04169.1000)</td>
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<tr>
<td>Indomethacin</td>
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<tr>
<td>Ionomycin calcium salt</td>
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<td>Laemmli Biorad</td>
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<td>LPS from E. coli O111:B4</td>
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<tr>
<td>Midori Green Advance DNA Stain</td>
<td>NIPPN Genetics Europe</td>
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<td>Milk powder</td>
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<td>Monensin</td>
<td>eBioscience (#00-4505-51)</td>
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<tr>
<td>PageRuler™ Unstained Protein Ladder</td>
<td>Thermo Scientific (#26615)</td>
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<tr>
<td>peqGOLD TriFast</td>
<td>Peqlab (#30-2020)</td>
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<tr>
<td>Phorbol 12-myristate 13-acetate (PMA)</td>
<td>Sigma-Aldrich (#P8139)</td>
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<tr>
<td>qPCR™ Mastermix Plus for SYBR Green</td>
<td>eurogentec</td>
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<tr>
<td>Roti Histofix®</td>
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<td>Rotiphorese® Gel 40</td>
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<td>Thymidine [methyl-3H]</td>
<td>Hartmann Analytic (#ART 0178)]</td>
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<td>TWEEN 20</td>
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### 2.1.6 Cytokines

<table>
<thead>
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</thead>
<tbody>
<tr>
<td>Recombinant Human TGF-β1 (carrier-free)</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Recombinant Mouse IL-6 (carrier-free)</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Recombinant Mouse IL-2 (carrier-free)</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Recombinant Mouse IL-4 (carrier-free)</td>
<td>BioLegend</td>
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<tr>
<td>Recombinant Mouse IL-12 (p70) (carrier-free)</td>
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<tr>
<td>recombinant human GM-CSF</td>
<td>Cell Genix</td>
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<tr>
<td>recombinant IL-4</td>
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<td>Recombinant Human IL-6</td>
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<td>PGE₂</td>
<td>Pfizer</td>
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### 2.1.7 Antibodies

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<tr>
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<td>Anti-human CD11c APC</td>
<td>B-ly6</td>
<td>BD Pharming (#559877)</td>
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<td>Anti-human CD25 PE-Cy7</td>
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<td>BD Pharming (#557741)</td>
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<td>BD Pharming (#555657)</td>
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<td>HB15e</td>
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<td>Vendor</td>
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<td>3/23</td>
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<td>Anti-mouse CD86</td>
<td>GL-1</td>
<td>BioLegend</td>
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<td>Anti-mouse Foxp3 PE</td>
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<td>Anti-mouse IFN-γ FITC</td>
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<tr>
<td>TruStain fcX™ (Fc-Block)</td>
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<td>BioLegend</td>
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</table>

**Western Blot antibodies**

- Anti-12 Lipoxigenase: Rabbit polyclonal Abcam (#ab23678)
- Goat Anti-rabbit IgG-HRP: Goat polyclonal Dako(#P 0448)

**ChIP antibodies**

- NRF2 (D1Z9C) XP® Rabbit mAb: Cell Signaling (#12721)
- Normal rabbit IgG: Santa Cruz (#sc-2027)

**T cell skewing antibodies**

- LEAF™ purified anti-mouse CD3ε: 145-2C11 BioLegend (#100331)
- LEAF™ purified anti-mouse CD28: 37.51 BioLegend (#102111)
- LEAF™ purified anti-mouse IL-4: 11B11 BioLegend (#504115)
- LEAF™ purified anti-mouse IFN-γ: MIB-5E9.1 BioLegend (#508104)
### 2.1.8 Kits

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<td>R&amp;D Systems (#DY499)</td>
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<td>Mouse IL-23p19 DuoSet® ELISA Kit</td>
<td>R&amp;D Systems (#DY1887)</td>
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<tr>
<td>PGE₂ EIA Kit</td>
<td>Cayman Chemicals (#514010)</td>
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<tr>
<td>EasySep Mouse CD4+ T Cell Isolation Kit</td>
<td>STEMCELL Technologies (#19852)</td>
</tr>
<tr>
<td>Foxp3/Transcription factor staining buffer set</td>
<td>eBioscience (#005223-56)</td>
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### 2.1.9 Primer

All primer were obtained from Invitrogen™ Life Technologies.

<table>
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<tr>
<th>Gene name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>CTA CAC TGA GGA CCA GGT TGT CT (sense), CAG GAA ATG AGC TTG ACA AAG TT (antisense)</td>
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<td>β-Actin</td>
<td>TGT CCA CCT TCC AGC AGA TGT (sense), AGC TCA GTA ACA GTC CGC CTA GA (antisense)</td>
</tr>
<tr>
<td>12/15-LO</td>
<td>CTC TCA AGG CCT GTT CAG GA (sense), GTC CAT TGT CCC CAG AAC CT (antisense)</td>
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<td>IL23p19</td>
<td>GAC CCA CAA GGA CTC AAG GA (sense), TAG AAC TCA GGC TGG GCA TC (antisense)</td>
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<tr>
<td>IL-12p35</td>
<td>ATG ACC CTG TGC CTT GGT AG (sense), TCT CCC ACA GGA GGT TTC TG (antisense)</td>
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</tbody>
</table>
### Material and Methods

<table>
<thead>
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<th>Primer Sequence</th>
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**ChIP primer** (for promoter region containing putative ARE)

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2.1.10 Plastic-ware

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### Material and Methods

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<td>Active Motif</td>
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### Material and Methods

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<td>FACScalibur™</td>
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<td>Inotech, Dottikon, Switzerland</td>
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<td>Microplate Counter</td>
<td>Wallac</td>
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#### 2.1.12 Software

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<td>Tree Star Inc.</td>
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<tr>
<td>Kaluza® 1.2</td>
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</tr>
<tr>
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<td>GraphPad Software</td>
</tr>
<tr>
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<tr>
<td>7300 System Software</td>
<td>Applied Biosystems</td>
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<tr>
<td>Bio-Rad CFX Manager 3.0</td>
<td>Bio-Rad</td>
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<tr>
<td>QuantStudio™ 6 and 7 Flex</td>
<td>Applied Biosystems</td>
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<tr>
<td>Real-Time PCR System</td>
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<tr>
<td>Software Base v1.x</td>
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</tbody>
</table>
2.2 Methods

2.2.1 Animals

Animal experiments were approved by the government of Mittelfranken. Mice were housed in the animal facility of the University of Erlangen-Nuremberg. Alox15\(^{-/-}\) mice were purchased from The Jackson Laboratory; C57BL/6 mice were purchased from Charles River Laboratories. All experiments comparing Alox15\(^{-/-}\) and WT mice and DCs were confirmed using littermates. NRF2-deficient (Nrf2\(^{-/-}\)) mice were previously described (Itoh, Chiba et al. 1997).

2.2.2 Genotyping

For genotype determination a small piece of tail was digested in tail lysis buffer and genomic DNA was isolated. The following Primers and PCR programs were used for genotyping:

<table>
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<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>1</td>
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<tr>
<td>2</td>
<td>AGG TGA GAT GAC AGG AGA TC</td>
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<td>3</td>
<td>CGT GGT TGA AGA CTC TCA AGG</td>
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<table>
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<tr>
<td>Red Taq Master Mix</td>
<td>6,25\mu l</td>
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<tr>
<td>Primer 1</td>
<td>1,00\mu l</td>
</tr>
<tr>
<td>Primer 2</td>
<td>1,00\mu l</td>
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<td>Primer 3</td>
<td>1,00\mu l</td>
</tr>
<tr>
<td>Primer 4</td>
<td>1,00\mu l</td>
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</tbody>
</table>

WT 266bp  Mut 280bp
2.2.3 Isolation and generation of cells

Bone marrow-derived dendritic cells

Bone marrow cells were flushed from the femurs and tibias of C57BL/6 or Alox15−/− mice and cultured for 8 days in R10 medium at a concentration of 2 × 10^6 cells per 10-cm dish (bacterial quality) and granulocyte macrophage colony-stimulating factor (GM-CSF). R10 culture medium is composed of RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), l-glutamine (2 mM), 2-mercaptoethanol (50 mM), and 10% heat-inactivated FCS. GM-CSF supernatant (1:10) from a cell line transfected with the murine GM-CSF gene was used (Zal, Volkmann et al. 1994). At day 3, a volume of 9 ml R10 medium containing 1 ml GM-CSF supernatant was added to the cultures. 50% of the culture supernatant was removed at day 6, and cells were fed again with fresh 10 ml of 1:10 diluted R10 medium containing GM-CSF supernatant. On day 8, nonadherent cells, which were >95% CD11c+, were harvested and used for the different experiments (Lutz, Kukutsch et al. 1999).

Human monocyte-derived dendritic cells

<table>
<thead>
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</tr>
</thead>
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<td><strong>Program</strong></td>
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<tr>
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</tr>
<tr>
<td>94°C 0:20 min</td>
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<tr>
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</tr>
<tr>
<td>72°C 0:35 min</td>
<td></td>
</tr>
<tr>
<td>94°C 0:20 min</td>
<td></td>
</tr>
<tr>
<td>58°C 0:30 min</td>
<td>25 cycles</td>
</tr>
<tr>
<td>72°C 0:35 min</td>
<td></td>
</tr>
<tr>
<td>72°C 2:00 min</td>
<td></td>
</tr>
<tr>
<td>10°C hold</td>
<td></td>
</tr>
</tbody>
</table>
Human moDCs were generated as described previously (Pfeiffer, Zinser et al. 2013). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from leukoreduction system chambers of healthy donors by density centrifugation using Lymphoprep. PBMCs were seeded on tissue culture dishes for 1 hour. The nonadherent fraction was washed off after 1 hour with RPMI 1640 without any supplement. The adherent cell fraction was cultured for 4 days in DC medium consisting of RPMI 1640 supplemented with 1% (vol/vol) of each of the following: heat-inactivated human serum type AB, Penicillin/Streptomycin/L-Glutamine, and 10 mM HEPES as well as 800 IU/ml (day 0) or 400 IU/ml (day 3) recombinant human GM-CSF and 250 IU/ml (day 0 and 3) recombinant IL-4. On day 4, immature DCs were used for further experiments.

2.2.4 Cell preparation

Maturation of murine BMDCs

On day 8, maturation of BM-DC cultures was induced by overnight treatment with 100 ng/ml LPS. In the case of LPS pulse stimulation, BM-DCs were incubated with 100 ng/ml LPS for 6 hours. Following incubation, medium was removed and adherent cells were washed twice with PBS. Cells were supplied with fresh R10 medium an incubated at 37°C until supernatants were harvested for determination of the cytokine content.

Phospholipid treatment of murine BMDCs

For lipid pretreatment, BM-DCs were incubated for 4 hours at 37°C before addition of maturation stimuli. oxPC was used at 50 μg/ml. For COX-2 inhibition, BM-DCs were pretreated with 10 μM indomethacin (4 hours, 37°C) prior to LPS-induced maturation.

Maturation of human DCs and treatment with Baicalein

Maturation of DCs was induced by the addition of a maturation cocktail consisting of 200 U/ml IL-1β (Cell Genix), 1,000 U/ml IL-6 (Cell Genix), 10 ng/ ml TNF-α, and 1 μg/ml PGE2 for 24 hours. Immature DCs were either incubated with 2 μM Baicalein
formulated in DMSO or treated with 2 μM Baicalein and subsequently matured as indicated above. DMSO on its own was used as control.

**T cell skewing and cocultures**

Mouse splenic CD4+ T cells were isolated by immunomagnetic separation using EasySep and cultured in IMDM medium. For cytokine mediated Th differentiation, CD4+ cells were cultured with plate-bound 5 μg/ml αCD3 antibody and IMDM medium supplemented with 3 μg/ml αCD28 antibody. Cytokines and neutralizing antibodies were added at the following concentrations where indicated: (Th17) 5 ng/ml TGF-β, 10 ng/ml IL-6, 10 μg/ml αIFNγ antibody; (Th2) 20 ng/ml IL-2, 50 ng/ml IL-4; (Th1) 5 ng/ml IL-2, 10 ng/ml IL-12, 10 μg/ml αIL-4; and (regulatory T cells) 5 ng/ml IL-2, 5 ng/ml TGF-β; for Th0 conditions, no cytokines or blocking antibodies were added. For assays of BM-DC–mediated T cell differentiation, DCs were generated as described above, followed by LPS (100 ng/ml) maturation for 14 hours. DCs were washed extensively and cultured with enriched CD4+ T cells at a ratio of 1:5 in the appropriate T cell–skewing conditions supplemented with or without 10 μg/ml oxPC. After 4 days of culture, T cells were collected and stimulated with PMA and ionomycin plus monensin for intracellular cytokine staining.

**Mixed lymphocyte reaction (MLR)**

For the MLR, allogenic lymphoid LN-T cells (4 x 10^5/well) were incubated in 96-well cell culture plates with graded numbers of mature WT and Alox15/-/- DCs for 5 days. The assay was performed in triplicates. Proliferation of T cells was monitored by measuring [methyl-3H]thymidine incorporation, added on day 4 of culture. Cells were pulsed with 1 μCi/well [methyl-3H]thymidine (Hartmann Analytik) for 16 h, harvested onto glass fiber filters (Printed Filtermat A; Wallac, Turcu, Finland) using an ICH-110 harvester (Inotech, Dottikon, Switzerland), and [methyl-3H]thymidine incorporation was determined using a microplate counter (Wallac).

### 2.2.5 Flow cytometric analysis

In general, measurements were performed with a GALLIOS cytofluorometer or a BD FACSCanto™ II and evaluated with FlowJo software.
Material and Methods

**Analysis of surface markers**

Following isolation and counting, cells were blocked by resuspending them in 20 µl PBS containing 5% FCS (FACS buffer) and FcBlock (1:100). After 10 min incubation at room temperature, 80 µl of staining solution containing the diluted antibodies in FACS buffer at a saturating concentration was added, and the cells were incubated in the dark, at 4°C for 30 min. Afterwards, the cells were washed with 300 µl FACS buffer and finally resuspended in 200-400 µl FACS buffer.

**Analysis of intracellular 12/15-LO expression**

To compare 12/15-LO expression in moDCs and conventional DCs, inguinal lymph nodes and spleens were harvested. Further, lymph nodes and spleens were digested for 25 to 30 minutes at 37°C with collagenase-DNase and then treated for 5 minutes with EDTA to disrupt T cell–DC complexes. After fixation with 4%PFA and surface staining, cells were permeabilized using Fixation/Permeabilization solution and blocked with Permeabilization buffer containing 10% goat serum. A polyclonal rabbit anti-mouse antibody against 12/15-LO was used in Perm/5% goat serum for 60 minutes at 4°C; for isotype control, 5% rabbit serum was used instead of primary antibody. After intense washing with Perm, secondary antibody against rabbit IgG was added at 1:400 for 30 minutes at 4°C.

**Analysis of T cell subsets**

Fresh isolated or cocultured T cells were stimulated for 5 hours in an appropriate medium (R10) with phorbol 12-myristate 13-acetate (PMA) and ionomycin in the presence of monensin before being stained according to the manufacturer’s instructions (eBioscience). The exclusion of dead cells was performed by staining with Fixable Viability Dye in parallel to surface staining (see above). For intracellular staining of T cell-specific cytokines, cells were fixed and permeabilized using “Foxp3/Transcription factor staining buffer set” according to the manufacturer’s instructions. Following permeabilization, cells were resuspended in Perm buffer containing the appropriate antibodies, and then incubated over night at 4°C.
Material and Methods

For analysis of surface markers, cells were stained in PBS containing 5% FCS for 30 minutes at 4°C. Cells were stained in various combinations of mAbs. For human moDCs, the following surface molecules were used: CD11c (B-ly6), CD25 (M-A251), CD86 (2331), CD83 (HB15e), CD80 (2D10), CD14 (M5E2), MHCII (L243), and LIVE/DEAD. For murine cells, we used the following: CD8a (53-6.7), CD11b (M1/70), CD25 (3C7), CD40 (3/23), CD86 (GL-1), Ly6C (HK1.4.), MHCII (M5/114.15.2), CD11c (N418), CD80 (16-10A1), and CD83 (Michel-19) for DCs; and CD3 (145-2C11) and CD4 (RM4-5) for T cells. For intracellular staining with αIL-17A (TC11), αFOXP3 (MF-14), αIFN-γ (XMG1.2), or αIL-4 (11B11), T cells were stimulated for 5 hours with phorbol 12-myristate 13-acetate (PMA) and ionomycin in the presence of monensin before being stained as described above. The exclusion of dead cells was performed by staining with Fixable Viability Dye in parallel to surface staining. Flow cytometry data were acquired on a Gallios Flow Cytometer or FACScan and were analyzed with FlowJo software.

2.2.6 Phospholipid preparation

Generation of oxPC has been previously described (Watson, Leitinger et al. 1997; von Schlieffen, Oskolkova et al. 2009). oxPC was generated by air oxidation of the corresponding nonoxidized precursor phosphatidylcholine (PAPC) until 20% of PAPC remained intact, while the rest transformed into oxidized products. The extent of oxidation was monitored by thin-layer chromatography, and the analysis of the oxidation products was performed by flow-injection electrospray ionization mass spectrometry. Phospholipid concentration was determined by phosphorus assay. Phospholipids were dissolved in chloroform and stored at -80°C until use. Immediately before an experiment, chloroform solutions of oxPC were evaporated under a stream of argon and resuspended in culture medium by vigorous vortexing for 30 seconds.

2.2.7 Quantification of phospholipids

Nonadherent BM-DCs (~3 × 106 cells) from WT and KO animals were harvested on ice, centrifuged, and washed twice with PBS supplemented with 2 mM EDTA and
0.01% butylated hydroxytoluene (PBS/BHT/EDTA). The suspensions were transferred into cryovials overlaid with argon and stored at –80°C. For cell culture supernatants, cells were centrifuged at 400 g, and 500 μl of the liquid phase was collected and supplemented with BHT to 0.01% and EDTA to 2 mM. Quantification of different 12/15-LO-derived oxidation products in WT and Alox15−/− BM-DCs was performed by liquid chromatography-tandem mass spectrometry as previously described (Uderhardt, Herrmann et al. 2012).

2.2.8 ELISA and EIA

To determine supernatant concentrations of IL-23p19 and IL-12/23p40 (R&D Systems), as well as PGE2 (Cayman), assays were performed according to the instructions in the manufacturer's protocols.

2.2.9 Western blot analysis

Cells and tissues were homogenized and lysed in Laemmli buffer. Protein concentration was determined using a RC/DC Protein Quantification Kit (Bio-Rad). Proteins were separated by electrophoresis in 10% SDS polyacrylamide gels. Proteins were blotted onto polyvinylidene difluoride membranes and, after blocking with 5% dry milk/0.1% Tween 20, incubated with primary and secondary peroxidase-conjugated antibodies and consequent chemiluminescent detection.

2.2.10 Quantitative real-time PCR

Real-time PCR was performed as previously described (Kronke, Bochkov et al. 2003). RNA was isolated using TRIzol reagent (Invitrogen). One microgram of total RNA was reverse transcribed with human leukemia virus reverse transcriptase using the Gene Amp RNA PCR Kit (Applied Biosystems) and oligo(dT) primers. mRNA levels were normalized to β-actin expression in the case of tissue or Gapdh in the case of BM-DC cultures.
2.2.11 Chromatin immunoprecipitation

ChIP analysis was performed using the ChIP-IT High-Sensitivity Kit (Active Motif). BM-DCs were treated with 100 ng/ml LPS or 50 μg/ml oxPC in RPMI containing 10% FCS. After 2 hours of incubation, cells were fixated and prepared for chromatin sonication according to the manufacturer's specifications. Chromatin shearing was performed using an EpiShear Probe Sonicator (Active Motif). Sonicated chromatin was incubated with antibodies (4 μg per 30 μg chromatin) against NRF2 and IgG. Before immunoprecipitation, 5% of the extract volume was removed and served as an input. The antibody-bound protein/DNA complexes were immunoprecipitated through the use of protein G agarose beads and washed via gravity filtration. Following immunoprecipitation, the DNA crosslinks were reversed and the DNA was purified according to the ChIP protocol. Purified immunoprecipitated and input DNA were analyzed by PCR. The following primers were used: for Hmox1 promoter region containing putative ARE (–352/–338), TTAGGAATCCGGAGCTGTC (sense), AGGGTTCAGTCTGGAGCAAC (antisense), and for NQO1 ARE (9510/9524), TCTAAGAGCAGAAGCAGCA (sense), AGTCACCTTTGCACGCTAGG (antisense). Data were expressed as fold enrichment of the ChIP samples relative to the IgG samples.

2.2.12 Induction of EAE

Female C57BL/6 mice and KO mice were immunized subcutaneously with 50 μg MOG peptide 35–55 (Charité Berlin) in 50 μl H2O emulsified in 50 μl CFA, which was enriched with 10 mg/ml Mycobacterium tuberculosis (H37Ra, Difco/PD PharMingen) at day 0 in order to induce EAE. In addition, 200 ng pertussis toxin (List/Quadratech) was administered intraperitoneally at day 0 and 2. EAE paralysis of mice was scored as follows: 0, no disease; 1, tail weakness; 2, paraparesis; 3, paraplegia; 4, paraplegia with forelimb weakness; 5, moribund or death. In a modified procedure, EAE induction was performed without the additional administration of pertussis toxin.
2.2.13 Statistical analysis

Data are shown as mean ± SEM. Group mean values were compared by 2-tailed Student’s t test. Mean clinical scores of the EAE experiments were determined based on at least 7 mice per group, and statistical differences between the groups were compared by Mann-Whitney's U test. The data shown are representative of at least 3 experiments generating similar results.
3 Results

3.1 Bone marrow derived DCs express 12/15-LO

Investigations of mice deficient for the Alox15 gene revealed important roles for the 12/15-LO protein in several inflammatory diseases. It was shown by in vitro studies that 12/15-LO derived products can act as coactivators, and modulators of gene expression, or regulators of cytokine generation (Gruber, Mayer et al. 2010; Mauerhofer, Philippova et al. 2016). However, the mechanisms by which 12/15-LO regulates physiological immune cell function are not yet entirely discovered (Kuhn and O’Donnell 2006). This is particularly important to gain more insight into the role of 12/15-LO in regulating innate and adaptive immune responses. Dendritic cells bridge both these arms of immunity, therefore they serve as ideal cell type to investigate the role of enzymatic lipid oxidation during the induction of adaptive immunity. In human monocyte-derived DCs (moDCs) that have been treated with IL-4, 15-LO is among the most prominently expressed genes, but a potential role of this enzyme in DC biology remains unclear (Spanbroek, Hildner et al. 2001). However, studies in dyslipidemic Apoe−/− mice showed that oxidized low-density lipoproteins (oxLDLs) can inhibit pro-inflammatory cytokine production, as well as up-regulation of costimulatory molecules by DCs. This provides indirect evidence, that lipid oxidation products might exert a major role in DC biology (Shamshiev, Ampenberger et al. 2007). Analysis of mRNA confirmed expression of the Alox15 gene in murine bone marrow-derived DCs (BM-DCs) (Figure 4A). Consequently these immature DCs also displayed a strong expression of 12/15-LO protein comparable to those extracted from peritoneal macrophages (Figure 4B). Furthermore, in response to lipopolysaccharide (LPS) Alox15 mRNA and 12/15-LO protein expression were gradually downregulated (Figure 4, A and B). This expression pattern during the DC maturation process, demonstrates an inverse correlation of 12/15-LO expression and the activation status of DCs.
To evaluate the functional enzymatic capability of the 12/15-LO protein in DCs, the content of 12/15-LO-derived oxidation products was determined. One substrate of 12/15-LO are esterified fatty acids in the form of phospholipids, which are a major component of cell membranes in DCs (Morgan, Dioszeghy et al. 2009). By applying mass spectrometry, the amount of specific 12/15-LO-derived phospholipid oxidation products was measured. These mass spectrometry measurements were performed in collaboration with the laboratory of Prof. Bochkov in Vienna. As expected, DCs generated from the bone marrow of WT mice were enriched in the 12/15-LO-derived oxidation products of phosphatidylcholine PAPC-OH and PAPC-OOH, which were normalized to a saturated internal standard 1, 2-dinonanoyl-sn-glycero-3-phosphocholine (DNPC). Furthermore, the accumulation of these oxidation products was reduced in DCs from Alox15−/− mice, indicating an enzymatically active form of the 12/15-LO protein in WT DCs (Figure 5).

Figure 4: BM-DCs express functionally active 12/15-LO.

(A) Quantification of Alox15 mRNA expression levels in immature BM-DCs from WT and Alox15−/− mice or of Alox15 mRNA expression in WT BM-DCs after stimulation with LPS (100 ng/ml) for indicated intervals. Error bars represent SEM. (B) Western blot analysis of 12/15-LO protein levels in extracts of BM-DCs and peritoneal macrophages (MΦ) isolated from WT and Alox15−/− mice and WT or Alox15−/− BM-DCs after maturation with LPS (100 ng/ml for 24 hours). Illustration adopted from Rothe et al. 2015.
Results

3.2 12/15-LO regulates the maturation process of murine and human DCs

Upon exposure to LPS, DCs undergo a rapid maturation process, which involves the up-regulation of co-stimulatory molecules and the production of pro-inflammatory cytokines. As 12/15-LO expression seems to potentially be linked to the maturation status of DCs, the next step was to elucidate its role in DC function. Therefore, the phenotype of WT and Alox15−/− DCs before and after LPS-induced maturation was investigated. Analysis of the expression of surface molecules on BM-DCs generated from WT and Alox15−/− mice by flow cytometry, revealed differential expression levels of the individual molecules. After maturation with LPS, Alox15−/− DCs showed an increase in the percentage of cells expressing MHC class II molecules, compared to WT DCs, as well as an increase in cells expressing costimulatory molecules and activation markers, including CD40, CD25, CD86, CD83 and CD80, on their surfaces (Figure 6).

Figure 5: BM-DCs are enriched 12/15-LO-derived oxidation products.

Determination of different 12/15-LO-derived oxidation products in WT and BM-DCs, as quantified by mass spectrometry. Peak area was normalized to an internal standard (1, 2-dinonanoylsn-glycerol-3-phosphocholine [DNPC]). Data shown are representative of 3 independent experiments (n = 3). Illustration adopted from Rothe et al. 2015.
Results

Figure 6: 12/15-lipoxygenase (12/15-LO) regulates the maturation process of DCs.

Flow cytometric-based analysis of the expression of MHCII or expression of the indicated co-stimulatory molecules on wild-type (Alox15\textsuperscript{+/+}) and Alox15\textsuperscript{-/-} CD11c\textsuperscript{+} bone marrow-derived dendritic cells (BM-DCs) after LPS-induced maturation (100ng LPS/ml). Percentage of maturated CD11c\textsuperscript{+} BM-DCs shown by dot plots of the indicated surface markers along with the corresponding bar graphs summarizing five independent experiments. Illustration adopted from Rothe et al. 2015.
Moreover, also the expression levels of the individual costimulatory molecules were elevated on the surface of *Alox15*<sup>−/−</sup> DCs, indicated by a higher mean fluorescence intensity (Figure 7). Notably, already in an immature state, *Alox15*<sup>−/−</sup> DCs displayed an increase in the percentage of cells positive for the expression of costimulatory molecules (Figure 6 and 7). Taken together, BM-DCs from *Alox15*<sup>−/−</sup> mice showed an enhanced maturation status.

![Flow cytometric-based analysis of the expression of MHCII or expression of the indicated co-stimulatory molecules on wild-type (Alox15<sup>+/+</sup>) and Alox15<sup>−/−</sup> CD11c<sup>+</sup> bone marrow-derived dendritic cells (BMDCs) after LPS-induced maturation (100ng LPS/ml). Comparison of surface marker expression levels on CD11c<sup>+</sup> BM-DCs by mean fluorescence intensity. Illustration adopted from Rothe et al. 2015.](image)

**Figure 7: 12/15-lipoxygenase (12/15-LO) regulates the maturation process of DCs.**

Flow cytometric-based analysis of the expression of MHCII or expression of the indicated costimulatory molecules on wild-type (Alox15<sup>+/+</sup>) and Alox15<sup>−/−</sup> CD11c<sup>+</sup> bone marrow-derived dendritic cells (BMDCs) after LPS-induced maturation (100ng LPS/ml). Comparison of surface marker expression levels on CD11c<sup>+</sup> BM-DCs by mean fluorescence intensity. Illustration adopted from Rothe et al. 2015.

Additionally, enzymatic inhibition of 15-LO with Baicalein in human moDCs was used to confirm the findings of 12/15-LO deficiency in murine DCs. Treatment with Baicalein alone resulted in a robust upregulation of costimulatory molecules and MHC class II molecules, which was comparable to those cells receiving only maturation cocktail, or inhibitor and cocktail in combination (Figure 8). This indicates that in the absence of an enzymatically active 15-LO, otherwise immature DCs spontaneously achieve a maturated status (Figure 8). These findings suggest an important role of 12/15-LO as a regulatory factor attenuating the maturation process of DCs.
Figure 8: Effects of pharmacologic inhibition of 15-LO in human moDCs.

Effects of the 15-lipoxygenase inhibitor Baicalein on the expression of indicated surface molecules on human monocyte-derived DCs in the absence and presence of maturation (mat) cocktail. Human moDCs were incubated overnight with mat. cocktail in combination w/o Baicalein. Comparison of surface marker expression levels on CD11c⁺ moDCs by mean fluorescence intensity, illustrated in histograms or bar graphs of the individual markers. Illustration adopted from Rothe et al. 2015.
3.3 12/15-LO-derived phospholipid oxidation products attenuate DC maturation

Besides free fatty acids, 12/15-LO also catalyses oxidation of fatty acids esterified to phospholipids (Uderhardt and Kronke 2012). To further address the mechanisms underlying 12/15-LO-mediated regulation of DC differentiation and to identify the enzymatic products responsible for the inhibitory effect on DC maturation, the effects of different known 12/15-LO–derived lipid mediators were examined on this process. Treatment of BM-DCs with the 12/15-LO–derived eicosanoids 12-HETE, 15-HETE, 13-HODE, and lipoxin A4 had no significant effect on LPS-induced DC maturation, neither on WT DCs nor on Alox15−/− DCs (Figure 9).

![Graph showing effects of eicosanoids on DC maturation](image)

**Figure 9:** Effects of 12/15-LO-derived eicosanoids on DC maturation.

Flow cytometry-based analysis of the expression of the costimulatory molecule CD86 on BM-DCs from WT and Alox15−/− CD11c+ MHCII+ positive BM-DCs after LPS-induced maturation (100 ng LPS/ml) in the absence and presence of the individual eicosanoids. BM-DCs were pre-treated overnight with either 12-HETE (100nM), 15-HETE (100nM), 13-HODE (1μM), or Lipoxin A (100nM) prior to LPS stimulation.

Since WT DCs were enriched in oxidized phosphatidylcholine (oxPC), as shown by mass spectrometry, also their effects on the DC differentiation were evaluated. In contrast to the eicosanoids tested before, treatment with oxPC potently inhibited the
maturation process of DCs and blocked the up-regulation of costimulatory molecules and of activation markers, such as MHC class II molecules, CD40, CD25, CD86, CD83, and CD80 (Figure 10 and Figure 11). Notably, oxPC treatment attenuated DC maturation and corrected the hyper-maturated phenotype of Alox15−/− DCs, whereas no effect was seen on immature non LPS-treated cells. These findings suggest that 12/15-LO derived phospholipid oxidation products such as oxPC counteract the LPS-induced maturation process and thereby stabilize DCs in a resting state.

Figure 10: oxPC reverses the increased activation of 12/15-LO-deficient DCs.

Flow cytometry-based analysis of the expression of (A) MHC class II molecules and (B) the costimulatory molecule CD86 on WT and Alox15−/− CD11c+ BM-DCs after LPS-induced maturation (100 ng LPS/ml) in the absence and presence of oxPC (50 µg/ml). Percentage of matured CD11c+ BM-DCs shown by (A) histograms or (B) dot plots of the indicated surface markers along with the corresponding bar graphs summarizing five independent experiments. Illustration adopted from Rothe et al. 2015.
Results

Figure 11: oxPC reverses the increased activation of 12/15-LO-deficient DCs.

Flow cytometry-based analysis of the expression the costimulatory molecules and activation markers on WT and Alox15−/− CD11c+ BM-DCs after LPS-induced maturation (100 ng LPS/ ml) in the absence and presence of oxPC (50 µg/ ml). Percentage of matured CD11c+ BM-DCs shown by dot plots of the indicated surface markers along with the corresponding bar graphs summarizing five independent experiments. Illustration adopted from Rothe et al. 2015.
3.4 12/15-LO-derived oxPC modulates DC maturation via activation of Nrf2

Although it has been shown in human moDCs that oxidized phospholipids can interfere with TLR-induced maturation, not much is known how this regulation is achieved on a mechanistic level (Bluml, Kirchberger et al. 2005; Bluml, Zupkovitz et al. 2009). Recent studies identified oxidized phospholipids as inducers of an electrophilic stress response, which is mediated to a major extent by the transcription factor erythroid 2 (NF-E2)-related factor 2 (NRF2) (Kadl, Meher et al. 2010; Bretscher, Egger et al. 2015). To address the mechanisms underlying the oxPC-mediated inhibition of DC maturation, the potential role of NRF2 during this process was investigated. In general, electrophilic lipids were shown to activate NRF2 by modifying its binding partner Kelch-like ECH-associated protein 1 (Keap1) (Espinosa-Diez, Miguel et al. 2015). This modification prevents the Keap1-dependent degradation of NFR2, which is then translocated to the nucleus where it binds to so-called antioxidant response elements (AREs) in the promoters of a set of genes involved in the antioxidative response, such as heme oxygenase-1 (Hmox1), quinone oxidoreductase 1 (NQO1), or glutamate-cysteine ligase (Gclc) (Al-Sawaf, Clarner et al. 2015). Using Chromatin Immunoprecipitation (ChIP) assays, binding of the transcription factor NRF2 to the promoters of these genes was determined. Compared to WT, binding of NRF2 to NQO1 and Hmox1 promoter regions, was markedly diminished in immature Alox15−/− DCs (Figure 12).

Figure 12: Diminished binding of NRF2 to antioxidative responsive genes.

ChIP-based analysis of the binding of NRF2 to the Nqo1 and Hmox1 promoter in WT and Alox15−/− DCs. Data represent binding of NRF2 in immature bone marrow-derived DCs without any further treatment. Illustration adopted from Rothe et al. 2015.
Results

Treatment with oxPC, on the other hand, strongly induced association of NRF2 to the promoters of these genes in WT DCs, but not in DCs deficient in the transcription factor NRF2 (Figure 13). Consistent with these findings, oxPC induced a robust increase in the mRNA expression of these genes in WT DCs but not Nrf2\(^{-/-}\) DCs (Figure 14).

![Graphs showing binding of NRF2 to NQO1 and Hmox1 promoters](image)

**Figure 13: oxPC induces binding of NRF2 antioxidative responsive genes.**

ChIP-based analysis of the oxPC-induced binding of NRF2 to the Nqo1 and Hmox1 promoter in WT and Nrf2\(^{-/-}\) DCs. BM-DCs were treated with LPS (100ng/ml) and/or oxPC (50µg/ml) for 2 hours. Illustration adopted from Rothe et al. 2015.

![Graphs showing expression of NQO1, Hmox1, and Gclc](image)

**Figure 14: oxPC induces expression of NRF2 target genes.**

OxPC-induced expression of Nqo1, Hmox1, and Gclc mRNA in WT and Nrf2\(^{-/-}\) DCs. BM-DCs were treated with 100ng/ml LPS and/or 50µg/ml oxPC for 2 hours. Expression levels of the indicated genes were quantified by real-Time PCR. Illustration adopted from Rothe et al. 2015.

Since oxPC was shown to induce NRF2 activation and target gene transcription, the question whether NRF2 was involved in the oxPC-induced block of DCs maturation
was determined. Therefore both WT and Nrf2−/− DCs were treated with oxPC w/o LPS and the effects of the phospholipids on the expression of costimulatory molecules was analysed. While in WT DCs oxPC treatment showed the expected inhibition of the maturation process, NRF2-deficient DCs showed high expression of costimulatory molecules and did not respond to oxPC treatment, that is, co-incubation of Nrf2−/− DCs with oxPC and LPS in combination continued to give rise to fully maturated DCs (Figure 15). These findings suggest that NRF2 functions as a key mediator of the oxPC-mediated immune-modulatory effects in DCs.

**Figure 15: oxPC modulates DC maturation via NRF2.**

Flow cytometry-based quantification of the oxPC-induced inhibition of the expression of the indicated surface molecules on WT and Nrf2−/− DCs. BM-DCs were maturated with LPS (100 ng/ml) in the absence and presence of oxPC (50 µg/ml). Displayed are the percentage of maturated CD11c+ BM-DCs shown by dot plots for CD86 and by bar graphs additionally for CD83 and CD40, summarizing three independent experiments. Illustration adopted from Rothe et al. 2015.
3.5 12/15-LO-deficiency alters the cytokine profile of DCs

Besides up-regulation of surface molecule, DCs start to express and secrete numerous cytokines during their maturation process, by which they shape the developing T cell response. One of the main group of cytokines secreted by conventional DCs are the members of the IL-12 family, such as IL-12 and IL-23. For example, IL-12 is involved in T helper 1 (Th1) differentiation, while IL-23 is critical for Th17 survival and expansion. (Vignali and Kuchroo 2012). Analysis of the mRNA expression of Alox15−/− DCs showed multiple alterations after LPS-induced maturation in comparison to the cytokine expression profile of WT DCs. Particularly, 12/15-LO-deficient DCs displayed a shift in the mRNA expression of different IL-12/IL-23 subunits, or more precisely an increased expression of the IL-23-specific subunit p19, whereas expression of the IL-12-specific subunit p35 was decreased. In turn, the expression of p40, which can form heterodimers with both p19 and p35, was not altered in Alox15−/− DCs. (Figure 16). Furthermore, expression of interferon-β (IFNβ) was increased, whereas expression of the anti-inflammatory cytokine IL-10 was decreased in 12/15-LO-deficient DCs (Figure 16).

![Graph showing mRNA expression changes](image)

**Figure 16: 12/15-LO deficiency alters the cytokine profile of DCs.**

Real-time PCR-based quantification of mRNA expression levels of the indicated cytokines in WT and Alox15−/− CD11c+ BM-DCs after LPS-induced maturation (100 ng LPS /ml for 16 hours). Illustration adopted from Rothe et al. 2015.
Results

To confirm that the altered mRNA expression profile of 12/15-LO-deficient DCs is also reflected on protein level, subsequently the amount of several cytokines secreted by pulse-stimulated BM-DCs from WT and Alox15^{-/-} were measured. Consistent with the mRNA data, 12/15-LO-deficient DCs displayed elevated levels of IL-23p19 in their supernatants, whereas the levels of IL-12/23p40 was not different compared to supernatants of WT cells (Figure 17 A). Moreover, Alox15^{-/-} DCs also showed increased production of the pro-inflammatory cytokines IL-1β and IL-6, indicating that stimulated 12/15-LO-deficient DCs provide a setting that would support Th17-differentiation (Figure 17 B).

Figure 17: 12/15-LO deficiency alters the cytokine profile of DCs.

ELISA-based analysis of cytokine production by BM-DCs from WT and Alox15^{-/-} mice. (A) Cells were pulse stimulated with LPS (100 ng/ml) for 6 hours, and the subsequent cytokine secretion was determined at the indicated time points. (B) BM-DCs were incubated with LPS (100 ng/ml) overnight and supernatants were analysed. Illustration adopted from Rothe et al. 2015.
3.6 12/15-LO-deficiency doesn’t enhance COX-2/PGE2 signaling

Besides 12/15-LO, also enzymes like cyclooxygenases (COX-1 and COX-2) can metabolize free arachidonic acids, leading to the formation of prostaglandins (PGs). Competition for arachidonic acid between the cyclooxygenase and lipoxygenase enzymes exists in cells and tissues containing both pathways, which also includes DCs (Harizi, Juzan et al. 2001; Harizi, Juzan et al. 2002; Fogel-Petrovic, Long et al. 2004). It has been shown for murine BM-DCs as well as for human moDCs, that the COX-2 (resembling the inducible form)-derived metabolite prostaglandin E2 (PGE2) can enhance IL-23 production and thereby promote the induction of Th17 responses (Sheibanie, Tadmori et al. 2004; Shi, Yin et al. 2015). This implicates the possibility that a deficiency in 12/15-LO enhances COX-2 mediated PGE2 production, which in turn could contribute or even be responsible for the increased IL-23 generation in Alox15−/− DCs. Analysis of mRNA expression of COX-2 in WT and Alox15−/− DCs revealed no significant differences in COX-2 expression after LPS stimulation. Furthermore, also no differences in the PGE2 levels of the corresponding supernatants could be observed (Figure 18).

![Figure 18: Effects of 12/15-LO deficiency on COX-2/ PGE2 pathway.](image)

(A) Real-time PCR-based quantification of mRNA expression levels of COX-2 and in (B) ELISA-based analysis of PGE2 production by WT and Alox15−/− bone marrow-derived dendritic cells before and after LPS-induced maturation (100ng/ml for 16 hours). Illustration adopted from Rothe et al. 2015.
In addition, the effects of COX-2 inhibition by Indomethacin on the expression levels of IL-23/IL-12 were investigated. Blockade of COX-2 did not ameliorate the increased IL-23p19 or decrease IL-12p35 expression in Alox15−/− DCs, but resulted in an additional exacerbation of this phenotype (Figure 19). These findings providing no evidence for a substrate competition between 12/15-LO and COX-2 during the observed effects in IL-23 production.

![Graphs showing IL-23p19 and IL-12p35 expression levels with and without Indomethacin treatment.](image)

**Figure 19: Effects of COX-2 inhibition by Indomethacin on the expression levels of IL-23/IL-12.**

Real-time PCR-based quantification of expression levels of IL-23p19 and IL-12p35 mRNA from WT and Alox15−/− bone marrow-derived dendritic cells. DCs were maturated with LPS (100ng/ml) with or without pre-treatment (4h) of 10µM Indomethacin. Illustration adopted from Rothe et al. 2015.

### 3.7 DC-intrinsic 12/15-LO activity attenuates Th17 T cell differentiation

Since DCs lacking 12/15-LO activity show a hyper-activated status as well as an altered cytokine profile after LPS-induced maturation, the question was addressed, if this has a functional consequence on the DC-mediated T cell response. Therefore, activated DCs isolated from WT and Alox15−/− mice (C57BL/6 background) were co-cultured with lymphocytes isolated from BALB/c mice, initiating an allogeneic
mixed-leukocyte reaction, to investigate the capacity of the DCs to induce T cell proliferation. Despite the increased maturation of \textit{Alox15}/- DCs, major differences in their T cell stimulatory capacity could not be observed (Figure 20 A). Furthermore, using ovalbumin (OVA) as a model antigen, also the specific DC-T cell interaction was examined. For this purpose, BM-DCs generated from WT and \textit{Alox15}/- mice (C57BL/6 background) were loaded with an OVA-peptide and syngeneically co-cultivated with T cells bearing an OVA-specific T cell receptor (OTII cells). Again, \textit{Alox15}/- DCs showed no increased capacity to induce T cell proliferation, but rather a tendency to a less pronounced T cell activation than the corresponding WT DCs (Figure 20 B).

\textbf{Figure 20: Effects of 12/15-LO activity on the T cell response in MLRs.}

Measurement of T-cell proliferation by [methyl-3H] thymidine incorporation after (A) allogeneic stimulation of BALB/c lymphocytes with immature or maturated (LPS-treated) bone marrow-derived dendritic cells (BM-DCs) isolated from wild-type (WT) and \textit{Alox15}/- mice (both C57BL/6 background) or after (B) specific DC- T cell interaction mediated by presentation of OVA-peptid (DCs) to OVA-specific transgenic T cell receptor (T cells). Data shown are representative for at least 3 independent experiments (n=3).

Using this experimental approach, it is not possible to generate specific insights into the T cell composition within the pool of proliferating T cells. Since the capacity to induce T cell expansion by \textit{Alox15}/- DCs is not changed, but these DCs displayed an
increased expression of IL-23p19, the question was addressed, if the expression of 12/15-LO has an effect on the differentiation of T helper cells, particularly on Th17 cell differentiation. Therefore, DCs were co-cultivated together with CD4+ T cells that were exposed to Th1-, Th2-, Th17-, or regulatory T cell–skewing conditions. Absence of 12/15-LO hardly affected the differentiation of T cells towards the Th1 or Th2 direction, but resulted in an increased differentiation of Th17 cells, whereas regulatory T cell development was diminished (Figure 21).

![Flow cytometric analysis of the frequency of the indicated Th cell subsets after co-cultivation of CD4+ T cells together with WT or Alox15-/- CD11c+ BM-DCs for 4 days under Th-skewing conditions. Illustration adopted from Rothe et al. 2015.](image1)

**Figure 21: DC-derived 12/15-LO regulates T cell differentiation.**

Flow cytometric analysis of the frequency of the indicated Th cell subsets after co-cultivation of CD4+ T cells together with WT or Alox15-/- CD11c+ BM-DCs for 4 days under Th-skewing conditions. Illustration adopted from Rothe et al. 2015.

The activation status of DCs was shown to be susceptible to phospholipid treatment, leading to the question of what effect oxPC would have on the DC-driven T cell differentiation. Applying oxPC to the co-culture did indeed interfere with differentiation of Th17 cells and restored the frequency of Th17 cells toward comparable levels between cells incubated either with WT or Alox15-/- DCs (Figure 22).
Results

To test whether oxPC not only has an indirect influence on Th17 cell differentiation via modulation of DC activation or also a direct effect on the T cells themselves, skewing experiments were performed in the absence of DCs. Interestingly, oxPC exerted also a direct inhibitory influence on Th17 differentiation, which suggests that lipid oxidation products of 12/15-LO such as oxPC can act as direct inhibitors of Th17 differentiation (Figure 23).

Figure 22: DC-derived 12/15-LO regulates Th17 T cell differentiation.

Effect of oxPC (10 μg/ml) on the differentiation of Th17 T cells during co-cultivation of CD4+ T cells together with WT or Alox15−/− CD11c+ BM-DCs under Th17-skewing conditions. Data shown are representative of at least 3 independent experiments (n = 3). Illustration adopted from Rothe et al. 2015.
Figure 23: DC-derived 12/15-LO regulates Th17 T cell differentiation.

Effect of oxPC (10 μg/ml) on the differentiation of Th17 T cells under Th0- or Th17-skewing conditions in the absence of BM-DCs. Data shown are representative of at least 3 independent experiments (n = 3). Error bars represent SEM. *P < 0.05, Student's t test. Illustration adopted from Rothe et al. 2015.
3.8 12/15-LO protein is expressed in different DC subsets in vivo

These *in vitro* studies clearly provided evidence for a regulatory function of 12/15-LO in activation and maturation of DCs, and that decreased or absent 12/15-LO in DCs also promotes Th17 cell differentiation. To determine if this *in vitro* phenotype also has consequences *in vivo*, it was essential to confirm 12/15-LO expression in mouse DCs *in vivo*. Thus, screening for 12/15-LO protein was performed by flow cytometry analysis of freshly isolated splenocytes and lymphocytes from WT mice. CD11c positive cells showed expression of 12/15-LO, whereas neither CD4 positive T cells nor CD19 positive B cells expressed this enzyme on or on mRNA level (Figure 24, A-C).

![Figure 24: 12/15-LO is expressed in DCs in vivo.](image)

(A) Flow-cytometric-based measurement of the 12/15-LO expression in splenocytes of naive wild-type (*Alox15*+/+) and *Alox15*−/− mice. (B) Quantification of the *Alox15* mRNA expression levels in enriched CD19+ or CD4+ splenocytes isolated from naive wild-type (+/+ ) and *Alox15*−/− (-/-) mice, compared to mRNA expression levels of wild-type bone marrow-derived DCs. (C) Agarose gel electrophoresis of the corresponding products of the performed real time PCR of the *Alox15* and *GAPDH* genes in the indicated cell types. Data shown are representative for at least 3 independent experiments (n=3). Illustration adopted from Rothe et al. 2015.

The spleen contains several common subsets of DCs. To study which of them does express 12/15-LO, splenic DCs were further subdivided into CD11b+ Ly6C+ CD11cint
monocyte-derived DCs (moDCs), CD11c⁺ CD11b⁻ CD8⁺ lymphoid DCs, or CD11c⁺ CD11b⁺ CD8⁻ myeloid DCs. All of the individual DC subsets showed expression of 12/15-LO, whereas no signal was detected in splenic DCs from 12/15-LO-deficient mice (Figure 25).

**Figure 25: 12/15-LO activity modulates maturation status of moDCs and myeloid DCs in vivo.**

Flow cytometry–based measurement of the 12/15-LO expression in indicated splenic DC subsets during steady state. DCs subsets were subdivided into CD11b⁺ Ly6C⁺ CD11cint monocyte-derived DCs (moDCs), CD11c⁺ CD11b⁻ CD8⁺ lymphoid DCs, or CD11c⁺ CD11b⁺ CD8⁻ myeloid DCs. Illustration adopted from Rothe et al. 2015.
3.9 Deletion of 12/15-LO protein results in increased DC maturation in vivo

After confirming 12/15-LO expression in DCs in vivo, it was further investigated whether this expression has also functional consequences. In order to achieve this, mice were immunized with MOG peptide to induce experimental autoimmune encephalomyelitis (EAE), an animal model which is used to study human CNS demyelinating diseases, including multiple sclerosis, in the murine system. EAE is considered as a prototypic Th17-mediated autoimmune disease (Constantinescu, Farooqi et al. 2011; Pierson, Simmons et al. 2012), but it also involves DC activation and antigen-presentation. After immunization with MOG peptide, the antigen is thought to be taken up by DCs, which in turn get activated and start to migrate to the lymphoid tissues to present the myelin-specific peptide to T cells. In accordance with the in vitro data, splenic DCs in Alox15⁻/⁻ animals displayed an enhanced maturation status following immunization, as reflected by increased expression of MHC class II molecules (Figure 26 A).

![Figure 26: 12/15-LO activity modulates maturation status of moDCs and myeloid DCs in vivo.](image)

(A) Flow cytometric analysis of MHC class II molecule expression on moDC, lymphoid DC, and myeloid DCs, in draining lymph nodes of Alox15⁺/⁺ and Alox15⁻/⁻ mice 3 days after induction of EAE. (B) Follow-up of 12/15-LO expression in spleen of WT mice of moDCs, lymphoid DC, and myeloid DCs after induction of EAE. Illustration partially adopted from Rothe et al. 2015.
This phenotype was more pronounced in the moDC and myeloid DC subsets, whereas the maturation status of lymphoid DCs showed no major changes. Additionally, upon induction of EAE, maturation of DC in the draining lymph nodes was paralleled by a gradual downregulation of 12/15-LO expression (Figure 26 B).

3.10 Alox15⁻/⁻ mice display an increased differentiation of T_h17 cells and an aggravation of T cell-dependent autoimmune responses

In the pathogenesis of EAE, activated Th17 cells and Th1 cells are thought to be the main drivers of disease. Priming of these cells occurs in peripheral lymphoid tissues, were MOG-peptide bearing DCs activate myelin-specific T cells leading to the differentiation of autoreactive effector T cells. After entering the blood circulation, these cells infiltrate the CNS. There they induce and perpetuate inflammation, eventually causing tissue damage. Finally this results in progressive paralysis. Since in vitro data indicate a strong tendency of 12/15-LO deficient DCs to promote Th17 cell differentiation, the potential of affecting the T cell response in vivo was investigated. After induction of EAE, draining lymph nodes of immunized WT and Alox15⁻/⁻ mice were screened for several cytokines connected to T cell differentiation. Analysis of the mRNA revealed an altered cytokine expression profile after induction of disease in Alox15⁻/⁻ mice. These mice displayed a significant increase in the expression of the IL-23 subunit p19, whereas expression levels of the IL-12/IL-23 subunits p35 and p40 were not significantly changed (Figure 23). In accordance with the data on 12/15-LO protein expression in the different DC subsets, expression of Alox15 mRNA decreased after disease initiation. Moreover, increased levels of IL-1b, which also drives Th17 cell differentiation, were observed. Finally, lymph nodes of Alox15⁻/⁻ mice exhibited elevated expression of the Th17-specific transcription factor RORyt (Figure 27).
Figure 27: Deletion of 12/15-LO results in altered cytokine expression profile in vivo.

Quantification of the mRNA expression profile of indicated cytokines, including \textit{Alox15} and \textit{Rorgt}, in draining lymph nodes of WT and \textit{Alox15}\textsuperscript{-/-} mice 3 days after induction of immunized (EAE) mice compared with non-immunized mice (control). Illustration adopted from Rothe et al. 2015.

Subsequently, the distribution of different T cell subsets in the spleens of WT and \textit{Alox15}\textsuperscript{-/-} mice, which were harvested 10 days after the initial immunization, was analyzed. Flow cytometry showed a significant increase in the number of Th17 T cells in \textit{Alox15}\textsuperscript{-/-} mice, whereas the distribution of other T cell subsets, such as FOXP3+ regulatory T cells, Th1 cells, or Th2 T cells, was not changed (Figure 28 A).
Results

In accordance with these findings, the clinical follow-up showed that \textit{Alox15–/–} mice suffered from an aggravated form of EAE. In a “classical” EAE model, which included application of pertussis toxin, we observed an increased clinical score in these mice (Figure 28 B). In a modified EAE protocol, performed without the administration of pertussis toxin, both the clinical score and the disease incidence were significantly increased in \textit{Alox15–/–} mice compared with those in WT controls (Figure 28 C).

\textbf{Figure 28: Deletion of 12/15-LO results in increased Th17 T cell differentiation as well as an aggravated form of EAE.}

\textbf{(A)} Flow cytometric analysis of the frequency of IL-17–expressing Th17 T cells, FOXP3–expressing regulatory T cells, IFN-γ–expressing Th1 T cells, and IL-4–expressing Th2 T cells in the spleens of WT or \textit{Alox15–/–} mice 10 days after induction of EAE. Data shown are representative of at least 3 independent experiments. Error bars represent SEM. *\(P < 0.05\), **\(P < 0.005\), ***\(P < 0.0005\), Student’s \(t\) test. \textbf{(B and C)} Analysis of the EAE severity score in WT and \textit{Alox15–/–} mice after immunization with MOG peptide in the \textbf{(B)} presence or \textbf{(C)} absence of pertussis toxin. Mean clinical scores were determined based on at least 7 mice per group (\(n = 7\)). ***\(P < 0.001\), Mann-Whitney’s \(U\) test. Illustration adopted from Rothe et al. 2015.
4 Discussion

The adaptive immune defense provides a specific response to a previously encountered antigen. This is mediated by expansion of antigen-specific T- and B-lymphocytes. Activation of T cells requires their interaction with antigen-presenting cells, of which dendritic cells (and B-cells) are the most potent. Residing in peripheral tissues, immature dendritic cells are equipped with toll-like receptors (TLRs), which recognize pathogen-associated molecular patterns (PAMPs) like LPS. Upon ingestion of invading pathogens, dendritic cells undergo a maturation process, which is accompanied by up-regulation of co-stimulatory molecules, and major histocompatibility complex molecules (MHC). After migration to the regional lymph nodes, dendritic cells present their processed antigens via MHC-molecules to T cell receptors (TCRs). In combination with the secretion of various cytokines, this initiates T cell activation and their effector functions. In this thesis, the enzyme 12/15-LO is identified to modulate this adaptive immune response cascade by acting on dendritic cell maturation as well as DC-derived cytokine production. 12/15-LO-derived phospholipid oxidation products inhibited expression of MHCII, and co-stimulatory molecules and caused a shift in the cytokine profile preventing Th17 cell differentiation. While deletion or blockade of 12/15-LO resulted in an enhanced maturation of mouse and human DCs, this effect was abrogated by 12/15-LO-derived products such as oxidized PC. Furthermore, it is shown, that this effect is mediated by the induction of the anti-oxidative stress response and activation of the transcription factor Nrf2 that interferes with DC maturation (Rangasamy, Williams et al. 2010; Al-Huseini, Aw Yeang et al. 2013). This provides evidence for an endogenous regulatory mechanism that involves the generation of 12/15-LO-derived phospholipid oxidation products that support a critical activation threshold to limit the spontaneous and excessive maturation of DCs. Additionally, absence of 12/15-LO enhanced the production of IL-23 promoting an increased Th17 cell differentiation, which in turn led to a higher susceptibility of 12/15-LO deficient mice to develop Th17-driven autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE).
4.1 Relevance of 12/15-LO-derived oxidation products in the maturation process of DCs

Although it is known that 12/15-LO is expressed in cells of the myeloid lineage, the previous research on that enzyme did strongly focus on macrophages in the context of atherosclerosis, but a potential role of this enzyme in dendritic cell biology and during the induction of adaptive immunity remained largely unexplored. Previous studies have described regulatory effect of oxidized phospholipids on human moDCs (Kuhn 2000; Bluml, Kirchberger et al. 2005; Bluml, Zupkovitz et al. 2009), but without intensively investigating a potential source of these lipids as well as downstream targets. The data in this manuscript confirm a strong expression of 12/15-LO in bone marrow-derived DCs as well as in several DC subsets in the spleen, including lymphoid, myeloid, and monocyte-derived DCs. Further, BM-DCs were greatly enriched in oxidized phospholipids, while 12/15-LO deficient DCs showed decreased oxPC level, indicating that 12/15-LO acts as an important enzymatic source for such bioactive lipids in DCs. In consequence of this lack of phospholipid oxidation products, 12/15-LO deficient DCs displayed hyper-activated status, revealed by increased up-regulation of MHCII, and co-stimulatory molecules after LPS-induced maturation. Interestingly, already the inhibition of the human ortholog 15-LO by Baicalein triggered a spontaneous maturation of moDCs. The reason for this difference in effect size could originate either from intrinsic cellular differences between human blood monocyte-derived DCs and murine BM-DCs or from substrate preferences and slight differences in catalytic properties between murine 12/15-LO and human 15-LO (Kuhn 2000). Additionally, DCs showed a gradual reduction of 12/15-LO protein during regular LPS-induced maturation. As a total block results in fully maturated DC, this downregulation might be a requirement for a controlled maturation of DCs.

In line with these results, previous studies of dyslipidemia, or, more precisely, hyperlipidemia in ApoE−/− mice with elevated serum levels of oxidized LDLs, revealed an inhibitory effect of oxidized LDLs on the TLR-induced maturation of CD8− DCs in vivo as well as on the development of Th17-mediated autoimmune disease (Angeli, Llodra et al. 2004; Shamshiev, Ampenberger et al. 2007; Asquith, Miller et al. 2010). These ApoE−/− mice, that have a genetic deficiency in the lipid
transport protein, apolipoprotein E (apoE), leading to dyslipidemia, were impaired in their response to TLR stimulation, resulting in a reduced production of pro-inflammatory cytokines, as well as an impaired up-regulation of costimulatory molecules culminating in shift of their T cell response. The major enzyme involved in the generation of these oxidized LDL particles in such hyperlipidemic mice in turn is 12/15-LO (Rankin, Parthasarathy et al. 1991; Kuhn, Belkner et al. 1994a; Benz, Mol et al. 1995; Cyrus, Pratico et al. 2001) and the biologic activity of oxidized LDL is strongly linked to LDL-associated oxidized phospholipids (Berliner, Subbanagounder et al. 2001; Navab, Berliner et al. 2001).

Hence, these findings are well in accordance with our presented data and support the concept of a regulative role of lipid oxidation during DC maturation and initiation of the adaptive immune response. The present study shows that the inhibitory effect of 12/15-LO-mediated lipid oxidation on the maturation of the CD8+ DC subsets and the Th17 response is independent from hyperlipidemia, as the mice in the here conducted experiments were not fed a high-fat diet. Therefore, both LDL-derived and endogenous cell membrane-derived lipids can act as the source for the 12/15-LO-mediated generation of lipid mediators that attenuate DC maturation.

4.2 Immuno-modulatory effects of oxidized phosphatidylcholine

Activation of DCs is promoted upon encountering pathogen-associated molecular patterns (PAMPs), such as LPS or other microbial products, which are recognized by pattern recognition receptors (PRRs) (De Smedt, Pajak et al. 1996; Medzhitov and Janeway 1997; Medzhitov 2001; Schnare, Barton et al. 2001). In addition to PAMPs, PRRs also recognize self-encoded molecules that are called damage-associated molecular patterns (DAMPs) (Kono and Rock 2008). Oxidized phospholipids like oxPC represent one class of DAMPs. While oxPC is an abundant lipid at the site of tissue damage and can exert pro-inflammatory effects (Imai, Kuba et al. 2008), it can also dampen inflammatory responses by inhibiting TLR activation (Bochkov, Kadl et al. 2002; Shirey, Lai et al. 2013). It was shown for human PBMC-derived DCs that treatment with oxPC blocked TLR-4-mediated induction of co-stimulatory molecules, and impaired lymphocyte stimulatory capacity (Bluml, Kirchberger et al. 2005; Bluml, Zupkovitz et al. 2009). In this case oxPC acts as a LPS-mimic by forming
a complex with LBP and thus prevents binding of LPS to LBP (von Schlieffen, Oskolkova et al. 2009). Consistent with these findings also 12/15-LO derived oxPC counteracted LPS-induced maturation in murine DCs, while free eicosanoids failed to attenuate this maturation process. Unlike eicosanoids, oxPC consist of a glycerol backbone and a phosphodiester polar head group, which might mediate the binding to LBP and explain the difference in their inhibitory effects. It was recently shown that oxPC is also able to induce an enhanced DC activity state (Zanoni, Tan et al. 2016). When primed from a first encounter with PAMPs, inducing TLR-signaling and T cell activation, DCs can achieve a second state of activation. This hyperactive state results from coincident encounters with PAMPs and oxPC, inducing a potent adaptive immune response. This underlines the importance of a balanced control of DC activation, with an appropriate response depending on the context, and highlights the important role of oxidized phospholipids in this process.

4.3 The role of NRF2 in mediating the properties of oxidized phospholipids

Although oxidized phospholipids are increasingly recognized as modulators of TLRs, the mechanisms underlying this process remain a matter of debate. 12/15-LO oxidation products like oxPC were shown to form adducts with KEAP1 and thereby activate the transcription factor NRF2 (Itoh, Mochizuki et al. 2004). Loss of NRF2 also leads to dysregulation of immune functions in DCs and T cell-mediated immune responses orchestrated by these DCs (Williams, Rangasamy et al. 2008; Rangasamy, Williams et al. 2010; Aw Yeang, Hamdam et al. 2012; Al-Huseini, Aw Yeang et al. 2013). Activation of NRF2 results in binding of the transcription factor to antioxidant response elements. Indeed, oxPC specifically activated NRF2 in BM-DCs and, thereby, exerted a feedback on the anti-oxidative response of the DC. While in WT BM-DCs, oxPC inhibited the maturation process, knockdown of the transcription factor NRF2 abrogated this effect, clearly identifying NRF2 as mediator of the oxPC-dependent regulation of DC activation. The results were confirmed by a recent publication (Bretscher, Egger et al. 2015), showing that oxPC-mediated inhibition of IL-12 production was abrogated in NRF2-deficient myeloid cells, that were
paralleled by a rapid inducting of NRF2-dependent transcription of the prototypic target genes.

### 4.4 Implication of DC mediated 12/15-LO activity in Th17 differentiation

Although 12/15-LO was identified as an important modulator of DC maturation, deficiency in 12/15-LO did not influence the capacity of the DCs to induce lymphocyte proliferation. T cell proliferation not only relies on co-stimulation, but also it requires cytokines that control differentiation of T cells. Hence, the current thesis also reveals a regulatory role of enzymatic lipid oxidation on the regulation of cytokine secretion, mainly affecting IL-12/IL-23 axis and the differentiation of Th17 T cells. Enzymatic activity of 12/15-LO attenuated production of IL-23, a member of the IL-12 family that is critical for Th17 survival and expansion (Vignali and Kuchroo 2012). In addition, expression of several cytokines that further contribute to favor Th17 direction, like IL-6, and IL-1β, are enhanced upon absence of 12/15-LO in DCs. Moreover, 12/15-LO-derived oxidation products also directly interfered with Th17 differentiation. It has been shown that oxidized phospholipids can inhibit T cell proliferation and induction of anergy (Schwartz 2003; Seyerl, Bluml et al. 2008), which may be explained by expression of a specific receptor on the T cell surface. Consequently, 12/15-LO activity in DCs diminished Th17 cell differentiation. On the other hand, regulatory T cell differentiation seems to be better supported by WT DCs, indicating a modulatory role of 12/15-LO to prevent excessive autoimmune responses.

However, previous reports showed 12/15-LO to selectively regulate IL-12 production in macrophages, in which deletion of this enzyme results in a reduced production of this cytokine (Zhao, Cuff et al. 2002; Middleton, Rubinstein et al. 2006). This would implicate a role of 12/15-LO in IL-12 driven type I immune responses. Deficiency of 12/15-LO activity in DC had no effect on Th2 cell differentiation. Although the authors claim to also detect no differences in IL-12 production in BM-DC derived from WT or Alox15−/−, these studies indicate a role of 12/15-LO in regulating the IL-12/ IL-23 balance in such professional antigen-
Discussion

presenting cells. Our current data thus clearly demonstrate a differential role of this enzyme in DC and macrophage biology.

An additional factor that influences the IL-12/IL-23 axis in DCs was reported to be the COX-2–derived prostaglandin E2 (PGE2) (Sheibanie, Tadmori et al. 2004). PGE2 induces strong expression of IL-23 in DCs and thereby promotes IL-17 secretion from activated CD4+ T cells. Thus, these findings indicate an inverse regulation of the IL-12/IL-23 ratio by COX-2 and 12/15-LO. As COX-2 and 12/15-LO can metabolize the same substrates, absence of 12/15-LO might result in increased production of PGE2 in Alox15−/− DCs. However, deletion of 12/15-LO neither resulted in increased COX-2 expression nor elevated PGE2 levels, excluding a potential substrate competition between the two enzymes. Furthermore, inhibition of COX-2 exacerbated IL-23 expression in Alox15−/− DCs, indicating that although PGE2 might induce IL-23 (Shi, Yin et al. 2015), other COX-2 metabolites seem to negatively affect IL-23 expression.

4.5 Role of 12/15-LO during autoimmune diseases

Consistent with our in vitro findings, Alox15−/− mice were prone to develop an exacerbated form of EAE as a prototypical Th17-driven autoimmune disease. These data support previous findings that described an enhanced susceptibility of Alox15−/− mice to the development of this disease (Emerson and LeVine 2004), although the authors of this previous study did not address the underlying mechanism. The data presented here point to an important role of 12/15-LO in counteracting an overwhelming autoreactive T cell response, indicated by enhanced DC maturation and increased IL-23 production in the absence of 12/15-LO that contributed to an augmented differentiation of Th17 T cells. Furthermore it was shown that mice suffering from EAE show high expression level of 12/15-LO during the resolution phase of disease (Kihara, Matsushita et al. 2009). Although the nature of the responsible cell population was not addressed, this suggests a second role for 12/15-LO during a later stage of the disease (Paintlia, Paintlia et al. 2006). In accordance with the finding that the transcription factor NRF2 might mediate the effect of 12/15-LO activity on the course of the disease, NRF2-deficient mice develop an exacerbated form of EAE as well, whereas pharmacological compounds activating
this transcription factor attenuate this disease in mice and were already successfully tested in the treatment of multiple sclerosis in humans (Johnson, Amirahmadi et al. 2010; Pareek, Belkadi et al. 2011; Bar-Or, Gold et al. 2013). 12/15-LO, can generate endogenous ligands for the peroxisome proliferator-activated receptor (PPAR)γ PPARγ. Interestingly, also PPARγ-deficient heterozygous (PPARγ+/−) animals develop an exacerbated EAE, whereas treatment with PPARγ agonists decreased the duration and clinical severity of EAE in mice (Natarajan and Bright 2002; Natarajan, Muthian et al. 2003). This suggests a potential role of PPARγ during the protective effects of 12/15-LO in EAE, although this hypothesis has to be addressed in more detail experimentally. Contradictory to that, inhibition of 12/15-LO by administration of Baicalein had a therapeutic effect on EAE progression as well (Xu, Zhang et al. 2013). The authors of the latter study propose that Baicalein mediated an increased expression PPAR β/δ in microglia of EAE mice. This might be in turn due to non-specific or side effects of Baicalein, such as inhibition of 5-LO that occurs at higher concentrations.

Furthermore, synthetic analogs of oxidized phospholipids like Lecinoxid, also called VB-201, were demonstrated to have anti-inflammatory and immunosuppressive action, leading to an amelioration of EAE (Mendel, Shoham et al. 2010). VB-201 impaired Th1 cell polarization and inhibited the infiltration of pathogenic CD4+ T cells into the central nervous system, while CD4+ T cell proliferation or generation of regulatory T cells was not affected. These oxidized phospholipid acts as small molecules that were also shown to inhibit development of atherosclerosis in ApoE−/− mice, where inhibition of monocytes and DCs seems to be involved (Mendel, Feige et al. 2014). Mechanistically, VB-201 binds directly to TLR-2 and CD14 to impair downstream cues and cytokine production. However, the role of 12/15-LO in the pathogeneses of atherosclerosis is still controversial, as 12/15-LO-deficient mice show a significant decrease in atherosclerotic lesions (Cyrus, Witztum et al. 1999). On the other hand, macrophage-mediated expression of human 15-lipoxygenase protects against atherosclerosis development in a transgenic rabbit model (Shen, Herderick et al. 1996).

Remarkably, 12/15-LO has also been implicated in the development of type 1 diabetes, in which deletion of Alox15 in NOD mice results in a markedly reduced rate
of diabetes (McDuffie, Maybee et al. 2008; Dobrian, Lieb et al. 2011; Green-Mitchell, Tersey et al. 2013). 12/15-LO deletion leads to decreased proinflammatory cytokine levels in macrophages, and in turn splenocytes of NOD-Alox15−/− mice were unable to transfer diabetes in an adoptive transfer model (Green-Mitchell, Tersey et al. 2013). In the absence of 12/15-LO, IL-12 production and STAT4 signaling were significantly reduced. As type 1 diabetes represents a prototypical Th1-driven disease, the observed reduction of the IL-12/IL-23 ratio might additionally contribute to attenuation of this disease in Alox15−/− mice. Additionally, 12/15-LO-induced oxidative stress and the direct cytotoxic effect of 12-HETE seem to contribute to mitochondrial dysfunction and altered insulin secretion.

4.6 Concluding Remarks

The immune system is constantly challenged by pathogens and the resulting immune responses have to be initiated in an adequate range to avoid overwhelming and harmful immune reactions. This thesis identifies 12/15-LO as an enzyme implicated in the fine-tuning of DC activation, which represent a starting point of immune responses. It could be shown, that 12/15-LO-derived oxidized phospholipids modulate DC maturation. These effects are mediated by the redox-sensitive transcription factor NRF2. In addition, 12/15-LO activity in DCs also affects the IL-12/IL-23 cytokine balance and directly interfered with Th17 differentiation. This is supported by the observation mice lacking the Alox15 gene developed an exacerbated from of EAE as a typical Th17 driven disease (illustrated in Figure 29).
Figure 29: Schematic overview of how 12/15-LO-mediated enzymatic lipid oxidation regulates DC activation and fine-tunes consecutive T cell responses, via the activation of the transcription factor NRF2.

Taken together, the present study provides evidence for a central role of enzymatic lipid oxidation during the modulation of DC function and the shaping of an adaptive immune response. Oxidized phospholipids are increasingly recognized not only to represent pro-inflammatory modulators, but also to exhibit protective effects against oxidative stress and inflammation. These insights might result in the identification of novel targets for the treatment of autoimmune diseases such as multiple sclerosis and rheumatoid arthritis.
Abbreviations

5 Abbreviations

12/15-LO  12/15-lipoxygenase
12-HETE  12-Hydroxyeicosatetraenoic acid
13-HODE  13-Hydroxyoctadecadienoic acid
15-HETE  15-Hydroxyeicosatetraenoic acid
5-LO     5-lipoxygenase
AA       arachidonic acid
AC       apoptotic cell
AREs     antioxidant responsive elements
BM       bone marrow
BM-DC    bone marrow-derived dendritic cell
CCL3     chemokine (C-C motif) ligand 3
cDC      classical dendritic cell
CDP      common dendritic cell progenitor
ChIP     chromatin immunoprecipitation
CLP      common lymphocyte progenitor
CMP      common myeloid progenitor
CNS      central nervous system
COX-2    cyclooxygenase-2
DC       dendritic cell
DNPC     1,2-dinonanoyl-sn-glycero-3-phosphocholine
ELISA    enzyme-linked immunosorbent assay
FACS     fluorescence-activated cell sorting
FCS      foetal calf serum
GCLC     glutamate-cysteine ligase catalytic subunit
GM-CSF   granulocyte-macrophage colony-stimulating factor
GMP      granulocyte-macrophage progenitor
HMOX1    heme oxygenase (decycling) 1
HO-1     heme oxygenase-1
HRP      horseradish peroxidase
i.p.     intraperitoneal injection
iNOS     inducible nitric oxide synthase
<table>
<thead>
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<th>Abbreviations</th>
<th>Description</th>
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<tbody>
<tr>
<td>LBP</td>
<td>LPS binding protein</td>
</tr>
<tr>
<td>LC</td>
<td>Langerhans cell</td>
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<td>LDL</td>
<td>low-density lipoprotein</td>
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<td>LN</td>
<td>lymph node</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>M-CSF</td>
<td>macrophage colony-stimulating factor</td>
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<tr>
<td>MDP</td>
<td>macrophage and DC progenitor</td>
</tr>
<tr>
<td>MOG</td>
<td>myelin oligodendrocyte glycoprotein</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NQO1</td>
<td>NAD(P)H dehydrogenase [quinone] 1</td>
</tr>
<tr>
<td>NRF2</td>
<td>nuclear factor erythroid 2-related factor</td>
</tr>
<tr>
<td>PAMPs</td>
<td>pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PAPC (PC)</td>
<td>1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E$_2$</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisomal proliferator-activating receptor</td>
</tr>
<tr>
<td>PRRs</td>
<td>pattern-recognition receptors</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acids</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous injection</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TLR</td>
<td>toll-like receptor</td>
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“If we knew what it was we were doing, it would not be called research, would it?”

Albert Einstein
7 References


References


References


Kensler TW, Wakabayashi N, et al. (2007). Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annual review of pharmacology and toxicology 47: 89-116. doi 10.1146/annurev.pharmtox.46.120604.141046


89759 [pii]


References


Natarajan C and Bright JJ (2002). Peroxisome proliferator-activated receptor-gamma agonists inhibit experimental allergic encephalomyelitis by blocking IL-12 production, IL-12 signaling and Th1 differentiation. Genes and immunity 3(2): 59-70. doi 10.1038/sj.gene.6363832


Pareek TK, Belkadi A, et al. (2011). Triterpenoid modulation of IL-17 and Nrf-2 expression ameliorates neuroinflammation and promotes remyelination in
autoimmune encephalomyelitis. Scientific reports 1: 201. doi 10.1038/srep00201


Tamoutounour S, Henri S, et al. (2012). CD64 distinguishes macrophages from dendritic cells in the gut and reveals the Th1-inducing role of mesenteric


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