

Cytokine-Dependent Expression Regulation of *ALOX15*

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Abstract

Lipoxygenases (LOX) are lipid-peroxidizing enzymes that play a role in cell differentiation, but have also been implicated in the pathogenesis of inflammatory, hyperproliferative and neurological disorders. They are widely distributed in plants and mammals but also occur sporadically in lower organisms. The human genome involves six functional LOX genes and a corrupted pseudogene. 20 years ago it was reported that expression of *ALOX15* was specifically induced in human peripheral monocytes by the classical Th2 cytokine interleukin 4 and later expression array profiles indicated that this enzyme is the most strongly upregulated gene product in human monocytes. Although the molecular basis for this IL4-dependent expression regulation has extensively been studied during the past 20 years, there are still a number of unsolved questions. This review is aimed at summarizing the current knowledge on the cytokine-dependent expression regulation of *ALOX15* with particular focus on the Th2 cytokines interleukin-4 and interleukin-13 in various cells and tissues and at critically evaluating the potential biological implication of this effect.

Keywords: Eicosanoids; Lipoxygenase; Leukotrienes; Inflammation; Energy metabolism

Introduction

According to the IUPAC recommendations lipoxygenases (LOXs) are classified as fatty acid dioxygenases since they introduce one molecule of atmospheric oxygen into the hydrocarbon tail of fatty acids [1,2]. LOXs are widely distributed in plants and mammals but they only occur sporadically in bacteria, fungi and lower marine organisms [3]. The first mammalian LOXs have been described in the mid 1970 in human blood platelets [4,5] and rabbit reticulocytes [6]. Since then a large number of LOX-isoforms have been described in various animal species. Complete sequencing of the human genome indicated the existence of six different LOX genes (*ALOX5*, *ALOX12*, *ALOX12B*, *ALOX15*, *ALOX15B*, *ALOXE3*), which encode for functionally distinct LOX-isoforms [7]. In addition, a corrupted pseudogene (7) has been detected in the human genome (*ALOXE12*). Free polyenoic fatty acids, such as linoleic acid (9,12-octadecadienoic acid), alpha- (9,12,15-octadecatrienoic acid) and gamma- (6,9,12-octadecatrienoic acid) linolenic acid, arachidonic acid (5,8,11,14-eicosatetraenoic acid), 5,8,11,14,17-eicosapentaenoic acid and 4,7,10,13,16,19-docosahexaenoic acid are preferred substrates of mammalian LOXs. Since free fatty acids do not abundantly occur in most mammalian cells the LOX-pathway is initiated by enzymatic hydrolysis of membrane ester lipids [1]. The primary products of the LOX reaction, the hydroperoxy fatty acids, are subsequently converted to a large array of bioactive mediators, which include leukotrienes [1], lipoxins [8], hepxilins [9], eoxins [10], resolvins [11], protectins [12] and others. However, LOXs exhibit their biological functions not only by producing bioactive mediators. As oxidizing enzymes they are also involved in the regulation of the cellular redox homeostasis, which strongly impacts the gene expression pattern. Moreover, since *ALOX15* orthologs are capable of oxidizing complex ester lipids such as

phospholipids [13] and cholesterol esters [14] they modify the functional characteristics of biomembranes and lipoproteins.

The expression of LOX-isoforms in general and of *ALOX15* in particular is highly regulated on transcriptional and translational levels [15]. In 1992 it was first reported that expression of *ALOX15* can be specifically induced in isolated human monocytes by the Th2-cytokine interleukin-4 (IL4) [16]. Two years later [17] another classical Th2 cytokine, interleukin 13 (IL13), was shown to upregulate *ALOX15* expression. Although the mechanistic basis for the IL4/IL13-dependent expression regulation has not extensively been studied in these early reports these papers mark the beginning of a new chapter in LOX research. A quick PubMed search with the key words “lipoxygenase” and “interleukin” revealed some 720 entries over the past 25 years. This review is aimed at summarizing the current knowledge on the cytokine-dependent expression regulation of *ALOX15* with particular emphasis on the Th2 cytokines IL4 and IL13. We will also critically evaluate the suggested mechanistic scenarios and the applicability of the robust *in vitro* effects for the *in vivo* situation.

The multiplicity of published and unpublished experimental data on this topic makes it impossible to consider all reports for this review. Although we did our best to balance the list of cited references, we might have overlooked important contributions. We apologize to those distinguished colleagues who significantly contributed to the field but whose work has not been referenced.

Lipoxygenases (LOX) as Lipid Peroxidizing Enzymes

LOXs are fatty acid dioxygenases, which introduce molecular dioxygen into the hydrocarbon chain of polyunsaturated fatty acids. The LOX-reaction consists of four elementary reactions (hydrogen abstraction, radical rearrangement, oxygen insertion, radical reduction) and leads to the formation of specific lipid hydroperoxides [18]. The stereochemistry of all four elementary reactions is tightly controlled so that from the mixture of theoretically possible

oxygenation products only one isomer is selectively formed. The product specificity depends on the chemical identity of the LOX-isoform. For instance, human *ALOX5* [19] converts arachidonic acid predominantly to 5S-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5S-HpETE), whereas other product isomers such as 5R-HpETE, 15S-HpETE or 11S/R-HpETE are not formed. In contrast, human *ALOX12* [20] oxygenates arachidonic acid almost exclusively to 12S-HpETE. The corresponding 12R-enantiomer and other isomers of hydroperoxy arachidonic acid are absent.

Diversity of LOX family and occurrence of LOX sequences in the domains of terrestrial life

LOXs form a very diverse family of enzymes. They occur in two (bacteria, eucarya) of the three domains of terrestrial life but have not been identified in archaea. Although a number of LOX-like sequences

are present in various archaea there is no report on the expression of a functional LOX enzyme in this domain. Similarly, there are a number of LOX-like sequences in viral genomes but so far no functional viral LOX has been identified. We recently cloned a LOX-like sequence from a giant virus (mimivirus), which infects *Acanthamoeba polyphaga*, and expressed the recombinant enzyme in *E. coli*. This protein consists of 565 amino acids and thus, is some 100 amino acids smaller than typical mammalian LOXs. Interestingly, the amino acid sequence of the mimivirus protein (Figure 1) involves two potential iron-ligand clusters: Cluster 1: His-Met-Arg-Lys-Thr, cluster 2: His-Ser-Lys-Asn-His (the putative iron liganding amino acids are marked in bold). Most functional LOXs contain two of such iron ligand clusters. Although the distance between the two putative iron clusters is somewhat bigger as in most mammalian LOX they may still adopt a 3D conformation suitable for iron liganding.

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1  mlkkigndls  fftgyltfrt  ydsiwrkatl  pmysklkenl  rkptktclpq  ndldyrlrht
61  lirqnarhyq  sikntfinip  tfelntflkk  efhsniilas  ptlsfsildy  lndgriinsd
121 kdirkiykkf  rldismtrlt  dynayqlnpa  idilkilddn  trnkllglfd  daifysldie
181 sfvtnfsdkk  yyfqdceain  kqfnvdvygl  yvpyddngkk  rlqietiilv  drrtdntkty
241 vfqsnsfsw  iirnniyinw  rtmssvyehq  lgsnvycqnt  lyHmrkTlak  thpitolmkp
301 fmegvyftnk  vftsfgisia  dtenevvnry  mdrvelfdls  nqtmiaaley  ihktdgykll
361 dykkvyheng  vddiyfeqkq  lledlyqivf  dlvtvnfeyy  yqstddyvkd  nelrdfylsi
421 knldtfvedl  qqkdnaikff  sniiflssir  HsknHinyay  lnsfydyalr  ktnfdllldk
481 ldngipfdek  dclstvgdfy  skyssgiyps  vpinlfgtgy  knlfadnevq  kfftdvtnkl
541 nqlkkntern  nyteflfrlq  nsntI
```

Figure 1: Amino acid sequence of the putative LOX from the *Acanthamoeba polyphaga* mimivirus. The cDNA sequence of the putative LOX from the *Acanthamoeba polyphaga* mimivirus was retrieved from the NCBI database (ADO18315.1). The open reading frame encodes for a 565 amino acid protein that involves two potential iron ligand clusters and a C-terminal Ile (black background). The amino acids given in capital letters might constitute the potential proteinogenic iron ligands. The one letter code for amino acids is employed.

In addition, in most mammalian LOXs the C-terminal amino acid is an Ile, which functions as a 5th proteinogenic iron ligand. The mimivirus protein does also carry C-terminal Ile and thus, should have a fully functional iron ligand sphere. We cloned the putative mimivirus LOX, expressed the corresponding protein as N-terminal his-tag fusion construct in *E. coli* and purified it by affinity chromatography on Ni-agarose. Unfortunately, when we incubated the purified enzyme with arachidonic acid or linoleic acid we could not detect the formation of typical LOX products. Moreover, when we determined the iron content of the purified enzyme we only quantified substoichiometric amounts of iron in the enzyme preparation (less than 10% of the expected value based on a 1:1 enzyme:iron stoichiometry). These data suggest that this LOX-like sequence of the *Acanthamoeba polyphaga* mimivirus does not encode a functional LOX and thus, there is still no report on the occurrence of a functional LOX in viruses.

Because of the diversity of the LOX family classification of these enzymes is a critical point. In the early days of LOX research the enzymes have been classified with respect to their reaction specificity of arachidonic acid oxygenation and three distinct enzyme subtypes (5-LOX, 12-LOX, 15-LOX) have been differentiated. Unfortunately, this arachidonic acid based classification is not applicable for all LOX-isoforms. Some plant LOXs do not accept arachidonic acid as substrate [21] and LOX-isoforms from lower marine organisms exhibit different reaction specificities [22-25]. Another disadvantage of the specificity-based nomenclature system is that it does not consider evolutionary

aspects of phylogenetic relatedness. For instance, human platelet 12-LOX (*ALOX12*) exhibits the same reaction specificity as mouse *ALOX15* but with respect to their evolutionary relatedness these two enzymes are far apart. In contrast, human *ALOX15* and mouse *ALOX15* share a much higher degree of phylogenetic relatedness but exhibit different positional specificities. Since mouse *ALOX15* converts arachidonic acid to 12S-HpETE and the human ortholog produces 15-HpETE, the two orthologous enzymes (functional equivalents in different species) are classified in different groups according to the specificity-based nomenclature system. This is highly confusing. On the other hand, the evolutionary concepts of LOX classification have the disadvantage that the degree of amino acid conservation is rather low when enzymes of evolutionary distant species are compared. For instance, the 12-lipoxygenating LOX1 of the zebrafish shares a similar degree of amino acid conservation with all human LOX-isoforms [26] and thus, it is impossible to assign this enzyme to any of the human isoforms based on the degree of amino acid conservation.

Mammalian *ALOX15* orthologs

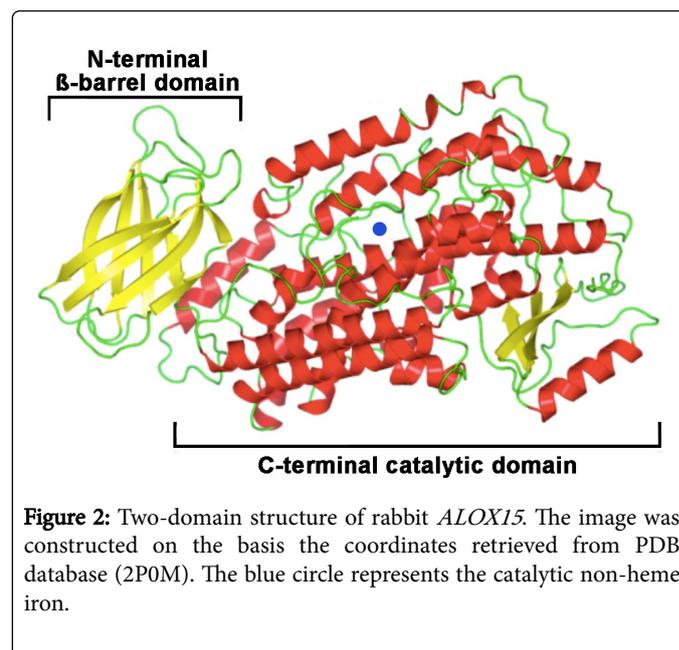
In mammals the situation is less confusing. As indicated above, the human genome involves 6 functional LOX genes and a corrupted pseudogen. In mice there is an ortholog for each human LOX gene (*ALOX15*, *ALOX15b*, *ALOX12*, *ALOX12b*, *ALOX5*, *ALOXE3*) but the human *ALOXE12* pseudogen is functional (*Aloxe12*) in mice. Thus,

mice express seven different LOX-isoforms. Functional inactivation of the different genes induced different phenotypes [7] and together with other findings these data suggest that the different LOX-isoforms exhibit different biological functions. Mammalian *ALOX15* orthologs have been implicated in cell differentiation [27,28] but also in the pathogenesis of cardio-vascular [29,30], inflammatory [31], hyperproliferative [32] and neurological [33] diseases. Blood platelets of *Alox12* knockout mice are more sensitive to agonist stimulation [34] and the water barrier of their epidermis [35] does not develop regularly. *Alox5* has also been implicated in cardio-vascular disorders [36] but also plays a role in allergic diseases [37,38]. *Alox12b* and *Alox3* are involved in epidermal differentiation and functional inactivation of the corresponding genes leads to dehydration of the newborns because of excessive loss of water [39,40]. In humans, mutations in the *ALOX12B* and *ALOXE3* genes have been related to autosomal recessive congenital ichthyosis, a severe hereditary skin disease [41]. Moreover, *Alox12b* appears to be involved in adipocyte differentiation [42]. For *ALOX15B* and *ALOXE12* knockout mice are currently not available.

Protein chemical and structural properties of mammalian *ALOX15* orthologs: Mammalian *ALOX15* orthologs are single polypeptide chain proteins, which consist of some 650 amino acids. The polypeptide chain folds into a two-domain structure with a small (some 110 amino acids) N-terminal β -barrel domain, which has been implicated in membrane binding and activity regulation [18,43]. The big (some 550 amino acids) alpha helical C-terminal domain harbors the catalytic center, which involves the catalytically active non-heme iron (Figure 2). In SDS PAGE these enzymes migrate in the 75 kDa molecular weight range. The two domains are covalently interconnected by a flexible linker peptide but tight together by non-covalent interactions. For rabbit *ALOX15* it has been reported that the two structural subunits may move relatively to each other [44] and the degree of this interdomain movement depends on pH and salt concentration [45]. The N-terminal beta-barrel unit is not essential for catalytic activity, but gene technical deletion of this structural motif lowers the oxygenase activity and impairs membrane binding [46]. In its resting state mammalian *ALOX15* orthologs contain ferrous non-heme iron at the active site and the iron ion is liganded by two clusters of amino acids (cluster A: His-A1-A2-A3-A4-His-, cluster B: His-B1-B2-B3-His), in which the His constitute the immediate iron ligands. The 5th proteinogenic iron ligand is the C-terminal Ile and truncation of this amino acid leads to a loss of the catalytic activity. A water molecule or a hydroxyl group completes the octahedral iron ligand sphere and this immediate iron ligand [47,48] is likely to be replaced by the fatty acid substrate. During the catalytic cycle the non-heme iron shuttles between its ferrous and its ferric form [43].

Enzymatic properties of mammalian *ALOX15* orthologs: Although mammalian *ALOX15* orthologs only have a single catalytic center they are multi-catalytic enzymes and exhibit at least three different catalytic activities [18]: i) lipoxygenase activity, ii) lipohydroperoxidase activity, iii) leukotriene synthase activity. Which reaction is catalyzed at a given time point depends on the availability of the reaction substrates and on the reaction conditions. For instance, under aerobic conditions and when free polyenoic fatty acids are available, *ALOX15* orthologs catalyze the lipoxygenase reaction converting the free polyenoic fatty acids to their corresponding hydroperoxides [49]. If these hydroperoxides still contain bisallylic methylene groups they can be lipoxygenated a second time, which leads to double or even multiple oxygenation products [50]. Such multiple oxygenation of free polyenoic fatty acids by different types of LOXs leads to the formation

of lipoxins [51,52], resolvins [53], protectins [11] and maresins [12], which exhibit anti-inflammatory properties.



If no free polyenoic fatty acids are available *ALOX15* orthologs can also oxygenate complex ester lipids such as phospholipids [6] and cholesterol esters [14] even if they are incorporated in complex lipid-protein assemblies such as biomembranes [13] and lipoproteins [54]. Although the biomembrane and lipoprotein oxygenase activity of *ALOX15* orthologs is at least one order of magnitude lower than their fatty acid oxygenase activity it can clearly be measured and has been suggested to be of biological relevance [27,55].

Hydroperoxy lipids can further be converted in the presence of polyenoic fatty acids to secondary hydroperoxidase products, such as short chain aldehydes, alkanes, ketodienes as well as oxygenated or non-oxygenated fatty acid dimers [56]. This hydroperoxidase activity of *ALOX15* orthologs also involves valency shuttling of the non-heme iron. It is initiated by homolytic cleavage of the peroxy group by the ferrous enzyme, which is paralleled by oxidation of the enzyme to its ferric form. To start the next catalytic cycle the enzyme must be reduced back to its ferrous form and free polyenoic fatty acids might serve as reducing agents [57]. The lipohydroperoxidase activity of *ALOX15* orthologs is strongly favored under anaerobic conditions but can also be detected during hypoxia [58]. The leukotriene synthase activity of *ALOX15* orthologs converts hydroperoxy fatty acids that still contain bisallylic methylenes, such as 15-HpETE, to secondary reaction products containing a conjugated triene system (50). This activity proceeds under aerobic and anaerobic conditions and does not require additional polyenoic fatty acids as reducing agents. The leukotriene synthase activity of *ALOX15* orthologs has been implicated in the formation of eoxins, which serve as pro-inflammatory mediators in allergic diseases [10,59].

Among these catalytic activities the lipoxygenase activity with free polyenoic fatty acids as substrate has most comprehensively been characterized. The reaction kinetics are rather complex and a typical kinetic progress curve starts with a kinetic lag-phase, which has been related to peroxide-dependent enzyme activation [60]. At later time points the progress curve slows down and this is due to irreversible

enzyme inactivation [61]. The molecular basis for this suicidal character of the *ALOX15* reaction has not been clarified but covalent modification of the enzyme by reactive reaction intermediates has been suggested [62].

All naturally occurring omega-6 and omega-3 polyenoic fatty acids serve as suitable substrates for *ALOX15* orthologs (low substrate specificity) but the reaction specificity of these enzymes is rather variable. *ALOX15* orthologs of higher primates including men [49] and orangutans [63,64] exhibit dominant omega-6 oxygenase activity. In contrast, *ALOX15* orthologs of lower mammals [mice [29,65], rats [66,67], pigs [68], cattle [69]] including lower primates [rhesus monkeys [63,64]] exhibit major omega-9 oxygenase activity. Gibbons, which are flanked in evolution by rhesus monkeys on one side and orangutans on the other, express an *ALOX15* ortholog that exhibits a pronounced dual positional specificity with almost equal distribution of omega-6 and omega-9 oxygenation (Adel et al., submitted). In other words, *ALOX15* reaction specificity was systematically changed during late primate evolution from arachidonic acid 12-lipoxygenation in lower mammals to arachidonic 15-lipoxygenation in higher primates (Adel et al., submitted). The evolutionary driving force for this alteration remains to be explored but arachidonic acid 15-lipoxygenating *ALOX15* orthologs have a higher biosynthetic capacity for anti-inflammatory lipoxins. Thus, this evolutionary switch in reaction specificity might be aimed at optimizing inflammatory resolution and thus may be considered a mechanism for fine-tuning the immune system of higher primates (Adel et al., submitted).

Mammalian *ALOX15* genes and tissue specific gene expression: The gene encoding human *ALOX15* is localized on chromosome 17 in a joint gene cluster, which also involves the genes for *ALOX15B*, *ALOX12*, *ALOX12B* and *ALOXE3*. The only human *ALOX* gene, which is not localized in this gene cluster, is that encoding for *ALOX5*. The human *ALOX15* gene consists of 14 exons and 13 introns. The mouse *ALOX 15* gene has a very similar exon/intron structure and is located in a syntenic region on chromosome 11. The genes encoding for other mammalian *ALOX15* orthologs look very similar. The promoter region of the human *ALOX15* gene [70] involves a number of potential transcription factor binding sites but lacks classical TATA- and CAAT boxes. However, it remains to be explored, which of these putative regulatory sequences are functional *in vivo*. When we evaluated the sequence data deposited in the database of the 1000 human genome project (www.1000genomes.org) we detected 78 single nucleotide polymorphisms in the *ALOX15* gene (allelic frequency >1%). These data indicate an average genetic variability [71]. In addition, some 90 non-synonymous coding variations and eight nonsense mutations have been identified, which occur with lower genetic frequency [71]. Functionally important amino acids, such as the immediate iron ligands and the reaction specificity determinants are rarely affected by amino acid exchanges [71].

Human *ALOX15* is high level expressed in reticulocytes, eosinophils and in airway epithelial cells [72]. Low level expression has been detected in polymorphonuclear leukocytes [73,74], alveolar macrophages [75], vascular cells [76,77], uterus [78], the male reproductive system [79], various parts of the brain [80,81] and in atherosclerotic lesions [82]. Resting human peripheral monocytes as well as B- and T-lymphocytes do not express significant amounts of *ALOX15*. In some human cancer cells *ALOX15* expression is silenced but can be reactivated by histone deacetylase inhibitors [83,84]. Mechanistic studies implicated the nucleosome remodeling and histone deacetylase repression complex (NuRD) in this transcriptional

regulation of *ALOX15* expression. In cancer cells NuRD appears to be recruited to the *ALOX15* promoter, which silences *ALOX15* expression [84].

In rabbits, a similar constitutive expression pattern of *ALOX15* was observed but experimental anemia strongly upregulated *ALOX15* expression in many cells and tissues including peripheral monocytes, lung, spleen, kidney and liver [85]. However, since the organs were not carefully saline- perfused to remove blood it might well be that the *ALOX15* activity detected in these organs may originate from contaminating reticulocytes. When elicited rabbit peritoneal macrophages were incubated with arachidonic acid, 5S-HETE and leukotriene B4 were identified as major oxygenation products. In contrast, only small amounts of 15-HETE were found. When similar experiments were carried out with cell homogenates formation of LTB4 and 5S-HETE was strongly down and 15-HETE was the major arachidonic acid oxygenation product (Kuhn et al., unpublished data). Unfortunately, the mechanistic basis for this switch in reaction specificity has not been explored.

In mice, peripheral eosinophils are a rich source of *Alox15* expression but as in humans the biological relevance of high-level *ALOX15* expression in these cells remains elusive. Similarly, high expression levels of *ALOX15* have been reported in residential peritoneal macrophages [29] and immunohistochemistry indicated that more than 80% of these cells were *ALOX15* positive. However, thioglycollate elicitation strongly decreased the share of *ALOX15* positive cells to about 10% [86]. Mouse blood monocytes, mouse alveolar macrophages and mouse bone marrow derived macrophages express *ALOX15* only at low levels [86], but the airway epithelium, which constitutes a major site of human *ALOX15* expression in humans [72] and cattle [69], has not been tested. Taken together, these data indicate that mammalian *ALOX15* orthologs exhibit species-specific expression patterns but the mechanistic details for the differences have not been explored in detail.

Biological implication of mammalian *ALOX15*: Mammalian *ALOX15* orthologs have been implicated in cell differentiation and maturation [red blood cells [87], epidermis [88,89], adipose tissue [42], sperms [90], neurons [91]] but they also play a role in the pathogenesis of various diseases [2]. A comprehensive review on the biological role of *ALOX15* has recently been published [2] and thus, there is no need to discuss this topic extensively in this paper. In various diseases *ALOX15* orthologs exhibit antagonizing activities depending on the disease model. For instance, in different types of animal inflammation models pro- [10,92,93] and anti-inflammatory [31,94] activities of *ALOX15* have been reported. A similar situation was found in animal atherosclerosis models and in different types of cancer. Experiments with *ALOX15* knockout mice suggested a pro-atherogenic activity of the enzyme [29,95-97] but overexpression studies revealed anti-atherogenic effects [85,98,99]. *ALOX15* overexpressing transgenics were protected from tumor growth and metastasis in two different mouse cancer models [100,101] and in different colorectal carcinoma cell lines *ALOX15* also exhibited anti-tumor properties [32,101]. On the other hand, overexpression of *ALOX15* in HCT116 colon carcinoma cells induced activation of ERK signaling, which upregulated the rate of cell proliferation. Treatment of these cells with a non-specific *ALOX* inhibitor blocked ERK activation, which is consistent with the pro-carcinogenic activity of the enzyme [102].

Cytokine-dependent Expression Regulation of Human *ALOX15*

IL4- and IL13-dependent expression regulation of *ALOX15* in human monocytes

In human circulating blood monocytes *ALOX15* is not expressed. However, when these cells were cultured *in vitro* in the presence of recombinant human IL4 (60-600 pM, which corresponds to 0.9-9 ng/ml) for 3 days *ALOX15* expression was strongly upregulated as indicated by immunohistochemistry, Northern blotting and activity assays [16]. The induced enzyme reacted with endogenous substrates since specific *ALOX15* products (15S-HETE) were detected in the membrane lipids. IL4-dependent *ALOX15* expression was time-dependent and maximal induction was reached after incubation periods longer than 48 h. These slow expression kinetics suggest that *ALOX15* does not belong to the immediate early genes of the IL4 response [16]. Two years later [17] it was reported that IL13, another classical Th2 cytokine, does also upregulate *ALOX15* expression. To explore the cell physiological context of IL4/13-induced *ALOX15* expression microarray-based expression profiles were recorded with human peripheral monocytes [103]. After 3 days of continuous IL4/13 exposure the six most strongly upregulated gene products were the following: *ALOX15*, fibronectin, monoamine oxidase-A, CD1c, CD23A and the coagulation factor XIII (transglutaminase). In fact, in IL4 treated monocytes *ALOX15* mRNA was almost 300-fold higher than in IL4-deficient control incubations [103]. In contrast to upregulation of *ALOX15* expression a number of classical pro-inflammatory gene products, such as tumor necrosis factor alpha, monocyte chemoattractant protein-1, IL1, IL6, IL8, IL18, cyclooxygenase-2, as well as enzymes and receptors of the leukotriene signaling cascade (*ALOX5*, *ALOX5*-activating protein, leukotriene B4 receptors, cysteinyl leukotriene receptor 2) were significantly downregulated. These expression regulation profiles are consistent with the hypothesis that IL4 treatment forces peripheral monocytes to adopt a resolving phenotype [103].

Specificity of cytokine-dependent expression regulation of LOX-isoforms: To explore the specificity of cytokine-dependent expression regulation of LOXs, two aspects have been investigated: i) Cytokine-specificity of *ALOX15* induction and ii) LOX-isoform specificity of IL4/13 induction.

i) *ALOX15* induction in human peripheral monocytes was IL4/13-selective since other cytokines such as interleukins-1, -2, -3, -5 and -6 (IL1, IL2, IL3, IL5, IL6) as well as interferon-gamma (IFN-g), granulocyte monocyte colony stimulating factor (GM-CSF), monocyte colony stimulating factor (M-CSF), platelet derived growth factor (PDGF), tumor necrosis factor (TNF), transforming growth factor (TGF) and phorbol myristate acetate (PMA) did not induce *ALOX15* expression [16]. Interestingly, incubation of the cells with IL4 in the presence of PMA or INFg did not lead to increased *ALOX15* expression suggesting an antagonizing effect of these cytokines [16]. IL10 is another classical Th2 cytokine that exhibits anti-inflammatory properties [104]. It binds to several subtypes of a cell surface receptor and activates STAT3-dependent intracellular signaling cascades. In contrast to IL4/13 this Th2-cytokine does not induce *ALOX15* expression in monocytes [17]. Erythropoietin is a hematopoietic cytokine, which regulates erythropoiesis on different levels [105]. It also exhibits activities outside the erythroid lineage [105] but does not induce expression of *ALOX15* in cultured human peripheral monocytes nor in A549 cells (H. Kuhn, unpublished data).

ii) To explore whether IL4/13 do also regulate the expression of other LOX-isoforms in human monocytes *ALOX12*, *ALOX15* and *ALOX15B* mRNA and protein was quantified during *in vitro* differentiation of isolated human monocytes [106]. Expression of *ALOX15B* was strongly upregulated (up to 10-fold) during the time course of *in vitro* incubation of the cells in the absence of any cytokines. This was not the case for *ALOX15* and *ALOX12*. In the presence of 10 ng/ml IL4/13, expression of *ALOX15* and *ALOX15B* was further upregulated whereas *ALOX12* expression was hardly altered. Comparison of the extent of IL4/13-dependent expression regulation of *ALOX15* and *ALOX15B* (fold change of mRNA concentrations in the absence and presence of cytokines) indicated that *ALOX15* was more strongly induced. In fact, in the presence of IL4 *ALOX15* expression was 300-fold upregulated whereas *ALOX15B* was only less than 3-fold up. A similar situation was observed for IL13 (80-fold upregulation of *ALOX15* vs. 3-fold upregulation of *ALOX15B*). These data are consistent with previous microarray and qRT-PCR data [103]. Interestingly however, if one quantifies the absolute copy numbers of *ALOX15* and *ALOX15B* mRNA in IL4 treated monocytes, similar expression levels were observed. In the presence of IL13, expression of *ALOX15B* mRNA was even dominating. Here the copy number of *ALOX15B* mRNA was higher than that of *ALOX15*. Taken together, these data indicate that IL4 and IL13 do not only induce expression of *ALOX15* in cultured human peripheral monocytes but also of *ALOX15B*. However, the extent of induction is higher for *ALOX15*. For the time being, the molecular mechanism of IL4-dependent *ALOX15B* expression has not been explored [106]. The different extents of induction, the different induction kinetics and the observation that expression of *ALOX15B* is already upregulated in the absence of any cytokine during *in vitro* cell culture (this is not the case for *ALOX15*) suggest mechanistic differences in the expression regulation of the two LOX-isoforms.

Cytokine-dependent *ALOX15* expression in other cells: To explore the mechanistic basis for IL4-dependent upregulation of *ALOX15* expression, a number of permanent cell lines were screened: HL60 (human myeloblastic), U937 (human promyelomonocyte), THP1 (human monocytic), MonoMac6 (human monocyte/macrophage), J774 and P388.D1 (mouse monocytic), A549, HTB56, HTB54 (all human lung carcinoma), HTB43 (human squamous head and neck carcinoma), HTB38 (human colon carcinoma), HMC1 cells (human mast cell). Unfortunately, most of them did not respond with upregulation of *ALOX15* expression when they were cultured in the presence of IL4 [107]. However, when the lung carcinoma cell line A549 was maintained in the presence of IL4 or IL13 for 24 h or longer *ALOX15* was expressed as indicated by RT-PCR, immunohistochemistry and activity assays. This effect was *ALOX15* specific, since expression of *ALOX5* and *ALOX12* remained low. The IL4 mutant Y124D, which constitutes an IL4 receptor antagonist, counteracted the effect of the wild-type cytokine. These data indicate that IL4-dependent expression regulation is cell specific and involves in A549 cells binding of the cytokine to the IL4/13 cell surface receptor [107].

IL4 and IL13 have been implicated in transdifferentiation of dendritic cells from hematopoietic precursor cells [108]. In the absence of IL4, dendritic cells that had been generated from CD34-positive precursors in response to a mixture of stem cell factor, granulocyte-macrophage colony stimulating factor and tumor necrosis factor alpha expressed high levels of *ALOX5* and *ALOX5* activating protein but hardly any *ALOX15*. Addition of IL4 to the cytokine mixture led to selective downregulation of *ALOX5* and strong upregulation of

ALOX15 [108]. Transforming growth factor beta1 counteracted the IL4-dependent suppression of *ALOX5* expression but did not alter *ALOX15* expression. These findings were consistent with the results of metabolomic studies, which indicated that in the absence of IL4 5-HETE and leukotriene B4 were the major eicosanoids produced by dendritic cells derived from CD34-positive precursors. In contrast, 15-HETE and 5S,15S-diHETE were the major eicosanoids formed in the presence of IL4. These data indicate that dendritic cells, which were transdifferentiated from peripheral monocytes *in vitro* in the presence of IL4, express large amounts of *ALOX15* [108]. However, it remains to be explored whether this effect is of any *in vivo* relevance. For the time being there is no convincing experimental evidence that *in vivo* differentiated dendritic cells express large amounts of *ALOX15*.

Orbital fibroblasts play a major role in tissue remodeling and have been implicated in the pathogenesis of Grave's disease, an autoimmune inflammatory disorder, which affects the orbit around the eye. Upper eyelid retraction, lid lag, bulging eyes and conjunctivitis are characteristic symptoms of this disease [109]. Resting orbital fibroblasts do not express *ALOX15*, but *in vitro* incubation of these cells in the presence of IL4/13 induce expression of the enzyme [110]. The intracellular signaling cascade involves Jak2 signaling since transient transfection of the cells with a dominant negative Jak2 mutant abolished *ALOX15* expression. Interestingly, IL4/13-dependent *ALOX15* induction was not detectable in dermal fibroblasts although these cells express the IL4 receptor and other elements of the intracellular IL4 signaling cascade [110].

Resting human umbilical vein endothelial cells (HUVEC) do not express *ALOX15* but when cultured in the presence of IL4 transcription of the *ALOX15* gene is induced [111]. However, activity assays and Western blotting did not provide any evidence for presence of the functional enzyme. Electrophoretic mobility shift assays indicated the activation of several transcription factors (STAT6, AP2, SP1, NF1), which have been implicated in expression regulation of *ALOX15* and these data suggest a functional IL4 signaling cascade in these cells [111]. The most plausible explanation for the apparent contradiction (presence of *ALOX15* mRNA vs. absence of functional *ALOX15* protein) is that IL4 in HUVEC induces transcription of the *ALOX15* gene, but that translation of the corresponding *ALOX15* mRNA is silenced. In immature red blood cells translation of *ALOX15* mRNA is inactivated by the binding of regulatory proteins to a repetitive sequence motif in the 3'-untranslated region of the mRNA [112,113]. If similar mechanisms of post-transcriptional regulation of *ALOX15* expression also occur in vascular endothelial cells, the experimental data obtained in this cellular system become plausible. Unfortunately, the presence of downregulatory proteins under these experimental conditions has not been explored.

For adipocytes, IL4 has been implicated in energy homeostasis [114]. It inhibits adipogenesis by downregulating the expression of peroxisome proliferator-activated receptor-gamma and the CCAAT/enhancer-binding protein-alpha [114]. In addition, IL4 upregulates the activity of hormone sensitive lipase (HSL) and thus, enhances lipolysis [114]. Unfortunately, it has not been explored yet whether *ALOX15* expression is upregulated in this cellular system and whether the enzymes might be involved in any of these processes.

Molecular mechanism of IL4/13-dependent expression regulation of *ALOX15*: IL4 and IL13 are two classical Th2 cytokines, which frequently exhibit anti-inflammatory activities. Both cytokines are capable of inducing *ALOX15* expression in peripheral human monocytes but the intracellular signaling mechanisms in these cells are

distinct [115]: i) IL4 induces Jak1 activation whereas the IL13-dependent signaling cascade involves Jak2 and Tyk2. ii) Tyk2 (IL13 response) controls STAT1 and STAT6 activation whereas Jak1 (IL4 response) upregulates STAT3 and STAT6. iii) IL13 utilizes both, the IL-4Ralpha/Jak2/Stat3 and IL-13Ralpha1/Tyk2/Stat1/Stat6 signaling pathways but IL4 can only employ the IL-4Ralpha/Jak1/Stat3/Stat6 cascade to upregulate the expression of *ALOX15*.

IL4-induced signalling: IL4 is typically liberated by activated Th2 cells (116). It binds to a cell surface receptor (IL4R), which consists of the common cytokine-receptor gamma subunit and the IL-4-binding chain (IL4R α). IL4R is mainly expressed on naive T cells and signals via activation of various transcription factors such as STAT6 (signal transduction and activator of transcription 6), GATA3 (GATA binding transcription factor-3), NFAT (nuclear factor of activated T cells), activating protein-1 (AP1) and others. Activation of these transcription factors upregulates expression of the constituents of the IL4 gene cluster on chromosome 5, which involves the genes encoding for IL4, IL5, and IL13 genes [116]. Thus, stimulation of naive T-cells with IL4 induces IL4 expression by these cells (autocrine loop), which strongly accelerates Th2 cell differentiation. However, Th2 cells do not express *ALOX15* and thus, the enzyme is unlikely to play a major role in T-cell differentiation.

The *ALOX15* promoter involves putative STAT6 binding sites [117] and serial promoter deletion studies as well as STAT6 binding site mutations suggested their functionality [118]. When we studied the molecular mechanisms of IL4-dependent expression regulation in A459 cells, we found that genistein (tyrosine kinase inhibitor) reduced phosphorylation of STAT6 and its recruitment to the *ALOX15* promoter [119]. Moreover, IL4 activated the histone acetyltransferase activity of the CREB-binding protein (CBP)/p300, which catalyzes acetylation of nuclear histones and of STAT6. This protein acetylation appears to be essential for the IL4-induced signaling cascade leading to *ALOX15* expression, since inhibition of the acetyltransferase activity of CBP/p300 by the E1A oncoprotein reduced histone and STAT6 acetylation and blocked transcriptional activation of the *ALOX15* gene. We also observed that the inhibition of histone deacetylases (addition of sodium butyrate) enhanced the IL4 induced *ALOX15* expression. Taken together, these data implicated STAT6 and the structure of nuclear histones in transcriptional activation of the *ALOX15* gene [119]. The critical role of the histone structure for IL4-dependent expression regulation of *ALOX15* was confirmed by recent studies on histone H3 trimethyl-lysine 27 (H3K27me3), which apparently interacts with the *ALOX15* promoter [120]. When A549 cells were incubated with IL4 demethylation of H3K27me3 occurs. This demethylation is catalyzed by a H3K27me2/3-specific demethylase (UTX) and siRNA-induced expression knockdown of UTX significantly attenuated H3K27me3 demethylation and *ALOX15* expression. The critical role of UTX in *ALOX15* expression was confirmed in human peripheral monocytes but here the H3K27me3-demethylase activity does not play a major role [120].

To identify additional regulatory elements in the IL4-induced signaling cascade, which lead to upregulation of *ALOX15* expression, differential protein binding studies were carried out [121]. For this purpose protein extracts of IL4-treated A549 cells and corresponding untreated controls were added to various promoter constructs and the binding proteins as well as their tryptic digest fragments were analyzed by MALDI-MS. The obtained proteinomic data identified the two major binding proteins as the Lupus KU autoantigens P86 and P70. Gel shift and supershift experiments employing monoclonal anti-Ku

antibodies confirmed the physical interaction of the Ku antigens with the *ALOX15* promoter and electroporation of neutralizing anti-Ku antibodies into A549 cells suppressed IL4-induced *ALOX15* expression [121].

As indicated above, IL4 has been related to the energy metabolism of adipocytes [114]. AMP-activated protein kinase (AMPK) is a key enzyme in the energy metabolism of all cells [122] but until recently no connection between IL4-dependent induction of *ALOX15* expression and AMPK has been described. Using primary human macrophages cultured *in vivo* in the presence of IL4 it has recently been shown, that activation of AMPK attenuated expression of *ALOX15* mRNA and protein [123]. Activators of AMPK (phenformin, aminoimidazole-4-carboxamide-1- β -D-ribofuranoside) also inhibited IL4-induced activation of STAT3, suggesting the involvement of this transcription factor in the IL4-dependent signaling cascade. Moreover, activation of AMPK (phenformin) prevented IL4-induced association of STAT6 and acetylation of histone H3 at the *ALOX15* promoter [123]. Taken together, these data suggest that activation of AMPK, which usually occurs under the conditions of energy deficiency, suppresses IL4-induced *ALOX15* expression and thus, interconnects the energy metabolism of cellular systems with the *ALOX15* pathway. Energy deficiency prevents IL4-dependent *ALOX15* expression or, if one sees it the other way around, IL4-dependent *ALOX15* expression might only occur in energy sufficient cells. Thus, the nutrition state of cells might impact their responsiveness to react with *ALOX15* expression when stimulated with IL4.

IL13-induced signalling: IL13 induces *ALOX15* expression in human peripheral monocytes [17] to a lesser extent than IL4 [106,124]. The IL13 induced intracellular signaling cascade, which leads to induction of *ALOX15* expression, is rather diverse and involves Jak2 and Tyk2 kinases as well as the transcription factors STAT 1, 3, 5, and 6 [125]. When cells are stimulated with IL13, serine phosphorylation of both STAT1 and STAT3, as well as activation of p38 mitogen-activated protein kinase (MAPK) was detected. Pharmacological inhibition of the MAPK pathway inhibited IL13-induced STAT1 and STAT3 phosphorylation as well as the DNA binding capabilities of the transcription factors [125]. These data suggest that IL13 induces p38 MAPK activation that upregulates STAT1 and STAT3 phosphorylation, which in turn activates the *ALOX15* gene.

In addition to MAPK another serine/threonine kinase, PKCdelta, has also been implicated in IL13-induced *ALOX15* expression [126]. When cells are treated with IL13, PKCdelta was rapidly phosphorylated. Rottlerin, an isoform specific PKCdelta inhibitor, blocked IL13-induced *ALOX15* expression but inhibitors of other PKC-isoforms were ineffective. Expression silencing of PKCdelta by siRNA oligonucleotides also inhibited *ALOX15* expression [126]. Interestingly, IL13-mediated activation of PKCdelta on one hand and IL13-dependent activation of p38 MAPK on the other are independent pathways, since inhibition of one kinase activity had no effect on the other. These data suggest that the two pathways act in parallel and may variably contribute to upregulation of *ALOX15* expression [126]. It remains to be worked out under which conditions the MAPK or the PKCdelta pathways prevail.

To make the situation even more complex, an ERK1/2-dependent signaling pathway has also been described [127]. When monocytes are exposed to IL13, rapid phosphorylation of ERK1/2 can be measured. Tyk2 kinase is required for ERK1/2 phosphorylation, which is independent of Jak2, p38MAPK and PKCdelta. To investigate the signaling mechanisms in more detail the possible involvement of

various transcription factors was also explored. In these experiments IL13 induces nuclear accumulation of the erythroblast transformation-specific gene related protein-1 (EGR1) and phosphorylation of the cAMP response element binding protein (CREB). These events are markedly attenuated by pharmacological inhibition of ERK1/2 [127]. Most importantly, expression silencing of EGR1 and CREB inhibited IL13-induced *ALOX15* expression. These results indicate an important regulatory role for ERK1/2 in mediating IL13-induced expression of *ALOX15* via the transcription factors EGR1 and CREB. This additional pathway broadens the intracellular multiplicity of IL13-induced signaling, which leads to upregulation of *ALOX15* expression.

***In vivo* relevance of IL4-induced *ALOX15* expression in humans:** The majority of studies carried out so far on IL4/13-induced *ALOX15* expression were performed in cellular *in vitro* systems, in which cultured human cells (primary cells or permanent cell lines) were incubated in the presence or absence of the cytokines. In most cases the observed effects were dramatic and robust but it still remains unclear whether they are of any *in vivo* relevance. IL4 is a classical Th2 cytokine, which is secreted in large amounts during the allergic reaction [128,129]. To answer the question of whether patients suffering from allergic diseases have elevated IL4 plasma levels and whether the IL4 concentrations reached under these conditions *in vivo* are sufficiently high to induce *ALOX15* expression, we employed a dual research strategy. First, we compared the IL4 concentrations required for *in vitro* induction of *ALOX15* in human monocytes with the plasma levels of IL4 measured in normal human beings and in patients with early rheumatoid arthritis. Here we found, that for *in vitro* induction of *ALOX15* expression IL4 concentrations of 1-10 ng/ml are typically needed (Table 1). In contrast, measurements of the IL4 concentrations in healthy humans [130] and in patients suffering from early rheumatoid arthritis [131] revealed concentrations ranging from 5-10 pg/ml. When we determined the IL4 plasma levels (see below) in normal human beings and in patients suffering from allergic diseases (bronchial asthma, allergic rhinitis, atopic ekzema) we obtained values ranging between 0.1 to 11 pg/ml. Thus, the IL4 plasma concentrations determined in our (Figure 3) and in previous studies [130,131] are up to three orders of magnitude lower than the values required for *in vitro* induction of *ALOX15*.

It should, however, be stressed that local IL4 concentrations, for instance in the bone marrow or in certain environments of peripheral tissue, might be much higher than equilibrium concentrations in the plasma. Moreover, unknown factors in the plasma might sensitize monocytes towards IL4. To address this problem we next quantified the arachidonic acid oxygenase activity of monocytes prepared from patients suffering from allergic diseases and from healthy controls. For this purpose we collected blood from patients suffering from different entities of allergic disorders (bronchial asthma, allergic rhinitis, atopic ekzema), prepared the peripheral monocytes and carried out *in vitro* activity assays measuring the formation of 15S-HETE from exogenous arachidonic acid. In parallel, we quantified the corresponding plasma levels of both IgE (readout parameter for the severity of the allergic disease) and IL4. As indicated in Table 2 the IgE levels of the patients suffering from the allergic diseases were significantly ($p < 0.001$) elevated indicating the activity state of the disease. Next, we compared the IL4 plasma concentrations of the patients with those of healthy controls and found elevated IL4 levels in allergic patients (Figure 3). Although the extent of elevation was not dramatic, the difference was highly significant ($p < 0.001$). Quantification of the arachidonic acid oxygenase activity of the monocytes revealed a higher activity of the "allergic monocytes" when compared with "healthy control" cells

(Table 3). However, more detailed analysis of the chemical structure of the oxygenation products (SP-HPLC) indicated that these products did not originate from the *ALOX15* pathway. In fact, a mixture of all major HETE isomers was detected and the 15-HETE identified in this mixture was racemic. These results are inconsistent with an *ALOX15* origin of the oxygenation products. We also attempted to detect the

ALOX15 protein in some of the monocyte preparations using our anti-*ALOX15* antibody (immunoblotting) but the amounts of *ALOX15* in the cells were below detection limits. Finally, we correlated the arachidonic acid oxygenase activity of the monocytes with the individual IL4 plasma concentrations but did not observe a significant correlation.

Reference	Cell type	IL4/13 (ng/ml)
Conrad et al. [16]	peripheral monocytes	1-10
Nassar et al. [17]	peripheral monocytes	2
Brinckmann et al. [107]	alveolar epithelial cells (A549)	10
Heydeck et al. [132]	peripheral monocytes, macrophages	15
Kelavkar et al. [121]	alveolar epithelial cells (A549)	0.2
Lee et al. [111]	human umbilical vein endothelial cells	10-50
Shankaranarayanan et al. [119]	alveolar epithelial cells (A549)	10
Spanbroek et al. [108]	dendritic cells (monocyte derived)	50
Chaitidis et al. [103]	peripheral monocytes	10
Chen et al. [110]	orbital fibroblasts	10
Wüst et al. [106]	peripheral monocytes	10
Bhattacharjee et al. [115]	peripheral monocytes	10
Han et al. [120]	alveolar epithelial cells (A549)	50
Namgaladze et al. [123]	peripheral monocytes	20

Table 1: IL4 concentrations used for *in vitro* induction of *ALOX15* expression.

Consequently, one has to conclude that for the time being there is no experimental evidence for an increased expression of *ALOX15* in the monocytes of allergic patients. Thus, it still remains to be explored of whether or not the IL4/13- dependent induction of *ALOX15* expression is of any biological relevance in humans.

IgE level (kU/ml)	n	median	25% percentile	75% percentile	Significance
Healthy controls	44	12	6	30	-
Allergic rhinitis	46	1028	165	1265	0.001
Bronchial asthma	20	473	156	1060	0.001
Atopic ekzema	15	564	347	1412	0.001

Table 2: Plasma IgE concentrations of patients suffering from allergic diseases and of healthy controls. EDTA blood was drawn from the patients, the plasma was prepared and aliquots (5 µl) were used to quantify the IgE levels by solid phase enzyme immunoassay of DPC Biermann. The experimental raw data were evaluated with the Mann-Whitney U test and the significance values p were calculated in comparison to the healthy controls.

IgE level (kU/ml)	n	median	25% percentile	75% percentile	Significance
Healthy controls	44	12	6	30	-
Allergic rhinitis	46	1028	165	1265	0.001
Bronchial asthma	20	473	156	1060	0.001
Atopic ekzema	15	564	347	1412	0.001

Healthy controls	42	2.5	1.1	8.8	-
Allergic rhinitis	45	12.9	6.6	34.9	<0.001
Bronchial asthma	19	6.9	2.4	13.6	<0.001
Atopic ekzema	13	6.0	3.1	7.8	<0.001

Table 3: Arachidonic acid oxygenation capacity of peripheral monocytes prepared from allergic patients and healthy controls. 50 ml of EDTA blood was drawn from the patients and the mononuclear cells were prepared by density gradient centrifugation (Ficoll gradient). The cells were plated in Petri-dishes overnight and the non-adhering cells were discarded. The adhering monocytes were scraped off, spun down and reconstituted in 1 ml of PBS. Arachidonic acid (100 µM final concentration) was added and the cells were incubated for 20 min at room temperature. 1 ml of methanol was added, protein precipitate was spun down and aliquots of the clear supernatant were injected to RP-HPLC for quantification of the arachidonic acid oxygenation products. A solvent system of methanol/water/acetic acid (85:15:0.1, by vol) was used to develop the chromatograms and the absorbance at 235 nm was recorded. The chromatographic scale was calibrated with known amounts of 15-HETE. The experimental raw data were evaluated with the Mann-Whitney U test and the significance values p were calculated in comparison to the healthy controls.

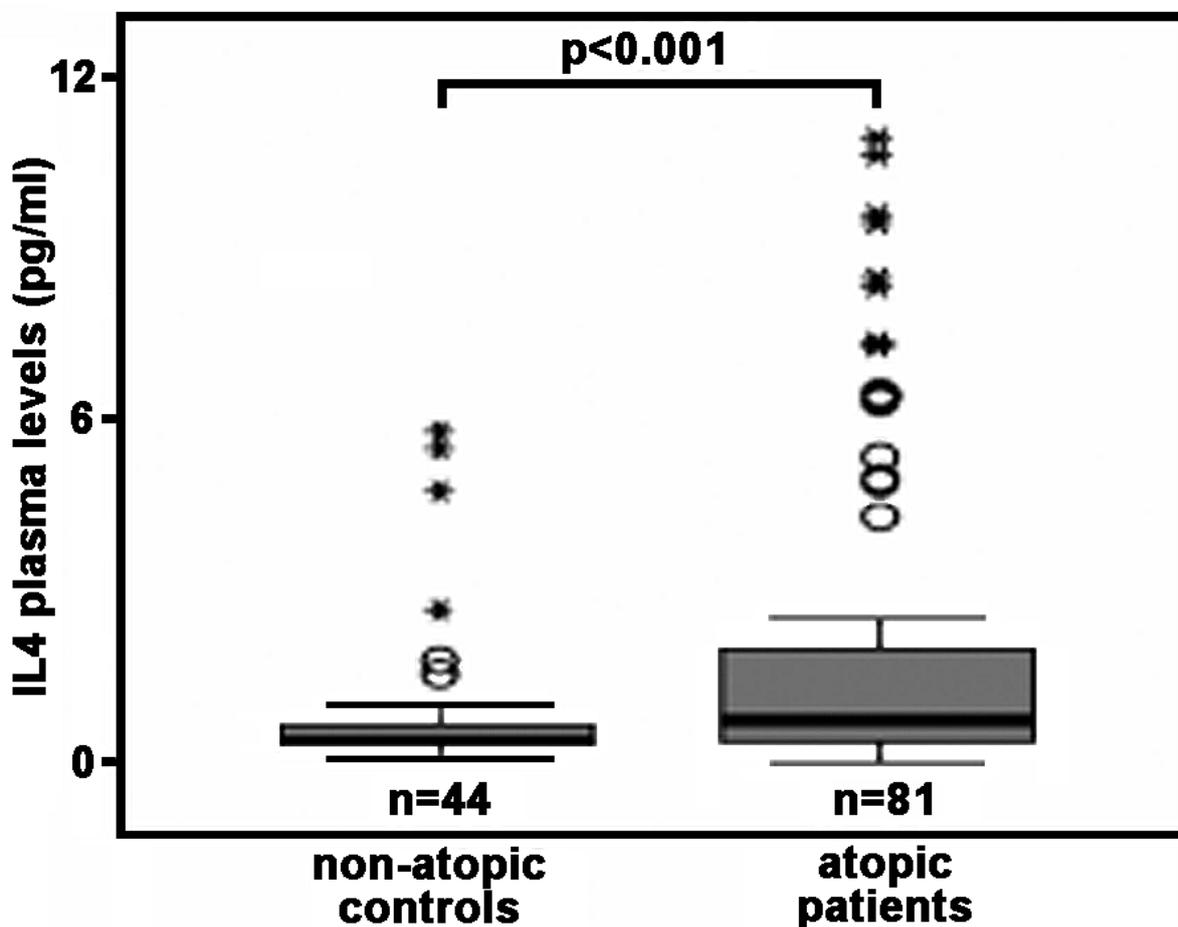


Figure 3: Comparison of the IL4 plasma levels of patients suffering from allergic diseases. The IL4 plasma levels were determined by ELISA for patients suffering from bronchial asthma, allergic rhinitis, atopic dermatitis (allergic patients) and from healthy controls. The level of statistical significance was calculated using the Mann-Whitney U test.

Regulation of ALOX15 in mice

ALOX15 expression in mouse monocytes and macrophages: In contrast to the human ortholog, mouse ALOX15 is a 12-lipoxygenating enzyme [65]. To explore whether human and mouse ALOX15 orthologs exhibit a similar sensitivity for cytokine dependent expression regulation, the impact of IL4/13 on ALOX15 expression was studied in mouse peritoneal macrophages and peripheral monocytes. Activity assays indicated that IL4 and IL13, but not IL10 upregulated ALOX15 activity in a dose-dependent manner [132]. Interferon gamma completely prevented the induction of ALOX15 [16,133]. In contrast, basal expression of ALOX15 in mouse peritoneal macrophages was not suppressed by interferon gamma [133]. Moreover, the time course of IL4-dependent expression regulation was dramatically different between men and mice. In human monocyte-derived macrophages ALOX15 expression is up for a time period of up to 72 h but then declines at longer incubation periods. In contrast, residential mouse peritoneal macrophages constitutively express ALOX15 but their activity declines by more than 90% when the cells are cultured in the absence of IL4. Interestingly, addition of IL4 prevented this decline [133].

In vivo relevance of the inducing effect IL4 on ALOX15 expression: Murine ALOX15 is constitutively expressed at high levels in peritoneal macrophages [65,132,134] and addition of IL4 to *in vitro* cell cultures augmented the expression level in peritoneal macrophages [132]. Interestingly, no upregulation of ALOX15 activity was observed when macrophages of STAT6-deficient mice were employed suggesting the involvement of STAT6 in the signaling cascade. In contrast, macrophages prepared from transgenic mice, which systemically overexpress IL4 exhibited a 3-4-fold higher ALOX15 activity when compared with cells prepared from control mice [132]. A similar upregulation was detected in other organs (heart, spleen, lung). Unfortunately, it has not been tested whether this gain in activity might be related to contaminating monocytes in the organs. These data suggest that as for human ALOX15, expression of the mouse ortholog can be upregulated *in vitro* by the addition of IL4.

However, this experimental setup did not answer the question of whether or not constitutive expression of ALOX15 in mouse peritoneal macrophages involves IL4 as decisive signaling molecule. This question was answered by testing the ALOX15 activity of peritoneal macrophages prepared from IL4^{+/+} and IL4^{-/-} mice [133].

Interestingly, there was no difference in the *ALOX15* activity of these two cell preparations and these data indicate that IL4 is not required for constitutive expression of this enzyme in mouse macrophages.

Open questions and perspectives

The classical Th2 cytokines IL4 and IL13 are powerful inducers of *ALOX15* expression in human [16,17] and mouse peripheral monocytes [132], although in mice constitutive expression of this enzyme in peritoneal macrophages is not IL4-dependent [133]. A number of other cytokines including IL1, IL2, IL3, IL4, IL5, IL6, IFN γ , GM-CSF, M-CSF, PDGF, TNF and TGF did not induce expression of this enzyme. However, most other cytokines have not been tested. The multiplicity of the cytokine family is constantly growing and it is about time to initiate a systematic screen of untested cytokines for their *ALOX15* inducing capacity. The assay systems (qRT-PCR, immunoblotting, activity assays) for such as screen have all been worked out and the required tools (specific amplification primers, *ALOX15* specific antibody, activity assay protocols) are available. The major problem for such experiments is the relatively high cost of the recombinant cytokines.

The recent finding that IL4-induced *ALOX15* expression is prevented by upregulation of AMPK interconnects the energy metabolism with eicosanoid biosynthesis, in particular with the *ALOX15* pathway. If such coupling can also be observed in other cellular systems, *ALOX15* might constitute part of cellular fuel sensors, which adapt the energy metabolism to fuel supply. If this is the case *ALOX15* inhibitors might interfere with the energy metabolism, which could be of biological relevance for lipid storage diseases, such as obesity or on the other hand, lipodystrophy. The recent finding that IL4 in adipocytes inhibits triglyceride synthesis and upregulates the activity of hormone sensitive lipase suggests the relevance of IL4 for storage lipid homeostasis [114]. However, a possible role of *ALOX15* in this metabolic scenario remains to be explored.

The molecular mechanisms for IL4/13-induced *ALOX15* expression have extensively been studied and the current mechanistic picture is already very complex. IL4/13 cell surface receptors are clearly involved but the downstream signaling cascades are quite diverse. A number of different protein kinases have been implicated, which lead to activation of several transcription factors. The current picture suggests that there is not a single but several distinct signaling cascades, which include modification of the histone structure. In complex regulatory networks like this there is always room for alternative or supplemental signaling events. One specific question that needs to be addressed is the molecular basis for AMPK-dependent suppression of IL4-induced expression regulation. It has been suggested that AMPK activation may directly interfere with STAT3 and/or STAT6 activation or histone acetylation [123] but for the time being there are no experimental data supporting this hypothesis.

The biological relevance of IL4/13-induced upregulation of *ALOX15* expression remains to be explored in more detail. In mice, constitutive expression of *ALOX15* in peritoneal macrophages does not depend on IL4 expression [133]. In humans preliminary data suggest that the plasma concentrations of IL4 are simply not high enough to induce expression of the enzyme in peripheral monocytes and it remains questionable whether IL4 concentrations in the range of 1-10 ng/ml, which are required for the *in vitro* effect, can be reached *in vivo*. One major problem in this respect is, that to the best of our knowledge, there is no major human disease, which is consistently associated with

strongly elevated plasma IL4 levels. In different allergic diseases (see 3.1.4.) we did not find sufficient elevations of the IL4 plasma levels. IL4 has been used in clinical trials for the treatment of chronic lymphatic leukemia [135]. In this study IL4 was well tolerated but the anti-tumor activity observed in previous *in vitro* studies could not be confirmed. Unfortunately, the impact of the administered IL4 on *ALOX15* expression in peripheral monocytes has not been explored in this study. Although the systemic IL4 concentrations in the treated patients have not been quantified, a rough estimate suggested that plasma concentrations in the lower ng/ml range should have been reached. IL4 was administered subcutaneously at doses varying between 2-6 μ g/kg day. If one neglects drug elimination and assumes homogenous distribution of the administered IL4 in the body water (which is probably not the case), IL4 concentrations in the one digit ng/ml range should have been reached. Since there are no reliable data characterizing distribution, compartmentation and elimination of exogenous IL4 in humans such model calculations neglecting these parameters may not be very informative.

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