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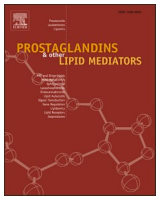

Formation of lipoxins and resolvins in human leukocytes

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ABSTRACT

Specialized pro-resolving lipid mediators (SPMs) such as lipoxins or resolvins are formed by the consecutive action of 5-lipoxygenase (5-LO, *ALOX5*) and different types of arachidonic acid 12- or 15-lipoxygenases using arachidonic acid, eicosapentaenoic acid or docosahexaenoic acid as substrate. Lipoxins are trihydroxylated oxylipins which are formed from arachidonic and eicosapentaenoic acid. The latter can also be converted to di- and trihydroxylated resolvins of the E series, whereas docosahexaenoic acid is the substrate for the formation of di- and trihydroxylated resolvins of the D series. Here, we summarize the formation of lipoxins and resolvins in leukocytes. From the data published so far, it becomes evident that FLAP is required for the biosynthesis of most of the lipoxins and resolvins. Even in the presence of FLAP, formation of the trihydroxylated SPMs (lipoxins, RvD1-RvD4, RvE1) in leukocytes is very low or undetectable which is obviously due to the extremely low epoxide formation by 5-LO from oxylipins such as 15-H(p)ETE, 18-H(p)EPE or 17-H(p)DHA. As a result, only the dihydroxylated oxylipins (5 *S*,15*S*-diHETE, 5 *S*,15*S*-diHEPE) and resolvins (RvD5, RvE2, RvE4) can be consistently detected using leukocytes as SPM source. However, the reported levels of these dihydroxylated lipid mediators are still much lower than those of the typical pro-inflammatory mediators including the monohydroxylated fatty acid derivatives (e.g. 5-HETE), leukotrienes or cyclooxygenase-derived prostaglandins. Since 5-LO expression is mainly restricted to leukocytes these cells are considered as the main source of SPMs. The low formation of trihydroxylated SPMs in leukocytes, the fact that they are hardly detected in biological samples as well as the lack of functional signaling by their receptors make it highly questionable that trihydroxylated SPMs play a role as endogenous mediators in the resolution of inflammation.

1. The 5-LO:12/15-LO pathway in SPM formation

Human 5-lipoxygenase (5-LO) catalyzes the conversion of arachidonic acid (AA) and eicosapentaenoic acid (EPA) to leukotriene A_4 (LTA_4) and LTA_5, respectively. LTA_4 is an unstable intermediate which can be further converted by LTA_4 hydrolase to leukotriene B_4 (LTB_4) or by LTC_4 synthases to LTC_4 [1]. Leukotrienes have been extensively studied and are considered as part of the innate immune system representing mediators of inflammation and allergy [2]. Alternatively, LTA_4 and LTA_5 released from leukocytes can be taken up by cells (e.g. platelets) that express 12/15-lipoxygenases and are then converted to lipoxins. This pathway consists of two consecutive oxidation steps catalyzed by 5-LO followed by a 12/15-LO which is referred to as the 5-LO:12/15-LO pathway. In contrast to the originally identified oxylipins like leukotrienes, lipoxins are classified as specialized pro-resolving mediators (SPMs) representing a group of oxylipins which have been suggested to be involved in the inhibition of inflammation and promotion of its resolution [3].

Within the 5-LO:12/15-LO pathway, 5-LO first converts AA, EPA and docosahexaenoic acid (DHA) to 5-HpETE, 5-HpEPE and 7-HpDHA, respectively. These oxylipins then serve as substrates for 12/15-lipoxygenating enzymes leading to a number of dihydroxylated products such as 5,15-diHETE, 5,15-diHEPE (RvE4) and 7,17-diHDHA (RvD5) (Fig. 1). Alternatively, 5-LO forms LTA_4 from AA which can also serve as substrate for the following 12/15-lipoxygenation step leading to lipoxins. A similar reaction occurs with EPA where 5-LO generates LTA_5

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which is then converted to the 5-series of lipoxins.

2. The 12/15-LO:5-LO pathway

12/15-LO:5-LO pathway is generally considered as the main pathway for SPM formation. Here, 5-LO together with 15-LO1 (*ALOX15*) or 15-LO2 (*ALOX15B*) may produce lipoxins, D-series resolvins and RvE4 from their corresponding PUFAs (Fig. 1) [4–6]. Initially AA, EPA or DHA are converted to 15-H(p)ETE, 15-H(p)EPE and 17-H(p)DHA, respectively by a 15-LO. 15-H(p)ETE and 15-H(p)EPE can be then further converted by 5-LO to 5,15-diHETE, LXA_4, LXB_4 and RvE4 (5 S, 15S-diHEPE), LXA_5 and LXB_5. 17-H(p)DHA which is formed from DHA

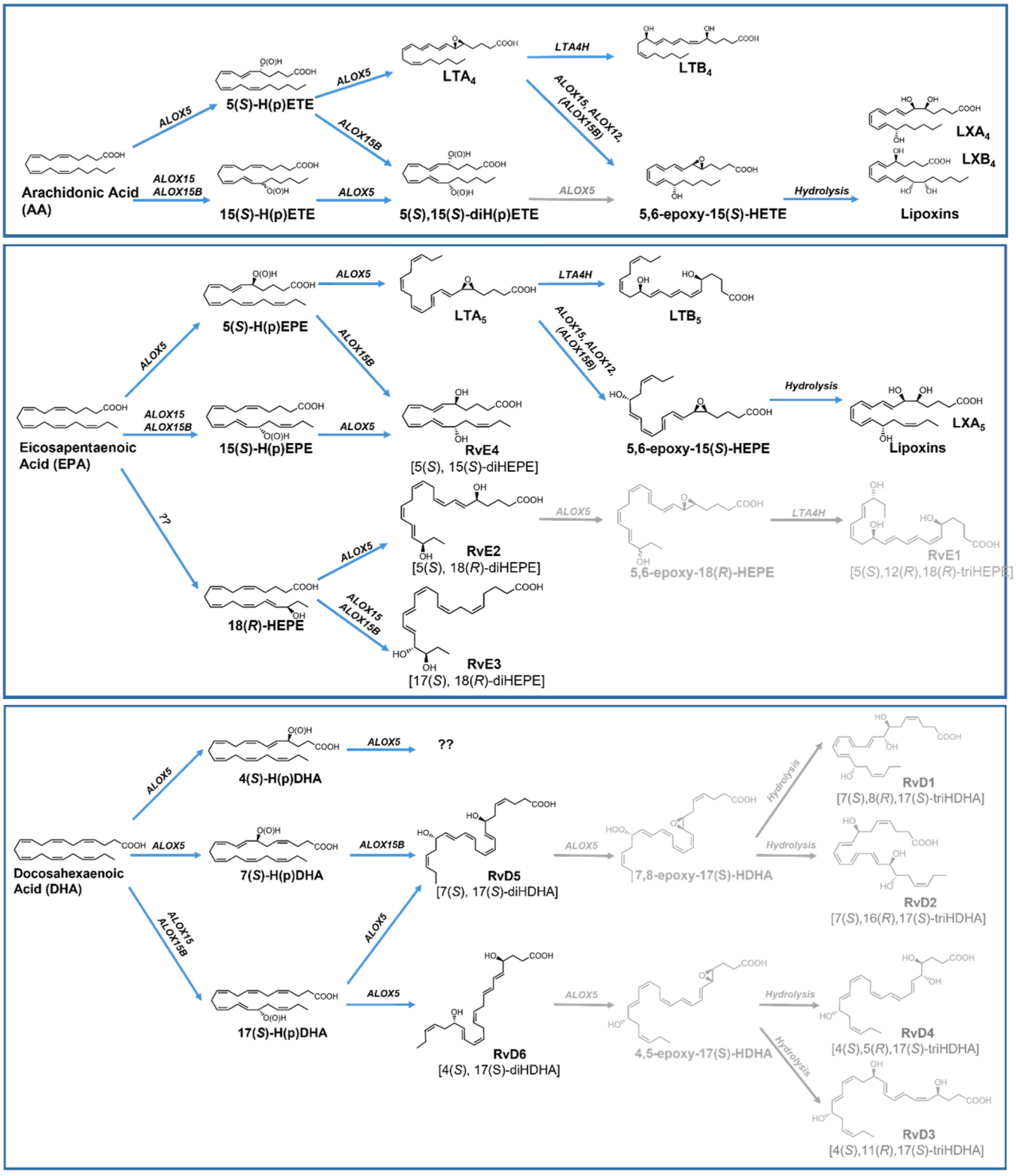

Fig. 1. SPM formation from AA, EPA and DHA in leukocytes via the 5-LO:12/15-LO pathway and the 12/15-LO:5-LO pathway. Inefficient biosynthesis routes for trihydroxylated SPMs are shown in gray (see also Table 2).

by 15-LO was reported to be converted to RvD1–6 [4,5]. Of note, in contrast to human 15-LO2, human 15-LO1 oxygenates DHA to a 1:1 mixture of 17-HDHA and 14-HDHA [7] (Fig. 1).

A hallmark of the proposed 12/15-LO:5-LO route of SPM formation is that the fatty acid substrates AA, EPA and DHA are initially oxygenated by 15-LO1/15-LO2 or other oxygenases such as CYPs to the corresponding monohydro(pero)xy-fatty acids (15-H(p)ETE, 15-H(p)EPE, 18-H(p)EPE or 17-H(p)DHA) and in a second step by 5-LO leading to lipoxins and resolvins of the D- and E-series (Fig. 1).

3. Biosynthetic routes of di- and trihydroxylated SPMs – enzymatic prerequisites

A prerequisite for the formation of SPMs via the 5-LO:12/15-LO pathway is the acceptance of substrates which carry a hydroxy or hydroperoxy group in position 5 (AA, EPA) or 7 (DHA) of the precursor PUFA by the 12- or 15-lipoxygenating enzymes. In case of lipoxins an epoxide at position 5 and 6 of AA or EPA has to be accepted. Indeed, both human 15-LO isoforms have been shown to accept these precursors (Table 1). 15-LO2 accepts 5-HETE, 5-HEPE and 7-HDHA for conversion to 5,15-diHETE, RvE4 and RvD5, respectively. Furthermore, the enzyme also accepts the corresponding hydroperoxides in a similar fashion [8–10] (Table 1). Human 15-LO1 also accepts 5-HEPE and 7-HDHA but the major reaction products are 5,12-diHEPE and 7,14-diHDHA instead of RvE4 and RvD5 [11]. In accordance with these data, RvE4 was formed in incubations of recombinant human 15-LO2 with 5-LO upon EPA supplementation but not in co-incubations of 5-LO with 12-LO and 15-LO1 [12]. This suggests that the formation of 5,15-diHETE, RvE4 and RvD5 via the 5-LO:12/15-LO pathway is 15-LO2 restricted.

Table 1
Comparison of enzyme kinetics of purified human lipoxygenases with different substrates.

Enzyme	Substrate	k_{cat} (s^{-1})	k_M (μM)	V_{max} (mol s^{-1} mol^{-1})	Reference
5-LO	AA	1.2 ± 0.4	3.3 ± 0.9	0.15 ± 0.3 0.17 ± 0.5 (V_{10})	[9,11]
5-LO	EPA			0.14 ± 0.01	[11]
5-LO	DHA			0.14 ± 0.03	[10]
5-LO	15*S*-HETE			0.0013 ± 0.0004 (V_{10})	[9]
5-LO	15*S*-HpETE			0.0020 ± 0.0001 (V_{10})	[9]
5-LO	15*S*-HEPE			0.0037 ± 0.0006	[11]
5-LO	14*S*-HDHA			0.00038 ± 0.0002	[10]
5-LO	14*S*-HpDHA			0.0015 ± 0.0006	[10]
5-LO	17*S*-HDHA			0.0012 ± 0.0004	[10]
5-LO	17*S*-HpDHA			0.0014 ± 0.0005	[10]
5-LO	5 *S*,15*S*-diHpETE			no conversion to LXA_4	[9]
15-LO1	AA	10 ± 1	5.0 ± 0.3		[9]
15-LO1	EPA				
15-LO1	DHA	0.95 ± 0.1	1.4 ± 0.3	0.84 ± 0.09	[10]
15-LO1	5*S*-HETE	1.1 ± 0.4	4.9 ± 0.6		[9]
15-LO1	5*S*-HpETE	1.8 ± 0.2	7.8 ± 2		[9]
15-LO1	7*S*-HDHA	3.1 ± 0.1	6.1 ± 0.5	1.9 ± 0.1	[10]
15-LO1	7*S*-HpDHA	0.57 ± 0.08	6.9 ± 0.8	0.30 ± 0.4	[10]
15-LO1	5,15-diHpETE	4.6 ± 0.1	23 ± 2		[17]
15-LO1	5,15-diHETE	no reaction			[17]
15-LO2	AA	0.96 ± 0.09	5.0 ± 0.2		[9]
15-LO2	EPA	2.1 ± 0.02	2.9 ± 0.03		[91]
15-LO2	DHA	3.0 ± 0.3	11 ± 2	1.3 ± 0.1	[10]
15-LO2	5*S*-HETE	2.1 ± 0.2	29 ± 2		[9]
15-LO2	5*S*-HpETE	2.0 ± 0.2	57 ± 3		[9]
15-LO2	7*S*-HDHA	5.8 ± 0.4	40 ± 4	1.2 ± 0.1	[10]
15-LO2	7*S*-HpDHA	3.4 ± 0.4	41 ± 7	0.67 ± 0.08	[10]

In addition to 5-H(p)ETE, LTA_4 is accepted as substrate by 15-LO1 for the formation of lipoxins [13]. Alternatively, platelet 12-LO efficiently converts LTA_4 to LXA_4 [14–16].

For the formation of SPMs via the 12/15-LO:5-LO pathway, 5-LO has to accept the monohydro(pero)xylated fatty acids as substrate. However, in the absence of 5-lipoxygenase activating protein (FLAP), the conversion rate of oxylipins by human 5-LO is low (Table 1). Conversion of 15-HpETE to 5,15-diHpETE by human 5-LO was found to be 10-fold lower compared to the conversion of AA to 5-HpETE and no conversion of 5,15-diHpETE to LXA_4 was observed [17]. The latter observation indicates that recombinant 5-LO cannot form the required 5,6-epoxide via its LTA_4 synthase activity. Similarly, 15-HETE is hardly converted to LXA_4 isomers by purified human 5-LO [16,18]. In fact, several studies with recombinant human 5-LO demonstrated that the conversion of the monohydro(pero)xylated precursors is around two orders of magnitude lower compared to the respective parent fatty acids AA and DHA [9,10,16] (Table 1). In our hands, 17-HDHA was not converted by recombinant human 5-LO and we could not detect resolvins such as RvD5 in the reaction mixture [18]. When 18 *R*,*S*-HEPE was offered as substrate to 5-LO, the dihydroxy derivative 5,18-diHEPE (RvE2) was formed in easily detectable amounts but RvE1 was only found at trace levels [12]. These data suggest that in the absence of FLAP oxylipins are poor substrates for purified human 5-LO in the usually applied in-vitro systems.

The biosynthesis of a number of E-series resolvins (RvE1, RvE2, RvE3) is dependent on 18*R*-HEPE, however the origin of this oxylipin in human cells is unknown so far. Considering the reaction specificity of human LO-isoforms, all known enzymes can be ruled out as a source for efficient 18-HEPE formation from EPA. In fact, when EPA reacted with the different human recombinant LOs (5-LO, 12-LO, 15-LO2, 15-LO1) only 15-LO1 formed minute amounts of 18-HEPE (*S*/*R*-ratio less than 3:1) showing that 18-HEPE formation via the 15-LO pathway is highly inefficient in humans [12]. Cytochrome P450 enzymes might constitute an alternative source for 18*R*-HEPE [19]. Unfortunately, no data are available at the moment to prove this hypothesis.

The EPA derivative RvE3 (17,18-diHEPE) is a SPM which is not dependent on 5-LO activity. We found that human recombinant 15-LO2 forms the 18 *S* stereoisomer of RvE3 in presence of EPA as well as 18 *R*/*S*-RvE3 when the 18 *R*/*S*-HEPE racemate is used as substrate, although the amounts formed were very low. Of note, recombinant human 15-LO1 did not produce significant amounts of RvE3 [12]. In contrast to this, release of RvE3 has been found in incubations of 18-HEPE-supplemented human eosinophils as well as in HEK293 cells transiently transfected with 15-LO1 upon stimulation with Ca^{2+} ionophore A23187 [20].

4. SPM formation in human granulocytes

Although lipoxin formation is very poor in enzyme-based in-vitro systems, lipoxin formation by 5-LO could be detected in human leukocytes [21,22]. For the 12/15-LO:5-LO pathway, it could be shown that 5-LO can accept 15-HETE as substrate in leukocytes when 5-LO interacts with FLAP which provides 5-LO with fatty acid substrates [23–25]. The finding that there is a FLAP-dependent conversion of certain oxylipins can explain the differences between the cellular and the enzyme and cell-based in-vitro experiments. Based on these observations, it was shown that FLAP is required for the biosynthesis of 5,15-diHETE, LXA_4, RvD1, RvD5 and RvE1 since their formation can be efficiently inhibited by the FLAP inhibitor MK-886 [16,18]. It was suggested that SPM formation mainly occurs by cytosolic 5-LO which might interact with

cytosolic 12/15-LOs (*ALOX15*, *ALOX15B*) [26]. This suggestion was mainly based on the observation that inhibition of p38 MAPK reduces nuclear localization of 5-LO and slightly reduces LTB_4 formation whereas LXA_4 formation was increased by 2-fold. However, the fact that the FLAP inhibitor MK-886 completely inhibits lipoxin formation in the 12/15-LO:5-LO pathway as well as in the 5-LO:12/15-LO pathway [16,18] precludes that there is cytosolic SPM formation by 5-LO as FLAP is located in the nuclear membrane [27]. Based on both observations, it is most likely that 5-LO, which translocates from the cytosol to FLAP at the nuclear envelope, is more efficient in SPM formation than 5-LO that approaches FLAP from the nuclear compartment.

Neutrophils which prominently express FLAP and 5-LO show only low or no expression of 12/15-LO and thus have low AA 12/15-lipoxygenating activity. Therefore, it is not surprising that no substantial amounts of AA- and DHA-derived SPMs were detected in ionophore-stimulated PMNL which mainly consist of neutrophils [18]. To mimic transcellular SPM formation where cells with 12/15-LO activity produce SPM precursors from AA, EPA and DHA and provide them to PMNL for further conversion by 5-LO/FLAP, 15-HETE, 17-HDHA and 18-HEPE were added to PMNL and the cells were stimulated with ionophore. With 15-HETE, LXA_4 and 6*S*-LXA_4 formation was about 75- and 110-times lower compared to 5,15-diHETE, suggesting that formation of the 5,6-epoxy-15-hydroxy intermediate is unfavorable [18] (Fig. 1). From 17-HDHA, only the dihydroxylated SPM RvD5 was formed in easily detectable amounts. When 18-HEPE was offered as substrate, the formed amounts of RvE1 and RvE2 were more than 30- and 90-fold lower as compared to 17-HDHA-derived RvD5 [18].

The data suggest that the 12/15-LO:5-LO pathway where the granulocyte 5-LO uses SPM precursors released from neighboring cells is an inefficient source of trihydroxy SPMs (lipoxins, resolvins D1–4, resolvin E1) (Fig. 1). The formation of dihydroxy SPMs, such as RvD5 (7,17-diHDHA), RvE2 (5,18-diHEPE), and RvE4 (5,15-diHEPE), appears to be more efficient [28], at least if DHA, EPA or the respective oxylipin precursors are added and the cells are stimulated with calcium-mobilizing agents. The low formation of trihydroxylated SPMs, even in these in-vitro systems, could be related to the fact that formation of the 5,6-epoxide from hydroperoxy precursors by 5-LO is rather slow. The pattern of SPM formation (high levels of dihydroxylated and minute amounts of trihydroxylated lipids) was comparable for different 5-LO stimuli tested (LPS/fMLF, S1P, phenol soluble modulin-α, Ca^{2+} ionophore and osmotic stress) [18]. Ca^{2+} ionophore was the most potent stimulus in PMNL, probably due to the strong mobilization of intracellular Ca^{2+}.

However, it should be considered that stimulation of PMNL with Ca^{2+} ionophore and addition of AA, EPA and DHA does not reflect the in vivo situation where weaker cell stimulation and lower substrate availability occurs. The AA, EPA and DHA levels in human cells depend on the diet [29]. In leukocytes from subjects with Western diet, AA is present and thus released at higher levels than EPA or DHA from cell membranes upon cell stimulation which would favor the formation of AA-derived oxylipins versus the EPA or DHA derived oxylipins [30,31]. Taken together, the data revealed that detectable formation of SPMs in PMNL is largely restricted to non-physiological conditions such as ionophore-mediated cell stimulation in combination with the exogenous supply of the respective polyunsaturated fatty acid substrates.

In contrast to neutrophils, human and mouse eosinophils prominently express 15-LO1. Accordingly, these cells biosynthesize high amounts of 14-HDHA and 17-HDHA, low amounts of the dihydroxylated SPM RvD5 but also low amounts of RvD4 in the presence of DHA. The levels of the other D-series resolvins were very low or undetectable [32]. Noteworthy, the stimulation of eosinophils with platelet-activating factor (which increases intracellular Ca^{2+} concentrations and thus 5-LO activity) did not increase the formation of D-series resolvins while increasing the leukotrienes.

Of note, efficient in vitro formation of lipoxins via the 5-LO:12/15-LO pathway takes place in platelet/leukocyte co-incubations treated with stimuli such as Ca^{2+} ionophore (A23187) or the combination of fMLF and thrombin in a transcellular fashion. Here, granulocytes release 5-LO-derived LTA_4 which is subsequently taken up by platelets followed by conversion to lipoxins by platelet-type 12-LO (*ALOX12*) [14–16] (Fig. 1). However, *Alox12* knockout mice do not show a resolution defect (for review see [33]) and the lipoxin levels in biological samples are low (see chapter 6) suggesting that this pathway does not seem to play a significant role in the resolution of inflammation.

5. SPM formation in human macrophages

Depending on their differentiation status, human monocytes/macrophages express AA 15-lipoxygenase paralogues (15-LO1, 15-LO2) together with 5-LO and FLAP [34]. 15-LO1 expression is strongly induced during differentiation of monocytes to M2-like macrophages by IL-4 or IL-13 [35–37] whereas the enzyme is absent in M1 macrophages. In addition, 15-LO1 expression is upregulated in macrophages by uptake of apoptotic cells via activation of LX receptors [38]. In contrast, 15-LO2 is expressed in minute levels in M1 and M2 macrophages and monocytes but expression is strongly potentiated after long-term stimulation with the TLR ligands LPS or zymosan as well as under hypoxic conditions in M2 macrophages [37,39,40]. Thus, M2 macrophages express all the proteins required for SPM biosynthesis. Furthermore, M2 polarization is associated with a strong decrease in PGE_2 formation (Table 2). Therefore, it has been proposed that the upregulation of 15-lipoxygenating enzymes (*ALOX15*, *ALOX15B*) leads to a lipid mediator switch which is an important step in the transition of a pro-inflammatory reaction into the resolution phase [41].

Due to the co-expression of 5-LO/FLAP and 15-LOs, human M2 macrophages should be capable of producing SPMs via both the 12/15-LO:5-LO as well as the 5-LO:12/15-LO pathway. However, their SPM forming capacity is very limited (Table 2). While stimulation of M1 macrophages by LPS or zymosan primarily triggers the release of pro-inflammatory oxylipins, polarization of the macrophages towards M2 leads to a strong upregulation of both 15-LO paralogues along with the prominent release of oxylipins derived from the 15-LO pathway (lipid mediator switch). In our hands, 15-HETE but no SPMs were found in the conditioned media of M2 macrophages (Table 2) and also after fMLF stimulation of these cells [37]. We found that an additional stimulation of human M2 macrophages with LPS for a prolonged time (16 h) further induces the 15-LO2 levels along with expression of 5-LO [37]. When these cells were stimulated with Ca^{2+} ionophore for 15 min together with AA, EPA and DHA, M2 macrophages released the dihydroxylated oxylipins 5,15-diHETE, RvE2 and RvD5 in detectable amounts [37]. Formation of the trihydroxylated SPMs (RvD1, RvD2, RvE1, LXA_4, LXB_4) was still very low or undectable (Table 2). Of note, the concentrations of these metabolites were about 2 or 3 orders of magnitude lower than those of LTB_4 or different monohydroxylated PUFAs in our hands. When the cells were incubated with LPS for 16 h without further stimulation, the cells mainly released cyclooxygenase-derived oxylipins and 15-HETE whereas 5-LO-derived oxylipin formation was low (Table 2). A possible explanation for this observation could be that LPS does not lead to an increase in intracellular calcium concentration so that 5-LO activation is low.

In line with our results on pathogen-associated molecular patterns (LPS, zymosan), it has been recently found that incubation of M1 and M2 macrophages with whole bacteria or bacterial toxins also leads to a strong upregulation of the 15-LO pathway, especially in M2 polarized macrophages. This was accompanied with 5-LO activation and the formation of high amounts of the SPM precursors 15-HETE and 17-HDHA whereas 18-HEPE formation was low [42–45].

A number of independent studies show that although there is a massive formation of SPM precursors in stimulated macrophages, production of the corresponding trihydroxylated SPMs is extremely low or undetectable (Table 2). Formation of LXA_4 was 2547-, 126-, 768-, 7246- and 2334-fold lower than 15-HETE [37,42–45], RvD1 and RvE1 were

Table 2
SPM formation in human macrophages. Trihydroxylated SPMs are shown in italic.

Macrophage differentiation	M1: GM-CSF/IFNγ M2: M-CSF/IL-4 16 h LPS (100 ng/ml) [37]				M1: GM-CSF M2: M-CSF 24 h LPS (10 ng/ml) [39]		M1: GM-CSF M2: M-CSF [47]		M1: GM-CSF/ IFNγ/LPS M2: M-CSF/IL-4 [42]		M1: GM-CSF/ IFNγ/LPS M2: M-CSF/IL-4 [43]		M1: GM-CSF/ IFNγ/LPS M2: M-CSF/IL-4 [44]		M1: GM-CSF/IFNγ/LPS M2: M-CSF/IL-4 [45]		M1: GM-CSF/ IFNγ/LPS M2: M-CSF/IL-4 [46]	
cell stimulation	–		Ca^{2+} ionophore A23178 + AA/EPA/ DHA 15 min		–		LPS/fMLF 20 min /10 min		living E. coli 90 min		living E. coli 180 min		Staphylococcus aureus, 180 min		Staphylococcus aureus conditioned medium, 180 min		–	
sample	conditioned medium		cells + supernatant		cells + conditioned medium (24 h)		cells + supernatant		cell supernatant		cell supernatant		cell supernatant		cell supernatant		cells + supernatant	
oxylipin	pg/μg protein		pg/μg protein		pg/10^6 cells		pg/10^6 cells		pg/10^6 cells		pg/10^6 cells		pg/10^6 cells		pg/10^6 cells		pg/10^6 cells	
	M1	M2	M1	M2	(M1)	(M2)	(M1)	(M2)	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2
TxB_2	684	64	479	518	39787	92457	5460	3150	890	1189	4185	7902	4502	5482	5684	8152	2200	888
PGD_2	12	n.d.	19	15	68324	93894	1540	581	34	76			34	34	111	67	16684	15696
PGE_2	638	64	313	35	183963	341291	462	434	2705	82	6980	344	1598	104	4606	234	9140	6292
$PGF_{2\alpha}$	333	8	109	72	413	498	420	231	223	123			355	242	411	239	45300	8432
5-HETE	18	35	420	130	n.d.	n.d.	1946	3962	197	118	812	579	74	124	1154	1342	12772	23628
5-HEPE			870	260			252	854	35	10			20	24	124	109	32380	85836
LTB_4	n.d.	n.d.	120	22	n.d.	n.d.	2128	2002	238	48	842	322	11	14	401	194	2672	1436
15-HETE	63	313	160	1350	3791	n.d.	91	147	77	756	637	20734	424	5797	1620	11669	15416	25320
15-HEPE			33	1640	218	n.d.	n.d.	n.d.	3	207	27	3476	32	729	78	941	440	980
18-HEPE			150	210	260	46	n.d.	n.d.	2	3			10	30			812	1564
14-HDHA			6.6	67			70	200	7	129	31	2016	8	334	56	757	4956	10760
17-HDHA			22	590	945	0	231	371	16	360	207	12645	168	1629	994	8264	3100	4956
5,15-diHETE			18	53			n.d.	n.d.	7	133	n.d.	856	7	202	58	362	12156	25568
LXA_4	*n.d.*	*n.d.*	*n.d.*	*0.53*	*n.d.*	*n.d.*	*n.d.*[1]	*n.d.*[1]	*0.7*	*6*	*n.d.*	*27*	*0.4*	*0.8*	*4*	*5*	*2700*	*3908*
LXB_4	*n.d.*	*n.d.*	*n.d.*	*0.2*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*			*n.d.*	*n.d.*	*n.d.*	*n.d.*	*30240*	*47000*
RvD1	*n.d.*	*n.d.*	*n.d.*	*0.14*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*0.6*			*n.d.*	*n.d.*	*n.d.*	*n.d.*	*1780*	*924*
RvD2	*n.d.*	*n.d.*	*n.d.*	*0.29*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*0.3*	*17*	*n.d.*	*89*	*0.3*	*6*	*n.d.*	*n.d.*	*5764*	*2428*
RvE1			*0.43*	*n.d.*	*n.d.*	*n.d.*			*0.1*	*0.1*			*n.d.*	*n.d.*	*n.d.*	*n.d.*	*1392*	*680*
RvD5			2.1	38					0.9	92	n.d.	895	1	299	5	198	172	784
RvE2			24	28	n.d.	n.d.			0.5	1			n.d.	n.d.	n.d.	n.d.	43640	82720
RvE3			n.d.	1.2	n.d.	n.d.			2	3			n.d.	n.d.	n.d.	n.d.[1]		
RvE4													n.d.	n.d.	18	28		

n.d. not detected (below LLOQ), 1peak detected but identity not unambiguously confirmed

mostly undetectable (Table 2). Formation of RvD2 was 2034-, 22-, 142- and 1054-fold lower than its precursor 17-HDHA [37,42–44]. Thus, regardless of the differentiation protocol used and the stimulus applied to the macrophages, trihydroxylated SPMs are either undetectable or their formation is two or three orders of magnitude lower than their precursors, clearly demonstrating that the monohydroxylated oxylipins are hardly converted to SPMs in macrophages. Table 2 lists the lipid mediator profiles of the different studies conducted by several independent groups. From these data it becomes evident that the SPM profiles are quite similar with the exception of a study by Dalli et al. [46]. In this report the values for lipoxins and resolvins are around three orders of magnitude higher than in the other studies (Table 2) although a similar or identical protocol for M1 and M2 macrophage differentiation and oxylipin extraction was employed. Of note, the incubation time is not specified in the paper. Furthermore, some data reported in this study are not in agreement with the biosynthetic pathways. For example, in M2 cells formation of 82,720 pg/million cells RvE2 was reported whereas the amount of the corresponding precursor (18-HEPE) was comparably low (1564 pg/million cells). In addition, the authors report very high levels of LXB_4 (47,000 pg/million cells) but no (below LLOQ) or only extremely low levels of LXB_4 could be detected in all the other studies (Table 2). These discrepancies deserve further investigation.

Interestingly, van Hegedus et al. found much higher amounts of TxB_2, PGD_2 and PGE_2 in their samples derived from M1 and M2 macrophages compared to the other studies (Table 2) [39]. One possible explanation for this difference is that the authors collected the supernatant for 24 h whereas all the other data were obtained in short term incubations of M1 and M2 cells from 15 up to 180 min. Thus, it seems possible that these prostaglandins accumulate in the samples during long term incubations.

Summarizing the above, the available studies with macrophages reveal that M2 polarization leads to a lipid mediator switch from the 5-LO to the 15-LO pathway [34,37,47]. However, despite the co-expression of 5-LO and 15-LOs, macrophages have only a low capacity for the synthesis of trihydroxylated SPMs. Only the dihydroxylated oxylipins 5 *S*,15*S*-diHETE, RvD5 and RvE2 are consistently detected in macrophages (Fig. 1, Table 2). The very low (if at all) formation of trihydroxylated SPMs is probably due to the inefficient conversion of oxylipin substrates to the corresponding epoxides by the LTA_4 synthase activity of 5-LO, regardless of the presence of FLAP.

In humans, 5-LO expression is mainly restricted to leukocytes. Therefore, these cell types can be considered as the main source of 5-LO products (e.g. 5-HETE, leukotrienes) and SPMs via the 5-LO:12/15-LO and the 12/15-LO:5-LO pathway. However, due to the extremely low enzymatic capacity for the formation of trihydroxylated SPMs in leukocytes, it is questionable on the basis of the currently available data, that these SPMs are formed in-vivo in sufficient amounts by leukocytes to counteract the functionality of the pro-inflammatory lipid mediators.

6. SPM analysis by LC-MS

Taking into account the low concentrations of SPMs as well as the large number of isomers, analytical methods are in the focus when their formation and occurrence are investigated. This is particularly the case for the analysis of SPM formation in leukocytes with limited amounts of cells. The only analytical method which has the required sensitivity and selectivity is (targeted) liquid chromatography tandem mass spectrometry [48]. Though slightly different analytical protocols are used, the applied techniques as well as instruments are similar [33,49]: For analysis, the cells are homogenized and, following the addition of internal standards, usually extracted by solid phase extraction. The analytes are reconstituted in a small volume of organic solvent and are injected into the liquid chromatography system. Separation of structurally similar oxylipins is carried out by reversed phase (RP) chromatography, using modern columns with small particle sizes leading to narrow peaks. For MS detection the evaporation of the solvent and ionization is carried out by electrospray-ionization in negative mode, where the slightly acidic oxylipins (oxidized fatty acids) form $[M\text{-}H]^-$ ions by deprotonation. In order to gain selectivity as well as sensitivity by reducing the noise, these ions are not used for quantification, but their specific fragments. For this purpose, the $[M\text{-}H]^-$ ion is selected by the first quadrupole, then fragmented in the second quadrupole of a triple-quadrupole analyzer (QqQ). Finally, the specific fragment ion is selected by the third quadrupole and reaches the detector, a process which is called selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) mode. Here, the MS continuously (or in case of scheduled MRM at the expected retention time) monitors this signal over the time of the chromatographic run. The presence of the compound as well as its concentration is evaluated based on the height of the chromatographic peak and its area. In order to highlight performance of a LC-MS method as well as the presence of a compound these chromatograms, which consist of the raw data of the intensity signal over the analysis time, are used in scientific publications. An example of the characterization of the limit of detection of our method is shown here (Fig. 2A, [49]). However, in the SPM field an uncommon way of showing peaks as illustrations (Fig. 2B) instead of chromatograms derived from original data is used by several groups, which is also the case in 2 out of 8 articles [42,46] reporting SPM formation in human macrophages (Table 2). It is obvious that this type of illustration contains no information about the sensitivity of the method (signal/noise ratio) nor the actual presence of a compound in a sample.

Exemplary raw LC-MS data of SPM analyses in macrophages are shown in Fig. 3. It becomes apparent, without quantitative evaluation, that no or only very low levels of trihydroxylated SPMs are formed by leukocytes (see above). The figure exemplarily depicts chromatograms from M2 macrophages stimulated with LPS and activated with Ca^{2+} ionophore in the presence of added AA/EPA/DHA (data from [37], quantitative information see Table 2). Clear large peaks can be found for the monohydroxy PUFAs 15-HETE, 18-HEPE and 17-HDHA which can serve as SPM precursors. The dihydroxy-PUFAs 5,15-diHETE, 5, 18-diHEPE (RvE2) and 7,17-diHDHA (RVD5) were found with significant lower intensities, still allowing a reliable quantification based on these peaks. However for the trihydroxylated SPMs, no signals were found indicating the formation of RvE1, and peak of RvD2 is at the edge of the method sensitivity which allows quantification only in a few samples. For LXA_4, higher signals were obtained. However, peak selection and thus quantification is challenging because of the high noise arising from isobaric interferences derived from co-eluting isomers [50].

It should be noted that the SPM concentrations calculated from these data are consistent in 7 out of 8 reports (Table 2) describing the formation of SPMs in macrophages. Even though the signal height of different oxylipins in MRM mode in LC-ESI-MS cannot be compared directly, this highlights that the concentration of monohydroxylated oxylipins exceeds the concentration of dihydroxylated PUFA by an order of magnitude or more. Trihydroxylated SPMs are one or two orders lower in concentration than dihydroxylated oxylipins, if their formation could be detected at all.

7. Biological activities of 5 S,15S-DiHETE, RvE2, RvE4 and RvD5

It is well established that there is a lipid mediator class switch ongoing during inflammation in humans which is mainly orchestrated by macrophages (also see Section 5). During this switch formation of pro-inflammatory LTs is attenuated while 15-LO-dependent monohydroxylated PUFAs such as 15*S*-HETE, 15*S*-HEPE and 17*S*-HDHA are released in high amounts from anti-inflammatory macrophages and eosinophils. The physiological consequences of these 15-LO-derived lipids on inflammation and its resolution in humans are not well understood and the mechanisms of action are poorly defined to date.

What is known so far is that 15-HETE, 15-HEPE as well as 17-HDHA and their carbonyl derivatives are endogenous agonists of peroxisome proliferator activated receptors (PPAR) which are involved in the

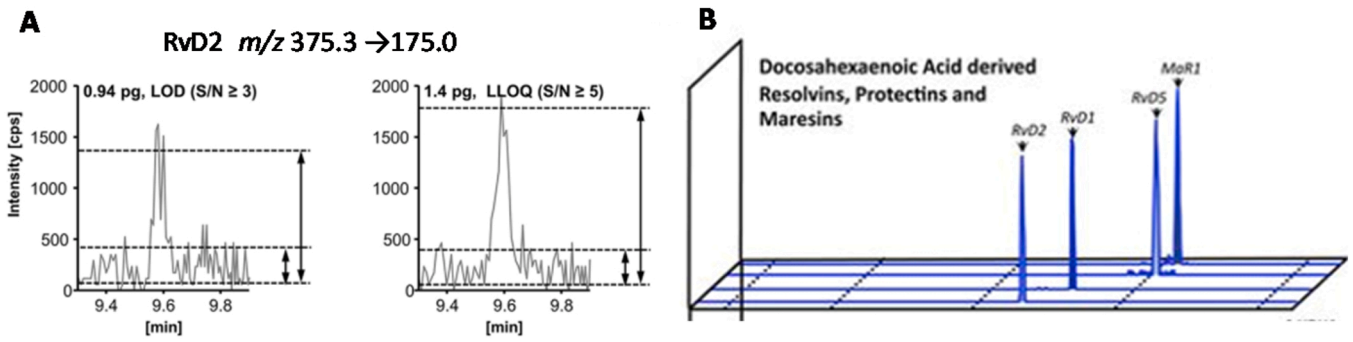

Fig. 2. Different ways of providing information about LC-MS method and analysis. **A)** LC-MS/MS chromatograms in MRM mode of different amounts of SPM injected showing the signal to noise ratio (S/N) used to define the sensitivity of the method (limit of detection, limit of quantification in LC-MS [49]. **B)** Typical illustration/artwork of "Representative MRM traces for the identified LMs" used in the SPM field to show LC-MS/MS data [46].

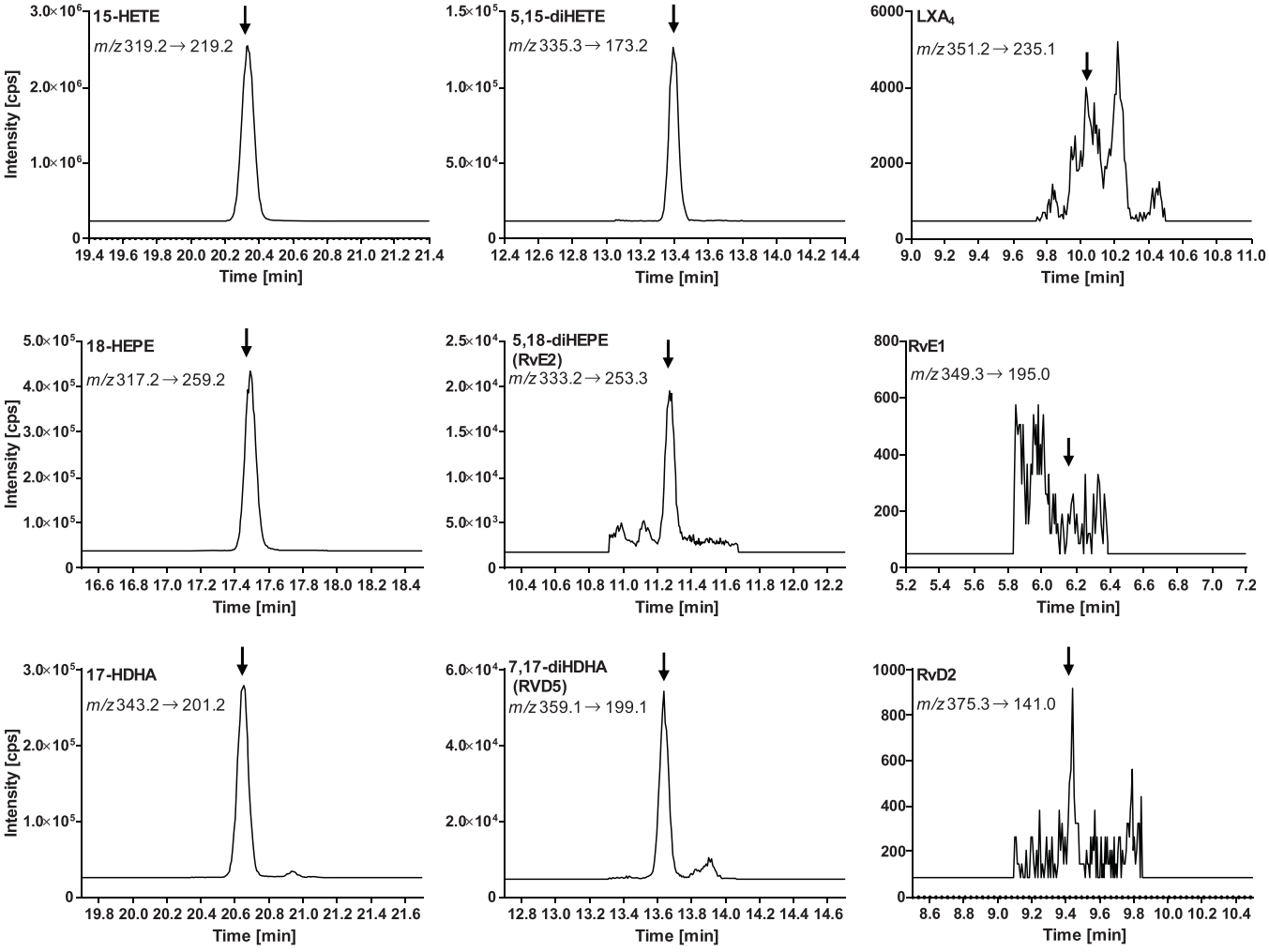

Fig. 3. Exemplary LC-MS/MS chromatograms of the analysis of M2 macrophages stimulated with LPS and activated with Ca^{2+} ionophore in the presence of added AA/EPA/DHA (data from [37], quantitative information see Table 2). The raw chromatograms of the MRM analysis from cell samples are shown highlighting the characteristics of these data: Peak presence, peak retention time, peak shape and height in relation to the height of the noise and peaks of interfering compounds. The arrow indicates the retention time of the respective standard compound. Dihydroxylated lipid mediators show a peak which is about 10 fold smaller than the peaks of the monohydroxy-PUFA. The signals of the trihydroxylated lipid mediators are again at least 10 fold lower than those of the dihydroxylated lipid mediators, not or only barely exceeding the height of the background noise.

differentiation of anti-inflammatory macrophages and resolution [51–54]. Furthermore, these oxidized lipids can be incorporated into cell membranes where they shape cell signaling, coagulation and innate immune responses [55].

In addition, 15-LO products directly interfere with LT/5-HETE biosynthesis by displacing AA from 5-LOs active center thus directly dampening 5-LO product-mediated leukocyte influx into the inflamed tissue. But 15-LO-derived oxylipins do not simply block 5-LO activity, they are themselves converted by the enzyme leading to the formation of dihydroxylated SPMs such as RvE4 (5 *S*,15*S*-diHEPE) and RvD5

(7 *S*,17*S*-diHDHA) in amounts comparable to LTs [56,57]. Curiously, 5 *S*,15*S*-diHETE, the AA analogue of RvE4, has never been assigned as SPM but is also formed in considerable amounts via this pathway [18,37]. As has already been conclusively discussed in this review, trihydroxylated oxylipins such as lipoxins and several other resolvins among them RvE1, RvE2, RvD1, RvD2 and RvD3 are only released in minute amounts, if at all, from human leukocytes. In accordance with this, the levels of these trihydroxylated oxylipins found in human tissues and body fluids are particularly low or undetectable [33,58].

In the following we summarize the biological activities of the dihydroxylated SPMs which are frequently reported to be released in substantial amounts from human leukocytes (5,15-diHETE, RvE2, RvE4, RvD5). Of note, the validity of the analytical methods used for oxylipin detection in these studies was not considered here.

AA-derived 5,15-diHETE is released along with LTB_4 from ionophore-stimulated PMNL derived from asthmatic patients while healthy donors only produce LTB_4 [59,60]. In line with this, the lipid was also released from PMNL of rheumatoid arthritis patients in contrast to healthy subjects and glucocorticoid or methotrexate treatment prevented this [61]. Furthermore, 5,15-diHETE is elevated in BAL fluids of COVID-19 patients in amounts comparable to LTB_4 and it is released from human skin blisters during the first 24 h following UV-killed E.coli challenge together with RvD5 [62,63]. In addition, it is known to influence granulocyte activity: Its oxidation product 5-oxo-15-hydroxyETE is a potent eosinophil-released eosinophil chemoattractant [64,65]. Furthermore, it was found to enhance neutrophil degranulation upon specific stimuli such as PAF. In contrast to this, fMLF-, PMA-, LTB_4- and ionophore-triggered degranulation was not affected by 5,15-diHETE [66]. Interestingly, 5-HETE showed the same effect, while 12-, 15- and 8,15-diHETE were not active in this setting. In addition, 5,15-diHETE is a postulated intracellular protein kinase C activator as it stimulates the purified enzyme *in vitro* [67] (Table 3). Unfortunately, the role of 5,15-diHETE has never been directly studied in models of human resolution such as macrophage efferocytosis, leukocyte pro-inflammatory gene expression, cytokine release and bacterial killing.

The EPA derivative RvE2 (5 *S*,18*R*-diHEPE) is reported to affect human PMNL cell shape and chemotactic velocity and reduces the LTB_4-stimulated actin polymerization in these cells (Table 3). Furthermore, it stimulates phagocytosis and IL-10 release from human GM-CSF-differentiated macrophages and activates CD18 in human whole blood in absence and presence of PAF [68]. In addition, RvE2 levels correlate with pain reduction in the synovial fluid of rheumatoid arthritis patients [69].

RvE4, the EPA analogue of 5 *S*,15*S*-diHETE, was recently found to be produced under hypoxic conditions termed 'physiologic hypoxia' (1% O_2) in M2 macrophages, neutrophils, macrophage-neutrophil co-incubations and macrophage-erythrocyte co-incubations (Table 3). In addition, it was found to stimulate PMNL and red blood cell efferocytosis by macrophages [6,70]. RvE4 was found in plasma from Ca^{2+} ionophore (50 μM A23187) stimulated whole blood samples from humans after ω-3-PUFA supplementation [12].

RvD5 (7 *S*,17*S*-diHDHA) can be found in the synovial fluid of rheumatoid arthritis patients and is elevated in psoriatic lesions [71,72]. Furthermore, elevated levels of this oxylipin have been found in the BAL fluids of intubated COVID-19 patients [63]. Interestingly, sepsis non-survivors show elevated RvD5 levels [49,73]. In addition, RvD5 can be found in the serum of patients with end stage renal disease [49] and the SPM is released during blood coagulation in vitro [74]. Furthermore, RvD5 has been found in human milk [75]. It is released from human M2 macrophages during the encounter with pathogenic bacteria and upon long-term stimulation with TLR-2 and TLR-4 ligands. RvD5 was found to stimulate the phagocytosis of *E.coli* by M1 and M2 macrophages as well as PMNL and reduces a number of inflammatory genes such as *PDE4B*, *PTGS2*, *NFκB* and *TNFα* during this process. In addition, an elevation in reactive oxygen species was found in PMNL during phagocytosis of *E. coli* [37,42,76]. In line with this, RvD5 was shown to reduce the expression of psoriasis-related cytokines such as IL-24 in human keratinocytes [72], downregulates IL-6 and CCL5 production in THP-1 cells and inhibits the LPS-stimulated phosphorylation of ERK and thus subsequent translocation of NFκB into the nucleus [77]. Furthermore, RvD5 is known to activate PLD2 in M2 macrophages during inflammation and resolution [78].

A comprehensive overview of the occurrence and published biological activities of dihydroxylated SPMs in humans can be found in Table 3.

8. Perspective

In order to act as a signaling molecule, an oxylipin has to be formed at concentrations that allow activation of the respective receptor(s). Furthermore, it has to be formed in sufficient quantities at the site of inflammation to mediate inflammation or resolution. *In vitro* investigations on the SPM-forming capacity of different leukocyte subsets revealed that these cells produce only minute amounts of trihydroxylated SPMs (RvD1, RvD2, RvE1, LXA_4, LXB_4) if at all. In contrast to this, formation of dihydroxylated SPMs such as RvE2, RvE4 and RvD5 is usually higher but still much lower compared to the classical reaction products such as monohydroxylated HETEs, HEPEs, HDHAs and the

Table 3
Formation and biological activities of dihydroxylated SPMs and 5,15-diHETE in humans.

	In vivo	*In vitro*
5,15-diHETE	Elevated in BAL fluids of COVID-19 patients[63] Released in skin blisters after E. coli challenge[62]	Elevated in stimulated PMNL from asthmatics[59,60] Elevated in stimulated PMNL from RA patients[61] Eosinophil chemoattractant[64,65] Enhances PMNL degranulation upon PAF stimulation[66] Protein kinase C activator[67]
RvE2	Correlates with pain reduction in the synovial fluid of RA patients[69]	Affects PMNL shape and chemotactic velocity[68] Inhibits LTB_4-stimulated actin polymerization in PMNL[68] Stimulates phagocytosis and IL-10 release in macrophages[68] Activates CD18 in whole blood[68]
RvE4	Formation in Ca^{2+} ionophore stimulated whole blood of human subjects after ω-3-PUFA supplementation[12]	Released from M2 macrophages, neutrophils and macrophage-erythrocyte co-incubations during hypoxia[70] Stimulates efferocytosis of PMNL and erythrocytes by macrophages[6]
RvD5	Present in synovial fluid of RA patients[71] Elevated in psoriatic lesions[72] Elevated in BAL of intubated COVID-19 patients[63] Elevated in sepsis non-survivors[49,73] Present in serum of patients with end stage renal disease[49] Present in human milk[75]	Released during blood coagulation[74] Released from M2 macrophages during pathogen stimulation[37,42,76] Stimulates phagocytosis of *E.coli* by M1 and M2 macrophages and PMNL[42,76] Activates phospholipase D2[78] Reduces inflammatory genes in macrophages[76] Reduces IL-6 and CCL5 in THP-1 cells via inhibition of NFκB translocation[77] Reduces psoriasis-related cytokines in keratinocytes[72]

BAL, bronchoalveolar lavage; PAF, platelet-activating factor; PMNL, polymorphonuclear leukocytes; RA, rheumatoid arthritis

leukotrienes. In addition, signaling of the trihydroxylated SPMs via their proposed receptors has recently been challenged (for review see [33]). It was initially found that LXA_4 binds to FPR2/ALX with high affinity [79] but studies from other groups could not show any functionality of the receptor with LXA_4 although the other established nonlipid ligands could be validated as agonists in the same studies [80,81]. Thus, due to the very low formation of trihydroxylated SPMs and the lacking functional validation of their receptors, it is questionable at the moment whether trihydroxylated SPMs are formed by human leukocytes in sufficient quantities via the 5-LO:12/15-LO or the 12/15-LO:5-LO pathway to act as endogenous mediators of the resolution of inflammation.

Changes in the lipid mediator profile of leukocytes are sometimes shown as heatmaps or similar illustrations which do not reflect the absolute oxylipin levels produced by the cells but only relative changes. However, these presentations of the data can be misleading due to the large differences in oxylipin formation in leukocytes (Table 2). Thus, it is rather unlikely that small increases in SPM formation counteract the massive release of pro-inflammatory mediators by the cells (Table 2). Furthermore, the prominent formation of pro-inflammatory oxylipins is sometimes simply not considered or the sum of several SPMs is shown to get larger amounts which can lead to a bias in the interpretation of the data.

In conclusion, manuscripts that report the formation of oxylipins including pro-resolving lipid mediators as heat-maps should always be accompanied by information on the absolute oxylipin levels. Furthermore, for the interpretation of the lipid mediator data in the context of the onset and resolution of inflammation, the levels of the typical pro-inflammatory oxylipins released alongside the pro-resolving oxylipins should be reported.

A general finding of the studies on macrophages was that stimulation of M1 and M2 macrophages leads to a different lipid mediator profile caused by the upregulation of 15-LO1 during M2 polarization, decreased PGE_2 formation and reduction of classical 5-LO metabolites such as 5-HETE and LTB_4, probably via inhibition of 5-LO activity by 15-LO1 reaction products such as 15-HETE [82,83]. From these data, it becomes evident that macrophage polarization to M2 leads to a lipid mediator switch from the 5-LO to the 15-LO pathway. However, it should be emphasized at this stage that stimulation of M1-polarized macrophages with LPS or zymosan induced 15-LO2 expression leading to an increased release of 15-HETE by these cells [37].

Up to now, the exact function of this lipid mediator switch is not fully understood and the oxylipin components that play a central role in the resolution of inflammation are not clarified. Stimulation of M2 macrophages leads to the very prominent release of 15-HETE and other monohydroxylated 15-LO products derived from EPA and DHA [37,44,84]. Also, a number of dihydroxylated oxylipins generated by the 5-LO:12/15-LO pathway and the 12/15-LO:5-LO pathway such as 5*S*, 15*S*-diHETE and RvD5 are consistently released by macrophages and granulocytes. However, the formation of trihydroxylated SPMs (LXA_4, LXB_4, RvD1, RvD2, RvD3, RvD4, RvE1) by these pathways in human leukocytes is extremely low or simply does not occur in detectable quantities even after extensive cell stimulation.

Although it is questionable whether trihydroxylated SPMs are formed by human leukocytes in amounts that promote the resolution of inflammation, they are interesting pharmacological tools. Up to now, a considerable number of papers have been published describing anti-inflammatory and pro-resolving properties of trihydroxylated SPMs when added at pharmacological concentrations which clearly exceed the endogenous levels (for review see [85]). It should also be considered that mono- and dihydroxylated oxylipins can share the pharmacological activity of trihydroxylated oxylipins [86] and that pharmacological application of trihydroxylated SPMs might mimic more abundant mono- or dihydroxylated oxylipins that endogenously promote resolution of inflammation.

Furthermore, oxylipins can act as ligands for nuclear receptors such as PPAR [87–89] or can rapidly be incorporated into lysophospholipids so that their esterified products could also contribute to the biological activities [90]. Thus, it is challenging to further elucidate the role of the lipid mediator switch from the 5-LO to the 15-LO pathway and to identify the active lipid mediators that drive the resolution of inflammation.

Data Availability

No data was used for the research described in the article.

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Author contributions statement

ASK, NHS, and DS structured the paper, ASK, NHS, and DS wrote the paper.

Conflict of interest statement

All authors declare no competing interests.

Contribution to the field statement

Formation of specialized pro-resolving lipid mediators (SPM) such as lipoxins or resolvins is suggested to play an important role in the resolution of inflammation. Typically, SPM formation relies on two sequential oxidation steps by two lipoxygenases (5-LO and 12/15-LO) where polyunsaturated fatty acids such as arachidonic acid, eicosapentaenoic acid or docosahexaenoic acid serve as substrates. One hallmark of SPM formation is that they are formed at much lower levels than typical pro-inflammatory mediators including the monohydroxylated fatty acid derivatives (e.g. 5-HETE), leukotrienes or the cyclooxygenase-derived prostaglandins. Of note, lipoxygenases have a low biosynthetic capacity for most of the SPMs. Due to the low sub-nanomolar SPM levels, the detection and quantification of these oxylipins in biological samples is challenging which led to the use of questionable peak definitions in LC-MS analyses in some reports in order to quantify SPM. Thus, this review critically summarizes the formation and the detection of lipoxins and resolvins in leukocytes.

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