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CYP450-derived oxylipins mediate inflammatory resolution

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Abstract

Resolution of inflammation has emerged as an active process in immunobiology with cells of the mononuclear phagocyte system being critical in mediating efferocytosis, wound debridement and bridging the gap between innate and adaptive immunity. A number of different lipid species have been shown to contribute to various aspects of inflammatory resolution. Here we investigated the roles of the CYP450 derived epoxy-oxylipins in a well-characterised model of sterile resolving peritonitis in the mouse. Epoxy-oxylipins were produced in a biphasic manner during the peaks of both acute (4h) and resolution phases (24-48h of the response). The epoxygenase inhibitor SKF525A (epoxI) given at 24h selectively inhibited arachidonic acid and linoleic acid derived CYP450-epoxy-oxlipins and resulted in a dramatic 3-fold influx in monocytes. The epoxI recruited monocytes were strongly GR-1+, Ly6c^{hi}, CCR2^{hi}, CCL2^{hi}, and CX3CR1^{lo}. In addition, expression of F4/80 and the recruitment of CD3^{+} T-cells, CD19^{+} B-cells and MHCII^{+} CD11c^{+} dendritic cells were suppressed. sEH (*Ephx2*)$^{-/-}$ mice which have elevated epoxy-oxylipins, demonstrated opposite effects to EpoxI treated mice: reduced Ly6c^{hi} monocytes, and elevated F4/80^{hi} macrophages and B-, T- and dendritic cells. EpoxI recruited cells had greatly elevated CCL2 levels, which could be inhibited *ex vivo* by 11,12-EET or 14,15-EET. Moreover, Ly6c^{hi}, Ly6c^{lo} monocytes, resident macrophages and recruited dendritic cells all showed a dramatic change in their resolution signature following *in vivo* epoxI treatment. In particular, markers of macrophage differentiation F4/80, CD11b, MerTK, and CD103 were reduced, and both monocyte-derived macrophages and resident macrophages showed greatly impaired phagocytosis of zymosan and efferocytosis of apoptotic thymocytes following epoxI treatment. These findings demonstrate that epoxy-oxylipins have a critical role in monocyte

linage recruitment and activity to promote inflammatory resolution and represent a novel internal regulatory system governing the establishment of adaptive immunity.

Significance statement:

A number of lipid mediators are known to contribute to inflammatory resolution. Fatty acid metabolites of CYP450 enzymes are found in abundance, however their roles in inflammatory resolution are not known. Targeted lipidomics revealed CYP450-epoxy-oxylipins were present during acute inflammation and inflammatory resolution. Using mice lacking soluble epoxide hydrolase, the major metabolizing pathway for CYP450-derived fatty acid mediators, and CYP450 epoxygenase inhibition specifically during resolution, we show CYP450-dervied lipids dramatically limit the accumulation of inflammatory monocytes during resolution. Moreover all cells of the monocyte lineage examined showed a dramatic alteration in their pro-resolution phenotype following epoxygenase inhibition. These findings demonstrate that the CYP450-epoxy-oxylipins pathway has a critical role in monocyte linage recruitment and resolution activity during inflammatory resolution.

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Introduction

Monocytes and monocyte-derived macrophages play a critical role in chronic inflammation in part via the production and release of lipid mediators (1). One such lipid precursor, arachidonic acid, is metabolised into families of biologically active mediators by cyclooxygenase, lipoxygenase and CYP450 (CYP) pathways (2, 3). CYPs metabolise arachidonic acid by: i) an epoxygenase activity that catalyzes the conversion of arachidonic acid to epoxyeicosatrienoic acids (EETs); ii) a lipoxygenase-like activity that metabolizes arachidonic acid to mid-chain hydroxyeicosatetraenoic acids (HETEs); and iii) ω- and ω-1-hydroxylase activity, which produces ω-terminal HETEs (3). In addition to arachidonic acid, CYPs with epoxygenase activity can also metabolise alternative polyunsaturated fatty acids such as linoleic acid and docosahexaenoic acid in to a series of products including epoxyoctadacamonoenoic acids (EpOME)s and 19,20-epoxydocosapentaenoic acid (EpDPE) respectively, whose functions remain poorly understood (3-5).

The main polyunsaturated fatty acid-metabolising CYPs belong to the CYP2 family, in particular, the CYP2J and CYP2C subfamilies (3, 4, 6, 7). Moreover, these CYP-lipid metabolizing enzymes are the primary sources of eicosanoids in small blood vessels, the kidney, liver, lung, intestines, heart, and pancreas (3, 7). In most organs, EETs and related epoxygenase products are metabolically unstable and are rapidly metabolised. The major pathway that regulates EET metabolism is that catalysed by epoxide hydrolases (8) which convert EETs to less biologically active dihydroxyeicosatrienoic acids (DHET)s (9). EpOMEs similarly get converted in to dihydroxyoctadecenoic acids (DiHOME)s while 19,20-EpDPE gets converted in to 19,20-dihydroxydocosapentaenoic acid (DiHDPA). Elevating the levels of endogenous CYP products by disrupting (knockout) or inhibiting soluble epoxide

hydrolase (sEH), reduces neointima formation (10), atherosclerosis, abdominal aortic aneurysm, dyslipidaemia (11), hypertension (12) and diabetes (13) in different mouse models; all of which to some extent one could argue have a degree of non-resolving inflammation.

Over the last 15 years there has been a vast increase in our knowledge of fatty acid mediators that regulate inflammatory processes, particularly newly identified mediators such as the resolvins that mediate the resolution of inflammation (14-16). However, unlike cyclooxygenase and lipoxygenase products, the roles of CYP450 pathways in chronic inflammation remains unclear. The arachidonic acid products of the CYP epoxygenases, the EETs, can regulate vascular tone, smooth muscle cell mitogenesis, platelet aggregation, steroidogenesis and endothelial and vascular smooth muscle cell activation (4, 5, 7, 17-19). We recently published that in human monocytes and macrophages, epoxygenases and some of their arachidonic acid products were anti-inflammatory through their ability to activate the peroxisome-proliferator activated receptor (PPAR), in particular PPARα (20, 21). Overexpression of epoxygenase enzymes CYP2J2 and CYP2C8, or genetic disruption of sEH ($sEH^{-/-}$) inhibit LPS induced pulmonary inflammation (22)(23), and $sEH^{-/-}$ mice or treatment with sEH inhibitors are highly effective against inflammatory and neuropathic pain (24-27).

Monocytes are heterogeneous in mice and in man.(28) In mice, monocyte subsets can be divided based on the expression of Ly6c, Gr1, CC-chemokine receptor 2 (CCR2), and CX3C-chemokine receptor 1 (CX3CR1). $Ly6c^{hi}$ monocytes are $Gr1^{+}$, $CCR2^{+}$, and $CX3CR1^{lo}$, whereas $Ly6c^{lo}$ monocytes are $Gr1^{-}$, $CCR2^{-}$, and $CX3CR1^{hi}$ (29, 30). Lipid mediators that regulate the recruitment and phenotype of monocytes are poorly understood.

Herein, using a sterile model of inflammatory resolution dependent on monocyte recruitment, we found CYP-epoxygenase products not only accumulate in a temporal

manner with monocyte recruitment, but also limit pro-inflammatory monocyte recruitment and promote a pro-resolution phenotype in cells of the monocyte linage.

Results

CYP450 derived lipid metabolites form part of the coordinated eicosanoid fatty acid metabolite response in acute inflammation and resolution. The naive mouse peritoneal cavity contained high levels of linoleic acid (9- and 13-HODE), and arachidonic acid (8-, 12- and 15-HETE) derived lipoxygenase products as well as 9,10-, and 12,13-DiHOME, which are epoxide diols of the linoleic acid epoxygenase products 9,10-EpOME and 12,13-EpOME (Figure 1). The COX, CYP450, 5-LO, and EPA/DHA derived mediators, including prostanoids, leukotrienes, EETs, and their metabolites were found at much lower levels (Figure 1). The LC/MS/MS assay we use was primarily designed to measure CYP450 and related products. We don't currently measure some of the other newly characterized lipid species known to regulate inflammatory resolution such as the resolvins, but clearly enzymatic pathways and lipid precursors for these pathways are all also present. During the acute inflammatory response elicited by zymosan (4h) there was the expected burst of prostanoids, increases in 5-, 11-, 19- and 20-HETE, and also increases in the CYP450 epoxygenase derived oxylipins 5,6-, 8,9-, 11,12-, 14,15-DHET and 17,18-DHEQ (Figure 1C, and 1D; supplemental Figure 1). The DHETs are the epoxide hydrolase diols of the P450 metabolites and could readily be detected, whereas parental EETs were not seen. The 15/12-lipoxygenase products initially found at high levels in the naive cavity dropped dramatically during acute inflammation (9- and 13-HODE and 8-, 12- and 15-HETE) with only 9-HODE and 8-HETE returning to baseline levels over the 48h time course (Figure 1C & E; supplemental Figure 1). After acute inflammation, which is driven primarily by PMNs, resolution begins and is associated with an accumulation of pro-resolution monocytes peaking 24-48h after the initial inflammatory

insult. After a drop at 24h, this influx in monocytes was associated with a second phase of epoxy-oxylipin production of arachidonic acid (DHETs), linoleic acid (DiHOMEs), EPA (17,18-DHEQ) and DHA (19,20-DiHDPA) epoxygenase products (Figure 2A and 2B). Moreover, this phase was associated with biosynthesis of new lipid products, as the addition of a selective epoxygenase inhibitor (epoxI; SKF525A; 30mg/kg i.p.) to the zymosan treated mice from 24h onwards led to a selective and specific inhibition of arachidonic acid and linoleic acid epoxy-oxylipins (Figure 2C). The epoxI had no significant effect on COX, LO or CYP450-lipoxygenase like products (supplemental table 2), indicating the epoxI used is highly selective for the CYP-epoxygenase pathway.

We screened the mouse *Cyp2* family to investigate which enzymes were present in the resolution phase inflammatory cell populations, and found *Cyp2c44*, *Cyp2j5*, *Cyp2j6*, *Cyp2j9*, *Cyp2j13* epoxygenases along with *Cyp2u1* (HETE and DHA metabolism) and *Cyp2s1* (xenobiotic and retinoic acid metabolism) (Table 1; supplemental figure 2).

Epoxygenase inhibition during resolution induces a profound accumulation of Ly6c^{hi} monocytes. Epoxygenase inhibition was accompanied by a 3-fold increase in monocyte numbers during the 24-48h period of inhibition, and a small but significant increase in PMNs (Figure 3A). These epoxI-recruited monocytes were strongly GR1^{+} (ly6c and Ly6g antigens, Figure 3B), and Ly6c^{hi} expression was confirmed by RT-PCR (Figure 3C). Moreover, these recruited cells were CCR2^{hi} (Figure 3D), CX3CR1^{lo} (Figure 3E), and CCL2^{hi} (Figure 3F) at the mRNA level. CCL2 peptide was found to be highly elevated in the peritoneal cavity of the epoxI treated mice (vehicle 8+/-8 pg/ml; epoxI 266+/- 190 pg/ml CCL2; n=3. Substantial levels of CCL2 were produced exclusively from cells elicited from epoxI treated mice *ex vivo* (Figure 3G), suggesting they could be a major source of this elevated CCL2. EETs are too

short lived to be given as a bolus dose *in vivo*, therefore to test whether EETs were anti-inflammatory in mouse monocytes, we added-back authentic 14,15-EET (1μM; 7h) to zymosan elicited cells *ex vivo*. 14,15-EET reduced *Ccl2* (Figure 3H), iNOS (Figure 3I), and *IL-12* (Figure 3J) mRNA expression in these cells *ex vivo*. Addition of 14,15-EET was not universally anti-inflammatory in these elicited cells as *TNFα* mRNA expression remained unaltered (Figure 3H). These results are consistent with our previous findings, which reported an anti-inflammatory role of epoxygenases in classically activated human monocytes and macrophages *in vitro* (21).

Soluble epoxide hydrolase (sEH)$^{-/-}$ mice have an opposing phenotype to epoxygenase inhibited mice during resolution. To confirm the role of epoxy-oxylipins during resolution we compared the findings of epoxI treated mice with sEH$^{-/-}$ mice. sEH is considered the main pathway for epoxy-oxylipin metabolism and inactivation. Compared to the epoxI treated mice, which have reduced epoxy-oxylipins, sEH$^{-/-}$ mice have elevated oxylipins (31). In zymosan treated mice, Ly6c^{hi} monocytes peaked at 48h with epoxI treatment (supplemental figure 3), whereas at 48h in sEH$^{-/-}$ mice, Ly6c^{hi} monocytes were reduced compared to wild type controls (Figure 4A). There was a small increase in Ly6g^{+} cells at 48h with epoxI, whereas in sEH$^{-/-}$ mice, Ly6g^{+} monocytes were slightly reduced compared to wild type controls (Figure 4B). With epoxI treatment, although there was no change in numbers of macrophages (F4/80) positive cells (5.4±0.5 compared to 5.9±2.2 cells /ml x10^{6}), there was a reduction in the levels of F4/80 expression on the epoxI-elicited macrophages (Figure 4C). In corollary, the opposite was found in sEH$^{-/-}$ mice: CD11b^{++} F480^{++} cells were greatly elevated compared to wild type controls (Figure 4C). CD19^{+} B-cells (Figure 4D), and CD3^{+} T-cells (Figure 4E) were reduced by epoxI, and elevated in sEH$^{-/-}$ mice. There was also a similar

trend to reduction of MHCII^{+} CD11c^{+} dendritic cells by epoxI, with a significantly elevation of these MHCII^{+} CD11c^{+} dendritic cells in sEH$^{-/-}$ mice. Therefore, in all the major indices of inflammatory cell accumulation tested, epoxy-oxylipin inhibition with epoxI showed opposing actions to epoxy-oxylipin elevation with the use of global sEH$^{-/-}$.

Cells of the monocyte lineage have a disrupted resolution phenotype following epoxygenase inhibition. Using a recently identified novel panel of qRT-PCR of rM markers (32) we examined the total inflammatory cell population for a changes in the resolution phenotype. The total epoxI elicited cell population were: *Timd4*^{lo}, *Tgfb2*^{lo} and *Plxdc2*^{lo} and *IL1f9*^{hi}, *CD86*^{hi} and *Ms4a7*^{hi} compared cells from vehicle / zymosan alone treated animals (supplemental Figure 3). At indicated time points there was also a significant difference in *Ccnb2* (increased with epoxI), and *Aspa* and *Stfa2l1* (decreased with epoxI; Supplemental Figure 2). Since epoxy-oxylipins appear to have the most dramatic effect on monocyte lineage cell recruitment, we examined these targets in FACs sorted Ly6c^{hi} and Ly6c^{lo} monocytes populations, resident macrophages and recruited dendritic cells (DCs) (Figure 5). Normal resolution Ly6c^{hi} monocytes were *Plxcd2*^{hi}, *Aspa*^{hi}, and *F5*^{hi}; Ly6c^{lo} monocytes were *Ccnb2*^{hi}, *Timd4*^{hi}, *Stfa2l1*^{hi}; resident macrophages *IL1F9*^{hi}, and dendritic cells *Ccr*2^{hi} and *IL1F9*^{hi} (Figure 5). Treatment with epoxI caused a significant down-regulation of *Plxcd2* in Ly6c^{hi} monocytes and DCs, *Ccna2* in Ly6c^{hi} and Ly6c^{lo} monocytes, resident macrophages and DCs , *Ccnb2* in Ly6c^{lo} monocytes, and DCs, *Aspa* in Ly6c^{hi} and Ly6c^{lo} monocytes, *F5* in Ly6c^{hi}, resident macrophages and DCs, *Tgfb2* in Ly6c^{hi} and Ly6c^{lo} monocytes, resident macrophages and DCs, and *Timd4* in Ly6c^{lo} monocytes, resident macrophages and DCs (Figure 5A); an upregulation of *Ccr2* in Ly6c^{lo} monocytes, resident macrophages and DCs, *Ccl2* in Ly6c^{hi} monocytes, resident macrophages and DCs, *Ms4A7* in Ly6c^{hi} monocytes, resident

macrophages and DCs, *CD86* in Ly6c^{lo} monocytes, and DCs, and *IL1F9* in Ly6c^{hi} and Ly6c^{lo} monocytes (Figure 5B), whereas *Stfa2l1* was upregulated in Ly6c^{hi} and resident macrophages but inhibited in Ly6c^{lo} monocytes. These findings collectively point towards a more inflammatory phenotype accompanying epoxygenase inhibition, with a clear induction in either or both *Ccl2* and *Ccr2* in all the cell types, and a decrease in resolution monocyte markers of apoptosis and repair (*Timd4* and *Tgfb2* respectively).

Epoxygenase inhibition disrupts resolution phase macrophage differentiation and leads to impaired phagocytosis and efferocytosis. To examine functional changes in the monocyte lineage we examined Ly6c^{+} monocytes, resident macrophages and monocyte-derived macrophage populations. As previously shown Ly6C+ monocytes were elevated, monocyte derived macrophages were reduced and resident macrophage levels remained unchanged (Figure 6A-C). Resident macrophages on a cell per cell level showed reduced expression of differentiation markers and phagocytosis receptors: F4/80, CD11b, MerTk, Timd4 and CD103, but an increase in CD64 (Figure 6C). A similar reduction in F4/80, MerTK, Timd4 and CD103 was also observed in monocyte-derived macrophages (Figure 6C). In contrast, Ly6c^{+} monocytes demonstrated a small but significant increases in markers of differentiation: CD11b, Timd4 and CD64, which was again accompanied by a decrease in CD103 (Figure 6C).

Using the ImageStream system we were easily able to identify phagocytosis from particles and bodies sticking to cells (Figure 7A). Ly6c^{+} monocytes had a small but significant decrease in ability to phagocytose zymosan, but not apoptotic cells (Figure 7B). In contrast, resident macrophages and monocyte-derived macrophages (Figure 7B) both showed greatly reduced ability to phagocytose FITC-labelled zymosan A and efferocytose apoptotic cells *ex vivo*.

Discussion

Our findings show epoxy-oxylipin generating pathways constitute an important control point limiting the accumulation of pro-inflammatory Ly6c^{hi} monocytes and the pro-inflammatory and clearance activity of cells of the monocyte lineage during inflammatory resolution. Similar to cyclooxygenases and lipoxygenases, the epoxygenase pathways of arachidonic acid metabolism are activated during both acute inflammation and resolution. There has been a great recent interest in the therapeutic potential of sEH inhibitors as novel anti-inflammatories. sEH inhibitors or genetic disruption of sEH in mice reduce inflammation in models of endotoxin induced pulmonary inflammation (33), ischemia-reperfusion injury (34, 35), subarachnoid hemorrhage (36), the murine ovalbumin model of asthma (37) and in more chronic models including atherogenic diet induced fatty liver disease and adipose tissue (38) and atherosclerosis (39, 40). In contrast, the expression and roles of epoxy-oxylipins during inflammatory resolution have not been investigated.

Human monocytes *in vitro* express CYP2J and CYP2C enzymes, and in particular CYP2J2 can be induced by TLR4 /LPS activation (21). Rodents have expanded CYP2J and CYP2C subfamilies compared to man, and we found the recently identified epoxygenase CYP2C44 (41) along with CYP2J6 and CYP2J9 to be present in all inflammatory exudates tested, though these findings do not rule out contributions from other local stromal or vascular cells. To test the role of epoxygenases we used the epoxI SKF525A, which we routinely use in our *in vitro* assays (18-20, 42, 43) and we found to have an IC50 for human CYP2J2 of 1μM (20). SKF525A is routinely used *in vivo* within the range of 5-50mg/kg (44-47), and although reported as a selective epoxygenase inhibitor, SKF525A had not been examined in the *in vivo* setting followed by a lipidomic analysis to confirm this. We choose SKF525A as our epoxI as it is soluble in aqueous solutions (in our case PBS), so could be

tolerated far better over longer periods with limited vehicle effect. In our experiments only epoxygenase products and not prostanoids or lipoxygenase products were inhibited (by approximately 50%) with 30mg/kg of the epoxI (supplemental table 2). The epoxI was given at 24h to look at resolution specifically, so the true levels of inhibition may be higher as the kinetics of preformed metabolite removal in the cavity over the 24-48h time period is not known.

The combination of these experiments using epoxI, and $sEH^{-/-}$ mice leads to the conclusion that epoxy-oxylipins are produced during resolution by the resident and incoming inflammatory cells and act in a paracrine/autocrine manner to limit $Ly6c^{hi}$ monocyte accumulation and monocyte macrophage pro-inflammatory phenotype. Following epoxI treatment at the onset of resolution, the elicited cells were predominantly $GR1^{+}$, $Ly6c^{hi}$, $CX3CR1^{lo}$, and produced large amounts of the major $Ly6c^{hi}$ chemokine CCL2. CCL2 was undetectable from zymosan alone elicited cells. Interestingly, while limiting $Ly6c^{hi}$ monocyte recruitment epoxy-oxylipins promoted the differentiation of monocytes in to $F4/80^{++}$ macrophages. I*n vitro* we previously found, epoxygenases and 11,12-EET promote macrophages bacterial phagocytosis which was associated with a reduction in CD11b (42). Similarly, here resident and monocyte derived macrophages had reduced CD11b, along with markers of the mature macrophage phenotype and phagocytosis MerTK and Timd4; the latter being particularly important for efferocytosis (48). These markers correlated with the both resident and monocyte-derived macrophages from epoxI treated mice to have a dramatic reduction in the ability to phagocytose fresh zymosan and efferocytose apoptotic cells. $LyC6^{+}$ monocytes had small but significantly elevated CD11b, and CD64 expression but reduced CD103. There was however little change in the ability of monocytes to phagocytose material apart from a small reduction in the ability to phagocytose zymosan *ex vivo*. These

findings suggest epoxy-oxylipins not only limit the recruitment of pro-inflammatory monocytes, but also limit resident and monocyte-derived macrophages ability to clear inflammatory stimuli and apoptotic cells further indicating a prominent role for epoxygenase pathways in promoting the resolution phenotype. Interestingly, $CD19^{+}$ B-cells and $CD3^{+}$ T-cells were also increased in $sEH^{-/-}$ mice (and reduced with epoxI treatment). Lymphocytes are dispensable for resolution in this model (49), but form an important part of a defense mechanism against reinfection (49). Although we have yet to investigate fully which lymphocyte subtypes are present, these findings nonetheless also provide the first evidence for a role for epoxy-oxylipins in acquired immune responses along with the roles in resolution reported here.

These findings all point to a pro-resolution role for epoxy-oxylipins on the pro-inflammatory monocyte lineage. CCL2 is known to be a central mediator of monocyte recruitment. EpoxI treated animals produced large amounts of CCL2, as did the inflammatory cell population taken *ex vivo*. When we examined individual cell types CCL2 was induced by epoxI in $Ly6C^{hi}$ monocytes, resident macrophages and DCs, whereas its receptor CCR2 (already expressed in $Ly6C^{hi}$ monocytes) was also induced in $Ly6c^{lo}$ monocytes, resident macrophages and DCs. Using gene array analysis we have previously identified a panel of novel resolution monocyte markers (32) including the T-cell co-stimulator *CD86*, the IL-1 family gene-9 (*IL-1f9*; *IL-36*), and the CD20-like family member *Ms4a7*; these were all elevated after epoxI treatment, while the phosphatidylserine apoptotic cell receptor *Timd4*, *Tgfb2* and *Plxdc2* were all decreased. The roles of a number of these novels markers are still not known in resolution, but clearly epoxI mediated changes in expression along with changes to functional clear antigens and apoptotic cells

represent cells of a phenotype of highly dysregulated resolution compared to the typical resolution monocyte cell population.

Recently, the major rat monocyte epoxygenase Cyp2j4 was knocked out using a zinc finger nuclease-mediated gene targeting approach (50). The resultant bone marrow derived macrophages showed a highly profibrotic enriched transcriptome and activity, and an induction of PPARγ (50). Although not an identical phenotype to those observed in our study, this adds to the literature showing lipid metabolizing CYPs have a critical role to play in the inflammatory phenotype of cells in the monocyte lineage.

EETs are too unstable to give as a bolus dose *in vivo*. Therefore, we tested the direct anti-inflammatory effects of monocyte epoxygenases *ex vivo*. 14,15-EET, the most abundant epoxygenase derived EET produced in the peritoneal cavity inhibited inflammatory cell activation *iNOS* and *IL-12 ex vivo,* from zymosan treated mice. Interestingly, the linoleic acid epoxygenase products 9,10-EpOME and 12,13-EpOME are highly expressed in the naive cavity, and with the exception of 9,10-EpOME, which drops rapidly during acute inflammation, change very little. Our results indicate therefore a potential change in substrate utilization by epoxygenases from linoleic acid in homeostasis to arachidonic acid during inflammation. Linoleic acid epoxygenase products are generally considered pro-inflammatory and cytotoxic (51-53), while the EETs are anti-inflammatory. How this concept fits with the high levels of EpOMEs and DiHOMEs in the naive peritoneal cavity is intriguing and points to novel as yet undiscovered homeostatic roles for these mediators in the peritoneal cavity. In contrast, to the EETs, which inhibited *Ccl2* in monocytes *ex vivo*, 9,10-EpOME induced *Ccl2* (supplemental figure 4), suggesting substrate utilization may be an additional layer of control over the effects of the epoxygenase pathway. High EpOME levels have been found in acute respiratory distress syndrome and in patients with extensive burns

(54), however, the roles of EpOMEs in inflammation and homeostasis remain relatively poorly understood, though it is becoming clearer that many of the cytotoxic effects attributed to them are in fact due to their sEH metabolite DiHOMEs (53, 55-57). Clearly more work is needed to elucidate the function of these linoleic acid epoxygenase products in homeostasis as well as pathophysiology. At this stage we cannot rule out potential effects of DHA and EPA CYP metabolites, but since we saw no significant change in their production with epoxI we have here focused on arachidonic acid and linoleic acid epoxy-oxylipins.

The levels of 12/15-lipoxygenase 9,10- and 12,13-HODE and 12-, and 15-HETE were high in the peritoneal cavity and consistent with the report that high levels of resident macrophages contain 12/15-lipoxygenase in mice where it regulates immune function. Our findings are also consistent with this report in that 12/15-lipoxygenase products drop during acute inflammation (in our experiments only 9-HODE returned back to basal levels by 48h), and support the findings that 12/15-lipoxygenase has an important homeostatic immunoregulatory role in this model (58).

In conclusion, we have characterized the epoxygenase pathways during a model of inflammatory resolution. Inhibition of epoxygenases during this resolution phase established a pro-inflammatory environment that allows the recruitment of pro-inflammatory Ly6c monocytes and macrophages with a highly dysregulated resolution/ clearance phenotype, while mice where epoxygenase products are elevated have an enhanced resolution phenotype. An efficient and active epoxygenase pathway therefore joins similar pro-resolution lipid pathways such as the resolvins in the requirement for effective inflammatory resolution. Drugs that elevate epoxygenase products, or epoxygenase product mimetics therefore represent novel resolution targets for chronic inflammatory disorders.

Materials and Methods

Materials: EETs were purchased from Cayman Chemical Company (Cambridge Bioscience, Cambridge, UK). SKF525A was purchased from Biomol (Affiniti Research Products, Exeter, UK. Cytokines and the MCP-1 ELISA were obtained from R&D Systems (Abingdon, Oxfordshire, UK). The TNFα ELISA was from eBioscience. (Hatfield, Herts, UK). Unless stated, all other reagents were purchased from Sigma-Aldrich (Poole, Dorset, UK).

Animal Models. All experiments were completed in adult male mice on a pure C57BL/6 background. Control C57BL6/J mice were bred under standard conditions and maintained in a 12 h light/dark cycle at 22°C and given food and tap water ad libitum in accordance with United Kingdom Home Office regulations. Mice with targeted disruption of sEH were re-derived and backcrossed onto a C57BL/6 genetic background for more than 10 generations, as previously described (31, 59, 60). The $sEH^{-/-}$ mice have significantly higher circulating epoxide:diol ratios compared to wild-type littermates consistent with functional sEH disruption (59, 61). Peritonitis was induced by i.p. injection of 1mg type A zymosan in 0.5ml PBS after 15 sec of sonication on ice. Inflammatory cells were retrieved at the time points described in the Results section by injecting 2ml sterile PBS. Cells were counted by hemocytometer, and exudates were stored at −80°C until further analysis. In some experiments the epoxI SKF525A (30 mg/kg) or sterile PBS (0.5ml vehicle), was given i.p. 24h after zymosan injection, and at 12h time points thereafter up until 96h. These studies received institutional review board approval for the use of mice from the United Kingdom Home Office.

Lipid analysis. Eicosanoids and other fatty acid metabolites were extracted from inflammatory exudates by solid-phase extraction and eluted in ethyl acetate, essentially as described (22); extended details in supplemental methods. Lipids were separated by reverse-phase HPLC on a 2-μ 150-mm, 5-μm Luna C18 column (Phenomenex, Torrance, CA, USA) and quantified using a MDS Sciex API 3000 triple quadrupole mass spectrometer (Applied Biosystems) with negative mode electrospray ionization and multiple reaction monitoring, as described (12). The relative response ratios of each analyte were used to calculate concentrations, while correcting for surrogate losses via quantification relative to internal standards. The sensitivity of analytes ranges from 0.25-25pg.

FACS analysis and cell sorting. Flow cytometry and cell sorting was done on LSR-II/LSR-Fortessa and FACSAria (BD Biosciences), respectively. Cells were incubated with Fc-Blocker (AbD Serotec) and fluorescent-labeled antibodies. Data were analyzed with FlowJo 7.0.1 software (Tree Star) using fluorescence minus one controls as the reference for setting gates. Antibodies were obtained from BD Biosciences (F4/80, CD11b, CD11c, Ly6c, Ly6g, Gr1, CD3, CD19, CD4, CD8, CD62l, CD44, MerTK, CD64, CD103, Timd4 and major histocompatibility complex (MHC)-II)(62). To identify resident macrophages (62) PKH26-PCL^{red} (2 mL of 500 nM; Sigma) was injected i.p. 3h before injection of zymosan. In cell sort experiments, monocytes and macrophages were sorted from a population of $CD19^-$ and $CD3^-$ cells as either $Ly6c^+F4/80^+$ and $Ly6c^-F4/80^+$. For identification of $Ly6g^+$ neutrophils and $Ly6c^+$ monocytes a combination of Gr-1 and anti-Ly6c or anti-Ly6g was also used. Resident macrophages were characterized as PKH^{red++}, DCs were characterized by PKH^{red-} MHC-II^+, $Ly6c^{hi}$ monocytes as $LyC6^{hi}$ PKH^{red+} MHC-II^-, and $Ly6c^{lo}$ as $Ly6c^{lo}$ PKH^{red-} as previously described (62).

Cell culture *ex vivo*. The peritoneal lavage was treated with ACK lysis buffer to remove erythrocytes. After being washed, peritoneal cells were suspended in DMEM supplemented with 10% FBS and 50 μg/ml penicillin/streptomycin. The cells ($2x10^6$) were seeded in wells of a 12-well plate and left to adhere for 45 min in a humidified CO_2 incubator. Non-adherent cells were removed by three washes using DMEM. Remaining adherent cells (approximately $1x10^6$ cells) were incubated in 0.5ml DMEM in the presence or absence of 1μM 14,15-EET or 11,12-EET or vehicle (0.3% EtOH). After 6h, cell-free supernatants were removed and cells lysed using Trizol for subsequent RNA extraction.

RT-PCR. Cells analyzed by qRT-PCR were lysed and RNA isolated using Trizol™ (Invitrogen). The panel of resolution markers: *Timd4* (T-cell immunoglobulin and mucin domain containing 4), *Tgfb2*, *Plxdc2* (Plexin domain-containing protein 2), *IL1f9* (Interleukin-1 family member 9), *CD86*, *Ms4a7* (membrane-spanning 4-domains, subfamily A, member 7), *Ccna2* (cyclin A2), *Ccnb2* (cyclin B2), *F5* (coagulation factor V), *Aspa* (aspartoacylase), and *Stfa2l1* (stefin A2 like 1) were measured by qRT-PCR as previously described (32). Resolution monocytes (r)M were previously found to be enriched for cell cycle/proliferation genes as well as *Timd4*, and *Tgfb2*, key systems in the termination of leukocyte trafficking and clearance of inflammatory cells. *Ly6c*, *CX3CR1*, *Ccl2*, *Ccr2*, *Cyp2j5*, *Cyp2j6*, *Cyp2j9*, *Cyp2j13*, *Cyp2c29*, *Cyp2c38*, *Cyp2c39*, *Cyp2c44*, *Cyp2c50*, *Cyp2c54*, *Cyp2c55*, *Cyp2a1*, *Cyp2u1*, *Cyp2s1* and *β-actin* were measured by RT-PCR. Primers are detailed in supplemental table 1.

Efferocytosis and Phagocytosis assays. Apoptotic cells (thymocytes) were produced from the thymuses of 3 naïve control c57/bl mice killed 24h pre-sort. Harvested thymuses were passed through a 70 μ mesh and then lysed with ACK buffer for 3 min. Cells were washed 2x

with RPMI 1640 with 100 U/ml pen-strep. To induce apoptosis, thymocytes were resuspended in media at 2 x 10^6 cells/ml and exposed to UV radiation (~300 nm) for 20 min followed by incubation for 16-24 h at 37^{o}C with 5% CO_2. Cells were then washed with PBS and labelled with 2uM CFSE according to manufacturer's protocol (Life technologies CellTrace CFSE).

Sorted cell populations, 2x10^5 cells/well were plated in 24 well plates and resuspended in X-Vivo 15 media (Lonza) containing 10% FBS, 2mM EDTA, and 1x pen-step. Cells were then challenged with apoptotic thymocytes (5: 1 monocyte / macrophage) or FITC labelled zymosan A BioParticles (10 particles: 1 monocyte / macrophage) for 30min at 37^{o}C; 5% CO_2. Phagocytosis was stopped by placing the plates on ice. Cells were detached using 10 mM EDTA containing 4 mg/ml lignocaine for 20 min. Cells were stained for either F4/80 or Ly6C (APC) for 20 min (see FACs sections above), before being washed 2x with PBS containing 2 mM EDTA. Cells were then fixed using 4% PFA in PBS and analysed on ImageStreamX Mk2 (MerckMillipore).

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Figure Legends

Figure 1. LC/MS/MS analysis of oxylipin generating pathways in the mouse peritoneal cavity during acute inflammation and resolution initiated by zymosan A. A) Absolute levels of oxylipins determined in a naive peritoneal cavity lavage fluid of male C57bl/6 mice. B) Sum of the oxylipins generated cyclooxygenase (COX), CYP450 (CYP), CYP450-lipoxygenase like (CYP-L) or lipoxygenase pathways (LO) using either arachidonic acid (AA), linoleic acid (LA), docosahexaenoic acid (DHA) or eicosapentaenoic acid as a substrate in the naïve peritoneal cavity. C) Heatmap showing fold changes in oxylipins formation following zymosan A treatment (1mg i.p.) from 0 to 4h (peak of acute inflammation), 24h and 48h (resolution). D) and E) show fold normalized (SQRT) of oxylipins produced by COX-AA (prostanoids), CYP-AA (DHETs), CYP-L-AA (19- and 20-HETE), CYP-DHA (19,20 EpDPE and 19,20-DiHDPA), CYP-EPA (17,18-DHEQ) and 5-LO-AA (5-HETE) pathways (D) and CYP-LA (EpOMEs and DiHOMEs), 8/12/15-LO (8-, 12- and 15-HETE), and LO-LA (HODEs) pathways (E) in response to zymosan A over 48h. Pathways in D and E represent two distinct responses to challenge with zymosan A. The data represents the mean ± s.e.m from n=4-8 mice per group.

Figure 2. Epoxygenase products form in a biphasic manner mirroring acute inflammation and inflammatory resolution: selective inhibition of CYP-AA and CYP-LA products by epoxI. Mean fold change in (A) CYP-AA products: DHETs and (B) CYP-LA (DiHOME), CYP-DHA (DiHDPA) and CYP-EPA (DHEQ) products 0-48h. Each mediator was normalized to its paired levels found in the naive cavity in each experiment; the levels found in the naive cavity given

an arbitrary value of 1. * denotes $p<0.05$ by one-sample t-test from 0h. † denotes $p<0.05$ by unpaired t-test between the values observed at 24 and 48h. (C) Mean fold change in epoxygenase product formation in the presence or absence of EpoxI (30mg/kg). EpoxI was given at 24h and 36h Epoxy-oxylipins were measured at 48h and represented as fold to the levels observed in the naive cavity at 0h. * denotes $p<0.05$ by unpaired t-test.

Figure 3. Epoxygenase inhibition during resolution causes Ly6c-hi monocyte recruitment, and Ccl2 generation. (A) Change in lymphocytes, monocytes/ macrophages and PMNs in the peritoneal cavity of mice 48h post-zymosan treatment. At 24h and 36h mice were given either sterile PBS or epoxI (30mg/kg; i.p.). Total cell numbers were counted on a heamo-cytometer and the proportion of each cell type determined by FACs. The data represents the mean ± s.e.m from n=6 mice per group. * denotes $p<0.05$ by unpaired t-test between vehicle (Cont; PBS) and epoxI treated mice. (B) Example of FACs analysis of total cell (top panels) and GR^+ populations (bottom panels) of cells elicited by zymosan alone (control; left) or in the presence of epoxI (right) in terms of size (FSc) and granularity (SSc), while bottom panels show the change in the $GR1^+$ cell population. Relative expression of Ly6c (C), Ccr2 (D), CXCR1 (E), and Ccl2 (F) mRNA compared to β-actin in elicited cells from zymosan+ control or epoxI treated mice taken at 48h. (G) CCL2 generation from cells from zymosan+ control or epoxI treated mice. Cells were elicited at 36h and left for a further 8h *ex vivo* and CCL2 measured by ELISA. (H) *Ccl2*, (I) *iNOS*, (J) *IL-12* and (K) *TNFα* mRNA expression in zymosan elicited cells at 36h treated with 14,15-EET or 11,12-EET. Cells were elicited at 36h and treated for a further 7h with EETs. The data represents the mean ± s.e.m from n=3-4 mice per group. * denotes $p<0.05$ by unpaired t-test between vehicle (Cont; PBS) and epoxI treated mice.

Figure 4. Endogenous oxylipins regulate Ly6c^{hi} monocyte, F4/80 macrophage, CD19 and CD3 cell populations during resolution. Changes in (A) Ly6c^{hi} cell populations, (B) Ly6g^{+} cell populations, (C) F4/80 expression (mean fluorescent intensity; mfi; left) and F4/80++ cell populations (right); (D) CD19^{+}, (E) CD3^{+} and (F) MHCII^{+} CD11c^{+} dendritic cell populations: in control and epoxI treated mice (solid black bars) in the left hand panels; and in wild type (wt) and sEH$^{-/-}$ mice in the right hand panels. The proportion of cells in each group were determined by FACs and related back to cell numbers. The data represents the mean ± s.e.m from n=4-5 mice per experimental group; * indicates $p<0.05$ by 2-way ANOVA, or unpaired t-test.

Figure 5. Epoxygenase inhibition alters the resolution phenotype of recruited cells of the monocyte lineage. Inflammation was initiated by zymosan (1mg i.p.), and mice treated with vehicle control (PBS) or epoxI (30mg/kg i.p.) at 24h and 36h. Cells were collected and pooled from n=10 mice and Ly6c^{hi}, Ly6c^{lo} monocytes, resident macrophages and recruited dendritic cells were sorted on a FACSAria as detailed in the Methods. A qRT-PCR resolution monocyte panel (n=3-6) was then used to examine the phenotype of each cell type. In the presence of epoxI: (A) *Plxcd2*, *Ccna2*, *Ccnb2*, *Aspa*, *F5*, *Tgfb2* and *Timd4* were found to be down-regulated in the cell types; (B) *Ccr2*, *Ccl2*, *Ms4A7*, *CD86* and *IL1F9* were up-regulated in the cell types; (C) *Stfa2l1* was up and down regulated in a cell type specific manner. (D) Top table summarizes the relative basal levels of each transcript in each cell type, the bottom table summarizes the effect of epoxI on the different transcripts. * indicates $p<0.05$ by unpaired t-test.

Figure 6. Epoxygenase inhibition regulates monocyte and macrophage differentiation. Inflammation was initiated by zymosan (1mg i.p.), and mice treated with vehicle control (PBS) or epoxI (30mg/kg i.p.) at 24h and 36h. Cells were collected at 48h and pooled from n=9-18 mice. Ly6c$^+$ monocytes, resident macrophages and monocyte-derived macrophages were sorted on a FACSAria as detailed in the Methods. (A) Representative Zebra Plots of the cell populations from control and epoxI treated mice expressing Ly6c and F4/80, with labeled populations representing [A] Ly6c$^+$ monocytes, [B] resident macrophages, and [C] monocyte derived macrophages. (B) Representative Dot Plots of the Ly6c$^+$, resident macrophage (Resi-Mϕ) and monocyte-derived macrophage (Mono-Mϕ) from control and epoxI treated mice expressing PKH-26 and CD11b (top panels) and CD64 and MerTK (bottom panels). (C) Changes in cell numbers, and expression (mean fluorescent intensity; MFI in relative light units; RLU) for F4/80, CD11b, MHCII, CD103, CD64, MerTK and Timd4 (TM4) in the Ly6c$^+$, Resi-Mϕ, Mono-Mϕ cell populations. Data is mean ± s.e.m from n=3-6 pooled samples and * indicates $p<0.05$ by unpaired t-test.

Figure 7. Epoxygenase inhibition reduces the phagocytic activity of monocyte-derived and resident macrophages. Inflammation was initiated by zymosan (1mg i.p.), and mice treated with vehicle control (PBS) or epoxI (30mg/kg i.p.) at 24h and 36h. Cells were collected at 48h and pooled from n=9-18 mice. Ly6c$^+$ monocytes, resident macrophages and monocyte-derived macrophages were sorted on a FACSAria as detailed in the Methods. Sorted cells were then tested for their ability to phagocytose CFSE labelled-apoptotic cells (thymocytes) or FITC-labelled-zymosan BioParticles over 30min. Cells were gated using F4/80 or LyC6 and on an ImageStreamX Mk2. (A) ImagesStream analysis differentiates cells which

phagocytosed apoptotic cells or zymosan (internalization score >0) from those where particles or bodies just stick to the cell (internalization score 0). (B) *Ex vivo* phagocytosis of apoptotic cells (left panels) and zymosan (right panels) by Ly6c^{+}, resident macrophage (Resi-Mϕ) and monocyte-derived macrophage (Mono-Mϕ) cell populations from control and epoxI treated mice. Data is mean ± s.e.m from n=3-6 pooled samples and * indicates $p<0.05$ by unpaired t-test.

Figure 8. Role of epoxy-oxylipins in the resolution. The resolution of zymosan initiated inflammation involves monocytes, dendritic cells, T and B cell recruitment and the differentiation of monocytes in to resolution type macrophages. In this process (as revealed by epoxI treatment and sEH -/- mice), epoxy-oxylipins, most likely EETs, limit Ccl2 and Ccr2 expression, Ly6c^{hi} monocyte accumulation, T and B cell recruitment and encourage the formation of mature phagocytotic resolution macrophages. Ly6c^{hi} monocytes, Ly6c^{lo} monocytes, dendritic cells, monocyte-derived and resident macrophages are all activated in the presence of epoxI.