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12/15-Lipoxygenase Regulates the Inflammatory Response to Bacterial Products In Vivo

Vincent Dioszeghy,* Marcela Rosas,* Benjamin H. Maskrey,* Chantal Colmont,* Nicholas Topley,* Pavlos Chaidisis,† Hartmut Kühn,† Simon A. Jones,* Philip R. Taylor,* and Valerie B. O’Donnell2*†

The peritoneal macrophage (Mφ) is the site of greatest 12/15-lipoxygenase (12/15-LOX) expression in the mouse; however, its immunoregulatory role in this tissue has not been explored. Herein, we show that 12/15-LOX is expressed by 95% of resident peritoneal CD11bhigh cells, with the remaining 5% being 12/15-LOX−. 12/15-LOX+ cells are phenotypically defined by high F4/80, SR-A, and Siglec1 expression, and enhanced IL-10 and G-CSF generation. In contrast, 12/15-LOX− cells are a dendritic cell population. Resident peritoneal Mφ numbers were significantly increased in 12/15-LOX−/− mice, suggesting alterations in migratory trafficking or cell differentiation in vivo. In vitro, Mφ from 12/15-LOX−/− mice exhibit multiple abnormalities in the regulation of cytokine/growth factor production both basally and after stimulation with Staphylococcus epidermidis cell-free supernatant. Resident adherent cells from 12/15-LOX−/− mice generate more IL-1, IL-3, GM-CSF, and IL-17, but less CCL5/RANTES than do cells from wild-type mice, while Staphylococcus epidermidis cell-free supernatant-elicited 12/15-LOX−/− adherent cells release less IL-12p40, IL-12p70, and RANTES, but more GM-CSF. This indicates a selective effect of 12/15-LOX on peritoneal cell cytokine production. In acute sterile peritonitis, 12/15-LOX+ cells and LOX products were cleared, then reappeared during the resolution phase. The peritoneal lavage of 12/15-LOX−/− mice showed elevated TGF-β1, along with increased immigration of monocytes/Mφ, but decreases in several cytokines including RANTES/CCL5, MCP-1/CCL2, G-CSF, IL-12-p40, IL-17, and TNF-α. No changes in neutrophil or lymphocyte numbers were seen. In summary, endogenous 12/15-LOX defines the resident Mφ population and regulates both the recruitment of monocytes/Mφ and cytokine response to bacterial products in vivo.

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Materials and Methods

Animals

All animal experiments were performed in accordance to the United Kingdom Home Office Animals (Scientific Procedures) Act of 1986. 12/15-LOX knockout mice generated as described previously and wild-type (WT) male C57BL/6 mice (25–30 g) from Charles River Laboratories were kept in constant temperature cages (20–22°C) and given free access to water and standard chow (9). HETE quantitation using liquid chromatography/mass spectrometry

12-HETE-d8 (10 ng) was added to each lavage before extraction, as internal standard. Hydroperoxides were then reduced to their corresponding stable alcohols using 1 mM SnCl2. Lipids were extracted by adding a solvent mixture (1 M acetic acid/2-isopropanol/hexane (2/20/30, v/v/v)) to the sample at a ratio of 2.5 ml of solvent mixture to 1 ml of sample.

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**Materials and Methods**

CD4, CD8, or CD45R (red) and 12/15-LOX (green) or mannose receptor (red), as described in Materials and Methods. Right panel, FACS analysis shows two populations of CD11b+ cells, characterized as F4/80+/CD11b+ (R1) or F4/80−/CD11b− (R2). Resident peritoneal lavage was analyzed for expression of CD11b or F4/80 as described in Materials and Methods. B, R1 and R2 cells are defined by 12/15-LOX or mannose receptor expression, respectively. Total lavage (left), FACS-sorted R1 cells (middle), and R2 cells (right) were immunostained for 12/15-LOX or mannose receptor (red), as described in Materials and Methods. Resident peritoneal lavage cells were cytospun onto glass slides and immunostained for 12/15-LOX or CD11b as described in Materials and Methods. Right panel, FACS analysis shows two populations of CD11b+ cells, characterized as F4/80+/CD11b+ (R1) or F4/80−/CD11b− (R2). Resident peritoneal lavage was analyzed for expression of CD11b or F4/80 as described in Materials and Methods. C, 12/15-LOX is not expressed by T or B cells. Total resident lavage cells were immunostained for CD4, CD8, or CD45R (red) and 12/15-LOX (green) as described in Material and Methods.

**Peritoneal inflammation**

Peritoneal inflammation was established in mice through i.p. administration of a defined 500 µl dose of SES, prepared from a clinical isolate of S. epidermidis (10). At defined intervals following SES administration, animals were sacrificed and the peritoneal cavity was lavaged with 2 ml of ice-cold PBS. Composition of the leukocyte infiltrate was assayed using a Coulter Z2 counter (Beckman Coulter), differential cell staining of cytospins with Accustain (Sigma-Aldrich), and flow cytometric analysis (see below). Lavage fluids were rendered cell-free by centrifugation for analysis of inflammatory mediators. In some experiments, 10 ng 12-HETE was administered at the same time as SES.

**Immunocytochemistry**

Cytospin preparations of peritoneal cells were methanol-fixed on glass slides, permeabilized using 0.1% (w/v) Triton X-100/PBS, and blocked using 1% (w/v) BSA/PBS. 12/15-LOX, F4/80, or mannose receptor (MR) expression was detected using guinea pig anti-12/15-LOX (generated in our laboratory), in combination with either rat anti-F4/80 or rat anti-MMR (both Serotec), with goat anti-rat IgG-Alexa 568 and goat anti-guinea pig IgG-Alexa 488 as secondaries (Molecular Probes). Negative controls used equivalent concentrations of isotype-matched rat or guinea pig IgG Ab. Nuclei were stained using DRAQ5 (Biostatus). Imaging was performed on an Axiovert 100 inverted microscope connected to a Bio-Rad MRC 1024ES laser scanning system (Bio-Rad Microscience) using standard analysis software (Lasersharp 2000, Bio-Rad Microscience). Images were acquired using a ×40 oil lens, with excitation at 488 nm and emission 522/35 nm, and excitation at 568 nm and emission 595/35 nm, at room temperature. For each slide, three separate regions were imaged and cells counted to calculate percentages.

**Flow cytometric analysis**

Leukocytes were incubated with mouse Fc block (BD Pharmingen) before immunolabeling for 30 min at 4°C. Cells were incubated 1 h with primary fluorochrome-conjugated or unconjugated Abs, as follows: anti-IgG (7/4, Serotec), anti-B220 (RA3-6B2, BD Pharmingen), anti-CD16/CD32 (2.4G2, BD Pharmingen), anti-CD4 (GK1.5, BD Pharmingen), anti-CD11b (M1/70, BD Pharmingen), anti-CD11c (HL3, BD Pharmingen), anti-CD14 (Sa2-8, e-Bioscience), anti-CD40 (3/23, BD Pharmingen), anti-CD54 (3E2, BD Pharmingen), anti-CD62L (MEL-14, BD Pharmingen), anti-CD80 (16-10A1, BD Pharmingen), anti-CD83 (Michel-17, e-Bioscience), anti-CD86 (PO3, BD Pharmingen), anti-CD115 (604B5 2E11, Serotec), anti-CD11b (281-2, BD Pharmingen), anti-CD206 (MRD53, Serotec), anti-CCR2 (E68, Abcam), anti-CCR5 (C54-3448, BD Pharmingen), anti-CX3CR1 (Torrey Pines Bios), anti-CXCR4 (2B11/CXCR4, BD Pharmingen), anti-ecto-5-D2 (D2.11E4) (11), anti-F4/80 Ag (Cl:A3-1, Serotec), anti-Gr-1 (RB6-8C5, BD Pharmingen), anti-I-A/I-E (2C9, BD Pharmingen), anti-MARCO (macrophage receptor with collagenous structure) (ED31, Serotec), anti-TLR2 (6C6, e-Bioscience), streptavidin-RPE (Serotec), streptavidin-RPE-Cy5 (Serotec), and RPE anti-rabbit IgG (Serotec). GM-CSF receptor was stained using a chimeric protein fusing the mouse GM-CSF to a mutated Fc region of human IgG1 (12). Where necessary, cells were incubated for a further 30 min at 4°C with appropriate fluorochrome-conjugated secondary Abs or streptavidin. Cells were washed and fixed with CellFix (BD Biosciences) and then analyzed by using a FACS Calibur flow cytometer and CellQuest software (BD Biosciences). Data were acquired from 50,000 events, and staining was compared with fluorochrome-conjugated isotype control Abs.

For cell cycle analysis, in brief, cells were fixed in 70% ethanol (2 h at −20°C) and then resuspended in 45 mM Na2HPO4, 2.5 mM citric acid, 0.1% Triton X-100, and blocked using 1% (w/v) BSA/PBS. 12/15-LOX, F4/80, or mannose receptor (MR) expression was detected using guinea pig anti-12/15-LOX (generated in our laboratory), in combination with either rat anti-F4/80 or rat anti-MMR (both Serotec), with goat anti-rat IgG-Alexa 568 and goat anti-guinea pig IgG-Alexa 488 as secondaries (Molecular Probes). Negative controls used equivalent concentrations of isotype-matched rat or guinea pig IgG Ab. Nuclei were stained using DRAQ5 (Biostatus). Imaging was performed on an Axiovert 100 inverted microscope connected to a Bio-Rad MRC 1024ES laser scanning system (Bio-Rad Microscience) using standard analysis software (Lasersharp 2000, Bio-Rad Microscience). Images were acquired using a ×40 oil lens, with excitation at 488 nm and emission 522/35 nm, and excitation at 568 nm and emission 595/35 nm, at room temperature. For each slide, three separate regions were imaged and cells counted to calculate percentages.

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acid, and 0.1% Triton X-100 (30 min at 37°C). Cells were stained by addition of 3.75 vol of 10 mM PIPES (pH 6.8), 100 mM NaCl, 2 mM MgCl₂, 0.1% Triton X-100, 40 g/ml RNase A, and 20 g/ml propidium iodide (30 min at room temperature in the dark) and analyzed by flow cytometry on a FACSCalibur flow cytometer and analyzed with CellQuest software.

**Cell population determination**

Peritoneal cell populations were identified as follows: M/monocytes (Mo) are CD11b/F4/80, dendritic cells (DC) are CD11b high/F4/80 med/CD11c, lymphocytes are CD11b low/F4/80 and show characteristic forward light scatter-side light scatter pattern, and neutrophils are CD11b high/F4/80 cells. Blood Mo were identified as CD11b/F4/80 cells in whole-blood samples.

**Ag presentation assays**

Cultures were established in 96-well U-bottom tissue culture plates (Costar). CFSE-labeled CD4 T cells (30,000 cells per well) from spleens of DO11.10 mice (TCR with specificity for an OVA peptide) were cultured with increasing concentrations of APCs (0, 2,500, 5,000, 10,000, and 20,000 cells) in RPMI 1640 supplemented with 10% low-endotoxin FBS and 50 μM -mercaptoethanol. OVA (Sigma-Aldrich) was used at final concentration of 0.5 mg/ml per well. Bone marrow-derived DC and the synthetic OVA peptide (323–339) were used as controls. After 72 h of incubation at 37°C, supernatants were collected and kept at 20°C for subsequent assay of IL-2 production by ELISA. The cell pellets were washed with PBS, resuspended in blocking buffer (PBS supplemented with 5% heat-inactivated rabbit serum, 0.5% BSA, 5 mM EDTA, and 2 mM NaN₃), and incubated for 1 h at 4°C. After three washes, cells were resuspended in 1% formaldehyde (in PBS) and analyzed on a FACSCalibur (Becton Dickinson). FACS analysis was performed using FlowJo software (Tree Star). CD3 T cells were gated and the CFSE fluorescence as a measure of T cell proliferation was determined. T cell proliferation data were expressed as a division index (average number of divisions that dividing cells underwent) as determined using FlowJo software.

**Cell isolation and culture**

Cell subpopulations were isolated from peritoneal lavages by MoFlo sorting. In brief, peritoneal cells from 15 to 20 mice were pooled and then labeled with F4/80-FITC and CD11b-allophycocyanin Abs. The cells were then washed three times with PBS, 0.5% BSA before flow-sorting based on their level of expression of CD11b and F4/80. Sorted cells were then cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM -glutamine, 10 U/ml penicillin, and 10 μg/ml streptomycin for 3 h. Nonadherent cells were removed and the remaining cells were cultured in fresh medium with or without SES (1/50). After 24 h of culture, cell culture supernatants were collected and stored at 20°C for future cytokine determination. Sorted cells were also used as APC for Ag presentation assays.

**Cytokine determination**

IL-1α, IL-1β, TNF-α, IL-10, G-CSF, GM-CSF, IL-13, MIP-1α/CCL3, IL-12p40, and IL-12p70 concentrations in culture supernatant fluids and in peritoneal fluids were measured by using a commercial Bio-Plex mouse cytokine assay kit (Bio-Rad). IL-6 and MCP-1/CCL2 concentrations in culture supernatant fluids and in peritoneal fluids were measured by using BD OptEIA mouse-specific ELISA set according to the manufacturer’s protocol (BD Biosciences). RANTES/CCL5 and KC/
Cytokine production of resident peritoneal Mϕ populations

<table>
<thead>
<tr>
<th>Cytokine/Growth Factor</th>
<th>Mean (±SEM), pg/ml</th>
<th>Two-way ANOVA (p-value)</th>
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<tr>
<td></td>
<td>R1</td>
<td>R1 + SES</td>
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<tr>
<td>IL-1α</td>
<td>52.58 (21.78)</td>
<td>391.20 (215.26)</td>
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<tr>
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<td>23.11 (6.39)</td>
<td>62.35 (21.50)</td>
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<td>5.093 (2.248)</td>
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<td>57.51 (15.58)</td>
<td>139.60 (49.95)</td>
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<td>14.96 (6.31)</td>
<td>38.69 (17.03)</td>
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<td>IL-12p70</td>
<td>15.05 (7.09)</td>
<td>48.30 (23.26)</td>
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<td>92.25 (77.02)</td>
<td>194.39 (139.26)</td>
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<td>1,412 (449)</td>
<td>2,289 (839)</td>
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<tr>
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<td>3.701 (2.884)</td>
<td>6.885 (3.747)</td>
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<tr>
<td>MCP-1</td>
<td>1.598 (456)</td>
<td>7.122 (4037)</td>
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<tr>
<td>KC</td>
<td>4.568 (2095)</td>
<td>11.117 (2933)</td>
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<tr>
<td>TNF</td>
<td>86.14 (68.95)</td>
<td>347.14 (159.82)</td>
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<tr>
<td>G-CSF</td>
<td>707 (330)</td>
<td>7,835 (2569)</td>
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<tr>
<td>GM-CSF</td>
<td>36.63 (2147)</td>
<td>174.67 (9877)</td>
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<th>Interaction</th>
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<th>Cell type</th>
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| 12/15-LOX (R1) & 12/15-LOX (R2) peritoneal populations were MoFlo sorted and cultured with or without SES for 24 h. Experiments were repeated four times (n) on separate occasions, except for MCP-1 (n = 3), and data were analyzed using a two-way repeated-measures ANOVA calculated in GraphPad Prism. Boldface type indicates statistically significant p-values, and underlined values are those that show a trend toward significance. Nonsignificant values are not shown. Interaction significant implies that R1 and R2 respond differently to SES. p-values for SES or cell type < 0.05 indicate that there is a significant effect of either on the response.

CXCL1 concentrations in culture supernatant fluids and in peritoneal fluids were measured by using mouse specific DuoSet ELISA kit according to the manufacturer’s protocol (R&D Systems). For TGF-β concentrations measurement, latent TGF-β1 was activated to immunoreactive TGF-β1 by adding 0.1 ml of 1 N HCl to 0.5 ml of sample for 10 min. Samples were then neutralized with 0.1 ml of 1.2 N NaOH/0.5 M HEPES.

Real-time PCR analysis of gene expression

Real-time PCR was conducted on a DNA Engine Opticon 2 (MJ Research/Biozym) using the QuantiTect SYBR Green PCR kit from Qiagen according to the recommendations of the vendor, and the following PCR protocol was applied: 15-min hot start at 95°C, followed by variable numbers of amplification cycles consisting of denaturation (30 s at 94°C), annealing (30 s at 60°C), and synthesis (30 s at 72°C) phases in a total volume of 20 μl. Amplicons were used as external standards for each gene product including GAPDH. For melting curve analyses, the temperature was elevated slowly from 60°C to 95°C. Data were acquired and analyzed with the Opticon Monitor software (version 2). The amplification kinetics were recorded as sigmoid progress curves, for which the fluorescence was plotted against the number of amplification cycles. Homogeneity of the amplified PCR products was tested by melting curve analyses, and the following primer pairs were used: GAPDH, 5'-CCA TCA CCA TCT TCC AGG AGC GA-3', GAPDH (reverse), 5'-GGA TGA TTC TCC CAG CTC TG-3'; COX1 (forward), 5'-CTG CGG CTC TCA AAG GAT GGG A-3', COX1 (reverse), 5'-GGA AGA GAA GGC ATC ACC CAG-3'; COX2 (forward), 5'-GTG CCT GAG CAT CTA CGG TTT G-3', COX2 (reverse), 5'-TGC TGG TCA ATG GAC TGT G-3'.

Statistical analysis

Data were analyzed using Student’s t test or two-way ANOVA, as indicated in the text. p-values of <0.05 were considered significant.

Results

12/15-LOX expression in peritoneal cells

Basally, ~95% of resident peritoneal Mϕ of WT C57BL/6 mice, identified by F4/80 and CD11b expression, express 12/15-LOX while 5% are negative (Fig. 1A, left and middle panels). Phenotypic characterization by FACS showed two distinct populations of CD11bhigh cells: one characterized by high levels of F4/80 (R1) and a second that had only medium levels of F4/80 (R2) (Fig. 1A, right panel). FACS sorting of the two Mϕ populations followed by immunostaining defined the R1 population as the 95% of cells expressing 12/15-LOX, whereas R2 cells are largely negative (Fig. 1B). In contrast, the 12/15-LOX− cells were positive for MMR (CD206), an Ag that is absent in 12/15-LOX+ cells (Fig. 1B). To determine whether lymphocytes that comprise most of the remaining lavage cells express 12/15-LOX, immunostaining was performed for CD4, CD8, or CD45R, along with 12/15-LOX. As shown, 12/15-LOX was not associated with any of these lymphocyte Ags, indicating that it is not expressed by these cells (Fig. 1C).

**FIGURE 4.** R2 cells present Ag and stimulate naive lymphocytes to proliferate and generate IL-2. A. Stimulation of T cell division by R1 or R2 cells. FACS-sorted R1 and R2 cells were used in Ag presentation assays with CFSE-labeled BALB/c.OD11.10 transgenic OVA-specific CD4+ T cells in the presence or absence of OVA. Histograms show the proliferation-induced reduction of CFSE labeling of the CD4+ T cells (gated on CD3) caused by the R2 cells, but not the R1 cells, in the presence of OVA. Histograms represent experiments with 10,000 APC and 30,000 CD4+ T cells in the presence or absence of 0.5 mg/ml OVA. B and C. Stimulation of T cell proliferation or IL-2 synthesis by R1 or R2 cells. Shown are summaries of T cell proliferation (B) (division index is the average number of divisions that the dividing cells underwent) and IL-2 generation (C) in the presence of OVA and the indicated numbers of APC (mean ± SEM of two independent experiments, each performed in triplicate).
Functional and phenotypic characterization of 12/15-LOX+ and 12/15-LOX− cells

A full phenotypic characterization of the cells was undertaken by FACS analysis (Fig. 2). 12/15-LOX− cells expressed higher levels of MMR (CD206), dectin-1, M-CSF-R, GM-CSF-R, CCR5, MHC class II, CD11c, Syndecan1, and MARCO than did the 12/15-LOX+ cells. Both 12/15-LOX+ cells express more SR-A and Siglec1 (CD169) than do 12/15-LOX− cells. Both cell types expressed comparable levels of CD14, CD40, CCR6, CCR2, CD49b, and CD83 (Fig. 2 and data not shown). These differences indicate that the expression of 12/15-LOX is associated with distinct monocyte cell populations and is not expressed by other peritoneal leukocyte populations.

Functional differences between these MΦ subpopulations were examined in vitro by cytokine profiling the responses of MoFlo-sorted cells to activation by SES, which acts predominantly via TLR2 (C. Colmont, N. Topley, S. Jones, M. Labeta unpublished). 12/15-LOX− MΦ produced greater quantities of IL-10 and G-CSF than did 12/15-LOX+ cells (Fig. 3, A and B, and Table I). There was a trend toward increased generation of IL-1α, IL-1β, IL-6, TNF-α, IL-12p70, IL-13, MCP-1/CCL2, and GM-CSF (Fig. 3C–J and Table I). In contrast, LOX− cells appeared to generate more IL-12p40 (Fig. 3K and Table I). Both cell populations secreted comparable levels of RANTES/CCL5, KC/CXCL1, and MIP-1α/CCL3 levels (Fig. 3L–N and Table I). There was, however, little or no production of IL-2, IL-3, IL-4, IL-5, IL-17A, and IFN-γ detected from either population (data not shown).

To assign an immunological function to these distinct monocyte populations, studies examined their capacity to present Ag to naive T cells. FACS-sorted 12/15-LOX− cells stimulated D011.10 CD4+ T cell proliferation in presence of OVA, but 12/15-LOX+ cells did not (Figs. 4, A and B). Furthermore, only 12/15-LOX− cells stimulated secretion of IL-2 by lymphocytes (Fig. 4C). Collectively, these studies show that 12/15-LOX expression defines two phenotypically and functionally distinct populations of resident MΦ-like cells in the murine peritoneal cavity, and they suggest that 12/15-LOX− cells represent a novel population of DC.

Comparison of MΦ numbers and phenotype between WT and 12/15-LOX−/− mice

There were 1.7-fold more CD11bhigh/F4/80high MΦ in the peritoneal cavity of 12/15-LOX−/− mice compared with WT animals (p = 0.00082). However, DC, lymphocytes, and blood Mo numbers were identical (Fig. 5A). Propidium iodide staining of total lavage cells or resident MΦ between WT and 12/15-LOX−/− mice was identical, indicating that the proportion of cells undergoing proliferation or apoptosis was the same for the two strains (Fig. 5B). This suggests that 12/15-LOX may exert selective negative effects on Mo/MΦ migration into the peritoneal cavity. Furthermore, in the light of recent reports regarding a role for 12/15-LOX in myeloid cell proliferation, we detected no alteration in spleen size for mice aged 10–12 wk between strains (Fig. 5C) (13).

CD11bhigh/F4/80high resident cells isolated by adhesion from 12/15-LOX−/− mice were phenotypically identical to those from
WT animals for expression of Ags including CCR5, MHC class II, dectin-1, dectin-2, MMR, CD11c, CD62L, ICAM-1, M-CSF-R, MARCO, and CD40 (Fig. 6). However, some differences were apparent in terms of cytokine generation in response to stimulation with or without bacterial products (SES) in vitro (Fig. 7). Specifically, 12/15-LOX /H11002/ cells isolated by adhesion generated more IL-1/H9251/, IL-3, GM-CSF, and IL-17, but less RANTES/CCL5, than did WT mice (Fig. 7 and Table II). SES-elicited cells behaved in a somewhat overlapping manner, with 12/15-LOX /H11002/ cells showing reduced RANTES and increased GM-CSF production, but also significantly reduced generation of IL-12p40 and IL-12p70 as compared with WT (Fig. 7 and Table III). Overall, elicited cells from either strain generated far greater quantities of cytokines in response to stimulation than did resident cells. All other cytokine levels remained unchanged between strains. Detailed statistical analyses of all cytokines analyzed in these populations are in Tables II and III.

Changes in MΦ populations during peritoneal inflammation

To characterize how 12/15-LOX / H11001/ cells responded to acute inflammation, mice were administered (i.p.) SES and the temporal changes in leukocyte numbers were recorded for 7 days. Following initiation of inflammation, peritoneal MΦ numbers rapidly declined during the first 3 h. However, these were replaced by CD11bmed/F4/80med Mo after 6 –12 h (Fig. 8, A and B). This population expressed 7/4 Ag, dectin-1, CD40, CCR5, and CX3CR1 (data not shown). Concurrent analysis of 12/15-LOX expression using immunohistochemistry showed a decline in 12/15-LOX expressing cells consistent with a decrease in the number of resident peritoneal MΦ (Fig. 8, C and D). The number of infiltrating Mo/MΦ increased during 12–72 h post-SES injection (Fig. 8, FIGURE 7. Deficiency of 12/15-LOX causes selective alterations in response to SES stimulation in vitro. Left panels. Altered cytokine generation by resident MΦ. Resident peritoneal MΦ were isolated by adhesion, then cultured for 24 h with or without SES, and culture fluids were assayed for cytokine generation by ELISA or Bio-Plex assay (n = 4, mean ± SEM, * p < 0.05 for effect of genotype using two-way ANOVA). Right panels. Altered cytokine generation by elicited MΦ. Mice were administered SES for 24 h before lavage. Elicited MΦ were isolated by adhesion, then cultured for 24 h with or without SES, and culture fluids were assayed for cytokine generation by ELISA or Bio-Plex assay (n = 4, mean ± SEM, * p < 0.05 for effect of genotype using two-way ANOVA). Full statistical analyses of all cytokines analyzed in these populations are in Tables II and III.
A and B). After 7 days, an F4/80high/CD11bhigh population was restored; however, a large population of F4/80med/CD11bmed monocyctic cells remained within the peritoneal cavity (Fig. 8B). Parallel analysis of 12/15-LOX expression within this emerging monocyctic infiltrate demonstrated a significant increase in the number of both F4/80+/12/15-LOX+ and F4/80+/12/15-LOX− cell types (Fig. 8, B and C).

By day 7, greater numbers of both 12/15-LOX+ and 12/15-LOX− Mo were found than at day 0 (Fig. 8C). It is possible that some of the infiltrating Mo already express 12/15-LOX, while other cells induce it during differentiation to a “resident-like” phenotype. Significantly, blood Mo do not express 12/15-LOX, suggesting that its expression in infiltrating Mo/MØ is induced early following their trafficking to the peritoneal cavity.

12/15-LOX activity profile generation during peritonitis

Mass spectrometry profiling of HETEs in lavage showed that 12-HETE is the predominant positional isomer, confirming its enzymatic production by the 12/15-LOX (Fig. 8E). Also, 12-HETE was virtually absent in lavage from 12/15-LOX−/− mice (Fig. 8E, right bars). Basically, low levels of 15-HETE, also formed by this isoform, were detected, but other isomers were largely absent. During SES peritonitis, the concentration of 12-HETE decreased from 7.4 ng to <1 ng per mouse after 24 h of inflammation (Fig. 8E). This decrease followed the disappearance of 12/15-LOX+ cells in the cavity and recovered partially by day 7. There was a small increase in 5-HETE levels during inflammation, but LXA4 was not detected (Fig. 8E and data not shown).

### Table II. Cytokine production by resident adherent peritoneal cells*  

<table>
<thead>
<tr>
<th>Cytokine/Growth Factor</th>
<th>Mean (±SEM), pg/ml</th>
<th>Two-way ANOVA (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>WT + SES</td>
</tr>
<tr>
<td>IL-1α</td>
<td>48.52 (6.17)</td>
<td>214.05 (5.64)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>24.35 (1.43)</td>
<td>52.51 (4.90)</td>
</tr>
<tr>
<td>IL-2</td>
<td>2.74 (0.67)</td>
<td>6.75 (1.02)</td>
</tr>
<tr>
<td>IL-9</td>
<td>33.24 (2.54)</td>
<td>65.12 (1.36)</td>
</tr>
<tr>
<td>IL-10</td>
<td>11.81 (0.80)</td>
<td>95.97 (5.21)</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>58.81 (13.48)</td>
<td>92.75 (9.88)</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>10.98 (0.76)</td>
<td>24.51 (0.97)</td>
</tr>
<tr>
<td>IL-13</td>
<td>41.95 (3.12)</td>
<td>84.12 (1.49)</td>
</tr>
<tr>
<td>IL-17</td>
<td>43.87 (18.34)</td>
<td>170.78 (52.23)</td>
</tr>
<tr>
<td>RANTES</td>
<td>250.52 (26.46)</td>
<td>559.18 (85.29)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>223.96 (12.57)</td>
<td>1,510.15 (52.67)</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>363.84 (26.55)</td>
<td>1,274.91 (119.07)</td>
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<tr>
<td>MIP-1β</td>
<td>223.49 (23.51)</td>
<td>2,126.59 (358.09)</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>500.60 (87.14)</td>
<td>1,461.34 (148.34)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>702.71 (208.87)</td>
<td>4,071.29 (3,162.84)</td>
</tr>
<tr>
<td>TNF</td>
<td>38.64 (7.71)</td>
<td>1,065.82 (353.42)</td>
</tr>
</tbody>
</table>

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<sup>*Resident cells isolated by adhesion from individual C57BL/6 mice were cultured with and without SES. Data were analysed using a two-way ANOVA calculated in GraphPad Prism (n = 3 separate mice). Boldface indicates significance (p < 0.05) and underlined numbers are those that show trend toward significance. Nonsignificant values are not shown. Interaction significant implies that WT and knockout (KO) respond differently to SES; p-values for SES or genotype of <0.05 indicate that there is a significant effect of either on the response.</sup>
12/15-LOX suppresses Mo/Mφ numbers in the peritoneal cavity during inflammation

Analysis of Mo/Mφ numbers (CD11b+/F4/80−) during SES-induced peritonitis in WT mice showed a decrease in numbers during the first 3 h followed by influx of Mo/Mφ (CD11b+/F4/80−), which peaked at 72 h (Figs. 8B and 9A). 12/15-LOX−/− mice displayed the same kinetics of Mo/Mφ migration into the peritoneal cavity, but with a 2-fold increase in the number of recruited cells after 24 h persisting to 7 days (p = 0.0477 and p = 0.0435 at 24 and 7 days, respectively) (Fig. 9A). Similarly, migration of DCs had significantly increased at 24 h, although numbers of these cells were still relatively low (Fig. 9B). In contrast, neutrophil and lymphocyte numbers in the peritoneal cavity were unaffected by 12/15-LOX deletion (Fig. 9, C and D).

12/15-LOX regulates cytokines and eicosanoid-generating enzymes during inflammation

Significantly lower levels of MCP-1/CCL2, RANTES/CCL5, IL-12p40, IL-17, G-CSF, and TNF-α were generated in 12/15-LOX−/− mice, particularly around 6 h after SES injection, which is the time of peak production of these inflammatory mediators in WT animals (Fig. 10A–F). Significantly higher levels of TGF-β1 were found in 12/15-LOX−/− mice 24 h after the induction of peritonitis, correlating with elevated numbers of inflammatory Mo/Mφ present at this time (see above) (Fig. 10G). No substantive differences in IL-1α, IL-9, IL-13 and MIP-1β/CCL4 were noted, while IL-2, IL-3, IL-4, IL-5, MIP-1α/CCL3, or IFN-γ were low or below the limit of detection (data not shown).

During inflammation, lavage cell expression of the eicosanoid-generating enzymes COX-1 and COX-2 were regulated in an inverse manner, with COX-2 being transiently induced and COX-1 being suppressed but recovering by 24–72 h, similar to 12/15-LOX (Fig. 10, H and I). In 12/15-LOX−/− mice, COX-1 and COX-2 expression followed a similar pattern during peritonitis, but, overall, expression of the two enzymes was lower than in WT mice (Fig. 10, H and I).

Discussion

The role of 12/15-LOX expressed by peritoneal Mφ is unknown. Some reports suggest a proinflammatory action of the enzyme in...
ascular actions of exogenously administered lipoxins and resolvins suggest an antiinflammatory role (7, 8, 16). Since the peritoneal cavity is by far the major site of 12/15-LOX expression in vivo, we sought to characterize its function in regulating inflammation in this organ. High concentrations of exogenous 12/15-LOX products or plant LOX are known to inhibit neutrophil migration into the peritoneal cavity (7, 8). However, unlike the murine enzyme that generates 12S-HETE, the plant LOX generates the 15S-positional isomer, and the role of the endogenous 12/15-LOX has not been explored in vivo. Herein, a model of sterile peritonitis was used where a cell-free supernatant from S. epidermidis, one of the principal causative agents of human peritonitis in peritoneal dialysis patients, is administered i.p. to create a well-characterized model of mild acute and resolving inflammation (17).

Basically, 12/15-LOX$^+$ cells accounted for 95% of the recovered CD11b$^{bigh}$ cells with their phenotype consistent with previous studies describing the expression pattern of resident peritoneal Mφ (11, 18–21) (Fig. 2). In contrast, 12/15-LOX$^-$ cells appeared to be DC (Figs. 1, 2, and 4) (22). Although it is known that a small number of human and rat peritoneal cells are DC, their phenotype has not been described before (23, 24). Unlike the 12/15-LOX$^+$ cells, the peritoneal DC expressed high levels of mannose receptor, previously only documented on specific murine DC located in T cell areas of lymphoid organs draining from the periphery (10, 12, 25). The comparison of SES response of purified resident peritoneal Mφ and DC populations in vitro demonstrates clear differences between the cell types. The greater generation of IL-10 and GM-CSF, with a trend toward increased production of several other inflammatory mediators, suggests that the peritoneal Mφ is likely to be the more important of these two populations as a source of these cytokines during the early stages of the inflammatory response (Fig. 3 and Table I). The lack of differences in Ag expression between WT and 12/15-LOX$^{-/-}$ resident Mφ indicates that the enzyme is not required for maintaining the major phenotypic characteristics of these cells (Fig. 6). However, the nearly 2-fold elevation in peritoneal Mφ number in 12/15-LOX$^{-/-}$ mice compared with WT mice, both before and during inflammation, shows that it exerts selective negative effects on the number of Mφ (Figs. 5A and 9A). The reason for this did not appear to be either increased proliferative capacity or decreased apoptosis of these cells within the peritoneal cavity (Fig. 5B), although only a slight increase in proliferation can mediate dramatic increases in peritoneal Mφ numbers and detecting a small change may be beyond the limit of detection for our method (26). Recently, it was proposed that 12/15-LOX is a suppressor of myeloproliferative disease (13). However, we found no increase in peripheral blood Mo nor splenomegaly in our mice (Fig. 5, A and C). Thus, the higher numbers of peritoneal Mφ could result from greater rates of inward migration and local differentiation or from slower rates of egress from the peritoneal cavity. The significantly lower generation of MCP-1/CCL2 and RANTES/CCL5 during acute inflammation in 12/15-LOX$^{-/-}$ mice indicates that the elevated Mo/Mφ levels are not due to elevated levels of classical chemokines (Fig. 10).

During peritonitis, the rapid clearance of 12/15-LOX-expressing Mφ as part of the “Mφ disappearance reaction” probably occurred via migration to draining lymph nodes and increased adhesion to peritoneal tissues (Fig. 8, A and B) (27, 28). Resorption of local expression may involve up-regulation of the enzyme in the recruited cells, as peripheral blood Mo do not express 12/15-LOX (data not shown). Since substantial recovery in the levels of 12/15-LOX products was not achieved until as long as 7 days poststimulation, it is possible that the enzyme is not fully active until later time points (Fig. 8E). By this time, cells with the phenotype of resident peritoneal Mφ have begun to repopulate the cavity. It is not clear whether these cells have been recruited and differentiated from peripheral blood Mo or if they are from an alternate source; however, their antigenic phenotype coupled with the repopulation of the cavity with 12/15-LOX$^{-/-}$ mice indicates that the elevated Mo/Mφ levels are not due to elevated levels of classical chemokines (Fig. 10).

To examine the impact of 12/15-LOX deficiency on the inflammatory response to SES in the context of the Mφ heterogeneity that exists in vivo, we examined the response of both resident- and SES-elicited adherent cells that consist primarily of Mφ to SES stimulation. This would model both the effects of initial in vivo challenge of the resident population and the impact of 12/15-LOX on the differentially programmed Mφ recruited from the periphery during the response. Our analysis highlighted some overlapping and distinct aspects of the effect of 12/15-LOX expression on these two cell types (Fig. 7 and Tables II and III). For example, both resident and SES-elicited cells exhibited similar increases in GM-CSF and reductions in RANTES in the absence of 12/15-LOX (Fig. 7, F and G). SES-elicited cells produced very high levels of IL-12p40 and IL-12p70, which...
in the case of IL-12p70 was further increased by in vitro SES stimulation (Fig. 7, C and D). Both IL-12p40 and IL-12p70 were significantly reduced in the 12/15-LOX−/− cells, consistent with previous reports of impaired IL-12 production in response to LPS of thioglycolate-elicited peritoneal Mφ (6). However, resident peritoneal cells produced minimal IL-12 even after in vitro SES stimulation, indicating that these cells are a different population in terms of response to bacterial products. Separately, the in vivo impact of 12/15-LOX deficiency was assessed by measuring the production of cytokines and COXs in peritoneal lavage of SES-challenged mice (Fig. 10). Consistent with our results using SES-elicited cells, IL-12p40 and RANTES were deficient in the absence of 12/15-LOX, but several additional cytokines were also decreased, including MCP-1/CCL2, IL-17, G-CSF, TNF-α, COX-1, and COX-2 (Fig. 10). These results suggest that infiltrating Mo may be the source of RANTES and IL-12p40 in vivo, but that others (MCP-1, IL-17, G-CSF, TNF-α) may originate from other cell types. The increase in TGF-β production in 12/15-LOX−/− mice at 24 h correlated with the marked increase in Mo/Mφ numbers present in the peritoneal cavity, suggesting they may be the source of this cytokine. Thus, the impact of 12/15-LOX on the production of inflammatory mediators is influenced by cellular heterogeneity and likely affects multiple cellular subsets in vivo.

Previous studies administering exogenous 12/15-LOX products and plant 15-LOX proposed a role for the enzyme in regulating neutrophil influx in peritoneal inflammation, through synthesis of LXA4 and related mediators from omega3 fatty acids. Our results show key differences: 1) there was no deficit in neutrophil influx, with instead a selective suppression of Mo/Mφ migration; and 2) LXA4 was not detected in our model even though the detection limit of our mass spectrometry assay is well below the levels previously determined using ELISAs in other studies (29–32). A caveat is that S. epidermidis-induced inflammation is bacterial rather than fungal, and in terms of neutrophil recruitment is less severe than the zymosan model (32, 33). The peak of LXA4 reported in zymosan peritonitis occurs at 2–4 h and decreases during resolution (32). In contrast, we found the opposite pattern, with 12/15-LOX and 12-HETE disappearing during inflammation (Fig. 8).

Pharmacological effects of lipoxin analogs on leukocyte recruitment utilize far higher doses (300 ng/mouse, i.p.) than reported to be generated in vivo (0.4 ng/mouse) (8, 32). In summary, a role for LXA4 derived from endogenous 12/15-LOX in regulating peritoneal responses to bacterial products appears unlikely.

Receptor-dependent stimuli for 12-HETE production by 12/15-LOX in Mφ are unknown. The high levels of 12-HETE in the lavage of naive mice indicate that the enzyme is generating products in vivo without inflammatory stimulation (Fig. 8E). Treatment of peritoneal lavage cells with SES in vivo or in vitro did not activate 12/15-LOX (data not shown). Several additional agents

FIGURE 10. 12/15-LOX regulates production of cytokines and COXs during inflammation. A–G. Generation of several cytokines is altered in 12/15-LOX−/− mice during peritonitis. Cytokine levels in peritoneal lavage were determined using Bio-Plex mouse cytokine assay kit, PCR (TNF-α), or ELISA (TGF-β) as described in Materials and Methods (n = 8–10 for both strains, mean ± SEM, *, p < 0.05 by Student’s t test). H and I. Expression of COX-1 (H) and COX-2 (I) was determined using PCR, as described in Materials and Methods (n = 4 for both strains, mean ± SEM, *, p < 0.05 using unpaired Student’s t test).
from organisms, including yeast and bacteria, also failed to stimulate significant 12/15-LOX activity in isolated peritoneal cells (including LPS, zymosan, β-glucan, flagelin, Pam3CSK4) (data not shown). Overall, the pattern of 12-HETE generation in peritoneal cells is not consistent with a classic proinflammatory pathway. Studies have suggested that formation of 12-HETE or any other known free acid product made by 12/15-LOX is not required for its bioactivity. Both IL-12-p40 response in LPS and phagocytosis of apoptotic thymocytes by elicited peritoneal macrophages require 12/15-LOX, but both phenomena occur in WT Mφ without significant 12/15-LOX generation (5, 6). Our data extend this idea, since while 12/15-LOX regulates the cellular and cytokine response to bacterial products in vivo, the enzyme was not acutely activated. Also, we could not restore the WT phenotype to 12/15-LOX−/− mice by i.p. administration of 12-HETE, live WT lavage cells, or WT lavage cell lipid extract (data not shown). In all of these experiments, lipids were added immediately before inflammatory activation, and thus an acute effect of exogenous lipids does not seem to account for the effect of 12/15-LOX. It is possible that the long-term absence of 12/15-LOX causes a fundamental change in the biology of the peritoneal cavity so that it responds differently to bacterial products. Alternatively, deletion of 12/15-LOX during development may play a role in macrophage phenotype. Thus, further aspects of the mechanisms by which 12/15-LOX regulates immune responses in vivo remain to be elucidated.

In summary, we show that endogenous 12/15-LOX is a marker for resident peritoneal Mφ that regulate Mo/Mφ homeostasis and cytokine production during acute inflammation. We propose that the enzyme is a central player in regulating the Mo/Mφ-dependent response to an inflammatory stimulus in vivo.

Disclosures
The authors have no financial conflicts of interest.

References