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12/15-Lipoxygenase Orchestrates the Clearance of Apoptotic Cells and Maintains Immunologic Tolerance

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SUMMARY

Noninflammatory clearance of apoptotic cells (ACs) is crucial to maintain self-tolerance. Here, we have reported a role for the enzyme 12/15-lipoxygenase (12/15-LO) as a central factor governing the sorting of ACs into differentially activated monocyte subpopulations. During inflammation, uptake of ACs was confined to a population of 12/15-LO-expressing, alternatively activated resident macrophages (resMΦ), which blocked uptake of ACs into freshly recruited inflammatory Ly6C^{hi} monocytes in a 12/15-LO-dependent manner. ResMΦ exposed 12/15-LO-derived oxidation products of phosphatidylethanolamine (oxPE) on their plasma membranes and thereby generated a sink for distinct soluble receptors for ACs such as milk fat globule-EGF factor 8, which were essential for the uptake of ACs into inflammatory monocytes. Loss of 12/15-LO activity, in turn, resulted in an aberrant phagocytosis of ACs by inflammatory monocytes, subsequent antigen presentation of AC-derived antigens, and a lupus-like autoimmune disease. Our data reveal an unexpected key role for enzymatic lipid oxidation during the maintenance of self-tolerance.

INTRODUCTION

Apoptosis as well as the consecutive removal of apoptotic cells (ACs) are essential to ensure regular tissue turnover and homeostasis (Elliott and Ravichandran, 2010). In this respect, the efficient and nonimmunogenic clearance of ACs is crucial to dispose self-antigens and to maintain self-tolerance (Elliott and Ravichandran, 2010; Muñoz et al., 2010). In the steady state and in noninflamed tissue, this task is primarily performed by alternatively activated, tissue-resident macrophages, which are involved in tissue repair and homeostasis (Geissmann et al., 2010; Gordon and Taylor, 2005). During inflammation and inflammatory tissue damage, however, inflammatory monocytes, which are defined as Ly6C^{hi}CD11b^{+}CD115^{+} cells, are rapidly recruited and invade the tissue (Geissmann et al., 2003; Shi and Pamer, 2011). These cells not only contribute to the uptake and eradication of pathogens but also have the capacity to ingest self-antigens and ACs. Notably, inflammatory monocytes differentiate into inflammatory macrophages and inflammatory dendritic cells (Auffray et al., 2009; Domínguez and Ardavín, 2010; Geissmann et al., 2010; León et al., 2007; Randolph et al., 1999), which subsequently migrate to lymph nodes, encounter T cells, and can initiate a protective immune response against pathogens (León et al., 2007; Randolph et al., 2008). Uptake and processing of ACs by inflammatory monocytes will therefore eventually result in an activation of self-reactive T cells and a break of self-tolerance (Green et al., 2009). Mechanisms that coordinate the clearance of ACs and thereby limit the uptake of self-antigens by inflammatory monocytes are still poorly understood, though.

The enzyme 12/15-lipoxygenase (12/15-LO) represents the murine ortholog of human 15-lipoxygenase and oxygenates both free and phospholipid-bound polyunsaturated fatty acids (Kuhn and Thiele, 1999). Expression of 12/15-LO is restricted to certain macrophage subsets and induced upon stimulation with interleukin-4 (IL-4) and IL-13. Such 12/15-LO-expressing macrophage populations include alternatively activated macrophages and macrophages participating in the resolution of inflammation (Brys et al., 2005; Gordon and Martinez, 2010; Heydeck et al., 1998; Kuhn and Thiele, 1999; Schif-Zuck et al., 2011; Stables et al., 2011). Although 12/15-LO has been recognized as a major factor promoting the oxidation of LDL particles and the pathogenesis of atherosclerosis, the physiological role of this enzyme has remained incompletely understood (Kuhn and Thiele, 1999). Several studies indicate that 12/15-LO can contribute to the generation of proresolving lipid mediators such as lipoxin A4, resolvin E1, and protectin D1. These lipid mediators, in turn, are identified as important factors initiating the resolution of inflammation and have been additionally implicated in the removal of apoptotic cells and the wound-healing process (Godson et al., 2000; Gronert et al., 2005; Krönke

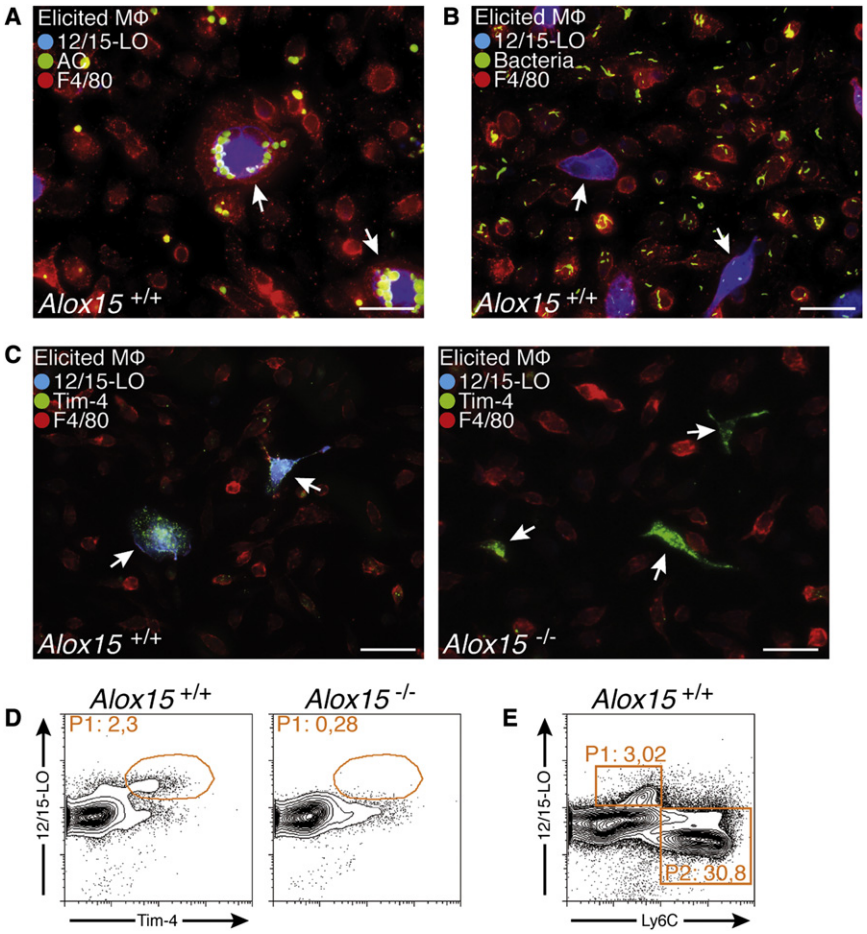

Figure 1. Uptake of ACs Is Confined to 12/15-LO-Expressing Macrophages

(A and B) Immunofluorescence (IF) microscopy analysis of the phagocytosis of (A) apoptotic cells (AC; green) and (B) heat-killed *E. coli* bacteria (green) by macrophages that were isolated from thioglycollate-induced peritonitis exudates. Staining for 12/15-LO (blue) and F4/80 (red) were performed as indicated.

(C) IF microscopy of 12/15-LO (blue) and Tim-4 (green) expression in macrophages isolated from peritonitis exudates of WT and *Alox15*$^{-/-}$ animals. Staining for Tim-4 allowed identifying resMΦ in exudates of *Alox15*$^{-/-}$ animals. Arrows point to the 12/15-LO- and Tim-4-expressing resMΦ.

(D and E) Flow cytometry analysis of the 12/15-LO-, Tim-4-, and Ly6C-expressing subpopulations of cells that are present in exudates of a thioglycollate-induced peritonitis (in WT and *Alox15*$^{-/-}$ mice) at day 3 after induction of peritonitis. The data shown here are representative for at least three independent experiments.

Scale bars represent 10 μm. See also Figure S1.

et al., 2009; Merched et al., 2008; Schwab et al., 2007; Serhan, 2010; Serhan et al., 2008). Interestingly, 12/15-LO is reported to translocate to the macrophages' plasma membrane during the phagocytosis of ACs (Miller et al., 2001). The implication behind this phenomenon as well as a potential role for 12/15-LO during the uptake of ACs has remained elusive, though.

Here we have identified the enzyme 12/15-LO as a crucial factor orchestrating the sorting of ACs during inflammation, where the clearance of ACs was primarily performed by a subpopulation of alternatively activated, 12/15-LO-expressing resident macrophages (resMΦ). These resMΦ inhibited uptake of ACs by freshly recruited inflammatory monocytes in a 12/15-LO-dependent manner. Furthermore, we have identified 12/15-LO-derived oxidation products of phosphatidylethanolamine as the corresponding inhibitory mediators generated by resMΦ. Absence of 12/15-LO, in turn, resulted in an unsorted clearance of ACs and enhanced uptake of apoptotic material by inflammatory monocytes, which triggered a subsequent T cell response against antigens derived from ACs. In line with these results, 12/15-LO-deficient animals displayed a break of self-tolerance and, with increasing age, developed a lupus-like autoimmune disease. On a mechanistic level, the identified 12/15-LO-derived phospholipid oxidation products were enriched in the plasma membrane of resMΦ where they sequestered and bound distinct soluble receptors for ACs such as milk fat globule-EGF factor 8 (MFG-E8), which were exclusively expressed and utilized by inflammatory monocytes and inflammatory macrophages. Thereby these oxidized phospholipids specifically interfered with the uptake of ACs into these proinflammatory cells.

Our data point toward a role for enzymatic lipid oxidation during the maintenance of self-tolerance and identify a mechanism that orchestrates the cell- and context-specific uptake of antigens by different subsets of phagocytes, imposing a paradigm in our understanding of the clearance of apoptotic cells.

RESULTS

Clearance of ACs Is Confined to 12/15-LO-Expressing Resident Macrophages

To study regulative mechanisms underlying the clearance of ACs during inflammation, we determined phagocytosis of ACs during thioglycollate-induced peritonitis. Apoptotic thymocytes were used as ACs in all experiments. Interestingly, in elicited macrophages of peritonitis exudates, phagocytosis of carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled ACs was largely confined to a certain subpopulation of macrophages, which highly expressed the enzyme 12/15-LO (Figure 1A). In contrast, phagocytosis of bacteria showed an inverse pattern of uptake and was mainly performed by the population of 12/15-LO-negative cells (Figure 1B). Immunofluorescence (IF) microscopy of elicited macrophages as well as flow cytometry analysis of the total cellular content in peritonitis exudates showed that the population of 12/15-LO-expressing macrophages coexpressed the phosphatidylserine-binding receptor Tim-4 and accounted for approximately 3% of total cells in exudates (Figures 1C and 1D, population P1). In contrast, the

major fraction of 12/15-LO-negative cells consisted of inflammatory Ly6C^{hi} monocytes, which coexpressed markers such as CCR2 and CD62L (Figure 1E, population P2; Figures S1A and S1B available online). The other cellular subsets in the exudates included neutrophils and B cells (data not shown).

The subsequent analyses identified the 12/15-LO-expressing macrophages within the peritonitis as resident peritoneal macrophages (resMΦ), which coexpressed 12/15-LO and Tim-4. After induction of peritonitis, these resMΦ were outnumbered by newly recruited inflammatory monocytes (Figures S1C–S1E).

12/15-LO Orchestrates the Cell Type-Specific Uptake of ACs during Inflammation

The confined phagocytosis of ACs by 12/15-LO-expressing resMΦ prompted us to elucidate a potential role for 12/15-LO during the clearance of ACs. Initial experiments showed that a pure homogenous population of Tim-4^{+} resMΦ, isolated from WT or 12/15-LO-deficient (*Alox15*$^{-/-}$) mice, ingested equally high amounts of ACs (Figures 2A and 2B). These data excluded an intrinsic role of 12/15-LO during the ingestion of ACs and showed that this enzyme was not required for the uptake of ACs into the subpopulation of 12/15-LO-expressing resMΦ.

The analysis of the clearance of ACs during peritonitis, however, revealed an unexpected and crucial role for 12/15-LO during the sorting of ACs into the different subpopulations of monocytes and macrophages. Although we consistently observed the confined uptake of ACs by 12/15-LO^{+}Tim-4^{+} resMΦ in exudates from WT animals, inflammatory exudates from 12/15-LO-deficient mice displayed an increased and distorted phagocytosis of ACs. Here, ACs were increasingly taken up by newly recruited inflammatory monocytes and macrophages (Figures 2C and 2D). We also observed this phenomenon in vivo, where the absence of 12/15-LO during peritonitis resulted in a redirection of the uptake of ACs toward the population of inflammatory Ly6C^{hi} monocytes (Figure 2E). Staining for Tim-4 allowed the identification of resMΦ in exudates from *Alox15*$^{-/-}$ mice. This analysis confirmed that, in the absence of 12/15-LO, the uptake of ACs was shifted to the population of recruited inflammatory cells, although resMΦ were still capable of ingesting ACs (Figure 2F).

These observations therefore prompted us to hypothesize that resMΦ possess the intrinsic capacity to actively inhibit the uptake of ACs by inflammatory monocytes in a paracrine- and 12/15-LO-dependent manner.

Resident Macrophages Utilize 12/15-LO to Generate Specific Phospholipid Oxidation Products

We consequently sought to determine the identity of potential 12/15-LO-derived mediator(s) in resMΦ, which interfere with the uptake of ACs by inflammatory monocytes. Although this enzyme can participate in the generation of different classes of eicosanoids, 12/15-LO was recently shown to preferentially oxygenate esterified fatty acids in the form of membrane-bound phosphatidylethanolamine (PE), although the biological consequence of this oxidation process remains largely elusive (Maskrey et al., 2007; Morgan et al., 2009). Analysis of phospholipids in resMΦ by mass spectrometry confirmed the presence of 12/15-LO-specific PE oxidation products in this macrophage subpopulation. WT resMΦ were enriched in PE hydroxides (PAPE-OH) and PE hydroperoxides (PAPE-OOH), which were both absent in *Alox15*$^{-/-}$ resMΦ (Figures 3A–3C). In contrast, we detected equal amounts of nonoxidized PE (PAPE) in WT and *Alox15*$^{-/-}$ resMΦ (Figure 3D). Quantification of PAPE-OH by mass spectrometry showed an increased production of PAPE-OH during later phases of the peritonitis in vivo coinciding with the resolution phase of inflammation (Figure 3E). 12/15-LO was shown to oxidize PAPE directly within the plasma membrane (Morgan et al., 2009). Concentrations of cell-associated PAPE hydro(pero)xides were calculated to reach 0.3 to 1.5 μmol/l within the resMΦ assuming a cellular volume of 460 fl (Nibbering et al., 1990).

Oxidation Products of PE Selectively Interfere with the Uptake of ACs into Inflammatory Monocytes

Consequently, we studied the capacity of various species of oxidized phospholipids to interfere with the ingestion of ACs by inflammatory monocytes. Indeed, intraperitoneal (i.p.) application of oxidized PE (oxPE) blocked the uptake of ACs by inflammatory Ly6C^{hi} monocytes during peritonitis in *Alox15*$^{-/-}$ mice (Figure 4A). In 12/15-LO-deficient animals, oxPE thereby ameliorated the uptake of ACs by inflammatory monocytes to amounts seen in WT mice. Additional in vitro experiments confirmed that oxidized phospholipids blocked the uptake of ACs in a dose-dependent manner (Figure 4B). Here we tested the effect of different species of phospholipid oxidation products including oxPE, oxidized phosphatidylcholine (oxPC), and oxidized phosphatidylserine (oxPS) on the uptake of ACs by inflammatory macrophages, which were isolated from peritonitis exudates. In this set of experiments, oxPE showed by far the highest potency and displayed high activity at low concentrations (1 μM). In addition, we performed site-specific enzymatic oxidation of PE by soybean 15-lipoxygenase, which resulted in the generation of specific PE oxidation products (15-PAPE-OH and 15-PAPE-OOH). Here, 15-PAPE-OOH exerted similar biologic activity as oxPE and potently blocked the uptake of ACs into inflammatory macrophages (Figure 4C). Oxidation of PE was a prerequisite for its biologic activity, as shown by the fact that nonoxidized PE had no effect on the ingestion of ACs (Figure 4D). Phagocytosis of latex beads, bacteria, mannosylated albumin, or dextran particles was not blocked (Figure 4E), demonstrating that oxPE did not interfere with the process of phagocytosis in general. Importantly, addition of oxPE did not affect the phagocytosis of ACs by the population of 12/15-LO^{+}Tim-4^{+} resMΦ. Here, oxPE had an effect neither on a culture of pure resMΦ (Figure 4F) nor on Tim-4-positive resMΦ, which were still present in the inflammatory exudates (Figure 4G). These data show that oxPE selectively acted on inflammatory monocytes and macrophages. In turn, the addition of oxPE rescued the selective phagocytosis of ACs by Tim-4^{+} resMΦ during peritonitis even in the absence of 12/15-LO (Figure 4G).

Together these data demonstrate that oxPE is able to exert a context- and cell type-specific inhibition of the phagocytosis of ACs. Furthermore, these results strongly indicate that oxPE largely accounts for the activity of 12/15-LO during the sorting of ACs into different macrophage and monocyte subpopulations.

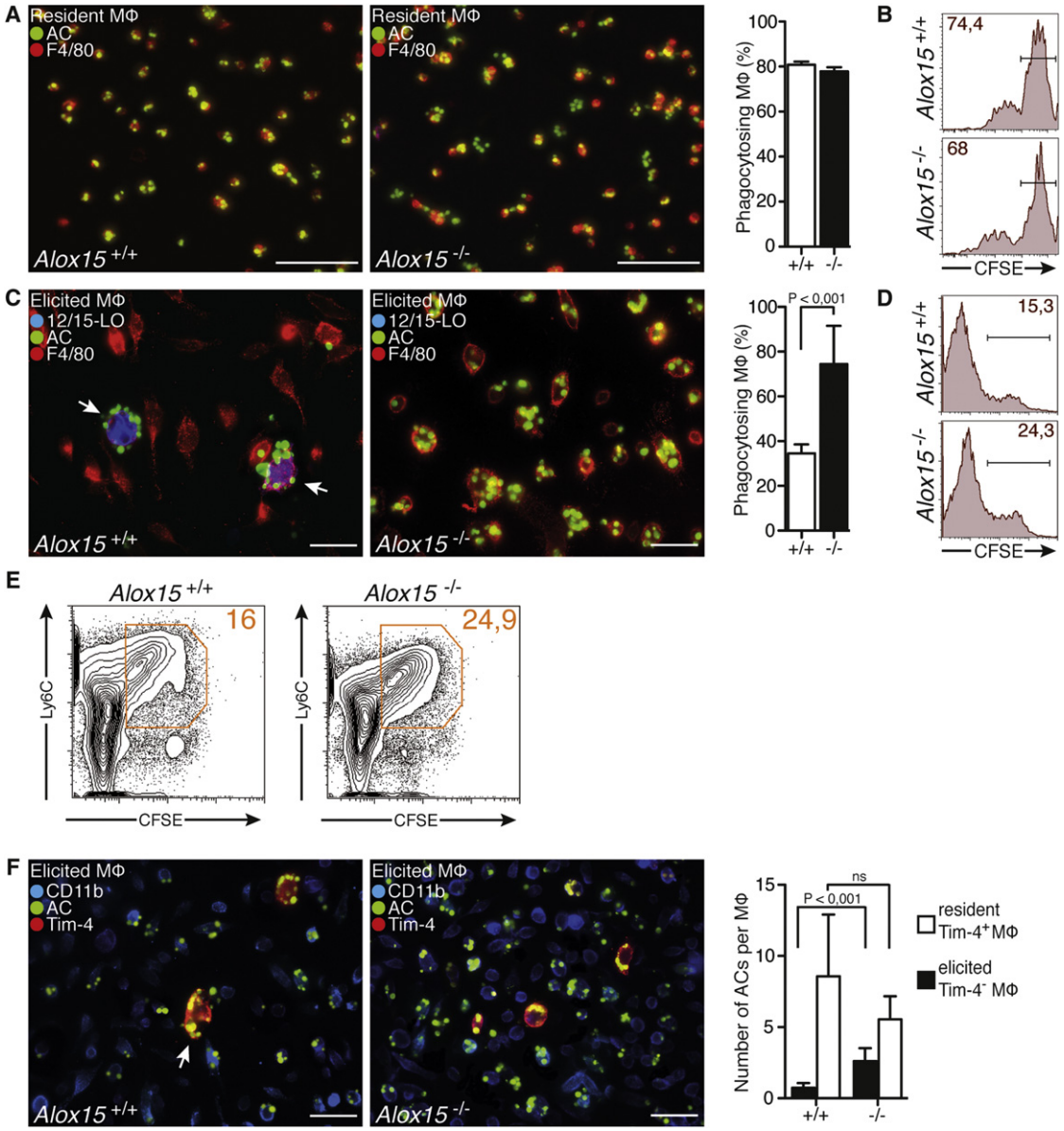

Figure 2. 12/15-LO Orchestrates the Phagocytosis of ACs

(A and B) IF microscopy-based (A) and flow cytometry-based (B) analysis of the phagocytosis of CFSE-labeled ACs (green) by a population of pure resMΦ from WT and *Alox15*$^{-/-}$ animals.

(C and D) IF microscopy-based (C) and flow cytometry-based (D) analysis of the phagocytosis of CFSE-labeled ACs by macrophages of peritonitis exudates of WT and *Alox15*$^{-/-}$ animals. Data here are representative for three independent experiments.

(E) Flow cytometry-based quantification of the phagocytosis of CFSE-labeled ACs by Ly6C^{hi} inflammatory monocytes in vivo. During thioglycollate-induced peritonitis, CFSE-labeled ACs were injected i.p. 30 min before phagocytosis of ACs was determined by flow cytometric analysis (representative of three independent experiments).

(F) IF microscopy-based analysis of the phagocytosis of ACs by macrophages of peritonitis exudates of WT and *Alox15*$^{-/-}$ animals. An antibody against Tim-4 (red) was used to identify resMΦ both in WT and *Alox15*$^{-/-}$ exudates and to differentiate these cells from recruited inflammatory macrophages. Arrows point at residual resMΦ. The data shown are representative for at least three experiments.

Error bars indicate SD. Scale bars represent 10 μm.

12/15-LO-Mediated Sorting of ACs Is Required for the Nonimmunogenic Disposal of Self-Antigens

To elucidate possible immunological consequences resulting from the sorting of ACs into different monocyte and macrophage subpopulations, we further characterized these populations of cells. After stimulation with LPS, resMΦ displayed characteristics of alternatively activated macrophages including elevated production of the anti-inflammatory cytokines IL-10 and TGF-β, whereas the subset of inflammatory macrophages was biased toward production of IL-12 and TNF-α (Figure 5A). These

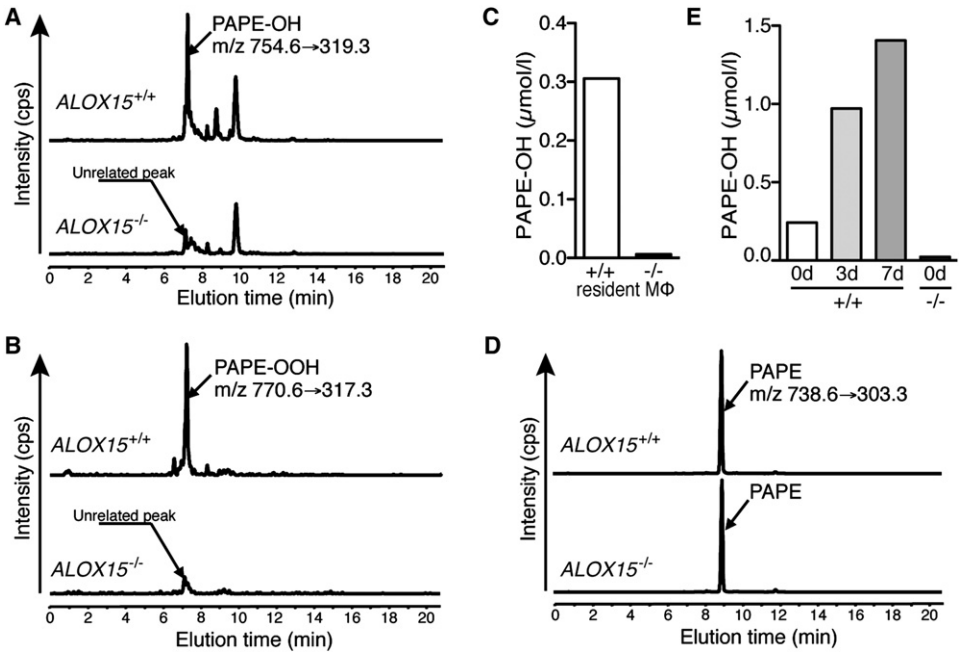

Figure 3. ResMΦ Are Enriched with 12/15-LO-Specific Oxidation Products

(A and B) Electrospray-ionization mass spectrometry (ESI-MS)-based analysis of (A) PE-hydroxides (PAPE-OH) and (B) PE-hydroperoxides (PAPE-OOH) in lipid extracts from WT and *Alox15*$^{-/-}$ resMΦ.

(C) After quantification of PAPE-OH in WT and *Alox15*$^{-/-}$ resident macrophages, cell-associated concentrations of this phospholipid were calculated, assuming a volume of 460 fl per macrophage.

(D) Analysis of nonoxidized PE (PAPE) in WT and *Alox15*$^{-/-}$ resident macrophages.

(E) ESI-MS-based quantification of the amounts of PAPE-OH at the indicated time points (0, 3, and 7 days) in cells of the peritoneal exudates of WT and *Alox15*$^{-/-}$ mice after thioglycollate challenge. Concentrations of cell-associated PAPE-OH within the resident macrophages were calculated on the basis of the number of resident macrophages present within the peritonitis exudates (and assuming a volume of 460 fl per macrophage).

See also Table S1.

data are consistent with a tolerogenic role of resMΦ (Geissmann et al., 2010; Murray and Wynn, 2011) and the proposed role of inflammatory Ly6C^{hi} monocytes, which can differentiate into inflammatory macrophages and dendritic cells, thereby gaining antigen-presenting capacity (Auffray et al., 2009; Domínguez and Ardavín, 2010; Geissmann et al., 2003; León et al., 2007; Randolph et al., 1999, 2008; Shi and Pamer, 2011). Antigens ingested by inflammatory monocytes and macrophages are therefore, to a certain extent, presented to T cells (Geissmann et al., 2003; Randolph et al., 2008). Hence, a nondirected sorting process of ACs during inflammation would eventually result in the presentation of autoantigens to potentially self-reactive T cells.

To test this hypothesis, we subsequently determined the consequences of a directed versus nondirected sorting of ACs on the immune response to AC-derived antigens in WT and 12/15-LO-deficient animals, respectively. ACs were generated from ovalbumin (OVA)-transgenic mice and incubated with different populations of macrophages. Thereafter, we measured the T cell response to OVA as an AC-derived antigen by monitoring the response of OVA-specific OT-II T cells in antigen-presentation assays. Phagocytosis of OVA transgenic-ACs by neither WT nor 12/15-LO-deficient resMΦ did induce a specific T cell response (Figure 5B). These data are in accordance with our previous results and confirm a tolerogenic role of resMΦ, which perform a nonimmunogenic disposal of AC-derived antigens in a 12/15-LO-independent manner. Next, we isolated macrophages from peritonitis exudates, thereby obtaining an assortment of resMΦ and inflammatory macrophages as potential antigen-presenting cells. Here, phagocytosis of OVA-transgenic ACs by macrophages that were isolated from WT exudates did not result in a T cell response to AC-derived OVA, either (Figure 5C). In contrast, we observed a specific T cell response to the AC-derived antigen when we used macrophages from exudates of *Alox15*$^{-/-}$ animals as antigen-presenting cells (Figure 5C). The addition of oxPE reversed this phenotype (Figure 5C). These results thereby confirmed that a directed 12/15-LO-mediated sorting process and uptake of ACs by resMΦ contributes to a nonimmunogenic disposal of AC-derived antigens. The absence of 12/15-LO during inflammation, which resulted in a distorted clearance and enhanced uptake of ACs by inflammatory monocytes, however, provoked a subsequent activation of antigen-specific T cells to AC-derived antigens. To exclude a cell-intrinsic effect as base for the increased stimulatory capacity of *Alox15*$^{-/-}$ cells, we subsequently exchanged soluble OVA for OVA-transgenic ACs as an antigen. Here, uptake and processing of soluble OVA by both WT and *Alox15*$^{-/-}$ exudates macrophages resulted in robust T cell responses (Figure 5D). In accordance, exudate macrophages of WT and *Alox15*$^{-/-}$ animals displayed a similar expression of costimulatory

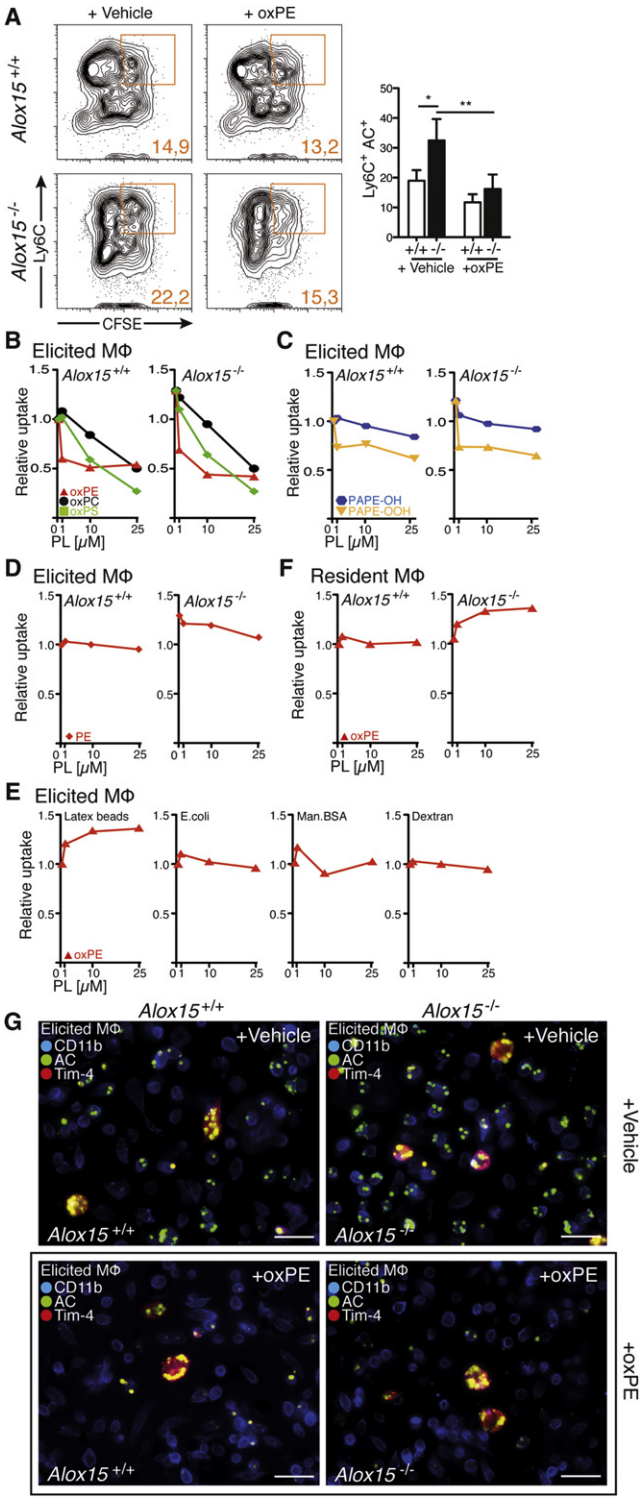

Figure 4. Oxidized Phosphatidylethanolamine Selectively Blocks the Uptake of ACs by Inflammatory Monocytes

(A) Flow cytometry-based in vivo analysis of the phagocytosis of CFSE-labeled ACs by Ly6C^{hi} inflammatory monocytes during thioglycollate-induced peritonitis after i.p. injection of ACs in the presence or absence of oxPE (10 μM). Error bars indicate SD.

(B–D) Flow cytometry-based quantification of the uptake of CFSE-labeled ACs by macrophages of peritonitis exudates of WT and *Alox15*$^{-/-}$ mice, in the presence of the indicated (B) oxidized, (C) enzymatically (soybean-15-LO) generated, or (D) nonoxidized phospholipid.

(E) Flow cytometry-based quantification of the uptake of labeled latex beads, *E. coli*, mannosylated BSA, or dextran by macrophages of peritonitis exudates in the presence of increasing concentrations of oxPE.

(F) Quantification of the uptake of apoptotic cells by a population of pure resMΦ in the presence of increasing amounts of oxPE.

(G) IF microscopy of WT and *Alox15*$^{-/-}$ macrophages of peritonitis exudates after incubation with CFSE-labeled ACs in the absence or presence of oxPE (10 μM). An antibody against Tim-4 (red) was used to identify resMΦ both in WT and *Alox15*$^{-/-}$ exudates and to differentiate these cells from recruited inflammatory macrophages. Scale bars represent 10 μm.

Data shown are representative of at least three experiments.

molecules (data not shown). Likewise, we detected no differences in the cytokine production of WT and *Alox15*$^{-/-}$ macrophages after ingestion of ACs (Figure 5E), which ruled out a role of 12/15-LO as a secondary mediator of potential anti-inflammatory effects of ACs. Together these results confirmed that 12/15-LO activity was crucially involved in the adjustment of a threshold for a T cell response against AC-derived self-antigens by coordinating the sorting of ACs.

We subsequently sought to determine whether the aberrant clearance of ACs in *Alox15*$^{-/-}$ animals would also result in a T cell response against AC-derived antigens in vivo. Therefore we transferred OT-II T cells into WT and *Alox15*$^{-/-}$ mice, induced peritonitis, and injected OVA-transgenic ACs into the peritoneum. In accordance with our in vitro data, we observed an OVA-specific T cell response in the draining mediastinal lymph nodes and spleens of *Alox15*$^{-/-}$ mice, whereas OT-II cells did not respond to this AC-derived antigen, when transferred to WT mice. OT-II cells in nondraining mesenteric lymph nodes showed no response at all (Figure 5F).

Absence of 12/15-LO Results in a Break of Self-Tolerance

Consequently, we analyzed whether the distorted clearance of ACs in *Alox15*$^{-/-}$ mice compromised their state of self-tolerance. Interestingly, aged 12/15-LO-deficient mice have been recently reported to display splenomegaly as well as a decreased survival when compared to their WT littermates (Middleton et al., 2006). Whether these findings can be attributed to a potential hematological disorder has been controversially discussed (Van Etten, 2007). The possibility of an autoimmune disorder in these mice had not been addressed, though.

Serum analysis of aged 12/15-LO-deficient mice revealed the emergence of anti-nuclear antibodies as a hallmark of systemic autoimmunity (Figure 6A). Consistent with a major role of 12/15-LO in the maintenance of self-tolerance, we detected a significant and spontaneous production of several forms of autoantibodies in aged 12/15-LO-deficient mice including anti-dsDNA, anti-cardiolipin, and anti-histone autoantibodies (Figure 6B). Likewise, 12/15-LO-deficient mice spontaneously developed signs of glomerulonephritis and showed deposits of complement and immunoglobulins within their glomeruli (Figures 6C, S3A, and S3B).

Consequently we performed the pristane model of experimental murine lupus, where simultaneous induction of sterile peritonitis and apoptosis trigger production of autoantibodies and the development of glomerulonephritis, all resembling key

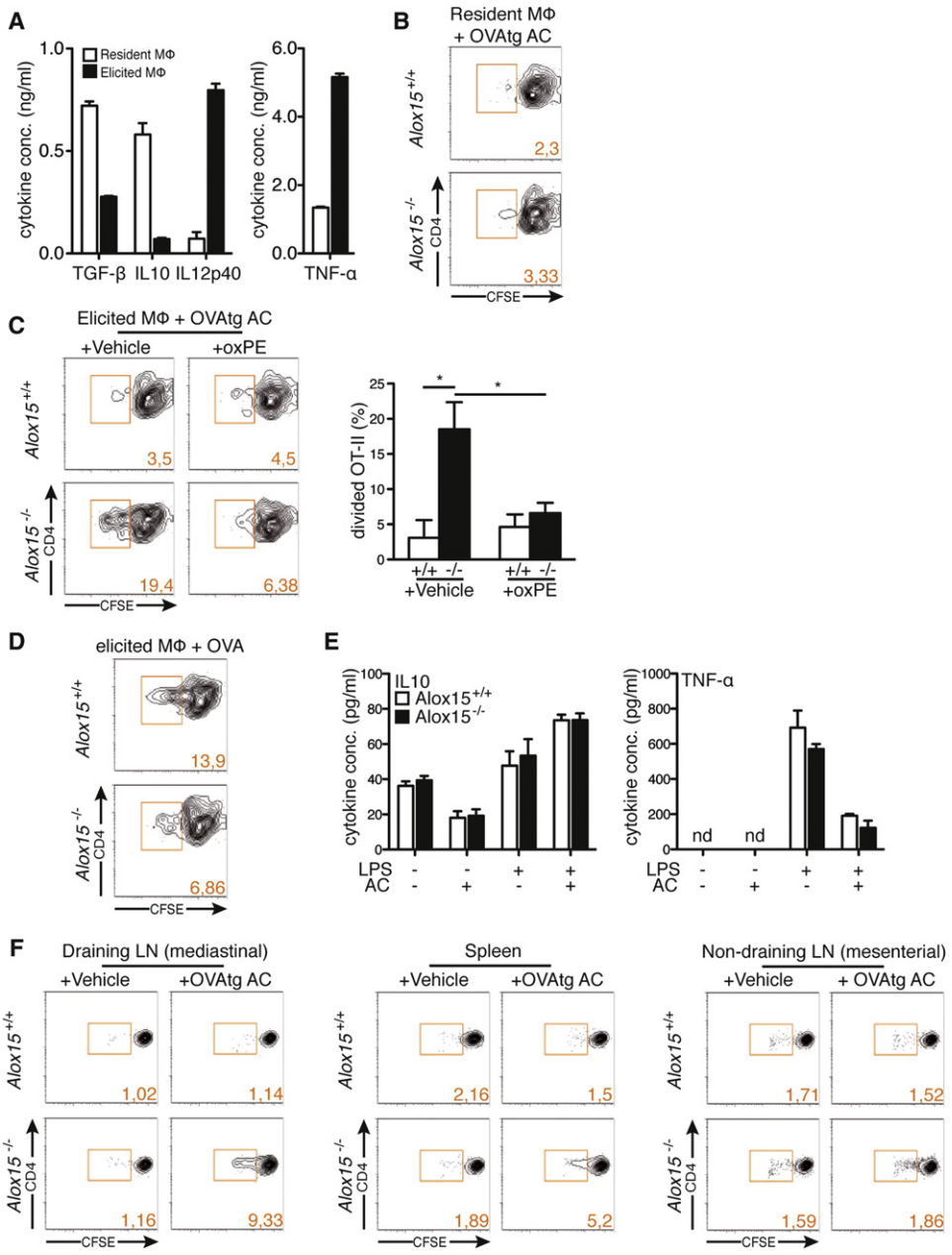

Figure 5. 12/15-LO Mediates the Nonimmunogenic Disposal of AC-Derived Antigens

(A) Determination of the cytokine profile of resMΦ and inflammatory macrophages. Concentrations of the indicated cytokines in the cellular supernatants were measured by ELISA after stimulation of macrophages with LPS (100 ng/ml; 12 hr).

(B and C) Determination of the OT-II T cell response against AC-derived OVA via CFSE-dilution assays. ACs of OVA-tg mice were incubated with (B) a population of pure resMΦ or (C) macrophages of peritonitis exudates in the absence or presence of oxPE (10 μM). After 24 hr, CFSE-labeled OT-II T cells were added and the T cell response was measured by CFSE dilution after an additional 72 hr as described in Experimental Procedures.

(D) Determination of the OT-II T cell response to soluble OVA that has been preincubated with macrophages of peritonitis exudates for 24 hr before the addition of OT-II T cells.

(E) ELISA-based measurement of the cytokine response of macrophages of peritonitis exudates, which had been preincubated with ACs for 2 hr before being stimulated with LPS (100 ng/ml; 6 hr).

(F) In vivo quantification of the T cell response to OVA-tg ACs, which had been i.p. injected into the inflamed peritoneum. T cell proliferation of transferred CFSE-labeled OT-II T cells in draining mediastinal lymph nodes, the spleen, and nondraining mesenterial LN was determined by flow cytometric analysis.

Data shown are representative for three experiments. Error bars indicate SD. See also Figure S2.

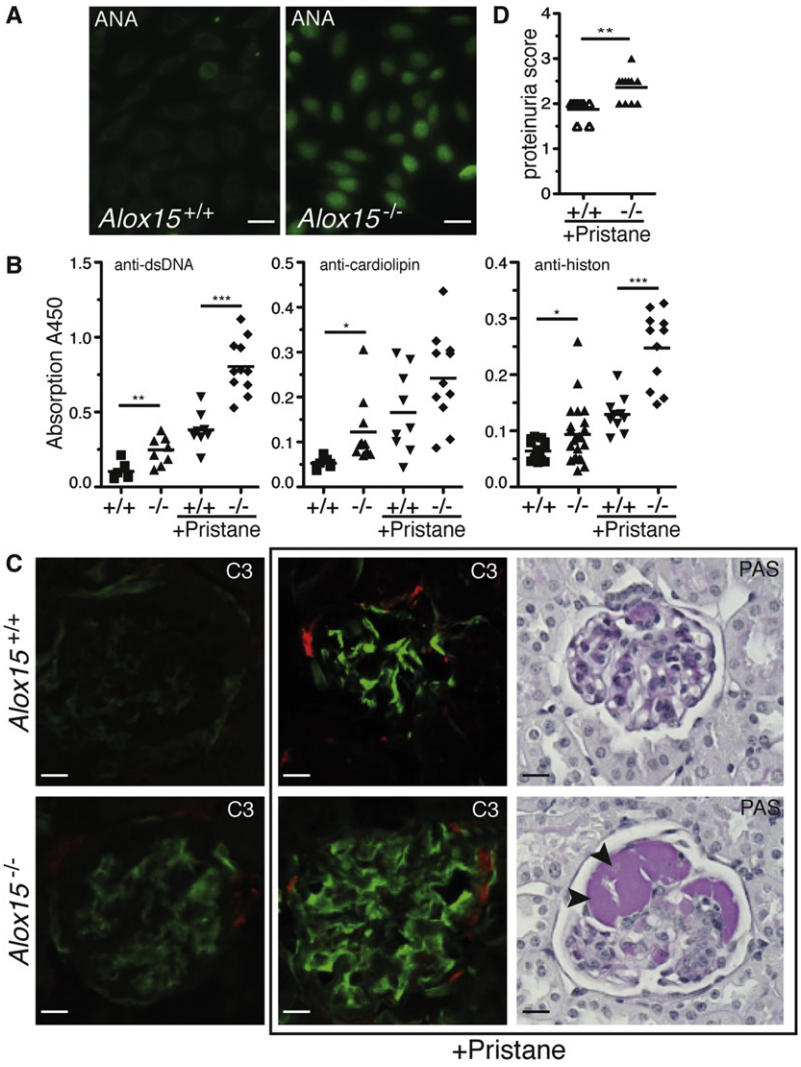

Figure 6. 12/15-LO Deficiency Results in a Break of Self-Tolerance

(A) HEp-2 cells were incubated with serum derived from 24-week-old WT and *Alox15*$^{-/-}$ mice to detect antinuclear antibodies (green).

(B) Measurement of the amounts of indicated autoantibodies in sera of WT and *Alox15*$^{-/-}$ mice. Analysis of all mice was performed at the age of 24 weeks (either in unchallenged mice or in mice 12 weeks after injection of pristane oil).

(C) IF microscopy determining complement (c3) deposits (green) and PAS staining of kidneys from WT and *Alox15*$^{-/-}$ mice. Analysis of all mice was performed at the age of 24 weeks (either in unchallenged mice or in mice 12 weeks after injection of pristane oil); F4/80 (red).

(D) Quantification of proteinuria in WT and *Alox15*$^{-/-}$ mice 12 weeks after injection of pristane oil.

Scale bars represent 10 μm. See also Figure S3.

features of human systemic lupus erythematosus (SLE) (Reeves et al., 2009). As expected, 12/15-LO deficiency provoked an additional exacerbation of the production of autoantibodies and glomerulonephritis (Figures 6B, 6C, S3A, and S3B). Here, *Alox15*$^{-/-}$ mice displayed prominent deposits of PAS-positive material in their glomeruli (Figure 6C) and increased proteinuria (Figure 6D).

Oxidation Products of PE Interfere with MFG-E8-Mediated Uptake of ACs into Inflammatory Monocytes

The ingestion of ACs relies on the receptor-mediated recognition of phosphatidylserine (PS), which is exposed on the outer leaflet of the membranes of ACs (Ravichandran, 2010). PS, in turn, is recognized by a multitude of receptors such as MFG-E8 and Tim-4 (Hanayama et al., 2002; Miyanishi et al., 2007), which are differentially expressed by distinct phagocyte subsets.

To further delineate the mechanism underlying the cell-type-specific inhibition of phagocytosis by 12/15-LO-generated oxPE, we performed a gene expression analysis of resMΦ and inflammatory macrophages to compare their repertoires of known PS-binding proteins (Figure 7A). Indeed, the two populations of macrophages displayed clear differences in their PS-receptor profiles. As reported previously (Miyanishi et al., 2007), expression of the PS receptor Tim-4 was confined to resMΦ, whereas expression of the secreted PS-binding protein MFG-E8 was restricted to inflammatory macrophages. MFG-E8 serves as a bridging molecule between PS on the surface of the apoptotic cell and αvβ3-integrin on the macrophage (Hanayama et al., 2002). MFG-E8 was shown to be essential for the phagocytosis of ACs by inflammatory macrophages (Hanayama et al., 2004), whereas resMΦ rely on different molecules such as Tim-4 (Miyanishi et al., 2007; Wong et al., 2010). We therefore determined whether oxPE can interfere with the binding of MFG-E8 to PS, which would account for the oxPE-mediated selective block of phagocytosis in inflammatory macrophages. In competitive binding assays, we tested for the potency of different phospholipid vesicles to interfere with the binding of MFG-E8 to a PS-coated surface (Figure 7B). Indeed, vesicles containing oxPE potently blocked binding of MFG-E8 to PS, whereas vesicles containing nonoxidized PE did not (Figure 7B). As expected, PS-containing vesicles blocked binding of MFG-E8 to PS in an oxidation-independent manner (Figure 7B). These data indicate an oxidation-dependent and competitive binding of oxPE to the PS-binding molecule MFG-E8. The addition of increasing amounts of recombinant MFG-E8, in turn, reconstituted the ability of inflammatory macrophages to ingest ACs in the presence of oxPE (Figure 7C), confirming a role of MFG-E8 as a functional target of oxPE.

Upon 12/15-LO-mediated oxidation, PE was shown to translocate to the outer leaflet of the plasma membrane in resMΦ (Morgan et al., 2009). We therefore determined binding of recombinant MFG-E8 to the plasma membrane of resMΦ. These experiments were performed in the presence of RGD peptides to exclude integrin-dependent binding of MFG-E8. Indeed, MFG-E8 was strongly bound by 12/15-LO-expressing resMΦ, whereas deletion of 12/15-LO abrogated MFG-E8 binding in

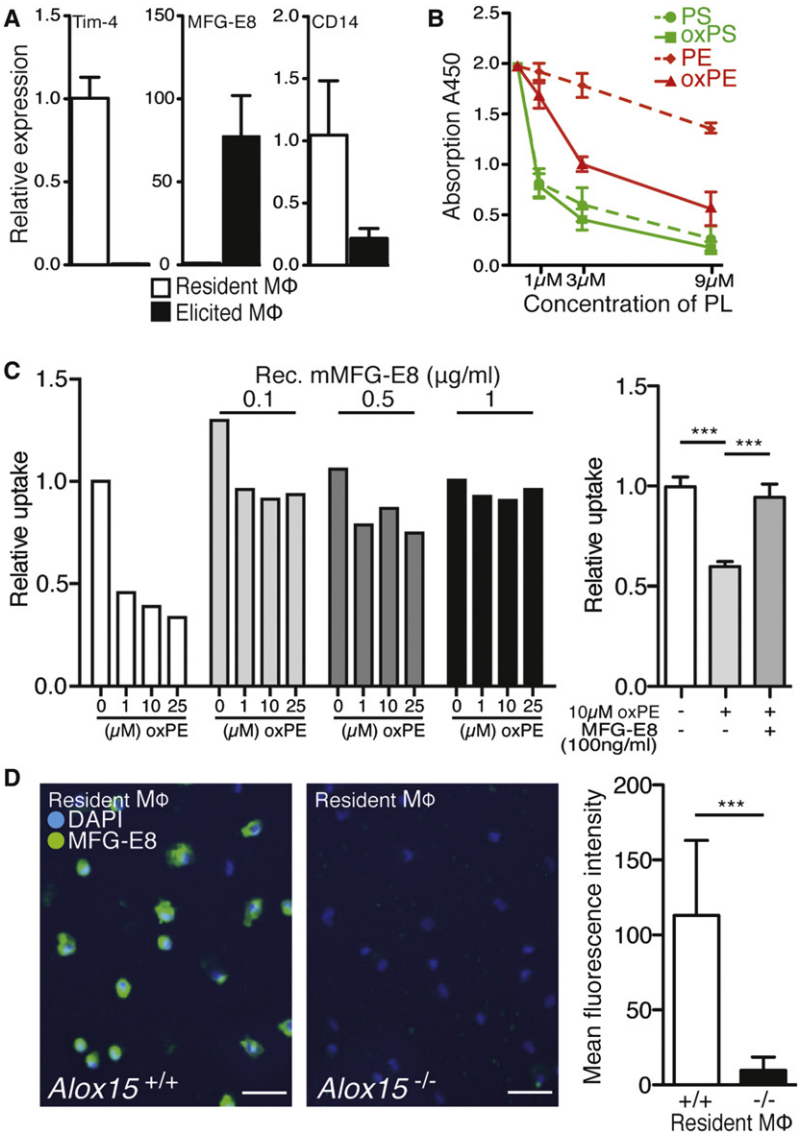

Figure 7. OxPE Blocks the Interaction between MFG-E8 and Phosphatidylserine

(A) Differential expression of various receptors for ACs in resMΦ and inflammatory macrophages.

(B) Binding of recombinant MFG-E8 to a PS-coated surface in the presence of vesicles containing the indicated phospholipids as competitors.

(C) Flow cytometry-based quantification of the uptake of CFSE-labeled apoptotic cells by macrophages of peritonitis exudates in the presence of increasing concentrations of oxPE and different concentrations of recombinant MFG-E8, respectively.

(D) Binding of recombinant MFG-E8 (green) to the surface of WT and *Alox15*$^{-/-}$ (pure) resMΦ in the presence of RGD peptides; DAPI (blue). Scale bars represent 10 μm.

Error bars indicate SD for triplicates.

Alox15$^{-/-}$ resMΦ (Figure 7D). Together these observations indicate that upon exposure of oxPE, resMΦ sequester soluble MFG-E8 in a 12/15-LO-dependent manner and thereby selectively interfere with the phagocytosis of ACs by inflammatory monocytes and macrophages (Figure S3).

DISCUSSION

Efficient elimination of pathogens and the maintenance of self-tolerance are key objectives during the immune response. Failure to do so results in fatal infection or autoimmune disease, respectively (Green et al., 2009). During inflammation and tissue damage, the innate immune system is simultaneously confronted with both pathogens and self-antigens in the form of ACs. Therefore, regulative mechanisms controlling the uptake and processing of ACs are crucial in the maintenance of tolerance (Muñoz et al., 2010). Our current data show that at a very early stage, and in particular during inflammation, the innate immune system performs a sorting of pathogens and ACs into distinct subsets of phagocytes. During the last two decades, multiple, and probably equally important, "tolerance checkpoints" have been identified, which cooperate to prevent a break of self-tolerance. Such tolerance-prevailing mechanisms include direct anti-inflammatory effects of apoptotic cells, which thereby modulate the phagocyte's cytokine profile directly (Fadok et al., 1998; Voll et al., 1997). Likewise, recent data suggest that a selection and sorting process of antigens, which are derived from ACs and pathogens, respectively, is also performed at the level of phagosome maturation. Here, the activation of Toll-like receptors influences the fate of the single phagosome within the phagocyte (Blander and Medzhitov, 2004, 2006a, 2006b). Therefore it is obvious that multiple mechanisms regulating the sorting, uptake, and processing of ACs have evolved in response to the evolutionary pressure exerted by the "horror autotoxicus." Together these mechanisms set up a certain threshold for self tolerance, which might considerably vary in a context-dependent manner as observed during different types of infection, during sterile inflammation, or in the tumor microenvironment.

Indeed, our current data provide evidence that the sorting of ACs is decisive for the quality of the subsequent immune response to AC-derived antigens. ResMΦ and inflammatory monocytes, which both have the capacity to participate in the uptake of ACs, are of distinct origin and activation status. Alternatively activated tissue-resident macrophages, which express both TGF-β and IL-10, have already been implicated in the clearance of ACs, tissue repair, the resolution of inflammation, and the maintenance of immunological tolerance (Geissmann et al., 2010; Gordon and Taylor, 2005; Green et al., 2009; Murray and Wynn, 2011). Nevertheless, direct evidence for their role during these processes has been scarce so far. In contrast, Ly6C^{hi} inflammatory monocytes, which are actively blocked in their capacity to engulf ACs in a 12/15-LO-dependent manner, are well-known precursors of inflammatory macrophages and

monocyte-derived dendritic cells (Auffray et al., 2007; Domínguez and Ardavín, 2010; Geissmann et al., 2010; Gordon and Taylor, 2005; Randolph et al., 1999, 2008). In accordance with this scenario, we observed that an aberrant sorting process and ingestion of ACs by inflammatory monocytes resulted in the presentation of AC-derived antigens to T cells and in a break of self-tolerance as observed in 12/15-LO-deficient mice.

On a molecular level, our data suggest that the crosstalk between resMΦ and their inflammatory counterparts is accomplished by exposure of oxidized phospholipids in a 12/15-LO-dependent manner. Here, oxidized phospholipids generated by alternatively activated macrophages selectively interfere with the uptake of ACs by proinflammatory monocytes. Besides the oxygenation of such membrane phospholipids, 12/15-LO has been implicated in the production of different classes of proresolving lipid mediators such as lipoxin A4 (Gronert et al., 2005; Merched et al., 2008; Serhan et al., 2008). Lipoxin A4 was previously shown to enhance the nonphlogistic phagocytosis of apoptotic neutrophils by human monocyte-derived macrophages (Godson et al., 2000). Together with our present data, these findings suggest that distinct 12/15-LO-derived eicosanoids such as lipoxin A4 might facilitate uptake of AC by alternatively activated macrophages, whereas other 12/15-LO-derived lipid oxidation products such as oxPE simultaneously block uptake of ACs by inflammatory monocytes. Both mechanisms would eventually contribute to a sorted and noninflammatory clearance of ACs by the alternatively activated macrophage.

The production both of proresolving eicosanoids and of oxPE coincides with the resolution of inflammation. Although most eicosanoids have been identified as high-affinity ligands for specific receptors and exert their effects at low concentrations in the nM range (Serhan et al., 2008), our current data suggest that 12/15-LO-derived oxidized phospholipids might act in a different manner. OxPE seems to exert its biological activity in the low μM range, at least in part, via sequestering and blocking distinct soluble pattern recognition receptors (PRRs) such as MFG-E8, which are otherwise selectively utilized by inflammatory monocytes for the purpose of phagocytic uptake of ACs. Even single oxPE species such as PAPE-OH (PAPE reflects around 20%–30% of total PE) are detected in μM concentrations within resMΦ. Moreover, oxPE is enriched in the outer membrane leaflet in a 12/15-LO-dependent manner (Morgan et al., 2009), which might yield even higher local concentrations.

Although we identify MFG-E8 as a major target for oxPE, previous studies have demonstrated that related species of oxidized phospholipids can also interact with other PRRs such as CD36 and CD14, which have been implicated in the binding and uptake of ACs as well (Blüml et al., 2005; Bochkov et al., 2002; Devitt et al., 1998; Greenberg et al., 2006; von Schlieffen et al., 2009). Future studies are needed to define the exact contribution and role of these different PRRs during the sorting of ACs.

MFG-E8 is produced by various cells including follicular dendritic cells, monocyte-derived dendritic cells, and inflammatory macrophages (Ravichandran, 2010). Because of a defective clearance of ACs in the germinal centers of the spleen, MFG-E8-deficient mice themselves spontaneously develop autoimmunity (Hanayama et al., 2004). On the other hand, intravenous or intraperitoneal injection of recombinant MFG-E8 into WT mice equally results in a break of tolerance and triggers the development of autoantibodies (Asano et al., 2004; Yamaguchi et al., 2010). Moreover, sustained or elevated serum concentrations of MFG-E8 are associated with an increased risk for human SLE (Yamaguchi et al., 2008, 2010). Together with our current data, these findings indicate that MFG-E8-dependent phagocytosis of ACs by macrophages and dendritic cells has to be carefully regulated in a site-, context-, and cell-specific manner. During infection, MFG-E8-dependent uptake of ACs by dendritic cells and inflammatory monocytes might be important to mount an immune response to efficiently eradicate intracellular pathogens. Uncontrolled ingestion of ACs by inflammatory antigen-presenting cells critically lowers the threshold of self-tolerance, though. Our current data may provide an explanation for the redundancy in PS-binding molecules. Here, the cell type-specific expression patterns of proteins like Tim-4 and MFG-E8 are a prerequisite for the observed crosstalk between resMΦ and inflammatory monocytes and allow a rapid and flexible regulation of the clearance of ACs.

Previous studies highlighted a major role of 12/15-LO during the oxidation of LDL and the pathogenesis of atherosclerosis (Cyrus et al., 1999, 2001; George et al., 2001). Nevertheless, the physiological role of this evolutionary highly conserved enzyme has remained elusive so far (Kuhn and Thiele, 1999). Our current findings on the role of 12/15-LO in the maintenance of tolerance highlight a role of lipid oxidation, which lies beyond cardiovascular disease. Hence it is compelling to speculate that enhanced lipid oxidation and the consecutive development of atherosclerosis are part of the price we pay for immunological tolerance.

EXPERIMENTAL PROCEDURES

Animals

Animal experiments were approved by the government of Mittelfranken. Mice were housed in the animal facility of the University of Erlangen-Nuremberg. 12/15-LO-deficient (*Alox15*$^{-/-}$), ovalbumin transgenic (OVAtg), and OT-II mice were purchased from The Jackson Laboratory; C57BL/6 mice were purchased from Charles River Laboratories.

Phagocytosis Assay

Resident macrophages were isolated from the peritoneum of naive 10- to 12-week-old mice. Inflammatory macrophages were isolated from peritonitis exudates, 72 hr after intraperitoneal injection of 2.5 ml of 3% Brewer's thioglycollate (Sigma-Aldrich) into WT and *Alox15*$^{-/-}$ mice at the age of 10–12 weeks. Both elicited and resident peritoneal macrophages were obtained by peritoneal lavage with ice-cold 4% FCS in PBS and plated in 48-well plates for flow cytometry analysis and on coverslips in 24-well plates for immunofluorescence microscopy, respectively. Macrophages were allowed to rest overnight at 37°C at 5% CO_2 before starting experiments. To generate apoptotic cells, thymocytes isolated from 6- to 8-week-old female C57BL/6 mice were labeled with CFSE (Sigma-Aldrich) and afterwards incubated for 6 hr in 1 μM dexamethason, washed several times with PBS, spun through a FCS cushion to eliminate the dexamethason, and resuspended in 10% FCS in RPMI. Macrophages were incubated with apoptotic cells in 10% FCS in RPMI for 30 min at a ratio of 1:5 at 37°C. For phagocytosis of bacteria, *E. coli* were heat inactivated, CFSE labeled, and incubated with macrophages at a ratio of 1:50 for 30 min at 37°C. Afterward, unbound cells were gently washed away with ice-cold PBS. For flow cytometry, macrophages were detached with trypsin and EDTA, transferred into plastic tubes, and stained if indicated at 4°C for 30 min. Analysis was performed with a GALLIOS cytofluorometer (Beckmann Coulter) and FlowJo software. The number of CFSE-positive macrophages was determined and relative uptake was calculated by normalization to the WT control (=1). For immunofluorescence microscopy, macrophages on

coverslips were stained as indicated and mounted on glass slides. Microscopy was performed with an Eclipse-80i microscope (Nikon) and a monochromatic camera (DS-Qi1MC; Nikon). Pictures are displayed with indicated pseudocolors by NIS elements software BR3.0 (Nikon).

In Vivo Phagocytosis Assay

20×10^6 CFSE-labeled apoptotic cells with or without oxPE (10 μM) were injected i.p. 3 days after induction of peritonitis with thioglycollate. 30 min after injection of ACs, mice were sacrificed and periteonal lavage was performed as described above. Cells were washed three times with ice-cold PBS and transferred into plastic tubes. Antibody staining was performed as indicated for 30 min at 4°C and flow cytometry was performed.

Detailed information on phagocytosis assays with the different particles can be found in the Supplemental Information.

In Vitro OT-II-Proliferation Assay

Macrophage cultures were obtained from peritonitis exudates or resident peritoneum as described above. Cells were incubated with either ovalbumin-transgenic apoptotic cells (MΦ:AC-ratio 1:5) or soluble ovalbumin (500 μg/ml) in 96-well plates for 24 hr (100,000 cells/well) in the presence of LPS (100 ng/ml) with or without oxPE (10 μM). Afterwards, cells were thoroughly washed five times with PBS and incubated with enriched (CD4-MACS; Milteny) CFSE-labeled (CFSE: 5 μM for 5 min at room temperature) OT-II cells for 72 hr (MΦ:T cell ratio 1:5). Afterwards, OT-II cells were transferred into plastic tubes and stained for CD4 and cytofluometry was performed. OT-II proliferation was measured by CFSE dilution of CD4-positive cells.

In Vivo OT-II-Proliferation Assay

2 days after induction of peritonitis with thioglycollate, 4×10^6 enriched (CD4-MACS; Milteny) CFSE-labeled OT-II cells were transferred into wild-type and *Alox15*$^{-/-}$ mice by intravenous injection. 24 hr later, apoptotic thymocytes were generated from ovalbumin-transgenic mice (OVAtg) as described above. 20×10^6 OVAtg ACs were injected intraperitoneally in each mouse. After 72 hr, mice were sacrificed, and lymph nodes and spleens were taken. Draining (mediastinal) lymph nodes were identified by intraperitoneal injection of 10% indian ink 4 hr before isolation (Figure S2). Single-cell solutions were stained for CD4 and cytofluometry was performed. OT-II proliferation was measured by CFSE dilution of CD4-positive cells.

Phospholipid Preparation

The detailed protocol is described in the Supplemental Experimental Procedures.

Quantification of PE-OH and PE-OOH in Resident Macrophages by Mass Spectometry

The detailed protocols are described in the Supplemental Experimental Procedures.

In Vitro MFG-E8-Binding Assay

For the solid-phase ELISA for MFG-E8-binding to phospholipids, 96-well plates (Nunc MaxiSorp) were coated with 100 μl of solution of PS at a concentration of 3 μg/ml in methanol and air-dried overnight at 4°C. The wells were blocked with 1% BSA in PBS. Indicated oxidized and unoxidized phospholipids were freshly mixed with DMPC at a ratio of 1:8 to create phospholipid vesicles. Vesicles were added together with recombinant murine MFG-E8 (10 nM; R&D Systems) to the wells and incubated at room temperature for 1 hr. After intense washing with 0.05%-Tween20 in PBS, MFG-E8 bound to the PS-coated wells was quantified by ELISA with a biotinylated MFG-E8 antibody and peroxidase-conjugated streptavidin (R&D Systems). Binding of recombinant MFG-E8 to the surface of resident peritoneal macrophages was performed in 8-well chamber slides and determined by incubating the cells with MFG-E8 (100 ng/ml) at 4°C after fixation with 4% PFA. Bound MFG-E8 was detected by immunofluorescence microscopy, with a biotinylated MFG-E8 antibody (R&D Systems) and Cy3-labeled streptavidin (Biolegend).

Pristane Model

The detailed protocol is described in the Supplemental Experimental Procedures.

Quantitative Real-Time PCR

The detailed protocols as well as primer sequences are described in the Supplemental Experimental Procedures.

Statistical Analysis

Data are shown as means ± SD. Group mean values were compared by two-tailed Student's t test. The data shown are representative of at least three experiments producing similar results. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at doi:10.1016/j.immuni.2012.03.010.

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