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Polarization of Macrophages toward M2 Phenotype Is Favored by Reduction in iPLA$_2\beta$ (Group VIA Phospholipase A$_2$)*

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Jason W. Ashley[‡1], William D. Hancock[§¶], Alexander J. Nelson[§¶], Robert N. Bone[‖], Hubert M. Tse[¶], Mary Wohltmann[‡‡], John Turk[‡‡], and Sasanka Ramanadham[§¶2]**

*From the [‡]Department of Biology, Eastern Washington University, Cheney, Washington 99004, [§]Department of Cell, Developmental, and Integrative Biology, [¶]Comprehensive Diabetes Center, and **Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294, [‖]Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana 46202, and [‡‡]Department of Medicine, Washington University School of Medicine, St. Louis, Missouri 63110*

Macrophages are important in innate and adaptive immunity. Macrophage participation in inflammation or tissue repair is directed by various extracellular signals and mediated by multiple intracellular pathways. Activation of group VIA phospholipase A$_2$ (iPLA$_2\beta$) causes accumulation of arachidonic acid, lysophospholipids, and eicosanoids that can promote inflammation and pathologic states. We examined the role of iPLA$_2\beta$ in peritoneal macrophage immune function by comparing wild type (WT) and iPLA$_2\beta^{-/-}$ mouse macrophages. Compared with WT, iPLA$_2\beta^{-/-}$ macrophages exhibited reduced proinflammatory M1 markers when classically activated. In contrast, anti-inflammatory M2 markers were elevated under naïve conditions and induced to higher levels by alternative activation in iPLA$_2\beta^{-/-}$ macrophages compared with WT. Induction of eicosanoid (12-lipoxygenase (12-LO) and cyclooxygenase 2 (COX2))- and reactive oxygen species (NADPH oxidase 4 (NOX4))-generating enzymes by classical activation pathways was also blunted in iPLA$_2\beta^{-/-}$ macrophages compared with WT. The effects of inhibitors of iPLA$_2\beta$, COX2, or 12-LO to reduce M1 polarization were greater than those to enhance M2 polarization. Certain lipids (lysophosphatidylcholine, lysophosphatidic acid, and prostaglandin E$_2$) recapitulated M1 phenotype in iPLA$_2\beta^{-/-}$ macrophages, but none tested promoted M2 phenotype. These findings suggest that (*a*) lipids generated by iPLA$_2\beta$ and subsequently oxidized by cyclooxygenase and 12-LO favor macrophage inflammatory M1 polarization, and (*b*) the absence of iPLA$_2\beta$ promotes macrophage M2 polarization. Reducing macrophage iPLA$_2\beta$ activity and thereby attenuating macrophage M1 polarization might cause a shift from an inflammatory to a recovery/repair milieu.

Macrophages are important in inflammation. These mononuclear myeloid hematopoietic lineage cells contribute to both innate and adaptive immunity (1). In the innate immune response, macrophages phagocytose invading pathogens and modulate recruitment and activation of inflammatory cells with secreted factors such as tumor necrosis factor α (TNFα) and prostaglandin E$_2$ (PGE$_2$)[3] (2, 3). Macrophages facilitate adaptive immunity primarily as antigen-presenting cells (4). Phagocytosed proteins processed into small fragments can be presented to CD4$^+$ T cells via the major histocompatibility class II receptor (MHC II) (5). The critical role of macrophages in initiation and resolution of infection can be observed in models of macrophage depletion; in cases of macrophage insufficiency, experimental mice have an increased susceptibility to infection, and existing infections progress further than in macrophage-sufficient animals (6). Macrophages also contribute to the pathogenesis of cancer progression, rheumatoid arthritis, diabetes, and atherosclerosis; macrophages are thus targets of emerging therapeutic regimens (7–10).

Macrophages also participate in autoimmune-mediated destruction of β-cells and type 1 diabetes (T1D). In diabetes-prone individuals, immune cells including macrophages migrate to pancreatic islets and secrete proinflammatory cyto-

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[2] To whom correspondence should be addressed: Dept. of Cell, Developmental, and Integrative Biology, University of Alabama at Birmingham, SHEL 1205, 1720 2nd Ave. S, Birmingham, AL 35294-2182. Tel.: 205-996-5973; Fax: 205-996-5220; E-mail: sramvem@uab.edu.

[3] The abbreviations used are: PGE$_2$, prostaglandin E$_2$; T1D, type 1 diabetes; T2D, type 2 diabetes; PLA$_2$, phospholipase A$_2$; ALOX12, 12-lipoxygenase (12-LO); CCL2, monocyte chemoattractant protein-1; CCL5, C-C motif chemokine 5 precursor; CDC, cinnamyl-3,4-dihydroxy-α-cyanocinnamate; CD68, cluster of differentiation; CHIL3, chitinase-like3 (YM1); CXCL10, chemokine (C-*X*-C motif) ligand 10; COX, cyclooxygenase; iNOS, inducible nitric oxide; cPLA$_2$, cytosolic phospholipase A$_2$; iPLA$_2\beta$, group VIA phospholipase A$_2\beta$; iPLA$_2\gamma$, group VIA phospholipase A$_2\gamma$; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPS, lipopolysaccharide; MRC1, mannose receptor 1c type precursor; NF-κB1, nuclear factor κ-light chain enhancer of activated B-cells (NF-κB p105, p50 precursor); NOD, non-obese diabetic; NOX4, NADPH oxidase 4; PTGS2, mouse cyclooxygenase 2 (COX2); RT-qPCR, real time quantitative PCR; RELA, Rel-associated protein (NF-κB p65); ROS, reactive oxygen species; *S*-BEL, *S*-enantiomer of bromoenol lactone; STAT1 and STAT6, signal transducer and activator of transcription 1 and 6, respectively; Arg, arginase.

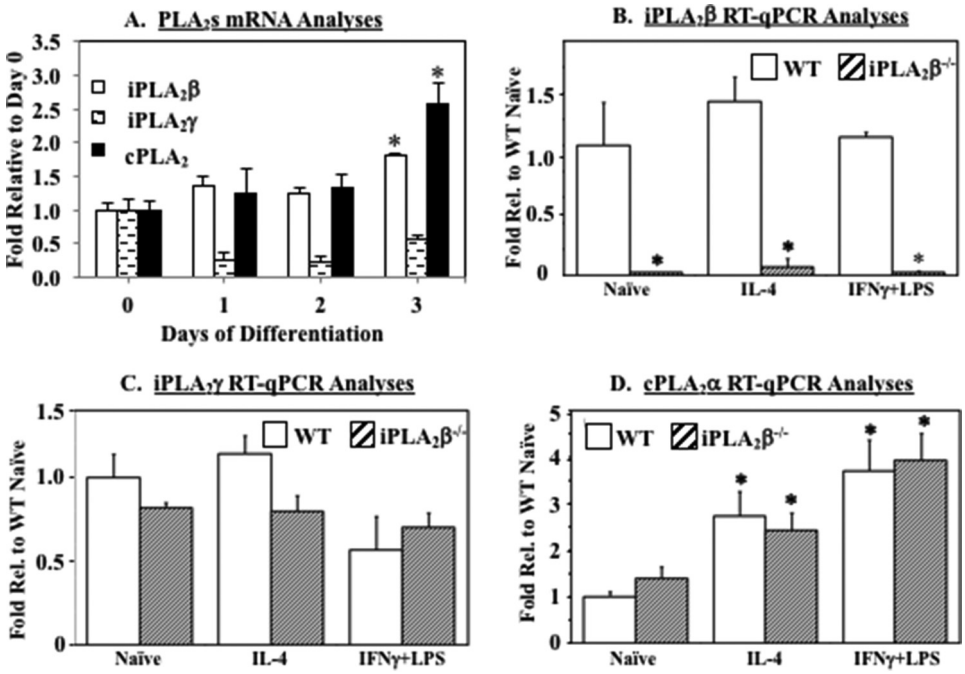

FIGURE 1. **Phospholipases A_2 expression in naïve, M2, and M1 macrophages.** RNA was isolated from macrophages, and cDNA was prepared for RT-qPCR analyses. *A*, expression of PLA_2 messages in differentiating macrophages. *, significantly different from corresponding 0 day, $p < 0.05$. *B–D*, mRNA expression in peritoneal macrophages after classical (*IFN*γ + *LPS*) or alternative (*IL-4*) activation. RNA was isolated from the macrophages, cDNA was prepared for RT-qPCR analyses, and data are the means ± S.E. generated from 4–11 independent measurements. *B*, iPLA$_2\beta$ (*, iPLA$_2\beta^{-/-}$ group significantly different from corresponding WT, $p < 0.05$). *C*, iPLA$_2\gamma$. *D*, cPLA$_2\alpha$ (*, significantly different from corresponding naïve group, $p < 0.05$).

kines and reactive oxygen species (ROS) that result in β-cell death (11). Two different activation states of macrophages have been described: M1 proinflammatory macrophages (12), which are classically activated (*e.g.* by interferon-γ (IFNγ), lipopolysaccharide (LPS), TNFα), and M2 macrophages, which are alternatively activated (*e.g.* by IL-4 or IL-10) (13). Whereas M1 macrophages are recognized causative factors in T1D development (14), M2 macrophages appear to protect against T1D (15). Recent studies suggest that ROS can modulate macrophage polarization and that reduction in ROS generation promotes an M2 macrophage phenotype, blunts M1 macrophage phenotype, and dramatically delays T1D onset (16).

Lipid signaling is increasingly recognized to modulate inflammation and immune responses (17). Phospholipases A_2 (PLA_2s) are important in generating lipid mediators and include secretory PLA_2 (sPLA$_2$), cytosolic (cPLA$_2$), and Ca^{2+}-independent cytosolic (iPLA$_2\beta$) and membrane-associated (iPLA$_2\gamma$) (18) enzymes. The PLA_2s hydrolyze the *sn*-2 substituent from membrane phospholipids to generate a free fatty acid and a 2-lysophospholipid. The fatty acid arachidonic acid can be further metabolized by cyclooxygenases (COXs) and lipoxygenases (LOs) to generate bioactive eicosanoids that include prostaglandins and leukotrienes (19). Several eicosanoids are proinflammatory and linked to macrophage phagocytosis, adhesion, apoptosis, and amplification of macrophage-derived eicosanoid release (20–23). Lysophospholipids (24) generated by PLA_2s (*e.g.* lysophosphatidic acid (LPA)) are implicated in monocyte survival and migration (25–27).

Expression of iPLA$_2\beta$ is elevated in pancreatic islets from diabetic subjects and rodent models of diabetes (28, 29). Stresses (endoplasmic reticulum, proinflammatory cytokines, oxidative) associated with T1D development that cause β-cell death also increase iPLA$_2\beta$ expression, and genetic or pharmacologic reduction of iPLA$_2\beta$ activity ameliorates β-cell apoptosis (30–35). We recently reported that immune cells, including $CD4^+$ T- and B-lymphocytes, express iPLA$_2\beta$, and selective inhibition of iPLA$_2\beta$ reduces immune responses and leukocyte infiltration of islets, preserves β-cell mass, and lowers T1D (29). Macrophages also express iPLA$_2\beta$ (23, 29), and its activation was recently reported to regulate ROS production from macrophages exposed to diabetic metabolic stress (36). The potential impact of iPLA$_2\beta$-derived lipid signals in affecting macrophage phenotype has not previously been addressed.

We have examined this using iPLA$_2\beta^{-/-}$ and wild type (WT) mouse peritoneal macrophages and report here that iPLA$_2\beta$ deficiency reduces macrophage expression of eicosanoid and ROS-generating enzymes and favors M2 over M1 macrophage phenotypic polarization. These findings suggest that genetic or pharmacologic reduction of iPLA$_2\beta$ activity could reduce inflammation and delay disease progression by shifting macrophage polarization away from proinflammatory and toward an alternatively activated phenotype, thereby reducing proinflammatory lipid and ROS signal generation.

Results

PLA_2 Expression in Macrophages—PLA_2s are ubiquitously expressed and activated in inflammatory settings (17, 37, 38), and as expected, *iPLA$_2\beta$* and *cPLA$_2$* mRNA species increase during macrophage differentiation from their bone marrow-derived precursors (Fig. 1*A*). Analogously, mRNA species encoding iPLA$_2\beta$ (Fig. 1*B*), iPLA$_2\gamma$ (Fig. 1*C*), and cPLA$_2$ (Fig. 1*D*) are evident in already differentiated WT naïve peritoneal

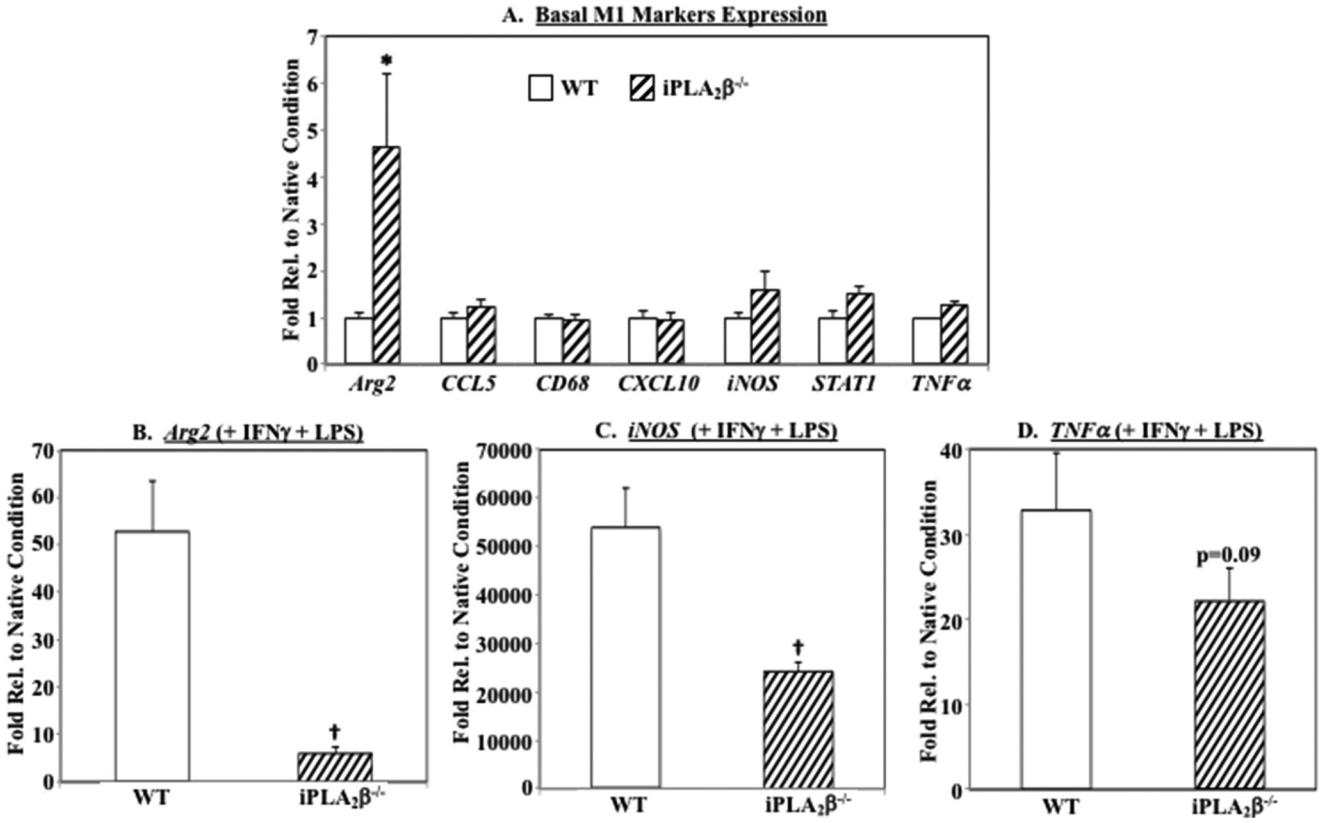

FIGURE 2. **M1 markers in peritoneal macrophages from WT and iPLA$_2\beta^{-/-}$ mice.** Macrophage RNA was isolated under naïve conditions and after classical activation, and cDNA was prepared for RT-qPCR analyses of M1 markers. *A*, naïve conditions. -Fold expression of each marker, relative to WT naïve, is presented. *, iPLA$_2\beta^{-/-}$ group significantly different from corresponding WT, $p < 0.05$. *B*, *Arg2*. *C*, *iNOS*. *D*, *TNFα*-fold induction by IFNγ + LPS relative to their own naïve conditions is presented. †, iPLA$_2\beta^{-/-}$ significantly different from WT, $p < 0.005$. Data are the means ± S.E. (n = 7–11 independent measurements).

macrophages. As expected, macrophages from iPLA$_2\beta^{-/-}$ mice did not express iPLA$_2\beta$ mRNA (Fig. 1*B*), but their expression of iPLA$_2\gamma$ (Fig. 1*C*) and cPLA$_2$ (Fig. 1*D*) mRNA was similar to WT. Neither classical nor alternative activation with IFNγ + LPS and IL-4, respectively, increased expression of iPLA$_2\beta$ mRNA in WT macrophages (Fig. 1*B*), and iPLA$_2\gamma$ expression with activation was similar between the two genotypes (Fig. 1*C*). In contrast, cPLA$_2$ expression was increased under both classical and alternative activation, but its induction was similar between WT and iPLA$_2\beta^{-/-}$ macrophages (Fig. 1*D*).

M1 Phenotype Markers in Peritoneal Macrophages—To examine the impact of iPLA$_2\beta$ on polarization of macrophages toward the M1 phenotype, macrophages were treated with IFNγ + LPS, total RNA was isolated, and cDNA was prepared for real-time quantitative PCR (RT-qPCR) analyses of various recognized markers of M1 macrophage phenotype. These included *Arg2*, *CCL5*, *CD68*, *CXCL10*, *iNOS*, *STAT1*, and *TNFα*. Of these, only *Arg2* was significantly different between the two genotypes (Fig. 2*A*) under naïve conditions. After classical activation, *CCL5*, *CD68*, *CXCL10*, *STAT1*, and *TNFα* did not differ significantly between the two genotypes (data not shown). However, robust increases in *Arg2* (Fig. 2*B*), *iNOS* (Fig. 2*C*), and *TNFα* (Fig. 2*D*) in WT peritoneal macrophages were evident. In contrast, induction of all three was blunted in the iPLA$_2\beta^{-/-}$ group.

Although *Arg1* is recognized as an M2 macrophage marker, there is controversy regarding whether *Arg2* is pro- or anti-inflammatory (39–41). Our analyses revealed (Fig. 3) *Arg1* induction in WT peritoneal macrophages with alternative, but not classical activation. In contrast, *Arg2* was induced by classical, but not alternative activation. These observations led us to suggest that *Arg2* is associated with an M1 macrophage phenotype in our study model. Collectively, these findings suggest that iPLA$_2\beta$ deficiency mitigates polarization of macrophages toward an M1 phenotype.

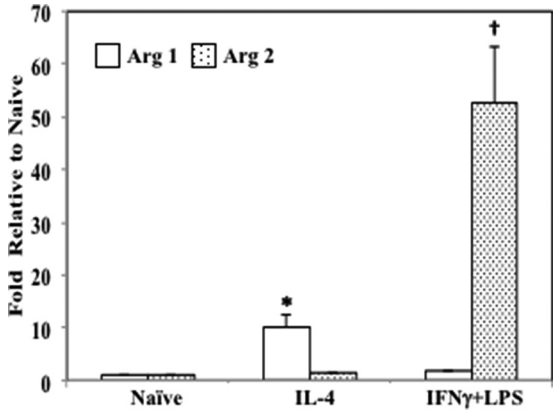

FIGURE 3. **Effects of activation on *Arg1* and *Arg2* in WT peritoneal macrophages.** Macrophage RNA was isolated under naïve conditions and after activation, and cDNA was prepared for RT-qPCR analyses for *Arg1* and *Arg2*. -Fold expression of each, relative to corresponding naïve, is presented. Data are the means ± S.E. (n = 7–11 independent measurements). * and †, significantly different from corresponding naïve group, $p < 0.0005$, and $p < 0.00005$, respectively.

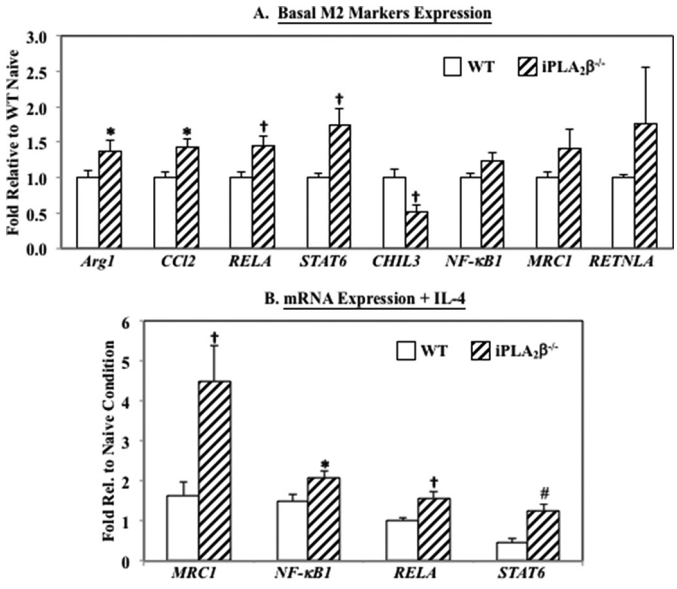

FIGURE 4. **M2 markers in peritoneal macrophages from WT and iPLA$_2\beta^{-/-}$ mice**. Macrophage RNA was isolated under naïve conditions and after alternative activation, and cDNA was prepared for RT-qPCR analyses. *A*, naïve conditions. -Fold expression of each marker relative to WT naïve is presented. *B*, -fold induction by IL-4 relative to their own naïve conditions is presented. Data are the means ± S.E. ($n = 7$–11 independent measurements). *, †, and #, iPLA$_2\beta^{-/-}$ group significantly different from corresponding WT, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

M2 Phenotype Markers in Peritoneal Macrophages—Next, we examined whether iPLA$_2\beta$ expression influenced polarization of macrophages toward the M2 phenotype. Macrophages were treated with IL-4 and processed for RT-qPCR analyses of various M2-related markers. These included *Arg1*, *CCL2*, *RELA*, *STAT6*, *CHIL3*, *NF-κB1*, *MRC1*, and *RETNLA*. Under naïve conditions, *NF-κB1*, *MRC1*, and *RETNLA* were unchanged, and *CHIL3* decreased in the iPLA$_2\beta^{-/-}$ group relative to WT group (Fig. 4*A*). However, *Arg1*, *CCL2*, *RELA*, and *STAT6* were significantly higher in the iPLA$_2\beta^{-/-}$ group relative to the WT group. Furthermore, under M2 polarizing conditions induction of *MRC1*, *NF-κB1*, *RELA*, and *STAT6* was significantly higher in the iPLA$_2\beta^{-/-}$ group, relative to WT group (Fig. 4*B*). These findings suggest that the M2 phenotype is predominant with iPLA$_2\beta^{-/-}$ macrophages.

ALOX-12 (12-LO) and PTGS2 (COX2) Expression in Peritoneal Macrophages—Induction of lipid-metabolizing enzymes and the corresponding accumulations of eicosanoids have been reported at the onset of insulitis and initial stages of diabetes (42–45). Consistent with this, we find that both *ALOX-12* (Fig. 5*A*) and *PTGS2* (Fig. 5*B*), which metabolize arachidonic acid to generate various oxidized lipids, are induced by classical but not alternative activation in the WT macrophages. We, therefore, compared expression of *ALOX12* (12-LO) and *PTGS2* (COX2) in macrophages from WT and iPLA$_2\beta^{-/-}$ mice. Under naïve conditions there was no statistical difference in *ALOX12* mRNA, but *PTGS2* mRNA was significantly higher in the iPLA$_2\beta^{-/-}$ group (Fig. 6*A*). However, under classical activation conditions, there were dramatic increases in both *ALOX12*, and *PTGS2* mRNA in the WT group (Fig. 6, *B* and *C*). In comparison, induction of both was markedly blunted in the iPLA$_2\beta^{-/-}$ group. These findings suggest that downstream generators of proinflammatory lipid signals are subject to modulation by iPLA$_2\beta$ expression.

Effects of iPLA$_2\beta$, COX, and 12-LO Inhibitors on M1 Markers—In view of the observed decreases in M1 markers and eicosanoid-generating enzymes in macrophages from iPLA$_2\beta$-KO mice, it was of interest to determine which pathway influenced M1 polarization. To address this, macrophages from WT mice were classically activated in the absence and presence of inhibitors of iPLA$_2\beta$ ((*S*)-bromoenol lactone (*S*-BEL)), COX (indomethacin), or 12-LO (cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC)), and select M1 markers were assessed. As expected, in the presence of IFNγ + LPS, both *Arg2* (Fig. 7*A*) and *iNOS* (Fig. 7*B*) mRNA were dramatically increased, and such increases were significantly inhibited by all three inhibitors. At the protein level, media accumulation of TNFα (Fig. 8*A*) and IL-1β (Fig. 8*B*) were increased by activation. Although *S*-BEL and indomethacin inhibited TNFα production by the macrophages, CDC had no effect (Fig. 8*A*). IL-1β accumulation was modestly decreased by *S*-BEL and CDC (~25%, $p = 0.1$) but was significantly inhibited by indomethacin (Fig. 8*B*). In contrast, IL-12, which was also increased by activation, was not affected by any of the inhibitors (data not shown). Furthermore, media accumulation of nitrite, a spontaneous oxidized product of iNOS-generated nitric oxide, which was markedly increased by activation in WT peritoneal macrophages (Fig. 8*C*), was inhibited by both *S*-BEL and indomethacin. Consistently, supplementation of the media during activation of macrophages from iPLA$_2\beta^{-/-}$ mice with PGE$_2$, lysophosphatidylcholine (LPC), or LPA, but not arachidonic acid, significantly elevated nitrite levels in the media (Fig. 8*D*). In agreement with these observations, endogenous LPA content is lower in macrophages from iPLA$_2\beta^{-/-}$ mice compared with WT (Fig. 8, *E* and *F*). These findings suggest that some, but not all, M1 markers are impacted by iPLA$_2\beta$ activation and subsequent generation of COX-derived lipid species.

Effects of iPLA$_2\beta$, COX, and 12-LO Inhibitors on M2 Markers—To assess the impact of eicosanoid-generating enzymes on eliciting an M2 phenotype, the effects of *S*-BEL, indomethacin, and CDC on select M2 markers (*Arg1*, *MRC1*, *STAT6*, *CCL2*) in the absence and presence of alternative activation were examined in WT peritoneal macrophages. In general, the inhibitors alone had no effect on M2 mRNA markers; however, *S*-BEL alone increased both *STAT6* and *CCL2*. Unlike their impact on M1 markers, the profile of M2 markers with activation in the presence of the inhibitors was variable. Although all were stimulated by IL-4, none of the inhibitors had an effect on *Arg1* (Fig. 9, *A* and *E*). *MRC1* was inhibited by *S*-BEL and CDC (Fig. 9*B*) but was increased by indomethacin (Fig. 9*F*). *STAT6* mRNA was increased by all three inhibitors (Fig. 9, *C* and *G*). *CCL2* was increased by *S*-BEL (Fig. 9*D*), decreased by CDC (Fig. 9*D*), and unchanged by indomethacin (Fig. 9*H*). At the protein level, TGFβ (Fig. 10*A*) and IL-10 (Fig. 10*B*) production were increased by IL-4. Whereas TGFβ was further increased in the presence of *S*-BEL, neither indomethacin nor CDC had any effect. None of the inhibitors had an effect on IL-10 production (Fig. 10*B*). Moreover, supplementation of media provided to iPLA$_2\beta^{-/-}$ macrophages with indi-

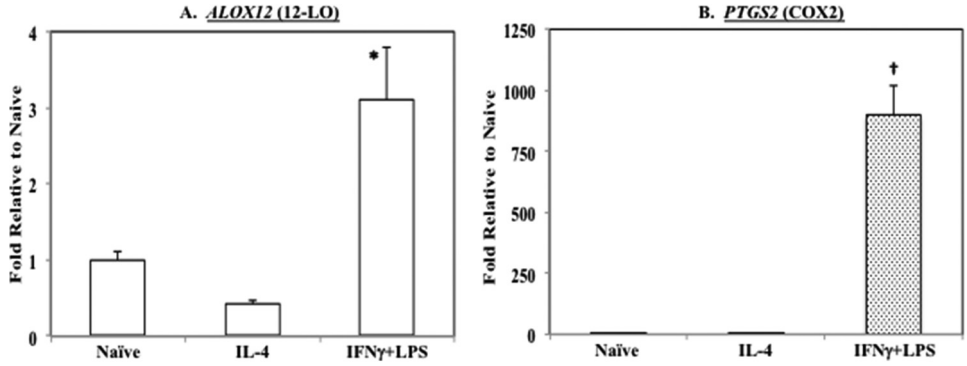

FIGURE 5. **Effects of activation on *ALOX12* (12-LO) and *PTGS2* (COX2) in WT peritoneal macrophages.** Macrophage RNA was isolated under naïve conditions and after activation, and cDNA was prepared for RT-qPCR analyses. *A*, *ALOX12*. *B*, *PTGS2*. -Fold expression of each marker, relative to corresponding naïve, is presented. Data are the means ± S.E. ($n = 7$–11 independent measurements). * and †, significantly different from other groups, $p < 0.05$ and $p < 0.0001$, respectively.

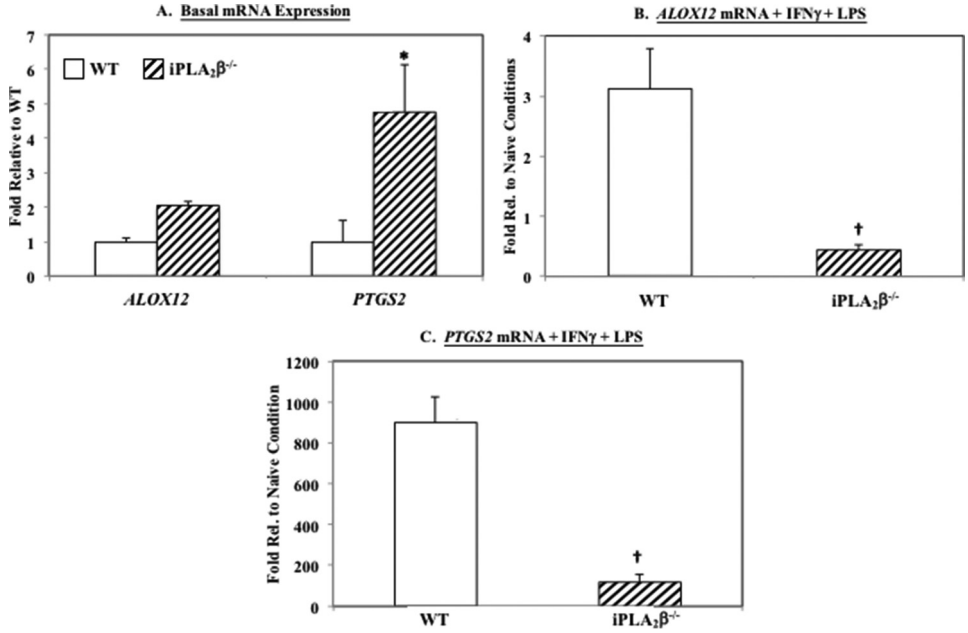

FIGURE 6. ***ALOX12* (12-LO) and *PTGS2* (COX2) in naïve and activated WT and iPLA$_2\beta^{-/-}$ peritoneal macrophages**. Macrophage RNA was isolated under naïve conditions and after classical activation, and cDNA was prepared for RT-qPCR analyses. Data are the means ± S.E. ($n = 7$–11 independent measurements). *A*, naïve conditions. -Fold expression of each marker relative to WT naïve is presented. *, significantly different from WT group, $p < 0.05$. *B*, *ALOX12*. *C*, *PTGS2*. -Fold induction by IFNγ + LPS relative to its own naïve conditions is presented. †, significantly different from WT group, $p < 0.0005$.

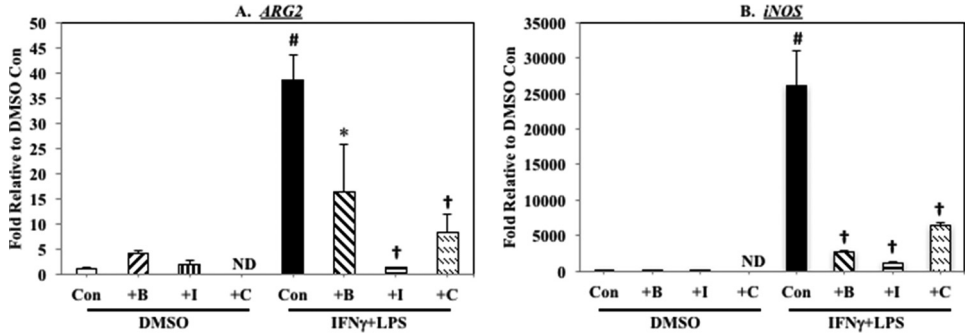

FIGURE 7. **Effects of inhibitors of lipid-metabolizing enzymes on M1 markers in peritoneal macrophages.** Macrophages from WT mice were treated as in Fig. 2 in the absence or presence of *S*-BEL (*B*, 1 μM), indomethacin (*I*, 50 μM), or CDC (*C*, 1 μM) before message analyses for *Arg2* (*A*) and *iNOS* (*B*). Data are the means ± S.E. ($n = 4$–13 independent measurements). #, significantly different from DMSO control (*Con*), $p < 0.001$; * and †, significantly different from IFNγ+LPS control, $p < 0.05$, $p < 0.01$; ND, not determined.

vidual lipid species did not promote significant increases in any of the M2 mRNA markers (data not shown). These findings suggest that although some of the M2 marker expression is impacted by iPLA$_2\beta$ activation, not all are, which raises the possibility that other PLA$_2$s or lipid species, not tested, contribute to their expression.

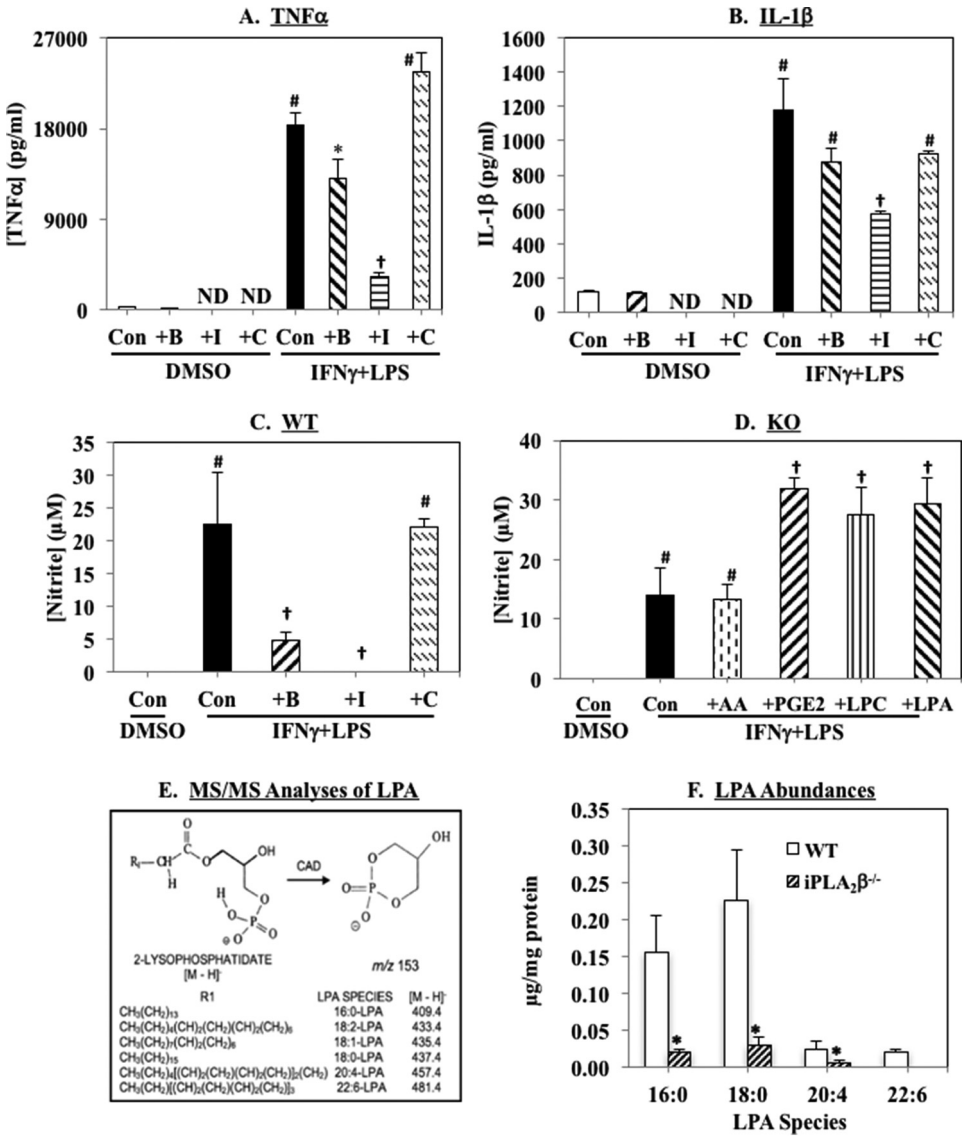

FIGURE 8. **Modulation of M1-related factors by inhibitors of lipid-metabolizing enzymes and evidence for involvement of PGE_2 in peritoneal macrophages.** Aliquots of media collected from macrophages treated in Fig. 7 were used for ELISAs for TNFα (A), IL-1β (*B*), and nitrite (*C*). #, significantly different from DMSO control (*Con*), $p < 0.001$; *, significantly different from IFNγ+LPS control, $p < 0.05$, $p < 0.01$; †, significantly different from IFNγ+LPS control, $p < 0.01$. *D*, nitrite generated by classically activated iPLA$_2\beta^{-/-}$ peritoneal macrophages in the absence or presence of arachidonic acid (*AA*, 0.5 μM), PGE_2 (1 μM), LPC (10 μM), or LPA (1 μM). #, significantly different from DMSO Con, $p < 0.001$. †, significantly different from IFNγ + LPS control and +arachidonic acid, $p < 0.01$; *ND*, not determined. *E*, MS/MS analyses of lysophosphatidic acid by multiple reaction monitoring. *F*, quantification of LPA molecular species in macrophages. *, significantly different from WT, $p < 0.05$. Data are the means ± S.E. ($n = 3$–4 independent measurements). *B*, *S*-BEL; *I*, indomethacin; *C*, CDC.

Feedback Modulation between ROS and iPLA$_2\beta$—Recently, macrophage migration was reported to be promoted by iPLA$_2\beta$ via induction of NOX4 (36). We find that under naïve conditions, *NOX4* is reduced by 50% in iPLA$_2\beta^{-/-}$ relative to WT peritoneal macrophages (Fig. 11*A*), Furthermore, even in the presence of classical activation with IFNγ + LPS, *NOX4* was 70% lower in iPLA$_2\beta^{-/-}$, relative to WT macrophages. These findings, taken together with nitrite accumulation presented in Fig. 8, *C* and *D*) suggest that downstream generators of proinflammatory ROS are subject to modulation by iPLA$_2\beta$. Consistently, we find that relative to immunodeficient and diabetes-resistant non-obese diabetic (NOD) *Rag* mice (29), iPLA$_2\beta$ is increased in islets from diabetes-prone NOD mice but not in islets from NOD mice that are deficient in NOX-derived superoxide (NOD.*Ncf1*^{m1J}) (Fig. 11*B*). These mice exhibit a marked delay in developing T1D relative to NOD mice (16). These findings suggest an additional component in the inflammatory process, wherein feedback regulation of iPLA$_2\beta$ involves ROS.

Discussion

In their role as immune cells, macrophages demonstrate remarkable diversity and plasticity. In addition to acting as phagocytic and antigen-presenting cells, macrophages can promote or modulate inflammation through classical and alternative activation pathways, respectively (46). Under different stimuli, macrophages produce multiple lipid mediators including lysophospholipids and eicosanoids, and these bioactive lipids can impact cells of surrounding tissues and the function of macrophages themselves (20, 36, 47). A major source of lyso-

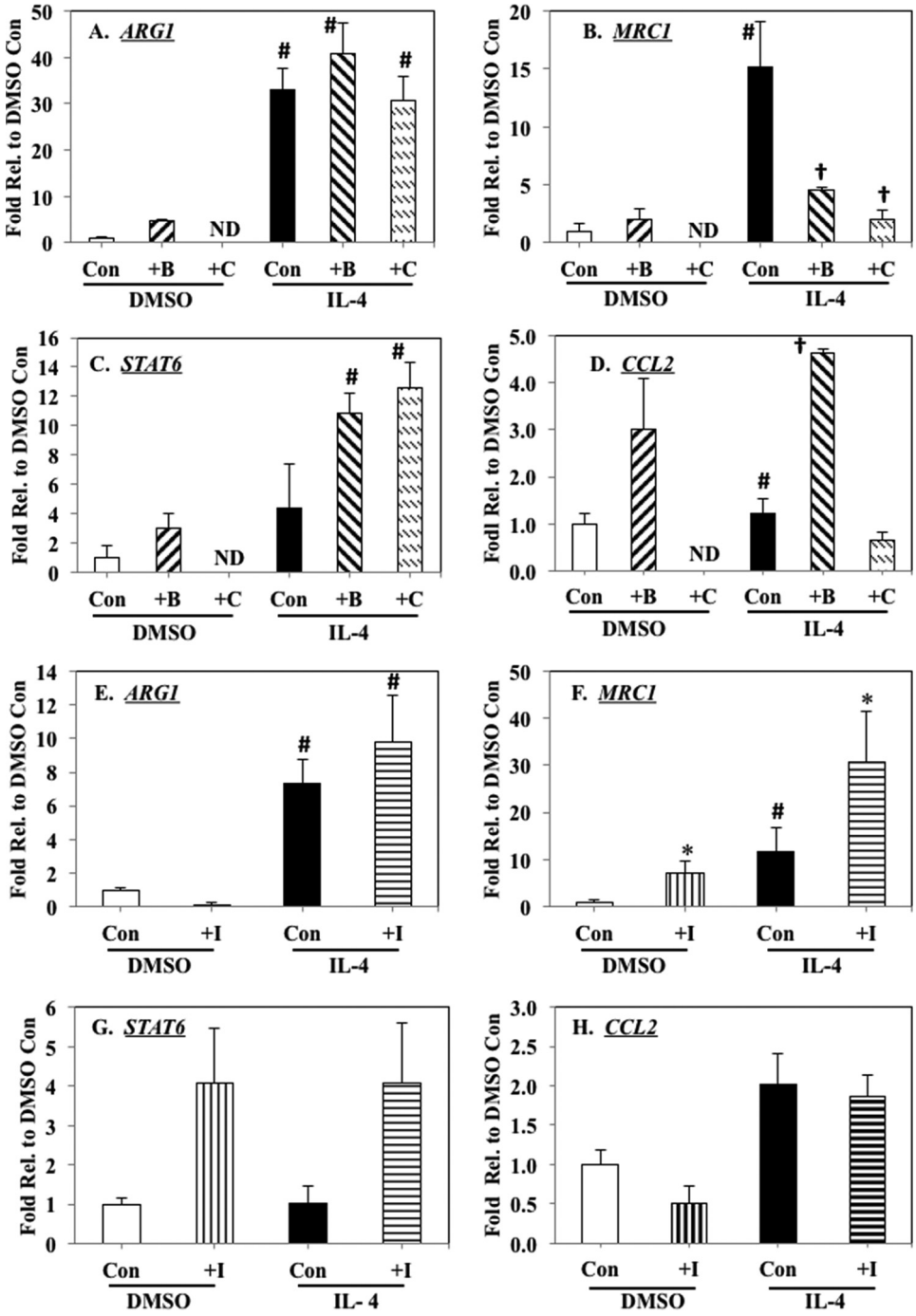

FIGURE 9. **Effects of inhibitors of lipid-metabolizing enzymes on M2 markers in peritoneal macrophages.** Macrophages from WT mice were treated as in Fig. 4 in the absence or presence of *S*-BEL (*B*, 1 μM), indomethacin (*I*, 50 μM), or CDC (*C*, 1 μM) before mRNA analyses for *Arg1* (*A* and *E*), *MRC1* (*B* and *F*), *STAT6* (*C* and *G*), *CCL2* (*D* and *H*). #, significantly different from DMSO control (Con), $p < 0.001$; †, significantly different from IL-4 Con, $p < 0.05$, $p < 0.01$; *ND*, not determined. Data are the means ± S.E. ($n = 3$–13 independent measurements.)

phospholipids and arachidonic acid, the common eicosanoid precursor, is the lipase activity of PLA$_2$s (19).

The family of PLA$_2$s has been implicated in inflammatory responses and contribution to onset and/or progression of autoimmune-mediated disease (48, 49), and iPLA$_2\beta$ has recently been linked to diabetes (29). Many components of the immune system, including macrophages (50–54), monocytes (55), neutrophils (56, 57), mast cells (58), and T-cells and B-cells (59), express iPLA$_2\beta$ activity. Among its proposed roles in macrophages, iPLA$_2\beta$ has been implicated in playing a major role in free fatty acid accumulation in macrophages (60–63) leading to apoptosis. iPLA$_2\beta$, but not cPLA$_2$, has also been reported to promote macrophage proliferation (37). Furthermore, iPLA$_2\beta$ appears to be required for maintenance of macrophage spreading and adhesion (64), which would contribute to inflammation by increasing macrophage retention at inflammation sites (22).

In view of the evidence for iPLA$_2\beta$ involvement in inflammatory processes related to macrophages and the recent reports

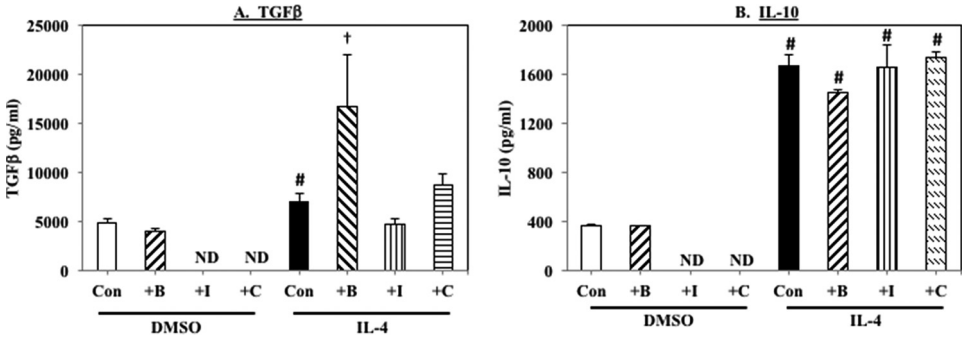

FIGURE 10. **Modulation of M2 marker proteins by inhibitors of lipid-metabolizing enzymes.** Aliquots of media collected from WT macrophages treated in Fig. 7 were used for ELISAs for TGFβ (*A*) and IL-10 (*B*). #, significantly different from DMSO control (*Con*), $p < 0.05$; †, significantly different from both controls, $p < 0.05$; *ND*, not determined. Data are the means ± S.E. ($n = 3$–4 independent measurements. *B*, *S*-BEL; *I*, indomethacin; *C*, CDC.)

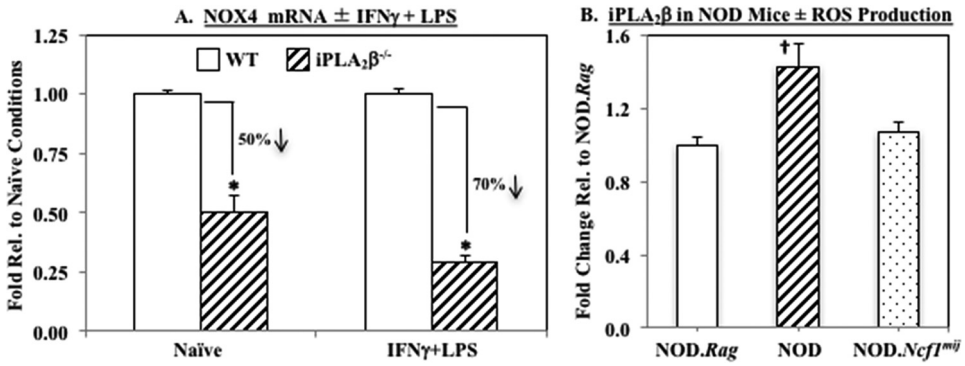

FIGURE 11. **Feedback modulation between ROS and iPLA$_2\beta$.** *A*, macrophage RNA was isolated under naïve conditions and after classical activation, and cDNA was prepared for RT-qPCR analyses of NOX4. -Fold induction relative to WT is presented. Data are the means ± S.E. ($n = 7$–11 independent measurements). *, significantly different from WT, $p < 0.01$. *B*, pancreatic islets were isolated from 7-week-old female immunodeficient NOD.*Rag*, diabetes-prone NOD and NOX-derived superoxide-deficient NOD (NOD.*Ncf1*^{m1j}) mice. RNA was isolated, and cDNA was used in RT-qPCR analyses of iPLA$_2\beta$. The means ± S.E. of -fold expression relative to NOD.*Rag* are presented. †, significantly different from the other groups, $p < 0.05$, $n = 3$.

that iPLA$_2\beta$ activation contributes to ROS generation by macrophages (36) and ROS influence macrophage polarization (16), we explored the impact of iPLA$_2\beta$ on macrophage function and polarization. We find that iPLA$_2\beta$ activation (*a*) participates in determining the direction of macrophage polarization and (*b*) modulates downstream expression of eicosanoid- and ROS-generating enzymes.

To explore the potential role of iPLA$_2\beta$ on macrophage activation, peritoneal macrophages from WT and iPLA$_2\beta^{-/-}$ mice were treated with IFNγ + LPS, which stimulates classical (M1) macrophage activation or with IL-4, which stimulates alternative (M2) macrophage activation. The absence of iPLA$_2\beta$ in the iPLA$_2\beta^{-/-}$ preparations was verified by PCR analyses, which also revealed that neither classical nor alternative activation induce iPLA$_2\beta$ mRNA in WT macrophages. The absence of iPLA$_2\beta$ also did not alter activated macrophage morphology (data not shown).

For the purpose of identifying susceptibility of peritoneal macrophage polarization to iPLA$_2\beta$ activation, we used various analyses to compare expression of multiple recognized markers of M1 (*Arg2*, *CCL5*, *CD68*, *CXCL10*, *iNOS*, *NOX4*, *STAT1*, and *TNFα* mRNA; TNFα, IL-1β, and IL-12 protein; nitrite accumulation) and M2 (*Arg1*, *CCL2*, *RELA*, *STAT6*, *CHICL3*, *NF-κB1*, *MRC1*, and *RETNLA* mRNA; TGFβ and IL-10 protein) macrophage phenotype (13, 16).

Under naïve conditions *Arg2* was higher in the iPLA$_2\beta^{-/-}$ macrophages, but all other M1 markers examined were similar between WT and iPLA$_2\beta^{-/-}$ macrophages. However, induction of *Arg2*, *iNOS*, and *NOX4* by classical activation was significantly blunted in iPLA$_2\beta^{-/-}$ macrophages. In contrast, several M2 markers (*Arg1*, *CCL2*, *RELA*, *STAT6*) were elevated under naïve conditions in iPLA$_2\beta^{-/-}$ macrophages, and induction of *MRC1*, *NF-κB1*, *RELA*, and *STAT6* with alternative activation was significantly greater in the absence of iPLA$_2\beta$. These findings suggest that iPLA$_2\beta$-derived lipids promote macrophage polarization favoring an M1 macrophage phenotype.

Arginase (Arg) and iNOS are predicted to exert contrary effects with respect to macrophage function (65). iNOS generates nitric oxide from the amino acid arginine, and Arg inhibits nitric oxide synthesis by reducing available arginine via conversion to ornithine and urea (66). However, there are two isoforms of arginase that are encoded by different genes (67): *Arg1*, which is cytoplasmic, and *Arg2*, which is mitochondrial. Macrophages express both *Arg1* and *Arg2* (39), and although *Arg1* is an established marker of M2 macrophage anti-inflammatory functionality (68), the role of *Arg2* is controversial. Both pro (39)- and anti-inflammatory (40) consequences have been linked with *Arg2* expression. A recent report used genetic approaches to demonstrate a proinflammatory role of *Arg2* in the development of type 2 diabetes (T2D) and atherosclerosis (41). Our findings of higher *Arg1* and blunted induction of *Arg2* in the iPLA$_2\beta^{-/-}$, relative to WT macrophages support the possibility that *Arg2* is a proinflammatory marker under classical activation conditions, as might exist in an *in vivo* inflamma-

tory milieu such as T1D. Consistent with a proinflammatory role of *Arg2*, we find that it is induced under classical activation but not alternative activation, suggesting that it is associated with an M1 macrophage phenotype in our study system.

Collectively, these observations suggest that iPLA$_2\beta$ deficiency disfavors M1 macrophage polarization. Because the products of PLA$_2$ catalysis are substrates for various oxygenases, we sought to identify contribution of specific eicosanoid-generating pathways and lipid species that may impact macrophage polarization. To address this, select markers were assessed in activated WT macrophages exposed to various inhibitors. These included *S*-BEL, which inhibits iPLA$_2\beta$ to limit hydrolysis of the *sn*-2 fatty acyl substituent and generation of lysophospholipids, indomethacin, which inhibits COX, and CDC, which is an inhibitor of 12-LO. These approaches revealed that all three inhibitors decreased M1 markers *Arg2* and *iNOS*, suggesting that COX- and 12-LO-derived lipids participate in M1 polarization. Among the M1-related proteins assessed, TNFα production from WT macrophages was also inhibited by *S*-BEL, similar to our earlier findings in CD4$^+$ T-cells (29), and indomethacin, but not CDC. Nitrite accumulation, a reflection of iNOS-catalyzed generation of nitric oxide, from activated WT macrophages was inhibited by both *S*-BEL and indomethacin, but not CDC. Consistently, nitrite accumulation from iPLA$_2\beta^{-/-}$ macrophages was significantly elevated by the addition of PGE$_2$, LPC, or LPA but not other lipids including arachidonic acid. In support of a role for LPA in contributing to the M1 phenotype are the findings of (*a*) decreased LPA content in macrophages from iPLA$_2\beta^{-/-}$ mice in the present study along with earlier demonstrations that (*b*) peritoneal fluid content of LPA is markedly reduced in iPLA$_2\beta$-null compared with wild type mice (69), and (*c*) stimulus-induced formation of LPA is greatly reduced in peritoneal macrophages isolated from iPLA$_2\beta^{-/-}$ compared with WT mice (36). These findings suggest that products of iPLA$_2\beta$ activity (lysophospholipids) and arachidonic acid, which is metabolized to COX products, contribute to M1 phenotype. In contrast, production of M1-related IL-12 or IL-1 protein is not impacted by any of the inhibitors, suggesting that not all M1-related factors are susceptible to lipids arising from PLA$_2$ activation or to lipid signaling directly.

Similar analyses of M2 markers revealed a lack of effect of any of the inhibitors on *Arg1*, whereas all three increased *STAT6*, and only *S*-BEL increased *CCL2*. Furthermore, accumulation of TGFβ protein in the media of activated macrophages was increased by *S*-BEL but not indomethacin or CDC. In contrast, IL-10 production by macrophages was not affected by any of the three inhibitors. These findings suggest that all M2 markers are also not affected by iPLA$_2\beta$. However, *STAT6* appears to be modulated by products of both COX- and 12-LO-catalyzed oxidation of arachidonic acid derived through iPLA$_2\beta$ activation. The findings that *CCL2* mRNA and TGFβ production are elevated by *S*-BEL, but not by CDC or indomethacin, raises the possibility that they are influenced by other eicosanoid-generating pathways, possibly involving 15- or 5-LO products. Unlike the M1 markers, none of the M2 markers in macrophages from iPLA$_2\beta^{-/-}$ mice were increased by the addition of any of the lipids tested. It is tempting to speculate that iPLA$_2\beta$-derived lipids do not directly promote an M2 phenotype but rather shift macrophage polarization toward an M1 phenotype. Conversely, in the absence or inhibition of iPLA$_2\beta$, stimuli to promote a M1 phenotype are reduced, giving rise to a more permissive environment for other factors to engage and drive macrophages toward an M2 phenotype.

Studies utilizing inhibitors and lipid supplementation highlight the complexity of the contribution of eicosanoids to immune responses (1, 46, 70). It is recognized that some of these oxidized lipids can have inflammatory effects, whereas others are anti-inflammatory. We find that the inducible COX2, which is expressed under inflammatory conditions (71), is up-regulated by classical activation in WT but not in iPLA$_2\beta^{-/-}$ macrophages. In addition to PGE$_2$, products of COX2 include PGD$_2$, PGF$_2\alpha$, prostacyclin, and thromboxane. It might be speculated that modulation of these other products is a factor in propagating an inflammatory response. Moreover 12-LO, which generates proinflammatory lipids (*i.e.* 12-*S*-hydroxyeicosatetraenoic acid) and is not detected in healthy islets but is in both T1D and T2D islets (42), is induced to a much higher level in WT macrophages than in iPLA$_2\beta$-deficient macrophages. The importance of 12-LO in promoting macrophage recruitment and activation and causing detrimental effects on islet function and β-cell mass is supported by the reports that deletion of 12-LO protects against T1D development (44, 45). Our findings, therefore, suggest that iPLA$_2\beta$ activation, in addition to skewing macrophage polarization toward M1, may also preserve functionality of downstream lipid-metabolizing enzymes. The net effect may, therefore, rely on preferential or selective generation of one lipid species over another. This also identifies a limitation in the studies performed with exposure of macrophages to individual lipids. It is very likely that the net impact on macrophage polarization may arise from combinations of different lipid species.

Equally important to consider are a separate class of lipids, collectively designated "specialized pro-resolving mediators," that facilitate recovery and repair from inflammatory episodes (72). Such lipids arise from oxidation of arachidonic acid by 15-LO or 5-LO (lipoxins), eicosapentaenoic acid (E series resolvins), or docosahexaenoic acid (D series resolvins, protectins, or marisins). In view of this, it is plausible to speculate that the net impact of lipid signaling on macrophage polarization is dependent on the pathway triggered and lipid species generated under activating conditions.

Furthermore, the hydrolyzed *sn*-2 fatty acid may arise from the actions of other PLA$_2$s (with different substrate preferences), and this may be the cause for the greater changes we observed in some marker abundances with indomethacin than *S*-BEL. For instance, cPLA$_2$, which has a preference for arachidonic acid-containing phospholipids is similarly induced in WT and iPLA$_2\beta^{-/-}$ macrophages. cPLA$_2$ co-localizes with COX1 (73), which is constitutively expressed and manifests a homeostatic protective role (71). Macrophages also express membrane-associated iPLA$_2\gamma$, which manifest similar activity as iPLA$_2\beta$ (74). However, its expression is not altered in the iPLA$_2\beta^{-/-}$ macrophages nor is it induced under classical or alternative activation in either WT or iPLA$_2\beta^{-/-}$ macrophages. In view of the observed predominance of M2 macro-

phage phenotype associated with the iPLA$_2\beta^{-/-}$ genotype, we propose that the predominant PLA$_2$ with impact on macrophage polarization is iPLA$_2\beta$.

We also recognize that the current study was performed in a non-diseased model; nevertheless, as it was in the absence of iPLA$_2\beta$ specifically, the findings reveal the potentially important participation of iPLA$_2\beta$-derived lipid signals in deciding the fate of macrophage polarization. We previously demonstrated that selective inhibition of iPLA$_2\beta$ in an autoimmune model of spontaneous diabetes ameliorates T1D, in association with reduced immune responses (29). The findings in the present study, therefore, provide strong motivation to examine the impact of selective inhibition of iPLA$_2\beta$ on polarization of macrophages toward M2 phenotype in a model that is prone to developing diabetes.

Cytokines induce NOX in a 12-LO product-dependent manner to increase generation of ROS (75). Here we find that under both naïve and classical activation conditions, NOX4 is decreased in iPLA$_2\beta^{-/-}$ relative to WT macrophages. This is consistent with a recent report demonstrating that NOX4 activity and subsequent macrophage chemotaxis were regulated by iPLA$_2\beta$ activity (36). Intriguingly, evidences of iPLA$_2\beta$ induction by ROS (76, 77) and iPLA$_2\beta$-mediated ROS generation (36) suggest a feedback link between the two that could lead to amplified inflammatory responses. Furthermore, ROS induce the chemoattractant *CCL2* (MCP-1) (36), and chemotaxis in response to MCP-1 requires iPLA$_2\beta$ activation (26). Consistent with a link between ROS and iPLA$_2\beta$ is our finding that, relative to diabetes-resistant mouse strains, an increased expression of iPLA$_2\beta$ mRNA is evident in islets from diabetes-prone NOD female mice, an autoimmune model of T1D, but not in islets from mice deficient in NOX-derived superoxide (NOD.*Ncf1*^{m1J}). These mice exhibit a marked delay in T1D development (16).

In summary, we report for the first time an impact of iPLA$_2\beta$ on macrophage polarization, where iPLA$_2\beta$ activation favors an M1 proinflammatory macrophage phenotype, and decreased activation favors an anti-inflammatory M2 phenotype. We acknowledge that not all markers of M1 and M2 macrophages were impacted by the absence of iPLA$_2\beta$, and this may be related to the stimuli used that are likely to differ from those present under *in vivo* inflammatory conditions. Nevertheless, the collection of marker changes observed in our study strongly suggests that iPLA$_2\beta$ activation participates in macrophage polarization away from the M2 phenotype. We further demonstrate that iPLA$_2\beta$ activation can modulate expression of downstream enzymes that generate proinflammatory lipid and ROS signals. As central regulators of diverse cellular functions, there is growing interest in therapeutic targeting of PLA$_2$s in the management of multiple inflammation-associated disorders (*i.e.* atherosclerosis, neurodegenerative, autoimmune, cancers) (48, 49). In this context, mitigating iPLA$_2\beta$ expression/activity may hold significant promise in countering the onset and/or progression of inflammatory-based diseases.

Experimental Procedures

Animals—Breeders (C57BL/6 background) obtained from Dr. John Turk (Washington University School of Medicine, St. Louis, MO) were used to generate WT and iPLA$_2\beta^{-/-}$ mice at the University of Alabama at Birmingham. The NOD mice were all generated as described (16, 29). Before experimentation, the mice were genotyped as described (30, 78). Animal experiments were conducted according to approved Institution Animal Care and Use Committee (IACUC) guidelines at the University of Alabama at Birmingham.

Isolation and Culture of Peritoneal Macrophages—Mice (6–16 weeks of age) were sacrificed by CO_2 inhalation and cervical dislocation. Peritoneal macrophages were obtained by filling the peritoneal cavity with cold 5-ml PBS containing 2% FBS, massaging gently, and withdrawing the cell-containing solution. Cells were pelleted at 300 × *g* for 5 min and resuspended in growth medium (Eagle's minimum essential medium (Sigma, M0894), 2.0 mg/ml sodium bicarbonate (Fisher, BP328-500], 2 mM L-glutamine (Life Technologies, 25030-081), 100 units/ml penicillin-100 μg/ml streptomycin (Life Technologies, 15140-122), and 10% heat-inactivated fetal bovine serum (Life Technologies, 16000044)) supplemented with 10% L929 cell-conditioned medium (source of M-CSF). Macrophages from a single collection were sufficient to seed six 60-mm non-treated culture dishes. Adherent macrophages appeared after 16 h of culture. All experiments were performed with expanded freshly isolated peritoneal macrophages under classical and alternative activation conditions as described below in the absence or presence of inhibitors of 12-LO, COX, or iPLA$_2\beta$, 1 μM CDC (ENZO Life Sciences, BML-EI211-0010), 50 μM 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid (indomethacin, Sigma, I7378), or 1 μM *S*-BEL (Cayman Chemical, 10006801), respectively, for 30 min. In certain experiments, macrophage media was supplemented with individual lipid species. These (from Cayman) included 1 μM 12(*S*)-hydroxyleicosatetraenoic acid (34570), 1 μM 5(*S*)- hydroxyleicosatetraenoic acid (34230), 1 μM 11,12-epoxyeicosatrienoic acid (50511), 1 μM 14,15-epoxyeicosatrienoic acid (59651), 0.5 μM arachidonic acid (9001886), 1 μM prostaglandin E_2 (14010), 10 μM lysophosphatidylcholine (10172), or 1 μM lysophosphatidic acid (857130).

Macrophage Activation—This was accomplished according to previously published methods (70). For classical activation, macrophages were treated with 15 ng/ml recombinant IFNγ, R&D Systems, 485-MI-100) for 8 h in growth medium followed by the addition of 10 ng/ml ultrapure LPS (InvivoGen, tlrl-3pelps) and incubated for 16 h at 37 °C. For alternative activation, macrophages were treated with 8 ng/ml recombinant IL-4 (R&D Systems, 404-ML-010) in growth medium for 16 h. Naïve macrophages, which received no activation stimuli, were maintained in growth medium with no additional treatment.

Macrophage mRNA Target Analyses—Macrophages cultured in 60-mm non-tissue culture-treated dishes were lysed in 1 ml of TRIzol (Life Technologies, 15596-026). Total RNA was prepared and purified using RNeasy mini kits (Qiagen, 74104), and 1 μg RNA was converted to cDNA using the Superscript III first strand synthesis system (Life Technologies, 18080-051) according to manufacturer's instructions. The cDNA was diluted 10-fold and used as a template in conventional or RT-qPCR. cDNA transcripts were amplified with primers (Table 1),

TABLE 1
Primers listing for targets analyzed by RT-qPCR
F, forward; R, reverse.

Name	Sequence (5′ to 3′)	T_m (salt)	Target	Product size
				bp
msRETNLA_qRT.F	CAGCTGATGGTCCCAGTGAAT	60	Resistin-likeα (Fizz1)	212
msRETNLA_qRT.R	TCCCAAGATCCACAGGCAAA	59		
msARG1_qRT.F	AGCACTGAGGAAAGCTGGTC	60	Arginase, liver (Arg1)	131
msARG1_qRT.R	CAGACCGTGGGTTCTTCACA	60		
msCHIL3_qRT.F	AAGCTCTCCAGAAGCAATCCT	59	Chitinase-like3 (YM1)	187
msCHIL3_qRT.R	GAGTACACAGGCAGGGGTCA	61		
msSTAT6_qRT.F	AGTTTTTAGGGCCAGCCCAG	60	Signal transducer and activator of transcription6 (STAT6)	265
msSTAT6_qRT.R	AAGCATCTGAACCGACCAGG	60		
msMRC1_qRT.F	GTCAGAACAGACTGCGTGGA	60	Macrophage mannose receptor 1 C-type precursor (Mrc1)	281
msMRC1_qRT.R	AGGGATCGCCTGTTTTCCAG	60		
msCCL5_qRT.F	GTGCCCACGTCAAGGAGTAT	60	C-C motif chemokine 5 precursor (CCL5)	103
msCCL5_qRT.R	TTCTCTGGGTTGGCACACAC	60		
msTNF_qRT.F	GGTGCCTATGTCTCAGCCTC	60	Tumor necrosis factor (TNF)	177
msTNF_qRT.R	GCTCCTCCACTTGGTGGTTT	60		
msSTAT1_qRT.F	CCTGCGTGCAGTGATCGTTT	62	Signal transducer and activator of transcription1 (STAT1)	285
msSTAT1_qRT.R	TGGGCCAGGTACTGTCTGAT	60		
msPtgs2_qRT.F3	TGAGTGGGGTGATGAGCAAC	60	Cyclooxygenase 2	178
msPtgs2_qRT.R3	TTCAGAGGCAATGCGGTTCT	60		
msNfkb1_qRT.F	GGTCACCCATGGCACCATAA	60	NF-κB p105 (p50 precursor)	231
msNfkb1_qRT.R	AGCTGCAGAGCCTTCTCAAG	60		
msRela_qRT.F	GAACCTGGGGATCCAGTGTG	60	NF-κB p65	266
msRela_qRT.R	AGTTCCGGTTTACTCGGCAG	60		
CCL2_qRT.F	CTGGAGCATCCACGTGTTGG	61	MCP-1	198
CCL2_qRT.R	CATTCCTTCTTGGGGTCAGC	59		
ALOX12_qRT.F	GGCTATCCAGATTCAGCCCC	60	12-Lipoxygenase	271
ALOX12_qRT.R	CCGGCTTCGCGTGTTAATTT	60		
CYBB_qRT.F	TTCTTCATCGGCCTTGCCAT	60	NADPH oxidase2 (NOX2)	226
CYBB_qRT.R	GCCAAAACCGAACCAACCTC	60		
NOX4_qRT.F	CATTCACCAAATGTTGGGCCT	60	NADPH oxidase4 (NOX4)	229
NOX4_qRT.R	GGCTACATGCACACCTGAGA	60		
CD68_qRT.F	GGGGCTCTTGGGAACTACAC	60	CD68	167
CD68_qRT.R	GTACCGTCACAACCTCCCTG	60		
msINOS_qRT.F	CAGGTCTTTGACGCTCGGAA	60	iNOS	167
msINOS_qRT.R	GCCTGAAGTCATGTTTGCCG	60		
msARG2_qRT.F	GCAAATTCCTTGCGTCCTGA	60	Arginase2 (Arg2)	254
msARG2_qRT.R	AGGCCCACTGAACGAGGATA	60		
msCXCL10_qRT.F2	ATGACGGGCCAGTGAGAATG	60	Chemokine (C-X-C motif) ligand 10 (Cxcl10)	249
msCXCL10_qRT.R2	GAGGCTCTCTGCTGTCCATC	60		
EMR1_qRT.F2	CTCTTCTGGGGCTTCAGTGG	60	F4/80	273
EMR1_qRT.R2	GCAGACTGAGTTAGGACCACA	60		
PLA2G4A_qRT.F	ACGTGCCACCAAAGTAACCA	60	cPLA$_2\alpha$	97
PLA2G4A_qRT.R	CCTGCTGTCAGGGGTTGTAG	60		
PLA2G6_qRT.F	GGCAGAAGTGGACACCCCAA	60	iPLA$_2\beta$	130
PLA2G6_qRT.R	CATGGAGCTCAGGATGAACGC	60		
PNPLA8_qRT.F	AATGAGTTGGAGCCATGCGT	60	iPLA$_2\gamma$	115
PNPLA8_qRT.R	TACCTTAGGACATGCGGGGT	60		

designed using NCBI Primer-BLAST (www.ncbi.nlm.nih.gov). RT-qPCR was carried out using SYBR Select Mastermix (Life Technologies, 4472908) according to the manufacturer's instructions. Relative gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method.

ELISA and Nitrite Assays—After macrophage exposures to the various treatments, media concentrations of M1- and M2-related proteins were assessed by ELISA and of nitrite by Griess assay, according to manufacturer's instructions. ELISA kits (R&D Systems) included those for ancillary ELISA reagent (DY008), IL-12 (DY419), TNFα (DY410), IL-10 (DY417), TGFβ (DY1679), and IL-1β (DY401). Nitrite quantitation was performed using a Griess reagent kit (ThermoFisher, G7921).

NOD Islet iPLA$_2\beta$ RT-qPCR Analyses—Islets were isolated from female spontaneous diabetes-prone NOD, immune-deficient and diabetes-resistant NOD.*Rag*, and NOX-derived superoxide-deficient NOD (NOD.*Ncf1*^{m1j}) mice, as described (30). Total RNA was isolated and cDNA was prepared for iPLA$_2\beta$ RT-qPCR analyses as described (29).

Mass Spectrometric Determination of LPA Molecular Species—Isolated mouse peritoneal macrophages were homogenized in a mixture of $CHCl_3$ (1 ml), 17:0 LPA (internal standard, 200 ng, Avanti, 857127), and 0.1 M HCl (0.5 ml). The homogenate was sonicated on ice (20% power, 5-s bursts for 60 s; Vibra Cell probe sonicator; Sonics and Materials, Danbury, CT). $CHCl_3$ (1 ml) and 0.1 M HCl were then added, and vortex-mixing and centrifugation (2800 × *g*, 5 min) were performed. The organic phase was removed, concentrated to dryness under N_2, and reconstituted in 80% CH_3OH (100 μl). An aliquot (10 μl) was analyzed by liquid chromatography-tandem tandem mass spectrometry (LC-MS/MS) on a Surveyor HPLC (ThermoElectron) using a modified gradient (79) on a C_8 column (15 cm × 2.1 mm; Sigma) interfaced with the ion source of a ThermoElectron Vantage triple quadruple mass spectrometer with extended mass range operated in negative ion mode as described (80). Multiple reaction monitoring transitions were monitored for $[M\text{-}H]^-$ ions of the most abundant LPA species in mouse macrophage lipid extracts: 409.0/153.0 (16:0-LPA),

423.0/153.0 (17:0-LPA internal standard), 433.0/153.0 (18:2-LPA), 435.0/153.0 (18:1-LPA), 437.0/153.0 (18:0-LPA), 457.0/153.0 (20:4 LPA), and 481.1/153.0 (22:6 LPA).

Statistical Analysis—Data are presented as the means ± S.E. Statistical significances between groups were determined using Student's *t* test. Values for $p < 0.05$ for RT-qPCR (2-tailed) and ELISA/Griess (1-tailed) were considered significant.

Author Contributions—J. W. A. conceived and coordinated the study, performed experiments, analyzed data, and wrote the paper. W. D. H., A. J. N., and R. N. B. performed experiments, analyzed data, and edited the paper. J. T. and M. W. generated the iPLA$_2\beta^{-/-}$ mice and provided breeding mates. J. T. provided MS data and edited the paper. H. M. T. provided expertise on marker assessment and edited the paper. S. R. conceived and coordinated the study, analyzed data, contributed to preparation of the figures, and edited the paper.

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