Lipid mediators elicit the activation of *IL23A* transcription via ATF2 phosphorylation

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ABSTRACT

The cytokines IL-12 p70 and IL-23 released by dendritic cells (DC) polarize the immune response into the Th1 and Th17 types, respectively. These cytokines share a common chain, p40 (gene *IL12B*) and differ in a specific subunit, p35 (gene *IL12A*) for IL-12 p70, and p19 (gene *IL23A*) for IL-23. The balance IL-12 p70/IL-23 is relevant to the development of fungal infection (1) and autoimmunity (2). As regards *IL12A*, a mechanism of transcriptional repression has been reported via the NAD^+-dependent deacetylation of histones by SIRT1 (3, 4). Likewise, the transcription of *IL23A* is regulated by c-Rel and ATF2, which in turn depends on the complementary phosphorylation of its Thr-71 and Thr-69 by PKC and MAPKs (5).

To disclose the mechanism of activation of *IL23A* transcription by fungal patterns; biochemical, pharmacological, and analytical approaches were conducted. The fungal mimic zymosan induced a massive release of arachidonic acid (AA), and the production of PAF (1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), leukotriene B_4, and cysteinyl-leukotrienes. The combined antagonism of the receptors CysLT1, BLT1, and PAF-R inhibited the binding of P-Thr71-ATF2 to the *IL23A* promoter and *IL23A* transcription in response to zymosan. Unlike zymosan, the combination of agonists of those receptors increased the binding of P-Thr71-ATF2 to the *IL23A* promoter and *IL23A* transcription in response to LPS, thus suggesting that LPS elicits the production of those mediators at a concentration below the threshold necessary for an optimal response. These data indicate that fungal pattern receptors induce a rapid activation of the cytosolic phospholipase A_2, which generates a massive release of AA, available for the biosynthesis of the eicosanoids and lyso-PAF (1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine) for the biosynthesis of PAF, together with acetyl-CoA generated by stimulated DC. The engagement of G-protein coupled receptors by their cognate ligands gathers an array of autocrine signals that favor activation of *IL23A* transcription through the phosphorylation of ATF2.

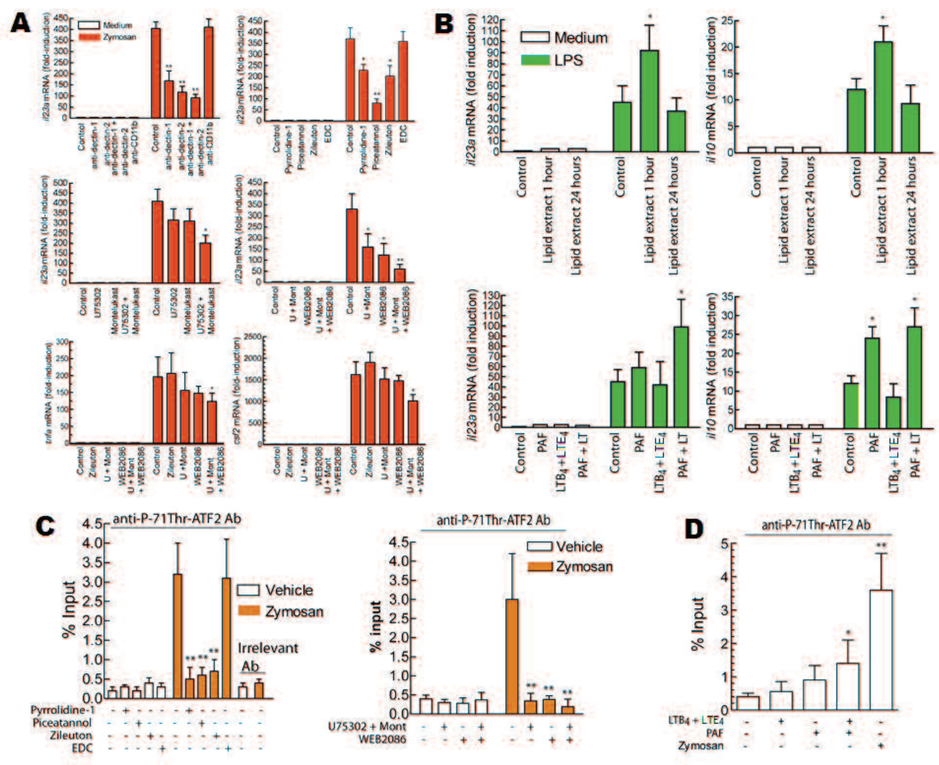

FIG 1. Response of inhibitors and lipid mediators on the *IL23A* transcription and binding of P-Thr71-ATF2 to the ATF2 site of the *IL23A* promoter. (**A**) DC were incubated for 30 min prior to the addition of 1 mg/ml zymosan with 10 µg/ml of either anti-dectin-1 or anti-dectin-2 Ab, and combination thereof. Anti-CD11b Ab was used as a control for specificity. Four hours after the addition of zymosan, the RNA was extracted and used for the assay of *IL23A* mRNA. Similar experiments were carried out in the presence of the BLT1 antagonist U75302 1 µM, the CysLT1 antagonist montelukast 10 µM, the 5-LO inhibitor zileuton 10 µM, the 12/15-LO inhibitor EDC 3 µM, the phospholipase A_2 inhibitor pyrrolidine-1 2 µM, the SYK kinase inhibitor piceatannol 25 µM, and the PAF receptor antagonist WEB2086 30 µM. The effect of different treatments was also assayed on the transcription of *TNFA* and *CSF2* to show specificity. (**B**) DC were stimulated in the presence of 1 mg/ml zymosan for 1 or 24 h. At the end of these times, the supernatants were collected and their lipid fractions extracted in C-18 reversed-phase cartridges. The lipid extract was evaporated to dryness, resuspended in culture medium and used to address its effect on the response elicited by LPS. After 4 h of incubation in the presence of 10 µg/ml LPS, the RNA was extracted and used for the assay of the mRNA of the cytokines. The effect of the addition of exogenous lipid mediators on the transcription of *IL23A* and *IL10* was also assayed in DC stimulated for 4 h in the presence of LPS. The concentrations of the lipid mediators are 0.1 µM LTB_4, 0.5 µM LTE_4, and 1 µM PAF. (**C**) DC were incubated with the indicated additions at the concentrations showed in A for 30 min, stimulated with 1 mg/ml zymosan for 1 h, and then collected for ChIP assays. Pharmacological modulation of P-Thr71-ATF2 binding to the *IL23A* promoter mirrors the changes on *IL23A* transcription. Anti-P-71Thr-ATF2 and irrelevant control Ab were used at a concentration of 10 µg/ml. (**D**) Effect of 0.1 µM LTB_4 and 0.5 µM LTE_4, 1 µM PAF, and combination thereof. Results show mean ± S.E.M. of three to six experiments. Mont indicates montelukast. *p < 0.05. **p < 0.01.

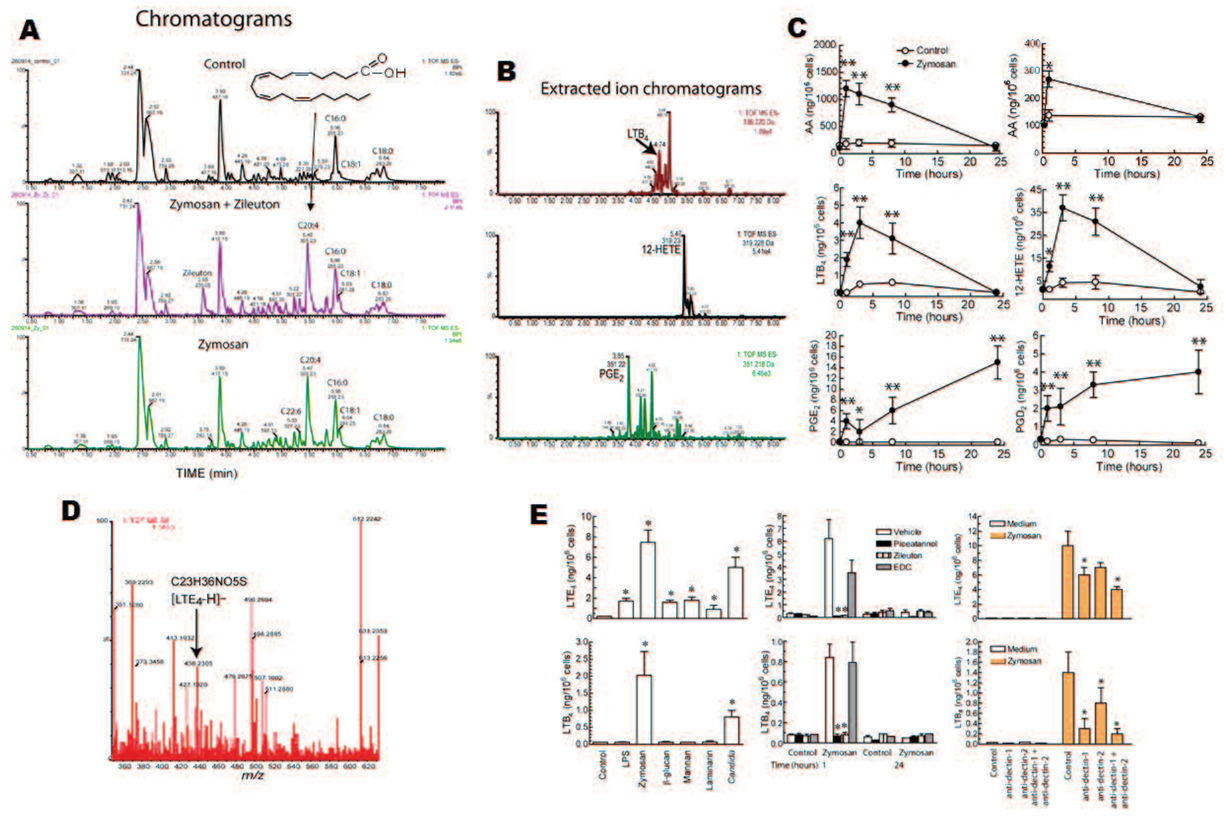

FIG 2. Analysis of DC supernatants by MS and effect of pharmacological treatments. (**A**) The culture medium of resting DC and DC stimulated for 1 hour with zymosan in the absence and presence of zileuton was extracted in C-18 reversed-phase cartridges and used for UPLC/MS assays. C16:0, C18:0, C20:4, C18:1, and C22:6 refer to palmitic acid, stearic acid, arachidonic acid, oleic acid, and docosahexaenoic acid, respectively. C20:4 shows the highest increase upon activation. Unlike C16:0 and C18:0, C22:6 and C18:1 also increased, consistent with the release of insaturated fatty acids at the *sn*-2 position of phospholipids (**B**) Extracted ion chromatograms of the eicosanoids released by DC. (**C**) DC were incubated for the times indicated in the presence and absence of 1 mg/ml zymosan, and the supernatants collected for lipid extraction. At late times only PGE_2 and PGD_2 are increase, AA is reacylated and LTs are degraded. Results show mean ± S.E.M. of 4 independent experiments. *p < 0.05. **p < 0.01. (**D**) Mass spectra of LTE_4. (**E**) DC were incubated with 10 µg/ml LPS, 1 mg/ml of the different zymosan components, and with *Candida* at a ratio of 50 yeasts per DC. The supernatants were collected at the times indicated for the ELISA assay of LT. The effect of compounds acting on SYK, 5-LO and 12/15-LO, and blocking Ab acting on dectin-1 and dectin-2 was assessed by incubating the DC in the presence of the indicated additions for 30 min prior to zymosan addition. Concentrations were as described in the legend to Fig. 1A.

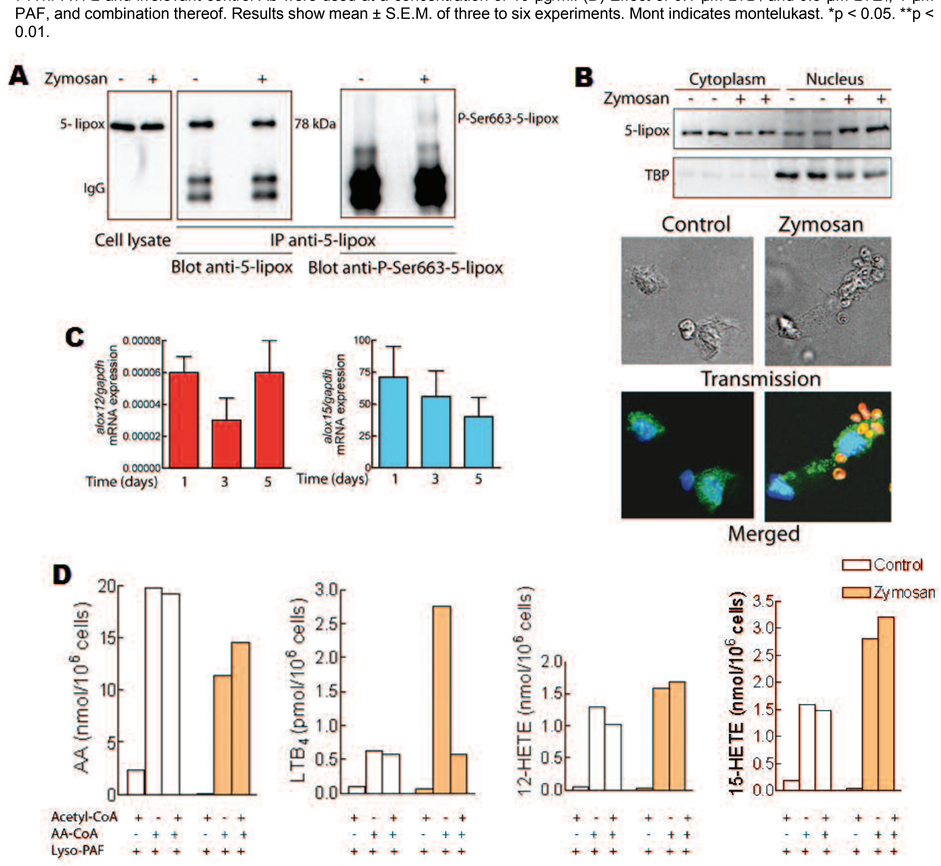

FIG 3. 5-Lipoxygenase phosphorylation and nuclear translocation, *ALOX12* and *ALOX15* expression, and eicosanoid assay on microsomal fractions. (**A**) Expression of 5-LO in cell lysates and phosphorylation in Ser663 in an immunoprecipitation assay after stimulation with zymosan for 15 min. (**B**) Translocation of 5-LO into the nucleus in DC stimulated for 1 h with zymosan. TBP indicates TATA box-binding protein. Immunofluorescence confocal microscopy of 5-LO (green staining) in control DC and in DC stimulated with zymosan particles (red staining). DAPI was used to stain the nuclei (blue staining). (**C**) Monocytes were cultured in the presence of GM-CSF and IL-4 for the indicated days and used for the assay of the *ALOX12* ("platelet type") and *ALOX15* ("leukocyte type") expression. DC only expresses *ALOX15*. Results represent mean ± S.E.M. of 3 experiments. (**D**) Unesterified AA, LTB_4, 12-HETE, and 15-HETE were assayed by UPLC/MS in microsomal fractions supplemented with 20 µM 1-hexadecyl-2-lyso-glycero-3-phosphocholine (lyso-PAF) and 100 µM acetyl-CoA or 100 µM AA-CoA and combination thereof as indicated. The lipid extract was used for the UPLC/MS assay. Results disclose the use of AA-CoA by 5-lipoxygenase and 12/15-lipoxygenase. IP indicates immunoprecipitation. WB indicates Western blot. Results show mean ± S.E.M. of 3 independent experiments. *p < 0.05. **p < 0.01.

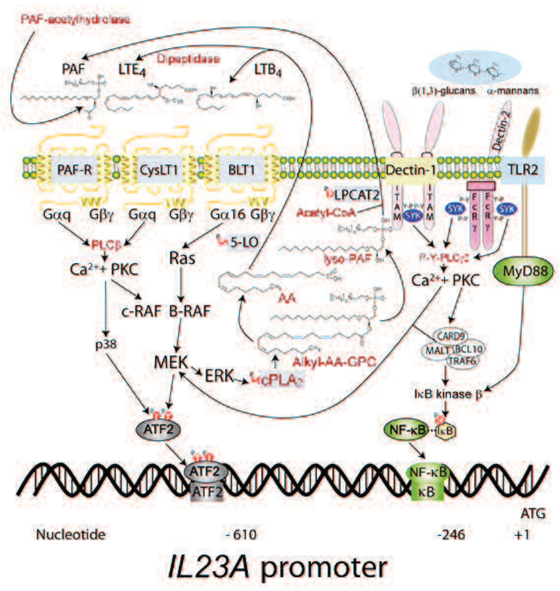

FIG 5. Lipid mediators and ITAM-signaling cooperate to *trans*-activate the *IL23A* promoter. Binding of fungal patterns activates the proteins of the NF-κB family and a phosphorylation cascade of $cPLA_2$ at Ser505, 5-LO at Ser663, and LPCAT2 at Ser34 after phospholipase Cγ activation. P-Ser505-$cPLA_2$ hydrolyses 1-alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (Alkyl-AA-GPC) and generates both free AA and 1-alkyl-2-lyso-glycero-3-phosphocholine (lyso-PAF) that by the action of 5-LO and LPCAT2 increase LTB_4, cysteinyl-LT, and PAF. The preferential detection of LTE_4 and the increased concentrations of PAF upon darapladib treatment can be explained by the presence of dipeptidase and PAF-acetylhydrolase activities. The lipid mediators activate the phosphorylation of ATF2 (5) by phospholipase Cβ signaling through their cognate receptors. The combined action of NF-κB and ATF2 on the *IL23A* promoter allows its transactivation. FcRγ, Fc receptor γ-chain; ITAM, immunoreceptor tyrosine-based activation motif; PLCβ, phospholipase Cβ; PLCγ2, phospholipase Cγ2; P, phosphate; P-Y, phosphotyrosine; SYK, spleen tyrosine kinase; TLR2, Toll-like receptor 2.

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FIG 4. PAF biosynthesis, *LPCAT* and *PTAFR* expression, and acetyl-CoA assay (**A**) DC were incubated in the presence of 0.1 µM of the PAF-acetylhydrolase inhibitor darapladib for 30 min and in the presence or absence of zymosan for one hour. At the end of this time, deuterated standards were added to the supernatants. The extracted ion chromatogram (EIC) of PAF C16:0 is shown using different scales. In the absence of zymosan, the EIC shows a 110.01 *m/z* compound and in zymosan-conditioned medium PAF C16:0 is detected at a high concentration (104.7 ± 10 pmol/10^6 DC) as compared to controls (13.3 ± 7 pmol/10^6 DC). In the mass spectrum corresponding to zymosan-treated DC, the peak of endogenous PAF C16:0 were detected at higher level than the deuterated standard. Results are representative of 3 independent experiments. (**B**) DC were incubated in the presence and absence of zymosan for the times indicated and then used for the assay of the expression of the different *LPCAT* enzymes. DC express all enzymes to similar extents. LPCAT2 acetylates lyso-PAF and decreases over time whereas LPCAT1 increases over time. At early times, PAF may be formed, whereas at later times, reacylation with long chain fatty acids is most likely. (**C**) The transcription of *PTAFR* was assayed by conventional RT-PCR with two forward primers and a reverse primer to address the expression of the different transcript variants. The PAF receptor is highly expressed in DC and show the same feed-back inhibition than LPCAT2 in stimulated DC. (**D**) Acetyl-CoA was assayed in DC lysates at different times after addition of zymosan. Anaerobic glycolysis is enhanced during phagocytosis and allows the production of acetyl-CoA in the cytoplasm from cataplerotic citrate. (**E**) LPCAT activity was assayed in microsomes of both resting and zymosan-treated DC supplemented with lyso-PAF, acetyl-CoA, and AA-CoA at the concentrations indicated in the legend to Fig. 3D. Results are consistent with the activation of LPCAT2 and the preferential acylation of lyso-PAF with acetyl-CoA in zymosan treated cells. *p < 0.05.

CONCLUSIONS

1. Dectin receptors trigger a parallel release of arachidonic acid and lyso-PAF by $cPLA_2\alpha$. It leads to a partial conversion of arachidonic acid into the products of oxidative metabolism by 5 and 12/15-lipoxygenase.
2. Acetylation of lyso-PAF by LPCAT2 produces the phospholipid mediator PAF and consumes acetyl-CoA, the disposal of which is increased by metabolic reprogramming and citrate cataplerosis.
3. The combined action of LTB_4, cysteinyl-leukotrienes and PAF exerts an autocrine role on *IL23A* induction. Their G protein-coupled cognate receptors trigger a signaling cascade involving Ras and phospholipase Cβ/PKC, which co-opt the activating phosphorylations of ATF2.

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