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Research Article

Innate Immunity

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Proteinase-activated receptor 2 activation promotes an anti-inflammatory and alternatively activated phenotype in LPS-stimulated murine macrophages

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Abstract

Proteinase-activated receptor 2 (PAR_2), a 7-transmembrane G protein-coupled receptor, contributes to inflammation either positively or negatively in different experimental systems. Previously, we reported that concurrent activation of PAR_2 and TLRs in human lung and colonic epithelial cells resulted in a synergistic increase in NF-κB-mediated gene expression, but a down-regulation of IRF-3-mediated gene expression. In this study, the effect of PAR_2 activation on LPS-induced TLR4 signaling was examined in primary murine macrophages. The PAR_2 activation of wild-type macrophages enhanced LPS-induced expression of the anti-inflammatory cytokine, IL-10, while suppressing gene expression of pro-inflammatory cytokines, TNF-α, IL-6, and IL-12. Similar PAR_2-mediated effects on LPS-stimulated IL-10 and IL-12 mRNA were also observed *in vivo*. In contrast, $PAR_2^{-/-}$ macrophages exhibited diminished LPS-induced IL-10 mRNA and protein expression and downstream STAT3 activation, but increased KC mRNA and protein. PAR_2 activation also enhanced both rIL-4- and LPS-induced secretion of IL-4 and IL-13, and mRNA expression of alternatively activated macrophage (AA-Mϕ) markers, e.g. arginase-1, mannose receptor, Ym-1. Thus, in the context of a potent inflammatory stimulus like LPS, PAR_2 activation acts to re-establish tissue homeostasis by dampening the production of inflammatory mediators and causing the differentiation of macrophages that may contribute to the development of a Th2 response.

Keywords

Proteinase-activated receptor 2, Toll-like receptor 4, macrophage, anti-inflammatory, alternative activation

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Introduction

Pattern-recognition receptors (PRRs) of the innate immune system detect infection by recognizing pathogen-associated molecular patterns (PAMPs), evolutionarily conserved structural motifs that are shared among microbes, *e.g.* LPS, lipopeptides, flagellin, and microbial nucleic acids.[1,2] Pattern-recognition receptors also sense tissue damage by responding to endogenous, host-derived danger-associated molecular patterns (DAMPs).[3] Classical PRRs, *e.g.* TLRs, provide surveillance by recognizing various PAMPs and DAMPs as ligands. In contrast, non-classical PRRs,[4] such as the protease activated receptors (PARs), respond to infection and tissue damage by sensing pathogen- or host-derived proteolytic enzymes, as well as certain allergens with intrinsic protease activity.[5,6] In macrophages, PRR activation primarily drives development of antimicrobial and pro-inflammatory responses that are typically associated with the 'classically activated' ('M1') differentiation phenotype that favors development of T helper cell type 1 (Th1)-skewed cytokine production, *e.g.* IL-12 and IFN-γ.[2,7] However, a temporally delayed, anti-inflammatory transcriptional program leading to expression of genes such as IL-10, IL-4,

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and IL-13 is also activated in macrophages by certain pathogens and promotes tissue homeostasis by counteracting induction of pro-inflammatory cytokines and by leading to the development of macrophages with an AA-Mϕ (also referred to as 'M2') differentiation phenotype that dampens the pro-inflammatory response.[8–11] Such macrophages produce a 'Th2-like' cytokine milieu and are often associated with tissue repair, wound healing, and responses to allergens and parasitic infections.

Toll-like receptors represent a large family of germline-encoded, single-transmembrane, classical PRRs. They are distributed ubiquitously in the body and are expressed on many innate immune cell types, including epithelial cells and macrophages.[2,12] Direct or indirect binding of TLR-activating ligands to the TLR N-terminal ectodomain induces TLR dimerization that brings the intracytoplasmic 'Toll/interleukin-1 receptor resistance' (TIR) domains into close proximity. This interaction facilitates the recruitment of TIR-domain-containing adapters (*e.g.* MyD88, TRIF), kinases, and other signaling molecules to the 'signaling platform' generated by the initiating TLR TIR dimer. Activation of the Gram-negative bacterial LPS transducing receptor, TLR4, results in nuclear translocation of NF-κB through the 'MyD88-dependent' signaling pathway and of interferon regulatory factor-3 (IRF-3) via the 'MyD88-independent' signaling pathway. These two key transcription factors are required for expression of many inflammatory and immunomodulatory chemokines and cytokines.[2,12]

Proteinase-activated receptor 2 (PAR_2) belongs to a family of four seven-transmembrane G protein-coupled receptors (7-TM GPCRs).[5,13] It is expressed highly in the respiratory and gastrointestinal (GI) tracts,[14,15] and, like the TLRs, is also expressed on epithelial cells and macrophages. PAR_2 mediates the cellular effects of trypsin and trypsin-like serine proteases, including mast cell tryptase, coagulation factors VIIa and Xa, and several pathogen-derived proteinases. The PAR-activating enzymes cleave each PAR irreversibly at a specific site in the extracellular N-terminus to expose a tethered neo-ligand that binds intramolecularly to the second extracellular loop (ECL2) of the GPCR to trigger receptor activation. Synthetic PAR agonist peptides (AP) that bear the hexapeptide sequences of the tethered neo-ligands of PAR_1, PAR_2, and PAR_4 mediate signaling non-enzymatically by binding directly to the ECL2 of their respective native, uncleaved PAR. We have reported that the human PAR_2 AP, SLIGKV-NH_2, induces both NF-κB and IRF-3 reporter activities in PAR_2-expressing HEK293T cells, but not in pcDNA3.1 empty vector-transfected cells, and that PAR_2 co-immunoprecipitated with TLR4 in the presence of AP, suggesting that these two receptors may physically associate in response to PAR_2 activation.[16] In addition, the PAR_2 AP, SLIGKV-NH_2, but not a scrambled control peptide, induced footpad edema in wild-type C57BL/6J mice, but not in $PAR_2^{-/-}$ or $TLR4^{-/-}$ mice.[4] These findings indicate that the PAR_2 AP specifically activates its target receptor and that TLR4 is required for this PAR_2-mediated inflammatory response. Activation of PAR_2 in 'sentinel' cells of the innate immune system, *e.g.* epithelial cells, endothelial cells, and monocytes, induces antimicrobial and inflammatory responses.[16–20]

Previous studies indicate that PAR_2 is involved in pro-inflammatory and allergic responses at anatomical sites that interact with protease-rich environments, *e.g.* inflamed tissues, the gut lumen, and the respiratory tract.[21–23] In addition, PAR_2 signaling intersects with TLRs to augment pro-inflammatory responses in epithelial cells, endothelial cells, and monocytes.[4,16,24–26] However, cytoprotective and anti-inflammatory functions have also been ascribed to PAR_2 signaling. For example, PAR_2 activation exerts powerful bronchoprotection in the airway and also protects the gastric mucosa against the injurious effects of non-steroidal anti-inflammatory drugs and acid or ethanol solutions.[27,28] *In vivo*, PAR_2 AP treatment inhibits Th1 cytokine production and protects mice from 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis and lethality.[29] Intranasal PAR_2 AP administration inhibits LPS-induced pulmonary neutrophil influx.[30] In this study, we hypothesized that the cytoprotective, anti-inflammatory outcomes observed *in vivo* for PAR_2 may depend on PAR_2-mediated activation of macrophages, as members of several other 7-TM GPCR families, *e.g.* β-adrenergic and adenosine A2a receptors, have been shown to dampen inflammation induced by LPS via TLR4 in myeloid cells.[7,31–33] In fact, activation of these GPCRs in macrophages has been shown to augment TLR4-induced expression of the anti-inflammatory cytokine, IL-10, while down-regulating the expression of various pro-inflammatory mediators, *e.g.* IL-1β, IL-6, TNF-α, and IL-12.[7,31–33] This study sought to define the capacity of PAR_2 to modulate TLR4 signaling in murine macrophages.

Materials and methods

Reagents, mice, and tissue culture

Human PAR_2 AP, SLIGKV-NH_2, and an inactive, control reverse peptide (RP), VKGILS-NH_2, were synthesized (>96% purity) by Phoenix Pharmaceuticals (Belmont, CA, USA). PAR_2 fAP (2-furoyl-LIGRLO-NH_2; >98% purity), a stabilized PAR_2 agonist, was purchased from Calbiochem (San Diego, CA, USA). Protein-free, phenol/water-extracted LPS from *Escherichia coli* K235 was purified as described previously.[34] Murine recombinant IL-4 (rIL-4) was purchased from R&D Systems (Minneapolis, MN, USA).

Wild-type C57BL/6J mice and $PAR_2^{-/-}$ mice (backcrossed onto a C57BL/6 background [N5]) were

obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were used between 6–10 wk of age. All experiments were conducted with institutional approval. *In vivo* treatments with PAR_2 AP and LPS were carried out as indicated in the figure captions. Primary murine thioglycollate-elicited peritoneal macrophages or bone marrow-derived macrophages, obtained by expanding bone marrow cells in rCSF-1 (10 ng/ml; R&D Systems), were prepared for *in vitro* studies as described previously.[35,36] Human embryonic kidney (HEK) 293T cells were transfected with PAR_2 expression vector, together with NF-κB-luciferase and β-galactosidase reporter constructs, and then stimulated with medium or the indicated peptides. Transfection conditions and measurement of reporter activities were carried out as previously described.[16]

Quantitative real-time PCR (qPCR)

Total RNA from tissue cultures or organs was extracted, and oligo(dT)-primed cDNA was synthesized as previously described.[35] The qPCR primers were designed and synthesized as previously described.[9,35] qPCR was carried out on ABI Prism® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as previously described.[35]

Flow cytometry analysis

C57BL/6J and $PAR_2^{-/-}$ thioglycollate-elicited macrophages were treated with medium, harvested by gentle scraping, fixed with 4% *p*-formaldehyde (PFA) and washed 3 times with PBS. Cells were blocked and permeabilized for 30 min with PBST (PBS, 1% BSA, 1% normal donkey serum, 0.3% Triton X-100) at room temperature. F4/80 and CD11b were detected using monoclonal antibodies directed against the proteins, followed by Cy2-conjugated donkey anti-mouse IgG. Cells were washed in PBST and suspended in PBS for immediate analysis using a FACSCalibur. Analytic gates were set to exclude cellular debris and aggregates. CELLQuest software (Becton Dickinson) was used to analyze the data.

Western analysis

At the indicated times, whole-cell lysates were prepared and resolved by gel electrophoresis, transferred to polyvinylidene difluoride membranes, probed with antibodies, and target protein bands detected by enhanced chemoluminescence as described.[35] Primary and horseradish peroxidase-conjugated secondary antibodies used in this study were purchased from Cell Signaling Technology (Danvers, MA, USA) and used at 1 : 1000 and 1 : 2000 dilutions, respectively. Densitometric analysis of Western blots was carried out using NIH software, Image J (<http://rsbweb.nih.gov/nih-image/>).

Analysis of secreted proteins by ELISA

The concentrations of secreted proteins were determined by enzyme-linked immunosorbent assay (ELISA) using kits from R&D Systems or through the Cytokine Core Laboratory (UMB).

Statistical analysis

Using GraphPad PRISM v4.0 (GraphPad Software, San Diego, CA, USA), one-way analysis of variance (ANOVA) with Tukey's post-test or two-way ANOVA with Bonferroni post-test was performed to assess statistical significance (P values < 0.05).

Results

Activation of PAR_2 mediates an anti-inflammatory response in LPS-stimulated murine macrophages

PAR_2 and TLR4 are expressed on many cell types in the body, including epithelial cells and macrophages.[2,5,12,13] Like TLR4, PAR_2 has been strongly implicated in the induction of pro-inflammatory responses in a variety of cell types in various species.[5,13] We reported recently that PAR_2 activation in HEK293T-PAR_2 transfectants by its activating enzyme, trypsin, or its AP, SLIGKV-NH_2, elicited NF-κB-dependent and IRF-3-dependent luciferase reporter activities.[16] We also reported that PAR_2 activation in HEK293T-PAR_2 transfectants and in human A549 lung and SW620 colonic epithelial cell lines induced expression of the NF-κB-dependent neutrophil chemokine, IL-8.[4,16] However, when PAR_2 is activated in the presence of a TLR agonist in these epithelial cell lines, NF-κB-dependent gene expression and secretion was further increased, while IRF-3-dependent gene expression induced by LPS (a TLR4 agonist) or poly I:C (a TLR3 agonist) was repressed.[4]

In contrast to the positive co-operation that was observed between PAR_2 AP and LPS for the induction of pro-inflammatory responses in mucosal epithelial cell lines, simultaneous treatment of primary murine peritoneal macrophages with PAR_2 AP (SLIGKV-NH_2) and LPS resulted in a dose-dependent down-regulation of several key LPS-inducible pro-inflammatory cytokines, *e.g.* TNF-α, IL-6, and IL-12 p40 (Figure 1). In contrast, LPS-induced production of the potent anti-inflammatory cytokine, IL-10, was increased in the presence of PAR_2 AP (Figure 1). We confirmed and extended these findings in murine macrophages using a chemically modified PAR_2 AP, 2-furoyl-LIGRLO-NH_2 (fAP).[37] PAR_2 fAP is metabolically stable,

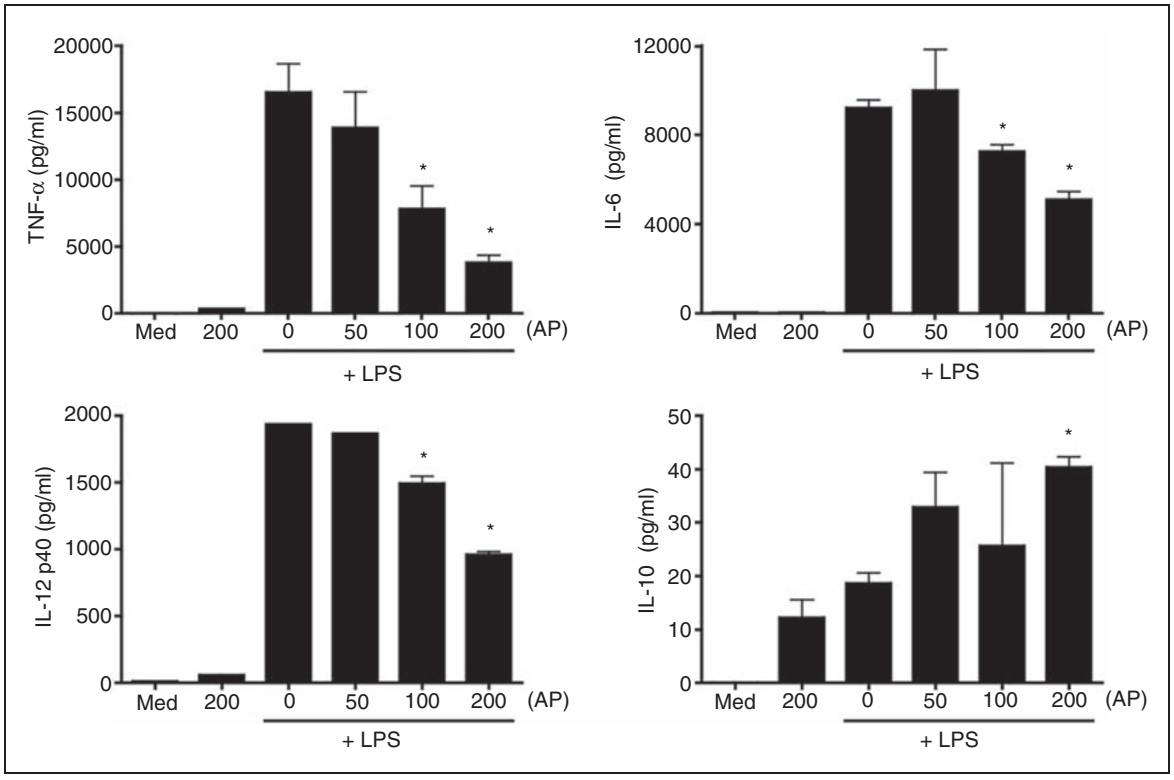

Figure 1. PAR_2 AP differentially modulates LPS-induced cytokine production in primary murine macrophages to promote an anti-inflammatory response. Thioglycollate-elicited peritoneal macrophages from C57BL/6J mice were stimulated for 24 h with medium only (Med), PAR_2 AP (SLIGKV-NH_2; 200 μM), LPS (100 ng/ml), or LPS in the presence of increasing concentrations of PAR_2 AP (50–200 μM). Supernatants were analyzed for cytokine production by ELISA. Data are presented as the mean ± SD of duplicate samples and are representative of one of two separate experiments. $^*P < 0.05$.

resistant to aminopeptidases, and exhibits high PAR_2 selectivity and potency.[37] Like native PAR_2 AP, fAP induced comparable NF-κB-luciferase reporter activities in HEK293T-PAR_2 transfectants (Figure 2A), but not in pcDNA3.1-transfected HEK293T cells (data not shown). In agreement with the results obtained using the native PAR_2 AP, SLIGKV-NH_2, PAR_2 fAP also enhanced LPS-induced IL-10 mRNA expression synergistically, while attenuating LPS-induced IL-12 p40 (Figure 2B), IL-12 p35, IL-6, and TNF-α mRNA levels (Figure 2C). The control reverse peptide (RP), 2-furoyl-OLRGIL-NH_2, had no effect on the LPS response (data not shown). This same pattern of gene expression, *i.e.* increased IL-10 mRNA and decreased IL-12 p40 mRNA, was also observed in bone marrow-derived macrophages (Figure 2D).

Next, the extent of PAR_2 and TLR4 signaling cross-talk was examined *in vivo*. Analysis of livers of mice injected i.p. with LPS in the absence or presence of PAR_2 AP (SLIGKV-NH_2) revealed enhanced expression of IL-10 mRNA that was followed by a significant decrease in IL-12 p40 mRNA levels in mice that received both PAR_2 AP and LPS (Figure 3). Co-administration of PAR_2 AP and LPS also led to a modest reduction in the expression of TNF-α, IL-6, and IL-12 p35 compared to that induced by LPS alone (data not shown). Taken together, our findings in murine macrophages *in vitro* and in livers, where the Kupffer cell resident macrophages represent the predominant LPS-responsive cells leading to the expression of IL-10 and IL-12 p40,[38] show that PAR_2 and TLR4 signaling pathways intersect such that PAR_2 promotes development of an anti-inflammatory IL-10 response while dampening the Th1-like pro-inflammatory response induced by LPS.

$PAR_2^{-/-}$ *macrophages exhibit altered LPS responses*

In the next series of experiments, $PAR_2^{-/-}$ macrophages were analyzed for their responsiveness to LPS. They were similar to wild-type C57BL/6J macrophages based on cellular morphology and protein expression of the macrophage markers F4/80 and CD11b (Figure 4A). The LPS-stimulated $PAR_2^{-/-}$ macrophages produced significantly less IL-10 mRNA (Figure 4B), while mRNA expression of the potent neutrophil chemokine, CXCL1/KC, was up-regulated (Figure 4B). The effects of PAR_2 deficiency on LPS-induced IL-10 and CXCL1/KC mRNAs in peritoneal exudate macrophages were confirmed at the level of secreted protein (Figure 4C). It has been reported that in response to LPS, secreted IL-10 acts back on

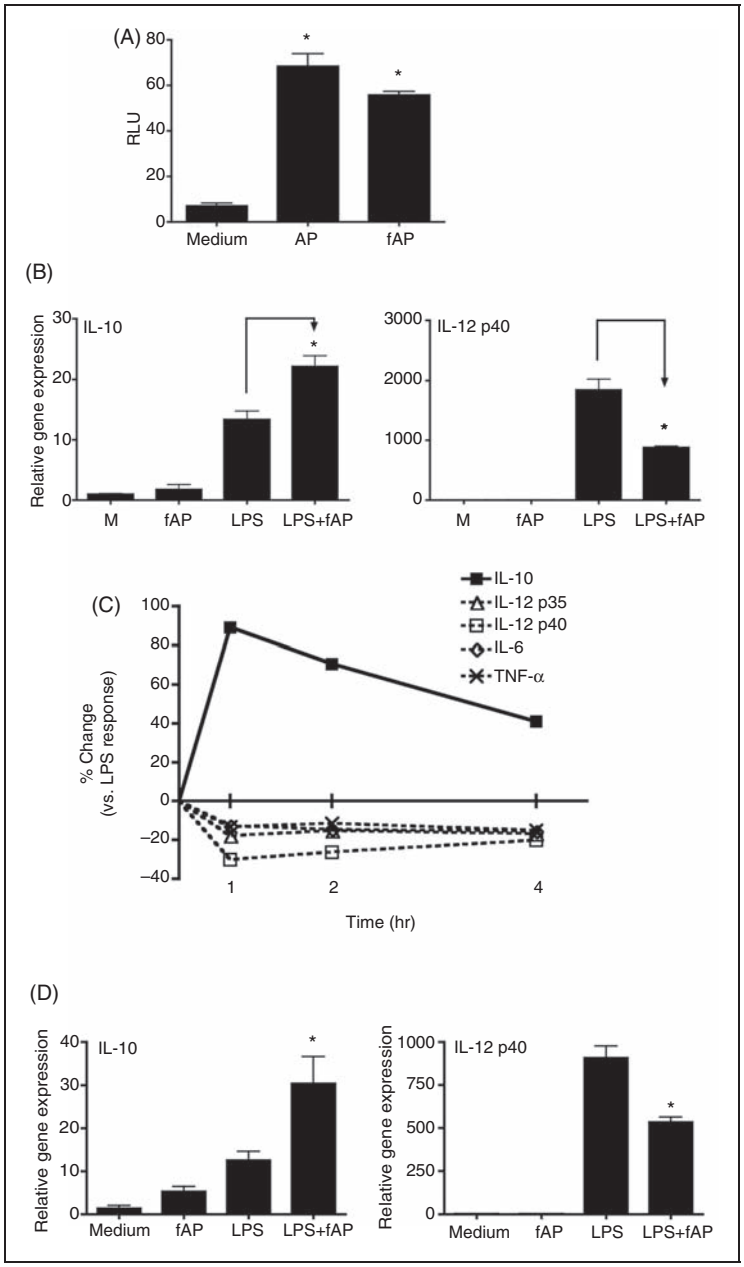

Figure 2. Both PAR_2 AP and fAP differentially modulate LPS-induced gene expression and cytokine production in primary murine macrophages to promote an anti-inflammatory response. (A) Induction of NF-κB activity by PAR_2 AP and fAP in PAR_2-expressing HEK293T transfectants. HEK293T cells were transiently transfected with PAR_2, together with NF-κB-luciferase and β-galactosidase reporter constructs. Cells were treated with PAR_2 AP (SLIGKV-NH_2; 200 μM) or PAR_2 fAP (2-furoyl-LIGRLO-NH_2; 200 μM) for 16 h. Relative luciferase units (RLU) represent luciferase reporter activity normalized to β-galactosidase reporter activity for each sample. Results are presented as the mean ± SD of duplicate samples and are representative of one of three separate experiments. $^*P < 0.05$. (B) PAR_2 fAP differentially modulates LPS-induced cytokine mRNA expression in primary murine macrophages. Thioglycollate-elicited peritoneal macrophages from C57BL/6J mice were stimulated for 2 h with medium (Med), PAR_2 fAP (2-furoyl-LIGRLO-NH_2; 200 μM), LPS (10 ng/ml), or both. Relative IL-10 and IL-12 p40 gene expression was analyzed by qPCR. Data are presented as the mean ± SD and are representative of one of five separate experiments, each with similar outcomes. $^*P < 0.05$. (C) PAR_2 fAP differentially modulates LPS-induced cytokine mRNA expression in primary murine macrophages (time course). Thioglycollate-elicited peritoneal macrophages from C57BL/6J mice were stimulated for the indicated times as described in (B). Relative gene expression was analyzed by qPCR. The effects of fAP on the LPS-induced mRNA responses are presented as the percentage change versus the response to LPS only for each gene at each time point. Data represent the average of three separate experiments. (D) PAR_2 AP differentially modulates LPS-induced cytokine mRNA expression in primary murine bone marrow-derived macrophages. Bone marrow-derived macrophages from C57BL/6J mice were stimulated for 2 h with medium (Med), PAR_2 fAP; 200 μM), LPS (10 ng/ml), or both. Relative IL-10 and IL-12 p40 gene expression was analyzed by qPCR as described in the caption to (B). Data are presented as the mean ± SD and are derived from a representative experiment ($n = 2$), each with similar outcomes. $^*P < 0.05$.

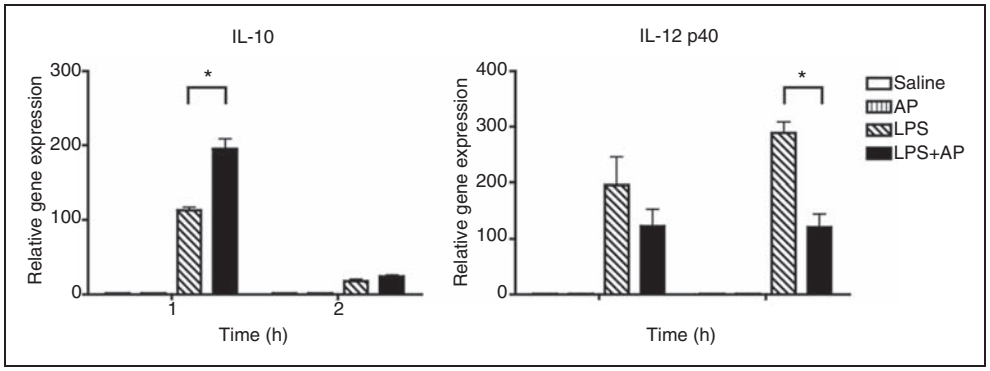

Figure 3. PAR_2 AP differentially modulates LPS-induced cytokine gene expression to promote an anti-inflammatory response *in vivo*. C57BL/6J mice were injected i.p. with 0.5 ml of saline, PAR_2 AP (SLIGKV-NH_2; 500 μM), LPS (25 μg), or both. At the indicated times, livers were harvested, and relative gene expression was analyzed by qPCR. The results are the mean ± SEM ($n = 3$ for each group at each time point) of a representative experiment ($n = 2$). *$P < 0.05$.

the macrophages through autocrine and paracrine pathways to activate an intracellular signaling mediator downstream of the IL-10 receptor known as signal transducer and activator of transcription 3 (STAT3).[39] In agreement with our findings showing diminished LPS-induced IL-10 in $PAR_2^{-/-}$ macrophages, STAT3-Tyr705 phosphorylation was also reduced in LPS-stimulated $PAR_2^{-/-}$ macrophages when compared to the wild-type response (Figure 4D), findings confirmed by densitometry (Figure 4E). Together, the findings in Figure 4 suggest that the absence of PAR_2 favors an LPS-inducible pro-inflammatory skew in the macrophages. Collectively, these results suggest that PAR_2 is activated in response to LPS, and that it counter-regulates LPS-induced signaling through the enhanced production of anti-inflammatory mediators such as IL-10.

Activation of PAR_2 synergistically augments expression of alternatively-activated/M2 macrophage markers

Recently, Porta et al.[40] reported that LPS-treated macrophages exhibited elevated expression of AA-Mϕ markers, *e.g.* IL-10, CCL2, CCL17, CCL22, and arginase-1 that followed the induction of pro-inflammatory cytokine gene expression. Since IL-4 and IL-13 drive the induction of AA-Mϕ,[8–11] we first measured the effect of PAR_2 AP and/or rIL-4 or LPS on the production of IL-4 and IL-13. PAR_2 fAP was a poor inducer of IL-4 and IL-13 by macrophages, but significantly enhanced production of both IL-4 and IL-13 induced by rIL-4 or LPS at 48 h (Figure 5A).

Figure 5B illustrates that macrophages stimulated with rIL-4 (5 ng/ml) or LPS (10 ng/ml) for 48 h exhibited increased mRNA expression of the prototypical AA-Mϕ markers, *i.e.* arginase-1, Ym-1, and mannose receptor.[8] The LPS-induced mRNA expression of the AA-Mϕ markers was significantly lower than that induced by treatment with rIL-4. Nevertheless, these data confirm the findings of Porta et al.[40] and indicate that, like rIL-4, LPS has the capacity to induce AA-Mϕ differentiation in primary murine macrophages.

Since concurrent PAR_2 activation up-regulated LPS-induced IL-4, IL-13, and IL-10 production, while decreasing pro-inflammatory cytokine expression, we sought to determine if PAR_2 activation would also augment rIL-4- or LPS-induced differentiation of AA-Mϕ. Consistent with the data shown in Figure 5A, Figure 5B also shows that PAR_2 fAP synergistically enhanced both LPS- and rIL-4-induced mRNA expression of the AA-Mϕ markers, arginase-1, Ym-1, and mannose receptor.

Discussion

Previous studies have demonstrated that concurrent stimulation of mucosal epithelial cells through PAR_2 and TLR4 enhanced expression of many NF-κB-dependent, pro-inflammatory genes, while inhibiting IRF-3-driven gene expression.[4,16,25] However, in contrast to the epithelial cell response, concurrent PAR_2 and TLR4 stimulation of murine macrophages resulted in a down-regulation of TLR4-induced pro-inflammatory gene and protein expression, augmented TLR4-driven production of the potent Th2-skewing, anti-inflammatory cytokine, IL-10, and promoted TLR4- or IL-4 receptor-mediated differentiation of AA-Mϕ phenotype, that has been strongly associated with wound healing, tissue repair, allergic and anti-parasitic Th2 immune responses.[8,10] These findings were confirmed and extended by showing that in PAR_2-null macrophages, the response to LPS was skewed toward a pro-inflammatory cytokine profile. Collectively, these data suggest that PAR_2 activation occurs in response to LPS signaling and attenuates the macrophage response to LPS both *in vitro* and *in vivo* through the production of counter-regulatory cytokines such as IL-10.

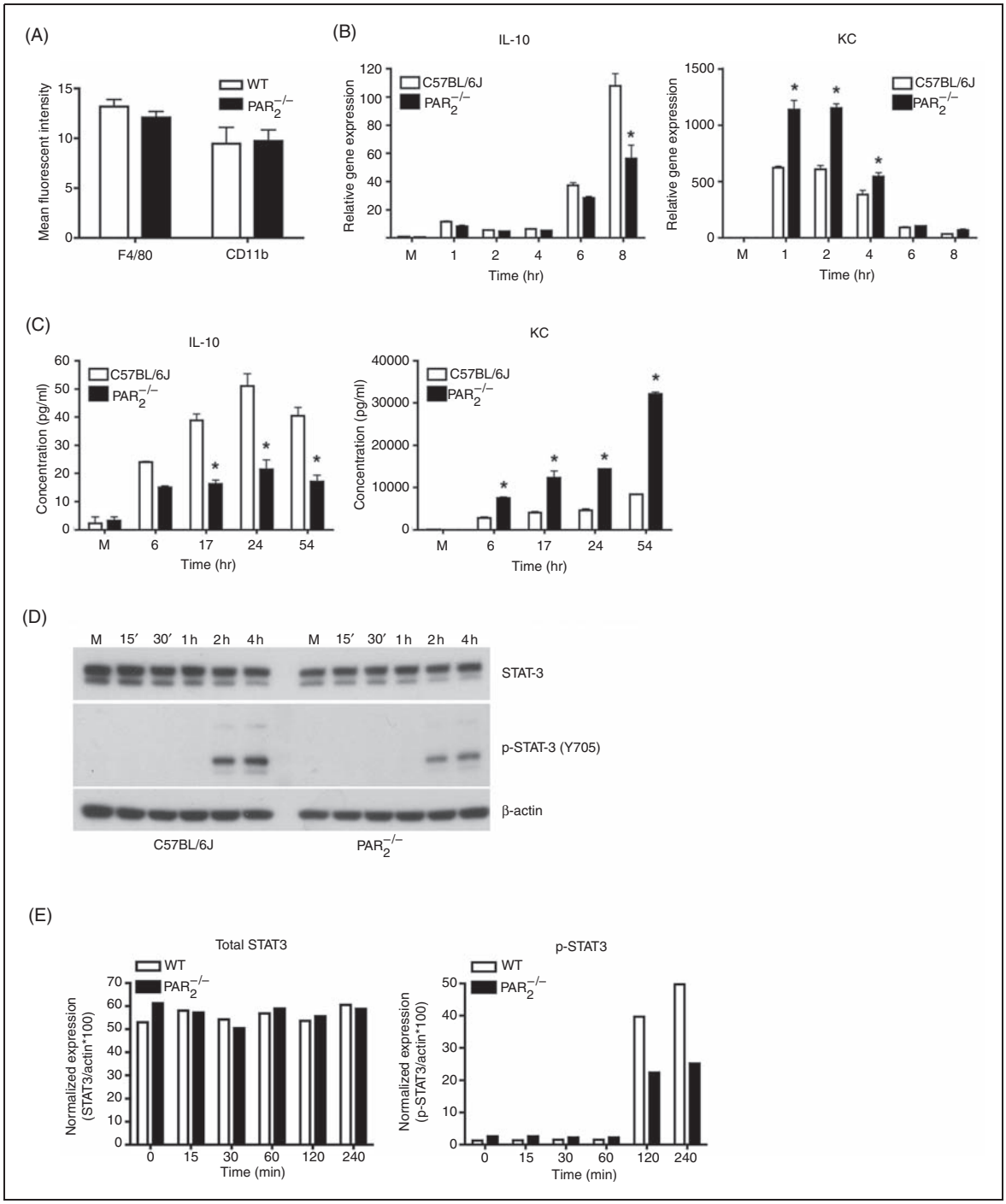

Figure 4. $PAR_2^{-/-}$ macrophages exhibit normal expression of macrophage markers, but altered responsiveness to LPS. (A) Wild-type and $PAR_2^{-/-}$ macrophages express comparable levels of F4/80 and CD11b. Thioglycollate-elicited peritoneal macrophages from wild-type and $PAR_2^{-/-}$ mice were stained for F4/80 and CD11b and analyzed by FACS analysis as described in the Materials and Methods. (B) $PAR_2^{-/-}$ macrophages exhibit decreased IL-10 and increased KC mRNA. Thioglycollate-elicited peritoneal macrophages from C57BL/6J and $PAR_2^{-/-}$ mice were stimulated with medium (M) or LPS (10 ng/ml) over the indicated time points. Relative gene expression was analyzed by qPCR. Data are presented as the mean ± SD and are representative of one of three separate experiments, each with similar outcomes. *$P < 0.05$. (C) $PAR_2^{-/-}$ macrophages exhibit decreased IL-10 and increased KC protein. Thioglycollate-elicited peritoneal macrophages from wild-type C57BL/6J and PAR2$^{-/-}$ mice were stimulated with medium (M) or with LPS (100 ng/ml) over the indicated time points. Supernatants were collected at each time point and were analyzed for secreted cytokines by ELISA. Data are presented as the mean ± SD and are representative of one of three separate experiments, each with similar outcomes. *$P < 0.05$. (D) $PAR_2^{-/-}$ macrophages exhibit altered LPS-mediated intracellular signaling. Thioglycollate-elicited peritoneal macrophages from wild-type C57BL/6J and $PAR_2^{-/-}$ mice were stimulated with medium (M) or with LPS (10 ng/ml) over the indicated time points. Whole cell lysates were subjected to Western analysis. Data are representative of one of two separate experiments. (E) Densitometric analysis of two separate experiments similar to that shown in (D) in which total STAT3 and phospho-STAT3 densitometry measurements were normalized for the expression of β-actin in the same samples using NIH software, Image J.

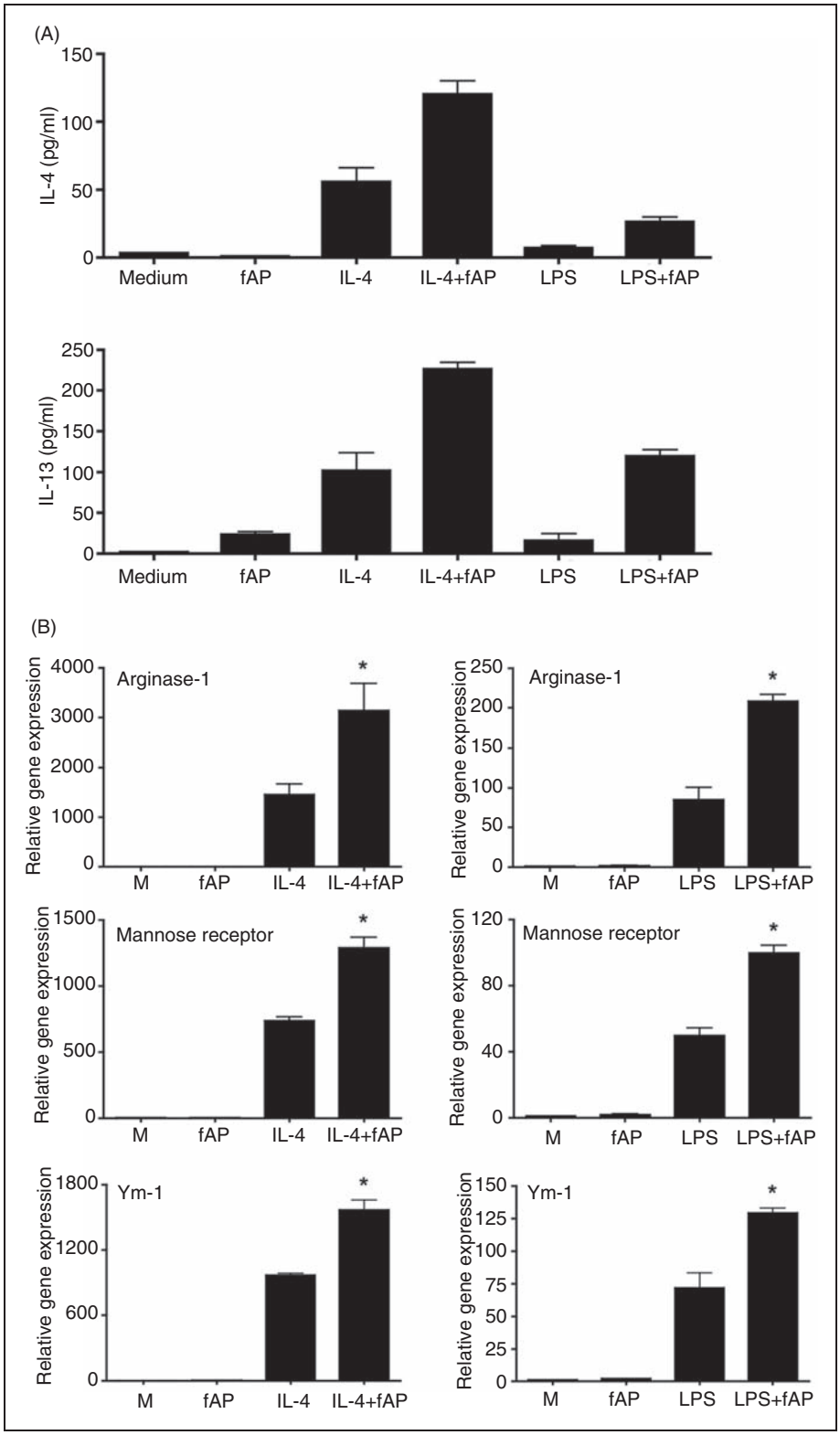

Figure 5. PAR_2 fAP synergistically augments rIL-4- or LPS-induction of IL-4, IL-13, and alternative activation of murine macrophages. (A) PAR_2 fAP synergizes with rIL-4 and LPS to induce IL-4 and IL-13. Thioglycollate-elicited peritoneal macrophage from C57BL/6J mice were stimulated for 48 h with medium (M), PAR_2 fAP (2-furoyl-LIGRLO-NH_2; 200 μM), recombinant IL-4 (5 ng/ml), LPS (10 ng/ml), or the indicated treatment combinations. Supernatants were analyzed for cytokine production by ELISA. Data are presented as the combined mean ± SEM in two separate experiments. (B) PAR_2 fAP synergistic induction of alternatively activated macrophage genes. Thioglycollate-elicited macrophages were treated as in (A). Relative gene expression was analyzed by qPCR. Data are presented as the combined mean ± SEM in two separate experiments. *$P < 0.05$.

Activation of PAR_2 has been reported to exhibit opposite effects in several models of inflammation,[21,22,28,41] and perhaps this is attributable to the balance of synergistic or antagonistic effects reported herein between PAR_2 and activation of other immune modulating receptors in different cell types. Our results in macrophages showing a preferential induction of an anti-inflammatory phenotype mediated by concurrent PAR_2 and TLR4 activation support and extend earlier studies. For example, a study using an ovalbumin-induced asthma model, in which $CD4^+$ Th2 cells are strongly implicated, reported that $PAR_2^{-/-}$ mice had decreased broncho-alveolar eosinophil infiltration, suppressed airway hyper-reactivity to methacholine, and diminished serum IgE levels.[21] In contrast, these same responses were reversed in PAR_2-overexpressing transgenic mice,[21] indicating that PAR_2 activation contributes to the development of Th2-like mediated immune responses. Consistent with our results showing a skew toward a pro-inflammatory cytokine expression profile for LPS-stimulated $PAR_2^{-/-}$ macrophages, PAR_2-deficient $CD4^+$ T-cells were found to be skewed toward a Th1 cytokine profile, accompanied by elevated T-bet mRNA expression, with diminished IL-4 and increased IFN-γ expression in a mouse model of ovalbumin-induced airway inflammation.[42] These findings support the notion that PAR_2-deficient T cells are preferentially Th1-polarized, again implying that PAR_2 signaling promotes a Th2-skewed inflammatory response. In addition, Devlin et al.[43] showed that PAR_2 activation promoted a Th2-skewed inflammatory response with augmented IL-10 production by lymphocytes. Consistent with the Th2-skewing capacity of PAR_2 in $CD4^+$ T-cells reported in these studies, our results in murine macrophages show that PAR_2 activation both enhances IL-4 and IL-13 production induced by rIL-4 and LPS, and furthermore, promotes rIL-4- or LPS-induced differentiation of AA-Mϕ that may also contribute to the development of Th2 responses.

The induction of Th2 differentiation is not limited to the intercellular interactions between antigen-presenting cells, *e.g.* macrophages and dendritic cells, and T cells; epithelial cell-derived thymic stromal lymphopoietin (TSLP) can also trigger dendritic cell-mediated Th2-type inflammation.[44] The Th2-promoting role for PAR_2 is further supported by two recent studies showing that PAR_2 activation in epithelial cells induces TSLP.[45,46] In addition, allergens that possess intrinsic protease activity can activate PAR_2 on epithelial cells to induce a Th2-type allergic inflammatory response.[6,47] Taken together, the results reported herein and by others[21,42,43,46] indicate that PAR_2 activation favors an immune deviation from the classical Th1-like pro-inflammatory response to one that is more Th2-like.

Clearly, these observations suggest that PAR_2 signaling in different cell types can differentially affect the outcome of an inflammatory response. PAR_2 activation has been observed to exert both protective and pathogenic effects in different cell types, *i.e.* glial cells and neurons, in Alzheimer's disease.[48] Differential expression of PAR_2 on different cell types, coupled with the co-expression of other PAR_2-interacting adapter proteins, *e.g.* G proteins, β-arrestins,[49–51] and the TLR adapters,[16] or PAR_2-interacting receptors, *e.g.* TLR4,[16] may also dictate the cell type-specific outcome of an inflammatory response. Future experiments will be required to dissect the relative contributions of these various interacting partners of PAR_2 to signaling in different cell types.

Conclusions

The results from this study provide a mechanism that potentially explains the opposite inflammatory roles observed for PAR_2 in different experimental models. We propose that the types of cell being activated and the timing of the experimental outcome likely contribute to the dual roles observed for PAR_2 signaling in inflammation. The results from our study suggest that manipulation of the extracellular protease/anti-protease balance, as well as cell-type specific inhibition or activation of PAR_2 signaling, may represent future novel therapeutic approaches to treating inflammatory PAR_2-dependent disorders.

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