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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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SUMMARY

Proteinase-activated receptor 2 (PAR₂), 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 PAR₂ and TLRs in human lung and colonic epithelial cells resulted in a synergistic increase in NF- κ B-mediated gene expression, but a down-regulation of IRF-3-mediated gene expression. In this study, the effect of PAR₂ activation on LPS-induced TLR4 signaling was examined in primary murine macrophages. PAR₂ activation of wild-type macrophages enhanced LPS-induced expression of the anti-inflammatory cytokine, IL-10, while suppressing gene expression of proinflammatory cytokines, TNF- α , IL-6, and IL-12. Similar PAR₂-mediated effects on LPS-stimulated IL-10 and IL-12 mRNA were also observed *in vivo*. In contrast, PAR₂^{-/-} macrophages exhibited diminished LPS-induced IL-10 mRNA and protein expression and downstream STAT3 activation, but increased KC mRNA and protein. PAR₂ activation also enhanced both rIL-4- and LPS-induced secretion of IL-4 and IL-13, and mRNA expression of alternatively activated macrophage (AA-M ϕ) markers, *e.g.*, arginase-1, mannose receptor, Ym-1. Thus, in the context of a potent inflammatory stimulus like LPS, PAR₂ activation acts to reestablish 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

INTRODUCTION

Pattern-recognition receptors (PRRs) of the innate immune system detect infection by recognizing pathogen-associated molecular patterns (PAMPs), evolutionarily conserved

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DECLARATION OF CONFLICTING INTERESTS

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structural motifs that are shared among microbes, *e.g.*, LPS, lipopeptides, flagellin, and microbial nucleic acids.^{1,2} PRRs 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 PRRs⁴, 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 proinflammatory 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, and IL-13 is also activated in macrophages by certain pathogens and promotes tissue homeostasis by counteracting induction of proinflammatory cytokines and by leading to the development of macrophages with an AA-M ϕ (referred to as “M2”) differentiation phenotype that dampens the proinflammatory 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.

TLRs represent a large family of germline-encoded, single-transmembrane, classical PRRs. TLRs 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 nuclear factor (NF)- κ B 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}

PAR₂ belongs to a family of four seven-transmembrane G protein-coupled receptors (7-TM GPCRs).^{5,13} PAR₂ is expressed highly in the respiratory and gastrointestinal (GI) tracts^{14,15}, and, like the TLRs, is also expressed on epithelial cells and macrophages. PAR₂ 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. 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 PAR₁, PAR₂, and PAR₄ mediate signaling non-enzymatically by binding directly to the ECL2 of their respective native, uncleaved PAR. We have reported that the human PAR₂ AP, SLIGKV-NH₂, induces both NF- κ B and IRF-3 reporter activities in PAR₂-expressing HEK293T cells, but not in pcDNA3.1 empty vector-transfected cells, and that PAR₂ co-immunoprecipitated with TLR4 in the presence of AP, suggesting that these two receptors may physically associate in response to PAR₂ activation.¹⁶ In addition, the PAR₂ AP, SLIGKV-NH₂, but not a scrambled control peptide, induced footpad edema in wild-type (WT) C57BL/6J mice, but not in PAR₂^{-/-} or TLR4^{-/-} mice.⁴ These findings indicate that the PAR₂ AP specifically activates its target receptor and that TLR4 is required for this PAR₂-mediated inflammatory response. PAR₂ activation 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 PAR₂ is involved in proinflammatory and allergic responses at anatomic sites that interact with protease-rich environments, *e.g.*, inflamed tissues, the gut lumen, and the respiratory tract.^{21–23} In addition, PAR₂ signaling intersects with TLRs to augment proinflammatory responses in epithelial cells, endothelial cells, and monocytes.^{4,16,24–26} However, cytoprotective and anti-inflammatory functions have also been ascribed to PAR₂ signaling. For example, PAR₂ 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*, PAR₂ AP treatment inhibits Th1 cytokine production and protects mice from 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis and lethality.²⁹ Intranasal PAR₂ AP administration inhibits LPS-induced pulmonary neutrophil influx.³⁰ In this study, we hypothesized that the cytoprotective, anti-inflammatory outcomes observed *in vivo* for PAR₂ may depend on PAR₂-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 proinflammatory mediators, *e.g.*, IL-1 β , IL-6, TNF- α , and IL-12.^{7,31–33} This study sought to define the capacity of PAR₂ to modulate TLR4 signaling in murine macrophages.

MATERIALS AND METHODS

Reagents, mice, and tissue culture

Human PAR₂ AP, SLIGKV-NH₂, and an inactive, control reverse peptide (RP), VKGILS-NH₂, were synthesized (>96% purity) by Phoenix Pharmaceuticals (Belmont, CA). PAR₂ fAP (2-furoyl-LIGRLO-NH₂; >98% purity), a stabilized PAR₂ agonist, was purchased from Calbiochem (San Diego, CA). 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).

WT C57BL/6J mice and PAR₂^{−/−} mice (backcrossed onto a C57BL/6 background (N5)) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were used between 6–10 weeks of age. All experiments were conducted with institutional approval. *In vivo* treatments with PAR₂ AP and LPS were carried out as indicated in the figure legends. Primary murine thioglycollate-elicited peritoneal macrophages or bone marrow-derived macrophages, obtained by expanding bone marrow cells in rCSF-1 (10 ng ml^{−1}; R&D Systems), were prepared for *in vitro* studies as described previously.^{35,36} Human embryonic kidney (HEK) 293T cells were transfected with PAR₂ expression vector, together with NF- κ B-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 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) as previously described.³⁵

Flow cytometry analysis

C57BL/6J and PAR2^{-/-} thioglycollate-elicited macrophages were treated with medium, harvested by gentle scraping, and then 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) and used at 1:1,000 and 1:2,000 dilutions, respectively. Densitometric analysis of Western blots were carried out using NIH software, Image J (<http://rsbweb.nih.gov/ni-image/>).

Analysis of secreted proteins by ELISA

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

Statistical analysis

Using GraphPad PRISM 4.0 (GraphPad Software, San Diego, CA), 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

PAR₂ activation mediates an anti-inflammatory response in LPS-stimulated murine macrophages

PAR₂ and TLR4 are expressed on many cell types in the body, including epithelial cells and macrophages.^{2,5,12,13} Like TLR4, PAR₂ has been strongly implicated in the induction of proinflammatory responses in a variety of cell types in various species.^{5,13} We reported recently that PAR₂ activation in HEK293T-PAR₂ transfectants by its activating enzyme, trypsin, or its AP, SLIGKV-NH₂, elicited NF- κ B-dependent and IRF-3-dependent luciferase reporter activities.¹⁶ We also reported that PAR₂ activation in HEK293T-PAR₂ transfectants and in human A549 lung and SW620 colonic epithelial cell lines induced expression of the NF- κ B-dependent neutrophil chemokine, IL-8.^{4,16} However, when PAR₂ is activated in the presence of a TLR agonist in these epithelial cell lines, NF- κ B-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 cooperation that was observed between PAR₂ AP and LPS for the induction of proinflammatory responses in mucosal epithelial cell lines, simultaneous treatment of primary murine peritoneal macrophages with PAR₂ AP (SLIGKV-NH₂) and LPS resulted in a dose-dependent down-regulation of several key LPS-inducible proinflammatory 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 PAR₂ AP (Figure 1). We confirmed and extended these findings in murine macrophages using a chemically modified PAR₂ AP, 2-furoyl-LIGRLO-NH₂ (fAP).³⁷ PAR₂ fAP is metabolically stable, resistant to aminopeptidases, and exhibits high PAR₂ selectivity and potency.³⁷ Like native PAR₂ AP, fAP induced comparable NF- κ B-luciferase reporter activities in HEK293T-PAR₂ transfectants (Figure 2A), but not in pcDNA3.1-transfected HEK293T cells (data not shown). In agreement with the results obtained using the native PAR₂ AP, SLIGKV-NH₂, PAR₂ 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-NH₂, 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 PAR₂ and TLR4 signaling crosstalk was examined *in vivo*. Analysis of livers of mice injected i.p. with LPS in the absence or presence of PAR₂ AP (SLIGKV-NH₂) 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 PAR₂ AP and LPS (Figure 3). Co-administration of PAR₂ 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 p40³⁸, show that PAR₂ and TLR4 signaling pathways intersect such that PAR₂ promotes development of an anti-inflammatory IL-10 response while dampening the Th1-like proinflammatory response induced by LPS.

PAR₂^{-/-} macrophages exhibit altered LPS responses

In the next series of experiments, PAR₂^{-/-} macrophages were analyzed for their responsiveness to LPS. PAR₂^{-/-} macrophages were similar to WT C57BL/6J macrophages based on cellular morphology and protein expression of the macrophage markers F4/80 and CD11b (Figure 4A). LPS-stimulated PAR₂^{-/-} 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 PAR₂ 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 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 PAR₂^{-/-} macrophages, STAT3-Tyr705 phosphorylation was also reduced in LPS-stimulated PAR₂^{-/-} macrophages when compared to the WT response (Figure 4D), findings confirmed by densitometry (Figure 4E). Together, the findings in Figure 4 suggest that the absence of PAR₂ favors an LPS-inducible proinflammatory skew in the macrophages. Collectively, these results suggest that PAR₂ 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.

PAR₂ activation synergistically augments expression of alternatively-activated/M2 macrophage markers

Recently, Porta *et al.* reported that LPS-treated macrophages exhibited elevated expression of AA-M ϕ markers, *e.g.*, IL-10, CCL2, CCL17, CCL22, and arginase-1 that followed the induction of proinflammatory cytokine gene expression.⁴⁰ Since IL-4 and IL-13 drive the induction of AA-M ϕ ⁸⁻¹¹, we first measured the effect of PAR₂ AP and/or rIL-4 or LPS on

the production of IL-4 and IL-13. PAR₂ 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 hours (Figure 5A).

Figure 5B illustrates that macrophages stimulated with rIL-4 (5 ng ml⁻¹) or LPS (10 ng ml⁻¹) for 48 h exhibited increased mRNA expression of the prototypical AA-Mφ markers, *i.e.*, arginase-1, Ym-1, and mannose receptor.⁸ 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 PAR₂ activation upregulated LPS-induced IL-4, IL-13, and IL-10 production, while decreasing proinflammatory cytokine expression, we sought to determine if PAR₂ 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 PAR₂ 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 PAR₂ 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 PAR₂ and TLR4 stimulation of murine macrophages resulted in a down-regulation of TLR4-induced proinflammatory 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 PAR₂-null macrophages, the response to LPS was skewed toward a proinflammatory cytokine profile. Collectively, these data suggest that PAR₂ 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.

PAR₂ activation 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 PAR₂ 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 PAR₂ 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 PAR₂^{-/-} mice had decreased bronchoalveolar eosinophil infiltration, suppressed airway hyperreactivity to methacholine, and diminished serum IgE levels.²¹ In contrast, these same responses were reversed in PAR₂-overexpressing transgenic mice²¹, indicating that PAR₂ activation contributes to the development of Th2-like mediated immune responses. Consistent with our results showing a skew toward a proinflammatory cytokine expression profile for LPS-stimulated PAR₂^{-/-} macrophages, PAR₂-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 PAR₂-deficient T cells are preferentially Th1-polarized, again implying that PAR₂ signaling promotes a Th2-skewed inflammatory response. In addition, Devlin *et al.*

showed that PAR₂ activation promoted a Th2-skewed inflammatory response with augmented IL-10 production by lymphocytes.⁴³ Consistent with the Th2-skewing capacity of PAR₂ in CD4⁺ T cells reported in these studies, our results in murine macrophages show that PAR₂ 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 PAR₂ is further supported by two recent studies showing that PAR₂ activation in epithelial cells induces TSLP.^{45,46} In addition, allergens that possess intrinsic protease activity can activate PAR₂ 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 PAR₂ activation favors an immune deviation from the classical Th1-like proinflammatory response to one that is more Th2-like.

Clearly, these observations suggest that PAR₂ signaling in different cell types can differentially affect the outcome of an inflammatory response. PAR₂ 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 PAR₂ on different cell types, coupled with the co-expression of other PAR₂-interacting adapter proteins, *e.g.*, G proteins, β-arrestins^{49–51}, and the TLR adapters¹⁶, or PAR₂-interacting receptors, *e.g.*, TLR416, 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 PAR₂ to signaling in different cell types.

In summary, the results from our study provide a mechanism that potentially explains the opposite inflammatory roles observed for PAR₂ in different experimental models. We propose that the type of cells being activated and the timing of the experimental outcome likely contribute to the dual roles observed for PAR₂ 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 PAR₂ signaling, may represent future novel therapeutic approaches to treating inflammatory PAR₂-dependent disorders.

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References

1. Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu Rev Immunol.* 2002; 20:197–216. [PubMed: 11861602]
2. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell.* 2006; 124:783–801. [PubMed: 16497588]
3. Bianchi ME. DAMPS, PAMPS and alarmins: all we need to know about danger. *J Leukoc Biol.* 2007; 81:1–5. [PubMed: 17032697]

4. Nhu QM, Shirey K, Tejjaro JR, Farber DL, Netzel-Arnett S, Antalis TM, et al. Novel signaling interactions between proteinase-activated receptor 2 and Toll-like receptors *in vitro* and *in vivo*. *Mucosal Immunol.* 2010; 3:29–39. [PubMed: 19865078]
5. Shpacovitch V, Feld M, Bunnett NW, Steinhoff M. Protease-activated receptors: novel PARTners in innate immunity. *Trends Immunol.* 2007; 28:541–550. [PubMed: 17977790]
6. Wills-Karp M, Nathan A, Page K, Karp CL. New insights into innate immune mechanisms underlying allergenicity. *Mucosal Immunol.* 2010; 3:104–110. [PubMed: 20032970]
7. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol.* 2003; 3:133–146. [PubMed: 12563297]
8. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol.* 2003; 3:23–35. [PubMed: 12511873]
9. Shirey KA, Cole LE, Keegan AD, Vogel SN. *Francisella tularensis* live vaccine strain induces macrophage alternative activation as a survival mechanism. *J Immunol.* 2008; 181:4159–4167. [PubMed: 18768873]
10. Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol.* 2009; 27:451–483. [PubMed: 19105661]
11. Shirey K, Pletneva LM, Puche AC, Keegan AD, Prince GA, Blanco JC, et al. Control of RSV-induced lung injury by alternatively activated macrophages is IL-4R α -, TLR4-, and IFN- β -dependent. *Mucosal Immunol.* 2010; 3:291–300. [PubMed: 20404812]
12. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol.* 2004; 4:499–511. [PubMed: 15229469]
13. Steinhoff M, Buddenkotte J, Shpacovitch V, Rattenholl A, Moormann C, Vergnolle N, et al. Proteinase-activated receptors: transducers of proteinase-mediated signaling in inflammation and immune response. *Endocr Rev.* 2005; 26:1–43. [PubMed: 15689571]
14. Vergnolle N. Review article: proteinase-activated receptors-novel signals for gastrointestinal pathophysiology. *Aliment Pharmacol Ther.* 2000; 14:257–266. [PubMed: 10735917]
15. Cocks TM, Moffatt JD. Protease-activated receptor-2 (PAR2) in the airways. *Pulm Pharmacol Ther.* 2001; 14:183–191. [PubMed: 11448145]
16. Rallabhandi P, Nhu QM, Toshchakov VY, Piao W, Medvedev AE, Hollenberg MD, et al. Analysis of proteinase-activated receptor 2 and TLR4 signal transduction: a novel paradigm for receptor cooperativity. *J Biol Chem.* 2008; 283:24314–24325. [PubMed: 18622013]
17. Sun G, Stacey MA, Schmidt M, Mori L, Mattoli S. Interaction of mite allergens Der p3 and Der p9 with protease-activated receptor-2 expressed by lung epithelial cells. *J Immunol.* 2001; 167:1014–1021. [PubMed: 11441110]
18. Chung WO, Hansen SR, Rao D, Dale BA. Protease-activated receptor signaling increases epithelial antimicrobial peptide expression. *J Immunol.* 2004; 173:5165–5170. [PubMed: 15470061]
19. Johansson U, Lawson C, Dabare M, Syndercombe-Court D, Newland AC, Howells GL, et al. Human peripheral blood monocytes express protease receptor-2 and respond to receptor activation by production of IL-6, IL-8, and IL-1 β . *J Leukoc Biol.* 2005; 78:967–975. [PubMed: 16000389]
20. Kida Y, Inoue H, Shimizu T, Kuwano K. *Serratia marcescens* serralyisin induces inflammatory responses through protease-activated receptor 2. *Infect Immun.* 2007; 75:164–174. [PubMed: 17043106]
21. Schmidlin F, Amadesi S, Dabbagh K, Lewis DE, Knott P, Bunnett NW, et al. Protease-activated receptor 2 mediates eosinophil infiltration and hyperreactivity in allergic inflammation of the airway. *J Immunol.* 2002; 169:5315–5321. [PubMed: 12391252]
22. Cenac N, Coelho AM, Nguyen C, Compton S, Andrade-Gordon P, MacNaughton WK, et al. Induction of intestinal inflammation in mouse by activation of proteinase-activated receptor-2. *Am J Pathol.* 2002; 161:1903–1915. [PubMed: 12414536]
23. Hansen KK, Sherman PM, Cellars L, Andrade-Gordon P, Pan Z, Baruch A, et al. A major role for proteolytic activity and proteinase-activated receptor-2 in the pathogenesis of infectious colitis. *Proc Natl Acad Sci USA.* 2005; 102:8363–8368. [PubMed: 15919826]
24. Chi L, Li Y, Stehno-Bittel L, Gao J, Morrison DC, Stechschulte DJ, et al. Interleukin-6 production by endothelial cells via stimulation of protease-activated receptors is amplified by endotoxin and tumor necrosis factor- α . *J Interferon Cytokine Res.* 2001; 21:231–240. [PubMed: 11359654]

25. Ostrowska E, Sokolova E, Reiser G. PAR-2 activation and LPS synergistically enhance inflammatory signaling in airway epithelial cells by raising PAR expression level and interleukin-8 release. *Am J Physiol Lung Cell Mol Physiol*. 2007; 293:L1208–L1218. [PubMed: 17766588]
26. Uehara A, Imamura T, Potempa J, Travis J, Takada H. Gingipains from *Porphyromonas gingivalis* synergistically induce the production of proinflammatory cytokines through protease-activated receptors with Toll-like receptor and NOD1/2 ligands in human monocytic cells. *Cell Microbiol*. 2008; 10:1181–1189. [PubMed: 18182086]
27. Cocks TM, Fong B, Chow JM, Anderson GP, Frauman AG, Goldie RG, et al. A protective role for protease-activated receptors in the airways. *Nature*. 1999; 398:156–160. [PubMed: 10086357]
28. Kawabata A, Kinoshita M, Nishikawa H, Kuroda R, Nishida M, Araki H, et al. The protease-activated receptor-2 agonist induces gastric mucus secretion and mucosal cytoprotection. *J Clin Invest*. 2001; 107:1443–1450. [PubMed: 11390426]
29. Fiorucci S, Mencarelli A, Palazzetti B, Distrutti E, Vergnolle N, Hollenberg MD, et al. Proteinase-activated receptor 2 is an anti-inflammatory signal for colonic lamina propria lymphocytes in a mouse model of colitis. *Proc Natl Acad Sci USA*. 2001; 98:13936–13941. [PubMed: 11717450]
30. Moffatt JD, Jeffrey KL, Cocks TM. Protease-activated receptor-2 activating peptide SLIGRL inhibits bacterial lipopolysaccharide-induced recruitment of polymorphonuclear leukocytes into the airways of mice. *Am J Respir Cell Mol Biol*. 2002; 26:680–684. [PubMed: 12034566]
31. Panina-Bordignon P, Mazzeo D, Lucia PD, D'Ambrosio D, Lang R, Fabbri L, et al. β_2 -agonists prevent Th1 development by selective inhibition of interleukin 12. *J Clin Invest*. 1997; 100:1513–1519. [PubMed: 9294119]
32. Braun MC, Kelsall BL. Regulation of interleukin-12 production by G-protein-coupled receptors. *Microbes Infect*. 2001; 3:99–107. [PubMed: 11251296]
33. Pinhal-Enfield G, Ramanathan M, Hasko G, Vogel SN, Salzman AL, Boons GJ, et al. An angiogenic switch in macrophages involving synergy between Toll-like receptors 2, 4, 7, and 9 and adenosine A_{2A} receptors. *Am J Pathol*. 2003; 163:711–721. [PubMed: 12875990]
34. McIntire FC, Sievert HW, Barlow GH, Finley RA, Lee AY. Chemical, physical, biological properties of a lipopolysaccharide from *Escherichia coli* K-235. *Biochemistry*. 1967; 6:2363–2373. [PubMed: 4867999]
35. Nhu QM, Cuesta N, Vogel SN. Transcriptional regulation of lipopolysaccharide (LPS)-induced Toll-like receptor (TLR) expression in murine macrophages: role of interferon regulatory factors 1 (IRF-1) and 2 (IRF-2). *J Endotoxin Res*. 2006; 12:285–95. [PubMed: 17059692]
36. Rowe DC, McGettrick AF, Latz E, Monks BG, Gay NJ, Yamamoto M, et al. The myristoylation of TRIF-related adaptor molecule is essential for Toll-like receptor 4 signal transduction. *Proc Natl Acad Sci*. 2006; 103:6299–6304. [PubMed: 16603631]
37. McGuire JJ, Saifeddine M, Trigg CR, Sun K, Hollenberg MD. 2-furoyl-LIGRLO-amide: a potent and selective proteinase-activated receptor 2 agonist. *J Pharmacol Exp Ther*. 2004; 309:1124–1131. [PubMed: 14976230]
38. Salkowski CA, Neta R, Wynn TA, Strassmann G, van Rooijen N, Vogel SN. Effect of liposome-mediated macrophage depletion on LPS-induced cytokine gene expression and radioprotection. *J Immunol*. 1995; 155:3168–3179. [PubMed: 7673730]
39. Carl VS, Gautam JK, Comeau LD, Smith MF Jr. Role of endogenous IL-10 in LPS-induced STAT3 activation and IL-1 receptor antagonist gene expression. *J Leukoc Biol*. 2004; 76:735–742. [PubMed: 15218058]
40. Porta C, Rimoldi M, Raes G, Brys L, Ghezzi P, Di Liberto D, et al. Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor κ B. *Proc Natl Acad Sci USA*. 2009; 106:14978–14983. [PubMed: 19706447]
41. D'Agostino B, Roviezzo F, De Palma R, Terracciano S, De Nardo M, Gallelli L, et al. Activation of protease-activated receptor-2 reduces airways inflammation in experimental allergic asthma. *Clin Exp Allergy*. 2007; 37:1436–1443. [PubMed: 17883723]
42. Shichijo M, Kondo S, Ishimori M, Watanabe S, Helin H, Yamasaki T, et al. PAR-2 deficient CD4⁺ T cells exhibit downregulation of IL-4 and upregulation of IFN- γ after antigen challenge in mice. *Allergol Int*. 2006; 55:271–278. [PubMed: 17075267]

43. Devlin MG, Gasser RB, Cocks TM. Initial support for the hypothesis that PAR₂ is involved in the immune response to *Nippostrongylus brasiliensis* in mice. *Parasitol Res.* 2007; 101:105–109. [PubMed: 17458579]
44. Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol.* 2002; 3:673–680. [PubMed: 12055625]
45. Briot A, Deraison C, Lacroix M, Bonnart C, Robin A, Besson C, et al. Kallikrein 5 induces atopic dermatitis-like lesions through PAR₂-mediated thymic stromal lymphopoietin expression in Netherton syndrome. *J Exp Med.* 2009; 206:1135–1147. [PubMed: 19414552]
46. Kouzaki H, O'Grady SM, Lawrence CB, Kita H. Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. *J Immunol.* 2009; 183:1427–1434. [PubMed: 19561109]
47. Reed CE, Kita H. The role of protease activation of inflammation in allergic respiratory diseases. *J Allergy Clin Immunol.* 2004; 114:997–1008. quiz 1009. [PubMed: 15536399]
48. Afkhami-Goli A, Noorbakhsh F, Keller AJ, Vergnolle N, Westaway D, Jhamandas JH, et al. Proteinase-activated receptor-2 exerts protective and pathogenic cell type-specific effects in Alzheimer's disease. *J Immunol.* 2007; 179:5493–5503. [PubMed: 17911636]
49. Lefkowitz RJ, Shenoy SK. Transduction of receptor signals by β -arrestins. *Science.* 2005; 308:512–517. [PubMed: 15845844]
50. Wang P, DeFea KA. Protease-activated receptor-2 simultaneously directs β -arrestin-1-dependent inhibition and G α_q -dependent activation of phosphatidylinositol 3-kinase. *Biochemistry.* 2006; 45:9374–9385. [PubMed: 16878972]
51. Goon Goh F, Sloss CM, Cunningham MR, Nilsson M, Cadalbert L, Plevin R. G-protein-dependent and -independent pathways regulate proteinase-activated receptor-2 mediated p65 NF- κ B serine 536 phosphorylation in human keratinocytes. *Cell Signal.* 2008; 20:1267–1274. [PubMed: 18424071]

Figure 1. PAR₂ 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), PAR₂ AP (SLIGKV-NH₂; 200 μM), LPS (100 ng ml⁻¹), or LPS in the presence of increasing concentrations of PAR₂ AP (50 – 200 μM). Supernatants were analyzed for cytokine production by ELISA. Data are presented as the mean ± s.d. of duplicate samples and are representative of one of two separate experiments. *, *p* < 0.05.

Figure 2. Both PAR₂ 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- κ B activity by PAR₂ AP and fAP in PAR₂-expressing HEK293T transfectants. HEK293T cells were transiently transfected with PAR₂, together with NF- κ B-luciferase and β -galactosidase reporter constructs. Cells were treated with PAR₂ AP (SLIGKV-NH₂; 200 μ M) or PAR₂ fAP (2-furoyl-LIGRLO-NH₂; 200 μ M) 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 $\pm$ s.d. of duplicate samples and are representative of one of three separate experiments. *, $p < 0.05$.
(B) PAR₂ 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), PAR₂ fAP (2-furoyl-LIGRLO-NH₂; 200 μ M), LPS (10 ng ml⁻¹), or both. Relative IL-10 and IL-12 p40 gene expression was analyzed by qPCR. Data are presented as the mean $\pm$ s.d. and are representative of one of five separate

experiments, each with similar outcomes. *, $p < 0.05$. (C) *PAR₂ 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 percent change *versus* the response to LPS only for each gene at each time point. Data represent the average of 3 separate experiments. (D) *PAR₂ 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), PAR₂ fAP; 200 μ M), LPS (10 ng ml⁻¹), or both. Relative IL-10 and IL-12 p40 gene expression was analyzed by qPCR as described in the legend to Figure 2B. Data are presented as the mean $\pm$ s.d. and are derived from a representative experiment (N = 2), each with similar outcomes. *, $p < 0.05$.

Figure 3. PAR₂ 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, PAR₂ AP (SLIGKV-NH₂; 500 μ M), LPS (25 μ g), or both. At the indicated times, livers were harvested, and relative gene expression was analyzed by qPCR. The results are the mean $\pm$ s.e.m (n = 3 for each group at each time point) of a representative experiment (N = 2). *, $p < 0.05$.

Figure 4. $PAR_2^{-/-}$ macrophages exhibit normal expression of macrophage markers, but altered responsiveness to LPS

(A) WT and $PAR_2^{-/-}$ macrophages express comparable levels of F4/80 and CD11b.

Thioglycollate-elicited peritoneal macrophages from WT and $PAR_2^{-/-}$ mice were stained for F4/80 and CD11b and analyzed by FACS analysis as described in the 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⁻¹) over the indicated time points. Relative gene expression was analyzed by qPCR. Data are presented as the mean $\pm$ s.d. 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 $PAR_2^{-/-}$ and C57BL/6J mice were stimulated with medium (M) or with LPS (100 ng ml⁻¹) 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 $\pm$ s.d. 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 WT C57BL/6J and PAR₂^{-/-} mice were stimulated with medium (M) or with LPS (10 ng ml⁻¹) 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. PAR₂ fAP synergistically augments rIL-4- or LPS-induced alternative activation of murine macrophages

Thioglycollate-elicited peritoneal macrophage from C57BL/6J mice were stimulated for 48 h with medium (M), PAR₂ fAP (2-furoyl-LIGRLO-NH₂; 200 μ M), recombinant IL-4 (5 ng ml⁻¹), LPS (10 ng ml⁻¹), or the indicated treatment combinations. Relative gene expression was analyzed by qPCR. Data are presented as the combined mean $\pm$ SEM in two separate experiments. *, $p < 0.05$.