The contribution of PARs to inflammation and immunity to fungi

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During inflammation, host- and microbial-derived proteases trigger the activation of protease-activated receptors (PARs), a family of G-protein-coupled receptors. We report here that activation of Toll-like receptors (TLRs) by fungi unmasks an essential and divergent role for PAR_1 and PAR_2 in downstream signaling and inflammation. TLRs activated PARs and triggered distinct signal transduction pathways involved in inflammation and immunity to *Candida albicans* and *Aspergillus fumigatus.* Inflammation was promoted by PAR_1 and PAR_2 activation in response to *Candida* and by PAR_2 inhibition in response to *Aspergillus*. This occurred by TLR regulation of PAR signaling, with TLR2 promoting PAR_1 activity, and TLR4 suppressing PAR_2 activity. Thus, tissue injury and pathogens induce signals that are integrated at the level of distinct TLR/PAR-dependent pathways, the exploitation or subversion of which contributes to divergence in microbial promotion of inflammatory response.

INTRODUCTION

Toll-like receptors (TLRs) control the inflammatory response to fungal infection, which is characteristic of innate immunity.[1] However, cooperation between innate immune receptors is of critical importance to regulating and shaping antimicrobial immunity.[2–4] During inflammation, host- or fungal-derived proteases are released into the extracellular environment.[5–7] Certain extracellular proteases can specifically cleave and trigger protease-activated receptors (PARs), a family of four G-protein-coupled receptors.[5,6] Thus, PARs are viewed as an integral component of the host antimicrobial alarm system capable of affecting host defense and immunity.[8] Four PARs have been cloned and they all share the same basic mechanism of activation: the proteolytic unmasking of a tethered peptide ligand resides in the receptor's N-terminal exodomain, and synthetic peptides that mimic this sequence function as agonists that activate PARs independent of receptor cleavage. Through their unique ability to sense serine proteases, such as thrombin, trypsin, and mast cell tryptase, PARs act as "sensors" of extracellular protease gradients.[5,6] Thrombin activates PAR_1, PAR_3, and PAR_4, whereas trypsin and mast cell tryptase activate PAR_2. However, certain microbial proteases can also activate mammalian PARs.[9] Activated PARs couple to signaling cascades that affect, among others, coagulation and inflammatory responses.[10] The role of PARs in inflammation is complex, as individual PARs have both proinflammatory and protective roles in the airway[11] and the gastrointestinal tract[6,12–15] as well as in the brain,[16] depending on disease context and cellular type.

Several observations suggest that PARs contribute to the host responses to fungal infections. First, serine proteases are activated when mannan lectins bind to fungi.[17] Second, fungal proteases, in common with PAR agonists (PAR-APs), initiate inflammatory responses.[18,19] Third, fungi are able to interact with cells and pathways of the coagulation cascade, as evidenced by the stimulation of tissue factor activity by *Aspergillus* conidia and the massive intravascular thrombosis at foci of *Aspergillus* infection.[20] However, the involvement of PARs in fungal infections has not been directly examined.

In the present study, we used PAR-APs, PAR antagonists (PAR-ANTs), and mice lacking or overexpressing PARs and TLRs to assess the PAR/TLR cross talk in infections caused by *Candida albicans* and *Aspergillus fumigatus,* two major fungal pathogens.[1] We found that PAR_1 and PAR_2 have opposing roles in governing the inflammatory response and pathology to *Aspergillus* or *Candida* and this occurred by delegation of different TLRs. Thus, the study identifies a previously unknown cross talk between PARs and TLRs in fungal infections.

RESULTS

Fungi regulate expression of PAR_1 and PAR_2 in PMNs *in vitro*

To establish whether fungal infections affect PAR expression, we studied polymorphonuclear neutrophils (PMNs) because they are essential for the initiation and execution of the acute inflammatory response to fungi and human PMNs express

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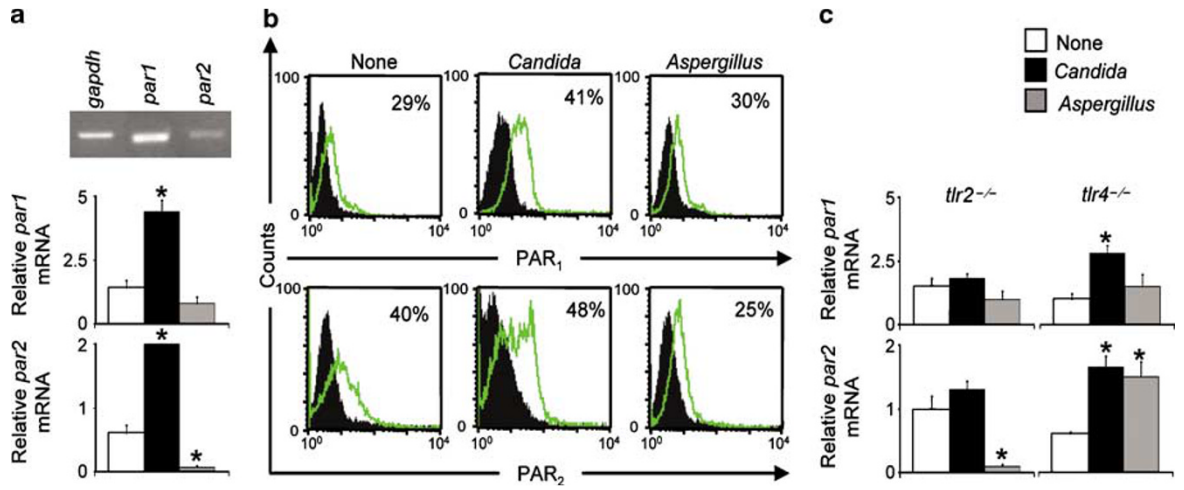


Figure 1 Fungi modulate *par1* and *par2* expression *in vitro*. (**a**) Purified Gr-1$^+$ PMNs from wild-type mice were stimulated with *Candida* yeasts or *Aspergillus* conidia for 30 min before *par1* and *par2* gene expression was assessed by real-time PCR. The *gapdh* mRNA-normalized data were expressed as relative *par* mRNA. *$P<0.05$, stimulated vs. unstimulated (none) cells. (**b**) Surface staining by flow cytometric analysis of PMNs stimulated as in **a**. Black histograms refer to cells stained with control antibody. Numbers refer to the percentage of positive cells over total cells analyzed. (**c**) *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ PMNs were stimulated and assessed as in **a**. Bars are s.e. *$P<0.05$, stimulated vs. unstimulated cells. PMN, polymorphonuclear neutrophil.

functional PAR_2.[21] We found that murine PMNs expressed both *par1* and *par2* mRNA and PAR_1 and PAR_2 proteins (**Figure 1a**,**b**). Exposure of PMNs to fungi differentially regulated PAR_1 and PAR_2 expression. *Candida* yeasts increased the expression of *par1* and *par2* mRNA and immunoreactivity, whereas *Aspergillus* conidia decreased *par2* expression (**Figure 1a**,**b**).

Fungi regulate PAR expression through TLR-dependent mechanisms

In mice, the fungicidal activity and inflammatory pathology of PMNs are strictly dependent upon distinct TLR signaling pathways.[22] As TLR2 and TLR4 exert distinct effects on the inflammatory responses of mice with candidiasis or aspergillosis,[23] we assessed the involvement of TLR2 and TLR4 in the modulation of *par* expression by fungi. *Candida* failed to increase *par1* and *par2* gene expression in *tlr2*$^{-/-}$ PMNs; *Aspergillus* instead increased *par2* expression in *tlr4*$^{-/-}$ PMNs (**Figure 1c**). These data demonstrate that fungi regulate expression of *par1* and *par2* in a manner that is differentially dependent upon TLR2 and TLR4.

Fungi regulate expression of *par1* and *par2 in vivo*

To assess whether modulation of *par* expression occur also *in vivo* during infection, we evaluated *par1* and *par2* expression in the stomach of mice with gastrointestinal candidiasis or the lungs of mice with pulmonary aspergillosis. Consistent with our *in vitro* findings, *par1* expression was increased in the stomach of mice with candidiasis, whereas *par2* expression was decreased in the lungs of mice with aspergillosis (**Figure 2**). However, *par2* was not upregulated in the stomach of *Candida*-infected mice as it was in isolated PMNs. The expression of *par1* was not increased in *tlr2*$^{-/-}$ mice with candidiasis, and *par2* expression was upregulated in the lungs of *tlr4*$^{-/-}$ mice with aspergillosis (**Figure 2**). Thus, TLR signaling, in response to fungi, modulates *par* expression both *in vitro* and *in vivo,* even though the expression *in vivo* likely reflects higher levels of complexity.

Host proteases regulate the expression and function of PAR

Serine proteases from PMNs, such as elastase and cathepsin G, which are stored in and released from azurophilic granules, can cleave PAR_1 and PAR_2 at sites that disable these receptors, and may thereby act in an autocrine and paracrine fashion to downregulate PAR signaling.[24] Fungi control secretory responses of PMNs, including protease activity, through TLR signaling.[22,23] Therefore, we hypothesized that fungi, through TLR activation, stimulate the release of proteases from PMNs that cleave PAR_1 and PAR_2 and modulate their activity. Because *par1* was upregulated by *Candida* and *par2* downregulated by *Aspergillus*, we analyzed PAR_1 or PAR_2 activation in response to *Candida* or *Aspergillus*, respectively. To test the hypothesis, we analyzed calcium mobilization in HEK293 cells, which naturally express both PAR_1 and PAR_2,[25] exposed to the following stimuli: (i) elastase and cathepsin G; (ii) supernatants from wild type (WT) PMNs exposed to *Candida* or *Aspergillus* (each containing 20 ng ml^{-1} protease activity); and (iii) supernatants from *tlr2*$^{-/-}$ or *tlr4*$^{-/-}$ PMNs challenged with the above fungi. We subsequently stimulated HEK cells with thrombin or trypsin and measured $[Ca^{2+}]_i$ and receptor surface expression to assess the activation of PAR_1 or PAR_2, respectively. We found that (i) thrombin and trypsin stimulated a prompt and transient increase in $[Ca^{2+}]_i$ in untreated HEK293 cells, which suggests activation of PAR_1 and PAR_2 (**Figure 2a**); (ii) the combination of elastase and cathepsin G also increased $[Ca^{2+}]_i$ and this treatment had no effect on responses to thrombin (**Figure 3a**, upper panel) but abolished responses to trypsin (**Figure 3a**, lower panel), a finding in line with the PAR_2 disarming ability of neutrophil serine proteases;[24] (iii) supernatants from WT, *tlr2*$^{-/-}$, and *tlr4*$^{-/-}$ PMNs also increased $[Ca^{2+}]_i$, although we do not know whether this response is mediated by PARs or

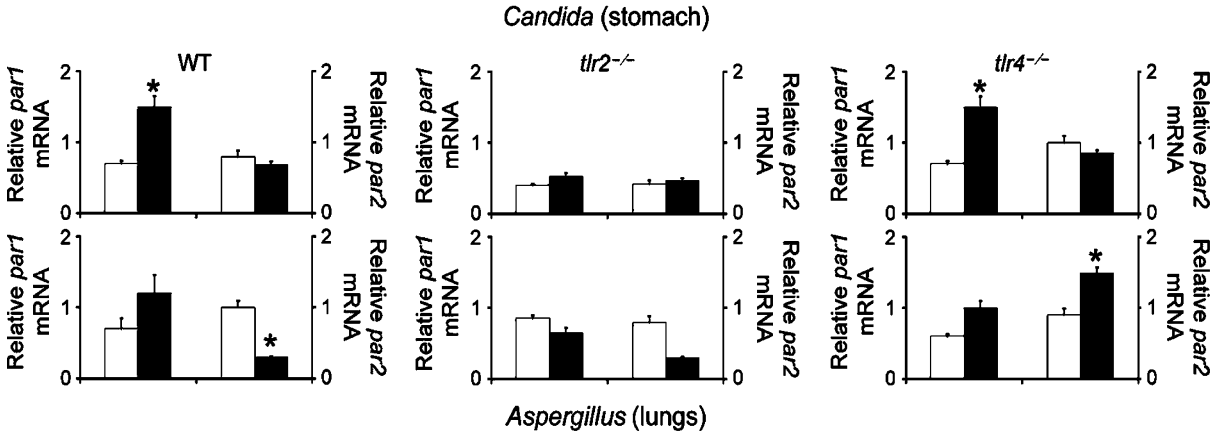


Figure 2 Fungi modulate *par1* and *par2* expression *in vivo*. *par1* and *par2* gene expressions were evaluated by real-time PCR in the stomach or lungs of wild type (WT) C57BL6 (n=6), *tlr2*$^{-/-}$ (n=4), or *tlr4*$^{-/-}$ (n=4) mice infected with 10^8 *Candida* yeasts intragastrically or 1×10^8 *Aspergillus* conidia intratracheally, respectively, 4 days before. The results are expressed as in **Figure 1**. (□) uninfected and (■) infected mice. *P<0.05, infected vs. uninfected.

other mechanisms; and (iv) pre-exposure to supernatants from *Candida*-exposed PMNs, either WT or TLR-deficient, did not affect thrombin signaling (**Figure 3a**, upper panel), but supernatants from *Aspergillus*-stimulated WT and *tlr2*$^{-/-}$, but not *tlr4*$^{-/-}$, PMNs, prevented trypsin signaling (**Figure 3a**, lower panel). Cytofluorimetric analysis confirmed the disparate activity of the different supernatants on PAR activation. Exposure to thrombin or trypsin reduced the levels of immunoreactive PAR_1 and PAR_2 at the cell surface, which indicates cleavage, activation, and internalization of these receptors (**Figure 3b**). The supernatant alone from *Candida*-exposed PMNs increased the surface expression of PAR_1, a finding suggestive of receptor exocytosis (**Figure 3b**, top panel). Subsequent exposure to thrombin decreased the immunoreactivity of PAR_1 at the cell surface, indicating an intact mechanism of receptor activation. In contrast, subsequent exposure to trypsin did not cause a decrease in immunoreactivity of PAR_2 at the cell surface after treatment with supernatant from *Aspergillus*-exposed PMNs, suggesting that the supernatant had prevented PAR_2 activation (**Figure 3b**, lower panel). Together, these results indicate that fungi activate PMNs in a TLR-dependent fashion to release factors that modulate the activity of PAR_1 and PAR_2.

Fungal proteases contribute to PAR activation

As both fungi secrete a variety of proteases *in vivo* and *in vitro*,[7] and microbial proteases are known to activate PAR receptors,[9] we assessed whether the ability of *Aspergillus* culture supernatant to inhibit trypsin signaling was sensitive to protease inhibition. For this purpose, supernatants from both *Candida* and *Aspergillus* were treated with protease inhibitors and used to assess $[Ca^{2+}]_i$ mobilization in HEK293 in response to thrombin and trypsin, respectively. The results show that, while treatment with protease inhibitors did not modify the activity of *Candida* culture supernatants, it abolished the ability of supernatants from *Aspergillus* cultures to inhibit trypsin signaling (**Figure 3c**). These findings suggest that *Aspergillus* proteases are involved in PAR_2 deactivation. Recombinant fungal proteases (the *Aspergillus* serine protease Aspf18 and *Candida* aspartyl protease SAP2) failed to show any activity (data not shown). Further studies in HEK293 cells exposed to *Candida* supernatant or SAP2 revealed that neither stimuli affected *par* mRNA expression. With *Aspergillus*, despite the ability to inhibit trypsin signaling, culture supernatant did not affect either PAR expression while, similar to other protease allergens,[26] Aspf18 downregulated the expression of *par1* (**Supplementary Figure S1** online). Thus, *Candida* activates PAR_1 and PAR_2 by a TLR2-dependent mechanism, an effect that is not mediated by fungal proteases, while *Aspergillus* proteases may contribute to the downregulation of trypsin-induced activation of PAR_2, which occurs by a TLR4-dependent mechanism.

PAR signaling depends on the presence of TLRs

To investigate the contribution of TLRs to PAR signaling and *vice versa*, we first evaluated the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated kinases (MAPKs) as well as NF-κB in WT PMNs exposed to PAR-APs or PAR-ANTs, as these pathways are known to be involved in PAR[5,6] and TLR[27,28] signaling and to mediate PMN antimicrobial effector functions.[29] We also studied *tlr2*$^{-/-}$ or *tlr4*$^{-/-}$ PMNs and PMNs lacking myeloid differentiation primary-response protein 88 (MyD88$^{-/-}$), since TLRs couple ligand binding to cell activation through members of the MyD88 family.[27,28] **Figure 4a** shows that PAR_1-AP and PAR_2-AP-stimulated, and the corresponding antagonists prevented ERK1/2 and p38 phosphorylation and NF-κB activation in WT PMNs. PAR_1-AP and PAR_2-AP did not phosphorylate ERK1/2 or activate NF-κB in *tlr2*$^{-/-}$ or *myd88*$^{-/-}$ PMNs, whereas *tlr4*$^{-/-}$ PMNs, despite a strong basal level of phosphorylation, responded normally (**Figure 4b**). In contrast, p38 phosphorylation by both PAR agonists was totally abolished in *tlr4*$^{-/-}$ PMNs and retained in *tlr2*$^{-/-}$ or *myd88*$^{-/-}$ PMNs (**Figure 4b**). Because phorbol esters stimulated phosphorylation of ERK1/2 and p38 similarly in TLR-deficient PMNs (**Figure 4c**), this indicates that these signaling pathways are not intrinsically defective in TLR- or MyD88-deficient cells. Therefore, the downstream pathways activated by PARs in PMNs depend

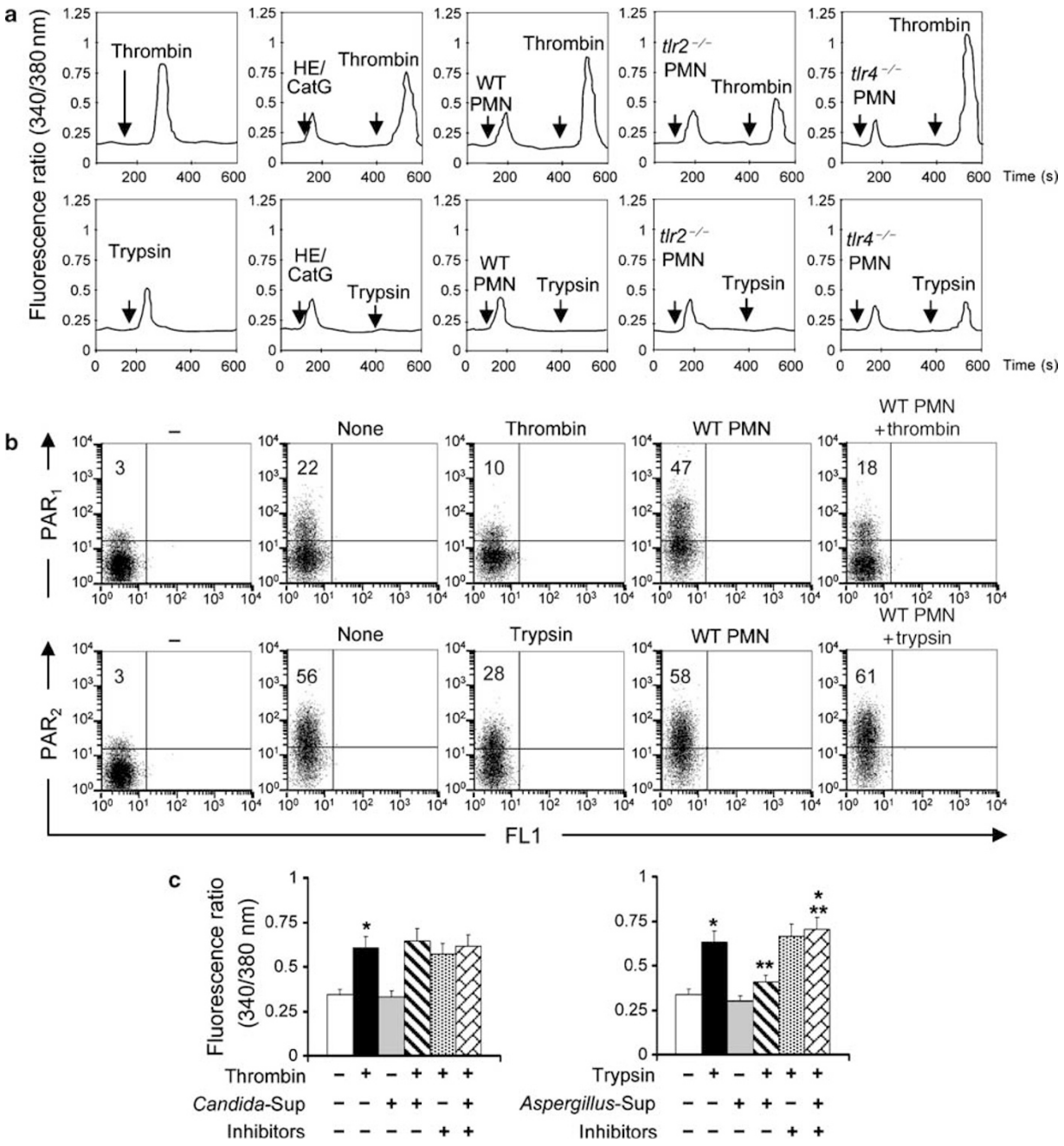


Figure 3 Host and fungal proteases regulate the expression and function of PARs. (**a**) Calcium mobilization in HEK293 cells pretreated (arrows) with elastase E + catepsin G (HE/CatG, each at 600 nM) or supernatants from degranulated WT (20 ng ml^{-1} of protease), *tlr2*$^{-/-}$ or *tlr4*$^{-/-}$ PMNs exposed to *Candida* (upper panel) or *Aspergillus* (lower panel), and stimulated (arrows) with thrombin or trypsin, 4 min later. The results, representative of three experiments, are expressed as 340/380 nm ratio. (**b**) Surface expression of PAR_1 or PAR_2 in HEK293 cells unexposed (none) or exposed to thrombin or trypsin alone or after preincubation with supernatants from WT PMNs exposed to *Candida* (upper panel) or *Aspergillus* (lower panel). (–), cells stained with irrelevant Ab. Numbers refer to the percentage of positive cells over total cells analyzed. (**c**) Calcium mobilization in HEK293 cells pretreated with *Aspergillus* or *Candida* culture supernatants (Sup, 50 μl containing 50 ng of protease activity) and stimulated with thrombin (3 U ml^{-1}) or trypsin (100 nM), 4 min later. For inhibitors, culture supernatants were preincubated with the serine protease inhibitor, phenylmethylsulfonyl fluoride, and the cathepsin B inhibitor, leupeptin. Data are the mean±s.e. from two independent experiments. Bars are s.e. *P<0.05, thrombin- or trypsin-stimulated vs. unstimulated cells; **P<0.05, trypsin + *Aspergillus*-Sup vs. trypsin alone. ***P<0.05, trypsin + *Aspergillus*-Sup, with and without inhibitors. Ab, antibody; PAR, protease-activated receptor; PMN, polymorphonuclear neutrophil; WT, wild type.

upon distinct TLR signaling. Agonists of PAR_1 and PAR_2 activate either ERK1/2 and NF-κB in a TLR2-/MyD88-dependent manner or p38 in a TLR4-dependent but MyD88-independent manner. As a matter of fact, the basal level of p38 phosphorylation was undetectable in *tlr4*$^{-/-}$ PMNs. This finding is consistent with the existence of divergent signaling pathways, originating upon TLR4 activation,[30,31] which control MAPK activity.[32]

TLR signaling depends on the presence of PARs

The above results showed how the activation of PARs depends upon TLR signaling. To assess instead how PARs contribute to TLR signaling, we silenced the expression of *par1* and *par2* using short interfering RNA (siRNA) in TLR2- or TLR4-transfected HEK293 cells, respectively, and assessed the effects of the TLR2 ligand, zymosan, or the TLR4 ligand, lipopolysaccharide, on activation of NF-κB. While the levels of the p65 and p50 NF-κB proteins were unmodified by siRNA treatments (**Figure 4d**), zymosan promoted NF-κB activation in TLR2-transfected cells and *par1* silencing inhibited this activation (**Figure 4d**). In contrast, lipopolysaccharide also activated NF-κB in TLR4-transfected cells, but *par2* silencing further increased this stimulation (**Figure 4d**). These data show that downstream pathways activated by TLRs depend on the expression of *par1* and *par2*. Altogether, the data indicate that PAR signaling depends on the expression of TLRs and *vice versa*.

Signaling pathways activated by PARs are subverted by fungi

Given that fungi modulate PAR expression in infection *in vitro* and *in vivo*, we assessed whether signaling pathways activated by either PAR are also subverted by fungi. For this purpose, we sequentially exposed WT PMNs to fungi and PAR-APs. **Figure 4e** shows that stimulation with *Candida* and either PAR-AP markedly enhanced ERK1/2 phosphorylation and NF-κB activation. However, disparate results were obtained by concomitant stimulation with *Aspergillus*. ERK1/2 phosphorylation and NF-κB activation were still promoted by PAR_1-AP but inhibited by PAR_2-AP. Interestingly, PAR_2-AP markedly enhanced p38 phosphorylation. As activation of p38 has been associated with inhibition of NF-κB activation,[33] we assessed whether SB202190, a highly specific p38 MAPK inhibitor,[33] could restore ERK1/2 and NF-κB activation by PAR_2-AP. The results showed that p38 inhibition was associated with restored ERK1/2 phosphorylation and NF-κB activation (**Figure 4e**), a finding implicating p38 in the inhibitory action of PAR_2 in the presence of the fungus. Experiments in *myd88*$^{-/-}$ PMNs confirmed that ERK1/2 phosphorylation and NF-κB activation occurred in an MyD88-dependent pathway and p38 phosphorylation in an MyD88-independent pathway (**Figure 4e**). Therefore, PAR_1 and PAR_2 activate distinct pathways in response to fungi. Consistent with the effects of the fungus on PAR_1 expression, PAR_1-AP promoted *Candida*-induced ERK1/2 and NF-κB activation, which occurs by a TLR2- and MyD88-dependent mechanism. In contrast, PAR_2 had divergent effects depending on the fungus. PAR_2-AP enhances *Candida*-induced activation of ERK1/2 and NF-κB but suppresses these pathways in response to *Aspergillus* through a p38-dependent mechanism.

Divergent role of PAR_1 and PAR_2 in PMNs' inflammatory response

Further studies showed that the agonistic and antagonistic effects of PAR_1 and PAR_2 on fungal-induced TLR signaling elicited distinct inflammatory responses in PMNs. Consistent with the pattern of ERK1/2 activation, PAR_1-AP increased (by 25–30%) and PAR_1-ANT decreased the respiratory burst of WT PMNs in response to both fungi, but particularly to *Candida* (80% inhibition compared to 30% to *Aspergillus*) (**Figure 4f**). PAR_2-AP greatly inhibited the respiratory burst (by ~60%) in response to *Aspergillus* but not to *Candida,* and the PAR_2-ANT did the opposite (**Figure 4f**). In terms of degranulation, PAR_1-AP enhanced the production of matrix metalloproteinase 9 (MMP9) in response to *Candida* and *Aspergillus*, and PAR_2-AP dampened it in response to *Aspergillus* (**Figure 4f**). PAR_1 agonistic activity was lost in *tlr2*$^{-/-}$ and *myd88*$^{-/-}$ PMNs and retained in *tlr4*$^{-/-}$ PMNs, whereas the PAR_2 inhibitory activity was retained in *tlr2*$^{-/-}$ and *myd88*$^{-/-}$ PMNs and lost in *tlr4*$^{-/-}$ PMNs (**Figure 4g**).

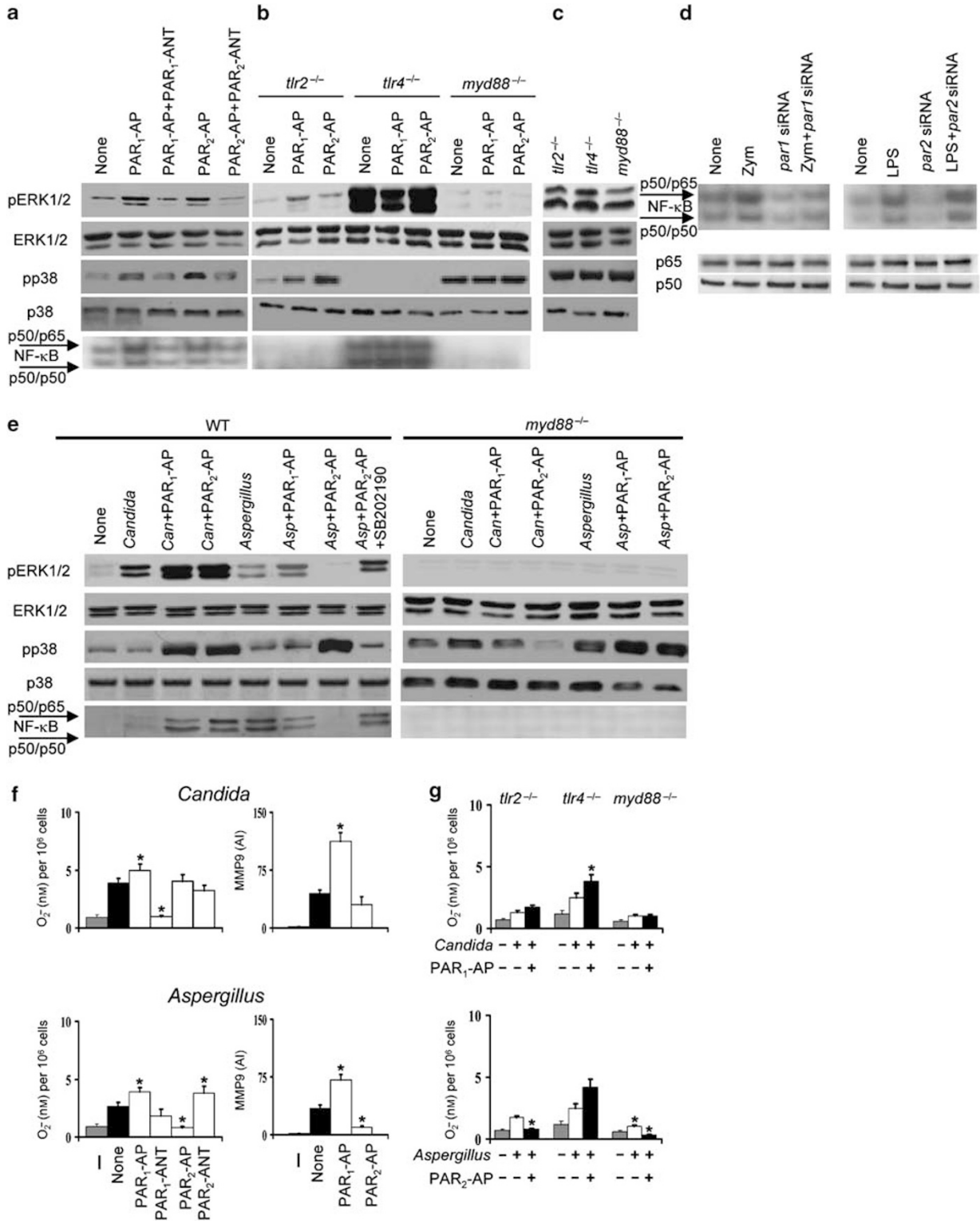
Considered together, these results suggest the existence of functional interactions between TLRs and PARs that modulate inflammatory signaling. The coengagement by TLRs and PARs of downstream adaptor modules may explain the proinflammatory role of PAR_1 and the anti-inflammatory role of PAR_2. If extrapolated to host responses in infections, this will predict that (i) PAR_1 promotes the inflammatory response to *Candida*, which is contingent upon TLR2 activation; (ii) PAR_2 dampens inflammation to *Aspergillus* in a TLR4-dependent fashion; and (iii) PAR_2 shows disparate effects in inflammation and immunity to each fungus. These predictions were all confirmed by the following *in vivo* studies.

PAR_1 promotes inflammation and immunity to *Candida* through TLR2

Wild type and *tlr2*$^{-/-}$ mice were infected with *C. albicans* and treated with selective agonists or antagonists of PAR_1, which

Figure 4 Distinct signal transduction pathways are activated in PMNs by PAR_1 and PAR_2 and are subverted by fungi. WT (**a**), *tlr2*$^{-/-}$, *tlr4*$^{-/-}$, or *myd88*$^{-/-}$ (**b**) PMNs were stimulated with PAR agonists (PAR-APs) with and without antagonists (PAR-ANTs) for 30 min. Blots of cell lysates were incubated with rabbit anti-ERK or phosphorylated (p) ERK and anti-p38 or pp38 MAPK antibodies. None, cells left unstimulated for 30 min. (**c**) PMNs stimulated with phorbol 12-myristate β-acetate for 30 min. (**b**) Zymosan was used to stimulate CD14/TLR2/HEK293 cells transfected with *par1*siRNA and LPS to stimulate CD14/TLR4/HEK293 cells transfected with *par2* siRNA. EMSA was performed 2 h later. The specificity of bands was verified by using antibodies to p65 and p50. (**e**) WT or *myd88*$^{-/-}$ PMNs were sequentially exposed to fungi (30 min) and PAR-AP (30 min) before blotting (none, unstimulated cells). Cells were pre-exposed to the p38 inhibitor SB202190 (10 μM) for 60 min before stimulation with *Aspergillus* + PAR_2-AP. Initial experiments have shown that MAPK phosphorylation in response to agonists or fungi alone was optimal at 30 min and declined at 60 min (**Supplementary Figure S2** online). (**f**) Antagonistic effects of PAR_1-AP and PAR_2-AP on the respiratory burst (O_2^-) and matrix metalloproteinase 9 (MMP9) production of PMNs in response to *Candida* or *Aspergillus*. PMNs were stimulated with PAR agonists/antagonists and fungi as above. (AI, arbitrary index of scanning densitometry) **P*<0.05, treated vs. untreated PMNs. –, unstimulated cells. (**g**) PMNs from *tlr2*$^{-/-}$, *tlr4*$^{-/-}$, and m*yd88*$^{-/-}$ mice were exposed to PAR agonists and fungi and assessed for oxidant production as above. Bars are s.e. **P*<0.05, treated vs. untreated. EMSA, electrophoretic mobility shift assay; LPS, lipopolysaccharide; MAPK, mitogen-activated kinase; PAR, protease-activated receptor; PMN, polymorphonuclear neutrophil; WT, wild type.

have been shown to modulate PAR_1 function in mice with colitis.[34] PAR_1-AP exacerbated and PAR_1-ANT attenuated the inflammatory pathology to the fungus, as revealed by signs of acanthosis, parakeratosis, recruitment of inflammatory cells, and local production of oxidants and MMP9 in the stomach of infected WT mice (**Figure 5a**). Treatment with PAR_1-AP was also associated with a significant increase of fungal growth (from 2.1×10^4 to 3.9×10^4 CFU, $^*P < 0.05$, untreated vs. treated mice). Consistent with the low level of *par1* expression (**Figure 2**), local inflammatory and secretory responses were not increased in the stomach of infected *tlr2*$^{-/-}$ mice as compared to WT mice and were not modified upon treatments (**Figure 5b**). Interestingly, both treatments oppositely affected parameters of adaptive immunity to the fungus, such as the interleukin



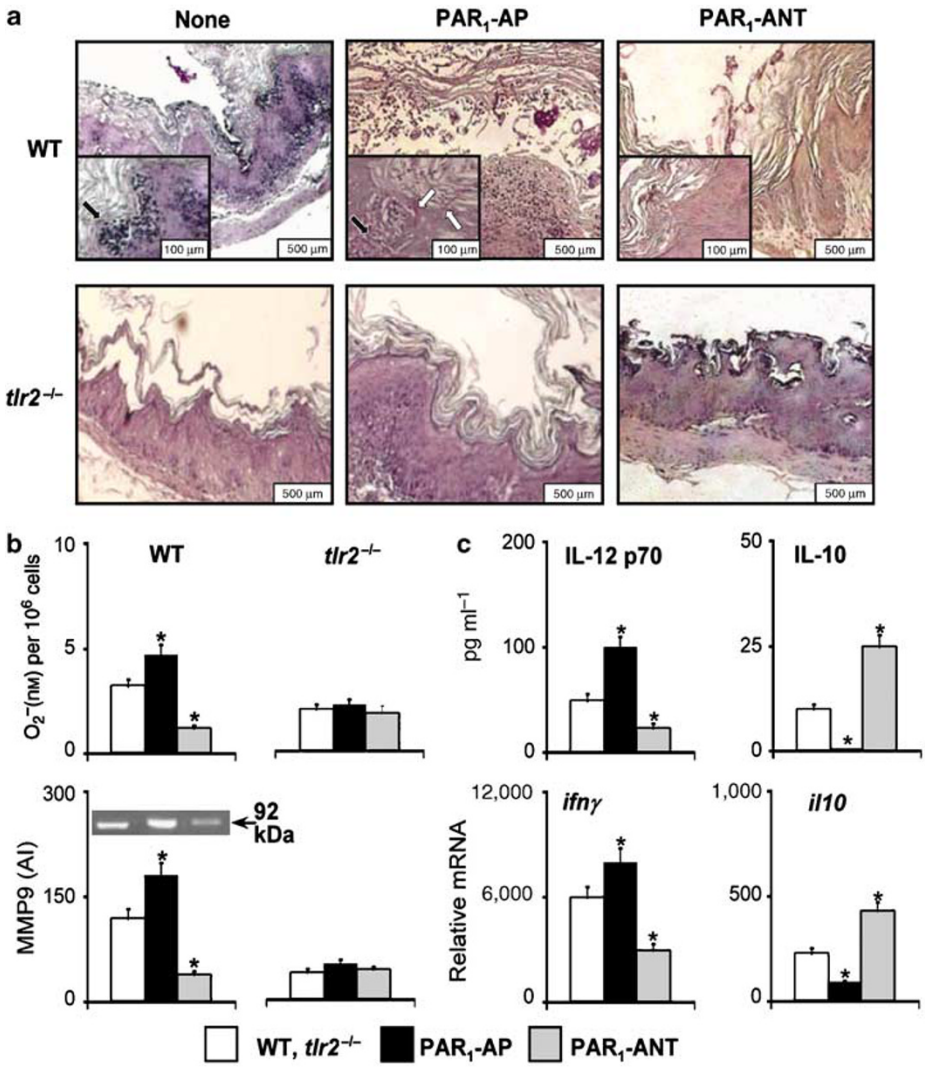


Figure 5 PAR_1 promotes inflammation and immunity to *Candida* through TLR2. WT C57BL6 or $tlr2^{-/-}$ (**a**) mice ($n=8$, each group) were infected with *C. albicans* and treated with PAR_1 agonists or antagonists (PAR_1-AP and PAR_1-ANT) as described in Methods. Periodic acid Schiff staining of sections from stomachs (6 days after the infection) shows the presence of fungal elements (white arrows, inset) and inflammatory cells (black arrows, inset). Bars indicate magnification. One representative of three experiments is shown. (**b**) The production of oxidant (O_2^-) and MMP9 was assessed in the lungs or stomach homogenates of infected mice (AU, arbitrary index of scannin densitometry). The bands show the active 92 kDa MMP9. Levels of O_2^- and MMP9 in uninfected controls were undetectable. (**c**) Cytokines (pg ml^{-1}) were measured by enzyme-linked immunosorbant assay in culture supernatants (24 h) of dendritic cells isolated from Peyer's patches of uninfected mice and pulsed with live yeasts *in vitro* for 2 h, before the addition of amphotericin B to prevent fungal overgrowth[35] in the presence of 3×10^{-5} M PAR_1-AP and PAR_1-ANT. Bars are s.e. *ifnγ* and *il10* gene expression was assessed by real-time PCR on $CD4^+$ T cells purified from mesenteric lymph nodes a week after infection.[35] *$P<0.05$, treated vs. untreated. MMP9, matrix metalloproteinase 9; PAR, protease-activated receptor; PMN, polymorphonuclear neutrophil; WT, wild type.

(IL)-12/IL-10 production by Peyer's patch dendritic cells, known to be regulated by PAR signaling,[8] and the pattern of *ifnγ* or *il10* gene expression in $CD4^+$ T cells from mesenteric lymph nodes of WT mice. PAR_1-AP promoted the IL-12/Th1 response and PAR_1-ANT promoted the IL-10^+ T regulatory cell response[35] to the fungus (**Figure 5c**). Thus, the upregulated expression of PAR_1 in candidiasis correlates with the occurrence of local and adaptive inflammatory responses. Similar treatments of mice with aspergillosis revealed that PAR_1-AP exacerbated the lung inflammatory pathology and the local oxidant/MMP9 production, which were largely unaffected by treatment with PAR_1-ANT (**Supplementary Figure S3** online).

PAR_2 attenuates inflammation and immunity to *Aspergillus* through TLR4

Wild type and $tlr4^{-/-}$ mice were infected with *A. fumigatus* and treated with agonists and antagonists of PAR_2 *in vivo*.[12] PAR_2-ANT exacerbated and PAR_2-AP slightly decreased the inflammatory response to *Aspergillus* in WT mice. The number of abscesses consisting of inflammatory cells associated with signs of parenchymal destruction and the oxidant/MMP9 production were both higher in the lungs after treatment with PAR_2-ANT (**Figure 6a**). Although the high level expression of *par2* (**Figure 2**) would predict a low inflammatory and secretory response in the lungs of infected $tlr4^{-/-}$ mice, foci of inflammation

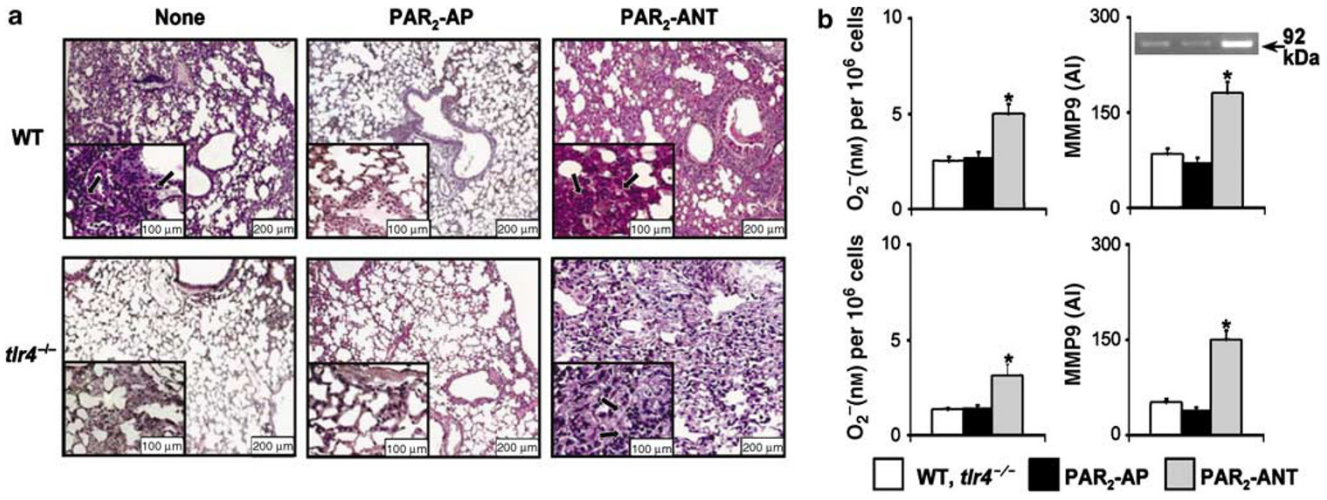


Figure 6 PAR_2 attenuates inflammation and immunity to *Aspergillus* through TLR4. WT C57BL6 or $tlr4^{-/-}$ (**a**) mice ($n=8$, each group) were infected with *A. fumigatus* and treated with PAR_2 agonists or antagonists (PAR_2-AP and PAR_2-ANT) as described in Methods. Periodic acid Schiff staining of sections from lungs (4 days after the infection) showing the presence of inflammatory cells (black arrows, inset). Bars indicate magnification. One representative of three experiments is shown. The production of oxidant (O_2^-) and MMP9 (**b**) was assessed in the lungs or stomach homogenates of infected mice (AU, arbitrary index of scanning densitometry). The bands show the active 92 kDa MMP9. Levels of O_2^- and MMP9 in uninfected controls were undetectable. $^*P<0.05$, treated vs. untreated. MMP9, matrix metalloproteinase 9; WT, wild type.

(inset of **Figure 6a**) could be detected, a finding confirming the contribution of TLR2 to inflammation in aspergillosis.[22,23] However, both inflammatory and secretory responses were augmented by PAR_2-ANT and left unaffected by PAR_2-AP (**Figure 6b**), a finding suggesting that PAR2 negatively regulates inflammation in pulmonary aspergillosis. Since *par2* expression was downregulated by *Aspergillus* through TLR4, our findings suggest that inhibition of PAR_2 is a mechanism through which host anti-inflammatory pathways could be subverted to promote inflammation in aspergillosis.

Divergent role of PAR_2 in inflammation and immunity to fungi *in vivo*

Studies in mice with genetic deficiency of ($par2^{-/-}$) or overexpressing (*par2*-Tg) *par2* confirmed that PAR_2 perturbations mainly affected inflammation and immunity to *Aspergillus* but not to *Candida*. PAR_2 deficiency greatly exacerbated fungal growth (from 2.5×10^4 CFU of WT to 2.2×10^6 CFU of $par2^{-/-}$) and the inflammatory pathology in the lungs of mice with aspergillosis but not in the stomach of mice with candidiasis. Numerous abscesses and fungal elements as well as severe signs of parenchymal destruction were present in the lungs of mice with aspergillosis but not in the stomach of mice with candidiasis (**Figure 7a**). In contrast, the inflammatory pathology and fungal growth were both significantly reduced, compared to WT mice, in lungs (from 2.5×10^4 to 1.0×10^4 CFU, $^*P<0.05$, *par2*-Tg vs. WT) and stomach (from 2.9×10^4 to 1.8×10^4 CFU, $P<0.05$, *par2*-Tg vs. WT) of *par2*-Tg mice with aspergillosis or candidiasis, respectively (**Figure 7a**). Concomitantly, the local generation of oxidants and MMP9 was significantly increased in $par2^{-/-}$ mice with aspergillosis and impaired in *par2*-Tg mice with either infection (**Figure 7b**). Interestingly, oxidant generation by $par2^{-/-}$ PMNs was greatly decreased upon exposure to PAR_1-ANT (inset), a finding indicating a possible reciprocal PAR regulation. PAR_2 also affected the production of cytokines known to reflect the innate immune response to *Aspergillus in vivo*.[1] Tumor necrosis factor-α production was increased and IL-10 production decreased in lung homogenates from infected $par2^{-/-}$ mice; the opposite pattern was observed in *par2*-Tg mice (**Figure 7c**). As a whole, these data suggest that PAR_1 and PAR_2 serve an opposite role in governing the inflammatory and immune responses to each fungus and that the pattern of PAR_1 and PAR_2 expression at sites of infection may contribute to the occurrence of local inflammation.

DISCUSSION

Our results reveal novel interactions between TLRs and PARs that contribute to signal diversity in inflammation and host antimicrobial responses to fungal infections. PAR_1 has a dominant role in determining inflammation and Th1 immunity to fungi upon which PAR_2 may exert an inhibitory control. These effects may depend on the functional interactions between PARs and TLRs, which control inflammatory signaling of PMNs. Because PAR_1 or PAR_2 has disparate effects on the fungicidal activity of PMNs (unpublished data), a combined action of PARs on inflammation and fungicidal activity may account for disparate inflammatory pathology and fungal burdens in conditions of either PAR deficiency or overexpression.

The PAR–TLR interactions exhibit distinct features. First, fungi differentially regulate PAR expression through TLR2 and TLR4, both in PMNs *in vitro* and in the stomach and lungs of infected mice. Second, proteases released from PMNs, in a TLR-dependent manner, and fungal proteases can cleave PARs and alter their capacity to signal. Third, PMN TLRs are required for PAR activation of downstream signaling pathways, and *vice versa*. Finally, the inflammatory responses of infected animals are dramatically altered by pharmacological (agonism and antagonism) or genetic (deletion and overexpression)

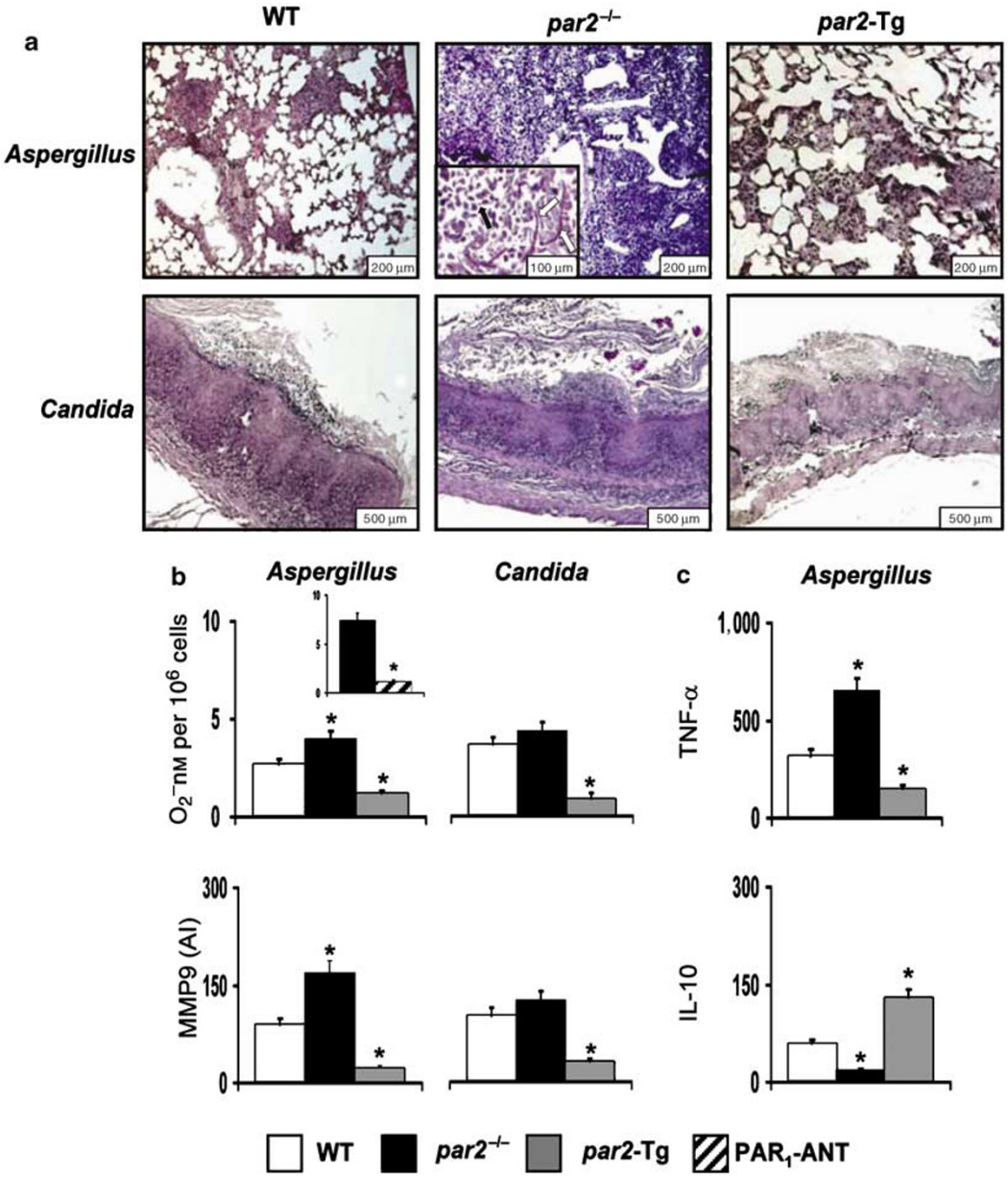


Figure 7 Divergent role of PAR_2 in *A. fumigatus* or *C. albicans* infection. WT C57BL6 ($n=12$), PAR_2-deficient ($par2^{-/-}$) ($n=8$) or overexpressing (*par2*-Tg) ($n=8$) mice were infected as described in the legend of **Figure 2**. (**a**) Histologic analysis of lungs (4 days after the *Aspergillus* infection) showing signs of exaggerated inflammatory reaction (black arrows, inset), fungal growth (white arrows, inset), and parenchymal destruction in $par2^{-/-}$ mice and attenuation in *par2*-Tg mice. Histological analysis of the stomach (6 days after the *Candida* infection) showed reduced inflammatory reaction in *par2*-Tg mice and no exacerbation in $par2^{-/-}$ mice. The tissue inflammatory pathology in response to either fungus was not different between WT C57BL6 and BALB/c mice (the corresponding WT of *par2*-Tg mice), and organs from uninfected $par2^{-/-}$ or *par2*-Tg mice showed no obvious signs of tissue alteration compared to WT mice (data not shown). Bars indicate magnification. One representative of three experiments is shown. (**b**) Oxidant (O_2^-) and MMP9 production were assessed in the lungs or stomach homogenates of infected mice (AU, arbitrary index of scanning densitometry). Shown in the inset is the O_2^- production *in vitro* of $par2^{-/-}$ PMNs in response to conidia upon exposure to PAR_1 antagonist. (**c**) Opposite pattern of TNF-α/IL-10 production in lung homogenates from $par2^{-/-}$ or *par2*-Tg mice. Levels of cytokines (pg ml^{-1}, by enzyme-linked immunosorbant assay) in lung homogenates from uninfected mice were comparable among groups. *$P<0.05$, $par2^{-/-}$ or *par2*-Tg vs. WT mice and treated vs. untreated PMNs. IL-10, interleukin 10; MMP9, matrix metalloproteinase 9; PAR, protease-activated receptor; PMN, polymorphonuclear neutrophil; TNF-α, tumor necrosis factor-α; WT, wild type.

manipulation of PARs. We have thus characterized a novel level of cooperation between innate immune receptors in infections, which may establish a new paradigm of recognition at the fungus–host interface. After microbial recognition by TLRs, PARs may become activated to sense tissue injury, mediate inflammatory responses, and modulate the activity of TLRs. Thus, fungi recognition by TLRs may be licensed by damage-associated molecular pattern recognition of the host, as has been suggested.[36] Therefore, proteolytic events associated with PARs may be the missing "activator" of mammalian TLRs, for which

no extracellular proteolytic events have been demonstrated upstream of the receptor.[30,31] These events identify an indirect mode of non-self recognition that has been recently described in the mechanics of the perception system of plants.[37,38] Indeed, a dual-sensor system to detect fungal infection seems to work throughout evolution,[39] because *Drosophila* senses fungi by sensing both the fungal cell wall and the activity of proteolytic virulence factors.[40]

Several possible mechanisms have been described that may underlie the potential for receptor transactivation within the PAR or TLR family.[3,6] Our results suggest that TLR2 implicates PAR_1 and MAPK to activate the PMN's inflammatory pathway to *Candida*. Conversely, PAR_2 is apparently deactivated on PMNs by *Aspergillus* through TLR4. There is a good precedent of TLR4-dependent desensitization of G-protein-coupled receptors on PMNs.[41] PMN proteases triggered by fungi could be responsible for PAR_1 activation as well as PAR_2 deactivation, a finding consistent with the ability of proteases to contribute to fungal septic shock through vascular damage and plasma leakage associated with tissue destruction in the lungs.[42] TLR4 and TLR2 induced distinct patterns of degranulation against either fungus,[22] and degranulated PMNs from TLR2- or TLR4-deficient PMNs have disparate activity on PAR functioning. It is therefore conceivable that an action on the protease/antiprotease balance may contribute to the ability of TLRs to condition heterologous or homologous PAR activation/desensitization at the infectious site. Interestingly, PAR activity was differently modulated by PMN degranulated in response to fungal hyphae more than conidia or yeasts (data not shown), a finding linking fungal morphogenesis to virulence through host inflammatory responses.[1]

The subversion of the host p38-dependent anti-inflammatory pathway by *Aspergillus* implies that the host anti-inflammatory pathways could be exploited therapeutically to attenuate signs of inflammatory pathology in fungal infections and sepsis. Not only is p38 a critically important mediator in the activation of Interferon regulatory factor 3,[30,31] a transcription factor of the MyD88-independent pathway associated with TLR4 signaling, but also there is mounting evidence for a negative cross-signaling between this and other MAPK pathways in inflammation.[33] p38MAPK has a central role in the activation of homeostatic cyclooxygenase-2 in the airways[11] and in the inhibition of NF-κB activation by salicylates.[43] Thus, PAR_2 agonists will share with salicylates a common mechanism of anti-inflammatory action and, at the same time, will rescue the host from toxicity associated with glucocorticoids, which are known to antagonize both p38MAPK[44] and PAR_2.[19]

The ability of PARs and TLRs to have *cis*- and *trans*-interactions with other receptors as well as the redundancy in their signaling pathways[6] precludes a definite mechanistic view of events regulating the inflammatory response at the sites of infection. However, our study is consistent with a model in which the inflammatory response is regulated by positive or negative signals that originated from the TLR2/PAR_1- or the TLR4/PAR_2-dependent pathway, the relative contribution of each receptor pair being dependent on the fungus

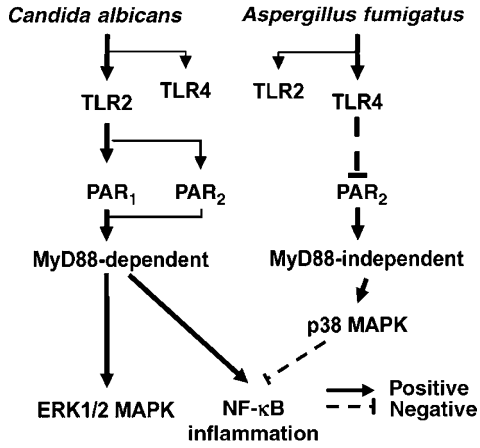


Figure 8 Cross talk between PARs and TLRs in inflammation to fungi. Shown is a proposed model in which inflammation is promoted by PAR_1 and PAR_2 activation in response to *Candida* and by PAR_2 inhibition in response to *Aspergillus*. This occurs by TLR regulation of PAR signaling, with TLR2 promoting PAR_1 activity, and TLR4 suppressing PAR_2 activity. Thus, the exploitation or subversion of distinct TLR/PAR-dependent pathways contributes to divergence in the promotion of inflammatory response in fungal infections. PAR, protease-activated receptor; TLR, Toll-like receptor.

(**Figure 8**). Inflammation is promoted through a PAR_1/ERK/NF-κB-dependent pathway and inhibited through a PAR_2/p38-dependent pathway. This occurs by delegation from TLRs, since PAR_1 activity is promoted by TLR2 through the MyD88-dependent pathway and PAR_2 activity is promoted by TLR4 through an MyD88-independent pathway. The TLR2/PAR_1-dependent pathway was promoted by *Candida* but the TLR4/PAR_2-dependent pathway was subverted by *Aspergillus*, a finding indicating divergence in pathway exploitation by fungi in the promotion of host inflammatory response.

METHODS

Mice. Female C57BL6 and BALB/c mice (8–10 weeks old) were from Charles River (Calco, Italy). Breeding pairs of homozygous *tlr2*$^{-/-}$, *tlr4*$^{-/-}$,[23] and *par2*$^{-/-}$ mice, raised on a C57BL6 background and breeding pairs of mice overexpressing PAR_2 (*par2*-Tg) raised on a BALB/c background,[45] were bred under specific pathogen-free conditions at the breeding facilities of the University of Perugia, Italy. Experiments were performed according to the Italian Approved Animal Welfare Assurance A-3143-01.

PAR agonists and antagonists. Since proteases can activate multiple PARs and exert effects by many other mechanisms, synthetic peptides mimicking the tethered ligand domains of PARs (PAR-activating peptides, PAR-APs) are commonly used to selectively activate these receptors.[5,6] The tethered ligand of PAR_2 (SLIGRL-NH_2) and an analog of the tethered ligand of PAR_1 (Tfllr-NH_2), both of which selectively activate these receptors, were used as PAR-APs. Scrambled sequences, which do not activate these receptors (PAR-sAP), were used as controls.[12,34] PAR_1-ANT and PAR_2-ANT (ENMD-1068) were as described.[46,47]

Microorganisms, infections, and treatment. The strains of *C. albicans* and *A. fumigatus* have been described.[23] For protease production *in vitro*, 10^6 yeasts or conidia/ml were grown in YEPD broth (1% yeast extract, 2% peptone, 2% dextrose) or minimum essential medium with 10% fetal bovine serum, respectively, for 60 h, the time at which the

protease activity (QuantiCleave Protease Assay Kit, Pierce, Milan, Italy) of supernatant was 142 ng ml^{-1} (*Candida*) or 264 ng ml^{-1} (*Aspergillus*), respectively. The lyophilized supernatant was resuspended in RPMI before use. For *Aspergillus* infection, conidia were given intratracheally (1×10^8 per 20 μl saline) to mice immunosuppressed with cyclophosphamide (150 mg kg^{-1} intraperitoneally) 6 h before and anesthetized by intraperitoneally injection of 2.5% avertin (Sigma). For the gastrointestinal candidiasis, 10^8 *Candida* yeasts were administered by gavage.[23] PAR_2-AP and PAR_2-ANT (1.5 mg kg^{-1} 20 μl^{-1})[12,34] were administered intratracheally the day of the *Aspergillus* infection and intranasally twice daily for 2 days. In candidiasis, PAR_1-AP and PAR_1-ANT (1.5 mg kg^{-1} 200 μl^{-1}) were administered intraperitoneally the day of the infection and twice daily for 5 days. PAR agonists and antagonists were dissolved as described.[12,34] Neither PAR agonist nor antagonist modified the inflammatory status when injected into uninfected mice (data not shown). Vehicles alone had no effects on infections (data not shown). Fungal growth was quantified and tissues were stained for Periodic Acid Schiff as described.[22]

PMN stimulation, respiratory burst, and zymography. Purified Gr-1$^+$ PMNs (>98% pure on flow cytometric analysis) were obtained by positive selection from the peritoneal cavity of thioglycolate-injected mice.[22,23] PMNs were stimulated at 37 °C with 3×10^{-5} M PAR agonists and/or antagonists and/or live fungi (at a PMN:fungus ratio of 10:1) for 30 min for each single stimulation or for a total of 60 min in sequential stimulation for MAPK phosphorylation and NF-κB activation.[34,46] Production of oxidants was performed by quantifying the release of superoxide anion (O_2^-) through the measure of the superoxide dismutase-inhibitable reduction of cytochrome *c*.[22] Experiments were performed in triplicate and the results were expressed as nanomoles $O_2^-/10^6$ cells. Gelatinolytic activity of MMP9 was assessed by gelatin zymography and determined by scanning the lysis band in the 72-kD area using a BioRad Gel DOC 1000 imaging densitometer (BioRad, Milan, Italy). The protease activity (200 or 140 ng ml^{-1} in *Candida*- or *Aspergillus*-stimulated PMNs, respectively) was quantified by the QuantiCleave Protease Assay Kit (Pierce).

Flow cytometric analysis. For surface staining, PMNs were incubated with fungi for 30 min and HEK293 cells with human thrombin (3 U ml^{-1}) or trypsin (100 nM) (Sigma) for 10 min. HEK293 cells were also pre-exposed to supernatants (containing 20 ng ml^{-1} of protease) from fungus-exposed WT PMNs for 30 min, at the time at which peak activity was observed. Cells were stained with goat polyclonal C-18, recognizing thrombin receptor of both human and mouse origins, followed by rabbit anti-goat IgG-PE or with the anti-murine PAR_2 SAM11-PE antibody (all from Santa Cruz Biotechnology) raised against amino acids 37–50 of human PAR_2, and evaluated by the FACScan flow cytofluorometer (Becton Dickinson, Mountain View, CA) equipped with Lysis II software. Before surface staining, cells were incubated at room temperature with 5 μg of anti-FcγR mAb (2.4G2; PharMingen, Palo Alto, CA).

ERK/p38 MAPK phosphorylation and electrophoretic mobility shift assays. Extracellular signal-regulated kinase and p38 phosphorylation and NF-κB activation were assessed on 20×10^6 PMNs stimulated as above. Blots of cell lysates were incubated with rabbit polyclonal Abs recognizing the unphosphorylated form of ERK and p38 or the phospho-p38 MAPK (Thr180/Tyr182) and phospho-p44/42 MAPK (Thr202/Tyr294) antibodies (Cell Signaling Technology, Milan, Italy) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology), as per the manufacturer's instructions. Blots were developed with the Enhanced Chemiluminescence detection kit (Amersham Pharmacia Biotech, Milan, Italy). Cells were pre-exposed to the p38 inhibitor SB202190 (Calbiochem, San Diego, CA) at 10 μM in 0.1% dimethylsulphoxide for 60 min. For electrophoretic mobility shift assay (EMSA), the double-stranded probe containing an NF-κB consensus site 5-agttgaggggactttcccaggc-3 was terminally labeled with T4 PNK. The EMSA experimental reaction contains 10 μg of nuclear extracts, 2 μg of nonspecific competitor poly (dI-dC), 200 ng of single-stranded oligonucleotide. The binding reaction mixture was made with 50,000 c.p.m. (40 fmol) of radiolabeled probe for 20 min in 20 mM HEPES (pH 7.6), 100 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1× complete protease inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN), and 5% glycerol. Complexes were resolved on a 6% native polyacrylamide gel for 120 min at 170 V in TBE (0.5×). After electrophoresis, the gel was dried and processed for autoradiography. For band specificity, the nuclear extracts were incubated with the antibodies to p65 and p50 (Santa Cruz Biotechnology, Santa Cruz, CA) for 45 min at 4 °C, before the probe was added.

Short interfering RNA. The human HEK293 embryonic kidney cell lines stably transfected with human CD14/TLR2 or CD14/TLR4 were maintained as described.[1] Synthetic RNA sequences of PAR_1 and PAR_2 were designed, synthesized, and purified by Dharmacon Research (siGENOME SMART pool: human *par1* cat. no. M005094-01 and human *par2* cat. no. M-061445-00). Transfections of siRNA (at 13 nM) were performed using the Transit TKO Transfection reagent, as per the manufacturer's instructions (Mirus, Madison, WI). siRNA direct against human β-actin was used as control (siGENOME SMART pool: human ACTB cat. no. M003451-01). The relative amounts of *par1* and *paR2* mRNA in cells transfected with *par1* or *par2* siRNA, but not scrambled siRNA, were found to be reduced by 80% compared to WT cells 48 h post-transfection, as assessed by quantitative reverse transcriptase-PCR. At this time, CD14/TLR2/HEK293 cells transfected with siRNA PAR_1 were stimulated with 10 μg ml^{-1} zymosan and CD14/TLR4/HEK293 cells transfected with siRNA PAR_2 were stimulated with 10 μg ml^{-1} lipopolysaccharide for 2 h before EMSA. Western blotting with specific polyclonal antibodies (Santa Cruz Biotechnology) was performed to assess the level of p50 and p65 upon siRNA in cells lysed in buffer containing Tris-Hcl 1 M (pH 6.8), 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 μg ml^{-1} leupeptin, and phosphatase inhibitors cocktail 1 (Sigma).

Calcium mobilization. Confluent HEK293 cells were nonenzymatically collected and suspended at 2×10^6 cells/ml in phosphate-buffered saline. Cells were loaded with 1 μM Fura -2-AM (Molecular Probes, Eugene, OR) and shaken for 30 min at room temperature. A total of 2×10^6 cells/ml loaded cells were transferred to stirred quartz cuvettes in an LS-50B spectrofluorimeter (Perkin Elmer, Padova, Italy), pretreated with elastase E + catepsin G (HE/CatG, each at 600 nM) or supernatants from degranulated WT (20 ng ml^{-1} of protease), *tlr2*$^{-/-}$ or *tlr4*$^{-/-}$ PMNs, and stimulated with human thrombin (3 U ml^{-1}) or trypsin (100 nM) (Sigma) 4 min later. Fura 2 fluorescence was measured at 340 and 380 nm excitation and 510 nm emission, and the ratio of the fluorescence at the two excitation wavelengths, which is proportional to $[Ca^{2+}]_i$, was calculated. For inhibitory studies, culture supernatants were preincubated with 4 mM of the serine protease inhibitor, phenylmethylsulfonyl fluoride, dissolved in ethanol, and 10 μg ml^{-1} of the cathepsin B inhibitor, leupeptin, dissolved in dimethyl sulfoxide (both from Sigma, St Louis, MO) for 15 min at 7 °C. The addition of each diluent alone did not modify calcium mobilization in HEK293.

Cytokine assay. The levels of cytokines in tissue homogenates and culture supernatants from dendritic cells purified from Payer's patches by magnetic-activated sorting using CD11c MicroBeads and MidiMacs (Miltenyi Biotec, Bergisch Gladbach, Germany) were determined by Kit ELISA (R&D Systems, Milan, Italy). The detection limits (pg ml^{-1}) of the assays were < 32 for tumor necrosis factor-α, < 10 for IL-12p70, and < 3 for IL-10.

Reverse transcriptase-PCR and real-time PCR. Total RNA from organs or purified CD4$^+$ T cells[35] was extracted with TRIZOL (Invitrogen SRL Life Technologies, Milano, Italy). Synthesis and PCR of cDNA were performed as described.[12,34] The forward and reverse PCR primers used for murine and human *par* and *gapdh* and cycles

were as described.[12,34,35] Semiquantitative PCR was performed using the "primer-dropping" method, in which *gapdh* was coamplified as an internal control in all reactions. Band intensity was quantified using laser scanning densitometry and ratios of *par1* or *par2* to *gapdh* were plotted for each autoradiogram. Results are representative of three experiments.

Statistical analysis. Student's *t*-test was used to determine differences between the experimental groups (significance was defined as $P < 0.05$). *In vivo* groups consisted of four to six animals. The data reported were pooled from three experiments. Blots are from one representative experiment out of three.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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