Interleukin receptor activates a MYD88–ARNO–ARF6 cascade to disrupt vascular stability

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The innate immune response is essential for combating infectious disease. Macrophages and other cells respond to infection by releasing cytokines, such as interleukin-1β (IL-1β), which in turn activate a well-described, myeloid-differentiation factor 88 (MYD88)-mediated, nuclear factor-kB (NF-kB)-dependent transcriptional pathway that results in inflammatory-cell activation and recruitment1–4. Endothelial cells, which usually serve as a barrier to the movement of inflammatory cells out of the blood and into tissue, are also critical mediators of the inflammatory response5,6. Paradoxically, the cytokines vital to a successful immune defence also have disruptive effects on endothelial cell–cell interactions and can trigger degradation of barrier function and dissociation of tissue architecture7–9. The mechanism of this barrier dissolution and its relationship to the canonical NF-kB pathway remain poorly defined. Here we show that the direct, immediate and disruptive effects of IL-1β on endothelial stability in a human in vitro cell model are NF-kB independent and are instead the result of signalling through the small GTPase ADP-ribosylation factor 6 (ARF6) and its activator ARNO nucleotide binding site opener (ARNO; also known as CYTH2). Moreover, we show that ARNO binds directly to the adaptor protein MYD88, and thus propose MYD88–ARNO–ARF6 as a proximal IL-1β signalling pathway distinct from that mediated by NF-kB. Finally, we show that SecinH3, an inhibitor of ARF guanine nucleotide-exchange factors such as ARNO, enhances vascular stability and significantly improves outcomes in animal models of inflammatory arthritis and acute inflammation.

A defining characteristic of the cytokine-induced inflammatory response is the destabilization of endothelial barriers resulting in vascular permeability10–13. To dissect the pathway(s) involved in this tissue disruption, we treated cultured monolayers of human dermal microvascular endothelial cells (HMVEC-d) with IL-1β, and detected an increase in endothelial permeability within 15 min (Fig. 1a). The canonical IL-1β pathway involves ligand-stimulated activation of interleukin-1 receptor (IL-1R), which recruits MYD88 to its cytoplasmic tail10. The subsequent signalling cascade through IRAK1 results in the phosphorylation of IkB-α by the IkB kinase (IKK) complex, leading to translocation of NF-kB to the nucleus and the eventual transcription of target genes that promote inflammatory-cell responses4,14 (Supplementary Fig. 1). To test the involvement of this pathway in IL-1β-induced vascular permeability, cells were treated with the IKK inhibitor SC-514 (ref. 11). Although SC-514 prevented IL-1β-induced nuclear localization of NF-kB, it was unable to rescue either IL-1β-induced permeability or disruption of vascular endothelial (VE)-cadherin surface localization (Fig. 1b, c and Supplementary Fig. 2a–c). We also wondered whether IL-1β-induced vascular permeability required other known MYD88-mediated downstream signalling mechanisms, including ERK1/2, p38 and JNK (also known as MAPK3/MAPK1, MAPK14 and MAPK8, respectively)15,16. Although ERK1/2, p38 and JNK were activated by IL-1β stimulation of endothelial cells, small-molecule inhibitors of each of these pathways were unable to prevent IL-1β-induced vascular permeability or IL-1β-induced disruption of VE-cadherin cell-surface localization (Supplementary Fig. 2d–h). Although specific NF-kB targets, such as VEGFA, COX-2 (also known as PTGS2) and the COX-2 product prostaglandin E2 are modulated by IL-1β, their activation had no effect on IL-1β-induced endothelial permeability14,16 (Supplementary Fig. 3a–e). Finally, treatment with actinomycin D or cycloheximide effectively inhibited transcription or translation, respectively, of NF-kB targets, but did not blunt IL-1β-induced permeability (Fig. 1d and Supplementary Fig. 3f, g). These data strongly support a role for the immediate and destabilizing effects of IL-1β on endothelial stability through signalling pathways independent of NF-kB, transcription and translation.

IL-1β can disrupt VE-cadherin cell-surface localization by promoting endocytic internalization17. We proposed that IL-1β might use ARF6, a known regulator of adherens protein localization18,19. Indeed, IL-1β activated ARF6 in HMVEC-d within 1 min, a response accompanied by increased endocytosis of VE-cadherin within 5 min and an increase in monolayer permeability within 15 min (Fig. 1a, e and Supplementary Fig. 4a, b). IL-1β treatment did not affect total VE-cadherin messenger RNA or protein levels (Supplementary Fig. 4c, d). Adenoviral-mediated overexpression of constitutively active ARF6 (ARF6(Q67L))20 elicited a dose-dependent increase in endothelial permeability, as well as a disruption of VE-cadherin cell-surface localization (Fig. 1f, g and Supplementary Fig. 4e, f). A similar dose-dependent loss of total VE-cadherin was also observed, probably through internalization and subsequent degradation (Supplementary Fig. 4g). Interestingly, at lower doses of adenovirus at which permeability was still induced, loss of total VE-cadherin was not observed, but a dose-dependent loss of cell-surface VE-cadherin occurred (Fig. 1h and Supplementary Fig. 4g). Moreover, short interfering RNA (siRNA) knockdown of ARF6 enhanced VE-cadherin cell-surface localization and prevented both IL-1β-induced disruption of VE-cadherin and IL-1β-induced endothelial permeability (Fig. 1i–k and Supplementary Fig. 4h, i). Collectively, these data link ARF6 as a critical regulator of VE-cadherin trafficking by controlling cell-surface localization and the immediate and disruptive effects of IL-1β-induced vascular permeability.

The ARF6 activation state is decreased through interaction with GTPase-activating proteins (GAPs) and increased through interaction with guanine nucleotide-exchange factors (GEFs). Consistent with this, treatment of endothelial cells with the ARF-GAP inhibitor QS11 evoked an increase in ARF6–GTP, a decrease in VE-cadherin cell-surface localization and increased permeability (Fig. 2a–d and Supplementary Fig. 5a, b).

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Supplementary Fig. 5a, b)39. We noted that a class of ARF-GEFs, the cytosines, is highly expressed in multiple types of endothelial cells (Supplementary Fig. 5c). Accordingly, treatment of HMVEC-d with SecinH3, a cytosine inhibitor, significantly increased endothelial cell-surface localization of VE-cadherin (Supplementary Fig. 5d-f)39. Notably, SecinH3 inhibited IL-1β-induced ARF6–GTP, as well as IL-1β-induced disruption of VE-cadherin cell-surface localization and endothelial permeability (Fig. 2e-g and Supplementary Fig. 5g, h). To determine which GEF might be uniquely involved, we used siRNA to knockdown CYTH1, ARNO and CYTH3 (coding for cytosines 1, 2 and 3, respectively) and GEP100 (also known as IQSEC1) (Supplementary Fig. 6a), and found that only siRNA targeting ARNO completely blocked IL-1β-induced endothelial permeability and phenocopied the knockdown of ARF6 (Fig. 2h and Supplementary Fig. 6b). Furthermore, ARNO siRNA inhibited IL-1β-induced ARF6–GTP formation, IL-1β-induced disruption of VE-cadherin cell–cell contacts and IL-1β-induced internalization of surface VE-cadherin (Fig. 2i, j and Supplementary Fig. 6c, g); all of these effects were rescued by the expression of siRNA-resistant ARNO (Supplementary Fig. 6d–g). In cells treated with ARNO siRNA, viral expression of siRNA-resistant ARNO, but not viral expression of siRNA-resistant ARNO(E156K) (carrying a mutation in the Sec7 domain), rescued the disruption of IL-1β-induced ARF6–GTP formation and permeability39 (Supplementary Fig. 6d, e). These data demonstrate that ARNO is a critical ARF-GEF necessary for IL-1β-induced activation of ARF6 and subsequent induction of vascular permeability, but does not rule out the role of other GEF family members in similar responses in different cell types or in response to different cytokines.

The signalling components in the NF-κB pathway downstream of IL-1β-induced activation of IL-1R are well characterized (Supplementary Fig. 1). Although the inhibition of the NF-κB pathway at the level of IRAK1 by siRNA did not inhibit IL-1β-induced ARF6 activation, siRNA knockdown of MYD88 inhibited both IL-1β-induced permeability and ARF6–GTP activation, suggesting a bifurcation of
IL-1β-induced signalling at the point of MYD88 (Fig. 3a–c and Supplementary Fig. 7a, b). The proposed bifurcation was further verified by the pharmacological uncoupling of the two pathway arms: although SecinH3 blunted IL-1β-induced permeability, it did not significantly inhibit IL-1β-induced NF-κB nuclear localization or NF-κB-dependent expression or localization of cell-surface adhesion molecules (Fig. 3d and Supplementary Fig. 7c, d). Furthermore, SecinH3 was unable to inhibit IL-1β-induced polyomorphonuclear leukocyte rolling and adherence under shear stress on an endothelial monolayer (Supplementary Fig. 7e). Mechanistic support for this novel signalling arm was provided by the demonstration of an interaction between MYD88 and ARNO by co-immunoprecipitation in both overexpression and endogenous settings (Fig. 3e, f). Our hypothesis that ARNO is the critical GEF in IL-1β-induced permeability in endothelial cells was further strengthened by our inability to detect an interaction between MYD88 and other potentially relevant ARF-GEFs including CYTH1, CYTH3 and GEP100 (Supplementary Fig. 7f, g).

An effective therapeutic strategy to combat numerous inflammatory conditions is to target pro-inflammatory cytokines proximal to the NF-κB pathway. However, this strategy can result in undesired pleiotropic effects. We wanted to know whether targeting a single arm in this pathway—the one mediated by ARNO–ARF6—could inhibit acute or chronic inflammation in vivo in two animal models of inflammation. The first model we tested was rheumatoid arthritis, a disease characterized by a dysregulated cytokine response causing excessive inflammation and tissue damage and treated therapeutically in humans with the anti-cytokine tumour necrosis factor receptor (TNFR)–Fc fusion protein etanercept (Enbrel)22–25. A standard animal model of arthritis through which a TNFR fusion approach has been proven effective is collagen-induced arthritis (CIA)24–26. Exposure of animals to the cytokines inhibitor SecinH3 after the onset of CIA reduced vascular permeability in the joints, but had no effect on global cytokine levels at 24 h after treatment initiation (Fig. 4a and Supplementary Fig. 8a, b). In addition, a significant inhibition in the increase in arthritic index, comparable to that achieved by treatment with Enbrel, was observed. The arthritic index is a scoring system determined by the number of digits or joints that are oedematous or erythematous. The significance of our findings was verified by histologic scoring of inflammation, pannus development, cartilage damage and bone damage (Fig. 4c, d). A similar effect of SecinH3 was confirmed in a second model of inflammation, the carrageenan air-pouch model. Six hours after an inflammatory stimulus, a time at which substantial inflammation was induced in the positive control mice, treatment with SecinH3 decreased exudate volume as well as leukocyte concentration in the exudates (Supplementary Fig. 8c, d). Collectively, these data identify MYD88–ARNO–ARF6 as a valid target for inflammatory conditions confirming a relevant role for manipulation of this pathway in vivo to modulate inflammatory processes and in the treatment of disease.

**Figure 3** | The immediate IL-1β-induced permeability pathway diverges at MYD88. a, IRAK1-siRNA-treated HMVEC-d, stimulated with IL-1β, subjected to ARF6–GTP pull-down and immunoblotted for ARF6. b, c, MYD88-siRNA-treated HMVEC-d stimulated with IL-1β, showing permeability and ARF6 activation. d, NF-κB p65 (also known as RELA) immunofluorescence in HMVEC-d stimulated with IL-1β and SecinH3. Arrows denote nuclear localization. e, Cell lysates from Ad-ARNO–MYC-infected HMVEC-d immunoprecipitated (IP) with anti-MYC antibodies and immunoblotted with anti-MYD88 antibodies. f, Lysates from HMVEC-d immunoprecipitated with anti-MYD88 antibodies and immunoblotted with anti-ARNO antibodies. n ≥ 3. Error bars denote s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001.

**Figure 4** | Inhibition of ARF-GEFs decreases collagen-induced vascular permeability and arthritis in mice. a, Arthritis-induced vascular permeability in the joint measured by Evans Blue leak 7 days after treatment initiation in the presence of SecinH3 or Enbrel. n = 14 per group. b, Chronological arthritic assessment. n = 10 per group. Significance values are measured against CIA plus vehicle. c, Haematoxylin and eosin staining of sections through joints. Bn, bone; HC, histologic changes of inflammation, pannus, cartilage and bone damage of indicated treatment. Control, n = 5; Enbrel and SecinH3, n = 10. Error bars bars denote s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 versus disease group.
Chronic inflammation causes tissue destruction through dysregulated cytokine release, inflammatory cell recruitment and vascular permeability; yet each of these mechanisms has critical roles in many physiologic processes, including the immune response.\(^5,7\) We have identified a novel pathway that uncouples cytokine effects on vascular stability from other critical functions of the canonical NF-κB transcription program (Supplementary Fig. 1). Our model suggests the potential for inhibition of vascular leak without modulation of immune-cell adhesion or other critical NF-κB-dependent responses.

The activation of many inflammatory cytokine receptors disrupts cell–cell interactions, precipitating tissue oedema and destruction.\(^3,9,27\) Toll-like receptors and the interleukin receptor also use MYD88, and the mechanism described here may well apply.\(^11,30\)

Interestingly, TNFR1 (also known as TNFReSF1A) does not use MYD88 yet still activates ARF6–GTP after stimulation (Supplementary Fig. 9). Whether ARNO or another ARF-GEF binds directly to TNFR1 or its adaptor protein TRADD is unknown, as is the possibility that ARF-GEF–ARF6–cadherin serves as a common signalling module exploited by multiple cytokines. Although this study focused on the endothelium, the concept of cytokine receptor–ARF–cadherin may also apply to the epithelial barrier, which expresses these constituents and is also compromised by cytokines including IL-1β.\(^16\)

Inhibition of this novel vascular-stability pathway, which is aimed at enhancing the resilience of the host to the cytokine response, shows effects commensurate to those of class-leading drugs that target cytokines upstream of NF-κB and seek to blunt the cytokine response of the immune system outright. This approach may be particularly useful in arthritis, as the current medical therapy can render a patient immunocompromised and susceptible to reactivation of infectious disease such as tuberculosis.\(^6\) Application of these findings to other diseases characterized by excessive acute or chronic inflammatory states, including sepsis, Crohn’s disease, ulcerative colitis, scleroderma and psoriasis, should also be considered.\(^25,30\)

METHODS SUMMARY

Transwell permeability. HMVEC-d cells were seeded on 1.0-μm Costar transwell inserts coated with fibronectin. Cells were grown to confluency and treated with SecinH3 for 3 h or MAPK/NF-κB transfection/transcription inhibitors for 30 min followed by treatment with 10 ng ml\(^{-1}\) IL-1β. Alternatively, cells were infected with Ad-GFP or Ad-ARF6(Q67L) for 48 h. siRNA knockdown was performed as described in Supplementary Methods, and cells were treated with IL-1β 72 h after the second siRNA transfection. Two hours later, HRP was added to the top chamber at a final concentration of 100 μg ml\(^{-1}\); Medium was removed after 60 min from the lower chamber. For time-course transwell assays and transcription/translation-inhibitor experiments (Fig. 1a, c, d), HRP was added to the insert at the same time as IL-1β. Transwell inserts were moved to fresh wells after each time point, and the concentration of HRP in the bottom chamber was measured for monolayer-permeabilized cells. The concentration of HRP in the bottom chamber was measured for monolayer-permeabilized cells, and is also compromised by cytokines including IL-1β.

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