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ARF6 Inhibition Stabilizes the Vasculature and Enhances Survival during Endotoxic Shock

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The vascular endothelium responds to infection by destabilizing endothelial cell–cell junctions to allow fluid and cells to pass into peripheral tissues, facilitating clearance of infection and tissue repair. During sepsis, endotoxin and other proinflammatory molecules induce excessive vascular leak, which can cause organ dysfunction, shock, and death. Current therapies for sepsis are limited to antibiotics and supportive care, which are often insufficient to reduce morbidity and prevent mortality. Previous attempts at blocking inflammatory cytokine responses in humans proved ineffective at reducing the pathologies associated with sepsis, highlighting the need for a new therapeutic strategy. The small GTPase ARF6 is activated by a MyD88–ARNO interaction to induce vascular leak through disruption of endothelial adherens junctions. In this study, we show that the MyD88–ARNO–ARF6–signaling axis is responsible for LPS-induced endothelial permeability and is a destabilizing convergence point used by multiple inflammatory cues. We also show that blocking ARF6 with a peptide construct of its N terminus is sufficient to reduce vascular leak and enhance survival during endotoxic shock, without inhibiting the host cytokine response. Our data highlight the therapeutic potential of blocking ARF6 and reducing vascular leak for the treatment of inflammatory conditions, such as endotoxic shock. The Journal of Immunology, 2014, 192: 000–000.

The innate immune system is the first line of defense against pathogenic microbes. It facilitates the recognition of microbial components, such as endotoxin, and initiates an inflammatory response that clears the invading organism and promotes reconstruction of damaged tissues. People with sepsis often have a frenetic inflammatory response and associated excess vascular leak that leads to tissue edema, organ failure, shock, and often death (1–3). Current treatment options are limited to supportive care and antibiotic therapies (4). Unfortunately, even with these options mortality still occurs in >25% of septic patients and occurs with even greater incidence in patients whose condition progresses to septic shock (5, 6).

The vast majority of therapeutic interventions for sepsis, outside of antibiotic therapies and supportive care, have focused on reducing the inflammatory and cytokine responses (7). These approaches, which include immunosuppression by steroids (8), inhibition of the inflammatory TLR4 with eritoran (9), and, in particular, direct inhibition of cytokines, such as TNF-α (10–12), have been successful in some animal models, but they have produced conflicting or negative outcomes in human phase III clinical trials. Because of this, alternative inflammatory pathways important to the pathology of sepsis need to be identified to exploit their potential as therapeutic targets.

The recognition of microbial components by TLRs is critical to the inflammatory response during sepsis. TLRs are expressed in many cell types and stimulate a MyD88-mediated cascade, which leads to activation of the inflammatory transcription factor NF-κB and to the subsequent cytokine storm observed during sepsis (13, 14). We recently identified an association between MyD88 and the guanine nucleotide exchange factor ARNO. IL-1β requires this association to activate ARF6 and to induce vascular leak in a process independent of MyD88’s canonical role in NF-κB–mediated inflammatory gene expression (15). This MyD88–ARNO–ARF6 cascade promotes enhanced vascular permeability through the internalization of vascular endothelial-cadherin (VE-cadherin). MyD88 is a critical adapter protein used by numerous other inflammatory pathways, including IL-1R and most of the described TLRs (14). Therefore, we hypothesized that TLR stimulation may induce vascular permeability independent from cytokine expression, that this permeability is mediated by a MyD88–ARNO–ARF6 cascade, and that blocking ARF6 would enhance survival in models of sepsis.

Materials and Methods

Reagents

Human dermal microvascular endothelial cells (HMVEC-Ds) were purchased at passage 0 from Lonza; experiments were performed at passages...
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VE-cadherin internalization

Internalization assays were adapted from previous protocols (15, 17). HMVEC-Ds were seeded on eight-well, glass-bottom chamber slides coated with human fibronectin. Cells were incubated in dialyzed VE-cadherin BV6 Ab solution (Millipore; MABT134) that was diluted in label-free BSA 4 [Sigma] + 5% fatty acid-free BSA in 0.1 mM HEPES) at a concentration of 5 μg/ml for 1 h at 4°C. Media were replaced with EGM-2MV with 100 ng/ml LPS or vehicle to stimulate endocytosis and 0.6 mM Primaquine (Sigma) to block recycling of vesicles back to the plasma membrane. Cells were fixed three times in HBSS (Intronogen) + Ca2+ + Mg2+ + 50 mM glycine (pH 2.7) to remove noninternalized Ab and then fixed in 4% paraformaldehyde for 10 min on room temperature. Fixation was washed three times and permeabilized with 0.3% Triton in HBSS for 5 min, followed by blocking in 5% nonfat milk + 10% normal donkey serum in HBSS for 1 h. Detection of VE-cadherin was performed with Alexa Fluor 488–conjugated anti-mouse IgG (Invitrogen), and cells were mounted in Antifade medium + 2 μg/ml DAPI. Five random fields/well were imaged on an Olympus FV1000 confocal microscope at 60× with three individual Z-slices, 0.3 μm apart. To quantify the images, each cell with >10 vesicles stained positive for VE-Cadherin was tallied and divided by the total number of DAPI-stained nuclei in the field. To score the image, the total number of cells with greater than 10 488 nm positive vesicles divided by the total number of cells (DAPI-stained nuclei)/field of a collapsed Z-projection was calculated. Statistics were performed, using two-way ANOVA, in Prism software.

Colocalization with EE1A was performed in the same manner, but the rabbit early endosome Ag1 (EE1A) primary Ab (Abcam) was added immediately after the blocking step and incubated for 1 h at room temperature.

Immunoprecipitation

The coding sequence of each functional domain of full length of MyD88 and ARNO was amplified from IMAGE cDNA clones by PCR and ligated into pcDNA3.1 vector after enzyme digestion. The ARNO and functional-domain constructs contained a Myc and His epitope, whereas MyD88 constructs contained an HA epitope. 293T cells were transfected with the indicated MyD88 or ARNO constructs. Cells were lyzed in ice-cold 50 mM Tris (pH 7.5), 750 mM MgCl2, 1% Nonidet P-40, and 10% glycerol supplemented with protease and phosphatase inhibitors (Thermo; 861282) and incubated with immobilized anti-HA high-affinity rat mAb (clone 12CA5) for 60 min at 4°C. Complexes were washed three times with lysis buffer and analyzed via Western blot.

Cytokine assay

HMVEC-D supernatants were treated as described in the figure legends. Cytokine expression was measured with Millipore Milliplex magnetic bead assays, per the manufacturer’s instructions. To assess murine plasma TNF-α concentrations, 3–4-mo-old mice that had been treated i.p. with 25 mg/kg LPS at 30 min and 0.4 mg/kg PMN-killed E. coli at 90 min were euthanized. Blood was centrifuged, and the plasma was obtained and stored at −80°C until analysis. TNF-α concentrations were analyzed using the Mouse TNF-α DuoSet ELISA (R&D Systems; DY410).

Leukocyte rolling and adherence assay

The protocol was adapted from previous studies (18, 19). HMVEC-Ds were seeded into each well of a human fibronectin–coated parallel plate flow chamber (U–plate 0.4 V1 Fibronectin; Ibidi) at a density of 3 × 105 cells/chamber. Media were changed daily until the cells had grown to confluence.

Human polymorphonuclear leukocytes (PMNs) were isolated using previously described protocols (20, 21). Human peripheral venous blood was collected from healthy, medication-free adult subjects and drawn into acid-citratedextrose (14% final concentration) through standard venipuncture technique and used immediately upon collection. All subjects provided informed consent, and Institutional Review board approval was received for blood collection at the University of Utah. Platelet-rich plasma was removed upon centrifugation of whole blood at 150 × g for 20 min at room temperature. The remaining red/WBC mixture was resuspended back to the original volume with 0.9% saline solution. Six percent dextran 70 was added to the cell mixture and left for 1 h. The leukocyte-rich supernatant was removed and centrifuged at 400 × g for 5 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 0.2% NaCl immediately followed with an equal part 1.6% NaCl. Cells were centrifuged
at 400 × g for 5 min at 4°C. The pellet was resuspended in HBSS supplemented with 1% human serum albumin (HBSS/A) and layered over an equal volume of Ficoll-Paque Plus. The suspension was centrifuged at 4°C for 30 min at 400 × g. The mononuclear leukocyte layer was removed, followed by the RBC layer, HBSS/A layer, and Ficoll-Paque layer. The remaining pellet containing ≥95% PMNs was washed with HBSS/A and resuspended to 10^6 cells/ml in warm Ultrasaline.

Endothelial cells previously seeded into flow chambers were treated with LPS (100 ng/ml) and/or SC-514 for 3 h in complete media. A syringe pump (Harvard Apparatus) was used to propel isolated and unstirred primary human leukocytes through the parallel plate flow chambers (at 1 dyne/cm² [typical venous shear stress (22)]) in Ultrasaline. Differential interference contrast microscopy images were taken one time per second for 30 s using an Olympus inverted microscope and a 10× objective, which is sufficient for visualizing a large number of endothelial cells. The image area was in the center (in both length and width) of each well, a point confirmed by the manufacturer using computational fluid dynamics to demonstrate the most uniform flow characteristics. The total number of PMNs rolling and adhered in all 30 images was quantified using MetaMorph software. Data presented are a quantification of the cumulative number of cells observed to be adhered or rolling on the endothelial monolayer in each image (total leukocytes adhered/30 s × 1000/cm²).

VE-cadherin area quantification

HMVEC-Ds were seeded on BD Falcon clear-bottom 96-well tissue culture plates (catalog number 353219) at a density of 10,000 cells/well. Cells were incubated with the indicated treatments for 5 h and then fixed with 4% paraformaldehyde in PBS for 10 min. Cells were permeabilized with 0.5% Triton-X 100 for 2 min, blocked with Odyssey Blocking Buffer (LI-COR Biosciences) for 30 min at room temperature, and incubated with a mouse monoclonal VE-Cadherin Ab (BD; 555661) overnight at 4°C. Cells were rinsed with PBS and incubated with Alexa Fluor 488 donkey anti-mouse secondary Ab and Hoechst 33258 dye for 4 h at room temperature. Cells were rinsed, and the ImageXpress Micro XLS (Molecular Devices) collected nine nonoverlapping images at the center of each well with a 20× objective. The VE-cadherin area of each image was quantified with ImageXpress by batch thresholding all images from an individual experiment and quantifying the number of pixels that were VE-cadherin+ within each image. The VE-cadherin area was divided by the number of nuclei/image and normalized to the Mock treatments. Data were compiled from three independent experiments with three replicate wells/condition in each experiment and are composed of nine images/well, for a total of 81 images/condition.

Organ permeability

This assay was adapted from previously published protocols (1, 23, 24). Male C57BL/6 mice, between 8 and 12 wk of age (The Jackson Laboratory) were anesthetized with ketamine and given 40 mmol/kg peptide, followed by 40 mg/kg Evans Blue dye in saline delivered i.v. via retro-orbital sinus injection. Anesthesia was reversed with 50 µl 500 µg/ml Antisedan (atipamezole) administered s.c. Six hours later, mice were euthanized via CO2 asphyxiation, and the mass of each organ was measured. All animal experiments were approved by the Animal Care and Use Committee of the University of Utah under protocol number 12-10002.

Endotoxemia survival

Eight- to twelve-week-old male C57BL/6 mice (The Jackson Laboratory), given 40 mmol/kg peptide i.v. via retro-orbital sinus injection and 25 mg/kg of LPS i.p., were monitored at least four times each day until all living mice resumed normal behaviors. To reduce death as an end point, all mice observed to be unresponsive to touch were euthanized immediately by CO2 asphyxiation.

Statistics

Unless otherwise noted, statistics and p values were calculated using one-way ANOVA, with the appropriate post hoc test, in PRISM software version 5.0f.

**Results**

TLRs induce vascular leak by activation of ARF6

To determine whether ARF6 is involved in TLR-mediated endothelial permeability, we used a well-characterized endothelial cell,
HMVEC-D, which is known to respond to TLR stimulation and is widely used in endothelial cell--permeability assays (25–27). We treated primary HMVEC-Ds with LPS, the well-characterized agonist of TLR4 (28), and found that LPS was sufficient to induce ARF6 activation, as measured by the increased presence of ARF6-GTP (Fig. 1A, 1B) to biologically relevant levels, consistent with other documented ARF6 functions (29–31). We also found that siRNA directed against ARF6 was sufficient to reduce permeability increased by LPS in a Transwell system (Fig. 1C).

During the IL-1β–signaling cascade in endothelial cells, the guanine nucleotide exchange factor ARNO interacts with the inflammatory adapter protein MyD88 to activate ARF6 (15). We hypothesized that the LPS-mediated increase in permeability was controlled by this MyD88–ARNO interaction. Indeed, siRNAs directed against either MYD88 or ARNO were sufficient to prevent LPS-induced endothelial permeability (Fig. 1D, 1E).

To probe the interaction of MyD88 and ARNO, we first wanted to confirm the relationship by defining their interacting domains. MyD88 is composed of three domains: the Toll/IL-1R (TIR) domain, the intermediate domain (ID), and the death domain (DD) (Fig. 1F). The TIR domain interacts with both the cytoplasmic tail of TLRs and IL-1R; the DD binds IL-1R–associated kinase; and the binding partners of ID are ill-defined (32). Interestingly, ARNO was immunoprecipitated with both the DD and the TIR domain of MyD88 only when ID was present. Furthermore, the coiled-coil and pleckstrin homology domains of ARNO were
found to be sufficient for ARNO’s interaction with MyD88 (Fig. 1G, 1H).

A MyD88–ARNO–ARF6–controlled permeability hypothesis suggests that, like IL-1β, modulation of ARF6 may be independent of LPS-induced changes in gene expression and that those changes may be dispensable for LPS-induced permeability. Indeed, siRNAs targeting ARF6 were unable to block LPS-induced TNF-α expression in endothelial cells (Fig. 2A). Additionally, blocking LPS-induced transcription factor NF-κB or IRF3 activation using SC-514 or BX-795, respectively, failed to inhibit LPS-induced permeability, but it blocked other LPS-induced responses, including leukocyte rolling and TNF-α and IL-6 expression (Fig. 2B–F) (33, 34).

**LPS induces permeability through VE-cadherin disruption**

ARF6 was implicated in the regulation of the internalization of the adherens junction protein VE-cadherin, which suggests that the LPS-mediated increase in endothelial permeability occurs in a paracellular manner. To test this, we assessed the leak of 2-MDa FITC-dextran, a reporter too large for transcellular transport across endothelial monolayers (35). We observed enhanced movement of the 2-MDa dextran across the monolayer upon LPS stimulation, suggesting that LPS enhances paracellular leak across the endothelium (Fig. 3A). LPS also induced VE-cadherin internalization, as shown by the enhanced presence of VE-cadherin in endocytic vesicles containing EEA1 (Fig. 3B–D). This internalization was abolished upon treatment of endothelial cells with siRNA directed against ARF6 mRNA, further supporting the hypothesis that LPS mediates permeability through ARF6-mediated adherens junction internalization (Fig. 3E, 3F).

**Inhibition of ARF6 with its N terminus reduces vascular leak**

Having established that activation of ARF6 plays a critical role in the enhancement of endothelial permeability, we wanted to determine whether deactivation of ARF6 would enhance endothelial barriers and reduce leak. A property of the ARF family of GTPases is that peptides constructed of their N termini are proposed to inhibit ARF GDP–GTP exchange (36, 37). We synthesized a myristoylated peptide composed of amino acids 2–13 of ARF6 (MyrARF6 2–13). The ARF6 N-terminal peptide, but not scrambled (MyrSCR 2–13) or nonmyristoylated (ARF6 2–13) controls, was sufficient to reduce ARF6 activation in HMVEC-Ds (Fig. 4A, 4B).

Our model predicts that reduced ARF6 activation would result in enhanced endothelial barrier function. The ARF6 N terminus, but not nonmyristoylated or scrambled controls, reduced permeability across an endothelial monolayer (Fig. 4C). This reduction in permeability corresponded to an increase in VE-cadherin at cell junctions (Fig. 4D, 4E). This ARF6 inhibitory function was also active in vivo and prevented the leak of Evans Blue dye from the blood into both the lungs and the kidneys (Fig. 4F, 4G). These data suggest that the synthetic ARF6 N terminus may be useful as a novel therapeutic to prevent pathologies resulting from excess inflammatory vascular leak.

**The synthetic N terminus of ARF6 enhances survival during endotoxic shock**

Sepsis is a condition characterized by immense vascular leak that leads to tissue dysfunction, shock, and, eventually, death. We sought to determine whether the stabilizing activity of the ARF6 N terminus could enhance survival of mice in the endotoxemia model of sepsis. Lethal doses of LPS were administered i.p. immediately following i.v. injection of peptides. Mice were monitored at least four times per day until they resumed normal grooming behaviors for ≥24 h. After 48 h, only 20% of the mice treated with LPS plus...
vehicle or a nonmyristoylated peptide were alive. However, 85% of the mice treated with LPS plus ARF6 N-terminal peptide were alive at 48 h, and >70% of the mice recovered completely (Fig. 5A). Interestingly, peptide treatment enhanced survival but did not significantly affect serum cytokine levels (Fig. 5B). The observation that peptide treatment does not affect cytokine levels provides further evidence that ARF6 pathway inhibition can protect mice from the deleterious effects of inflammation while allowing canonical inflammatory cascades to continue (Fig. 6).

Discussion

We defined a signaling cascade in endothelial cells by which LPSs induce vascular leak. We also described a peptide inhibitor of this pathway, MyrARF6 2–13, which stabilized the vasculature in both in vitro and in vivo assays and enhanced survival in a mouse model of endotoxic shock.

The endothelial cell monolayer is the critical barrier between blood and the peripheral tissues. It regulates the migration of fluid, nutrients, and cells from blood into the interstitial space. The integrity of this barrier is maintained by a balance of opposing cues that regulate endothelial cell transcytosis and cell–cell junctions (38). For example, homophilic interactions of VE-cadherin at the cell–cell junction are enhanced upon stimulation by TIE2-dependent angiopoietin signaling (39) and ROBO4-dependent SLIT signaling (1), but they are disrupted after stimulation with destabilizing cues, such as VEGF (17), IL-1β (15), or LPS, as described in this article. The ability of the stabilizing receptor ROBO4 to prevent leak and pathologic angiogenesis is dependent on its ability to recruit the GTPase activating protein, GIT1, to inactivate ARF6 (40). Conversely, the ability of the inflammatory mediators LPS and IL-1β to destabilize the vasculature is dependent on their ability to activate ARF6 (15). These observations, combined with the data from this study, place ARF6 at the nexus of multiple pathways that disrupt the endothelium. ARF6 activation leads to an unstable, leaky vasculature, whereas ARF6 inhibition leads to a stable, less permeable vasculature. This tightly regulated balance of endothelial stability is disrupted in conditions such as sepsis or pulmonary infections. During these conditions, the inflammatory response induces intractable vascular leak, hypovolemic shock, and/or acute respiratory distress syndrome, all of which can precipitate death. By blocking ARF6 with its N-terminal peptide and reducing vascular leak, we were able to enhance the survival of mice in the endotoxia model of sepsis.

Our in vitro and in vivo data demonstrate that ARF6 regulates permeability independent from downstream inflammatory gene expression. Knockdown of ARF6 expression in endothelial cells, using specific siRNAs and the systemic in vivo administration of the ARF6 N-terminal peptide, did not block LPS-induced TNF-α secretion. Thus far, all of the evidence in animal models, including the current study, suggests that ARF6, although necessary for disease pathology, does not regulate cytokine release (15). In contrast, it was proposed that inhibition of ARF6 in fibroblasts, macrophages, and dendritic cells decreases LPS-induced cytokine production by preventing the association of MyD88 with TIRAP and TLR4 (41, 42). However, LTA-induced cytokine expression, which also requires the association of MyD88 with TIRAP, was not affected by ARF6 inhibition, suggesting a greater complexity in these TLR-signaling pathways (42). Furthermore, ARF6 inhibition had no effect on LPS-induced cytokine expression in dendritic cells (43). Therefore, the preponderance of in vitro evidence, as well as that from all in vivo studies published to date, suggests that ARF6 inhibition does not block cytokine expression or release.

FIGURE 6. LPS uses a Myd88–ARNO–ARF6 cascade to induce VE-cadherin internalization and vascular permeability independent from cytokine expression. LPS stimulates TLR4, which recruits the adapter proteins Myd88 and TRIF to facilitate downstream transcriptional regulation, such as cytokine expression. LPS also stimulates an independent and divergent pathway through the Myd88–ARNO–ARF6–signaling axis that dissociates VE-cadherin from the cell–cell junctions, allowing for vascular leak to occur. MyrARF6 2–13 blocks this divergent pathway by deactivating ARF6 and reducing vascular leak.
Aside from enhancing vascular stability, inhibition of a highly conserved molecule, such as ARF6, may have a number of developmental effects. For example, genetic ablation of ARF6 leads to the failure of proper hepatic cord formation and is embryonically lethal (44). However, the specific ablation of ARF6 in adults has not been described. In adult mice, inhibition of the ARF family of GTPases prevented signal transduction of the insulin-receptor complex, leading to transient insulin resistance (45). Additionally, inhibition for 2 wk showed no obvious toxicity, but it decreased inflammatory pathologies in mouse models of arthritis (15). ARF inhibition also reduced invasion and metastasis of melanoma xenografts (31). Furthermore, ARF6 inhibition blocked proper VEGF receptor signaling and angiogenesis (40, 46), as well as altered platelet activation (36, 47). Determining the effects of ARF6 inhibition on other tissues, especially in the context of hyperinflammation and sepsis, is an important and ongoing area of research in our laboratory and others.

This study identified a novel host–pathogen interaction through which bacterial LPSs activate a MyD88–ARNO–ARF6 cascade that induces endothelial leak independent of cytokine expression after stimulation (Fig. 6). We characterize an ARF6 inhibitor that reduces vascular leak and increases survival during lethal LPS-induced endotoxicemia. MyD88 is a central effector of innate immunity; blocking the MyD88–ARNO–ARF6 cascade might serve as a mechanism for translational control of transcriptional events. J. Exp. Med. 200: 671–680.

We expect that this study will open new avenues for the development of therapeutics that could be used for the treatment of inflammatory processes related to infection, as well as any inflammatory process associated with vascular instability, including trauma, epilepsy, and autoimmune diseases, such as multiple sclerosis (48–50).

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Disclosures

D.Y.L. and the University of Utah own intellectual property regarding the use of ARF6 inhibitors.

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