ARF6 Inhibitors for Disorders of Vascular Leak

February 2018

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Vascular Leak Is a Critical Pathologic Component of Many Serious Conditions

Examples

Acute Disorders:

- Acute Respiratory Distress Syndrome
- Sepsis
- Myocardial Infarction and Stroke
- Cytokine Release Syndrome

Chronic Disorders:

- Diabetic Retinopathy
- Rheumatoid Arthritis

ARF6 Regulates Vascular Stability

- ARF6 plays a critical role in control of neovascularization and inflammation
- ARF6 is a small GTPase of the Ras superfamily
- ARF6 is a convergence point for signaling through a number of receptors involved in angiogenesis and vascular leak pathways, including IL-1R, IL-6R, TLR4, and VEGFR
- ARF6 activation destabilizes surface cadherin-catenin complexes, inducing vascular permeability

Zhu et al. Nature 2012 Dec 13;492(7428):252-5.
Davis et al. J Immunol. 2014 Jun 15;192(12):6045-52.
Zhu et al. JCI 2017 Oct 23 doi:10.1172/JCI91770.

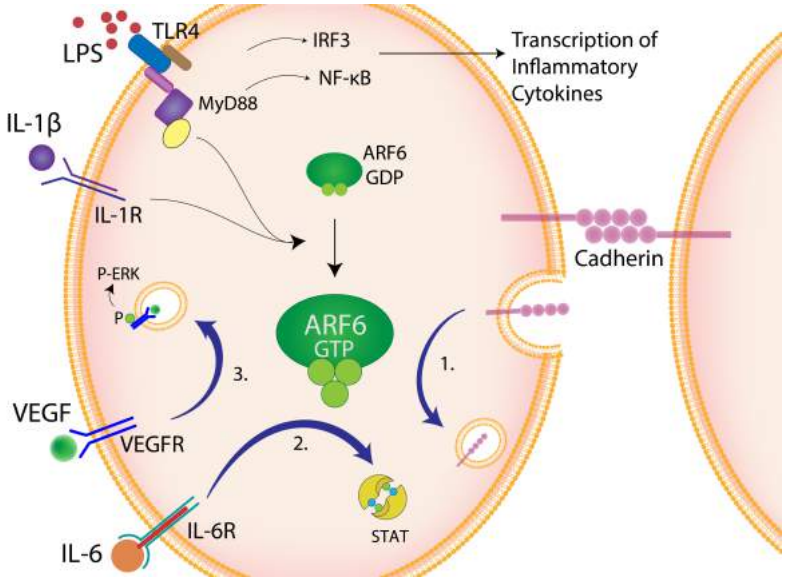

Figure description: ARF6 is activated by a number of inflammatory mediators (LPS) and cytokines (IL-1b, Il-6 and VEGF). Active GTP-bound form of ARF6 mediates VE-cadherin internalization downstream of TLR4 (LPS) and IL-1b pathways (1), potentiates IL-6-induced JAK/STAT signaling (2), and leads to VEGFR internalization and P-ERK signaling (3). When ARF6 is in its inactive GDP-bound form, the adherens junctions and vasculature are stabilized.

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Effects of ARF6 Inhibition *In Vivo:* Animal Models of Vascular Leak

ARF6 Knockout Significantly Reduces Vascular Leak in LPS-Induced Acute Lung Injury

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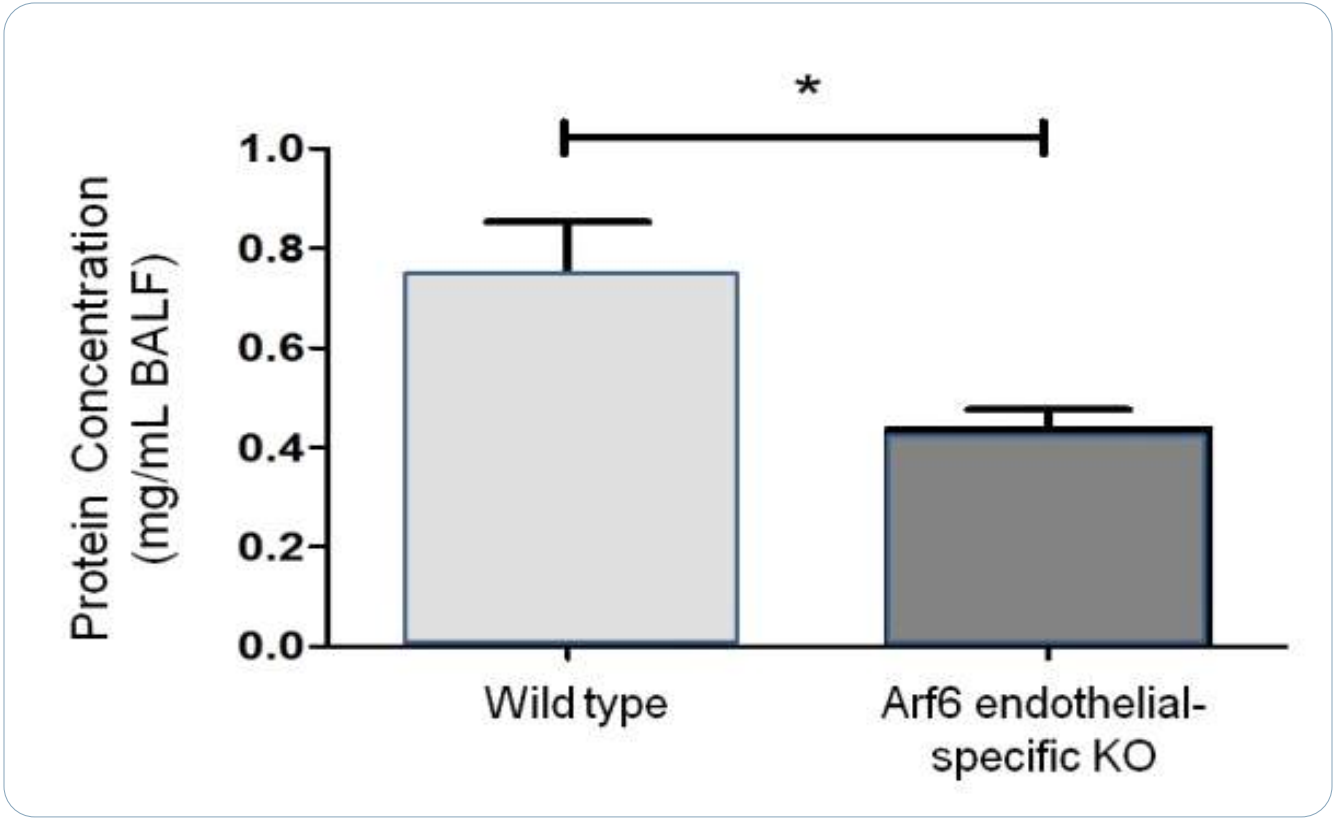

Genetic knockout of ARF6 leads to reduction in vascular leak in mice with LPS-induced acute lung injury. Mice containing an endothelial-specific knockout of ARF6 are more resistant to LPS-induced acute lung injury as measured by the concentration of protein in lung fluid. *, $p<0.05$.

NAV-5171 and NAV-5172 Reduce Vascular Leak in LPS-Induced Lung Injury

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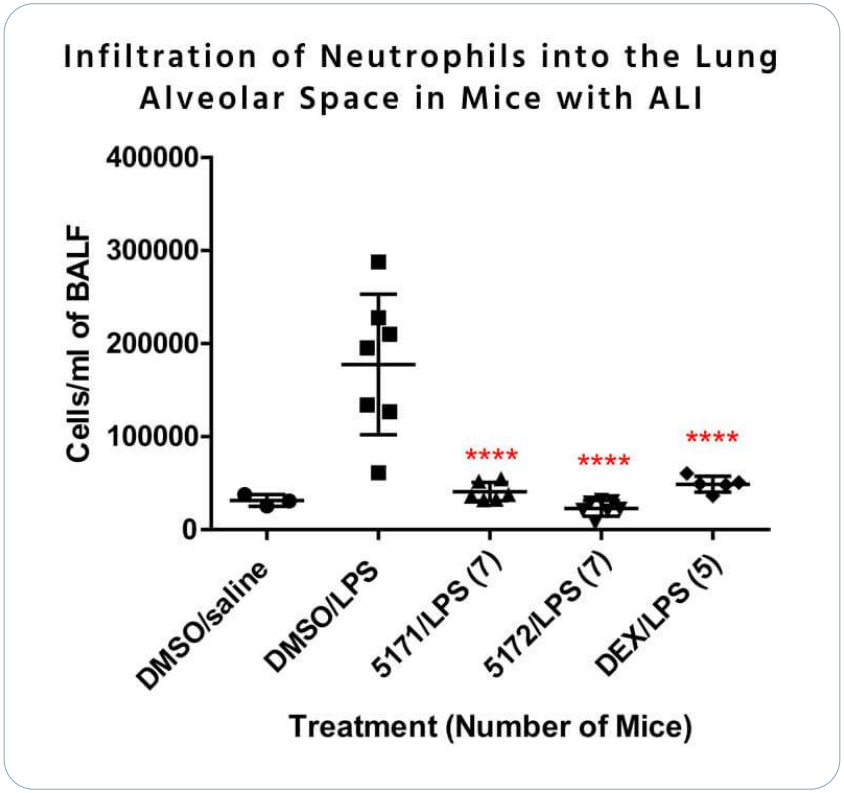
Reduction in LPS-induced bronchoalveolar lavage fluid (BALF) cell count by NAV-5171 and NAV-5172 (IP). ARF6 inhibitor (30 mg/kg) was given by intraperitoneal (IP) injection 3 hours after intratracheal administration of LPS. Dexamethasone (5 mg/kg) was given by IP injection immediately after LPS instillation and 6 hours later. Each data point represents one mouse. ****, $p < 0.0001$ compared to DMSO/LPS. Data were analyzed by 1-way ANOVA followed by Tukey's test for multiple comparisons.



Also see: Mueller A., et al, ARF6 Inhibitors for Acute Lung Injury; (441). Presented at ASM Microbe, June 2016, Boston.

NAV-5093 Improves Survival in Animals with Multi-Drug-Resistant Bacterial Pneumonia

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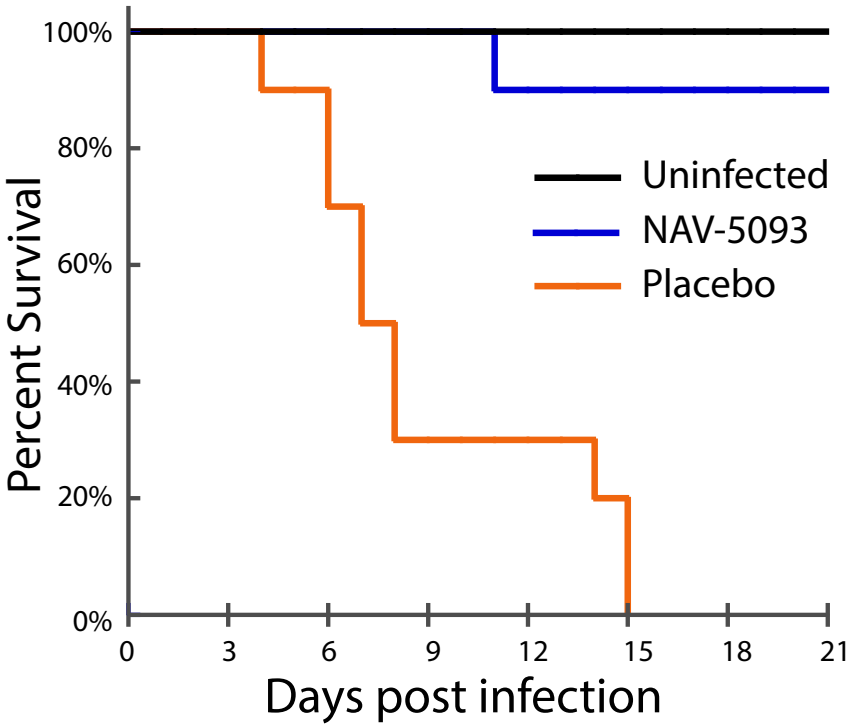
Treatment with NAV-5093, (43 mg/kg IP once daily for 7 days; first dose administered 3 hours after infection) resulted in a highly significant ($p<0.001$) 90% survival rate for neutropenic mice whose lungs were infected with *Acinetobacter baumannii* (AB) (n=10 mice per group).



Also see: Ibrahim A., et al, Targeting MYD88/ARF6 Pathway as a Novel Pathway for Multi-Drug Resistant Acinetobacter Infection; (440). Presented at ASM Microbe, June 2016, Boston.

NAV-5093 Improves Survival in Severe Cerebral Malaria

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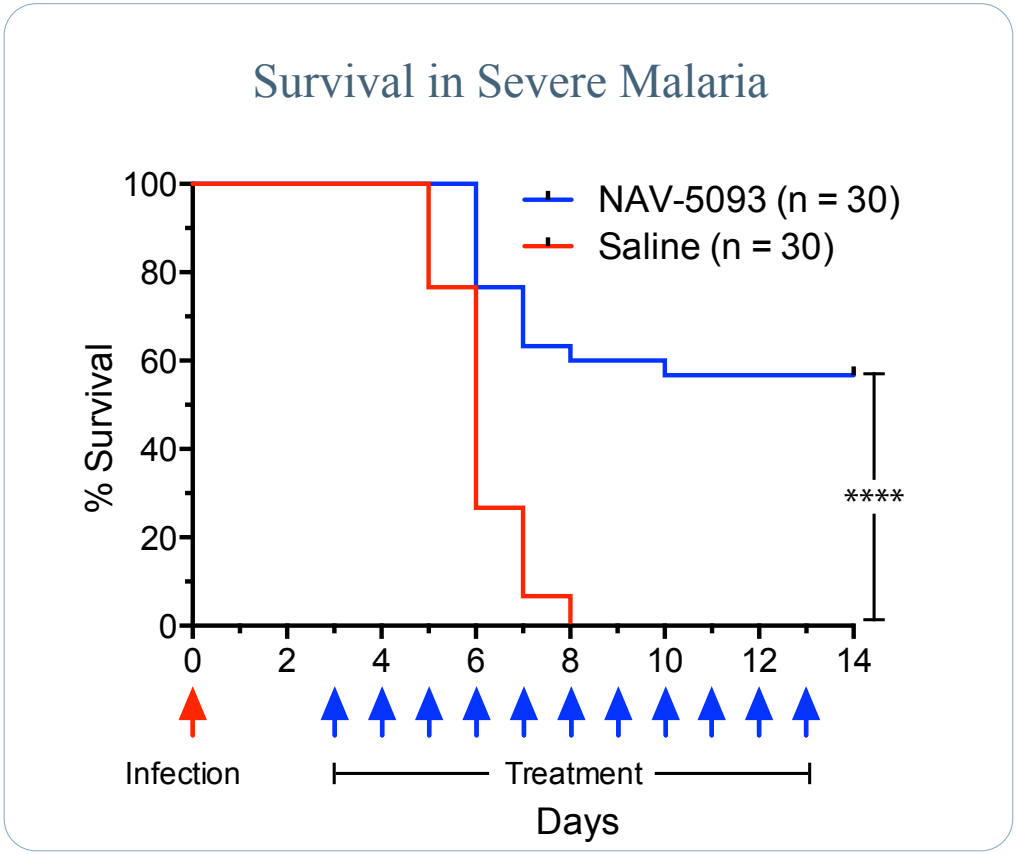
Animals infected with *Plasmodium berghei* ANKA at Day 0 (IP injection). NAV-5093 (IP, 43 mg/kg once daily) starting at Day 3. Combined data from three experiments.
****, p<0.0001



NAV-5093 Reduces Brain Vascular Permeability in Severe Cerebral Malaria

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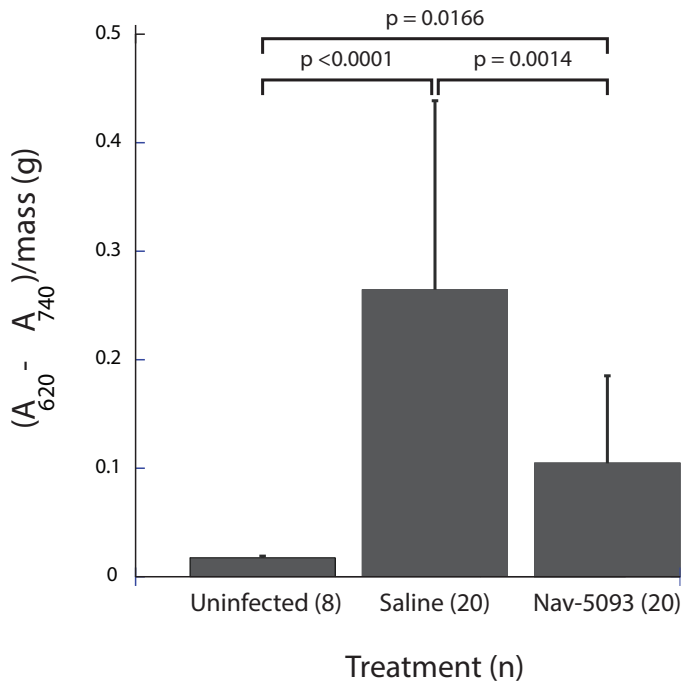
NAV-5093-treated mice showed reduced brain permeability compared to saline-treated and uninfected controls. Brain permeability measured by extravasation of Evans blue dye into brain tissue.

Brain Permeability – Normalized to Weight



Mean and SD (Error bar) of combined data from two studies; Kruskal-Wallis, Dunn's multiple comparison test

NAV-5093 Improves Survival in Mice with Cecal Ligation and Puncture (CLP)-Induced Sepsis

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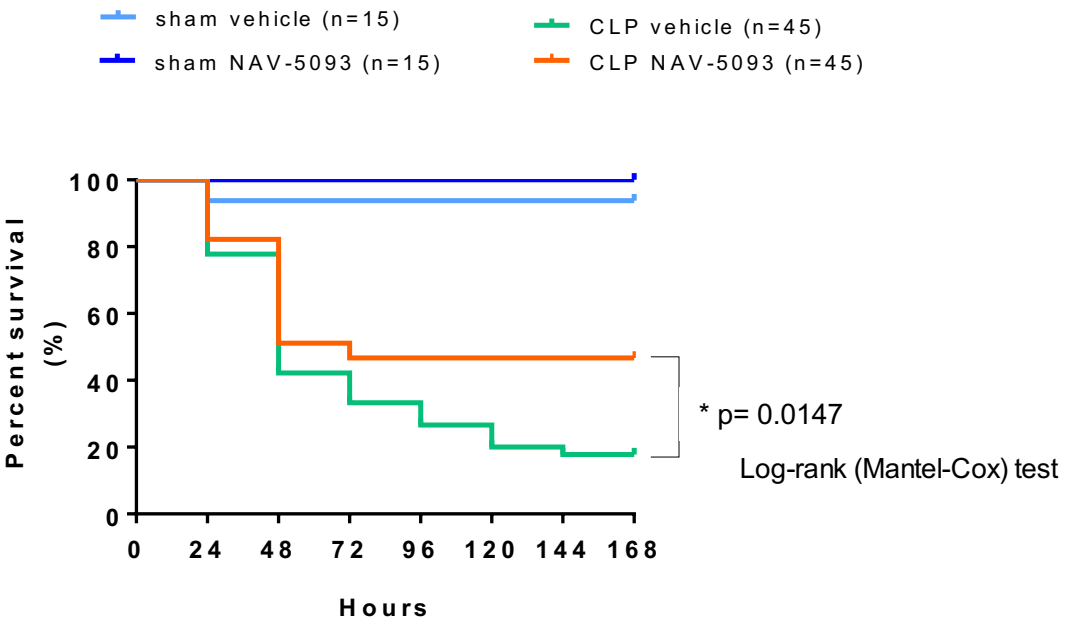
Treatment with NAV-5093 for 3 days (43 mg/kg SC once daily; first dose given 3 hours prior to CLP) significantly improves survival in septic mice. Data from three experiments combined.

Sham vehicle and Sham NAV-5093: n=5 per experiment.
CLP vehicle and CLP NAV-5093: n=15 per experiment.
One sham mouse died after chewing out his stitches.



NAV-5093 Improves Survival in Systemic Candidiasis

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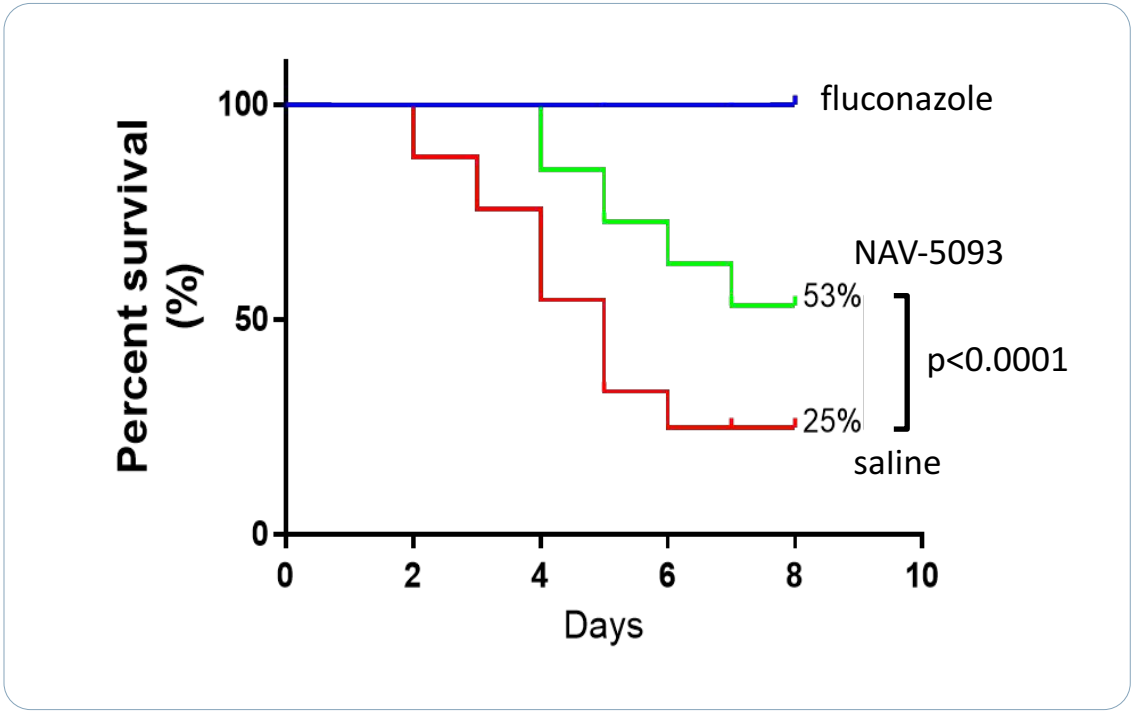

NAV-5093, 43 mg/kg once daily by SC injection for 5 days (first dose administered 3 hours post-infection), produced a significant improvement in survival of Balb/c mice infected with *Candida albicans* (IP injection). n=33 animals/group

ARF6 Inhibitors Improve Survival in MRSA Sepsis

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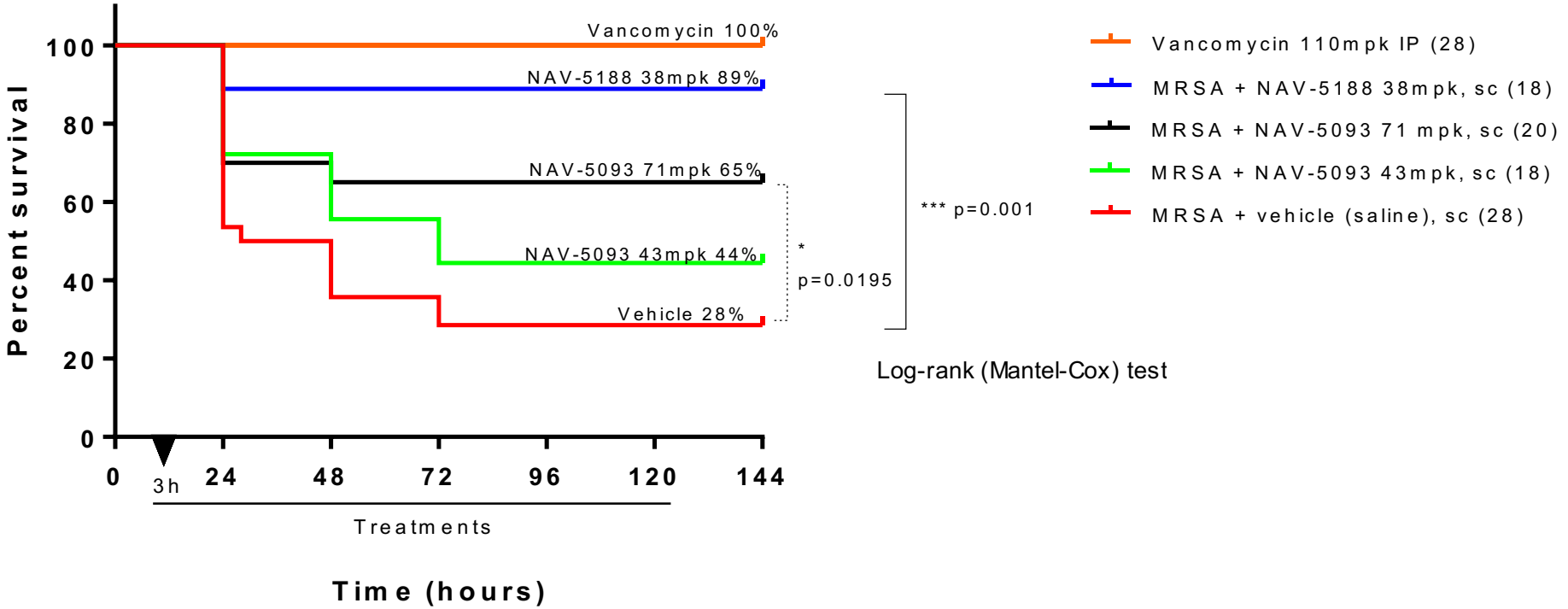

Administration of ARF6 inhibitor by SC injection once daily for 5 days improves survival of mice infected with MRSA. The first dose was given 3 hours after infection.

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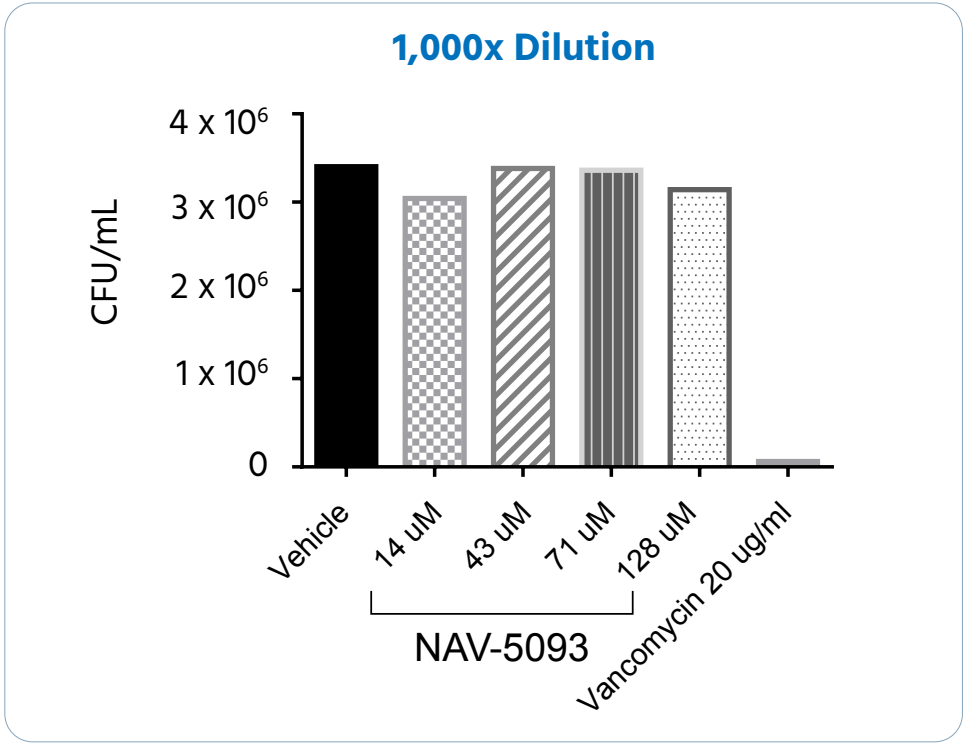
- ARF6 inhibitors do not act directly on the pathogen.
- Rather, they reduce vascular leak without affecting the native immune system, thus allowing time for the host to attack and eliminate the pathogen.

NAV-5093 Has No Direct Effect on MRSA Growth



MRSA (5X10^4 cells/mL) was incubated at 37°C for 4 hours with saline, vancomycin (20 ug/mL), or NAV-5093 (14, 43, 71, or 128 mM) and then plated in duplicate onto blood agar (50 uL at 1000-fold dilution). CFUs were counted 48 hr later. Vancomycin totally inhibited growth of MRSA, whereas NAV-5093 in different concentrations did not show any effect. The numbers in the graph represent the average of the duplicates.

NAV-5093 Reduces Kidney Vascular Permeability in MRSA-Infected Mice

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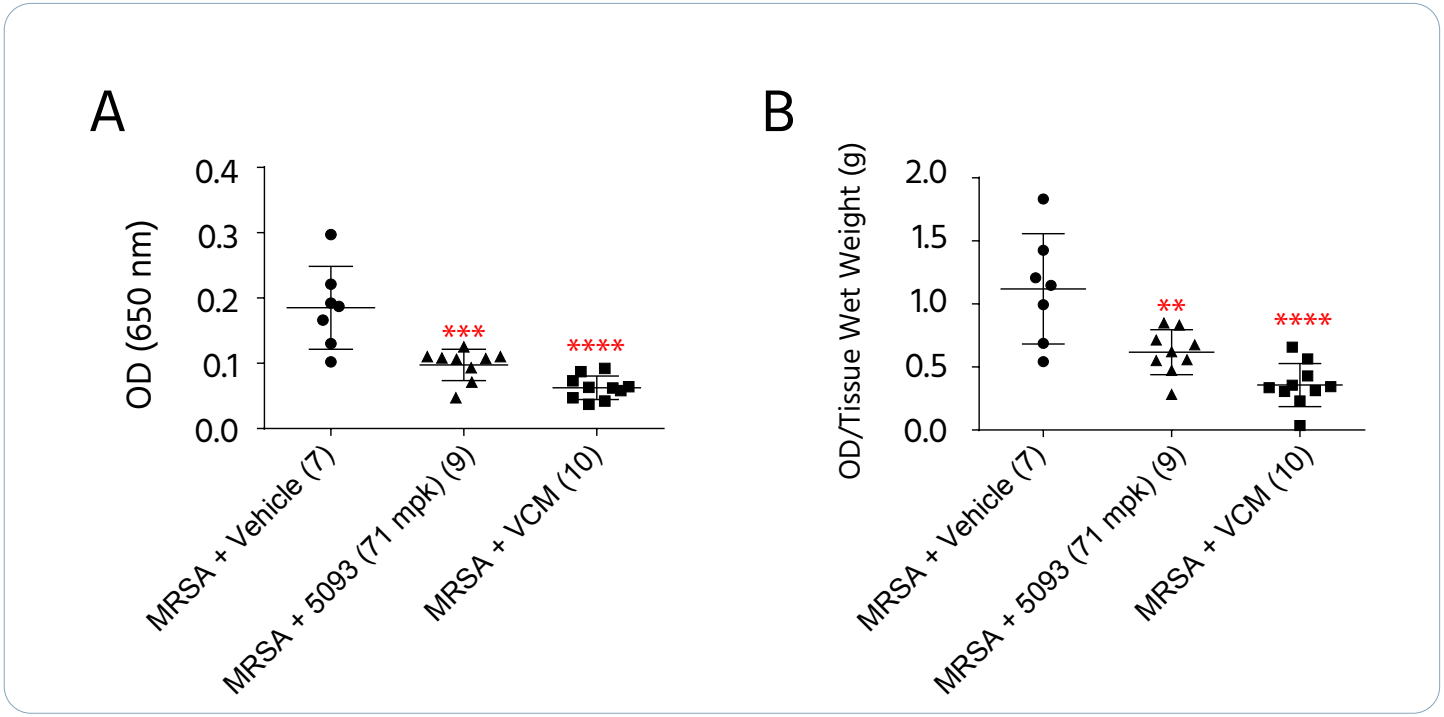

The amount of Evans blue dye (EB) in kidney was measure 18 hours post-inoculum in mice treated with: vehicle (saline, SC), NAV-5093 (71 mg/kg, SC) or vancomycin (VCM, 110 mg/kg, IP). Treatments started 3 hours post-inoculum. (A) OD values represent the EB extravasation from the vasculature into kidneys in MRSA-infected mice. (B) OD values normalized by kidney wet weight. **, $p<0.01$ compared to vehicle control; ***, $p<0.001$ compared to vehicle control; ****, $p<0.0001$ compared to vehicle control; 1-way ANOVA followed by Tukey's test for multiple comparisons.

NAV-5093 Leads to Reduced Bacteremia in MRSA-Infected Mice

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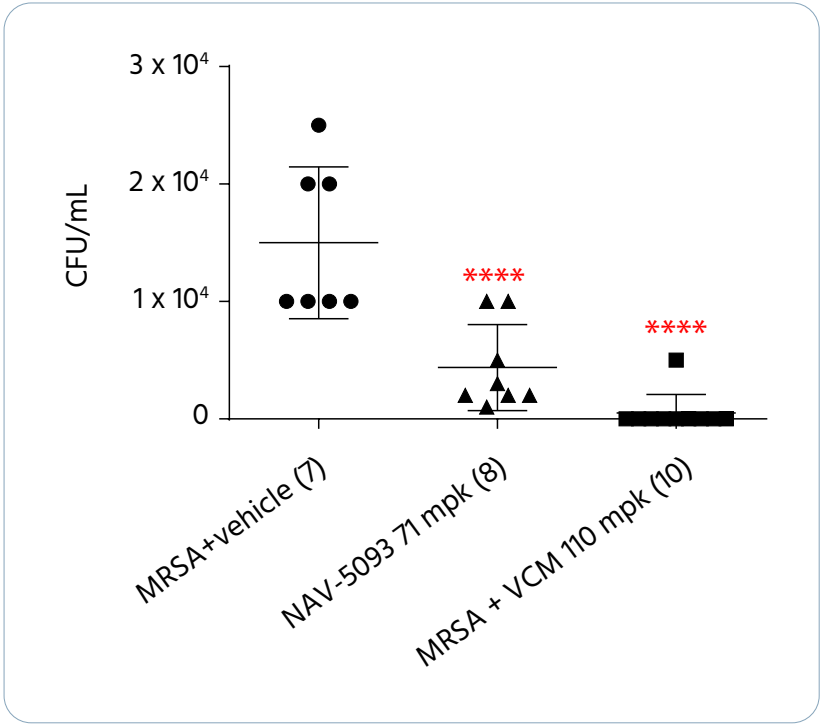

Blood sample was collected from MRSA-infected mice at 18 hr post-inoculation and placed on agar plates for bacterial growth represented by CFU. Mice treated with saline vehicle presented significant bacteremia at 18 hr PI. Mice treated with NAV-5093 (71 mg/kg) or vancomycin (110 mg/kg) showed significant decreases in bacteremia. ****, $p<0.0001$ compared to vehicle control; 1-way ANOVA followed by Tukey's test for multiple comparisons. Data are from 2 identical experiments combined.

NAV-4422 Improves Clinical Score in a Mouse Model of Rheumatoid Arthritis (CIA-induced arthritis)

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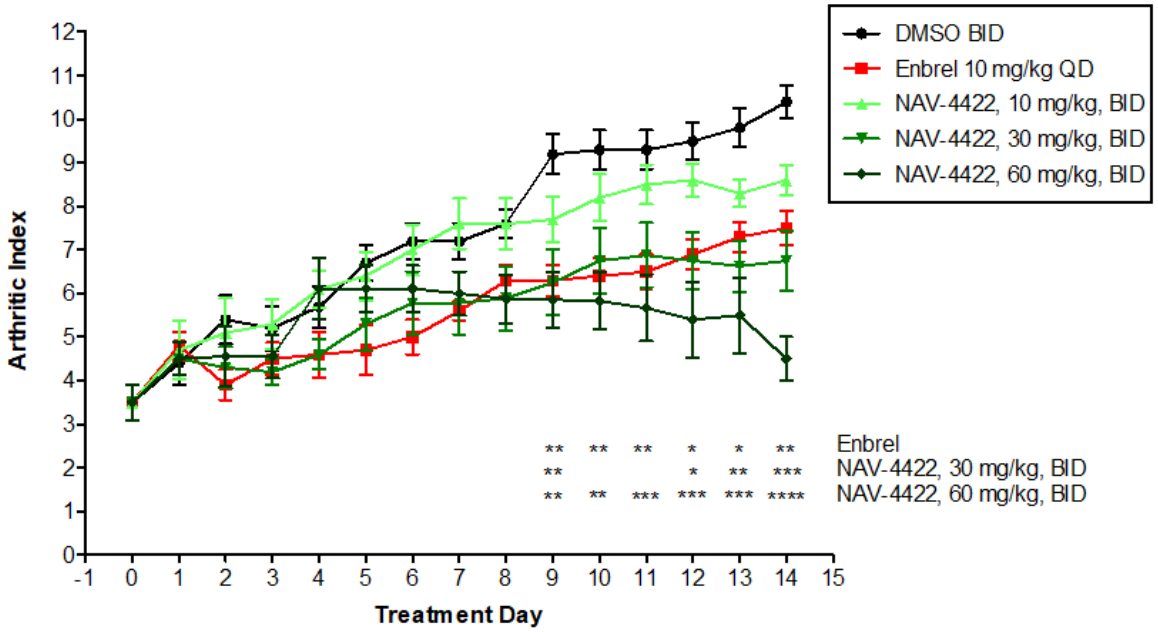

Error bars = SEM
Two-Way ANOVA, Treatment $p < 0.0001$
Bonferroni's post hoc test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

Mice were immunized on Days 1 and 21, and randomized to treatment groups on or around Day 28 for 14 days of therapy (NAV-4422 injected IP BID).

ARF6: A Novel Target for Vascular Eye Disease

- ARF6 is a convergence point of multiple retinal vascular leak pathways, including VEGFR, IL-1R, and TLR4.
- ARF6 is upregulated in the eyes of human diabetes patients, suggesting this pathway is involved in diabetic retinopathy.
- Endothelial ARF6 knockout protects against vascular hyperpermeability (leak) in mouse models of diabetic retinopathy and age-related macular degeneration.
- A6 and its collaborators have shown that small-molecule inhibitors of ARF6 reduce retinal permeability in animal models of vascular eye disease (VEGF-induced retinal permeability, choroidal neovascularization, oxygen-induced retinopathy, and STZ diabetic-induced retinopathy), providing a strong rationale for targeting ARF6 in retinal vascular diseases.

NAV-2729 Inhibits Vascular Hyperpermeability in the STZ Mouse Model of Diabetic Retinopathy

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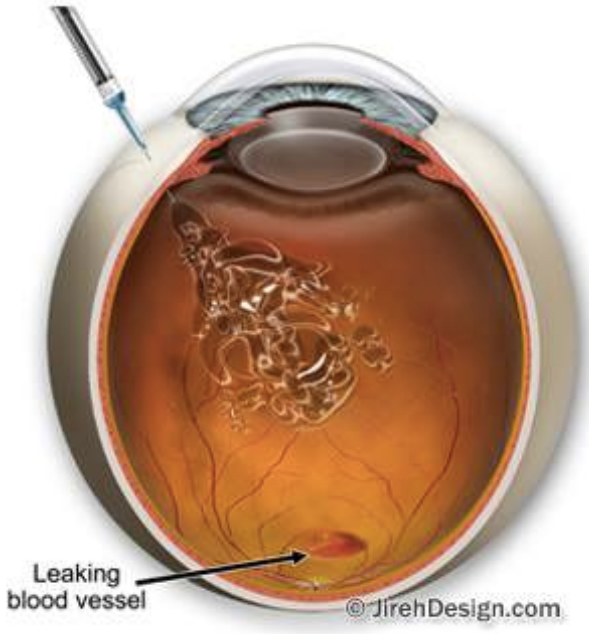

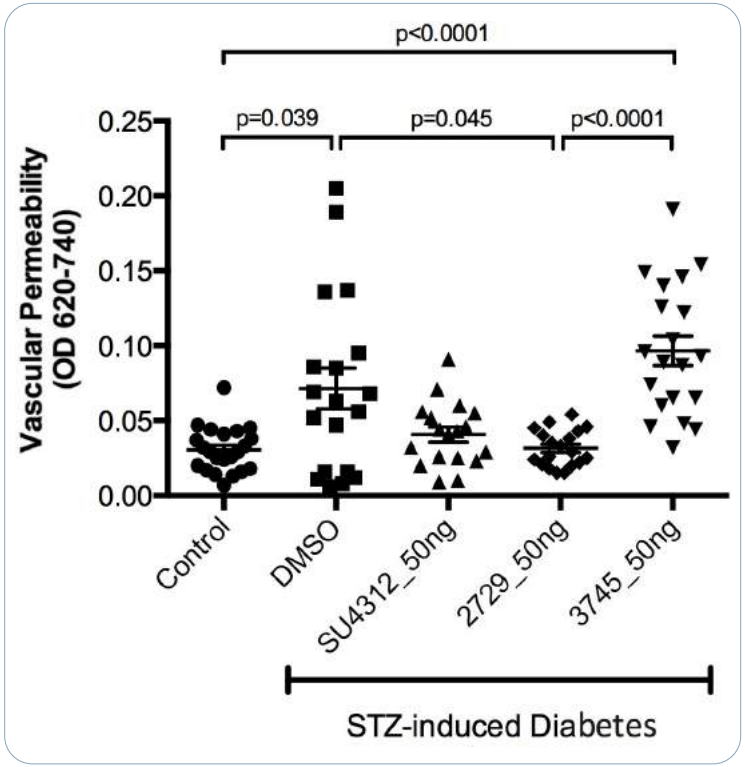

Streptozotocin (STZ)-induced diabetic permeability in mice treated with DMSO, SU4312, NAV-2729, or NAV-3745 (negative control), n=20 eyes/group. SU4312 is a VEGFR/PDGFR inhibitor.

JCI 2017 Oct 23 doi:10.1172/JCI91770

NAV-2729 Rescues Retinal Vascular Hyperpermeability in the STZ Rat Model of Diabetic Retinopathy

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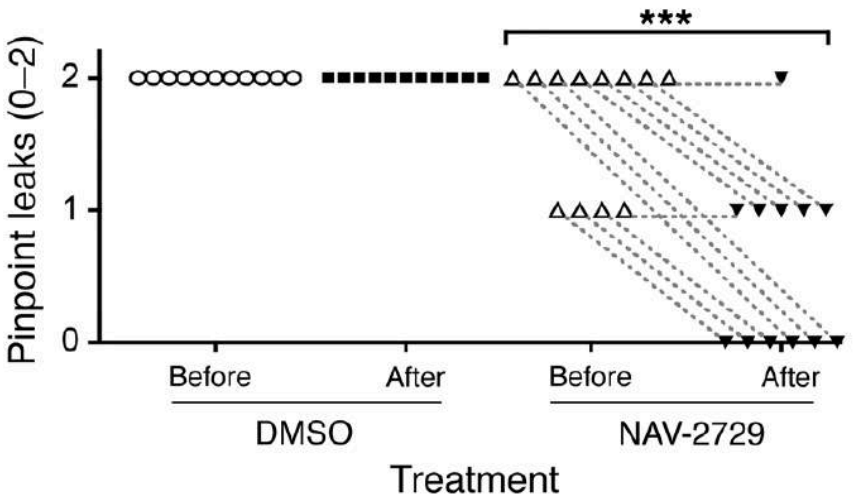
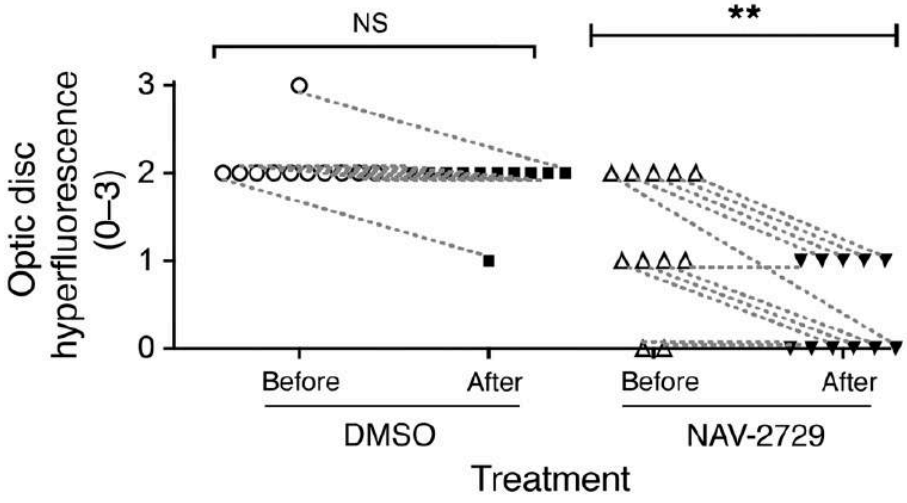
- In diabetic rats, small-molecule ARF6 inhibition significantly improved measures of diabetic retinopathy, including pinpoint leaks and optic disc hyperfluorescence in the retinae, without changing blood glucose levels





Three-month-old diabetic (STZ) male brown Norway rats were injected intravitreally with NAV-2729 (5 ug/5 ul) on day 0 and day 7, and then scored for pinpoint leaks and optic disc hyperfluorescence.

JCI 2017 Oct 23 doi:10.1172/JCI91770

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ARF6-Mediated Pathways Leading to Vascular Leak

ARF6 is Critical to IL-1β-Induced Vascular Leak and Inflammation

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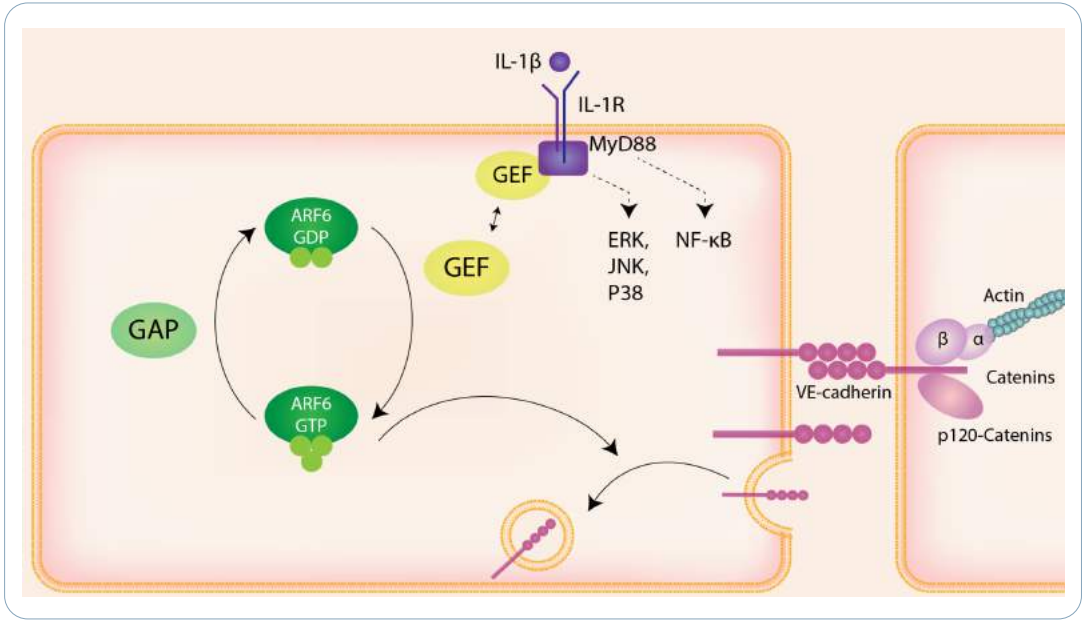
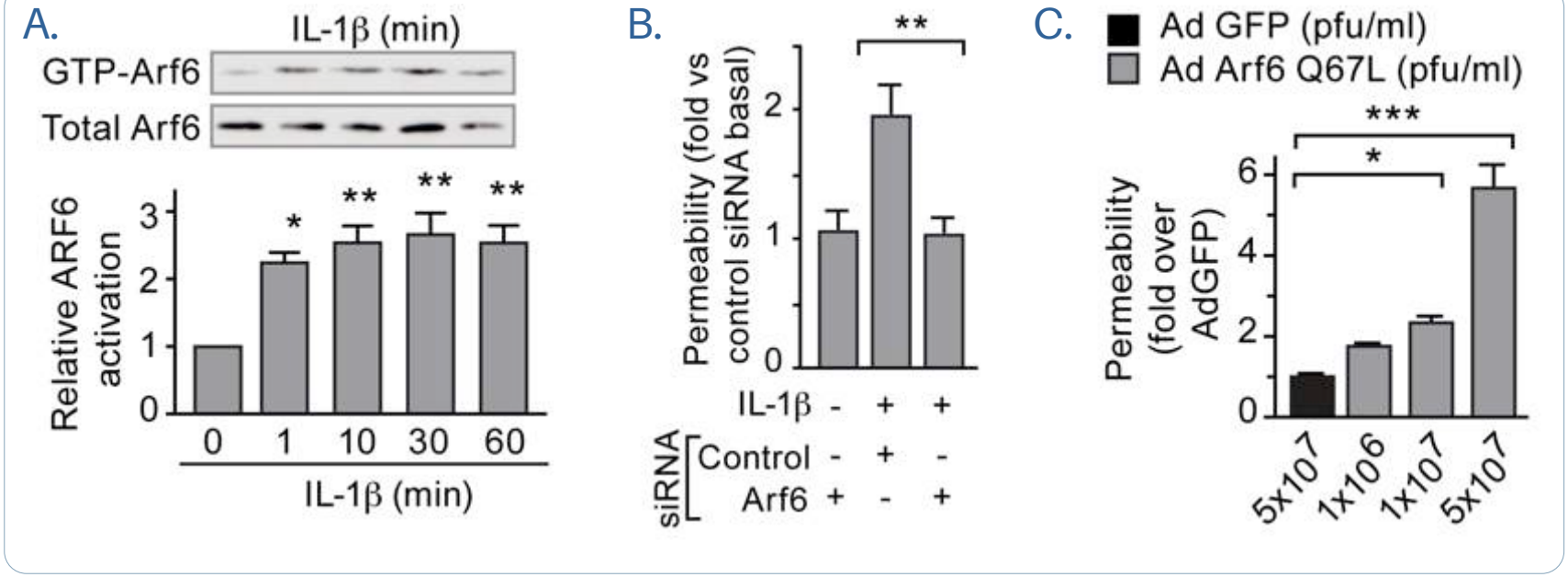
- Disruptive effects of IL-1β on endothelial stability are mediated by ARF-6-induced VE-cadherin internalization
- VE-cadherin internalization dissociates VE-cadherin from the cell-cell junctions, resulting in vascular leak
- Small molecule inhibitors of ARF6 enhance vascular stability by blocking this effect of IL-1β



Zhu et al. Nature 2012 Dec 13;492(7428):252-5

Il-1β Disrupts Endothelial Barrier via ARF6



A. IL-1β-stimulated human dermal micro-vascular endothelial cell (HMVEC-d) lysates were precipitated with glutathione S-transferase (GST)-GGA3 and immunoblotted for ARF6.

B. Monolayers of HMVEC-d were stimulated with IL-1β (10 ng/mL) and assayed for permeability to horseradish peroxidase (HRP) in presence of control or Arf6 siRNA.

C. HMVEC-d monolayers infected with adenovirus (Ad) containing green fluorescent protein (GFP) or ARF6(Q67L) were assayed for permeability to HRP.

Error bars denote SEM. *P<0.05, **P<0.01, ***P<0.001

Zhu et al. Nature 2012 Dec 13;492(7428):252-5

ARF6 is Critical to TLR4 Signaling – Another Important Pathway for Inflammation and Vascular Leak

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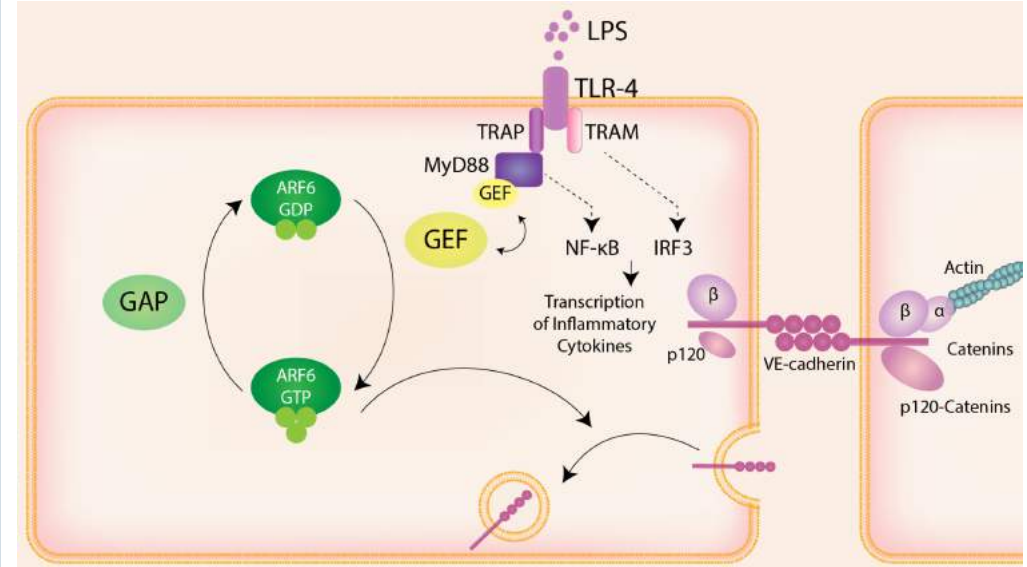
- TLR4 signaling, which induces VE-cadherin internalization and vascular leak, is mediated by ARF6
- TLR4 responds to microbe-derived ligands such as LPS (as illustrated) and endogenous ligands associated with tissue injury and inflammation, including heat shock proteins, fibrinogen, fibronectin, biglycan, and beta amyloid



Davis et al. J Immunol. 2014 Jun 15;192(12):6045-52

LPS Stimulates TLR4 to Induce Vascular Leak via ARF6

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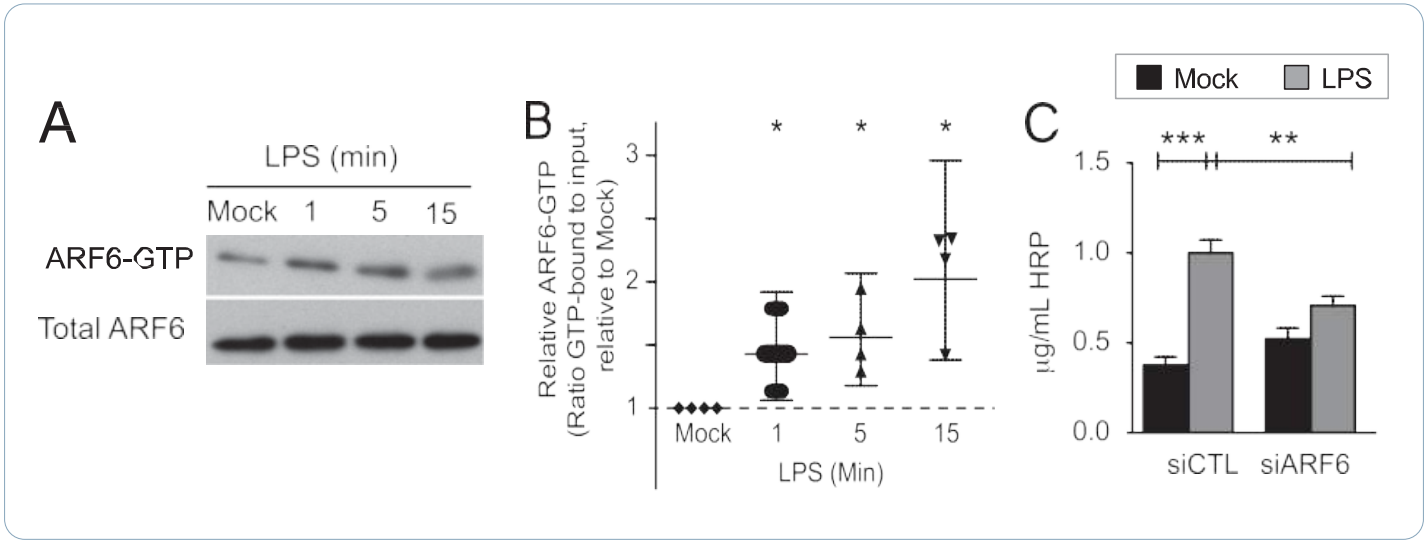

A) HMVEC-Ds were exposed to 100 ng/ml LPS, and active, GTP-bound ARF6 was assessed by GGA3 immunoprecipitation assay and compared with total ARF6 in the lysate.

B) Quantification of data in (A) showing the geometric mean and 95% confidence interval; asterisks denote statistically significant results for which the lower bound of the 95% confidence interval for the normalized ratio does not cross 1 (the null value, represented as a dashed line).

C) HMVEC-Ds treated with siRNAs directed toward ARF6 or a nontargeting control RNA (siCTL) were assessed for their ability to reduce the leak of an HRP reporter after treatment with 100 ng/ml LPS.

*p<0.05, **p<0.01, ***p<0.001.

Davis et al. J Immunol. 2014 Jun 15;192(12):6045-52

ARF6 is Critical to VEGF-Induced Vascular Leak and Inflammation

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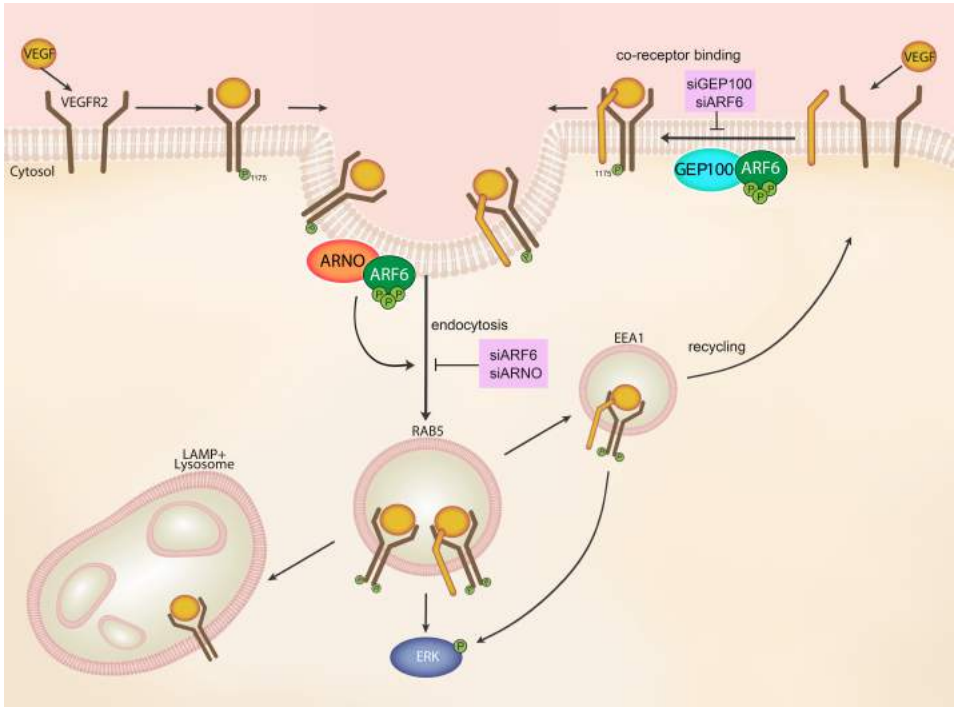
- VEGF induces ARF6 activation
- Activated ARF6 mediates VEGF receptor internalization and recycling
- ARF6 inhibition blocks pathological VEGF signaling, reducing vascular leak



Zhu et al., 2017, J. Clin. Invest. https://doi.org/10.1172/JCI91770.

ARF6 siRNA Blocks VEGFR Internalization and Signaling

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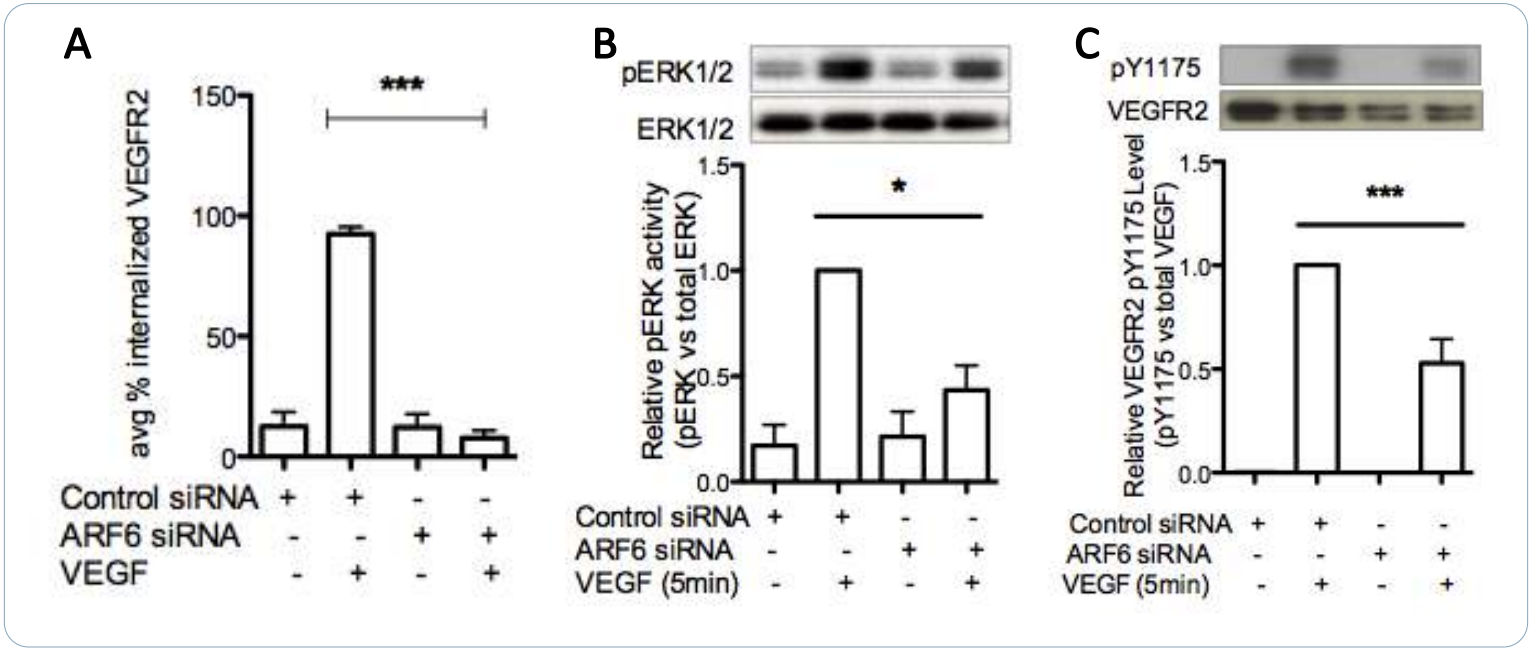

(A) ARF6 siRNA-treated HRECs were labeled with biotin, stimulated with VEGF for 5 minutes, and assayed for VEGFR2 internalization. (B, C) ARF6 siRNA-treated HRECs were simulated with VEGF for 5 min and assayed for ERK and VEGFR2 phosphorylation, respectively. *p<0.05 and ***p<0.001.

Zhu et al., 2017, J. Clin. Invest. https://doi.org/10.1172/JCI91770.

ARF6 Inhibitors and Disorders of Vascular Leak

- Vascular leak is a significant factor in the pathogenesis of many important disorders
- ARF6 plays a central role in regulating vascular permeability
- First-in-class ARF6 inhibitors developed by A6 have been shown to be effective in animal models of numerous disorders associated with vascular leak. These include:
  - Acute lung injury
  - Multidrug-resistant Gram-negative bacterial pneumonia
  - Systemic MRSA infection
  - Severe cerebral malaria
  - Polymicrobial sepsis
  - Systemic candidiasis
  - Rheumatoid arthritis
  - Diabetic retinopathy

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