Maintenance of vascular integrity via ARF6-GTP inhibition protects mice from MDR *Acinetobacter* infection

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ARF6-GTP Inhibitors: Navigen Pharmaceuticals, Salt Lake City
Introduction

• Septicemia due to Multidrug resistant (MDR) Gram negative bacteria (GNB) such as *Acinetobacter baumannii* (AB) is a predominant cause of healthcare-associated infections world-wide with high mortality rates (*Boucher et al.*, *CID* 2009).

• GNB septicemia is treated with few highly toxic antibiotics and in many cases are untreatable (*Boucher et al.*, *CID* 2009).

• Hence, novel approaches of treatment are urgently needed which can be facilitated by understanding the pathogenesis of the infection.
Introduction

• LPS acute lung injury triggers a TLR4-mediated activation of the MyD88/NF-kB cascade leading to robust inflammatory immune response (Zhu…Li., Nature 2012; Davis….Li, J Immunolol. 2014).

• LPS also triggers a MyD88/ARF6 activation pathway that leads to increased vascular leak via internalization of VE-cadherin intracellularly (London…Li Sci Transl Med 2010).

• The increased vascular leak results in tissue edema, organ failure, and ultimate death which is a common feature of septicemia.

• LPS plays a major role in pathogenesis of many GNB including AB (Lin et al. mBio 2012)
Aims

• We sought to determine the role of GNB LPS, using *AB* as a prototype bacterium, in activation of MyD88/ARF6-GTP pathway and its consequence of vascular permeability *in vitro* (using umbilical vein endothelial cells [HUVEC]) and in mice.

• Given its convergence point in destabilizing vascular integrity, we wanted to investigate the role of novel ARF6-GTP inhibitors in protecting against *AB*-induced infections in murine models.
Methods

• **AB**-mediated ARF6-GTP formation in HUVEC and the effect of ARF6-GTP inhibitors was studied by immunoprecipitation (IP) and trans-well permeability assays.

• HUVEC VE-Cadherin expression was tracked by immunofluorescence.

• Contribution of ARF6-GTP to **AB** virulence *in vitro* and *in vivo* was studied by reduction of ARF6-GTP expression (siRNA) and by using ARF6⁻⁻ mice, respectively.

• ARF6-GTP Inhibitors were evaluated for their protective effect in neutropenic mice with **AB** pneumonia.
Results

(AB LPS induces HUVECs permeability via TLR-4 signaling)

(A) Endothelium Permeability (relative to no treatment)

<table>
<thead>
<tr>
<th></th>
<th>HUMC1</th>
<th>HUMC1 Sup</th>
<th>ATCC17978</th>
<th>E. coli LPS</th>
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<td>cells</td>
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(B) Endothelium Permeability (relative to no treatment)

<table>
<thead>
<tr>
<th></th>
<th>HUMC1+</th>
<th>HUMC1+ Isootype Ab</th>
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<tr>
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(C) Endothelium Permeability (relative to no treatment)

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<thead>
<tr>
<th></th>
<th>HUMC1</th>
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<th>NAV4424</th>
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</table>
Results

(AB activates ARF6-GTP formation-Pull Down Assay)
Results

(AB compromises vascular stability via ARF6-mediated intracellular recruitment of VE-cadherin)
Results

(Down regulation of MyD88/ARNO/ARF6 genes attenuate HUVEC permeability in response to AB in vitro)

A

Fold Change (relative to control scramble siRNA)

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<tr>
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<tr>
<td>ARF6 siRNA</td>
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</tr>
<tr>
<td>ARNO siRNA</td>
<td>0.4</td>
</tr>
<tr>
<td>MyD88 siRNA</td>
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<tr>
<td>ROBO4 siRNA</td>
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B

ARF6-GTP Fold Change (relative to Scramble siRNA+AB)

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<tbody>
<tr>
<td>Scrambled siRNA+AB</td>
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</tr>
<tr>
<td>ARF6 siRNA+HUMC1</td>
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<tr>
<td>ARNO siRNA+HUMC1</td>
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<tr>
<td>MyD88 siRNA+HUMC1</td>
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C

Endothelium permeability (relative to no treatment)

<table>
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<th>Treatment</th>
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<tbody>
<tr>
<td>Scrambled siRNA+HUMC1</td>
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<td>ARNO siRNA+HUMC1+Slit2</td>
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<tr>
<td>MyD88 siRNA+HUMC1</td>
<td>4</td>
</tr>
<tr>
<td>HUVEC+AB</td>
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</table>
Results

(ARF6−/− mice are resistant to AB pneumonia)

![Graph showing % survival over days post infection for wild-type and ARF6 null mice.](image)

- Wild-type mice
- ARF6 null mice

P=0.003

N=13 wild-type mice and 14 ARF6 null mutant mice

![Images of lung sections stained with H&E under 325x magnification.](image)
Results

ARF6 inhibitors prolong survival of neutropenic mice with AB pneumonia without affecting the inflammatory immune response.

(A) % Survival

(B) Log CFU/g of lungs

(C) IL-6 (pg/ml)

(D) Lung permeability (µg Evans Blue/g tissue)

(E) Histological images of lungs and spleens.
Results

(Water soluble prodrug ARF6-GTP inhibitor is protective against AB pneumonia model)

NAV-5093 is the prodrug of NAV-4424

* P<0.004 compared to placebo or NAV-4424 treated mice (n=10 mice per arm).
Summary/Conclusions

• **AB** activates MyD88/ARNO/ARF6 pathway via TLR4 stimulation by LPS.

• Activation of ARF6-GTP formation results in enhanced endothelium vascular permeability through intracellular recruitment of VE-Cadherin.

• Down regulation of any of the MyD88/ARNO/ARF6 expression in HUVEC protect them from AB-induced vascular permeability in vitro.

• Conditional HUVEC ARF6 knockout mice are more resistant to **AB** pneumonia than wild-type mice.

• Treatment of wild-type neutropenic mice with ARF6-GTP inhibitors protect them from **AB** pneumonia via a mechanism that involves stabilizing vascular integrity.

• The ARF6-GTP inhibitors can potentially have an effect on other GNB infection and potentially any other organisms that activate the MyD88/ARF6 pathway (e.g. MRSA, *Candida* sepsis)

• Continued investigations of ARF6-GTP inhibitors as a novel treatment for MDR organisms are warranted.
The Model

Death
Organ failure
Tissue edema
Vascular leak

TLR4
VE-cadherin
MyD88
ARF6
GDP
GTP
NF-κB

Inflammatory Cytokines

Serum proteins

Leukocytes

AB

ARF6 inhibitors

MyD88

ARNO

NF-κB

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