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NLRP3 Inflammasome Is a Target for Development of Broad-Spectrum Anti-Infective Drugs

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We describe the molecular mode of action and pharmacodynamics of a new molecular entity (NME) that induces the NLRP3 inflammasome-mediated innate immune response. This innate response reduces the pathogen load in an experimentally induced methicillin-resistant Staphylococcus aureus infection, enhances survival in an experimentally induced Gram-negative bacteremia, and overrides the escape mechanism of an obligate intracellular pathogen, viz. Chlamydia pneumoniae. Furthermore, the NME is more effective than standard-of-care antibiotic therapy in a clinically established multifactorial bacterial infection. Analysis of transcriptional regulation of inflammasome signaling genes and innate/adaptive immune genes revealed consistent and significant host changes responsible for the improved outcomes in these infections. These studies pave the way for the development of first-in-class drugs that enhance inflammasome-mediated pathogen clearance and identify the NLRP3 inflammasome as a drug target to address the global problem of emerging new infectious diseases and the reemergence of old diseases in an antibiotic-resistant form.

Despite the benefit of antibiotics, it now appears that antibiotics have selected for bacterial resistance factors and have given rise to the so-called “superbugs.” The CDC has reported that more people now die from hospital-acquired infections than HIV (23), and methicillin-resistant Staphylococcus aureus (MRSA) is among the leading causative pathogens. In the past, MRSA was a problem that was largely confined to hospitals and extended care facilities; however, emergence and spread of community-acquired MRSA (CA-MRSA) is reaching epidemic proportions (10, 14).

The development of new antibiotics has recently slowed considerably, in large part due to the long lead time from discovery to market for new molecular entities (NMEs), the high failure rates of new drug candidates, the short treatment period, and the low reimbursement rates for antibiotics. Thus, medical practitioners are left in an ever-widening chasm between emerging new antibiotic-resistant pathogens and a dearth of therapeutic alternatives. Given the recent evidence that bacterial pathogens harbor resistance genes to all known classes of antibiotics (11), continued efforts to discover and develop new antibiotics are simply repeating the same experiment with the expectation of a different result.

Mammals have evolved tolerance mechanisms to accommodate beneficial microorganisms as well as potential pathogens and have evolved resistance mechanisms to reduce the pathogen burden during infection (reviewed in reference 33). Therefore, we have been exploring a strategy of modulating the host tolerance and resistance mechanisms as a means to ablate infection from progressing to disease.

The inflammasomes are the major regulator of resistance and tolerance in mammalian cells and are comprised of a family of cytosolic receptors, called NOD-like receptors (NLR), that are involved in innate immune recognition of pathogen-associated molecular patterns as well as intracellular and extracellular damage-associated molecular patterns (reviewed in reference 24). Thus far, more than 20 inflammasomes have been identified, and many are present in nearly every cell type. NLR protein 3 (NLRP3; also known as NALP3) is the most extensively studied inflammasome and has been found to be activated by a diverse range of stimuli, including microbe-derived products (8, 12, 18, 28, 35), environmental factors (6, 13, 22), and endogenous molecules (16, 25, 31, 39).

Herein, we show that regulation of the NLRP3 inflammasome is a safe and effective means to prevent Gram-positive and Gram-negative bacterial infections from progressing to disease (enhanced tolerance) and abrogate disease progression in established bacterial infection (enhanced resistance). The obvious advantage of this therapeutic strategy is that, by targeting the host tolerance and resistance mechanisms, selective pressure for the expression of pathogen resistance is greatly reduced or altogether absent.

Thacker and colleagues (34) reported the isolation, structure elucidation, and synthesis of an NME, 1-peptidyl-2-arachidonoyl-3-stearoyl-glyceride, derived from a caprine serum fraction similar to that which had been previously reported (2, 20, 29, 30). The chemical structure is shown in Fig. 1 and here is referred to with the acronym pDAG. In the previous report it was also shown that the biological activity of pDAG was attributable to the 18-amino-acid peptide moiety that is here referred to as acALY18. In this report we describe the molecular mechanism of action (MMOA) of acALY18 as a first-in-class, broad-spectrum anti-infective agent targeting the NLRP3 inflammasome.

(Presented in part at the National Meeting of the American Society for Microbiology.)
Association for Immunologists, San Francisco, CA, May 2011, abstract B235.)

MATERIALS AND METHODS

Details of the methods used are available in the supplemental material and are summarized here.

Animal care and use. Animals were handled in accordance with NIH policies for humane care and use of laboratory animals, and studies were conducted under specific protocols approved by the Institutional Animal Care and Use Committee of Lampire Biological Laboratories, Inc. (for antibody production and adjuvant experiments in rabbits), Covance, Inc. (for adjuvant experiment in rabbits and Salmonella bacteremia experiments in mice), and Drexel University College of Medicine (for the MRSA study of mice).

Test article. pDAG was isolated and synthesized as previously described, and acALY18 was synthesized by solid-phase methods as previously described (37).

Cell culture. Normal primary human fibroblasts (GM5659) obtained from the Coriell Institute (Camden, NJ) were cultured in 70-mm dishes as previously described (5). Mouse fibroblasts were established from the Coriell Institute (Camden, NJ) were cultured in 70-mm dishes as previously described (5). Mouse fibroblasts were established from the Coriell Institute (Camden, NJ) were cultured in 70-mm dishes as previously described (5). Fibroblasts and THP-1 monocytes were harvested, and RNA was purified using the Qiagen RNA isolation kit. Five micrograms of RNA was converted to cDNA and applied to the human innate and adaptive immune response RT2 Profiler PCR array and to the inflammasome array (SABiosciences). Gene expression was assayed using SYBR green as a reporter. The cycle threshold (Ct) of the genes was normalized to that of β-actin.

Cytokine and chemokine protein assay. Cytokines and chemokines in fibroblast supernatants were measured by enzyme-linked immunosorbent assay according to the manufacturer’s specifications (eBioscience). Cell lysates were assayed using the caspase-1 colorimetric assay kit (Abcam) according to the manufacturer’s protocol.

Tissue histology and bacteriology. The lesion tissue was collected using an 8-mm punch biopsy tool and weighed. Samples of the excised tissue for determining pathogen load were homogenized in 4 ml of sterile normal saline using an OMNI model HT homogenizer. The homogenate was centrifuged briefly at 100 g to pellet tissue debris, and the supernatant was serially diluted and plated on Trypticase soy agar plates supplemented with gentamicin and methicillin and incubated overnight at 37°C. Colonies were counted and are reported as CFU/g of tissue. Samples taken for tissue histology were fixed in 4% paraformaldehyde overnight, paraffin embedded, and sectioned onto positively charged microscope slides. Sections were stained with Masson’s trichrome according to standard protocols (5) to localize collagen fibers, gross structural changes within the tissue, and inflammatory infiltrate. Separate sections were deparaffinized as previously described (3), blocked with 5% donkey serum, and incubated with either rabbit antimyeloperoxidase or IgG-fluorescein isothiocyanate (FITC; 1:400; Abcam, Cambridge, MA), washed, then incubated with donkey anti-rabbit Cy3 diluted 1:1,000 (Jackson Immunochemicals, West Grove, PA), counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and viewed with an epifluorescence microscope. Images were taken at 100× and 400× magnification.

Monocyte activation. Primary normal human fibroblasts (GM5659; Coriell Institute) were transfected with or without 3 ng/ml acALY18 and cultured as previously described. Cells not receiving acALY18 were treated with Lipofectamine alone as a control. Human THP-1 monocytes were cultured in T75 flasks in RPMI plus 10% fetal bovine serum and 1% penicillin-streptomycin for 48 h and harvested after centrifugation (2,000 rpm for 5 min at 4°C). THP-1 monocytes were resuspended in the medium collected from the acALY18-treated or control-treated fibroblasts and incubated for an additional 72 h. In a separate experiment the harvested THP-1 monocytes were resuspended in the medium collected from the acALY18-treated or control-treated fibroblasts, added back to the respective fibroblast culture, and allowed to incubate for an additional 72 h. Monocytes were recovered from the respective cultures and centrifuged to remove the culture medium. Cells were resuspended in 4 ml of flow washing buffer (FWB) and centrifuged, and the supernatant was removed. Cells were counted, an aliquot of 1,000,000 cells was resuspended in 20 µl of FWB, and 5 µl of fluorescently labeled CD14 antibody was added to the cells for 20 min at 4°C. The cells were washed twice with 4 ml of FWB and then resuspended in 4 ml FWB. Cells were fixed with 200 µl of 1% paraformaldehyde for at least 30 min and then analyzed by flow cytometry (FACSaria; BD Biosciences).

Statistics. Statistical analyses were performed using GraphPad Prism (v. 5.03) and GraphPad InStat (v. 3.10). Tests for significance (P values) were calculated using Student’s t test for paired or unpaired data with or without Welch’s approximation to correct for unequal standard deviations when appropriate. Survival curves were analyzed using a contingency table.

RESULTS

Therapeutic proof of relevance. Nineteen cows were clinically diagnosed with mastitis (group B Streptococcus and Escherichia coli), and 9 were treated with pDAG (0.25 mg/kg of body weight, intramuscularly [i.m.], day 0 and day 3), and 10 received the standard of care (Polylex brand of ampicillin; 5 mg/lb, i.m., for 3 days). The standard practice in the dairy industry is to measure the leukocyte count in the milk of cows as a diagnostic metric (normal milk contains <500 cells/µl). pDAG reduced the leukocyte count from 2,176 ± 347.5 to 510.6 ± 97.42 cells/µl (means ± standard deviations; P = 0.0003), whereas the antibiotic treatment group was unchanged (P = 0.8). These results are presented in Fig. 2A.

Salmonellosis is a significant diarrheal disease in humans. Whereas E. coli and group B streptococci (present in the bovine mastitis study) are extracellular pathogens, Salmonella enterica can infect macrophages during disease and are considered facultative intracellular pathogens. To determine if pDAG could modulate disease caused by an intracellular pathogen, we studied the effects of pDAG on S. enterica bacteremia. pDAG enhanced survival of mice in an experimentally induced S. enterica bacteremia model (Fig. 2B). Infected mice (5 × 10⁷ CFU) either received a single dose of pDAG (0.25 mg/kg) or vehicle only (control) by subcutaneous injection at the time of infection. By day 10 all control mice died, whereas only 20% of pDAG-treated mice died (P = 0.0001).

acALY18 clears intracellular Chlamydia pneumoniae. Chlamydia pneumoniae causes an important community-acquired respiratory infection that persists in various human cells, including monocytes, where it is refractory to antibiotic treatment (17). The persistent form of C. pneumoniae has been implicated in the etiology of chronic diseases (19), and Appel and colleagues recently showed that C. pneumoniae suppresses autophagy and subsequent

FIG 1 Chemical structure of pDAG and acALY18. The chemical structure of the 1-peptidyl-2-arachidonoyl-3-stearoyl-sn-glyceride natural product is shown. The 18-amino-acid peptide moiety is acALY18.

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Antimicrobial Agents and Chemotherapy
lysosome-phagosome fusion to promote intracellular survival within the phagosome (3). We wanted to determine if acALY18 would override the escape mechanism of C. pneumoniae and induce clearance from infected monocytes.

Human THP-1 monocytes were infected (multiplicity of infection [MOI], 1) and incubated for 24 h, followed by addition of acALY18 (25, 50, and 100 ng/ml) and analysis 24 h posttreatment. THP-1 monocytes did not tolerate Lipofectamine well, necessitating a higher concentration of acALY18 in these experiments to ensure its intracellular delivery, as we have previously reported (34). A dose-response relationship was observed: ~90% of the untreated cells remained infected, and ~12% of the acALY18-treated cells remained infected at 100 ng/ml, ~35% at 50 ng/ml, and ~40% at 25 ng/ml. Figure 2C and D show the representative photomicrographs of infected, untreated monocytes and the in-fected, acALY18-treated (100 ng/ml) monocytes.

We examined gene expression from infected/acALY18-treated monocytes versus infected/untreated cells. At 48 h postinfection, 26 innate and adaptive immune genes were upregulated >4-fold in infected/treated cells, and 4 genes were downregulated at least 3-fold. Functional grouping of the increased gene expression included the following: (i) cytokines/chemokines, receptors, and signaling proteins (CCL2, INF1B, INFGR1, IL-1β, IL1F5, IL1F7, IL1F8, IL1F9, IL1F10, IL1RN, IL-6, MYD88, TLR1, TLR10, TLR6, TLR8, and TNF); (ii) host defense (DEFB4, DMBT1, NCF4, NLRC4, and S100A12); (iii) antibacterial response (COLEC12, CYBB, and LY96); (iv) modulators of the tissue response to inflammation (ADOR4A, PTAFR, SERPINA1, SERPINE1, and TREM1). These results are presented in Table S1 of the supplemental material.

acALY18 activates the inflammasome. We postulated that acALY18 induces a signaling cascade that is at least in part mediated by the inflammasome (34). Inflammasome effector mechanisms have recently been reviewed (24), and it is clear that the inflammasome is a key regulator of the innate immune response to cellular injury and infection. The IL-1 family of cytokines are expressed as procytokines, and secretion is dependent upon activation of the cysteine protease caspase-1. IL-1β, IL-18, and IL-33, all IL-1 family cytokines, can act in an autocrine or paracrine manner, and their receptors are present in a variety of cell types, including fibroblasts and keratinocytes as well as tissue-resident immune cells (4).
acALY18-treated fibroblasts expressed 2.5-fold more active caspase-1 than untreated fibroblasts, and this activity was ablated by the caspase-1 inhibitor Z-YVAD(OMe)-FMK. acALY18 induced a 5-fold increase in secreted IL-1β, a 2.1-fold increase in secreted IL-18, and a 1.9-fold increase in secreted IL-33 relative to untreated fibroblasts (Fig. 3). The caspase-1 inhibitor significantly reduced acALY18-induced IL-1β and IL-18 secretion when used alone. The caspase-1 inhibitor also reduced acALY18-induced IL-33 secretion to a concentration that was statistically equivalent to that secreted by untreated control cells. Data are presented as means of three separate experiments in duplicate ± the standard deviation.

acALY18-induced caspase-1-dependent secretion of inflammatory cytokines (IL-1β, IL-18, and IL-33) from primary human fibroblasts, suggesting that the effects of acALY18 are mediated through the inflammasome complex. Previous work by Artlett et al. demonstrated that the caspase-1 inhibitor Z-YVAD(OMe)-FMK does not induce apoptosis or alter fibroblast proliferation (5), suggesting that the observed effects are mediated through the inflammasome rather than cell-induced apoptosis.

The apoptosis-associated Speck-like protein containing a caspase recruitment domain (CARD; ASC) is a scaffold protein associated with an NLRP in many inflammasomes. NLRP3 is one of the most well-studied NLRPs and is generally believed to respond to damage-associated molecular patterns (DAMPs), like extracellular ATP, uric acid, or amyloid-β, as well as intracellular DAMPs, like reactive oxygen species (15, 32). Therefore, we examined the effects of acALY18, a presumptive DAMP, on fibroblasts from wild-type C57BL/6 mice and from ASC−/− and NLRP3−/− mice of the C57BL/6 background. Similar to the effects in human fibroblasts, acALY18 induced IL-1β, IL-18, and IL-33 secretion from C57BL/6 mouse fibroblasts and this was abrogated by Z-YVAD(OMe)-FMK. acLY18 did not induce significant secretion of IL-1β, IL-18, or IL-33 in the ASC−/− or NLRP3−/− fibroblasts, confirming that the effects of acALY18 are mediated, at least in part, by the NLRP3 inflammasome (Table 1).

acALY18 induces innate and adaptive immune gene transcription. We examined the specific inflammasome-related genes that were up- or downregulated more than 2-fold at 24, 48, and 72 h after treatment of primary human fibroblasts with acALY18 (3 ng/ml). At 24 h, 27 inflammasome-related genes were upregulated and 1 gene was downregulated, but none of these genes was found...
at levels significantly different from untreated cells. At 72 h, 39 genes were significantly upregulated, including six inflammasome platform genes (AIM2, NLRP3, NLRP4, NLRP5, NLRP9, and NOD2), and 36 genes were significantly downregulated, including apoptosis regulators (BCL2, BIRC2, and FADD). Other inflammasome-associated protein mRNAs were upregulated at 72 h, including the caspase recruitment domain family members CARD18 and CARD6 (214-fold and 1,600-fold, respectively) and Mediterranean fever pyrin (93-fold). Forty-eight genes associated with the innate and adaptive immunity were upregulated by 48 h, including caspase-1 and caspase-4 (9.9-fold and 5.8-fold, respectively), interleukin receptor-associated kinases 1 and 2 (26-fold, and 95-fold, respectively), MyD88 (5-fold), and NF-κB (27-fold). IL-1β mRNA expression was unchanged at 24 h but was upregulated nearly 50-fold at 48 h, and it declined to a 4-fold upregulation by 72 h. Several other cytokine genes were upregulated, including the type I interferons (IFN-α, 10.8-fold; IFN-β, 9.3-fold), IFN-γ (5.5-fold), IL-10 (17-fold), and IL-18 (2-fold), as were the genes for cellular defensive proteins, such as nitric oxide synthase (135-fold), C5 (10-fold) and C8 (10-fold), cathelicidin (10-fold), defensin-B4 (14-fold), and the lysosomal protease cathepsin B (34-fold). Clearly, the gene regulation that is induced by acALY18 enhances cellular defensive responses. It also appears that the inflammatory response to acALY18 reaches a maximum around 48 h and is downregulated by 72 h. The pharmacodynamic profiles for selected gene expression are depicted in Fig. 4, and summaries of the complete gene expression results are presented in Tables S2 and S3 of the supplemental material.

**acALY18 induces antigen-specific IgM.** A key feature of the innate immune response is production of opsonizing molecules, like complement. In primary human fibroblasts, acALY18 induced a 10-fold upregulation of C5 and C8 complement genes at 48 h (see Table S2 in the supplemental material). acALY18 promotes IL-33 secretion by fibroblasts, and IL-33 activates B1 cells in the mouse (1) to contribute to serum IgM levels (36). We examined IgM production in rabbits treated with either Freund’s complete adjuvant (FCA) as a positive control, Freund’s incomplete

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**TABLE 1** acALY18 requires a functional inflammasome for induction of IL-1β, IL-18, and IL-33 secretion*

<table>
<thead>
<tr>
<th>Cytokine measured and mouse line</th>
<th>Cytokine secretion (pg/ml, ± SD)</th>
<th>P value (comparison)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-1β</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td>41.26 ± 3.04</td>
<td></td>
</tr>
<tr>
<td>C57BL/6 + acALY18</td>
<td>71.47 ± 0.23</td>
<td>0.0006 (vs C57BL/6)</td>
</tr>
<tr>
<td>C57BL/6 + YVAD</td>
<td>33.1 ± 3.17</td>
<td>0.14 (vs C57BL/6)</td>
</tr>
<tr>
<td>C57BL/6 + YVAD + acALY18</td>
<td>53.57 ± 1.22</td>
<td>0.02 (vs C57BL/6 + acALY18)</td>
</tr>
<tr>
<td>ASC−/−</td>
<td>22.17 ± 2.91</td>
<td></td>
</tr>
<tr>
<td>ASC−/− + acALY18</td>
<td>19.80 ± 2.85</td>
<td>0.59 (vs ASC−/−)</td>
</tr>
<tr>
<td>NLRP3−/− + acALY18</td>
<td>22.63 ± 1.45</td>
<td></td>
</tr>
<tr>
<td><strong>IL-18</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td>51.63 ± 2.07</td>
<td></td>
</tr>
<tr>
<td>C57BL/6 + acALY18</td>
<td>63.23 ± 2.70</td>
<td>0.05 (vs C57BL/6)</td>
</tr>
<tr>
<td>C57BL/6 + YVAD</td>
<td>33.6 ± 1.27</td>
<td>0.004 (vs C57BL/6)</td>
</tr>
<tr>
<td>C57BL/6 + YVAD + acALY18</td>
<td>33.07 ± 0.47</td>
<td>0.002 (vs C57BL/6 + acALY18)</td>
</tr>
<tr>
<td>ASC−/−</td>
<td>33.53 ± 2.40</td>
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</tr>
<tr>
<td>ASC−/− + acALY18</td>
<td>35.76 ± 0.88</td>
<td>0.35 (vs ASC−/−)</td>
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<tr>
<td>NLRP3−/− + acALY18</td>
<td>32.77 ± 2.00</td>
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</tr>
<tr>
<td><strong>IL-33</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td>143.97 ± 2.52</td>
<td></td>
</tr>
<tr>
<td>C57BL/6 + acALY18</td>
<td>159.10 ± 0.95</td>
<td>0.03 (vs C57BL/6)</td>
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<tr>
<td>C57BL/6 + YVAD</td>
<td>118.13 ± 2.22</td>
<td>0.01 (vs C57BL/6)</td>
</tr>
<tr>
<td>C57BL/6 + YVAD + acALY18</td>
<td>137.93 ± 1.76</td>
<td>0.02 (vs C57BL/6 + acALY18)</td>
</tr>
<tr>
<td>ASC−/−</td>
<td>79.20 ± 6.04</td>
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<tr>
<td>ASC−/− + acALY18</td>
<td>93.93 ± 2.51</td>
<td>0.13 (vs ASC−/−)</td>
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<tr>
<td>NLRP3−/− + acALY18</td>
<td>86.10 ± 5.15</td>
<td></td>
</tr>
<tr>
<td>NLRP3−/− + YVAD + acALY18</td>
<td>94.76 ± 3.49</td>
<td>0.22 (vs NLRP3−/−)</td>
</tr>
</tbody>
</table>

*Experiments were conducted in triplicate using mouse fibroblasts derived either from wild-type C57BL/6 control mice or the corresponding knockout, also on a C57BL/6 background. ASC, Apoptosis associated Speck-like protein containing a CARD; ASC−/−, the ASC knockout; NLRP3, the NOD-like receptor protein 3; NLRP3−/−, the NLRP3 knockout; YVAD, the caspase 1 inhibitor Y-VAD(Ome)-FMK.
adjuvant (FIA) as a negative control, or acALY18 in FCA. FNN-21 is the universal tetanus toxin epitope, and it functions as an adjuvant; FNN-21 in FCA was also used as a mock control (37). The IgM titers in rabbits receiving acALY18 were increased by day 3, compared to rabbits receiving either FCA or FNN-21/FCA, and by day 5 this increase was 2-fold ($P < 0.01$). At days 10 to 14 acALY18 had increased the IgM titer by 3-fold ($P < 0.001$). Results are presented in Fig. 5.

Pharmacodynamics of acALY18. We evaluated the pharmacodynamics of modulating the host response to infection in a mouse model of a cutaneous MRSA infection. MRSA infection at 4.5 $\times$ 10$^8$ CFU/mouse produced visible lesion formation in the skin within 72 h in 80% of the mice, and pathogen was detected in the circulation and spleens in 10% of the mice, suggesting that systemic MRSA infection had occurred. The mice that were treated with acALY18 at a presumptive minimum effective dosage (100 $\mu$g/kg; subcutaneous) had a $>3$-log reduction in pathogen load at the infection site 24 h postinfection ($P < 0.05$), and by day 10 the response was reduced $>6$ logs ($P < 0.01$) relative to the untreated mice (Fig. 6).

Trichrome staining was performed as an assessment of inflammatory infiltrate and gross structural integrity of the skin lesions (Fig. 7). In the untreated mice, lesions appeared by day 3 with significant disruption to the skin architecture with inflammatory infiltrate and resolution of the lesions by day 10; however, substantial infiltrate remained in the upper and lower dermis regions. In mice treated 24 h prior to MRSA infection, with the exception of 1 mouse that had a small pustule of bacteria at day 1, lesions did not appear until day 5, and by day 10 the lesions that did appear were starting to resolve. In mice treated 24 h after MRSA infection, lesions were as advanced as in untreated mice; however, by day 10 lesions in treated mice had resolved, and few inflammatory cells remained in the upper dermis.

acALY18 can induce IL-33 secretion from human and mouse fibroblasts, and IL-33 has recently been shown to increase neutrophil influx and more efficient pathogen clearance in a cecal ligation and puncture model of sepsis in the mouse (1). IL-33 prevented downregulation of CXCR2 in neutrophils and enhanced the recruitment of activated neutrophils to the infection site. In our study, we demonstrated increased myeloperoxidase at the infection site (Fig. 8), possibly a result of acALY18-induced secretion of IL-33 and activation of recruited neutrophils. IL-33 also enhances IgM production from tissue resident B1 cells (1, 36). Mice infected with MRSA and administered acALY18 either 24 h prior to infection or 24 h postinfection had enhanced IgM deposition at the infection site (Fig. 9). IL-33-driven IgM production and opsonization in addition to neutrophil activation may play a role in the enhanced clearance of MRSA in acALY18-treated mice.

In addition to increased IL-33 secretion, Thacker et al. demonstrated that human fibroblasts secrete IL-8 in response to acALY18 (34). IL-8 is a potent chemotactic chemokine in humans that is responsible for the recruitment of neutrophils. Mice do not produce IL-8, and the corresponding neutrophil-recruiting cytokine is KC/GRO/CXCL1. Miller and colleagues (26) established that IL-1R/MyD88-dependent KC/GRO/CXCL1 production in mouse skin cells promoted neutrophil and phagocytic pathogen clearance. We measured an increased and prolonged presence of KC/GRO/CXCL1 in the circulation of mice that were adminis-
tered acALY18 (Fig. 10A), and tissue histology showed enhanced myeloperoxidase activity (Fig. 8). *S. aureus* infections can activate the inflammasome (9, 27), mediating increased secretion of IL-1β and IL-18, which is required for neutrophil recruitment (27). *S. aureus* also inhibits neutrophil function and can subvert the innate immune response (7, 38). The latter point is under investigation in our laboratories to determine if acALY18-induced inflammasome activation overrides *S. aureus* escape mechanisms in neutrophils.

Circulating cytokines and chemokines (monocyte chemoattractant protein 1 [MCP-1]/CCL2, IL-1β, IL-4, IL-6, KC/GRO/CXCL1, IL-10, IFN-γ, TNF-α, IL-12p40, and macrophage inflammatory protein 1α [MIP-1α]) were measured. IL-6, MCP-1/CCL2, KC/GRO/CXCL1, and IL-12p40 were the only cytokines/chemokines detected in the circulation of treated or untreated (control) mice over the 10-day study period (Fig. 10A to D). IL-12p40 was not significantly elevated in the serum of treatment or control mice until day 3, and levels remained comparable between the treatment and control groups at each time point (Fig. 10A).

IL-6 and KC/GRO/CXCL1 were elevated in the serum of both treatment and control mice 1 day after infection and were not statistically different. By day 3, however, IL-6 and KC/GRO/CXCL1 serum levels in the treated mice were significantly elevated relative to control mice (Fig. 10B and C). Both IL-6 and KC/GRO/CXCL1 remained in the circulation at statistically higher concentrations in treated mice through day 5. By day 10 postinfection, levels of IL-6 in all mice had returned to baseline. However, KC/GRO/CXCL1 was still elevated in treated mice at day 10, but it returned to baseline levels in control mice. MCP-1/CCL2 was elevated in the serum of treated mice relative to control mice 1 day after infection, but this difference was not quite statistically significant (Fig. 10D). MCP-1/CCL2 remained detectable through day 10 postinfection in both groups.

Recruitment, activation, and differentiation of monocytes are key features of the innate immune response. Thacker et al. previously showed acALY18 induces the secretion of monocyte chemoattractant protein (CCL2) from human fibroblasts (34). The CCL2 gene was upregulated 18-fold at 48 h and significantly downregulated (204-fold) at 72 h (see Table S2 in the supplemental material). Mice treated with acALY18 24 h prior to infection had elevated levels of CCL2 in the circulation compared to control mice.

**FIG 7** Trichrome staining of MRSA skin lesions. Representative trichrome stains of MRSA skin lesions in mice treated 24 h prior to or 24 h post-MRSA infection and vehicle controls during the 10-day study. With the exception of 1 mouse, mice treated with acALY18 24 h prior to infection had a 2-day delay in the appearance of the lesion. Lesions in mice treated 24 h postinfection appeared at the same time as in the vehicle control mice; however, there was increased bacterial clearance by day 10. All images were taken at 100× magnification.

**FIG 8** Myeloperoxidase staining (red) of MRSA skin lesions. Representative myeloperoxidase stains of MRSA skin lesions in mice treated 24 h prior to infection, 24 h postinfection, and vehicle controls during the 10-day study. Myeloperoxidase-positive cells were observed at day 3 in mice treated 24 h postinfection and at day 1 in vehicle control mice, but by day 10 these cells were absent in these groups. In contrast, in mice treated 24 h prior to infection, myeloperoxidase-positive cells were not seen until day 5 and were still present at day 10. All images were taken at 100× magnification (insets, 400× magnification).
the treated mice. (Fig. 10D), thus suggesting enhanced monocyte recruitment in all images were taken at 100 magnification.

In a separate in vitro experiment, we investigated activation of monocytes treated with conditioned medium from fibroblasts that were pretreated with acALY18, cocultured with acALY18-pretreated fibroblasts, or medium from untreated fibroblasts (control). THP-1 monocytes had a 2-fold increase in CD14 expression (P = 0.0008) when treated with the conditioned medium from fibroblasts previously treated with acALY18, compared to THP-1 monocytes treated with conditioned medium from the control fibroblasts. THP-1 monocytes that were cocultured with fibroblasts previously treated with acALY18 showed a 4-fold increase in CD14 expression (P = 0.0002), which was greater than levels observed in monocytes cultured in fibroblast-conditioned medium (P = 0.008). THP-1 monocytes also showed a 50% increase in the expression of CD69 when cocultured with the acALY18-pretreated fibroblasts (P < 0.05). These results suggest that acALY18 not only enhances recruitment of monocytes, but also that recruited monocytes differentiate into macrophages (Fig. 11).

**Discussion**

For more than 75 years the therapeutic paradigm for infectious diseases has been to administer a toxin that is more toxic to the pathogen than it is to the patient. Antibiotics have been of unquestionable benefit in terms of lives saved and the alleviation of suffering from infectious diseases, but this benefit has not been without unanticipated consequences. Present efforts toward new antibiotic drug development have not yielded NMEs to adequately address the current need and have arguably contributed to the current crisis of emergent antibiotic resistance.

The development of new anti-infective agents to address the epidemic of emerging antibiotic-resistant infections is a pressing unmet medical need. The strategy of modulating the host response to infection holds promise for development of new anti-infective drugs that are safe, effective, and less likely to promote resistant strains of pathogens (21). However, if we are to embark on this path toward the development of a new therapeutic strategy, that effort should be predicated upon a deeper understanding of mammalian resistance and tolerance mechanisms.

Mammals and their microbiota have symbiotically coevolved over millions of years. Potential pathogens, such as *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Neisseria meningitidis*, colonize specific anatomical compartments and are held in check by tolerance mechanisms. Further, at any given moment we are challenged by infections from any number or type of environmental pathogens, but the vast majority of these infections do not progress to disease, due to the effectiveness of our highly evolved resistance mechanisms.

Pathogens have also evolved a variety of defensive mechanisms to escape detection and eradication, and it is becoming increasingly evident that many of the chronic idiopathic diseases are clinical manifestations of an inflammatory state maintained by the cellular response to the presence of a persistent infection. It would seem prudent, therefore, to understand the host-microbe defense and escape mechanisms as a means to develop pharmaceutical strategies for drug intervention in infectious diseases and possibly many of the chronic diseases as well.

Our laboratories have been studying the host response to pathogen infection in order to develop a pharmaceutical intervention that has a broad spectrum in its effect and that can abrogate an infection from progressing to disease. Herein we have described an NME (acALY18) that can be administered to augment a host defense mechanism via the NLRP3 inflammasome and induce clearance of either a Gram-positive or Gram-negative bacterium or override the escape mechanism of an intracellular bacterial pathogen.

In an experimentally induced MRSA skin infection, the downstream effect of acALY18 modulation of inflammasome signaling is an enhanced innate immune response. These effects include cytokine- and chemokine-mediated activation of tissue resident immune cell phenotypes, recruitment and activation of professional phagocytic cells, and production of IgM to opsonize the invading pathogen, culminating in enhanced pathogen clearance.

acALY18-induced immune gene expression reaches a maximum by 48 h, and by 72 h it is significantly downregulated. The cytokine and chemokine expression profiles in the sera of MRSA-infected mice treated with acALY18 were consistent with the gene expression profile. This observation suggests that the cellular response to acALY18 administration is short-lived and is subject to...
inherent negative feedback control mechanisms and/or a short in vivo half-life for acALY18. These results also suggest that administration of a second dose of acALY18 at 48 to 72 h hours after the initial dose might prolong the innate immune response and enhance the efficacy for pathogen clearance. More detailed pharmacokinetic and pharmacodynamic studies will be required to answer these important questions.

Nevertheless, in light of the broad-spectrum effect, the pharmacodynamic properties of acALY18-mediated inflammasome modulation seem to be well-suited for adjunctive treatment or prophylactic treatment for antibiotic-resistant infections. Ongoing pharmacokinetic, toxicity, and pharmacological safety studies will establish the viability of this strategy and pave the way for first-in-humans clinical evaluations.

ACKNOWLEDGMENTS

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We thank Michele Kutzler (Drexel University College of Medicine) for her invaluable assistance in the conduct of the MRSA/SSTI studies. Lam-pire Biologics, Inc. (Pipersville, PA) and Covance Laboratories, Inc. (Denver, PA) conducted the rabbit studies, Covance Laboratories also

FIG 10 acALY18 promotes immune cell recruitment and activating cytokines and chemokines. Serum cytokines/chemokines elevated over the 10-day study for mice that were treated 24 h postinfection compared to vehicle-treated mice. Data are presented as means ± SEM of n = 3 for each sampling interval on study days 1, 3, 5, and 10. (A) Serum IL-12p40 levels were not significantly elevated in the serum of treatment (red *) or control (blue *) mice until day 3, and levels were comparable thereafter (P > 0.05). (B) Serum IL-6 levels in mice receiving acALY18 (red *) or the vehicle control (blue *). By day 3 serum levels of IL-6 in the treated mice were significantly elevated relative to untreated control mice (*, P < 0.01) and remained statistically elevated through day 5 (**, P < 0.05). By day 10 postinfection IL-6 in all mice had returned to baseline levels. (C) Serum CXCL1 levels in mice receiving acALY18 (red *) or the vehicle control (blue *). By day 3 serum levels of CXCL1 in treated mice were significantly elevated relative to untreated control mice (*, P = 0.0002) and remained significantly elevated through day 5. CXCL1 remained elevated in the serum of treated mice through day 10 but had returned to baseline levels in the untreated mice (**, P < 0.05). (D) MCP-1/CCL2 levels were elevated in the serum of treated mice (red *) relative to the untreated control mice (blue *) 1 day after infection, but this difference was not quite statistically significant (P = 0.06) and remained detectable through day 10 in both groups (P > 0.05).

FIG 11 acALY18-treated fibroblasts induce differentiation of recruited monocytes. Primary human fibroblasts were cultured in the presence of acALY18 (3 ng/ml) or the vehicle alone (control) for 48 h. In one experiment, the conditioned medium from the fibroblast culture was collected and added to cultured THP-1 monocytes for 72 h. In a separate experiment, THP-1 monocytes were added to the fibroblast culture pretreated with acALY18 (cocultured) along with fresh medium and incubated for an additional 72 h. Monocytes from both experiments were collected and analyzed for CD14 (A) and CD69 (B) expression by flow cytometry. *, P = 0.0002; **, P = 0.008; ***, P < 0.05.
conducted the bacteremia study of mice, and Lampire Biologicals prepared the rabbit polyclonal anti-acALY18 sera. J.D.T. is the principal stockholder and President/Chief Science Officer for TherimmuneX Pharmaceuticals, Inc. S. Sassi-Gaha, M. Purohit, C. M. Artlett, and R F. Rest are employees of Drexel University College of Medicine, which has a financial interest in TherimmuneX Pharmaceuticals, Inc.

REFERENCES


SUPPLEMENTAL INFORMATION

MATERIALS AND METHODS

Animal Care and Use. Animals used in these studies were handled in accordance with published NIH policies for humane care and use of laboratory animals and the studies were conducted under specific protocols approved by the Institutional Animal Care and Use Committee of Lampire Biological Laboratories, Inc (antibody production and adjuvant experiments in rabbits) and Covance, Inc. (adjuvant experiment in rabbits and Salmonella bacteremia experiment in mice, and Drexel University College of Medicine (MRSA/SSTI study in mice).

Test Article Preparation. The peptide, acALY18, was synthesized by solid-phase methods as previously described (34) and the full length tetanus universal epitope, FNN-21, was synthesized by solid-phase methods as previously described (37). The natural product, pDAG, was isolated and synthesized as previously described (34).

Fibroblast Culture Methods. Normal primary human fibroblasts (GM5659) obtained from the Coriell Institute (Camden, NJ) were cultured in 100 mm dishes in Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% FBS and penicillin/streptomycin at 37 °C and 5% CO₂. The culture medium was stored at -80 °C for ELISA (see below). Mouse fibroblasts were established from C57BL/6 (Jackson Laboratories), NLRP3⁻/⁻, and ASC⁻/⁻ mice (Genentech, San Francisco, CA). The mice were sacrificed and the skin was harvested, washed in 10X fungizone/antibiotics, and minced into small pieces. The pieces of tissue were placed in culture flasks and allowed to attach for 60 min, then the flask was flooded with DMEM supplemented with 20% FBS, fungizone, and antibiotics. The explants were allowed to grow at 37 °C in 5% CO₂ until confluent, passaged once then utilized in experiments. C57BL/6, NLRP3⁻/⁻, and ASC⁻/⁻ fibroblasts were seeded into dishes and cultured in DMEM/10% FBS/antibiotics until confluent then transfected with acALY18 peptide/lipofectamine overnight. Cells were cultured so that at the time of treatment they were 80 - 90% confluent. Five µL of the stock acALY18 was diluted in Opti-MEM Reduced Serum Medium to give a final concentration of 5 ng/ml. PLUS reagent (0.5 mL) and lipofectamine (1 mL) were then added to the peptide Opti-MEM solution, mixed, and incubated at room temperature for 30 min. Five hundred µL of complete DMEM were added to the fibroblasts and 0.6 mL of the peptide/lipofectamine were added and allowed to incubate overnight. RNA was extracted and processed as described below.

Gene Expression Arrays. Normal human fibroblasts were cultured in 100 mm dishes in DMEM supplemented with 10% FBS and antibiotics until confluent as described above. The cells were cultured for 72 hours with 5 ng/ml acALY18/lipofectamine supplemented media. Cells were harvested by trypsinization and the RNA was purified using the Qiagen RNA Isolation kit. Five-micrograms of RNA was converted to cDNA and applied to the Human Innate and Adaptive Immune Response RT² Profiler™ PCR Array and to the Inflammasome Array (SABiosciences)
and gene expression was assayed using SYBR green as a reporter. The CT of the genes was normalized to β-actin and a 2-fold change in expression was noted.

**ELISA for Cytokine/Chemokine Proteins.** Cytokine/chemokine protein was measured by ELISA according to the manufacturer’s specifications (eBioscience) in the supernatants of fibroblasts cultured in complete DMEM with 5 ng/ml acALY18/lipofectamine. Briefly, high-affinity binding plates were coated with anti-human cytokine/chemokine and incubated overnight at 4 °C. Wells were washed with Wash Buffer five times for 1 min at each wash and then blocked with Assay Diluent for 60 min at room temperature. Samples were added to the wells along with diluted standards and incubated for 2 hours. Wells were washed and detection antibody was applied for 30 min, washed, an Avidin-HRP antibody applied for 30 min, unbound antibody removed by washing as described, and tetramethylbenzidine substrate added for 15 min. The reaction was halted with Stop Solution and the plate read at 450 nm with wavelength subtraction at 570 nm.

**ELISA Analysis for Activated Caspase-1.** Primary normal human fibroblasts were cultured as previously described. When cells were ~90% confluent they were treated with 20 μM of the caspase-1 inhibitor Z-YVAD(OMe)-FMK (Enzo Life Sciences) for 48 hours. Cells were treated with acALY18 (3 ng/ml) plus lipofectamine as previously described for 48 hours. The culture media was collected and retained frozen at -80° C. Cells were harvested by trypsinization and pelleted by centrifugation. The supernatant was discarded and the cell pellet was resuspended in 100 μl of NP40 lysis buffer and 1 μl of protease inhibitor was added. Cell lysates were assayed using the Caspase1 Colorimetric Assay Kit (Abcam) according to the manufacturer’s protocol.

**Chlamydia pneumoniae Infected Monocytes.** THP1 human monocytes from the American Type Culture Collection (ATCC TIB202; American Type Culture Collection, Manassas, VA), were propagated at a concentration of 1x10^6 cells/ml in RPMI 1640 (ATCC) + 10% Fetal Bovine Serum (Cellgro 35-011-CV; Thermo Fisher Scientific, Pittsburgh, PA) growth media (GM) in a 37°C incubator with 5% CO2.

Prior to the addition to THP1 human monocytes, *Chlamydia pneumoniae*, laboratory strain AR-39 (ATCC 53592) was thawed and sonicated (iSonic Digital Ultrasonic Cleaner CD-4820; iSonic, Vernon Hills, IL) for 2 min. THP1 cells were spun down, washed with Hank’s Balanced Salt Solution, 1X (HBSS; ATCC 30-2213), and resuspended at a concentration of 1x10^6 cells in 1 ml of growth media (GM). *C. pneumoniae* AR-39 was used to infect these cells at a multiplicity of infection (MOI) of 1 (1x10^6 IFU). The cells were allowed to incubate for 1 h at 37°C with 5% CO2 in a T-25 flask upright, after which, 4 ml of GM was added for a final volume of 5 ml with the flasks in the horizontal for 48 hr.

*C. pneumoniae*-infected monocytes were treated at 24 hr post-infection with acALY18 at concentrations of 25, 50, or 100 ng/ml diluted in growth media. The cells were incubated for an additional 24 hr at 37°C with 5% CO2 followed by harvesting. For RT-PCR, cells from the 100 ng/ml treated flasks were pelleted at 1000 x g for 5 min, washed in HBSS, re-pelleted and then prepared for RNA extraction (see gene
expression assays). Comparable flasks left untreated were prepared in the same manner.

Samples from both infected/untreated and infected/acALY18 treated groups were used for cytopsins to be analyzed by immunofluorescence microscopy for level of infection. 10 slides, (Fisherbrand Superfrost 12-550-14; Thermo Fisher Scientific) with a concentration of $1 \times 10^5$ cells/400 µl HBSS per slide, were prepared using cytofunnels (Fisher EZ Single Cytofunnel A78710020) and were cytopspun at 500 RPM for 4 min with medium acceleration (Shandon CytoSpin III Cytocentrifuge; Thermo Fisher Scientific). The cells were fixed using Cytofix/Cytoperm (BD Cytofix/Cytoperm 554722; BD Biosciences, San Jose, CA) for 30 min and were washed 2x with Phosphate Buffered Saline (PBS; Sigma P3813; Sigma-Aldrich, St. Louis, MO), 5 min each. The cytopspun slides were rinsed with PBS then blocked with 1X Permwash/PBS (BD 10X Permwash 51-20915z) for 30 min and rinsed again with PBS. Cells were stained with a FITC-conjugated anti-chlamydia antibody, Fitzgerald 61C75 (Fitzgerald Industries International, Acton, MA) for 1 h at 37°C then counterstained using a bisBenzimide stain (Sigma bisBenzimide B2883) at a dilution of 1:1000 for 5 min. They were then rinsed with filtered de-ionized H2O 3x and finally cover slipped using Flourogel with tris buffer mounting media (Electron Microscopy Sciences 17985-10; Electron Microscopy Sciences, Hatfield, PA). The slides were then imaged on a Nikon E80i microscope using the NIS-Elements AR 3.0 software (Nikon Inc., Melville, NY). At minimum, 5 random fields per slide per experiment were acquired at 40X objective magnification and cells containing one or more inclusions of *C. pneumoniae* were counted as positive for infection.

**IgM Expression Study.** Twelve female New Zealand White rabbits weighing approximately 2.5 Kg were used in four groups of 3 rabbits. Rabbits were either administered either 250 µg of acALY18 or FNN-21 (100 µg/Kg) formulated in Freund’s Complete Adjuvant (FCA) or FCA alone as a positive control or Freund’s Incomplete Adjuvant alone as a negative control by sub-cutaneous injection. The injection volume was 0.25 mL for each compound. A second booster injection, also 0.25 mL of the respective test article, was administered on day 7. Arterial test bleeds were taken on days 0, 3, 5, 7, 10, 12, and 14. IgM specific for *M. tuberculosis* was measured by ELISA.

**Clinical Bovine Mastitis Study.** Nineteen lactating dairy cows in a working dairy herd in Southeastern Ohio were showing signs of clinical mastitis, i.e. abnormal milk appearance and elevated leukocytes as measured by flow cytometry (> 300,000 cells/ml) in the milk. A mixed population of *Eschericia coli* and *Streptococcus agalactiae* were the predominant pathogens cultured from milk samples. These 19 animals were randomly assigned to either a test or a control group. The control group animals (n = 10) were treated according to the dairy’s normal practice for treatment of clinical cases of mastitis, i.e. intramuscular injection of Polyxflex brand (Fort Dodge Animal Health) of ampicillin (5mg/lb x 3 days). The test group (n = 9) animals were treated with a 5-ml intramuscular injection of a 20 mg/ml solution (100 mg/cow, ~250 µg/Kg) of the isolated natural product, pDAG, prepared as described previously (34). The initial dose was
followed by a booster injection three days later. The leukocytes/ml of milk in the
treatment and control animals were compared 30 days after the initial treatment.

**Salmonella Bacteremia Study.** Four-week old, female Swiss Webster mice
weighing approximately 20 grams were purchased from Charles River Laboratories
(Wilmington, MA). The mice were acclimated for 2 weeks, fed and watered *ad libitum*. Mice were housed (5 mice per cage) in plastic boxes bedded with wood shavings. Four
cages (n=20 mice) were randomly assigned to either a treatment or control group.
*Salmonella typhimurium* (ATCC 14028) was used as the challenge organism after
passaging three times through a murine host with subsequent isolation and stored in
phosphate buffered saline with 10% glycerol at -80 °C as previously described (5). All mice were administered 0.1 ml of *Salmonella typhimurium* (~5.00 * 10³ bacteria/mouse) by intraperitoneal injection on day 0. At the time of infection, mice in the treatment
group were administered 0.25 ml (sub cu.) of a 20 µg/ml solution (~5 µg/mouse, 250 µg/Kg) of the isolated natural product, pDAG, prepared as described previously (23).
Mice in the control group received a normal saline placebo (0.25 ml) by subcutaneous
injection at the time of infection. Mice were monitored twice daily for weight loss and
weight loss > 15% was counted as mortality. Mice were euthanized by CO₂
asphyxiation followed by cervical dislocation.

**Cutaneous MRSA Study.** Female specific-pathogen-free C57Bl/6J mice with an
average weight of 20 grams were procured from Jackson Laboratories. Mice were
acclimated for one week, housed 2 mice per cage, placed on a 12 hour lighting cycle,
and given food and water *ad libitum*. The experiments were conducted in two cohorts of
30 mice each. *Staphylococcus aureus* resistant to gentamicin and methicillin was
procured from the ATCC (#43300) and grown overnight at 37°C in Trypticase Soy Broth
supplemented with gentamicin and methicillin. Working stocks in Trypticase Soy
Broth/10% glycerol were stored at -80°C. For administering 0.1 ml inoculates by
subcutaneous injection a working stock was diluted to ~4 X 10⁷ CFU/ml and the
bacterial concentration actually administered was determined using standard dilution
plate counts.

An initial probative study was conducted with 30 mice to optimize a non-lethal
infecting dose of MRSA and the vehicle for the test article (acALY18) delivery. In the
subsequent experiments, all mice received 0.1 ml of the MRSA inoculum (either 2 x 10⁶
CFU/mouse in Cohort 1 or 4.5 x 10⁶ CFU/mouse in Cohort 2) by subcutaneous injection
in a previously shaved area of the lower back on study day 0. The test article,
acALY18, was formulated as the acetate salt in a vehicle of 1% Dextran-40 in PBS, pH 7.2 to give a final concentration of 20 µg/ml. Mice were administered 0.1 ml (2 µg, 100 µg/Kg) of the test article, the presumptive minimum effective dosage, by subcutaneous injection either 24 h prior to infection or 24 h post-infection. Animals in the vehicle
control group received 0.1 ml of the vehicle blank.

Three mice from Cohort 1 (n = 15) were selected on study days 0, 1, 3, 5 and 10
and anesthetized by i.p. administration of 400 µl Avertin. Lesion tissue samples were
collected using an 8 mm punch biopsy and weighed. Samples of the excised tissue
from two mice at each sampling interval were separately homogenized in 4 ml of normal
saline using an OMNI model HT homogenizer. The homogenate was centrifuged and
the supernatant diluted and plated on Trypticase Soy Agar plates supplemented with gentamicin and methicillin and incubated for 24 h at 37 °C in ambient air. Colonies were counted and reported as CFU/g of tissue. Spleens were also harvested, weighed, and homogenized for assessment of bacterial load by the standard plate count method as previously described.

Tissue samples from one mouse at each sampling interval were fixed in 4% paraformaldehyde overnight, paraffin embedded, sectioned onto positively charged microscope slides. Sections were stained with Masson’s Trichrome according to standard protocols to localize collagen fibers, gross structural changes within the tissue, and inflammatory infiltrate. Tissues were assessed by brightfield illumination with a Nikon Eclipse 80i microscope and documented with a Spotcam digital camera.

Separate sections were deparaffinized as previously described (10), blocked with 5% donkey serum and incubated with either rabbit-anti-myeloperoxidase or IgM-FITC at a dilution of 1:100 (Abcam, Cambridge MA), washed, then incubated with donkey-anti-rabbit-Cy3 diluted 1:1000 (Jackson Immunochemicals, West Grove, PA), counterstained with bisBenzamide, and viewed with an epi-fluorescent microscope. Images were taken at 100X magnification blocked with 5% goat serum, washed and viewed at 100X magnification.

Three mice from Cohort 2 (n =15) were selected on study days 0, 1, 3, 5 and 10 and anesthetized by i.p. administration of 400 µl Avertin. Approximately 100 µl of blood was collected by retro-orbital bleed into a sterile, heparinized collection tube and 100 µl of bacterial culture media was added for plating and culture. Additional blood collected by retro-orbital bleeding was allowed to clot and serum obtained by centrifugation. Sera were assayed for inflammatory cytokines and chemokines (mJc/MCP-1/CCL2, IL-1β, IL-4, IL-6, KC/GRO/CXCL1, IL-10, INFγ, TNFα, IL-12p40, and MIP-1α) using the SearchLight® microarray (Aushon Biosystems, Billerica, MA).

The lesion site tissue from each mouse was collected using an 8 mm punch biopsy, weighed, homogenized, and the pathogen load in CFU/g was determined using the standard plate count method as described above.

Statistical Analysis. Statistical analyses were performed using GraphPad Prism® (v. 5.03) and GraphPad InStat® (v. 3.10). Tests for significance (p value) were calculated using Student’s t-test for un-paired data with or without Welch’s approximation to correct for unequal standard deviations when appropriate.
Table S1. Comparison of gene expression in THP1 infected monocytes with and without acALY18 treatment.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Fold-Change (Untreated)</th>
<th>Fold-Change (Treated)</th>
<th>P value</th>
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<tr>
<td>ADORA2A</td>
<td>Adenosine A2a receptor</td>
<td>7.3</td>
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Notes: Fold-change in green font indicates down-regulation and red font indicates up-regulation. P values in red are significant (< 0.05).
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Notes: Fold-change in **green** font indicates down-regulation and **red** font indicates up-regulation. P values in **red** are significant (< 0.05).
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<td>C8A</td>
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Notes: Fold-change in green font indicates down-regulation and red font indicates up-regulation. P values in red are significant (< 0.05).