Published in final edited form as:

Vaccine. 2011 December 9; 30(1): 9–13. doi:10.1016/j.vaccine.2011.10.054.

Control of Methicillin Resistant *Staphylococcus aureus* Infection Utilizing a Novel Immunostimulatory Peptide

Tamsin R. Sheen^{a,#}, Courtney K. Cavaco^{a,#}, Celia M. Ebrahimi^a, Marilyn L. Thoman^b, Sam D. Sanderson^c, Edward L. Morgan^b, and Kelly S. Doran^{a,d,*}

^aDepartment of Biology, Center for Microbial Sciences, San Diego State University, 5500 Campanile Drive, San Diego, CA 92182, USA

^bBioScience Center, San Diego State University, 5500 Campanile Drive, San Diego, CA 92182, USA

^cSchool of Allied Health Professions, University of Nebraska Medical Center, 984000 Nebraska Medical Center, Omaha, NE 68198, USA

^dDepartment of Pediatrics, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92037, USA

Abstract

The emergence of community-acquired methicillin-resistant *S. aureus* (CA-MRSA) is a serious health concern worldwide that requires new therapeutic approaches that extend beyond the development and use of new antibiotics. In this study, a conformationally-biased, response-selective agonist of human C5a, known as EP67, was used to induce host innate immunity as a therapeutic method of reducing CA-MRSA infections. Using a murine model of dermonecrosis we show that EP67 treatment effectively limits CA-MRSA infection by promoting cytokine synthesis and neutrophil influx. In contrast, EP67 was ineffective in reducing lesion formation in C5a receptor (CD88^{-/-}) knockout mice, indicating that EP67 activates host innate immunity by engagement of CD88 bearing cells. These results suggest that EP67 may serve as a novel immunotherapeutic for prevention and treatment of CA-MRSA dermal infection.

Keywords

Staphylococcus aureus; CA-MRSA; innate immunity; EP67; C5a agonist; PMN

1. Introduction

Staphylococcus aureus is a formidable human pathogen responsible for a variety of disease pathologies ranging from minor skin irritations to more severe infections such as septicemia, necrotizing pneumonia and necrotizing fasciitis [1, 2]. The emergence of multi-drug resistant strains of *S. aureus*, including community-acquired methicillin-resistant *S. aureus*

© 2011 Elsevier Ltd. All rights reserved.

*Corresponding author: Kelly S. Doran, San Diego State University Department of Biology and Center for Microbial Sciences, 5500 Campanile Drive, San Diego, CA 92182, USA. (619) 594-1867. kdoran@sciences.sdsu.edu.

#These authors contributed equally to this work

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

(CA-MRSA), has increased interest in the development of new vaccines and effective treatment strategies. Currently CA-MRSA is associated with severe disease and fatality in otherwise healthy individuals with closely related strains belonging to one pulse-field type, USA300, causing the majority infections in the United States [3, 4]. Much research has focused on the understanding of CA-MRSA pathogenesis and virulence as a prelude to the development of alternative approaches for controlling the current CA-MRSA epidemic. One therapeutic approach that has garnered considerable attention is selective activation of host innate immunity. This would not only induce the body's inherent first line of defense to infection, but would also minimize mutational pressures on the bacteria since the therapeutic effect would not have the potential of directly impacting mutable aspects of bacterial replication as observed with conventional antibiotics.

Toward this end we have developed a conformationally-biased, response-selective agonist of the biologically active region of human C5a, the C-terminal ten residues, C5a₆₅₋₇₄. C5a is a small (76-residues) fragment that is an important pharmacologically active byproduct of complement activation. The principal role of native C5a is to recruit inflammatory cells and lymphocytes to sites of tissue injury and infection, and to then activate various effector responses including neutrophil degranulation, inflammatory and chemotactic mediator release. The agonist, EP67, has the sequence Tyr-Ser-Phe-Lys-Asp-Met-Pro-(N-methylLeu)-D-Ala-Arg or YSFKDMP(MeL)aR. EP67 retains C5a-like activity in the engagement of C5a receptors (C5aR) and activation of antigen presenting cells (APCs) but not polymorphonuclear cells (PMNs) for cytokine synthesis and release [5-7]. We have also recently investigated the use of EP67 as a novel adjuvant in vaccine development [8].

Given this ability of EP67 to induce the release of cytokines and other mediators, the objective of this study was to evaluate the effectiveness of EP67 in inducing a protective innate immune response to dermal MRSA infections. Results of this study indicate that treatment strategies specifically designed to activate host innate immunity, such as EP67, represent effective approach for treating problematic MRSA infections.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

Methicillin-sensitive *Staphylococcus aureus* (MSSA) laboratory strain ISP479C [9] and CA-MRSA USA300 isolate (TCH1516-HOU-MR, ATCC accession number BAA-1717) [10] were grown aerobically in tryptone soy (TS) broth (Oxoid) at 37°C.

2.2. Peptide synthesis

EP67 (YSFKDMP(MeL)aR) and its inactive scrambled sequence (sEP67) ((MeL)RMYKPa-FDS) were generated by solid-phase methods using the Fmoc method of orthogonal synthesis, purified by analytical and preparative reverse-phase HPLC, and characterized by electrospray mass spectrometry according to previously published methods [11].

2.3. Mouse model of MRSA dermonecrotic infection

All animal work was carried out under the approval of the Office of Laboratory Animal Care (OLAC) at San Diego State University and adhered to accepted veterinary standards. Outbred female CD1 mice 8-12 weeks were purchased from Charles River Laboratories. CD88^{-/-} mice (Jackson Labs) were back-crossed at least 5 times onto the C57Bl/6 background. Prior to infection, hair was removed (n = 6-10) and sub-cutaneous (sc) *S. aureus* infection was carried out as described previously [12]. Briefly, 0.1 mL volumes of mid-logarithmic phase MRSA (~ 4 × 10⁷ CFU) diluted in cytodex bead-DPBS solution were injected sc into the right flank of prepared animals. Where indicated, 250 µg EP67, sEP67 or

an equivalent volume of DPBS (50 μ L) were injected sc into the right flank 24h and 4h prior to and 24h following bacterial infection. Ulcerative lesions were measured over time, harvested and homogenized using sterile 1 mm ceramic beads. Dilutions of the homogenate were plated on TS agar to enumerate bacterial cfu per g tissue. Mice (CD1) were rendered neutropenic as described previously [13]. Briefly, mice were injected ip with rat monoclonal anti-mouse Ly-6G antibody (RB6-8C5, eBioscience) or control rat immunoglobulin (IgG, eBioscience) 24 h prior to EP67 injection and subsequent bacterial infection, as described [14].

2.4. Measurement of cytokines

IL-6, IL-1 β , TNF- α , INF- γ and KC inflammatory cytokines were measured in tissue homogenates as previously described [7], or according to manufacturer's protocols (BD and R&D Systems). Samples were run in triplicate.

2.5. PMN chemotaxis and myeloperoxidase assay

PMN recruitment at the site of injection by EP67 or sEP67 was examined by histopathologic analysis and with an *in vivo* chemotaxis assay [15]. In one set of experiments, 250 μ g EP67 or sEP67 alone were injected subcutaneously into the right flank of CD1 mice. The injection was repeated after 24 h and 4 h later mice were euthanized and tissue excised and stained with hematoxylin-and-eosin. In subsequent experiments a similar dosing regime of EP67 or sEP67 was performed followed by bacterial infection 4 h post the last EP67/sEP67 treatment. Tissue was excised 1 h post bacterial infection for determination of myeloperoxidase (MPO) activity [15] or neutrophil filtration using FITC rat anti-mouse Ly6G as described previously [14]. These assays were carried out two times and the samples analyzed in duplicate.

2.6. Statistical analyses

Student's t test statistical analyses of results were carried using GraphPad Prism version 5. Significance was accepted at $P < 0.05$.

3. Results

3.1. EP67 treatment reduces *S. aureus* dermal lesion formation in mice

In order to examine the effects of EP67 on CA-MRSA disease progression, we utilized a mouse model of ulcerative dermal infection [12]. Infection with CA-MRSA strain USA300 resulted in the formation of pus-filled lesions following sc injection, with lesions reaching maximum diameter approximately 48-72 h post-infection. Prior to and post-MRSA infection mice were injected sc with 250 μ g EP67 or the scrambled peptide control, sEP67, as described in Materials and Methods. Disease progression was assessed via measurement of cutaneous lesion development. Lesion size was significantly reduced in animals treated with EP67 48 h post-bacterial inoculation, compared to the sEP67 control (Figure 1A-E). Images of lesions from representative mice are shown for EP67 treated animals (Fig. 1A, B) and sEP67 treated animals (Fig. 1C, D). Animals were sacrificed 5 days post-inoculation and lesion tissue excised and homogenized to determine bacterial CFU present within the lesions. A significant reduction in bacterial load was observed in MRSA infected animals treated with EP67 compared to those treated with the sEP67 (Figure 1F) or PBS (data not shown) controls.

3.2. Proinflammatory cytokines are enhanced by EP67 treatment

The decrease in lesion size observed in EP67-treated animals indicated a possible enhanced immune activation in these animals. In order to determine the nature of the immune response

activated by EP67 treatment, skin lesions were harvested 48 h post-infection and EP67 or sEP67 treatment, and subjected to ELISA to measure proinflammatory cytokines in tissue homogenates. Significantly higher levels of murine KC (homologue of human chemokine CXCL1), IL-6, and IL-1 β were observed in mice treated with EP67 compared to sEP67 (Fig. 1 G). EP67 treatment similarly resulted in an increase of TNF α and INF γ production during MRSA infection (data not shown).

3.3. PMN influx is essential for the protective action of EP67

As enhanced levels of cytokines, including the PMN chemoattractant KC, were detected in EP67-treated and infected animals, we hypothesized that EP67 independently may act to increase inflammatory infiltrate following administration. Histopathologic analysis of skin tissue from representative mice revealed normal pathology following injection of PBS (Fig. 2A) or sEP67 (Fig. 2B), but massive influx of inflammatory cells, including PMNs, following EP67 injection (Fig. 2C, D). We further analyzed PMN recruitment to the site of EP67 injection using an *in vivo* PMN recruitment assay as described previously [15]. PMN migration was assessed in skin homogenates during active bacterial infection following injection of EP67 or sEP67 by visualizing PMN infiltration and determining the level of the neutrophil enzyme myeloperoxidase (MPO), which serves as an effective indication of PMN infiltration [16]. PMN infiltration and MPO levels were significantly higher after injection with EP67 compared to the sEP67 control (Fig. 2E-I).

To further investigate the role of PMNs in EP67-mediated MRSA lesion reduction, we used rat monoclonal antibody (MAb) RB6-8C5 to induce PMN depletion in CD-1 mice 24 h prior to EP67 treatment and MRSA infection. RB6-8C5 binds to both Ly6G $^{+}$ and Ly6C $^{+}$ cells including neutrophils, dendritic cells and subsets of monocytes, macrophages, and lymphocytes [13]. We have shown previously that the dose of RB6-8C5 used in our study (50 μ g) induced sustained levels of neutropenia [14], and others have shown that this dose induced neutropenia without affecting the population of Ly6G $^{+}$ dendritic cells or other cell types [17, 18]. Consistent with our previous results, there was a significant reduction in lesion size in EP67-treated animals compared to sEP67-treated animals in the groups treated with the isotype IgG control antibody (Fig. 2J). In contrast, there was no quantifiable difference in lesion size between animals treated with EP67 and sEP67 and with the RB6-8C5 Mab (Figure 2J). In addition, both groups of neutropenic mice developed significantly larger lesions ($P < 0.001$) than mice treated with the IgG control Ab (Fig. 2J). These results indicate that PMN infiltration to the site of MRSA infection is essential for the reduction in lesion size mediated by EP67 treatment.

3.4. EP67 acts via the C5a receptor (C5aR) CD88

We have previously shown *in vitro* that EP67 selectively engages C5aR-bearing APCs [6]. This argues that the protective effects of EP67 *in vivo* are likewise C5aR-specific. To examine this hypothesis, we examined the effects of EP67 in CD88 $^{-/-}$ homozygous C57Bl/6 mice. CD88 $^{-/-}$ and CD88 $^{+/+}$ controls were infected sc with MRSA and treated with EP67 or sEP67 as described above. Consistent with the results seen in CD1 mice, wild-type C57Bl/6 CD88 $^{+/+}$ animals treated with EP67 had significantly smaller lesions compared to those treated with sEP67 (Fig. 2K). In contrast, there was no difference in lesion size between the two treatment groups in CD88 $^{-/-}$ animals (Fig. 2K), suggesting that EP67-mediated reduction of lesion size occurs via binding to CD88.

4. Discussion

MRSA is a significant human pathogen associated with severe infections that are resistant to most antibiotic therapies. Initially MRSA was limited to hospital settings being one of the

most common causes of nosocomial infections. However, the emergence of highly virulent community associated MRSA (CA-MRSA) strains has resulted in increased cases in otherwise healthy individuals throughout the population. CA-MRSA strains have caused a pandemic of mostly skin and soft tissue infections, being the most frequent cause of these types of infections reported in emergency rooms throughout the United States [19]. Although the molecular basis of CA-MRSA virulence has been controversial, it has been documented that CA-MRSA strains have a higher virulence potential, with USA300 being among the most virulent strains [20].

In this report, we demonstrate that a conformationally-biased agonist of C5a (EP67) is effective in limiting USA300 infection in a mouse model of dermonecrosis by virtue of its ability to enhance innate immunity. We have previously shown that EP67 induces the release of cytokines IL-6, TNF α , and INF γ , but not IL-2, IL-4, IL-5, IL-10 (Th2 cytokines) from splenic APCs obtained from C57BL/6 mice, and that induction requires the presence of the C5aR CD88 [7]. Here, we similarly observed that s.c. injection of EP67 resulted in increased production of pro-inflammatory cytokines and chemokines TNF α , INF γ , IL-6, IL-1 β , and KC during active bacterial infection. Also, EP67 in the absence or presence of bacteria, promoted the influx of inflammatory infiltrate that included neutrophils, as evidenced by increased levels of MPO and the visual presence of PMNs in skin tissue (Fig. 2). Additional experimentation is required to further determine the exact nature of the infiltrate during infection; however, our results clearly show PMN influx contributes to the EP67-mediated defense, as PMN depletion abrogates the therapeutic effect of EP67 (Fig. 2).

Our results demonstrating that PMNs play an important role in defense against CA-MRSA dermal infection are consistent with previous findings [21] as neutrophils are a critical component of the innate immune response during MRSA infection [22]. However, it has been well documented that MRSA is capable of resisting innate immunity, and specifically PMN killing, by various mechanisms [23]. Thus while additional experimentation is required to further elucidate specific mechanism(s) of EP67-mediated immune enhancement during infection, we speculate that EP67 engagement of APC populations results in cytokine/chemokines mediator release, and subsequent PMN recruitment to the infection site.

It is apparent that with the emergence of antibiotic resistant organisms, therapeutic approaches beyond the development of new antibiotics modalities need to be addressed. The activation of innate immunity is critical step for the control of problematic bacterial infections. Our data suggest that prophylactic use of EP67 may be advantageous for individuals at high risk of developing MRSA infection, such as those with wounds or burns. While further experimentation is required to determine if EP67 can be used to treat an established infection, our data support the continued investigation and advancement of EP67 as a candidate immunotherapeutic for CA-MRSA infection.

Acknowledgments

We thank Victor Nizet for providing the USA300 strain and Joy Phillips for helpful discussions. Histopathologic analysis was performed at the UCSD Core Facility, director Nissi Varki. This work was supported in part by grant R01GM095884 to J.P., R01AG02877 to M.L.T., the Presidential Research Grant from San Diego State University to E.L.M. and grants from the San Diego State Research Foundation and the California State University Program for Education and Research in Biotechnology (CSUPERB) to K.S.D.

References

1. Lowy FD. *Staphylococcus aureus* infections. N Engl J Med. 1998 Aug 20; 339(8):520–32. [PubMed: 9709046]

2. Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, Ray S, et al. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA*. 2007 Oct 17; 298(15):1763–71. [PubMed: 17940231]
3. DeLeo FR, Chambers HF. Reemergence of antibiotic-resistant *Staphylococcus aureus* in the genomics era. *J Clin Invest*. 2009 Sep; 119(9):2464–74. [PubMed: 19729844]
4. Kennedy AD, Otto M, Braughton KR, Whitney AR, Chen L, Mathema B, et al. Epidemic community-associated methicillin-resistant *Staphylococcus aureus* recent clonal expansion and diversification. *Proc Natl Acad Sci U S A*. 2008 Jan 29; 105(4):1327–32. [PubMed: 18216255]
5. Taylor SM, Sherman SA, Kirnarsky L, Sanderson SD. Development of response-selective agonists of human C5a anaphylatoxin: conformational, biological, and therapeutic considerations. *Curr Med Chem*. 2001 May; 8(6):675–84. [PubMed: 11281848]
6. Vogen SM, Paczkowski NJ, Kirnarsky L, Short A, Whitmore JB, Sherman SA, et al. Differential activities of decapeptide agonists of human C5a: the conformational effects of backbone N-methylation. *Int Immunopharmacol*. 2001 Nov; 1(12):2151–62. [PubMed: 11710544]
7. Morgan EL, Morgan BN, Stein EA, Vitrs EL, Thoman ML, Sanderson SD, et al. Enhancement of in vivo and in vitro immune functions by a conformationally biased, response-selective agonist of human C5a: implications for a novel adjuvant in vaccine design. *Vaccine*. 2009 Dec 11; 28(2):463–9. [PubMed: 19836478]
8. Morgan EL, Thoman ML, Sanderson SD, Phillips JA. A novel adjuvant for vaccine development in the aged. *Vaccine*. Dec 6; 28(52):8275–9. [PubMed: 20965299]
9. Pattee PA. Distribution of Tn551 insertion sites responsible for auxotrophy on the *Staphylococcus aureus* chromosome. *J Bacteriol*. 1981 Jan; 145(1):479–88. [PubMed: 6257640]
10. Highlander SK, Hulten KG, Qin X, Jiang H, Yerrapragada S, Mason EO Jr, et al. Subtle genetic changes enhance virulence of methicillin resistant and sensitive *Staphylococcus aureus*. *BMC Microbiol*. 2007; 7:99. [PubMed: 17986343]
11. Phillips JA, Morgan EL, Dong Y, Cole GT, McMahan C, Hung CY, et al. Single-step conjugation of bioactive peptides to proteins via a self-contained succinimidyl bis-arylhydrazone. *Bioconjug Chem*. 2009 Oct 21; 20(10):1950–7. [PubMed: 19788175]
12. Bunce C, Wheeler L, Reed G, Musser J, Barg N. Murine model of cutaneous infection with gram-positive cocci. *Infect Immun*. 1992 Jul; 60(7):2636–40. [PubMed: 1612733]
13. Daley JM, Thomay AA, Connolly MD, Reichner JS, Albina JE. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *J Leukoc Biol*. 2008 Jan; 83(1):64–70. [PubMed: 17884993]
14. Banerjee A, Kim BJ, Carmona EM, Cutting AS, Gurney MA, Carlos C, et al. Bacterial Pili exploit integrin machinery to promote immune activation and efficient blood-brain barrier penetration. *Nat Commun*. 2:462. [PubMed: 21897373]
15. van Sorge NM, Ebrahimi CM, McGillivray SM, Quach D, Sabet M, Guiney DG, et al. Anthrax toxins inhibit neutrophil signaling pathways in brain endothelium and contribute to the pathogenesis of meningitis. *PLoS One*. 2008; 3(8):e2964. [PubMed: 18698416]
16. Bradley PP, Priebat DA, Christensen RD, Rothstein G. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J Invest Dermatol*. 1982 Mar; 78(3):206–9. [PubMed: 6276474]
17. Tvinnereim AR, Hamilton SE, Harty JT. Neutrophil involvement in cross-priming CD8+ T cell responses to bacterial antigens. *J Immunol*. 2004 Aug 1; 173(3):1994–2002. [PubMed: 15265934]
18. Stephens-Romero SD, Mednick AJ, Feldmesser M. The pathogenesis of fatal outcome in murine pulmonary aspergillosis depends on the neutrophil depletion strategy. *Infect Immun*. 2005 Jan; 73(1):114–25. [PubMed: 15618146]
19. Moran GJ, Krishnasadan A, Gorwitz RJ, Fosheim GE, McDougal LK, Carey RB, et al. Methicillin-resistant *S. aureus* infections among patients in the emergency department. *N Engl J Med*. 2006 Aug 17; 355(7):666–74. [PubMed: 16914702]
20. Li M, Cheung GY, Hu J, Wang D, Joo HS, Deleo FR, et al. Comparative analysis of virulence and toxin expression of global community-associated methicillin-resistant *Staphylococcus aureus* strains. *J Infect Dis*. Dec 15; 202(12):1866–76. [PubMed: 21050125]

21. Molne L, Verdrengh M, Tarkowski A. Role of neutrophil leukocytes in cutaneous infection caused by *Staphylococcus aureus*. *Infect Immun*. 2000 Nov; 68(11):6162–7. [PubMed: 11035720]
22. DeLeo FR, Diep BA, Otto M. Host defense and pathogenesis in *Staphylococcus aureus* infections. *Infect Dis Clin North Am*. 2009 Mar; 23(1):17–34. [PubMed: 19135914]
23. Voyich JM, Braughton KR, Sturdevant DE, Whitney AR, Said-Salim B, Porcella SF, et al. Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *J Immunol*. 2005 Sep 15; 175(6):3907–19. [PubMed: 16148137]

Abbreviations

Individual amino acids in peptide sequences are denoted by their single letter designations. Uppercase letters denote the L-stereoisomeric form and lowercase letters the D-stereoisomeric form.

APC	antigen presenting cell
cfu	colony forming unity
C5aR/CD88	C5a receptor
CA-MRSA	community acquired methicillin resistant <i>Staphylococcus aureus</i>
HPLC	high performance liquid chromatography
IL	interleukin
INFγ	interferon-gamma
ip	intraperitoneal
KC	murine homologue of CXCL1
LPS	lipopolysaccharide
MAb	monoclonal antibody
Ab	antibody
PMN	polymorphonuclear cells
sc	subcutaneous
ip	intraperitoneal
Th1/Th2	T lymphocyte helper type 1/type II
TNFα	tumor necrosis factor-alpha

Highlights

> We study a conformationally-biased, response-selective agonist of human C5a, EP67, as a therapeutic method of reducing CA-MRSA infections. > We show that EP67 treatment effectively limits CA-MRSA infection by promoting cytokine synthesis and neutrophil influx. > These results suggest that EP67 may serve as a novel immunotherapy for CA-MRSA dermal infection.

Figure 1. EP67 administration reduces CA-MRSA dermal infection

CD-1 mice were treated with EP67 or sEP67 pre and post injection (s.c.) of CA-MRSA as described in Methods. Infection experiments were performed at least 5 times, a representative experiment is shown. Lesions from representative mice treated with EP67 (A, B) or sEP67 (C, D) and the lesion measurements over time following infection (E). Bacterial load (cfu) (F) and cytokine levels of KC, IL-6 and IL-1 β (G) in skin tissue homogenates were assessed. Statistically significant differences are relative to treatment with the sEP67 control. For ELISA data error bars indicate 95% confidence intervals of mean values from three wells. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$.

Figure 2. PMN influx and CD88 is essential for the protective action of EP67

Histopathology of hematoxylin-and-eosin-stained skin tissues (50X) of representative mice treated only with PBS (A), sEP67 (B) and EP67 (C, D) showing inflammatory infiltration following EP67 administration. Panel D is a magnification (1000X) of the boxed area in C. Neutrophil influx assessment by FITC rat anti-mouse Ly6G immuno staining in representative mice (50X) during bacterial infection and treatment with EP67 (E, F) or sEP67 (G, H). Panels F and H are magnifications (200X) of boxed areas in E and G respectively. Measurement of myeloperoxidase (MPO) activity in skin homogenates post administration of EP67 or sEP67 and subsequent bacterial infection as described in Materials and Methods (I). Bars indicate mean level of MPO activity. Lesion development in mice following i.p. treatment (24h post EP67 or sEP67 and subsequent bacterial injections) with 50 μ g of either neutrophil depleting Ab, RB6-8C5 or isotype control Ab (J). Bars indicate mean lesion size 48 hours post-bacterial infection. Lesion development in CD88^{-/-} mice compared to WT C57Bl/6 following EP67 or sEP67 treatment 48 hours post-bacterial infection (K). Statistically significant differences in lesion size are relative to that obtained in the WT CD88^{+/+} mice treated with EP67. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$.