(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 March 2013 (07.03.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/032964 A1

(51) International Patent Classification:
A01N 63/00 (2006.01) *C12N 5/00* (2006.01)

(21) International Application Number:
PCT/US2012/052421

(22) International Filing Date:
26 August 2012 (26.08.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/527,773 26 August 2011 (26.08.2011) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2013/032964 A1

(54) **Title**: COMPOSITIONS AND METHODS FOR PREVENTING AND TREATING BIOFILMS

(57) **Abstract**: Disclosed are materials, uses and methods for preventing and treating biofilm infection, such as a device-associated biofilm infection. Exemplary devices include catheters and artificial implants, such as artificial hips, knees, ankles, shoulders, elbows, wrists, and the like. The methods comprise administration of a prophylactically or therapeutically effective amount of activated macrophages to reduce the spread of infection, to reduce the growth of microbes associated with a biofilm, such as Staphylococcus aureus (e.g., MRS A), or to kill such microbes, thereby effectively preventing or treating a biofilm infection.

COMPOSITIONS AND METHODS FOR PREVENTING AND TREATING BIOFILMS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the priority benefit of provisional U.S. Patent Application Number 61/527,773 filed August 26, 2011, which is incorporated herein by reference in its entirety.

GOVERNMENT SUPPORT

[0002] This invention was made with government support under Grant No. P01 A1083211 awarded by the National Institutes of Health (NIH/NIAID). The government has certain rights in the invention.

BACKGROUND

[0003] Microorganisms, such as bacteria, can form biofilms, where organisms are encased in a complex three-dimensional matrix consisting of extracellular polysaccharides, DNA, and proteins. This structure protects the microbes from a variety of physical and chemical challenges, making biofilm treatment difficult. In addition, upon biofilm formation, some bacteria assume a metabolically dormant state, which protects them from antibiotic killing that requires active cell wall and/or protein synthesis.

[0004] Biofilm infections often lead to significant morbidity due to their chronicity and recalcitrance to antibiotics. Biofilms cause over 80% of infections and approximately 65% of nosocomial infections caused by microorganisms in the developed world involve biofilms.

[0005] One patient population particularly vulnerable to biofilm infection is the group of patients harboring foreign devices, such as implants (*e.g.*, artificial joints, subcutaneous pacemakers) or indwelling catheters. Biofilms are responsible for catheter infections that cause about 10,000 U.S. deaths and more than 11 billion US dollars in losses in hospital costs annually. Twenty percent of urinary catheters inserted into 5 million U.S. patients have developed biofilm infections, resulting in medical expenses of 1.6 billion U.S. dollars each year.

[0006] More specifically with respect to patients harboring foreign devices, *e.g.*, implants or catheters, the frequency of device-related biofilms continues to rise, with current infection rates totaling 1-2% of primary arthroplasties, 3-5% of all revisions, about 10-50% of short-term urinary catheterizations, and the majority of all patients with long-term catheterization. These numbers do not take into account the propensity of bacterial biofilms to colonize other

medical devices as well as natural body surfaces. It is estimated that the total cost of biofilm infections, in treatment, lost productivity and fouled equipment, is about 500 billion US dollars annually.

[0007] In addition to the significant cost associated with biofilm infections, devastating health consequences can result from such infections. As but one example, biofilms of a single potentially pathogenic bacterium, *Pseudomonas aeruginosa*, are the cause of 70,000 cases of cystic fibrosis worldwide. Between 5 to 7 billion U.S. dollars are spent in medical care and productivity losses in the United States due to 25 to 80 million cases annually of biofilm-related food-borne diarrhea and 8,000 to 18,000 cases of biofilm-related Legionnaire's disease.

[0008] Bacterial biofilm infections alter host immune responses to favor anti-inflammatory and pro-fibrotic pathways, which contribute to biofilm persistence. A variety of microbes, *e.g.*, bacteria, have been identified as being associated with, or causative agents of, biofilm. A number of these infectious microbes are major health scourges, including methicillin-resistant *Staphylococcus aureus* (MRSA), a common etiologic agent of biofilms that often cause chronic and recurrent infections when associated with indwelling medical devices, such as implants and catheters. The persistent biofilms caused by these microbes, moreover, are associated with a variety of diseases and medical conditions, including but not limited to, dental caries, periodontitis, infection complications associated with cystic fibrosis, pneumonia, infective endocarditis, muscle skeletal infections, necrotizing fasciitis, osteomyelitis, meloidosis, infectious kidney stones, bacterial endocarditis, airway infections, otitis media, biliary tract infections, chronic bacterial prostatitis, and infections associated with medical devices (*e.g.,* intravenous catheters, urinary catheters, artificial joints, contact lenses).

[0009] Current therapeutic options are ineffective in treating and controlling microbial burden, *e.g.*, bacterial burden, in biofilms. For example, conventional antibiotics are ineffective in treating or controlling bacterial burden in biofilms. The reasons for the ineffectiveness of antibiotics and other chemical-based approaches to biofilm control are not fully understood, but it is believed that the biofilm matrix or "slime" presents a barrier to antibiotics that is exacerbated by a host-derived fibrous capsule typically found associated with chronic biofilm infections. In addition, it is believed that the altered physiological state of microbes associated with a biofilm affect their susceptibility to many antibiotics. Regardless of the reasons, however, empirical evidence demonstrates that antibiotic treatment of biofilm infection is insufficient to address the problem. Nevertheless, antibiotics are

commonly used to control microbes, *e.g.*, bacteria, that escape the biofilm matrix in order to prevent their colonization of other tissue sites. Such uses of antibiotics impose selection pressures on microbes such as bacteria and increase the chance of antibiotic-resistant strains arising.

[0010] A relatively recent development regarding antibiotic treatment involves an administration schedule characterized by the delivery of intermittent low dosages of antibiotics to treat biofilm infection. The schedule is designed to better attack persister cells, which are biofilm-associated microbes that survive initial, high-dosage treatments. Persister cells have been offered as an explanation for the capacity of many biofilm infections to not only survive initial antibiotic treatments, but to propagate biofilm infection over time. This capacity of biofilm infections has been suggested as a basis for the chronic infections associated with biofilm. Although the intermittent, low-dose approach to biofilm infection treatment may help to treat persister cells associated with biofilm, this approach does not advance methods to prevent biofilm infection and it does not reduce the risk of developing resistant strains because it continues to apply selective pressure in the form of antibiotics.

[0011] For biofilm infections associated with artificial devices such as implants or catheters, current therapy involves a cycle of implant or catheter removal and replacement, which is inconvenient, ineffective, and typically undesirable. Management of implant- or device-associated biofilm infections involves a staged replacement of the hardware, either as a single-step exchange, whereby the entire implant is replaced in a single procedure or, more commonly, as a two-stage exchange. In the latter case, patients receive extended antibiotic regimens in addition to surgical management, which generally consists of device removal and replacement with an antibiotic-impregnated temporary spacer, followed by insertion of a new prosthesis after a two- to eight-week period. This is a long and debilitating process associated with significant morbidity and economic impact for patients. The difficulties of biofilm treatment are further underscored in the context of more permanent implants such as artificial joints (*e.g.*, hips, knees, shoulders, ankles, elbows, and wrists), procedures that are particularly common in the elderly, who grow increasingly less immune-responsive over time.

[0012] Accordingly, a need exists in the art for compositions and methods for preventing and treating biofilms that are efficacious and cost-effective without incurring significant risk of toxicity or other complications arising from prophylactic or therapeutic treatment, while minimizing the need for surgical intervention.

SUMMARY

[0013] The materials and methods (and uses) disclosed herein provide cells, such as autologous cells, that can be manipulated *ex vivo* and/or *in vivo* to become activated macrophages suitable for use in preventing or treating biofilm infections. In providing an immune-based approach to the prevention and treatment of biofilm, the disclosure provides materials and methods of wide applicability, *i.e.*, any biofilm associated with a native surface or "foreign" device such as an infectious microbe (*e.g.*, bacterium) is amenable to prevention or treatment with the disclosed materials and methods. In addition, the disclosed methods provide the benefit of phrophylactic and therapeutic approaches that will not contribute to the collective evolutionary problem of effectively selecting for infectious superbugs resistant to most treatments, such as methicillin-resistant *S. aureus* (*i.e.*, MRSA), because the methods take advantage of bacterial killing attributes inherent to the host's immune system.

[0014] In developing the disclosed materials and methods/uses, it was recognized that biofilms can skew the immune response to favor anti-inflammatory and pro-fibrotic pathways, which contribute to biofilm persistence. As a specific example, although macrophages are a prominent infiltrate in *S. aureus* biofilm infections, their penetration into the biofilm itself is impeded by a robust fibrotic response surrounding the infection. In addition, biofilm-associated macrophages are polarized towards an alternatively activated M2 phenotype that exhibits anti-inflammatory and pro-fibrotic properties that limit bacterial clearance. By extension, the programming of macrophages towards a microbicidal M1 response is diverted towards a M2 phenotype prior to physical contact with the biofilm. These findings led to methods of treating biofilms by exogenous administration of M1-activated macrophages directly into sites of biofilm infection. As disclosed herein, the administration overcame the local immunoinhibitory environment and bypassed the fibrotic barrier associated with biofilm infection.

[0015] Also contemplated are variations of the above-described methods/uses to prevent and/or treat biofilms further comprising administration of an effective amount of a macrophage-activating peptide, such as a C5a receptor agonist, a C5a analog, or a C5a fragment, wherein each of these peptides facilitates bacterial clearance by inducing a pro-inflammatory milieu. One exemplary C5a receptor agonist is EP67 (YSFKDMP(MeL)aR; SEQ ID NO:1; uppercase letters designate the L stereoisomeric form and lower case the D stereoisomeric form of the amino acids; (MeL) corresponds to N-methyl leucine)), which is a response-selective agonist of the human C5a receptor (C5aR) that preferentially elicits pro-inflammatory mediator production from C5aR-bearing macrophages without affecting C5aR-

bearing neutrophils. EP67 was derived from the C-terminal region of human complement component, C5a (Taylor SM, et al., Curr Med Chem 2001;8:675-684.). EP67 possesses potent immune-enhancing properties (Morgan et al., Vaccine, 28(2): 463-469 (2009)). The disclosure further provides a C5a analog comprising 1, 2, 3, 4, or 5 amino acid substitutions in the amino acid sequence set out in SEQ ID NO:1 wherein the peptide having the substituted amino acid sequence retains binding activity of the peptide set out in SEQ ID NO:1.

[0016] The C-terminus of C5a comprises amino acids 65-74 of C5a (*i.e.*, ISHKDMQLGR ("$C5a_{65-74}$") (SEQ ID NO:4). The disclosure further provides a C5a analog comprising 1, 2, 3, 4, or 5 amino acid substitutions in the amino acid set out in SEQ ID NO:4 wherein the peptide having the substituted amino acid sequence retains binding activity of the peptide set out in SEQ ID NO:4.

[0017] The C-terminal analog of C5a, EP54 (YSFKPMPLaR; SEQ ID NO:2) is a C5a receptor agonist that has been described previously (Duryee et al. (2009) Vaccine 27:2981-2988; Hegde et al. (2008) Int. Immunopharmacol. 8:819-827; and Ulrich et al. (2000) J. Immunol. 164:5492-5498). However, these studies described an EP54 construct wherein EP54 was conjugated to a specific antigen or hapten. The disclosure further provides a C5a analog comprising 1, 2, 3, 4, or 5 amino acid substitutions in the amino acid sequence set out in SEQ ID NO:2 wherein the peptide having the substituted amino acid sequence retains binding activity of the peptide set out in SEQ ID NO:2. Additional disclosure of C5a analogs is provided in USSN 61/359,444 and International (PCT) Patent Application No. PCT/US2011/042344 , each of which is incorporated herein by reference.

[0018] One or more of the macrophage-activating compounds disclosed herein, *e.g.*, a C5a receptor agonist, a C5a analog or a C5a fragment, is useful in methods of activating macrophages prior to, concurrent with, or after administration of macrophage to prevent or treat a biofilm infection. In a typical embodiment, the macrophage-activating compound contacts the macrophage *ex vivo* prior to administration of the macrophages. Given that, in some embodiments, administration will place the macrophages beyond the fibrotic barrier in the vicinity of the biofilm, it is contemplated that the macrophage-activating compound is co-administered with the macrophages or the macrophage-activating compound is administered after the administration of the macrophages, where administration beyond the fibrotic barrier and in the vicinity of the biofilm places the macrophage-activating compound(s) and macrophages in sufficiently close proximity to result in contact and macrophage activation *in vivo*.

[0019] Targeting macrophage pro-inflammatory activity with exogenous M1-activated macrophages inhibited *S. aureus* biofilm formation and provided long-lasting protection from catheter colonization and infection in surrounding tissues. This protection was not provided by neutrophils, consistent with the failure of these cells to become a prominent infiltrate during *S. aureus* biofilm infection in some settings. Collectively, these results indicate that immune cell-based therapy using activated macrophages overcomes the current difficulties associated with biofilm treatment and control.

[0020] The approaches to biofilm prevention and treatment disclosed herein exploit natural host immune mechanisms for therapeutic benefit. Targeting the host response rather than the pathogen itself offers certain advantages by largely avoiding selective pressures for the evolution of microbial resistance. Indeed, stimulating adaptive immunity through vaccination has remained effective in the face of microbial resistance over decades of clinical use, although not all pathogens have been amenable to vaccination strategies, most notably *S. aureus*. Furthermore, the fact that innate immune defenses are geared to rapidly recognize an infinite pathogen repertoire, indicates that immunomodulation will afford broad-spectrum protection against a range of microbial pathogens, enabling prophylactic use in high-risk groups and early treatment prior to the identification of causative infectious agents.

[0021] The local administration of classically activated M1 macrophages for biofilm prevention and treatment disclosed herein overcomes immune evasion by the causative microbes by triggering a pro-inflammatory milieu. Early administration of M1-activated macrophages significantly attenuated biofilm formation in a mouse model of MRSA catheter-associated infection. Several pro-inflammatory mediators were significantly elevated in biofilm-infected tissues from macrophage-treated animals, revealing effective reprogramming of the biofilm environment to a pro-inflammatory milieu. Treatment of established biofilm infections with activated macrophages also significantly reduced catheter-associated biofilm burdens compared to antibiotic treatment. A requirement for macrophage pro-inflammatory activity was demonstrated by the fact that transfer of MyD88-deficient macrophages had minimal impact on biofilm growth. Likewise, neutrophil administration had no effect on biofilm formation. Collectively, these results demonstrate that targeting macrophage pro-inflammatory activity can overcome the local immunoinhibitory environment created during biofilm infections and represents a promising therapeutic strategy to the prevention and treatment of biofilm infections.

[0022] The disclosure provides a convenient and effective way to prophylactically and/or therapeutically reduce the incidence and/or severity of microbial, *e.g.*, bacterial, biofilm

infections generally, and more particularly, of microbial, *e.g.*, bacterial, biofilm infections associated with catheters (*e.g.*, urinary, intravenous, an intraarterial catheters) and other artificial implants such as artificial joint components (*e.g.*, artificial components of hip, knee, ankle, shoulder, elbow and wrist joints).

[0023] One aspect of the disclosure is a composition comprising an isolated activated macrophage for the prevention or treatment of a biofilm. In some embodiments, the composition further comprises a macrophage-activating peptide selected from the group consisting of EP67 (SEQ ID NO:1), EP54 (SEQ ID NO:2) and $C5a_{65-74}$ (SEQ ID NO:4). Variants and derivatives of the EP67 peptide, the EP54 peptide, or the $C5a_{65-74}$ peptide that retain the capacity to beneficially affect endogenous macrophages, as disclosed herein, are also contemplated, and in particular those variants and derivatives of the EP67 peptide, EP54 peptide, and $C5a_{65-74}$ peptide that differ by 1, 2, 3, 4, or 5 residues from the relevant wild-type sequence, *i.e.*, SEQ ID NO:1 for EP67, SEQ ID NO:2 for EP54, and SEQ ID NO:4 for $C5a_{65-74}$ are within the scope of the compositions, and methods, of the disclosure.

[0024] A related aspect of the disclosure provides a kit comprising an isolated macrophage and a macrophage-activating agent. Also provided is a kit comprising a macrophage-activating agent. In some embodiments of the kit, the macrophage-activating agent is selected from the group consisting of a C5a receptor agonist, including but not limited to macrophage-activating peptides such as EP67 (SEQ ID NO:1), EP54 (SEQ ID NO:2), $C5a_{65-74}$ (SEQ ID NO:4) and analogs and variants thereof, interleukin-1β as well as combinations of interferon-γ and tumor necrosis factor (TNF), and interferon-γ and an inducer of TNF. An exemplary kit comprises a C5a receptor agonist that is selected from the group consisting of EP67 (SEQ ID NO:1), EP54 (SEQ ID NO:2) and $C5a_{65-74}$ (SEQ ID NO:4). As with the compositions according to the disclosure, kits according to the disclosure include variants and derivatives of the EP67 peptide, the EP54 peptide, or the $C5a_{65-74}$ peptide that retain the capacity to beneficially affect endogenous macrophages, as disclosed herein, and in particular, those variants and derivatives that differ by 1, 2, 3, 4, or 5 residues from the relevant wild-type sequence, *i.e.*, SEQ ID NO:1 for EP67, SEQ ID NO:2 for EP54, and SEQ ID NO:4 for $C5a_{65-74}$ are within the scope of the kits of the disclosure.

[0025] Another aspect of the disclosure is a method of providing biofilm therapy to a subject-in-need comprising: (a) isolating peripheral blood monocytes from a subject; (b) culturing the monocytes under conditions compatible with formation of macrophages; and (c) administering a prophylactically or therapeutically effective amount of the macrophages to a subject-in-need, wherein the macrophages are activated by contact with a macrophage-

activating agent prior to, simultaneously with, or after administration of the macrophages. In some embodiments of the method, the macrophages are activated by contact with a macrophage-activating agent prior to administration of the macrophages. A related aspect is drawn to the use of an activated macrophage in the treatment of an established biofilm infection in a subject-in-need. In some embodiments of the method and of the use, the subject-in-need is at risk of developing a biofilm infection (*i.e.*, the subject-in-need does not have an established biofilm infection), while in other embodiments, the subject-in-need comprises a biofilm infection (*i.e.*, the subject-in-need does have an established biofilm infection).

[0026] Also contemplated by the disclosure are embodiments wherein the subject-in-need is a human patient or a veterinary animal, such as a veterinary animal selected from the group consisting of a dog, a cat, a horse, a bovine (a bull or cow), a sheep, a goat and a pig. Embodiments of the method and use are contemplated in which the subject is the subject-in-need (*i.e.*, the isolated cells are autologous cells). In some embodiments, the dose or doses of macrophages, *e.g.*, activated macrophages, for administration according to the methods and uses of the disclosure are determined on an individual basis using factors known in the art, *e.g.*, general health, immunocompetency, age, weight, and the like. In general, the disclosure contemplates dosages within the range of about 10^5 to 10^8 macrophages, *e.g.*, 10^6 macrophage cells. Consistent with relative infectious microbe burden, lower dosages and/or a reduced schedule of administration of macrophages would be expected in prophylactic methods and uses compared to treatment methods and uses. Dosage scheduling is expected to range from about one to five doses of macrophages, with each dose separated from other doses by about one to five days. In some embodiments, multiple doses are administered to different sites around the expected or real periphery of a potential or existing biofilm infection. An exemplary dosage schedule for prophylactic methods and uses is about three daily doses of 10^5 to 10^8 macrophages, which is compatible with the average hospital stay for receiving a surgical implant, which places the patient at risk of a device-associated biofilm infection.

[0027] In some embodiments, the macrophage-activating agent is selected from the group consisting of a C5a receptor agonist, including but not limited to macrophage-activating peptides such as EP67 (SEQ ID NO:1), EP54 (SEQ ID NO:2), $C5a_{65-74}$ (SEQ ID NO:4), and analogs and variants thereof, interleukin-1β as well as combinations of interferon-γ and tumor necrosis factor (TNF), and interferon-γ and an inducer of TNF. An exemplary inducer of TNF is a TNF inducer selected from the group consisting of peptidoglycan and

lipopolysaccharide. An exemplary C5a receptor agonist is selected from the group consisting of EP67 (SEQ ID NO:1), EP54 (SEQ ID NO:2) and $C5a_{65-74}$ (SEQ ID NO:4). In some embodiments of the method and use, the macrophage-activating agent is a combination selected from the group consisting of interferon-γ and tumor necrosis factor (TNF) or interferon-γ and an inducer of TNF. An exemplary inducer of TNF is selected from the group consisting of peptidoglycan and lipopolysaccharide. Yet another stimulatory agent is the above-noted macrophage-activating peptide EP67, a C5a receptor/CD88 agonist that specifically induces pro-inflammatory properties of $CD88^+$ macrophages without any effects on $CD88^+$ neutrophils. Yet other embodiments of the method and use further comprise delivering to the subject-in-need an effective amount of a pro-inflammatory compound, such as interleukin-6, interleukin-12, monocyte chemoattractant protein-1 (MCP-1), monokine-induced by IFN-γ (MIG), interferon-regulatory protein-10 (IP-10), macrophage inflammatory protein-2 (MIP-2), and keratinocyte chemoattractant (KC). Also contemplated are macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) to encourage macrophage and neutrophil expansion at the site of biofilm infection, whereupon pro-inflammatory cytokines would be present to activate these cells to trigger their bactericidal activity.

[0028] It is also contemplated that, in some embodiments of both the method and use, the subject-in-need undergoes surgery to receive a surgical implant, such as an artificial joint component, a cranial plate (natural or artificial), a dental implant, or a subcutaneous pacemaker. Exemplary artificial joint components are an artificial hip component, an artificial knee component, an artificial ankle component, an artificial elbow component, an artificial shoulder component, and an artificial wrist component. In some embodiments of each of the disclosed method and use, the subject-in-need is scheduled to receive, or has received, a catheter. In some implementations of the method and of the use, the activating agent and the macrophage are co-administered to the subject-in-need. Yet other embodiments of the method or use further comprise delivery of a proteolytic enzyme, such as MMP-1, MMP-2, MMP-7, MMP-9, MMP-12 or collagenase to the subject-in-need, for example to prevent biofilm infection. Additional embodiments of the method or use further comprise use of a proteolytic enzyme, such as MMP-1, MMP-2, MMP-7, MMP-9, MMP-12, and collagenase, in the treatment of an existing biofilm infection in a subject-in-need. Without wishing to be bound by theory, the proteolytic enzyme is expected to degrade the fibrotic capsule often associated with biofilms, enhancing the access of activated macrophages to the biofilm.

[0029] In some embodiments of both the methods and uses according to the disclosure, the proteolytic enzyme is packaged in a delivery vehicle such as a microsphere, microparticle, nanosphere, nanoparticle, liposome, micelle, or gel. These delivery vehicles may be formulated to provide for the controlled, *e.g.*, slow, release of their contents, as would be known in the art. In various embodiments, the delivery vehicle is selected from the group consisting of a microsphere, a microparticle, a nanosphere, a nanoparticle, a liposome, a micelle and a gel, wherein the delivery vehicle comprises a macrophage and a macrophage-activating agent, or an activated macrophage, or a macrophage, a macrophage-activating agent and a proteolytic enzyme, or an activated macrophage and a proteolytic enzyme. Delivery vehicles suitable for the disclosed methods and uses include vehicles that are targeted to a biofilm, such as by exhibiting a binding partner to a marker displayed on the infectious organism, and vehicles that are targeted to the vicinity of a biofilm infection, for example by exhibiting a binding partner to a component of the fibrotic matrix frequently associated with biofilms. An exemplary proteolytic enzyme is collagenase, which may be packaged in any of the foregoing vehicles, such as a microsphere. In some embodiments, the method and use further comprise the delivery of at least one molecule that interferes with the formation of the fibrotic capsule surrounding biofilm infections, such as slow-release delivery vehicles (any of the above-described vehicles) containing at least one proteolytic enzyme (*e.g.*, collagenase or an MMP) and, optionally, at least one compound that interferes with the formation of a fibrotic capsule, such as a Lysyl Oxidase (*i.e.*, LOX) inhibitor (*e.g.*, BAPN). In general, the disclosure comprehends any delivery vehicle known in the art for use in packaging and delivering activated macrophages, or macrophages and at least one macrophage-activating agent, or activated macrophages and at least one proteolytic enzyme, or macrophages, at least one macrophage-activating agent and at least one proteolytic enzyme. Any of these formulations may further comprise a compound that interferes with the formation of the fibrotic capsule, such as BAPN or another Lysyl Oxidase (LOX) inhibitor.

[0030] Embodiments of the use of activated macrophages further comprise using a prophylactically or therapeutically effective amount of an EP67 peptide (SEQ ID NO:1) in the treatment of a biofilm infection in a subject-in-need. In some embodiments of each of the method and the use, the subject-in-need exhibits an inflammatory disorder. In some embodiments of each of the method and thus use, the subject-in-need exhibits a disorder or condition selected from the group consisting of atherosclerosis, infectious complications of cystic fibrosis, endocarditis, chronic sinusitis, nasal polyposis, chronic otitis media,

leptospirosis, kidney stones, osteomyelitis, necrosis of the jaw, a urinary tract infection, a bladder infection, a chronic wound, an artificial component of a joint, a temporary catheter and a permanent catheter.

[0031] Embodiments of the methods and uses for preventing or treating biofilm infections include methods and uses in which the subject-in-need exhibits an inflammatory disorder. An inflammatory disorder as used herein means any inflammatory disorder of humans or veterinary animals that is known in the art. Exemplary methods and uses comprise a subject-in-need that exhibits a disorder or condition selected from the group consisting of atherosclerosis, infectious complications of cystic fibrosis, endocarditis, chronic sinusitis, nasal polyposis, chronic otitis media, leptospirosis, kidney stones, osteomyelitis, necrosis of the jaw, a urinary tract infection, a bladder infection, a chronic wound, an artificial component of a joint, a temporary catheter and a permanent catheter.

[0032] Embodiments of the methods and uses for preventing or treating biofilm infections further comprise administering a prophylactically or therapeutically effective amount of an antibiotic. In some embodiments, the antibiotic is a member of a class of antibiotics selected from the group consisting of aminoglycosides, ansamycins, carbacephems, carbapenems, cephalosporins, glycopeptides, lincosamides, lipopeptides, macrolides, erythromycins, monobactams, nitrofurans, penicillins, penicillin combinations, antimicrobial polypeptides, quinolones, sulfonamides, tetracyclines and antimycobacterials. In some embodiments, the antibiotic is selected from the group consisting of aminoglycosides, e.g., ,amikacin (amikin), gentamicin (garamycin), kanamycin (kantrex), neomycin (neofradin), roxithromycin, netilmicin (netromycin), tobramycin (nebcin), paromomycin (humatin); ansamycins, e.g., geldamycin, herimycin; Carbacephems, e.g., loracarbef (lorabid); carbapenems, e.g., ertapenem (invanz), doripenem (doribax), imipenem or cilastatin (primaxin), meropenem (merrem); cephalosporins, e.g., cefadroxil (duricef), cefazolin (ancef), cefalotin or cefalothin (keflin), cefalexin (keflex), cefaclor (distaclor), cefamandole (mandol), cefoxitin (mefoxin), cefprozil (cefzil), cefuroxime (cetin, zinnat), cefonicid, cefixime (suprax), cefdinir (omnicef, cefdiel), cefditoren (spectracef, cefobid), cefotaxime (claforan), cefetamet, cefoperazone, cefpodoxime (vantin), ceftazidime (fortaz), ceftibuten (cedax), ceftriaxone (rocephin), cefepime (maxipime), ceftaroline or fasamil (teflaro), ceftobiprole (zeftera), cephalothin, cefmetazole, cefotetan, ceftizoxime, cefsulodin, cephalexin, cinoxacin; glycopeptides, e.g., teicoplanin (targocid), vancomycin (vancocin), telavancin (vibativ); lincosamides, e.g., clindamycin (cleocin), lincomycin (lincocin); lipopeptides, e.g., daptomycin (cubicin); macrolides, e.g., azithromycin (zithromax, sumamed, xithrone), clarithromycin (biaxin,

dynabac); erythromycin (erythocin, erythroped), erythromycin estolate, erythromycin ethyl succinate, erythromycin glucoheptonate, erythromycin lactobionate, erythromycin stearate, roximthromycin, trolandomycin (tao), telithromycin (ketek), spectinomycin (trobicin), spriamycin (rovamycine); monobactams, e.g., aztreonam (azactam); nitrofurans, e.g., furazolidone (furoxone), nitrofurantoi (macrodantin, macrobid); penicillins, e.g., amoxicillin (novamox, amoxil), ampicillin (principen), azlocillin, carbenicillin (geocillin), cloxacillin (tegopen), dicloxacillin (dynapen), flucloxacillin (floxapen), mezlocillin (mezlin), methicillin (staphcillin), nafcillin (unipen), oxacillin (prostaphlin), penicillin G (pentids, pfizerpen, negaban), penicillin V (veetids), piperacillin (pipracil), ticarcillin (ticar); penicillin combinations, e.g., amoxicillin and clavulanate (augmentin), ampicillin and sulbactam (unasyn), piperaccillin and tazobactam (zosyn), ticarcillin and clavulanate (timentin); antimicrobial polypeptides, e.g., bacitracin, colistin (colymycin S), polymyxin B; quinolones, e.g., ciprofloxacin (cipro, ciproxin, ciprobay), enoxacin (penetrex), gatifloxacin (tequin), levofloxacin (levaquin), lomefloxacin (maxaquin), moxifloxicin (avelox), ofloxacin (floxin, ocuflox), nalidixic acid (negram), norfloxacin (noroxin), ofloxicin (floxin, ocuflox), trovafloxacin (trovan), grepafloxacin (raxar), sparfloxacin (zagam), temafloxacin (omniflox); sulfonamides, e.g., mafenide (sulfamylon), sufonamidochrysoidine (prontosil), sulfacetamide (sulamyd, bleph-10), sufadiazine (micro-sulfon), silver sulfadiazine (silvadene), sulfamethizole (thiosulfil forte), sulfamethoxazole (gantanol), sulfanilamide, sulfasalazine (azulfidine), sulfisoxazole (gantrisin), trimethoprim-sulfamethoxazole (bactrim, septra); Tetracyclines, e.g., demeclocycline (declomycin), doxycycline (vibramycin), minocycline (minocin), oxytetracycline (terramycin), tetracycline (sumycin, achromycin V, steclin); and antimycobacterials, e.g., clofazimine (lamprene), dapsone (avlosulfon), capreomycin (capastat), cycloserine (seromycin), ethambutol (myambutol), ethionamide (trecator), isoniazid (INH), rifampicin or rifampin (rifadin, fimactane), rifabutin (mycobutin), rifapenine (priftin), streptomycin, arshenamine (salvarsan), chloramphenicol (chloromycetin), fosfomycin (monurol), fusidic acid (fucidin), linezolid (zyvox), metronidazole (flagyl); mupirocin (bactroban), platensimycin, quinupristin/dalfopristin (synercid), rifaximin (xifaxan), thiamphenicol, tigercycline (tigacyl) tinidazole (tindamax fasigyn); and other compounds, e.g., trimethoprim (proloprim, trimpex); fleroxacin, nitrofurantoin, and coamoxiclavuanate. Also contemplated are various forms of each of these antibiotics, including but not limited to their salts, acids, and bases.

[0033] Particular aspects of the disclosure are described in the following enumerated paragraphs.

[0034] 1. A composition comprising an isolated activated macrophage for the prevention or treatment of a biofilm.

[0035] 2. The composition according to paragraph 1 further comprising a macrophage-activating peptide selected from the group consisting of EP67, EP54 and $C5a_{65-74}$.

[0036] 3. A kit comprising an isolated macrophage and a macrophage-activating agent.

[0037] 4. A kit comprising a macrophage-activating agent.

[0038] 5. The kit according to paragraph 3 or 4 wherein the macrophage-activating agent is selected from the group consisting of a C5a receptor agonist, interleukin-1β, interferon-γ and tumor necrosis factor (TNF), and interferon-γ and an inducer of TNF.

[0039] 6. The kit according to paragraph 5 wherein the C5a receptor agonist is selected from the group consisting of EP67, EP54 and $C5a_{65-74}$.

[0040] 7. A method of providing biofilm therapy to a subject-in-need comprising:

(a) isolating peripheral blood monocytes from a subject;

(b) culturing the monocytes under conditions compatible with formation of macrophages; and

(c) administering a prophylactically or therapeutically effective amount of the macrophages to a subject-in-need, wherein the macrophages are activated by contact with a macrophage-activating agent prior to, simultaneously with, or after administration of the macrophages.

[0041] 8. The method according to paragraph 7 wherein the macrophages are activated by contact with a macrophage-activating agent prior to administration of the macrophages.

[0042] 9. A use of macrophages in the prevention or treatment of a biofilm infection in a subject-in-need, wherein the macrophages are activated by contact with a macrophage-activating agent prior to, simultaneously with, or after administration of the macrophages.

[0043] 10. The method according to paragraph 7 or 8 wherein the subject-in-need is at risk of developing a biofilm infection.

[0044] 11. The method according to paragraph 7 or 8 wherein the subject-in-need comprises an established biofilm infection.

[0045] 12. The method according to paragraph 7 or 8 wherein the subject-in-need is a human patient.

[0046] 13. The method according to paragraph 7 or 8 wherein the subject-in-need is a veterinary animal selected from the group consisting of a dog, a cat, a horse, a bovine (a cow or a bull), a sheep, a goat and a pig.

[0047] 14. The method according to paragraph 7 or 8 wherein the subject is the subject-in-need.

[0048] 15. The method according to paragraph 7 or 8 wherein the macrophage-activating agent is selected from the group consisting of a C5a receptor agonist, interleukin-1β, interferon-γ and tumor necrosis factor (TNF), and interferon-γ and an inducer of TNF.

[0049] 16. The method according to paragraph 15 wherein the inducer of TNF is selected from the group consisting of peptidoglycan and lipopolysaccharide.

[0050] 17. The method according to paragraph 15 wherein the C5a receptor agonist is selected from the group consisting of EP67 (SEQ ID NO:1), EP54 (SEQ ID NO:2) and $C5a_{65-74}$ (SEQ ID NO:4).

[0051] 18. The method according to paragraph 7 or 8 wherein the macrophages are administered at a dose of 10^5 to 10^8 cells.

[0052] 19. The method according to paragraph 18 wherein one to five doses of macrophages are administered.

[0053] 20. The method according to paragraph 15 further comprising delivering to the subject-in-need an effective amount of a pro-inflammatory compound selected from the group consisting of interleukin-6, interleukin-12, monocyte chemoattractant protein-1 (MCP-1), monokine-induced by IFN-γ (MIG), interferon-regulatory protein-10 (IP-10), macrophage inflammatory protein-2 (MIP-2), keratinocyte chemoattractant (KC), macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF).

[0054] 21. The method according to paragraph 7 or 8 wherein the subject-in-need undergoes surgery to receive a surgical implant.

[0055] 22. The method according to paragraph 21 wherein the surgical implant is selected from the group consisting of an artificial joint component, a cranial plate (natural or artificial), a dental implant and a subcutaneous pacemaker.

[0056] 23. The method according to paragraph 22 wherein the artificial joint component is selected from the group consisting of an artificial hip component, an artificial knee

component, an artificial ankle component, an artificial elbow component, an artificial shoulder component, and an artificial wrist component.

[0057] 24. The method according to paragraph 7 or 8 wherein the subject-in-need is scheduled to receive, or has received, a catheter.

[0058] 25. The method according to paragraph 7 wherein the macrophage-activating agent and the macrophage are co-administered to the subject-in-need.

[0059] 26. The method according to paragraph 7 or 8 further comprising delivery of a proteolytic enzyme to the subject-in-need.

[0060] 27. The method according to paragraph 26 wherein the proteolytic enzyme is selected from the group consisting of MMP-1, MMP-2, MMP-7, MMP-9, MMP-12 and collagenase.

[0061] 28. The method according to paragraph 7, 8, or 27 further comprising a delivery vehicle selected from the group consisting of a microsphere, a microparticle, a nanosphere, a nanoparticle, a liposome, a micelle and a gel, wherein the delivery vehicle comprises a macrophage and a macrophage-activating agent, or an activated macrophage, or a macrophage, a macrophage-activating agent and a proteolytic enzyme, or an activated macrophage and a proteolytic enzyme.

[0062] 29. The method according to paragraph 7 or 8 wherein the subject-in-need exhibits an inflammatory disorder.

[0063] 30. The method according to paragraph 7 or 8 wherein the subject-in-need exhibits a disorder or condition selected from the group consisting of atherosclerosis, infectious complications of cystic fibrosis, endocarditis, chronic sinusitis, nasal polyposis, chronic otitis media, leptospirosis, kidney stones, osteomyelitis, necrosis of the jaw, a urinary tract infection, a bladder infection, a chronic wound, an artificial component of a joint, a temporary catheter and a permanent catheter.

[0064] 31. The method according to paragraph 7 or 8 further comprising administering a prophylactically or therapeutically effective amount of an antibiotic.

[0065] 32. The method according to paragraph 31 wherein the antibiotic is a member of a class of antibiotics selected from the group consisting of aminoglycosides, ansamycins, carbacephems, carbapenems, cephalosporins, glycopeptides, lincosamides, lipopeptides, macrolides, erythromycins, monobactams, nitrofurans, penicillins, penicillin combinations, antimicrobial polypeptides, quinolones, sulfonamides, tetracyclines and antimycobacterials.

[0066] 33. The method according to paragraph 32 wherein the antibiotic is selected from the group consisting of amikacin, gentamicin, kanamycin, neomycin, roxithromycin, netilmicin, tobramycin, paromomycin, geldamycin, herimycin, loracarbef, ertapenem, doripenem, imipenem or cilastatin, meropenem, cefadroxil, cefazolin, cefalotin or cefalothin, cefalexin, cefaclor, cefamandole, cefoxitin, cefprozil, cefuroxime, cefonicid, cefixime, cefdinir, cefditoren, cefotaxime, cefetamet, cefoperazone, cefpodoxime, ceftazidime, ceftibuten, ceftriaxone, cefepime, ceftaroline or fasamil, ceftobiprole, cephalothin, cefmetazole, cefotetan, ceftizoxime, cefsulodin, cephalexin, cinoxacin; teicoplanin, vancomycin, telavancin; clindamycin, lincomycin, daptomycin, azithromycin, clarithromycin, erythromycin, erythromycin estolate, erythromycin ethyl succinate, erythromycin glucoheptonate, erythromycin lactobionate, erythromycin stearate, roximthromycin, trolandomycin, telithromycin, spectinomycin, spriamycin, Aztreonam, furazolidone, nitrofurantoi, amoxicillin, ampicillin, azlocillin, carbenicillin, cloxacillin, dicloxacillin, flucloxacillin, mezlocillin, methicillin, nafcillin, oxacillin, penicillin G, penicillin V, piperacillin, ticarcillin, amoxicillin and clavulanate, ampicillin and sulbactam, piperaccillin and tazobactam, ticarcillin and clavulanate; bacitracin, colistin, polymyxin B, ciprofloxacin, enoxacin, gatifloxacin, levofloxacin, lomefloxacin, moxifloxicin, ofloxacin, nalidixic acid, norfloxacin, ofloxicin, trovafloxacin, grepafloxacin, sparfloxacin, temafloxacin, mafenide, sufonamidochrysoidine, sulfacetamide, sufadiazine, silver sulfadiazine, sulfamethizole, sulfamethoxazole, sulfanilamide, sulfasalazine, sulfisoxazole, trimethoprim-sulfamethoxazole, demeclocycline, doxycycline, minocycline, oxytetracycline, tetracycline, clofazimine, dapsone, capreomycin, cycloserine, ethambutol, ethionamide, isoniazid, rifampicin or rifampin, rifabutin, rifapenine, streptomycin, arshenamine, chloramphenicol, fosfomycin, fusidic acid, linezolid, metronidazole, mupirocin, platensimycin, quinupristin or dalfopristin, rifaximin, thiamphenicol, tigercycline, tinidazole, trimethoprim, fleroxacin, nitrofurantoin, coamoxiclavuanate, and their salts, acids, and bases.

[0067] Other features and advantages of the present disclosure will become apparent from the following detailed description, including the drawing. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments, are provided for illustration only, because various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from the detailed description.

BRIEF DESCRIPTION OF THE DRAWING

[0068] Figure 1. Effect of activated macrophages on biofilm-causing bacteria. C57BL/6 mice were infected with 10^3 colony forming units (cfu) of methicillin-resistant *S. aureus* (MRSA) USA300 LAC in the lumen of surgically implanted catheters to establish biofilm infection. Animals were initially treated with 10^6 neutrophils or M1-activated macrophages derived from C57BL/6 mice at 12, 24 and 48 hours post-infection. Mice were sacrificed at day 3 following MRSA infection, whereupon catheters (A) and tissues surrounding the catheters (B) were removed to quantitate bacterial burdens. Results are expressed as the number of cfu per ml for catheters or cfu per mg tissue, to correct for differences in tissue sampling size. Significant differences are denoted by asterisks (*, $p <$ 0.05). Results are presented from individual animals combined from at least two independent experiments.

[0069] Figure 2. Long-term effect of activated macrophages on biofilm-causing bacteria. Growth of *S. aureus* on a catheter surface, measured on day 3 and again on day 14 as the $\log_{10}$ of the number of *S. aureus* colony forming units (cfu) per ml. Activated macrophages were administered at 12, 24 and 48 hours post-infection and no additional cells were administered beyond 48 hours following infection. Control mice received injections of vehicle (PBS) alone. Results showed that activated macrophages were capable of long-term attenuation of *S. aureus* biofilm growth *in vivo*.

[0070] Figure 3. Reduction of established *S. aureus* biofilm burden associated with catheters. *S. aureus* biofilms were established using a mouse s.c. catheter model with therapies initiated at day 7 following biofilm growth. A robust biofilm is formed within this time interval. Mice received either vehicle (PBS) or 10^6 activated macrophages (MΦ) within the catheter lumen at days 7 and 9 post-infection, whereas antibiotic-treated mice received i.p. injections of rifampin and daptomycin at the same intervals (25 and 5 mg/kg, respectively, which is a standard therapeutic cocktail for *S. aureus* infection). Mice were sacrificed at day 10 post-infection, whereupon catheter-associated biofilm burdens were determined by sonication and quantitative culture. Significant differences between mice receiving activated macrophage therapy versus vehicle control were observed. Each circle represents data from an individual animal. Left panel: Results from study 1, which compared activated macrophage therapy, antibiotic therapy, and vehicle alone as a control. Right panel: Results from a later study confirming the positive effect of activated macrophage therapy relative to vehicle alone as a control. (Additional studies had confirmed the relative ineffectiveness of antibody therapy.) The results establish that macrophage transfer therapy is effective at reducing established *S. aureus* biofilm burdens on foreign devices.

[0071] Figure 4. M1 macrophage polarization increases *S. aureus* biofilm phagocytosis and killing. A) Neutrophils, non-activated, and M1-activated (10ng/ml IFN-γ + 10μg/ml PGN) macrophages from C57BL/6 mice, as well as M1-activated macrophages from MyD88 knockout (KO) animals were labeled with CellTracker Orange (orange-red) or CellTracker Blue (blue) and co-cultured with mature MRSA biofilms or planktonic bacteria (green) for 2 hours and imaged to observe their phagocytic ability. B) After 24 hours following immune cell addition, biofilms were mechanically disrupted by triturating to evaluate the ability of the various immune cell populations to attenuate biofilm growth. Biofilms without the addition of immune cells were used as untreated controls. Arrows indicate phagocytic cells and significant differences are denoted by asterisks (**, $p < 0.01$; ***, $p < 0.001$).

[0072] Figure 5. *S. aureus* biofilms decrease macrophage cytokine secretion. Bone marrow-derived macrophages were incubated with 6-day-old biofilms or planktonic bacteria for 2 hours in glass chamber slides, whereupon viable macrophages were purified by FACS and then incubated at 37°C for 24 hours without any further stimulation after which supernatants were collected to quantitate TNF-α, IL-10, and IL-1RA by ELISA. Significant differences in secreted cytokine levels between biofilm and planktonic exposed macrophages are denoted by asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

[0073] Figure 6. Activated macrophages but not neutrophils impair MRSA biofilm formation *in vivo*. C57BL/6 mice were infected with 10^3 colony forming units (cfu) of methicillin-resistant *S. aureus* (MRSA) USA300 LAC in the lumen of surgically implanted catheters to establish biofilm infection. Animals were treated with 10^6 neutrophils, non-activated macrophage, or M1-activated macrophages derived from C57BL/6 mice at 12, 24 and 48 hours post-infection. Mice were sacrificed at day 3 following MRSA infection, whereupon catheters (A) and tissues surrounding the catheters (B) were removed to quantitate bacterial burdens. Results are expressed as the number of cfu per ml for catheters or cfu per mg tissue, to correct for differences in tissue sampling size. Significant differences are denoted by asterisks (*, $p < 0.05$). Results are presented from individual animals combined from at least two independent experiments.

[0074] Figure 7. The ability of M1-polarized macrophages to impair MRSA biofilm development is mediated by MyD88-dependent signals. C57BL/6 mice were infected with 10^3 colony forming units (cfu) of methicillin-resistant *S. aureus* (MRSA) USA300 LAC in the lumen of surgically implanted catheters to establish biofilm infection. Animals were initially treated with 10^6 activated macrophages derived from C57BL/6 or MyD88 knockout

(KO) mice at 12, 24 and 48 hours post-infection. Mice were sacrificed at day 3 following MRSA infection, whereupon catheters (A) and tissue surrounding the catheters (B) were removed to quantitate bacterial burdens. Results are expressed as the number of cfu per ml for catheters or cfu per mg tissue, to correct for differences in tissue sampling size. Results are presented from individual animals combined from at least two independent experiments. Significant differences are denoted by asterisks (*, $p < 0.05$).

[0075] Figure 8. Activated macrophage therapy alters the local inflammatory milieu in MRSA biofilm associated tissue. Animals were sacrificed at day 3 post-infection (prophylactic) or day 10 (established) following methicillin-resistant *S. aureus* (MRSA) exposure. Tissues surrounding MRSA biofilms from vehicle- and M1-activated macrophage treated mice were homogenized to quantitate CXCL9 (A and D), CCL5 (B), IFN-γ (C), CXCL2 (E), IL-17 (F) and IL-6 (G) expression by MILLIPLEX analysis. Results were normalized to the amount of total protein recovered to correct for differences in tissue sampling size. Significant differences between groups are denoted by asterisks (*, $p < 0.05$).

[0076] Figure 9. M1-activated macrophages attenuate MRSA biofilm catheter persistence in an established infection. C57BL/6 mice were infected with 10^3 colony forming units (cfu) of methicillin-resistant *S. aureus* (MRSA) USA300 LAC in the lumen of surgically implanted catheters to establish biofilm infection. On days 7 and 9 post-infection, animals received vehicle control (PBS), 10^6 activated macrophages, or antibiotic combination (rifampicin 25 μg/ml + daptomycin 5 μg/ml). Catheters (A) and surrounding host tissues (B) were removed on day 10 to quantitate bacterial burdens. Significant differences between groups are denoted by asterisks (*, $p < 0.05$).

[0077] Figure 10. M1-activated macrophage therapy attenuates arginase-1 expression in biofilms. Vehicle- (A & C) or M1 macrophage-treated (B & D) animals from the established biofilm paradigm were sacrificed at day 10 following methicillin-resistant *S. aureus* (MRSA) exposure, whereupon tissues surrounding infected catheters were subjected to immunofluorescence staining with Iba-1 (red) to identify macrophages, arginase-1 (green), and nuclear staining with DAPI (blue). Asterisks represent the original location of the catheter, which is non-adherent to glass slides. (E & F) Quantitation of arginase-1 and Iba-1 fluorescence surrounding *S. aureus* biofilms of vehicle- or M1 macrophage-treated animals from the established biofilm paradigm. Significant differences are indicated with asterisks (*, $p < 0.05$; **, $p < 0.01$).

[0078] Figure 11. Qdot labeling of M1-activated macrophages. (A) Mice received one dose of 10^7 M1-activated macrophages at the time of *S. aureus* challenge or at day 7 following infection, representing prophylactic and established therapies, respectively. The same cohort of animals was subjected to IVIS imaging daily to visualize macrophage persistence (red dots). Results are representative of 10 individual animals per group. (B) $F4/80^+$ macrophages were recovered at day 5 post-infection, whereupon M1 (IRF-5) or M2 (arginase-1) markers were assessed by FACS to determine if exogenously introduced Qdot-labeled macrophages retained their M1-activated state.

[0079] Figure 12. The macrophage-activating peptide EP67 attenuates *S. aureus* biofilm growth *in vivo*. EP67- or vehicle-treated mice were infected with 10^3 CFU of MRSA USA300 LAC in the lumen of surgically implanted catheters to establish biofilm infection. Animals were sacrificed at days 3 (A & B) or 14 (C & D) following *S. aureus* infection, whereupon catheters (A and C) or tissues surrounding the catheters (B and D) were removed to quantitate bacterial burdens. Data are expressed as the number of CFU per ml, or CFU per mg host tissue, for normalization. Results are presented from individual animals pooled from at least two independent experiments with bars representing the mean of each group. Significant differences in bacterial burdens between vehicle and EP67-treated mice are denoted by asterisks (*, $p < 0.05$).

[0080] Figure 13. EP67 augments inflammatory mediator expression in tissues surrounding MRSA biofilm infections. Tissues surrounding MRSA biofilms from EP67- or vehicle-treated mice were homogenized at day 3 post-infection to quantitate the effects of EP67 treatment on IL-12p40 (A), CCL5 (B), IL-17 (C), IL-1α (D) and IFN-γ (E) expression by MILLIPLEX analysis. Results were normalized to the amount of total protein recovered to correct for differences in tissue sampling size. Results are presented from individual animals combined from two independent experiments. Significant differences between EP67- vs. vehicle-treated catheter-associated tissues are denoted by asterisks (*, $p < 0.05$).

[0081] Figure 14. EP67 augments macrophage infiltration into MRSA biofilms. (A & B) Mice were sacrificed at day 3 post-infection, whereupon tissues surrounding infected catheters were subjected to immunofluorescence staining with Iba-1 (red) to identify macrophages and nuclear staining with DAPI (blue); asterisks represent the original location of the catheter, which is non-adherent to glass slides. (C & D) Macrophage infiltrates in tissues surrounding infected catheters from vehicle- or EP67-treated animals were quantitated by FACS to identify macrophage ($F4/80^+$) infiltrates. Results are expressed as the percentage of cells after correction for isotype control staining (*, $p < 0.05$; ***, $p < 0.001$).

DETAILED DESCRIPTION

[0082] The disclosure provides compositions and methods that are useful in preventing the formation of biofilm infections and in treating existing biofilm infections. Disclosed herein are methods of administering cells such as macrophages, including previously activated macrophages, macrophages being activated simultaneously with administration, or macrophages activated *in vivo* post-administration, to prevent and/or treat biofilms. In one embodiment, peripheral blood monocytes are collected from a patient prior to surgery, such as one week prior to surgery. Macrophages are prepared from this sample and are activated using various activating agents, including but not limited to, peptidoglycan (*e.g.*, *S. aureus*-derived peptidoglycan) and interferon-γ. The activated macrophages are then administered to the patient at the site of implantation to prevent biofilm formation. In another embodiment, activated macrophages are administered to a patient who already has an established biofilm to treat the biofilm infection. It is envisioned that one or multiple doses of activated macrophages could be administered to treat an established biofilm. In addition, evidence documenting that activated macrophages reduce bacterial burdens on infected devices, supports embodiments where activated macrophages are used in combination with antibiotics. Another aspect of treating biofilms is interfering with the extensive fibrotic matrices that form, which represents a barrier to effective treatment. To overcome this barrier, it is envisioned that proteolytic enzymes or drugs are co-administered with activated macrophages. In another embodiment, one or more compounds or factors that are capable of activating macrophages *in vivo* is/are co-administered or administered pre- or post-administration of activated macrophages to further enhance macrophage activation.

[0083] The use of immune cell-based therapy using activated macrophages overcomes at least one of the aforementioned disadvantages of preventing or treating biofilms. The data disclosed herein demonstrate that macrophages are superior to chemicals, such as antibiotics, and to such physical processes as wound debridement, for effective clearance of biofilm-associated bacteria, such as biofilm-associated bacteria from infected medical devices, as well as the surrounding tissue. Importantly, macrophage therapy provides long-lasting protection. In one embodiment, only three activated macrophage cell treatments were required for sustained bacterial containment (out to two weeks post-infection). Multiple cell injections would be feasible from a clinical perspective, given the fact that patients undergoing orthopedic joint surgeries are hospitalized for a minimum of two-three days. Consequently, given the growing prevalence of nosicomial infections and biofilm development, the increased use of procedures that can give rise to biofilm development, the

growing elderly population who will be receiving artificial implants and who exhibit progressively compromised immune responses, and the difficulties of treating and/or controlling bacteria within a biofilm, increasing demands will be placed on the use of macrophage-based immune cell therapy as a way of treating biofilms in both young and old.

[0084] The following definitions will be helpful in understanding the detailed description of the disclosure.

[0085] "Biofilm" or "biofilm infection" refers to an organized community of bacteria contained within a matrix or "slime," typically composed of bacterial polysaccharides, DNA, and proteins, within which is found at least one species of infectious microbe (*e.g.*, bacterium) and which typically exhibits a complex heterogeneous composition of microbes. Biofilms may exist within the body on natural surfaces (*e.g.*, the lining of bones and the heart) or inanimate objects inserted for therapeutic treatment (*e.g.*, indwelling devices or artificial joints).

[0086] A "subject" is a human being or a veterinary animal, including but not limited to, a dog, a cat, a horse, a bovine (a cow or a bull), a sheep, a goat and a pig, as well as a zoo animal and a game animal.

[0087] "Subject-in-need" means a subject, such as a human patient or veterinary animal including but not limited to a dog, a cat, a horse, a bovine (a cow or a bull), a sheep, a goat and a pig, that is in need of prevention for a possible biofilm infection or is in need of treatment for an existing biofilm infection.

[0088] "Isolating," in the context of isolating a cell, is given its ordinary and accustomed meaning in the art of separating that cell from at least one other component with which the cell is normally associated in the body of a subject.

[0089] "Peripheral blood monocyte" is given its ordinary and accustomed meaning in the art of a white blood cell in peripheral blood that has a single nucleus and can take in (ingest or phagocytose) foreign material. A monocyte, like a macrophage, is a phagocyte. Under the proper conditions, a monocyte can differentiate into a macrophage.

[0090] "Culturing" is given its ordinary and accustomed meaning of incubating a cell in the presence of nutrients and under conditions permissive for continued viability and/or growth.

[0091] "Contacting" means removing any barrier to a coming together, a locating in the immediate vicinity of, or a touching, such as between a cell and a compound(s) such as an activating agent.

[0092] A "macrophage-activating agent" is one or a combination of compounds that induce an activated state or phenotype in a macrophage. Exemplary macrophage-activating agents include macrophage-activating peptides such as EP67 (SEQ ID NO:1), EP54 (SEQ ID NO:2), $C5a_{65-74}$ (SEQ ID NO:4), and analogs and variations thereof, interleukin-1β as well as combinations of compounds selected from the group consisting of interferon-γ and tumor necrosis factor (TNF), and interferon-γ and an inducer of TNF.

[0093] A "macrophage" is a phagocyte having a single nucleus that ingests or phagocytoses foreign material, such as infectious microbes, including infectious bacteria. Macrophages are derived from monocytes by differentiation.

[0094] An "activated macrophage" is a macrophage that exhibits an activated phenotype relative to a resting macrophage. The activated macrophage becomes larger, more motile, adherent, expresses more MHCII proteins on its surface, contains more lysosomes and lysosomal enzymes, and secretes a variety of substances including interleukin-1 and tumor necrosis factor, relative to a resting macrophage. Again relative to a resting macrophage, an activated macrophage has increased phagocytic activity and increased bacterial killing via reactive oxygen intermediates and lysosomal enzymes. Unless otherwise noted, an activated macrophage as used herein is an "M1-activated macrophage" or a "classically activated macrophage."

[0095] A "prophylactically effective amount" is an amount effective in prevention of a disease, disorder or condition and is determined on an individual basis with consideration given to well-known factors such as weight, gender, general health, and the like.

[0096] A "therapeutically effective amount" is an amount effective in treatment of a disease, disorder or condition and is determined on an individual basis with consideration given to well-known factors such as weight, gender, general health, and the like.

[0097] "At risk of developing a biofilm infection" means a subject exhibiting a greater risk of developing a biofilm infection than the average subject, such as a subject with a compromised immune system, a subject with a documented history of recurrent bacterial infections, or a subject harboring a surgical implant such as an artificial component of a joint (*e.g.*, hip, knee, ankle, shoulder, elbow, wrist) or an implanted pacemaker.

[0098] A "veterinary animal" means any animal conventionally receiving treatment from a veterinarian and having an immune system,, including but not limited to a dog, a cat, a horse, a bovine (a cow or a bull), a sheep, a goat a pig, a zoo animal, and a game animal.

[0099] An "immunomodulatory cytokine" is a cytokine exhibiting the capacity to modulate an immune response under at least one set of conditions. Exemplary cytokines are pro-inflammatory cytokines, such as interleukin-6, interleukin-12, monocyte chemoattractant protein-1 (MCP-1), monokine-induced by IFN-γ (MIG), interferon-regulatory protein-10 (IP-10), macrophage inflammatory protein-2 (MIP-2), and keratinocyte chemoattractant (KC). In addition, macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are contemplated as these molecules encourage macrophage and neutrophil expansion at the site of biofilm infection, whereupon pro-inflammatory cytokines would be present to activate these cells to trigger their bactericidal activity.

[0100] A "surgical implant" is an artificial implant or device placed within the body of a subject and includes, but is not limited to, an artificial joint component (an artificial hip component, an artificial knee component, an artificial ankle component, an artificial elbow component, an artificial shoulder component, and an artificial wrist component), a cranial plate (natural or artificial), a dental implant, and a subcutaneous pacemaker.

[0101] "Catheter" is given its ordinary and accustomed meaning of a hollow, typically flexible, tube for insertion into a body cavity, duct, or vessel to allow the passage of fluids or to distend a passageway.

[0102] A "proteolytic enzyme" is an enzyme that catalyzes the proteolysis of at least one protein, polypeptide or peptide, and includes, but is not limited to, MMP-1, MMP-2, MMP-7, MMP-9, MMP-12 and collagenase.

[0103] An "EP67 peptide" is a peptide functioning as a C5a receptor agonist and exhibits the amino acid sequence set forth in SEQ ID NO:1, or a variant or derivative of that sequence. An "EP54 peptide" is a peptide functioning as a C5a receptor agonist and exhibits the amino acid sequence set forth in SEQ ID NO:2, or a variant or derivative of that sequence. An "$C5a_{65-74}$" is a peptide functioning as a C5a receptor agonist and exhibits the amino acid sequence set forth in SEQ ID NO:4, or a variant or derivative of that sequence.

[0104] "Inflammatory disorder" is a disorder characterized by an inflammatory response causing redness, swelling, heat and/or pain. Inflammatory disorders include, but are not limited to, atherosclerosis, infectious complications of cystic fibrosis, endocarditis, chronic

sinusitis, nasal polyposis, chronic otitis media, leptospirosis, kidney stones, osteomyelitis, necrosis of the jaw, a urinary tract infection, a bladder infection, and a chronic wound.

[0105] Terms used in this disclosure that are not expressly defined herein are given their ordinary and accustomed meanings in the art.

[0106] With these terms defined, disclosure of the compositions and methods for preventing and treating biofilm infection will be straightforward. Methods for prophylactic immune-cell therapy comprise administering a prophylactically effective amount of an immune cell, such as an activated macrophage. In some embodiments, the method involves administering a therapeutically effective amount of autologous activated macrophages. These cells must be obtained and, in some embodiments, activated. For example, a blood sample is obtained from a patient prior to implant placement, *e.g.*, approximately one week prior to the medical device placement procedure. The blood is sent to a laboratory where immune stimulation would take place, which would allow for quality control and standardization. Once cells are propagated, they are delivered to the recipient for local treatment at the implant site, making the cells immediately available to respond to any potential bacterial contaminant introduced during the surgical procedure (most commonly via skin microflora).

[0107] Macrophage-based immune-cell therapy is not only effective in treating/controlling microbe, *e.g.*, bacterial, burdens associated with biofilms, it has the added advantage of doing so by utilizing the host's own innate immune cells. In relying on a subject's own innate immune cells, the methods of the disclosure eliminate or minimize selection pressures imposed directly on the microbe, *e.g.*, bacteria, and decrease the likelihood of developing resistant strains. In addition, minimal side effects would be expected because autologous cells that won't survive long-term (*i.e.*, no longer than 7-14 days) are being introduced.

[0108] Biofilms have been shown to skew the immune response to favor anti-inflammatory and pro-fibrotic pathways, which likely contributes to biofilm persistence. To overcome this immune deviation and provide a treatment strategy for biofilm infections, antimicrobial activity has been augmented through the local administration of classically activated M1 macrophages, optionally supplemented by administration of a macrophage-activating peptide such as EP67, EP54, $C5a_{65-74}$, or their analogs or variants, provided such peptides retain the capacity to invoke macrophage pro-inflammatory responses. Early administration of M1-activated macrophages limited biofilm formation and treatment of established biofilm infections with activated macrophages also significantly reduced catheter-associated biofilm

burdens compared to antibiotic treatment. Several pro-inflammatory mediators were significantly elevated in tissues surrounding biofilm infections following M1-activated macrophage treatment, which may account for the observed antimicrobial effects. Furthermore, a role for macrophage pro-inflammatory activity was demonstrated by showing that transfer of neutrophils or macrophages from MyD88 knockout mice had minimal impact on biofilm growth *in vivo*. Based on this evidence, a therapeutic strategy has been developed to prevent and/or treat biofilm infections, including *S. aureus* (*e.g.*, MRSA) device- (*e.g.*, implant-) or catheter-associated biofilm infections, by exploiting macrophage activation.

[0109] These macrophage-based approaches boost pro-inflammatory activity, which is useful in preventing biofilm infection and in treating existing biofilm infections. These approaches are expected to be useful when administered to patients undergoing orthopedic surgery or other device-related implant procedures to prevent nosocomial infections, particularly for individuals who are at high-risk for developing infectious complications. M1-activated macrophage therapy provides a new strategy for preventing and treating biofilms, optionally supplemented with macrophage-activating agents such as macrophage-activating peptides like EP67 (SEQ ID NO:1), EP54 (SEQ ID NO:2), or $C5a_{65-74}$ (SEQ ID NO:4), or their analogs or variants. In addition, the activated macrophage therapies disclosed herein are suitable for combination with antibiotics, particularly for surgical-implant patients who are unable or unwilling to undergo additional surgery to manage the infection and thereby maintain the implanted device. The significance of this approach is even more pronounced against the backdrop of the rapidly increasing elderly population, which grows progressively less immunoresponsive and will be the primary recipients of more permanent artificial implants, such as hips and knees.

[0110] *S. aureus* (*e.g.*, MRSA) is a frequent etiological agent of biofilm infections on indwelling devices and orthopedic implants. The therapeutic potential of activated macrophages is supported by results demonstrating that early treatment with pro-inflammatory M1-activated macrophages significantly limited *S. aureus* biofilm growth *in vivo* and provided long-term protection from biofilm colonization. Likewise, activated macrophages were also effective at reducing *S. aureus* burdens in established biofilms by 100-fold. Without wishing to be bound by theory, this reduction in device-associated bacterial burdens may result from dispersal of organisms from the biofilm which, in turn, would restore the metabolic activity of the bacteria. By extension, it is expected that M1-activated macrophages will display synergy with antibiotics to maximize biofilm clearance,

because these organisms are no longer foreign body-associated and, thus, more amenable to antibiotic action.

[0111] There are several advantages associated with the activated macrophage approach to preventing or treating biofilm infections. Monocytes can be easily collected from patient's blood by elutriation, expanded, induced to differentiate into mature macrophages, and activated *in vitro* for introduction into the surgical site for infection prophylaxis. A relatively wide range of activated macrophage dosages are suitable for use in the prophylactic and therapeutic methods and uses according to the disclosure. Exemplary macrophage dosages are 10^5, 10^6, 10^7, and 10^8 activated macrophages per dose. Further, the methods and uses of the disclosure are amenable to a variety of dosing schedules, which can readily be determined by those of skill in the art. By way of example, the activated macrophage can be delivered in one to five doses, over one to five days. For multiple administrations, it is contemplated that administered doses may be placed in a region approximately surrounding the expected or actual location of a potential or existing biofilm.

[0112] Modulating macrophage effector responses represents a promising therapeutic avenue for the prevention and/or treatment of device-associated biofilm infections. Although initial efforts have been focused on demonstrating the therapeutic methods on MRSA biofilms, additional advantages are that activated macrophages possess potent anti-microbial activity and are capable of eliminating a wide spectrum of infectious pathogens based on their immunological characteristics. Stated in the alternative, the disclosed methods rely on elements of the immune response for efficacy. Any infectious agent will be perceived as foreign by the host's immune system, including all microbes such as bacteria, including all infectious gram-positive and gram-negative bacteria. This is far superior to antibiotics, which are typically only active against specific bacterial classes and are ineffective at clearing biofilm infections. Also of significance is that bacteria are less likely to develop mutations to escape immune-directed therapies, because activated macrophages are a primary source of microbial killing in the immune system and most pathogens have not evolved resistance to the effects of the immune system.

[0113] The disclosure provided herein establishes a previously unappreciated role for M1-activated macrophages in biofilm containment and bacterial clearance. By extension, it is not unexpected that biofilms such as MRSA biofilms have the capacity to thwart this response by skewing macrophages away from a pro-inflammatory M1 to an anti-inflammatory M2 phenotype, which could ensure biofilm persistence in an immunocompetent host. The implementation of activated macrophage transfer therapy would allow macrophages to be "on

site" to deal with possible contamination of the implant from normal bacterial flora on the skin. While conventional antibiotics are ineffective in treating/controlling biofilms, they are commonly used to control bacteria that escape the biofilm matrix thereby preventing their colonization of other tissue sites. Such uses of antibiotics impose mutational pressures on the bacteria and increase the risk of selecting antibiotic-resistant strains. Macrophage-based immune cell therapy not only appears to be effective in treating/controlling microbial, *e.g.*, bacterial, burdens associated with biofilms, it has the added advantage of doing so by utilizing the hosts own innate immune cells, thus eliminating or minimizing mutational pressures imposed directly on the microbes, such as bacteria, and decreasing the likelihood of promoting the growth of resistant strains.

Organisms Associated with Biofilm Infections

[0114] Investigations over the past 20-30 years have revealed that many, if not most, infections of humans and veterinary animals, including pets and domesticated farm animals, are caused by organisms capable of forming biofilm infections. Accordingly, the compositions and methods of the disclosure are contemplated as suitable for preventing or treating biofilm infections caused by, or associated with, any known infectious organism, such as microbes, including gram-positive and gram-negative bacteria, fungi, and yeast. Exemplary infectious microbes causing or associated with biofilm infections include gram-positive bacteria such as *Staphylococcus aureus*, *Enterococcus faecalis* and *Enterococcus faecium* and gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Haemophilus influenza*,. Other exemplary microbes causing or associated with biofilm infections include Leptospira species such as *Leptospira interrogans*, and the yeast, *Candida albicans*. These exemplary organisms are representative of the hundreds and hundreds of microbes capable of causing or being associated with biofilm infections. For example, dental plaque is known to involve a biofilm infection associated with the teeth of humans and veterinary animals. Investigations have shown that biofilms associated with dental plaque contain over 500 species of microorganisms. The disclosure contemplates the prevention or treatment of biofilm infections caused by or associated with each of these organisms, and other microbes capable of causing or being associated with biofilm infection. The biofilm-associated microbes of dental plaque are provided to illustrate the number and diversity of microbes associated with biofilm infections amenable to prevention or treatment with the disclosed compositions and methods, even if the administration of cells such as activated macrophages to prevent or treat biofilm infections

associated with dental plaque is not contemplated as a preferred embodiment of the disclosed subject matter.

Diseases, Disorders, and Conditions Associated with Biofilm Infection

[0115] A variety of diseases, disorders and conditions are known to be associated with biofilm infection, and the compositions and methods disclosed herein are contemplated as suitable for preventing or treating any of these biofilm infections. Exemplary diseases associated with biofilm infection include, but are not limited to, atherosclerosis, infectious complications of cystic fibrosis, endocarditis, chronic sinusitis, nasal polyposis, chronic otitis media or middle ear infections, leptospirosis, kidney stones, osteomyelitis, necrosis of the jaw, urinary tract infections, and bladder infections. Exemplary disorders include chronic wounds, and exemplary conditions include subjects, *e.g.*, human patients, that have or are receiving a prosthetic device such as an artificial component of a joint (*e.g.*, a component of a hip, knee, ankle, elbow, shoulder, or wrist joint), or a temporary or permanent catheter (*e.g.*, urinary, intravenous or intraarterial catheter). With respect to prosthetic implants and catheters, moreover, it is known that typically non-pathogenic microbes, *e.g.*, benign members of the normal skin flora, can form biofilm infections in association with these artificial surfaces, and the prevention or treatment of such biofilm infections is also contemplated. In addition, infectious diseases of veterinary animals are associated with biofilm infection and are comprehended as suitable for prevention or treatment using the disclosed compositions and methods. Given the prevalence and wide variety of biofilm infections, the disclosed compositions and methods are contemplated as suitable for preventing or treating any known biofilm infection.

Activating Agents

[0116] Activating agents suitable for use in activating a macrophage include any compound, or combination of compounds, known to activate macrophages. For ease of discourse, compounds effective in activating macrophages only in combination with at least one other compound are referred to herein as activating agents. Exemplary activating agents include macrophage-activating peptides such as EP67 (SEQ ID NO:1), EP54 (SEQ ID NO:2), $C5a_{65-74}$ (SEQ ID NO:4) and their analogs and variants, interleukin-1β as well as combinations of compounds such as interferon-γ and tumor necrosis factor (TNF), interferon-γ and an inducer of TNF. Exemplary inducers of TNF are peptidoglycan and lipopolysaccharide. Yet other embodiments of the method further comprise delivering to the patient in need an effective amount of a pro-inflammatory compound, such as interleukin-6, interleukin-12, monocyte chemoattractant protein-1 (MCP-1), monokine-induced by IFN-γ

(MIG), interferon-regulatory protein-10 (IP-10), macrophage inflammatory protein-2 (MIP-2), and keratinocyte chemoattractant (KC). In addition, macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are contemplated as useful in the methods and uses according to the disclosure to encourage macrophage and neutrophil expansion at the site of biofilm infection, whereupon pro-inflammatory cytokines would be present to activate these cells to trigger their bactericidal activity. A cell, *e.g.*, a macrophage, is exposed to one or more activating agents *ex vivo* or *in vivo*, and prior to, concurrent with, or following administration of the cell to the subject, such as a human patient or veterinary animal.

Administered Cells

[0117] A cell to be administered to a subject, such as a human patient or veterinary animal, is an activated macrophage or a macrophage. Each of these cell types results in a prophylactically and/or therapeutically effective active macrophage *in vivo*, at the site or in the region where a biofilm infection is to be prevented and/or treated. Relative to the recipient human patient or veterinary animal, a syngeneic cell is utilized, such as an autologous cell. Autologous cells are administered in order to minimize complicating influences from the host immune system and cell rejection.

[0118] The following examples illustrate embodiments of the disclosure. Example 1 provides the materials and methods used in the experiments disclosed herein. Example 2 established that activated macrophages are bactericidal towards biofilm-associated *S. aureus in vitro*. Example 3 showed that biofilm infections alter the gene expression profile of administered macrophage. Example 4 demonstrated that activated macrophages prevented the formation of a biofilm infection (*i.e.*, MRSA infection) *in vivo*. Example 5 established that activated macrophages reduced the bacterial burden of biofilm infections, while establishing a pro-inflammatory microenvironment at the biofilm site. Example 6 showed that administering a C5aR agonist, *e.g.*, EP67, successfully prevented or inhibited formation of a biofilm infection, *i.e.*, a MRSA biofilm infection. Without wishing to be bound by theory, this effect is believed to be due to the C5aR agonist reprogramming endogenous macrophages from an M2- to a pro-inflammatory M1-activation state, which would complement or enhance activated-macrophage therapy. Example 7 established that activated macrophages eliminated or reduced the bacterial burden of a catheter-associated biofilm infection *in vitro* (*i.e.*, MRSA infection).

Antibiotics

[0119] The compositions, methods and uses according to the disclosure are generally drawn to macrophages that are or can be activated for use in preventing or treating biofilm infections. As noted herein, the macrophages can be combined with macrophage-activating agents, including macrophage-activating peptides such as EP67, EP54 and $C5a_{65-74}$. In addition, the macrophages can be combined, alone or with a macrophage-activating agent, with one or more antibiotics. Further, the macrophages, with or without a macrophage-activating agent, can be administered separately or jointly with one or more antibiotics in the prophylactic or therapeutic methods and uses according to the disclosure. Any known antibiotic is expected to be suitable for these methods and uses. Suitable classes of antibiotics include , but are not limited to, aminoglycosides, ansamycins, carbacephems, carbapenems, cephalosporins, glycopeptides, lincosamides, lipopeptides, macrolides, erythromycins, monobactams, nitrofurans, penicillins, penicillin combinations, antimicrobial polypeptides, quinolones, sulfonamides, tetracyclines and antimycobacterials.

[0120] In some embodiments, the antibiotic is selected from the group consisting of amikacin, gentamicin, kanamycin, neomycin, roxithromycin, netilmicin, tobramycin, paromomycin, geldamycin, herimycin, loracarbef, ertapenem, doripenem, imipenem or cilastatin, meropenem, cefadroxil, cefazolin, cefalotin or cefalothin, cefalexin, cefaclor, cefamandole, cefoxitin, cefprozil, cefuroxime, cefonicid, cefixime, cefdinir, cefditoren, cefotaxime, cefetamet, cefoperazone, cefpodoxime, ceftazidime, ceftibuten, ceftriaxone, cefepime, ceftaroline or fasamil, ceftobiprole, cephalothin, cefmetazole, cefotetan, ceftizoxime, cefsulodin, cephalexin, cinoxacin; teicoplanin, vancomycin, telavancin; clindamycin, lincomycin, daptomycin, azithromycin, clarithromycin, erythromycin, erythromycin estolate, erythromycin ethyl succinate, erythromycin glucoheptonate, erythromycin lactobionate, erythromycin stearate, roximthromycin, trolandomycin, telithromycin, spectinomycin, spriamycin, Aztreonam, furazolidone, nitrofurantoi, amoxicillin, ampicillin, azlocillin, carbenicillin, cloxacillin, dicloxacillin, flucloxacillin, mezlocillin, methicillin, nafcillin, oxacillin, penicillin G, penicillin V, piperacillin, ticarcillin, amoxicillin and clavulanate, ampicillin and sulbactam, piperaccillin and tazobactam, ticarcillin and clavulanate; bacitracin, colistin, polymyxin B, ciprofloxacin, enoxacin, gatifloxacin, levofloxacin, lomefloxacin, moxifloxicin, ofloxacin, nalidixic acid, norfloxacin, ofloxicin, trovafloxacin, grepafloxacin, sparfloxacin, temafloxacin, mafenide, sufonamidochrysoidine, sulfacetamide, sufadiazine, silver sulfadiazine, sulfamethizole, sulfamethoxazole, sulfanilamide, sulfasalazine, sulfisoxazole, trimethoprim-

sulfamethoxazole, demeclocycline, doxycycline, minocycline, oxytetracycline, tetracycline, clofazimine, dapsone, capreomycin, cycloserine, ethambutol, ethionamide, isoniazid, rifampicin or rifampin, rifabutin, rifapenine, streptomycin, arshenamine, chloramphenicol, fosfomycin, fusidic acid, linezolid, metronidazole, mupirocin, platensimycin, quinupristin or dalfopristin, rifaximin, thiamphenicol, tigercycline, tinidazole, trimethoprim, fleroxacin, nitrofurantoin, coamoxiclavuanate, and their salts, acids, and bases.

Example 1

Materials and Methods

[0121] ***Animals.*** Male C57BL/6 mice (6–8 weeks old) were obtained from Charles River Laboratories (Frederick, MD) and MyD88 KO animals were purchased from the Centre de La Recherche Scientifique and have been backcrossed with C57BL/6 mice for over 10 generations (36-38). Mice were housed in restricted-access rooms equipped with ventilated microisolator cages and maintained at 21°C under a 12-hour light:12-hour dark cycle with *ad libitum* access to water (Hydropac™; Lab Products, Seaford, DE) and Teklad rodent chow (Harlan, Indianapolis, IN).

[0122] ***S. aureus strains.*** For *in vitro* biofilms, the *S. aureus* MRSA strain USA300 LAC was used and, for confocal microscopy studies, was transformed with the plasmid pCM11 to express GFP driven by the sarA P1 promoter (USA300 LAC-GFP) and plasmid expression was maintained with erm selection (10 μg/ml) (15, 39). For *in vivo* biofilm infections, the bioluminescent MRSA USA300 LAC::*lux* strain was used as previously described (15, 40, 41).

[0123] ***In vitro S. aureus biofilms.*** Static biofilms were generated as previously described (15). Briefly, sterile two-well glass chamber slides (Nunc, Rochester, NY) were treated with 20% human plasma in sterile carbonate-bicarbonate buffer (Sigma-Aldrich, St. Louis, MO) overnight at 4°C to facilitate bacterial attachment (40). Overnight cultures of MRSA USA300 LAC or USA300LAC-GFP were prepared in macrophage medium, supplemented with 10 mg/ml erm for the GFP strain. The following day, plasma coating buffer was removed and chambers were inoculated with bacteria, diluted in mammalian cell culture medium (RPMI-1640 supplemented with 10% fetal bovine serum) to an OD600 of 0.050 in 2 ml, and incubated at 37°C under static aerobic conditions for a period of up to 6 days. Medium was carefully replenished every 24 hours to prevent disruption of the biofilm structure.

[0124] ***Macrophage or neutrophil co-culture with S. aureus biofilms in vitro.*** MRSA USA300 LAC-GFP static biofilms and bone marrow-derived macrophages (BMDMs) from either WT or MyD88 KO mice were prepared as previously described (42, 43). Neutrophils were recovered from the bone marrow utilizing a three-layer Percoll gradient of 78%, 69%, and 52% Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) diluted in PBS (100% Percoll = 9 parts Percoll and 1 part 10X PBS), and centrifuged (1500 g, 30 minutes, room temperature without braking) as previously described (44). Neutrophils were further purified using a Miltenyi Anti-Ly6G MicroBead Kit (Miltenyi Biotec, Cambridge, MA) according to the manufacturer's instructions. Briefly, 50 µl Anti-Ly-6G-Biotin microbeads were added to 10^8 total bone marrow cells in 200 µl MACs buffer, and samples were vortexed and incubated for 10 minutes at 4°C. Subsequently, 150 µl MACs buffer and 100 µl of anti-biotin MicroBeads per 10^8 total cells were added and cells were incubated for 15 minutes at 4°C. After washing, labeled cells were then positively selected by magnetic separation using MS MACs columns and a MACs separator (Miltenyi Biotec). The column was washed three times with 500 µl of MACs buffer. After the column was removed from the MACs separator, the positive fraction containing Ly-6G neutrophils was eluted and collected in 1 ml of MACs buffer. BMDM and neutrophils were labeled with either 5 µM CellTracker Orange (CTO) or CellTracker Blue (CTB) (both from Molecular Probes, San Diego, CA) depending on the experimental setup. Co-cultures of mature *S. aureus* biofilms (six-day-old biofilms) with 10^7 neutrophils, non-activated macrophages, or activated macrophages (pre-treated with 10 ng/ml IFN-γ and 10 µg/ml *S. aureus*-derived peptidoglycan (*i.e.*, PGN) for 6 hours) were established at 37°C under static aerobic conditions and imaged by confocal microscopy using a Zeiss 510 META laser scanning microscope (Carl Zeiss, Oberkochen, Germany) at 2 and 24 hours following the addition of immune cells. Neutrophil or macrophage-biofilm co-cultures were harvested at 24 hours after immune cell addition by mechanical dissociation followed by sonication, whereupon bacterial enumeration was performed by serial dilution on TSA plates supplemented with 5% sheep blood (Hemostat Laboratories, Dixon, CA).

[0125] To determine the effect of biofilm exposure on macrophage activation, as measured by cytokine secretion, a separate set of experiments was performed. Co-cultures of mature *S. aureus* biofilms with 10^7 non-activated CTB-labeled macrophages were established at 37°C under static aerobic conditions for 2 hours following the addition of immune cells, as described above. To separate macrophages from the biofilm, FACs purification was performed using a BD FACSAria (BD Biosciences, San Diego, CA) after mechanical dissociation of the macrophage biofilm co-culture. Macrophages were distinguished from

bacteria based on their size and CTB staining. Following macrophage recovery by FACS, 200,000 cells/well were plated in a 96-well polystyrene microtiter plate (BD Biosciences) and incubated for 24 hours at 37°C with 5% CO_2. After 24 hours, supernatants were collected to compare the production of inflammatory mediators associated with biofilm– versus planktonic–exposed macrophages utilizing sandwich ELISA kits to quantitate TNF-α, IL-10 (OptiEIA; BD Biosciences, Franklin Lakes, NJ), and IL-1RA (DuoSet; R & D Systems, Minneapolis, MN) per the manufacturers' protocols.

[0126] ***Mouse model of S. aureus catheter-associated biofilm infection.*** *S. aureus* biofilm infections were performed as previously described (15, 40, 41). Briefly, a small s.c. incision was made in the mouse flank under avertin anesthesia and a blunt probe was used to create a pocket for insertion of a sterile 14-gauge teflon intravenous catheter 1 cm in length (Exel International, St Petersburg, FL). The incision was sealed using Vetbond Tissue Adhesive (3M, St. Paul, MN) and 10^3 cfu MRSA USA300 LAC::*lux* in 20 μl of sterile PBS was slowly injected through the skin into the catheter lumen to initiate infection. The health status of each mouse was regularly monitored throughout the course of infection and any moribund animals were immediately euthanized.

[0127] ***Immune cell administration into biofilm infections in vivo.*** Mice received injections of 10^6 neutrophils or activated WT or MyD88 KO macrophages (10 ng/ml IFN-γ + 10 μg/ml *S. aureus*–derived PGN) beginning at 12 hours, with repeat administration at 24 and 48 hours post-infection. Cells were introduced at four distinct sites surrounding infected catheters to ensure maximal immune cell access to the developing biofilm. A separate cohort of animals received activated macrophages or vehicle until 48-hours post-infection, whereupon no additional cells were administered to determine the longevity of early macrophage treatment on inhibiting biofilm growth until day 14 after infection. In some experiments, the numbers of neutrophils injected was increased by 1-log (*i.e.*, to 10^7) to confirm that these cells did not exhibit any beneficial effects on biofilm clearance. For established biofilm experiments, mice were infected with 10^3 colony forming units (cfu) of MRSA USA300 LAC in the lumen of surgically implanted catheters to establish biofilm infection and on days 7 and 9 post-infection, animals received vehicle control (PBS), 10^6 activated macrophages, or antibiotic treatment consisting of rifampicin (25 μg/ml) and daptomycin (5 μg/ml). In a separate experiment, animals received vehicle or 10^7 activated macrophages loaded with Quantum dots (Molecular Probe) at 24 hours post-infection for the prophylactic paradigm or at 7 days post-infection for established biofilm paradigms. The longevity of the administered macrophages was monitored daily in the same cohort of mice

using an *In vivo* Imaging System (IVIS Spectrum; Caliper Life Sciences, Hopkinton, MA) under isoflurane anesthesia until 5 days after administration, at which point animals were sacrificed to determine absolute bacterial burdens associated with catheters and surrounding tissues. In some experiments, antibiotic treatment, consisting of rifampicin (25 μg/ml) and daptomycin (5 μg/ml) given twice daily starting 12 hours post-infection and continuing until 48 hours post-infection, was used to compare experimental treatments with the standard of care typically administered in the clinic, which does not exhibit any beneficial effects on biofilm clearance.

[0128] ***EP67 Peptide-Activated Macrophage Combined Treatment.*** To demonstrate that macrophage-activating agents, such as macrophage-activating peptides, were suitable for combination with activated macrophage therapy in preventing or treating biofilm infection, the effect of administering a macrophage-activating peptide alone was assessed. Animals were initially treated locally at the site of *S. aureus* infection with EP67 or a biologically inactive scrambled derivative (sEP67) having the sequence set forth in SEQ ID NO:3. 200 μg of either peptide was injected into the catheter at the time of infection (time 0) followed by 800 μg peptide divided into four different injection sites surrounding the catheter 24 and 48 hours post-infection.

[0129] ***Recovery of Catheter and Associated Tissues for S.aureus enumeration.*** At day 3 or 14 following infection, animals were sacrificed and catheters removed and placed in 1 ml of PBS for sonication to dissociate bacteria from the catheter surface. Tissues surrounding infected catheters were also collected, weighed, minced, and disrupted in 500 μl homogenization buffer [PBS supplemented with 100 μl RNasin and a protease inhibitor tablet (Roche Diagnostics, Indianapolis, IN)] using a Bullet Blender (Next Advance Inc., Averill Park, NY). Bacterial titers associated with catheters and surrounding tissues were enumerated using TSA plates supplemented with 5% sheep blood and were expressed as Log_{10} cfu/ml for catheters or Log_{10} cfu/g wet tissue weight.

[0130] ***MILLIPLEX multi-analyte bead array.*** To evaluate the ability of M1-activated macrophages to alter the inflammatory milieu associated with MRSA biofilm infections, a custom-designed mouse microbead array was utilized according to the manufacturer's instructions (MILLIPLEX; Millipore, Billerica, MA), which detects the following inflammatory mediators: IL-1α, IL-1β, TNF-α, IFN-γ, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-15, IL-17, CXCL1, CXCL2, CXCL9, CXCL10, CCL2, CCL3, CCL4, and CCL5. Results were analyzed using a Bio-Plex workstation (Bio-Rad, Hercules, CA) and normalized to the amount of total protein recovered to correct for differences in tissue sampling size.

[0131] ***Flow cytometry.*** Tissues surrounding biofilm-infected catheters were collected and processed for flow cytometry as previously described (15). Cells were stained with the following antibodies: F4/80-PE-Cy7 and Ly6G-AF700 (all from BD Biosciences). Macrophages were identified as $F4/80^+$ and $Ly6G^-$, while neutrophils were identified as $F4/80^-$ and $Ly6G^+$.

[0132] ***Immunofluorescence staining and confocal microscopy.*** Tissues surrounding infected catheters were fixed in 10% formalin and embedded in paraffin, whereupon 10 μm thick sections were deparaffinized in xylene and a graded series of alcohols followed by antigen retrieval, as previously described (15). Sections were processed for immunofluorescence staining using primary antibodies specific for Iba-1 (macrophage-specific marker; Biocare Medical, Concord, CA) and arginase-1 (Santa Cruz Biotechnology, San Diego, CA), followed by donkey anti-rabbit-FITC or -biotinylated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA), and a streptavidin-594 conjugate for the latter (Invitrogen, Carlsbad, CA). Confocal imaging was performed using a Zeiss 510 META laser scanning microscope with staining specificity confirmed by tissues incubated with secondary antibodies only. Quantitation of arginase-1 or Iba-1 fluorescence was calculated from at least 10 random fields of view using AxioVision software 4.8 (Carl Zeiss, Oberkochen, Germany).

[0133] ***Statistical Analysis.*** Significant differences between experimental groups were determined using an unpaired two-tailed student's *t*-test in GraphPad Prism 4 (La Jolla, CA). For all analyses, a *p*-value of less than 0.05 was considered statistically significant.

[0134] ***Ethics Statement.*** This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center (Approval ID: 09-049-00-EP). All surgery was performed under avertin anesthesia, and every effort was made to minimize suffering.

Example 2

[0135] ***Activated macrophages exhibit S. aureus biofilm bactericidal activity in vitro.*** MRSA biofilms are capable of attenuating traditional pro-inflammatory responses, explaining, in part, why these infections persist in an immunocompetent host (15). To determine whether macrophages that were preprogrammed towards a pro-inflammatory M1 phenotype were capable of overcoming the immune inhibitory biofilm environment,

macrophages were stimulated with *S. aureus*-derived peptidoglycan (PGN) and interferon-gamma (IFN-γ) for 6 hours prior to their addition to MRSA biofilms or planktonic cultures as a control. Importantly, activated macrophages were capable of phagocytosing biofilm-associated bacteria, which resulted in significant reductions in bacterial burdens following a 24 hour co-culture period (Fig. 4B). The ability of activated macrophages to phagocytose biofilm-associated bacteria and reduce bacterial burdens required MyD88-dependent signals, because MyD88 KO macrophages treated with PGN + IFN-γ had no impact on biofilm growth (Fig. 4B).

[0136] To compare the efficacy of macrophages versus neutrophils in regulating MRSA biofilm formation, neutrophils were isolated from murine bone marrow and co-cultured with biofilms. Unlike macrophages, neutrophils were not pre-activated prior to biofilm addition, because this would lead to rapid degranulation and reduced cell viability. Interestingly, neutrophils were able to phagocytose MRSA biofilms, yet this did not translate into reduced bacterial numbers (Fig. 4B), revealing disconnect between the two processes. This may result from additional virulence determinants released by *S. aureus* during biofilm growth, because the organism is known to produce numerous factors that interfere with neutrophil function/survival (20-22). On a comparative basis, biofilm formation did afford some protection against phagocytic uptake compared to planktonic growth conditions, because both macrophages and neutrophils actively phagocytosed planktonic *S. aureus* but were less capable of internalizing biofilm-associated bacteria (Fig. 4A).

Example 3

[0137] ***Biofilms alter macrophage activation profiles.*** *S. aureus* biofilms skew macrophage gene expression towards an alternatively activated (M2) phenotype compared to planktonic bacteria (15, 23). To determine whether biofilms influence macrophage cytokine secretion profiles, macrophages were exposed to *S. aureus* biofilms or planktonic bacteria for 2 hours, separated from bacteria by FACS and incubated at 37°C for 24 hours, at which point supernatants were collected to determine cytokine secretion profiles. Macrophages co-cultured with biofilms demonstrated a decreased cytokine secretion profile with reductions in TNF-α, IL-10 and IL-1RA expression compared to macrophages exposed to planktonic bacteria (Fig. 5). These results further establish that biofilm growth dampens the classic M1 macrophage response (24).

Example 4

[0138] ***Introduction of activated macrophages prevents* MRSA *biofilm formation in vivo.*** Based on the *in vitro* studies demonstrating the ability of M1-polarized macrophages to phagocytose biofilm-associated *S. aureus* and reduce bacterial burdens, experiments to determine whether this would translate *in vivo* to overcome the immune attenuation observed during biofilm growth were designed and undertaken. These experiments utilized a mouse model of MRSA catheter-associated biofilm infection that limits macrophage invasion into biofilms and skews cells towards an alternatively activated M2 phenotype (15). Initially employed was a paradigm where activated cells were administered beginning at 12 hours following MRSA infection, with repeat injections occurring at 24 and 48 hours after bacterial exposure. Importantly, the introduction of activated macrophages directly into the biofilm infection site resulted in significant reductions in bacterial burdens on both infected catheters and in surrounding tissues at day 3 post-infection (Fig. 6A & B). More importantly, this early intervention with activated macrophages led to long-term inhibition of biofilm formation, since catheters and surrounding tissues showed little/no evidence of biofilm growth at day 14 without any additional macrophage treatment (Fig. 7). Similar to the *in vitro* studies, MyD88-dependent mechanisms played an important role in M1 macrophage-mediated biofilm clearance, because macrophages from MyD88 KO mice did not demonstrate any efficacy in controlling biofilm burdens on either infected catheters or surrounding tissues (Fig. 8). In addition, neutrophils had no impact on biofilm formation when injected at 10^6 cells per administration (Fig. 6), and even when the number of injected cells was increased to 10^7 per administration, which confirmed the *in vitro* findings and the fact that neutrophils do not significantly infiltrate catheter-associated MRSA biofilms.

[0139] MRSA biofilms attenuated the expression of numerous pro-inflammatory mediators compared to a sterile foreign body (15, 23). The introduction of M1-activated macrophages led to increases in CXCL9, CCL5, and IFN-γ expression within biofilm-infected tissues (Fig. 9), revealing successful re-direction of the macrophages towards a pro-inflammatory milieu. The pro-inflammatory activity of activated macrophages is expected to play an important role in limiting biofilm growth.

Example 5

[0140] ***Introduction of M1-activated macrophages for the treatment of established MRSA biofilm infections.*** Based on the efficacy of the early M1 macrophage treatment paradigm, it was next determined whether this would extend to attenuate bacterial growth in established MRSA biofilm infections. A strategy similar to the early treatment regimen for macrophage administration was employed, except that activated cells were initially given at

day 7 following *S. aureus* infection, with a repeat injection occurring at day 9. Similar to the early treatment paradigm, the introduction of activated macrophages directly into the biofilm infection site led to significant reductions in bacterial burdens on infected catheters at day 10 post-infection, although no effect was seen in surrounding tissues (Fig. 10A and B, respectively). In contrast to macrophage delivery, antibiotic treatment had no effect on biofilm formation (Fig. 10). Interestingly, M1-activated macrophage administration attenuated arginase-1 expression within the surrounding tissue, indicating that macrophages were able to modify the local tissue environment to favor a pro-inflammatory response (Fig. 11). Likewise, the introduction of M1-activated macrophages into established biofilms augmented CXCL9, CXCL2, IL-17, and IL-6 expression within established biofilm-infected tissues (Fig. 9D-G), again revealing the successful re-direction of macrophages towards a pro-inflammatory milieu. To establish the longevity of M1-activated macrophages after injection into the site of biofilm infection, 10^7 M1-activated macrophages were labeled with near-infrared Quantum Dots (QDots) and injected at either the time of infection or on day 7 in the early- and established-biofilm models, respectively. Animals were subjected to IVIS imaging immediately following macrophage transfer to confirm macrophage signals immediately following injection, and subsequently reimaged every 24 hours (Fig. 12A). Qdot-labeled M1 macrophages were still visible at 5 days post-treatment, at which point animals were sacrificed in order to recover tissues for FACs analysis. Data indicated a slight increase in $F4/80^+/IRF5^+$ macrophages in Qdot-labeled M1 macrophage-treated animals compared to vehicle-treated animals (Fig. 12B).

Example 6

[0141] ***EP67 impacts* MRSA *biofilm establishment in vivo and stimulates local pro-inflammatory responses.*** A supplementary approach to the introduction of exogenous M1-activated macrophages is the combined administration of M1-activated macrophages and a macrophage-activating compound, such as a macrophage-activating peptide like EP67 (SEQ ID NO:1), EP54 (SEQ ID NO:2), or $C5a_{65-74}$ (SEQ ID NO:4) or their analogs or variants as described herein. To establish that a macrophage-activating compound could have a beneficial effect, an experiment was designed to determine whether the administration of the C5aR agonist EP67 would re-program the endogenous macrophage infiltrate associated with MRSA biofilms *in vivo* from an anti-inflammatory M2 to a pro-inflammatory M1 phenotype to facilitate bacterial clearance. The *in vivo* efficacy of EP67 against MRSA was examined using a mouse model of catheter-associated biofilm infection, as previously described (17, 22, 23). Animals were initially treated with EP67 at the time of infection followed by a series

of injections at 24 and 48 hours, two at sites adjacent to the external catheter wall and two into each open end of the catheter. Bacterial burdens associated with biofilm-infected catheters as well as surrounding tissues were significantly decreased following EP67 treatment compared to animals receiving an inactive scrambled sequence of EP67 (sEP67) or vehicle control (Fig. 13). Importantly, early peptide treatment was key to preventing MRSA biofilm establishment, since minimal bacterial growth was detected at day 14 following infection, even though the last dosing interval of EP67 occurred at 48 hours (Fig. 13C and D).

[0142] To determine whether EP67 could restore the biofilm environment to a pro-inflammatory state, cytokine and chemokine expression were evaluated in biofilm-infected tissues. Several inflammatory mediators that are predominantly expressed by activated macrophages, such as IL-12p40 and RANTES, were significantly increased in EP67-treated animals compared to vehicle-treated animals (Fig. 14A and B). Similarly, IL-17, IL-1α, and IFN-γ may also have been elevated, although the observed increases did not reach statistical significance (Fig. 14C, D and E). To further investigate mechanisms of EP67 immune activation during biofilm infections, the degree of macrophage influx into tissues surrounding MRSA biofilms was examined by immunofluorescence staining and flow cytometry. Immunofluorescence staining revealed that macrophage accumulation into EP67-treated biofilms was significantly increased at day 3 post-infection compared to vehicle-treated animals (Fig. 15A and B). Importantly, while only a few macrophages were recruited to the biofilm surface in vehicle-treated mice, EP67 administration dramatically increased the numbers of macrophages that migrated into the biofilm (Fig. 15A). The ability of EP67 to augment macrophage infiltrates in MRSA biofilms was confirmed by FACS (Fig. 15C and D). Collectively, these findings demonstrate that EP67 induces a pro-inflammatory milieu by augmenting macrophage recruitment and cytokine/chemokine production, which effectively counteracts the anti-inflammatory environment elicited by MRSA biofilms. Experiments were also designed and conducted to determine whether EP67 treatment could impact established biofilms; however, the peptide did not exert any beneficial effects in this setting, suggesting its optimal use as a prophylactic modality.

Example 7

Activated Macrophage Effect on Biofilms

[0143] The local injection of macrophages pre-activated *in vitro* with *S. aureus*-derived PGN and IFN-γ into the area surrounding a catheter-associated *S. aureus* biofilm infection eliminated/reduced bacterial burden within the biofilm as well as the healthy tissue in the vicinity of the biofilm. This was demonstrated by the following experiment.

[0144] Briefly, an *S. aureus* biofilm (USA 300 LAC strain) was established on a hollow catheter (1 cm in length) inserted subcutaneously into C57BL/6 mice by introducing 1,000 colony forming units (CFUs) of *S. aureus* into the catheter lumen. At 12, 24 and 48 hours following infection, 10^6 macrophages were introduced directly into the catheter lumen. Samples of the biofilm and surrounding tissue were obtained at days 3 and 14 post-infection to quantitate the number of viable bacteria by standard culture methods. Mice treated with activated macrophages exhibited a significant decrease in bacterial burdens within the biofilm and surrounding tissue relative to mice treated with neutrophils or PBS vehicle.

[0145] Collectively, the studies disclosed in Examples 1-7 have established that M1-activated macrophages are effective in biofilm containment and bacterial clearance. Additionally, data disclosed herein supports the position that M1-activated macrophages and macrophage-activating compound such as a macrophage-activating peptide as disclosed herein, when administered in combination, are effective in the prevention and treatment of biofilms. By extension, it is not unexpected that biofilms, including MRSA biofilms, have the capacity to thwart this response by skewing macrophages away from a pro-inflammatory M1 phenotype to an anti-inflammatory M2 phenotype to ensure biofilm persistence in an immunocompetent host.

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[0190] Each of the references cited herein is incorporated by reference in its entirety or as relevant apparent from the context of its usage.

[0191] From the disclosure herein it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention.

CLAIMS

What is claimed is:

1. A composition comprising an isolated activated macrophage for the prevention or treatment of a biofilm.

2. A kit comprising an isolated macrophage and a macrophage-activating agent.

3. A kit comprising a macrophage-activating agent.

4. The kit according to claim 2 or 3 wherein the macrophage-activating agent is selected from the group consisting of a C5a receptor agonist, interleukin-1β, interferon-γ and tumor necrosis factor (TNF), and interferon-γ and an inducer of TNF.

5. A method of providing biofilm therapy to a subject-in-need comprising:

(a) isolating peripheral blood monocytes from a subject;

(b) culturing the monocytes under conditions compatible with formation of macrophages; and

(c) administering a prophylactically or therapeutically effective amount of the macrophages to a subject-in-need, wherein the macrophages are activated by contact with a macrophage-activating agent prior to, simultaneously with, or after administration of the macrophages.

6. The method according to claim 5 wherein the macrophages are activated by contact with a macrophage-activating agent prior to administration of the macrophages.

7. A use of macrophages in the prevention or treatment of a biofilm infection in a subject-in-need, wherein the macrophages are activated by contact with a macrophage-activating agent prior to, simultaneously with, or after administration of the macrophages.

8. The method according to claim 5 or 6 wherein the subject-in-need is at risk of developing a biofilm infection.

9. The method according to claim 5 or 6 wherein the subject-in-need comprises an established biofilm infection.

10. The method according to claim 5 or 6 wherein the subject-in-need is a human patient.

11. The method according to claim 5 or 6 wherein the subject-in-need is a veterinary animal selected from the group consisting of a dog, a cat, a horse, a bovine, a sheep, a goat and a pig.

12. The method according to claim 5 or 6 wherein the subject is the subject-in-need.

13. The method according to claim 5 or 6 wherein the macrophage-activating agent is selected from the group consisting of a C5a receptor agonist, interleukin-1β, interferon-γ and tumor necrosis factor (TNF), and interferon-γ and an inducer of TNF.

14. The method according to claim 13 wherein the inducer of TNF is selected from the group consisting of peptidoglycan and lipopolysaccharide.

15. The method according to claim 5 or 6 wherein the macrophages are administered at a dose of 10^5 to 10^8 cells.

16. The method according to claim 15 wherein one to five doses of macrophages are administered.

17. The method according to claim 13 further comprising delivering to the subject-in-need an effective amount of a pro-inflammatory compound selected from the group consisting of interleukin-6, interleukin-12, monocyte chemoattractant protein-1 (MCP-1), monokine-induced by IFN-γ (MIG), interferon-regulatory protein-10 (IP-10), macrophage inflammatory protein-2 (MIP-2), keratinocyte chemoattractant (KC), macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF).

18. The method according to claim 5 or 6 wherein the subject-in-need undergoes surgery to receive a surgical implant.

19. The method according to claim 18 wherein the surgical implant is selected from the group consisting of an artificial joint component, a cranial plate, a dental implant and a subcutaneous pacemaker.

20. The method according to claim 19 wherein the artificial joint component is selected from the group consisting of an artificial hip component, an artificial knee component, an artificial ankle component, an artificial elbow component, an artificial shoulder component, and an artificial wrist component.

21. The method according to claim 5 or 6 wherein the subject-in-need is scheduled to receive, or has received, a catheter.

22. The method according to claim 5 wherein the macrophage-activating agent and the macrophage are co-administered to the subject-in-need.

23. The method according to claim 5 or 6 further comprising delivery of a proteolytic enzyme to the subject-in-need.

24. The method according to claim 23 wherein the proteolytic enzyme is selected from the group consisting of MMP-1, MMP-2, MMP-7, MMP-9, MMP-12 and collagenase.

25. The method according to claim 5, 6, or 24 further comprising a delivery vehicle selected from the group consisting of a microsphere, a microparticle, a nanosphere, a nanoparticle, a liposome, a micelle and a gel, wherein the delivery vehicle comprises a macrophage and a macrophage-activating agent, or an activated macrophage, or a macrophage, a macrophage-activating agent and a proteolytic enzyme, or an activated macrophage and a proteolytic enzyme.

26. The method according to claim 5 or 6 wherein the subject-in-need exhibits an inflammatory disorder.

27. The method according to claim 5 or 6 wherein the subject-in-need exhibits a disorder or condition selected from the group consisting of atherosclerosis, infectious complications of cystic fibrosis, endocarditis, chronic sinusitis, nasal polyposis, chronic otitis media, leptospirosis, kidney stones, osteomyelitis, necrosis of the jaw, a urinary tract infection, a bladder infection, a chronic wound, an artificial component of a joint, a temporary catheter and a permanent catheter.

28. The method according to claim 5 or 6 further comprising administering a prophylactically or therapeutically effective amount of an antibiotic.

29. The method according to claim 28 wherein the antibiotic is a member of a class of antibiotics selected from the group consisting of aminoglycosides, ansamycins, carbacephems, carbapenems, cephalosporins, glycopeptides, lincosamides, lipopeptides, macrolides, erythromycins, monobactams, nitrofurans, penicillins, penicillin combinations, antimicrobial polypeptides, quinolones, sulfonamides, tetracyclines and antimycobacterials.

30. The method according to claim 29 wherein the antibiotic is selected from the group consisting of amikacin, gentamicin, kanamycin, neomycin, roxithromycin, netilmicin, tobramycin, paromomycin, geldamycin, herimycin, loracarbef, ertapenem, doripenem, imipenem or cilastatin, meropenem, cefadroxil, cefazolin, cefalotin or cefalothin, cefalexin, cefaclor, cefamandole, cefoxitin, cefprozil, cefuroxime, cefonicid, cefixime, cefdinir, cefditoren, cefotaxime, cefetamet, cefoperazone, cefpodoxime, ceftazidime, ceftibuten, ceftriaxone, cefepime, ceftaroline or fasamil, ceftobiprole, cephalothin, cefmetazole, cefotetan, ceftizoxime, cefsulodin, cephalexin, cinoxacin; teicoplanin, vancomycin, telavancin; clindamycin, lincomycin, daptomycin, azithromycin, clarithromycin, erythromycin, erythromycin estolate, erythromycin ethyl succinate, erythromycin glucoheptonate, erythromycin lactobionate, erythromycin stearate, roximthromycin, trolandomycin, telithromycin, spectinomycin, spriamycin, Aztreonam, furazolidone, nitrofurantoi, amoxicillin, ampicillin, azlocillin, carbenicillin, cloxacillin, dicloxacillin, flucloxacillin, mezlocillin, methicillin, nafcillin, oxacillin, penicillin G, penicillin V, piperacillin, ticarcillin, amoxicillin and clavulanate, ampicillin and sulbactam, piperaccillin and tazobactam, ticarcillin and clavulanate; bacitracin, colistin, polymyxin B, ciprofloxacin, enoxacin, gatifloxacin, levofloxacin, lomefloxacin, moxifloxicin, ofloxacin, nalidixic acid, norfloxacin, ofloxicin, trovafloxacin, grepafloxacin, sparfloxacin, temafloxacin, mafenide, sufonamidochrysoidine, sulfacetamide, sufadiazine, silver sulfadiazine, sulfamethizole, sulfamethoxazole, sulfanilamide, sulfasalazine, sulfisoxazole, trimethoprim-sulfamethoxazole, demeclocycline, doxycycline, minocycline, oxytetracycline, tetracycline, clofazimine, dapsone, capreomycin, cycloserine, ethambutol, ethionamide, isoniazid, rifampicin or rifampin, rifabutin, rifapenine, streptomycin, arshenamine, chloramphenicol, fosfomycin, fusidic acid, linezolid, metronidazole, mupirocin, platensimycin, quinupristin or dalfopristin, rifaximin, thiamphenicol, tigercycline, tinidazole, trimethoprim, fleroxacin, nitrofurantoin, coamoxiclavuanate, and their salts, acids, and bases.

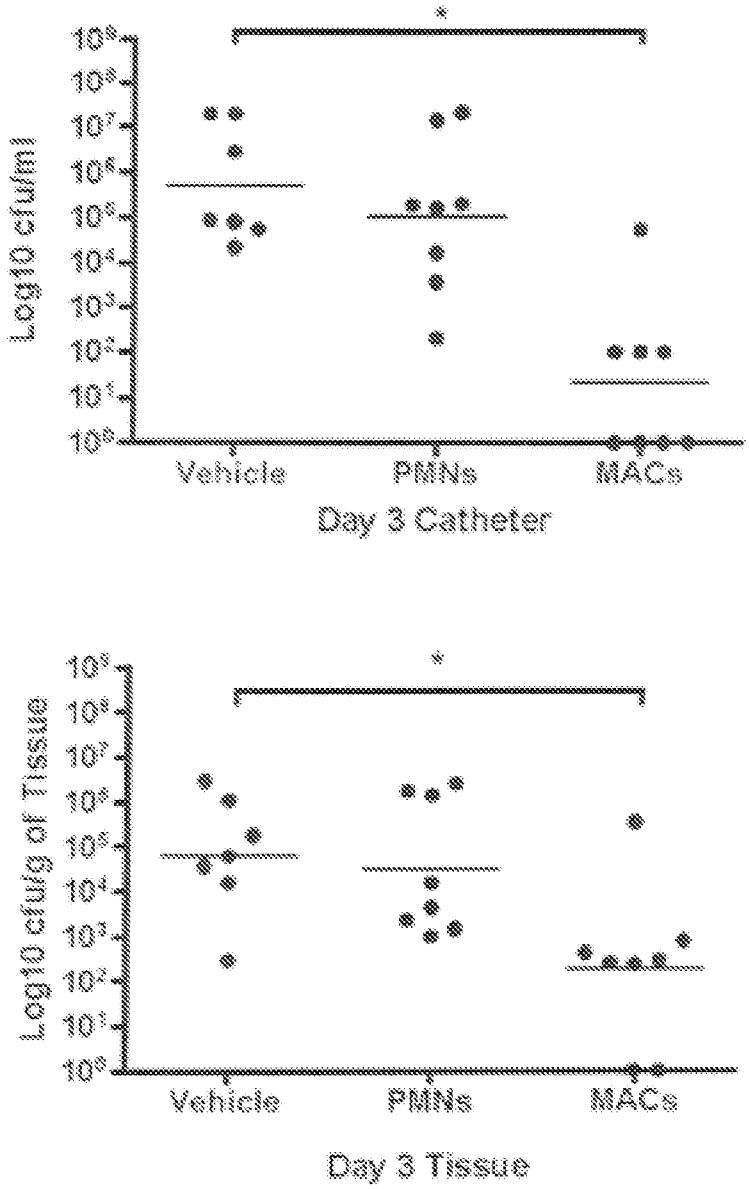

*
Log10 cfu/ml
Vehicle
PMNs
MACs
Day 3 Catheter
*
Log10 cfu/g of Tissue
Vehicle
PMNs
MACs
Day 3 Tissue

Figure 1

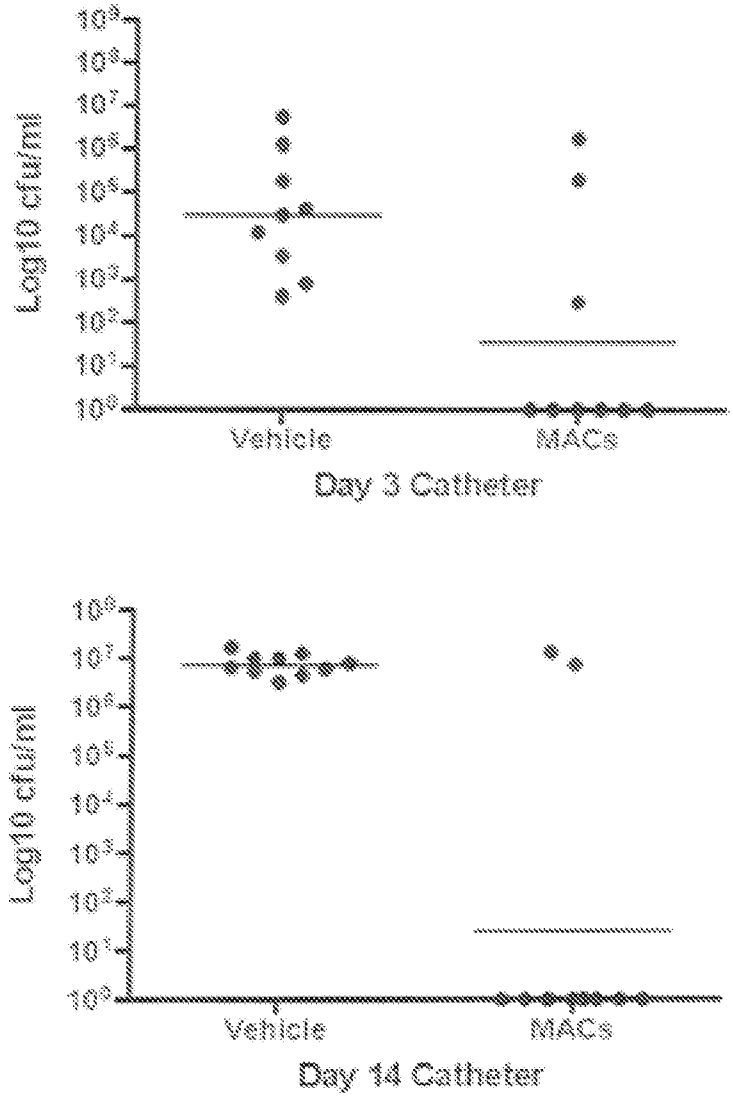

Log10 cfu/ml
Vehicle
MACs
Day 3 Catheter
Log10 cfu/ml
Vehicle
MACs
Day 14 Catheter

Figure 2

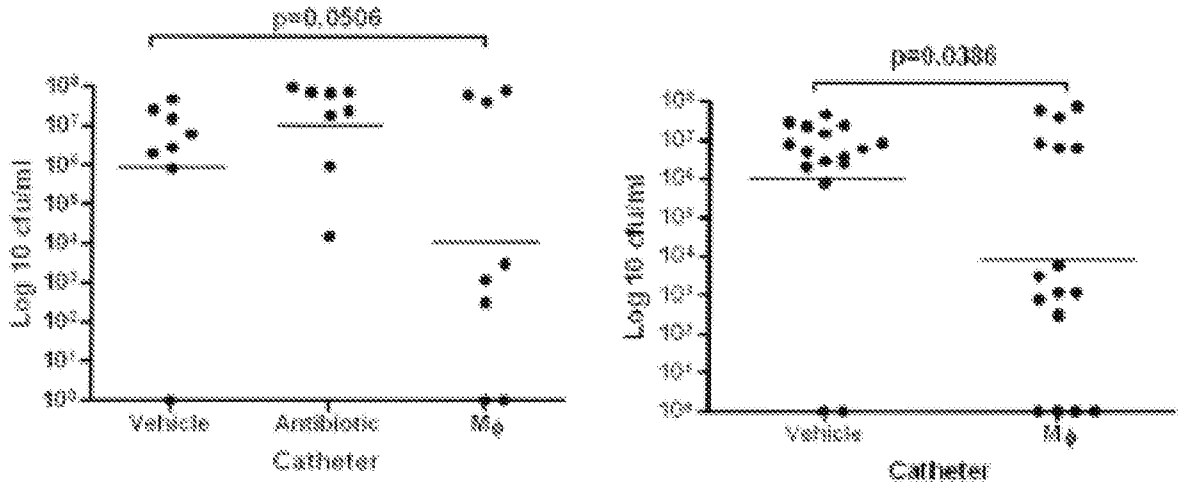

p=0.0506
Log 10 cfu/ml
Vehicle
Antibiotic
Catheter
p=0.0386
Log 10 cfu/ml
Vehicle
Catheter

Figure 3

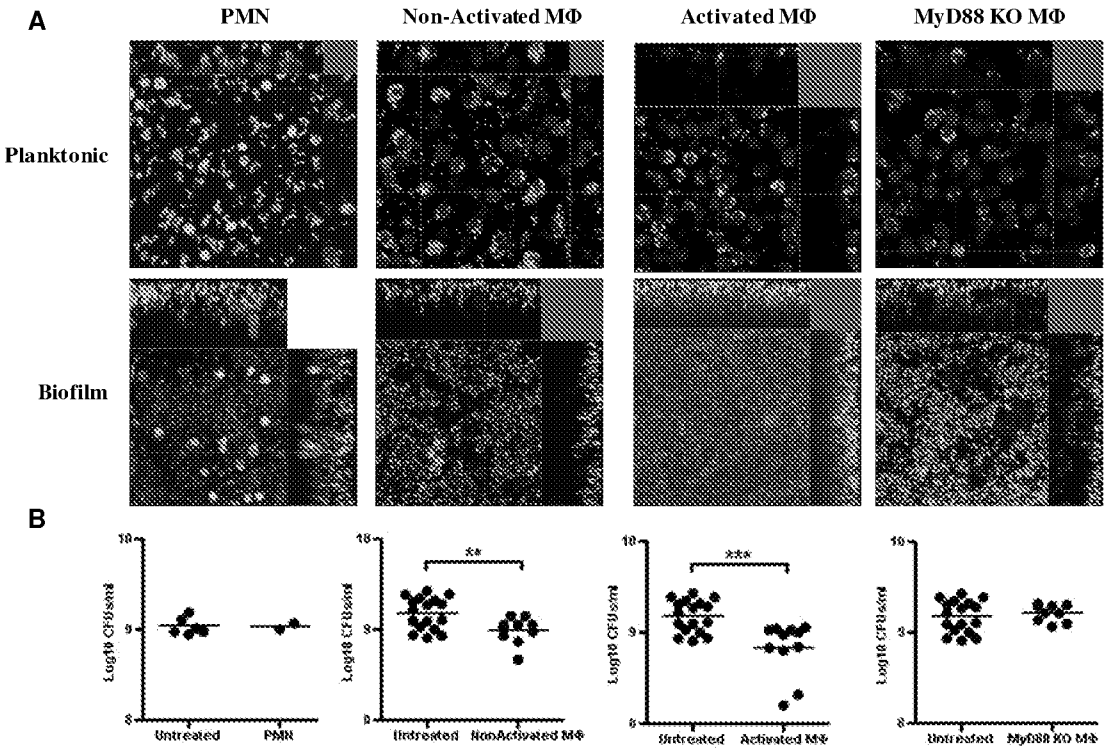

A
PMN
Non-Activated MΦ
Activated MΦ
MyD88 KO MΦ
Planktonic
Biofilm
B
Untreated
PMN
Untreated
Non-Activated MΦ
Untreated
Activated MΦ
Untreated
MyD88 KO MΦ

Figure 4

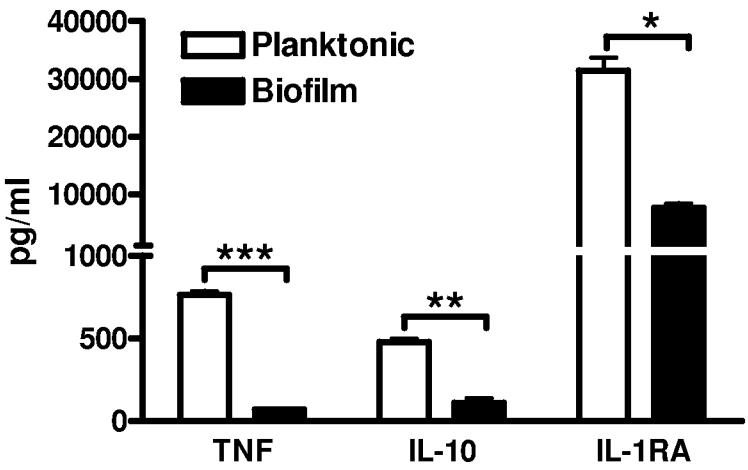

40000
30000
20000
10000
1000
500
0
pg/ml
Planktonic
Biofilm
***
**
*
TNF
IL-10
IL-1RA

Figure 5

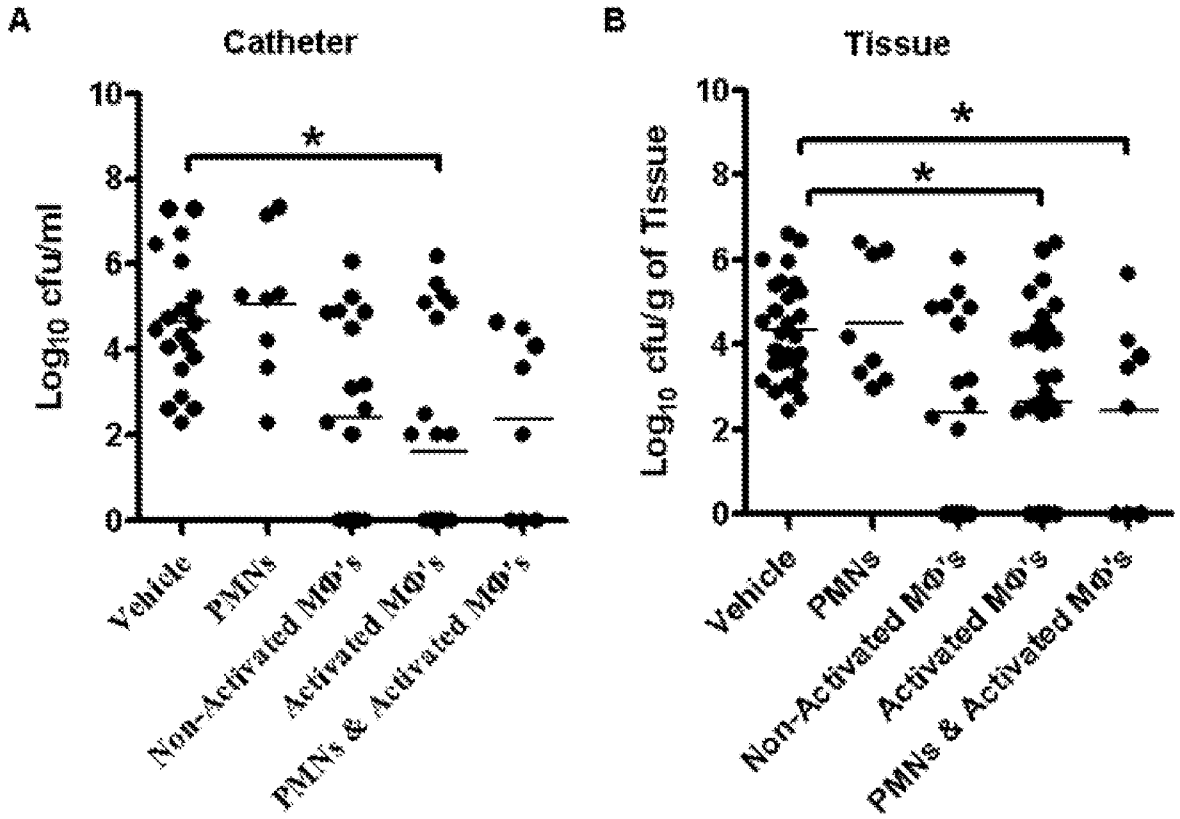

A
Catheter
Log10 cfu/ml
10
8
6
4
2
0
*
Vehicle
PMNs
Non-Activated MΦ's
Activated MΦ's
PMNs & Activated MΦ's
B
Tissue
Log10 cfu/g of Tissue
10
8
6
4
2
0
*
*
Vehicle
PMNs
Non-Activated MΦ's
Activated MΦ's
PMNs & Activated MΦ's

Figure 6

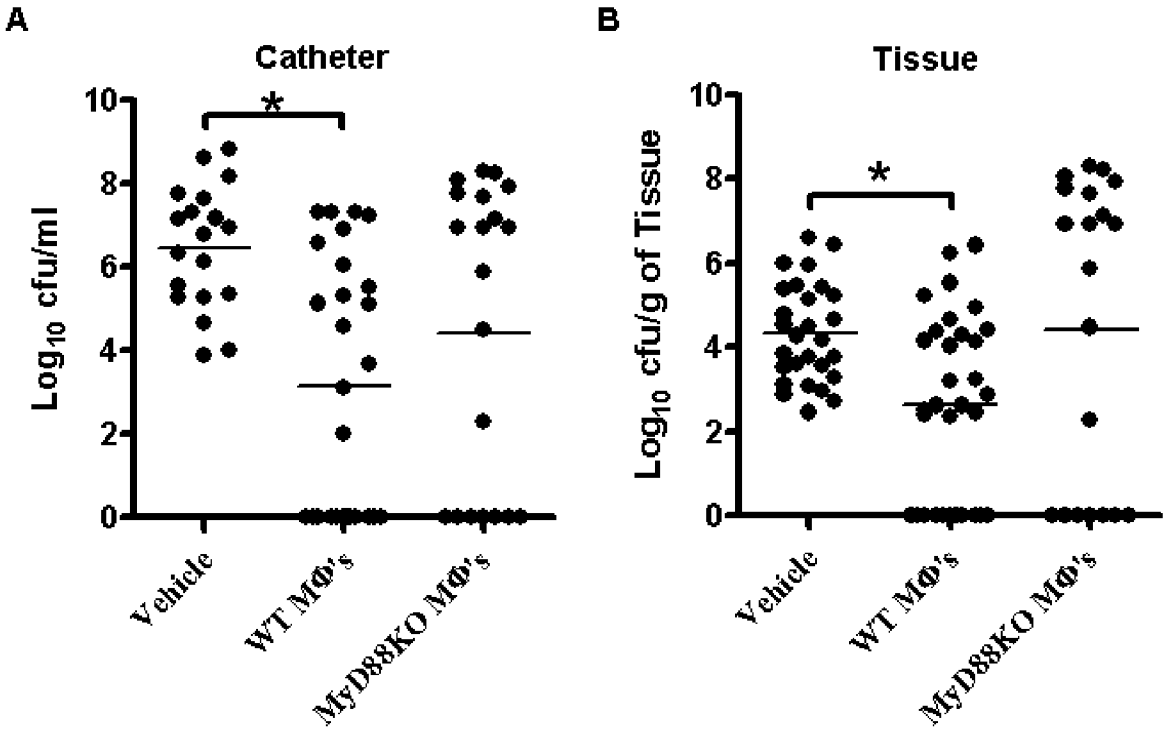
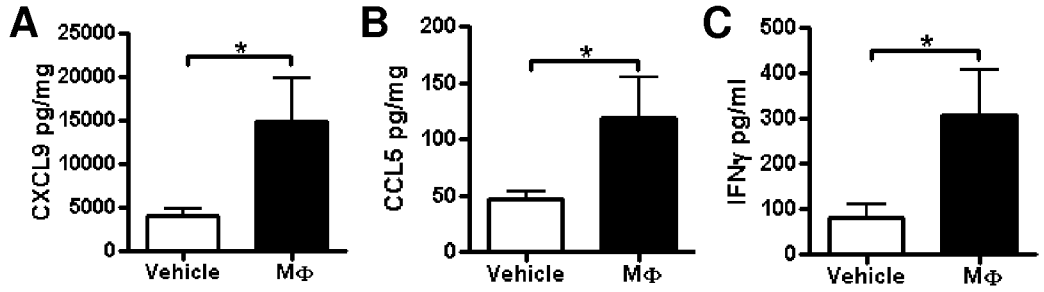

A
Catheter
Log10 cfu/ml
10
8
6
4
2
0
*
Vehicle
WT MΦ's
MyD88KO MΦ's
B
Tissue
Log10 cfu/g of Tissue
10
8
6
4
2
0
*
Vehicle
WT MΦ's
MyD88KO MΦ's

Figure 7

Prophylactic Treatment



Established Biofilm Treatment

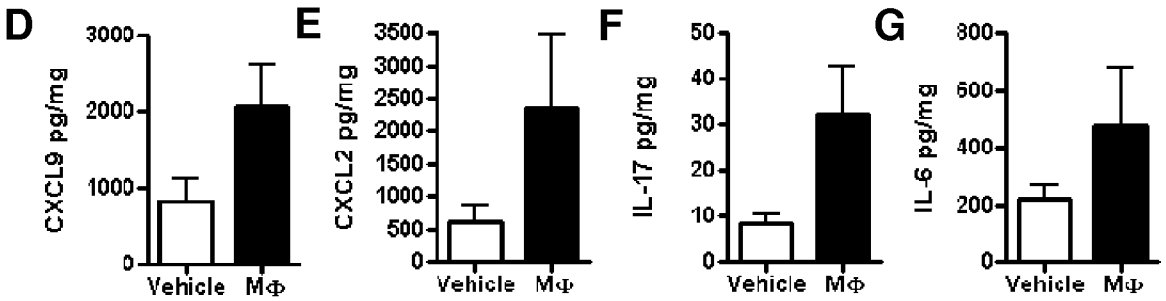


Figure 8

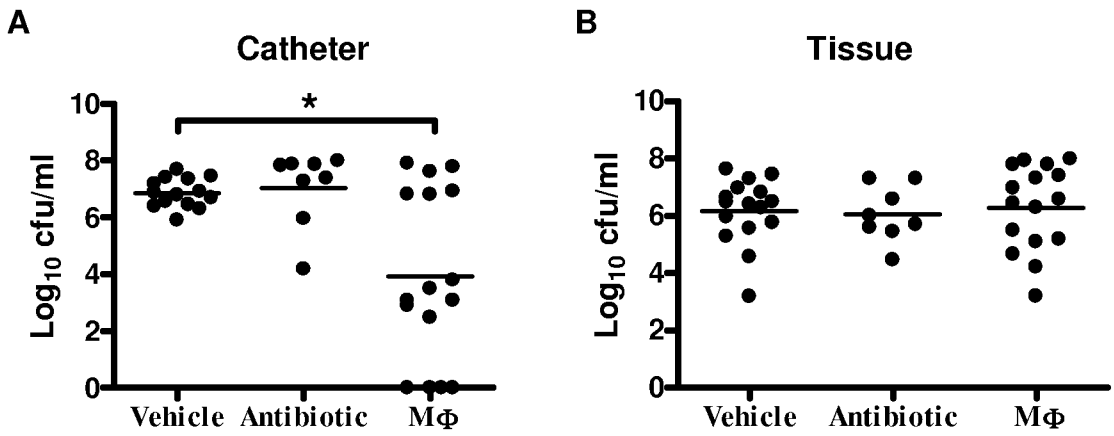

A
Catheter
*
Log10 cfu/ml
10
8
6
4
2
0
Vehicle
Antibiotic
MΦ
B
Tissue
Log10 cfu/ml
10
8
6
4
2
0
Vehicle
Antibiotic
MΦ

Figure 9

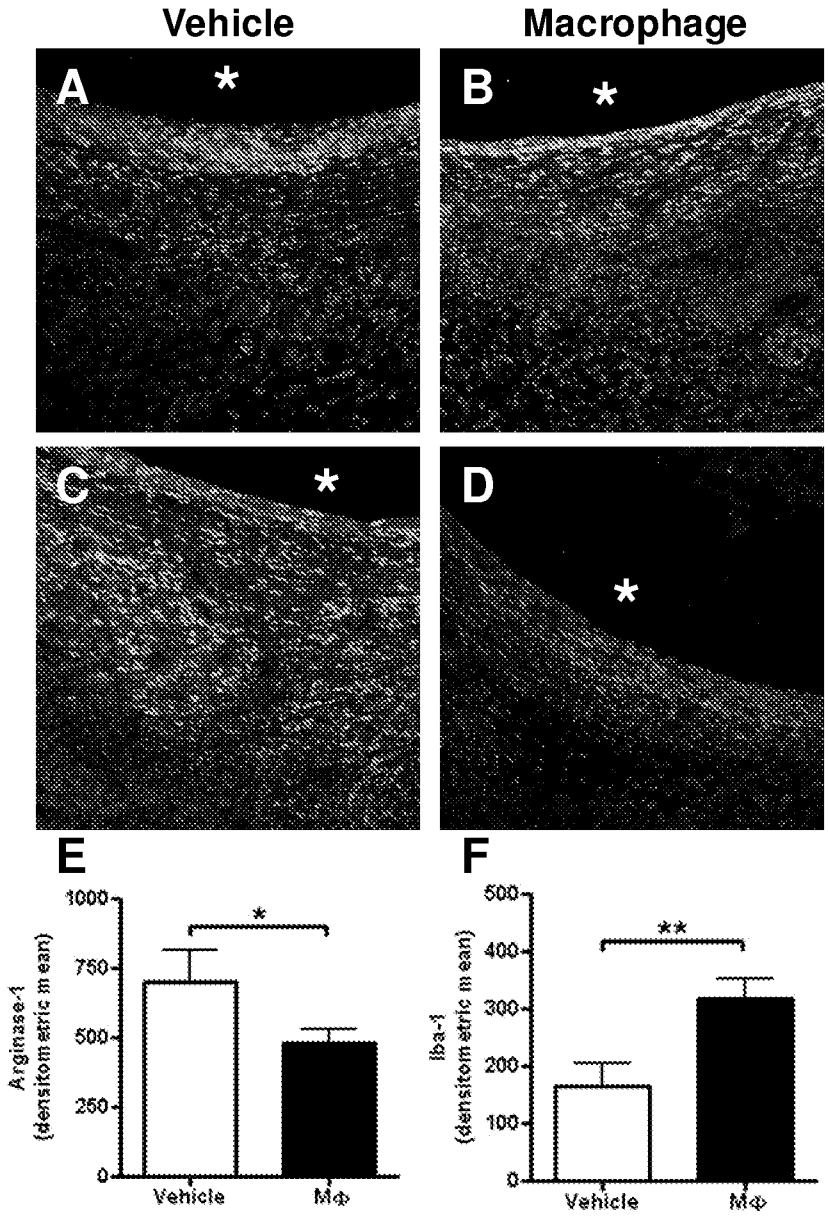

Vehicle
Macrophage
A
B
C
D
E
F
Arginase-1 (densitometric mean)
1000
750
500
250
0
*
Vehicle
MΦ
Iba-1 (densitometric mean)
500
400
300
200
100
0
**
Vehicle
MΦ

Figure 10

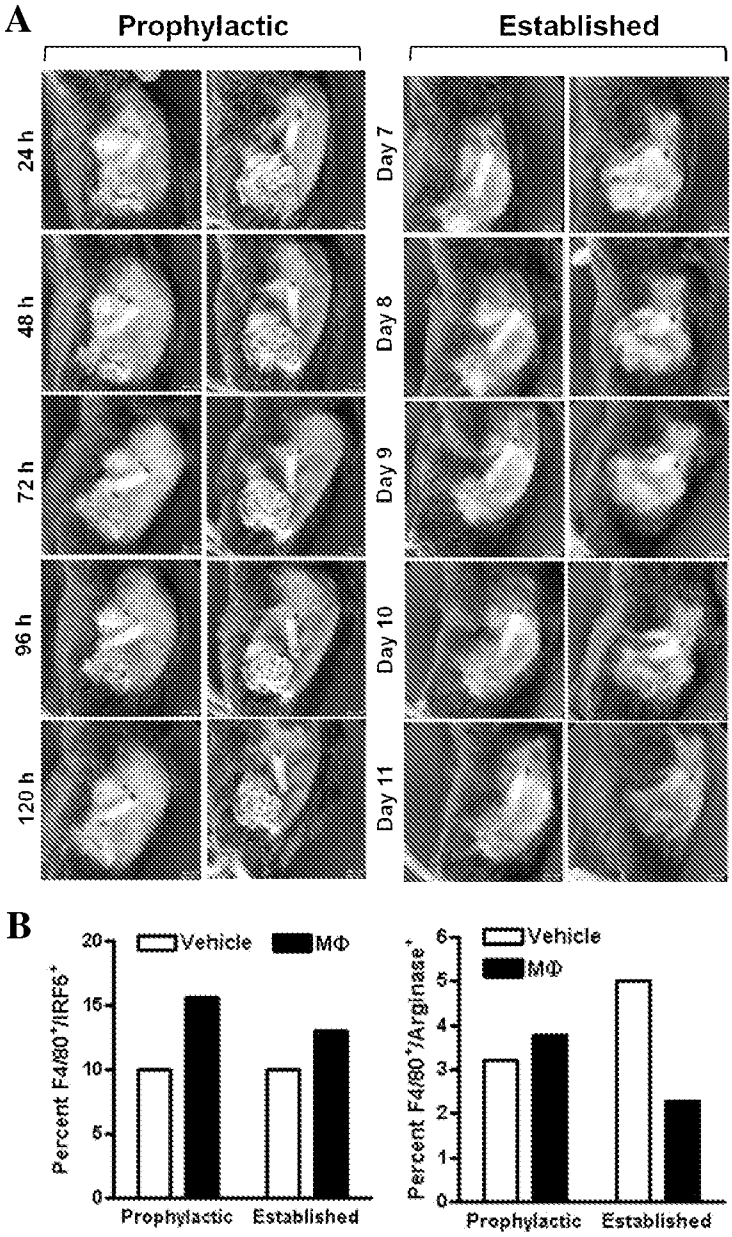

A
Prophylactic
Established
24 h
48 h
72 h
96 h
120 h
Day 7
Day 8
Day 9
Day 10
Day 11
B
Vehicle
MΦ
Percent F4/80+/IRF5+
Prophylactic
Established
Vehicle
MΦ
Percent F4/80+/Arginase+
Prophylactic
Established

Figure 11

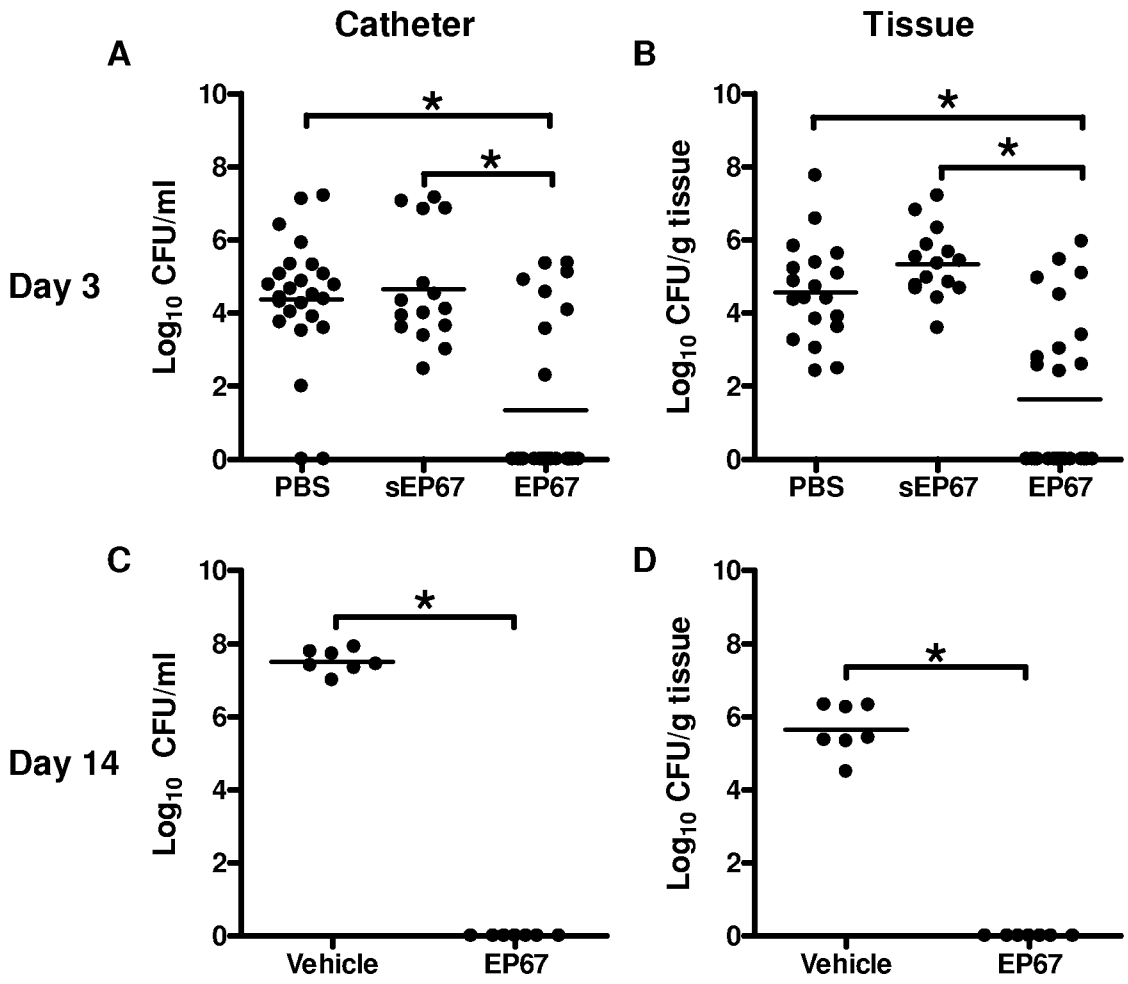

Catheter
Tissue
A
B
C
D
Day 3
Day 14
Log10 CFU/ml
Log10 CFU/g tissue
10
8
6
4
2
0
*
PBS
sEP67
EP67
Vehicle

Figure 12

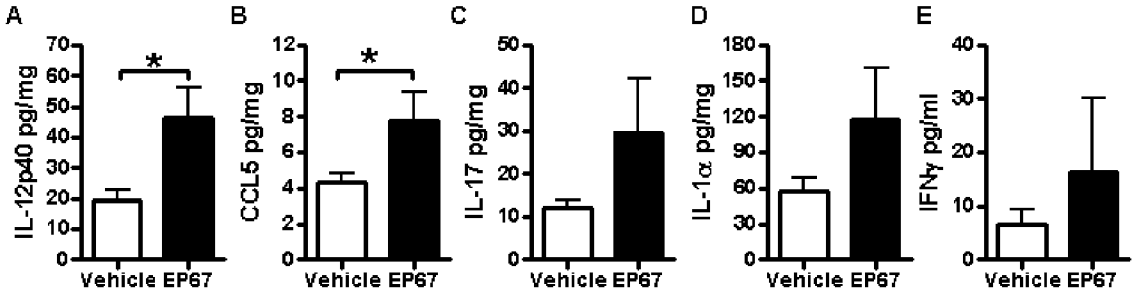

A
B
C
D
E
IL-12p40 pg/mg
CCL5 pg/mg
IL-17 pg/mg
IL-1α pg/mg
IFNγ pg/ml
Vehicle EP67
*
*

Figure 13

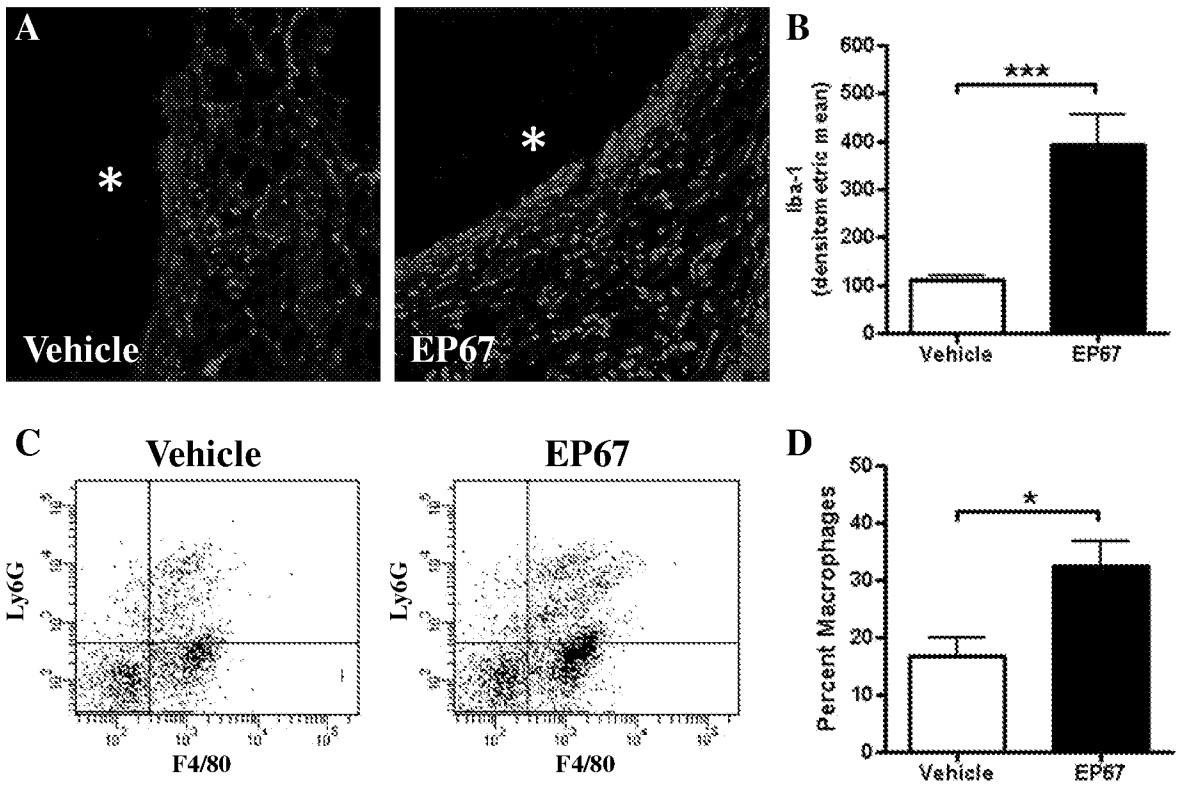

A
Vehicle
EP67
B
Iba-1 (densitometric mean)
600
500
400
300
200
100
0
***
Vehicle
EP67
C
Vehicle
EP67
Ly6G
F4/80
D
Percent Macrophages
50
40
30
20
10
0
*
Vehicle
EP67

Figure 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/52421

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A01N 63/00; C12N 5/00 (2012.01)
USPC - 424/93.71, 435/325
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC- 424/93.71, 435/325

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC- 435/375, 514/13.5

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(PGPB,USPT,USOC,EPAB,JPAB); Google Patents; Google Scholar: Macrophage, activated, primed, elicited murine macrophage, undifferentiated monocyte, autologous cell, ex vivo, in vivo, M1 macrophages, M2 macrophages, C5a receptor agonist, a C5a analog, or a C5a, EP67, activated MI macrophage

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Xu, et al. IFN-gamma and TNF regulate macrophage expression of the chemotactic S100 protein S100A8. J Immunol. 2000, 164(9):4916-23; pg 4917, 3rd para	1
A	US 2011/0195062 A1 (Timans) 11 August 2011 (11.08.2011) para [0220]	1
A	US 2011/0142812 A1 (Du Clos, et al.) 16 June 2011 (16.06.2011) para [0023]	1
A	Edwards, et al. Biochemical and functional characterization of three activated macrophage populations. J Leukoc Biol. 2006, 80(6):1298-307	1
A	Lin, et al. A novel exopolysaccharide from the biofilm of Thermus aquaticus YT-1 induces the immune response through Toll-like receptor 2. J Biol Chem. 20 May 2011, 286(20):17736-17745	1
A	Feola, et al. Azithromycin alters macrophage phenotype and pulmonary compartmentalization during lung infection with Pseudomonas. Antimicrob Agents Chemother. 2010, 54(6):2437-2447	1
A	Bystrom, et al. Resolution-phase macrophages possess a unique inflammatory phenotype that is controlled by cAMP. Blood. 2008,112(10): 4117-4127	1
A	Ling, et al. Vacuolar and plasma membrane stripping and autophagic elimination of Toxoplasma gondii in primed eff ector macrophages. J Exp Med. 2006, 203(9):2063-2071	1

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 December 2012 (21.12.2012)	22 JAN 2013
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/52421

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 25
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I: claim 1, drawn to a composition comprising an isolated activated macrophage for the prevention or treatment of a biofilm.
Group II: claims 2-4, drawn to a kit comprising an isolated macrophage and/or a macrophage-activating agent

Group III: claims 5-24, 26-30, drawn to a method of providing biofilm therapy to a subject in need by
(a) isolating peripheral blood monocytes from a subject;
(b) culturing the monocytes under conditions compatible with formation of macrophages; and
(c) administering a prophylactically or therapeutically effective amount of the macrophages to a subject in need, wherein the macrophages are activated by contact with a macrophage-activating agent prior to, simultaneously with, or after administration of the macrophages.

********** See Supplemental Sheet to continue **********

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

********** Supplemental Sheet **********

In Continuation of Box III. Observations where unity of invention is lacking:

The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The inventions of Group I and II do not include the inventive concept of a method of providing biofilm therapy to a subject in need by (a) isolating peripheral blood monocytes from a subject; (b) culturing the monocytes under conditions compatible with formation of macrophages; and (c) administering a prophylactically or therapeutically effective amount of the macrophages to a subject in need, wherein the macrophages are activated by contact with a macrophage-activating agent prior to, simultaneously with, or after administration of the macrophages, as required by Group III.

The inventions of Groups I and III do not include the inventive concept of a kit comprising an isolated macrophage, as required by Group II.

The inventions of Groups I-III share the technical feature of an activated macrophage. However, this shared technical feature does not represent a contribution over prior art as being anticipated by an article titled "Biochemical and functional characterization of three activated macrophage populations" by Edwards, et al. (J Leukoc Biol. 2006, 80(6):1298-307) (hereinafter "Edwards") that discloses said activated macrophage (Abstract, "Classically activated Mphi (Ca-Mphi) were primed with IFN-gamma and stimulated with LPS. Type II-activated Mphi (Mphi-II) were similarly primed but stimulated with LPS plus immune complexes. Alternatively activated Mphi (AA-Mphi) were primed overnight with IL-4"). As said activated macrophage was known in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups.

In addition, claim 1 is anticipated by an article titled "IFN-gamma and TNF regulate macrophage expression of the chemotactic S100 protein S100A8" by Xu, et al. (J Immunol. 2000, 164(9):4916-23) (hereinafter "Xu") that discloses a composition comprising an isolated activated macrophage (pg 4917, 3rd para, "Mac [elicited murine macrophages] isolation and culture. Quakenbush-Swiss mice ... were injected i.p. with 2 ml 5 nM NaIO4 or 10% TG broth and peritoneal cells were lavaged with cold HBSS containing 0.38% sodium citrate (15 ml) 4 days later. Washed cells ... were washed three times with warmed HBSS to remove nonadherent cells, and equilibrated in CM for 18 h. CM (3 ml) was replenished before activation and populations contained .98% Mac (>98% viable by trypan blue exclusion) and ,0.3% neutrophils. Mac were stimulated or up to 96 h with the agents indicated"; Fig 1 and its legend, "Northern blot analysis of S100A8 and S100A9 mRNA expression by TG-elicited Mac. A, Control unstimulated (2); cells stimulated with two different samples of... IL-1b (300 U/ml)").
Xu does not disclose that said composition is for the prevention or treatment of a biofilm. However, said limitation is inherently present in Xu, because Xu uses the same macrophage activating agent, i.e. IL-1b, for activation of macrophages as Applicant (instant application, para 0024], "the macrophage-activating agent is selected from the group consisting of ... interleukin-IL-1b...") thereby achieving the macrophage possessing the same properties as the claimed macrophage.
In addition or in the alternative, the limitation "for the prevention or treatment of a biofilm" is a functional limitation and therefore does not carry patentable weight.

Groups I-III therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.