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Effect of macrophage classical (M1) activation on implant-adherent macrophage interactions with *Staphylococcus epidermidis*: A murine *in vitro* model system

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Abstract

A model *in vitro* system was developed for eliciting classical (M1) activation of surface-adherent murine macrophages, which was then used to study the interaction of the M1 macrophages with *Staphylococcus epidermidis*. Glass substrata were first covalently grafted with a mixture of methoxy- and biotin-terminated silanated polyethylene glycol. Interferon (IFN)- γ and/or lipopolysaccharide (LPS), ligands known to induce the highly microbicidal M1 activation state in macrophages, were biotinylated and immobilized by way of a streptavidin intermediate to the biotin-PEG base substratum. Assessment of mouse bone marrow-derived macrophage (BMDM) interleukin (IL)-12(p40) and nitric oxide response to the fabricated surfaces confirmed that the model system achieved activation of adherent macrophage: IFN- γ -presenting surfaces primed cells for M1 activation, LPS-presenting surfaces elicited innate activation, and surface presenting a combination of IFN- γ and LPS induced M1 activation. The phagocytic and microbicidal capacity of activated, surface-adherent BMDM was evaluated using *S. epidermidis*, a bacterial species prevalent in implant-associated infections. Results indicate that M1 activation of implant-adherent macrophages trends towards diminishing their phagocytic capacity, but enhances their microbicidal capacity for *S. epidermidis*.

Keywords

immunostimulation; infection; inflammation; macrophage; surface grafting

INTRODUCTION

Macrophages are the predominant cell type present at the tissue/biomaterial interface,¹ and they are known to play a pivotal role in steering the outcome of implanted biomaterials. Following biomaterial implantation, macrophages infiltrate the site of injury and mount an inflammatory response to clear wound debris and any pathogens introduced during the implantation procedure; downstream, they mediate the foreign body reaction, dictating the integration of the implant into the surrounding tissue.² The early response of macrophages to bacterial challenge is critical not only for the prevention of catastrophic infections, but also to the proper healing and integration of the implant: persistent infections, even if asymptomatic, can cause prolonged inflammation at the implant site, leading to biomaterial degradation and/or damage to host tissue.³ Unfortunately, the presence of implanted foreign bodies has been shown to increase host susceptibility to infections.^{4,5} Surfaces of implanted

materials provide a potential locus for bacterial adhesion and formation of biofilms—complex, structured communities of sessile bacterial cells, which confer the bacteria resistance to antibiotics as well as to host immune defense mechanisms.⁶ Consequently, macrophage clearance of bacteria at the implant surface during the early postimplantation period is of key importance to the prevention of infections.

Macrophages comprise a heterogeneous population of cells with significant functional plasticity.^{7,8} Depending on the molecular cues present in their local microenvironment, they adopt distinct activation states with specific functional roles. Activated macrophages are most commonly categorized into two broad subtypes: classically activated (M1) and alternatively activated (M2) macrophages. While M2 macrophages are considered to play an important role in tissue repair, M1 macrophages are primarily responsible for microbial killing.⁹ M1 macrophages are characterized by markedly enhanced intracellular pathogen killing, as well as the production of reactive oxygen and nitrogen intermediates and proinflammatory cytokines such as tissue necrosis factor (TNF)- α , interleukin (IL)-12, IL-1, and IL-6.⁹ In the murine system, M1 macrophages are easily distinguished by their production of nitric oxide (NO).^{10,11} M1 activation requires two distinct signals: interferon (IFN)- γ , and a microbial stimulus such as bacterial lipopolysaccharide (LPS), which triggers the endogenous production of tissue necrosis factor (TNF)- α .^{9,12,13} Interestingly, recent work has revealed that implant sites are characterized by a predominantly immunosuppressive microenvironment.¹⁴ Areas of implants were shown to express high levels of antiinflammatory cytokines IL-4 and IL-10, while lacking expression of TNF- α . Notably, IFN- γ —the primary signal necessary for M1 activation—was not detected near the implant site at any time point.¹⁴ Considering the predominant role of M1 macrophages in pathogen clearance, it has been suggested that such an immunosuppressive microenvironment may result in the susceptibility of the implant surface to bacterial colonization.

However, the importance of M1 activation on macrophage microbicidal function has never been fully elucidated in the context of implant-associated infections. In fact, controversies exist in the literature regarding the effect of M1 activation on macrophage phagocytic and microbicidal capacity: for example, Speert et al.¹⁵ reported that IFN- γ treatment diminished both the phagocytosis and killing of *Pseudomonas aeruginosa* by human monocytes, whereas Gratchev et al.¹⁶ found that IFN- γ /LPS treatment decreased the overall phagocytic capacity of human monocytes, but increased their killing of *Escherichia coli*. Importantly, investigations of the effect of M1 activation on macrophage–bacteria interactions have rarely been performed using either adherent macrophages, whose immune function may or may not differ compared to non- or semiadherent monocyte/macrophages, or the bacterial species most frequently known to cause implant-associated infections, *Staphylococcus epidermidis*.^{5,17}

The goal of this research was to investigate the effect of M1 activation on surface-adherent macrophage interactions with bacteria relevant in implant-associated infections. To model the interaction of implant-adherent, M1-activated macrophages with bacteria, an *in vitro* system was developed for the elicitation of surface-adherent macrophage M1 activation, in which M1-activating ligands IFN- γ and LPS were immobilized to a model surface. Such a system was designed to enable the specific activation of adherent macrophages—and macrophages only—by the surface-immobilized ligands, throughout the duration of the macrophage–bacteria interaction assays. A biotin-functionalized, nonfouling PEG base substratum was used as a flexible platform for ligand presentation, which would preclude the confounding effects of nonspecifically adsorbed serum proteins on macrophage activation. M1-activating ligands IFN- γ and LPS were immobilized to the base substratum, each individually or in combination. Adherent macrophage response to the ligand-

functionalized surfaces was evaluated using primary mouse bone marrow derived macrophages (BMDM). Finally, the phagocytic and microbicidal capacity of surface-adherent BMDM was assessed using *S. epidermidis*. Ultimately, the aim of this study was to provide a preliminary assessment of the role of macrophage M1 activation in the context of implant-associated infections.

MATERIALS AND METHODS

Fabrication of biotin-PEG base substratum

Monomethoxy poly(ethylene glycol) (mPEG-silane), MW 2000, and biotinylated PEG-silane (biotin-PEG-silane), MW 3400, were purchased from Laysan Bio (Arab, AL), and used as received, without further purification. Circular glass coverslips ($d = 15$ mm; Bellco Glass, Vineland, NJ) were cleaned and activated in piranha solution (1:3 mixture of 30% H_2O_2 and H_2SO_4) for 30 min, rinsed three times in ultrapure dI water, then dried with a stream of nitrogen. mPEG-silane and biotin-PEG-silane were dissolved in toluene and mixed at various molar ratios, to a total PEG content of 2.5 mM. Cleaned, dried coverslips were immediately immersed in solutions of PEG-silane, and stirred at room temperature overnight (18 h). Coverslips were rinsed sequentially in toluene, acetone, and dI water, then dried with a nitrogen stream.

X-ray photoelectron spectroscopy (XPS) analysis of the fabricated base substrata was performed by the University of Washington National ESCA and Surface Analysis Center for Biomedical Problems (NESAC/BIO), using a SSX-100 spectrometer (Surface Science Instruments, Mountain View, CA) equipped with a monochromatic Al $\text{K}\alpha_{1,2}$ X-ray source.

^{125}I -Fibrinogen adsorption assay

Highly purified human fibrinogen was radiolabeled with ^{125}I (Na^{125}I ; Amersham, Arlington Heights, IL) using the iodine monochloride (ICl) technique, as previously described by Tsai et al.¹⁸ Briefly, fibrinogen and ICl were mixed at a molar ratio of 3:1, and reacted with 1 mCi ^{125}I per 0.5 mL protein. Labeled fibrinogen was separated from unincorporated ^{125}I via gel filtration, and stored for up to 2 weeks at -80°C until use.

Radiolabeled fibrinogen adsorption assays were performed on biotin-PEG-grafted glass coverslips, as in Zhang et al.¹⁹ Sample substrata were soaked for 45 min in citrate phosphate-buffered saline with 3 mM sodium azide and 10 mM sodium iodide (CPBSzI), then incubated with solutions of radiolabeled fibrinogen, 0.03 mg/mL, for 2 h at 37°C . Coverslips were rinsed thoroughly with fresh buffer to remove any loosely bound protein, then the retained radioactivity measured using a Cobra II Series Auto-Gamma counting system (Packard Instrument, Meriden, CT). The amount of adsorbed fibrinogen was calculated from the radioactivity measurements, using the specific activity of the fibrinogen solution and the sample surface area.

Determination of optimal biotin-PEG % for ligand immobilization

The optimal molar % of biotin-PEG for base substratum fabrication was determined via streptavidin binding studies. Glass coverslips were grafted with PEG-silane solutions of varying biotin-PEG content (0, 0.1, 1, 10, 100 molar %), soaked for 45 min in CPBSzI, then incubated for 1 h in solutions of ^{125}I -labeled streptavidin (Perkin Elmer, Waltham, MA), at 0.1 mg/mL. After thorough rinsing in fresh buffer, the amount of streptavidin bound to each coverslip was determined by measuring the radioactivity of the samples in a gamma counter.

The molar % of biotin-PEG yielding the highest density of bound streptavidin (10%) was chosen as optimal and used for all subsequent studies.

Ligand biotinylation and immobilization to biotin-PEG base substratum

Mouse recombinant IFN- γ (Peprotech, Rocky Hill, NJ), confirmed by the manufacturer to have less than 0.1 ng endotoxin per μg protein (1 EU/ μg), was biotinylated at free primary amino groups using succinimidyl-6-[biotinamidol]-hexanoate (EZ-Link NHS-LC-Biotin; Thermo Scientific, Rockford, IL). NHS-LC-Biotin was dissolved in dimethylsulfoxide (DMSO) to 10 mM, and immediately added to a 1 mg/mL solution of IFN- γ in PBS at molar ratios ranging from 1 to 10 (NHS-LC-Biotin:IFN- γ). The reaction was incubated at room temperature for 30 min, then the biotinylated protein purified using Zeba Desalt Spin Columns with a 7-kDa MWCO (Thermo Fisher).

LPS from *E. coli* 0111:B4 (Sigma, St. Louis, MO) was biotinylated at the core polysaccharide 2-keto-3-deoxyoctononic acid (Kdo) moieties via a previously reported method,²⁰ with modifications. Sodium meta-periodate (NaIO_4) was dissolved in cold 0.1 M sodium acetate buffer, pH = 5.5, and added to a 1 mg/mL LPS solution to final concentrations ranging from 0.1 to 10 mM. The reaction was allowed to proceed for 30 min at 4°C. Glycerol was added to stop the oxidation, and the oxidized LPS purified using 7 kDa MWCO Zeba Desalt Spin Columns. Biotin-LC-Hydrazide (Thermo Scientific) was dissolved to 50 mM in DMSO, then added to the oxidized LPS to a final concentration of 5 mM. The reaction mixture was incubated 2 h at room temperature with end-to-end rotation. Biotinylated LPS was purified by desalting.

The degree of biotinylation of the ligands was determined via a 4'-hydroxyazobenzene-2-carboxylic acid (HABA)/avidin displacement assay (Pierce Biotin Quantitation Kit; Thermo Scientific), where the displacement of HABA binding to avidin by biotin is measured by the decrease in absorbance at 500 nm. Biotinylation reaction conditions were carefully controlled to yield a degree of labeling (DOL) of ~1.0 for both IFN- γ and LPS (NHS-LC-Biotin:IFN- γ molar ratio of 2.7 for IFN- γ ; NaIO_4 concentration of 1 mM for LPS). The biological activity of the resultant biotin-IFN- γ and biotin-LPS was confirmed by testing their ability elicit IL-12(p40) and NO production in macrophages versus unmodified IFN- γ and LPS.

To assemble the ligand-presenting surfaces (from here on designated PEG, PEG:IFN- γ , PEG:LPS, and PEG:IFN- γ /LPS), biotin-PEG-grafted coverslips were incubated with 100 $\mu\text{g/mL}$ streptavidin for 1 h, rinsed thoroughly, then incubated with 2 μM solutions of biotin-IFN- γ and/or biotin-LPS for 1 h. For the combination surface, PEG:IFN- γ /LPS, biotin-IFN- γ and biotin-LPS were mixed at a molar ratio of 1:1. Ligand immobilization procedures were performed using materials and reagents confirmed by manufacturers to have been successfully tested for endotoxin contamination. To estimate the density of immobilized ligands, biotin-IFN- γ and biotin-LPS were fluorescently labeled with DyLight 488 NHS ester (Thermo Scientific) and Alexa Fluor 594 hydrazide sodium salt (Invitrogen, Carlsbad, CA), respectively, immobilized to the biotin-PEG base substrata as described, then their fluorescence measured in a plate reader. The amount of surface-bound ligand was estimated by extrapolation from standard curves of known quantities of fluorescently labeled ligands (ng/well).

Macrophage preparation and culture

All animal procedures were carried out following a protocol approved by the University of Washington Institutional Animal Care and Use Committee (IACUC). NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) have been observed. Bone marrow cells were harvested from C57BL/6 mice between 6 and 12 weeks of age (The Jackson Laboratory, Bar Harbor, ME) and differentiated into macrophages, as previously described.^{21,22} Briefly, bone marrow cells were flushed from the femurs with complete

RPMI 1640 medium, and cultured in RPMI supplemented with 0.5% penicillin/streptomycin, 20% heat-inactivated FBS, and 10 ng/mL recombinant macrophage-colony stimulating factor (M-CSF; Peprotech, Rocky Hill, NJ) for 6 days at 37°C/5% CO₂. On day 7, bone marrow-derived macrophages (BMDM) were harvested by trypsinization, then used for experiments. BMDM were maintained in RPMI/10% FBS supplemented with 5 ng/mL M-CSF.

Characterization of macrophage *in vitro* response to ligand-presenting surfaces

PEG, PEG:IFN- γ , PEG:LPS, and PEG:IFN- γ /LPS coverslips were placed in wells of a 24-well tissue culture polystyrene (TCPS) plate, in triplicates. Mouse BMDM were seeded at a density of 2×10^5 cells/well, and cultured for 24 h at 37°C/5% CO₂. Triplicate wells of cells seeded on TCPS were also treated with soluble IFN- γ and/or LPS (10 ng/mL). Macrophage culture supernatants were collected for cytokine and nitric oxide (NO) analysis, and the number of adherent cells was determined either via cell lysis (in 0.5% Triton X-100) followed by quantitation of total lactate dehydrogenase (LDH) release (CytoTox 96; Promega, Madison, WI). Macrophage viability was determined by quantitation of LDH content in the cell culture supernatants. IL-12(p40) secretion by the adherent cells was measured via ELISA (BD Biosciences, San Jose, CA). NO production was assessed indirectly via nitrite measurement, using the Griess reagent (0.8% sulfanilamide, 0.75% *N*-(naphthylethylene)diamine in 0.5M HCl) (Invitrogen). To assess macrophage activation on the ligand-presenting surfaces over prolonged culture, BMDM were cultured on PEG, PEG:IFN- γ , PEG:LPS, or PEG:IFN- γ /LPS for 72 h, with supernatant collection and media replenishment every 24 h. Supernatant samples were evaluated for IL-12(p40) and NO concentrations; the number of adherent cells was determined at every 24 h.

Phagocytosis assay

S. epidermidis strain RP62A (*SERP62A*; ATCC 35984) cultures were grown overnight in trypticase soy broth (TSB), at 37°C with shaking at 180 rpm. Overnight cultures were inoculated into fresh TSB, grown to mid- to late-log phase, and subsequently used for experiments.

SERP62A cells were labeled with pHrodo™ succinimidyl ester (pHrodo; Invitrogen), using methods described by Bernardo et al.²³ with slight modifications. Briefly, bacterial cells were resuspended to 1×10^9 cells/mL in 0.1M NaHCO₃, pH = 9. Ten microliter of 10 mM pHrodo™ was added to 1 mL of bacterial cells, to final concentration 0.1 mM; labeling reaction was allowed to proceed for 1 h at room temperature. Labeled bacteria were rinsed thoroughly in PBS.

For phagocytosis experiments, BMDM were precultured for 24 h on the ligand-presenting surfaces, as described previously. pHrodo™-labeled *SERP62A* cells, in DMEM without phenol red supplemented with 5% normal mouse serum from C57BL6 mice (Innovative Research, Novi, MI), were added to macrophage-containing wells at a bacteria:macrophage ratio of 20. Plates were centrifuged for 5 min at 250g, then incubated at 37°C, 5% CO₂ for up to 6 h; the fluorescence of the wells (ex 550 nm/em 595 nm) was measured in a plate reader at every hour. As a negative control for phagocytosis, triplicate wells of macrophages were treated with cytochalasin D (Sigma) 1 h before and during coinocubation with bacteria.

Bacterial killing assay

BMDM were precultured for 24 h on the ligand-presenting surfaces. *SERP62A* cells were added to wells at a bacteria:macrophage ratio of 1:1, in antibiotic-free RPMI supplemented with 5% mouse serum. Bacteria were also added to triplicates of wells without BMDM (on unmodified PEG base substrata). Plates were centrifuged at 250g for 5 min to sediment the

bacteria, then incubated at 37°C/5% CO₂ for 4 h. Subsequently, BMDM were lysed with 0.5% Triton X-100 (Sigma), serially diluted in cold dI H₂O, and plated onto TSB agar plates. Colony forming units (CFU) were enumerated after 18–24 h growth at 37°C.

RESULTS

Biotin-PEG base substratum

To create a base substratum resistant to nonspecific protein adsorption, with sites for subsequent ligand attachment, we grafted mixtures of mPEG-silane and biotin-PEG-silane onto glass coverslips (Fig. 1). Biotin-PEG-silane of a slightly higher molecular weight (3400 kDa) than mPEG-silane (2000 kDa) was chosen to increase the accessibility of the biotin functional groups to ligands. XPS analysis showed that glass coverslips were successfully modified with PEG. Compared to clean glass, all PEG-grafted coverslips showed a decrease in silicon and oxygen surface concentration, and an increase in carbon concentration (Table I). The high-resolution C1s spectra revealed a higher surface ether carbon and lower hydrocarbon content on all of the PEG-grafted coverslips versus clean glass (Table II). No significant differences in chemical composition were found among biotin-PEG substrata prepared using solutions of varying biotin-PEG %. To confirm the nonfouling properties of the base substratum, we quantified fibrinogen adsorption onto coverslips grafted with various molar % solutions of biotin-PEG. We found all PEG-grafted substrates to be ultra-low fouling (<5 ng/cm²) [Fig. 2(A)].

To optimize immobilized ligand density, we tested the binding of ¹²⁵I-labeled streptavidin to biotin-PEG substrata fabricated using 0–100% (molar) solutions of biotin-PEG. Maximal streptavidin binding (~150 ng/cm²) was achieved with ~10% biotin-PEG, with higher biotin-PEG content not yielding any further increase in streptavidin binding [Fig. 2(B)]; therefore, 10% biotin-PEG was chosen as the base substratum for all subsequent studies.

IFN- γ and LPS biotinylation and immobilization to biotin-PEG

To provide attachment sites to the base substratum, IFN- γ and LPS were labeled with biotin at their free primary amino groups and at the core oligosaccharide moieties, respectively. HABA/avidin displacement assays confirmed the successful attachment of biotin to both IFN- γ and LPS. To confirm that biotin labeling did not affect the biological activity of the ligands, biotin-IFN- γ and biotin-LPS (DOL = 1.0) were tested alongside unmodified IFN- γ and LPS for their ability to elicit macrophage IL-12(p40) production; results showed that the biotinylated ligands fully retained their activity (data not shown).

Immobilized ligand density was estimated using fluorescently labeled IFN- γ and LPS (Table III). Estimation of immobilized streptavidin density via this method yielded a quantity (2.5 ± 0.02 pmol/cm²) comparable to that determined using ¹²⁵I-labeled streptavidin (2.7 ± 0.2 pmol/cm²), confirming the validity of the method. The surface density of LPS was found to be ~5-fold lower than that of IFN- γ , likely due to differences in the positioning of biotin moieties in biotin-IFN- γ and biotin-LPS. Although IFN- γ molecules were biotinylated at the N-terminus, LPS molecules were biotinylated at the core in a central position, possibly resulting in higher steric blocking of surface binding sites during immobilization.

Macrophage response to ligand-presenting surfaces

Macrophage response to the ligand-presenting surfaces PEG:IFN- γ , PEG:LPS, and PEG:IFN- γ /LPS was determined using primary mouse BMDM. After 24–72 h culture on the ligand-presenting surfaces, adherent macrophage production of IL-12(p40) and NO was quantitated; both IL-12(p40) and NO are markers of M1 activation in the mouse, as their

production is specifically upregulated by M1-activated macrophages.^{9,13,24} Soluble ligand treatments (at 10 ng/mL, concentration previously optimized to yield maximal macrophage response) were also performed, as positive controls for macrophage response to the ligands. Experiments were performed multiple times, and yielded response levels that varied in magnitude from experiment to experiment due to biological variation; hence, results were analyzed with a focus on trends rather than on absolute quantities, and representative results are presented in Figures 3 and 4.

Ligand-presenting surfaces elicited macrophage responses generally matching those attained via treatment with soluble ligands, confirming the ability of surface-immobilized ligands to modulate macrophage response (Fig. 3). The unmodified PEG surface did not elicit significant increase in either IL-12(p40) or NO production. The dual-ligand surface, PEG:IFN γ /LPS, elicited high levels of both IL-12(p40) and NO production. PEG:IFN- γ did not markedly affect IL-12(p40) production, but caused a small increase in NO production (29% of the quantity produced on PEG:IFN- γ /LPS). PEG:LPS produced an intermediate increase in IL-12(p40) production (42% of the quantity produced on PEG:IFN- γ /LPS) and a small increase in NO production (21% of the quantity produced on PEG:IFN- γ /LPS). LDH assays revealed no significant cell death (<5%) in any of the treatments (data not shown).

BMDM activation on the ligand-presenting surfaces was shown to be moderated after the initial 24 h (Fig. 4). IL-12(p40) production dropped sharply after 24 h; NO production at 48 h decreased to less than 50% of the levels produced at 24 h. By 72 h, IL-12(p40) and NO were not detectable in the supernatants of cells cultured on any of the ligand-presenting surfaces.

Phagocytosis of *S. epidermidis* by surface-adherent macrophages

The phagocytosis of live *SERP62A* cells by implant-adherent, activated macrophages was studied using the model *in vitro* system developed. BMDM were precultured on the ligand-presenting surfaces for 24 h, then challenged with live, pHrodoTM-labeled *SERP62A* cells (Fig. 5). The assay method was shown to successfully detect bacterial internalization by macrophages, as evidenced by the lack of increase in the fluorescence signal in cytochalasin D-treated wells. For the rest of the wells, fluorescence signals increased during the first 3 h of assay, indicating increasing bacterial internalization, then decreased thereafter, likely due to the degradation of internalized bacteria and/or photobleaching of the dye over time.

No differences in phagocytosis were found between untreated BMDM (cultured on PEG) and those cultured on PEG:LPS. BMDM cultured on PEG:IFN- γ /LPS displayed lower phagocytosis than untreated BMDM for all observed time-points, though the differences were not statistically significant. At later timepoints in the assay ($t = 4$ h), BMDM cultured on PEG:IFN- γ also displayed a trend towards lower phagocytosis relative to BMDM cultured on PEG, though again the differences were not statistically significant.

Killing of *S. epidermidis* by surface-adherent macrophages

The microbicidal capacity of surface-adherent, activated macrophages was tested by challenging BMDM with *S. epidermidis* (Fig. 6). All BMDM-containing wells had significantly fewer surviving bacteria than bacteria-only wells, demonstrating that all BMDM, regardless of their activation states, achieved significant killing of *S. epidermidis* cells. Bacterial killing by BMDM cultured on PEG:LPS did not differ significantly from untreated BMDM (cultured on PEG). BMDM cultured on PEG:IFN- γ or PEG:IFN- γ /LPS displayed significantly increased bacterial killing, with both treatments resulting in ~40% decrease in surviving bacteria compared to PEG.

DISCUSSION

We report an *in vitro* model study of the interactions of M1-activated, implant-adherent murine macrophages and bacteria. While M1 is generally acknowledged to be the primary antimicrobial form of the macrophage, the effect of M1 activation on macrophage microbicidal capacity has rarely been investigated in the context of implant-associated infections. To study the immune function of surface-adherent, activated macrophages, we developed a series of model surfaces functionalized with M1 signaling molecules, IFN- γ and LPS. The two signals were immobilized individually or in combination, to model the full spectrum of M1 activation. IFN- γ and LPS were presented on a nonfouling PEG base, to inhibit macrophage interactions with nonspecifically adsorbed serum proteins; cells were expected to adhere directly to the ligands (via interaction with their respective receptors, IFN- γ R for IFN- γ and TLR4/CD14 for LPS), rather than to a layer of serum proteins adsorbed from the culture media. Visual observations of the cells supported this speculation: in contrast to cells cultured on ligand-presenting surfaces, cells cultured on unmodified, pure PEG surfaces displayed rounded morphology and were easily detached during experimental procedures, showing that cell adhesion depended on the presence of ligands.

The model surfaces elicited the expected responses from adherent macrophages. PEG:IFN- γ , on its own, did not markedly enhance the production of IL-12(p40) or NO, a result in line with the conventional knowledge that IFN- γ priming increases the sensitivity of macrophages to subsequent microbial stimuli, but does not itself elicit macrophage activation.⁹ PEG:IFN- γ /LPS enhanced the production of both IL-12(p40) and NO, indicating M1 activation of the adherent macrophages. PEG:LPS, on the other hand, elicited an intermediate increase in the production of IL-12(p40) but did not markedly affect NO production, indicating a macrophage phenotype distinct from M1 activation. Toll-like receptor ligation on its own is, in fact, known to give rise to a phenotype referred to as innate activation, characterized by a limited enhancement of pro-inflammatory cytokine production without the upregulation of microbicidal properties characteristic of M1 macrophages.^{24,25} Overall, ligand-presenting surfaces elicited lower levels of macrophage activation compared to treatment with soluble ligands. This may be attributed to differences in the total amounts of IFN- γ and LPS presented to the cells: while soluble ligands were added at 10 ng/well, much lower total amounts were provided in the surface-immobilized form (Table III). In addition, the presentation of surface-immobilized ligands to the cells may have been limited by the contact area of the cells with the surface, resulting in lower per cell activation.

The phagocytic capacity of IFN- γ -primed, innately activated, and M1-activated adherent macrophages was evaluated using live *S. epidermidis* cells. Live, rather than chemically- or heat-killed, bacteria were used for the present studies, since live and killed bacteria have been shown to induce significantly different innate immune responses.²⁶ Although the differences were not statistically significant, a consistent trend towards lower phagocytosis (relative to untreated macrophages) was observed in M1-activated macrophages at all timepoints. M1 activation has been shown to downregulate the expression of nonopsonic receptors such as mannose and scavenger receptors^{27,28}; our results suggest that nonopsonic pathways are important in the phagocytosis of *S. epidermidis*, since M1 activation appears to decrease their internalization.

While phagocytic capacity is an important parameter of immune function, the critical determinant for overall microbicidal function is the efficiency of intracellular killing. Bacterial killing assays were performed using a low bacteria:macrophage ratio (1:1), to minimize the influence of differential phagocytic rates on the assessment of killing efficiency. Compared to untreated macrophages, IFN- γ -primed and M1-activated

macrophages were shown to have increased ability to kill *S. epidermidis* cells; innate activation appeared to have no effect on *S. epidermidis* killing. These results are in accordance with the well-established view that M1 activation improves macrophage microbicidal capacity⁹; the upregulation of toxic oxygen species and NO, as well as the restriction of nutrients such as iron and tryptophan from the phagosome, are believed to be important mechanisms of enhanced microbial killing by M1 macrophages.^{29,30} While IFN- γ priming alone does not elicit M1 activation, the exposure to bacterial challenge appears to have provided IFN- γ -primed macrophages the secondary signal required for M1 activation; *S. epidermidis* does not possess LPS, but its analogous cell wall component, lipoteichoic acid (LTA), is also known to elicit macrophage activation.³¹

While the present findings suggest that M1 activation enhances the macrophage defense against *S. epidermidis* at an interface, these results should be considered in light of certain important limitations. First, the study was conducted using mouse, rather than human, macrophages. Previous studies have shown that mouse and human macrophages differ in important ways, including the expression of various surface markers and receptors (e.g., CD4, Toll-like receptors, FcR).³² Importantly, in contrast to mouse macrophages, human macrophages have not been consistently documented to produce NO in response to IFN- γ and LPS.³³ While mouse macrophages were useful for a preliminary assessment of macrophage function using the new *in vitro* model system presented here, the relevance and applicability of this data to humans is necessarily limited. Second, the model system excluded any potential effects of surface-adsorbed proteins on macrophage activation. *In vivo*, implanted materials instantaneously adsorb a layer of plasma proteins, which are believed to be central mediators of ensuing inflammatory and wound healing responses.² While the nonfouling PEG base allowed the study of specific interactions between adherent macrophages and the presented ligands, it precluded the examination of important *in vivo* processes in macrophage activation.

Nevertheless, the *in-vitro* model system presented in this work provides a useful framework for the further study of macrophage immune function in the context of implant-associated infections. Repetitions of the study using human monocyte/macrophages would yield more relevant insight into macrophage phenotype and function. The study may be further expanded to include other immune cell types important in mediating the early defense against implant-associated infections, such as neutrophils. In addition, the model system may be adapted to probe the effects of various different molecules of interest in the context of implant-associated infections. These may include signaling molecules known to elicit different macrophage activation states (e.g., IL-4, known to elicit M2 activation), or small molecules with potential for therapeutic applications (e.g., RGD peptides, known to enhance mammalian cell adhesion^{34,35}).

CONCLUSIONS

We have fabricated a series of model biomaterial surfaces immobilized with ligands known to stimulate macrophage classical (M1) activation, as a *in vitro* model system to study implant-adherent macrophage interactions with *S. epidermidis*. The response of surface-adherent BMDM was assessed via analysis of IL-12(p40) and NO production. Results confirmed that the model surfaces elicited the expected phenotypes in adherent macrophages: IFN- γ priming, innate activation, and M1 activation. Using the model system, the ability of surface-adherent, activated BMDM to phagocytose and kill *S. epidermidis* was assessed. Compared to resting BMDM, M1-activated cells trended towards having lower phagocytic capacity, but exhibited significantly higher bacterial killing. IFN- γ primed BMDM also displayed enhanced bacterial killing. Results suggest that M1 activation may enhance implant-adherent macrophage defense against *S. epidermidis*. A complementary

study, using this model system to solicit alternative M2 activation in adherent macrophage, is currently underway.

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FIGURE 1.

Fabrication of the biotin-PEG base substratum. mPEG-silane (MW 2000) and biotin-PEG-silane (MW 3400) are covalently grafted onto piranha-activated glass coverslips, from a 90% (molar) mPEG-silane/10% biotin-PEG-silane solution in toluene. Biotinylated, macrophage-activating ligands are subsequently immobilized to this substratum via streptavidin-biotin chemistry.

FIGURE 2.

Characterization and optimization of biotin-PEG base substratum. Glass coverslips grafted with mixtures of mPEG-silane and biotin-PEG-silane were tested for their resistance to nonspecific protein adsorption, and the optimal molar % of biotin-PEG determined via quantitation of streptavidin binding. (A) ¹²⁵I-fibrinogen adsorption to control glass and PEG-grafted coverslips. All samples were tested in at least triplicates; values are expressed as the mean $\pm$ SD. One-way ANOVA was performed to test significance of differences among mean values, with post-hoc testing via Tukey's test to compare all pairs of means; asterisks (*) denote values significantly different from glass ($p < 0.05$). (B) ¹²⁵I-streptavidin binding to 0, 0.1, 1, 10, and 100% biotin-PEG after 1-hour incubation with 0.1 mg/mL solution.

FIGURE 3.

BMDM response to ligand-presenting surfaces over 24 h. BMDM were cultured on the ligand-presenting surfaces or on TCPS with soluble IFN- γ $\pm$ LPS (10 ng/mL), for 24 h. Cell culture supernatant was collected and assayed for IL-12(p40) and NO; cell numbers were quantitated via LDH assay. (A) IL-12(p40) response, normalized by cell number; (B) NO response, normalized by cell number. All samples were tested in at least triplicates; values are expressed as the mean value $\pm$ SD. Representative results from multiple independent experiments are shown.

FIGURE 4.

BMDM response to ligand-presenting surfaces over 72 h. Cell culture supernatant was collected every 24 h, and media replenished; cell numbers were quantitated at every timepoint. (A) IL-12(p40) response, normalized by cell number; (B) NO response, normalized by cell number. All samples were tested in at least triplicates; values are expressed as the mean value $\pm$ SD. Representative results from multiple independent experiments are shown.

FIGURE 5.

BMDM phagocytosis of *S. epidermidis* RP62A. BMDM were pre-cultured on the ligand-presenting surface for 24 h, or with cytochalasin D ($10 \mu M$) for 1 h. pHrodo™-labeled *SE* RP62A cells were added to the macrophages at bacteria:macrophage ratio of 20:1, in 5% mouse serum, and incubated at $37^{\circ}C$, 5% CO_2 . The fluorescence of the wells (ex 550 nm/em 595 nm) was measured every hour for 6 h. All values are expressed as the mean value $\pm$ SD ($N=3$ for all treatments). One-way ANOVA was performed to test significance of differences among mean values; no significant differences were found (at $p < 0.05$).

FIGURE 6.

BMDM killing of *S. epidermidis* RP62A. BMDM were cultured on the ligand-presenting surface for 24 h, then challenged with *S. epidermidis* RP62A at a bacteria:macrophage ratio of 1:1 in the presence of 5% mouse serum. After 4h coincubation at 37°C, 5% CO₂, BMDM were lysed and surviving bacteria were quantitated via standard plate count method. Data is expressed as the mean colony forming unit count (CFU $\times 10^5$) per well $\pm$ SD. One-way ANOVA with *post hoc* testing was performed to test significance of differences among mean values. All macrophage-containing wells had significantly fewer surviving bacteria than bacteria-only wells ($p < 0.05$); asterisks (*) denote values significantly different relative to PEG ($p < 0.05$).

TABLE I

Elemental Composition of mPEG-silane/Biotin-PEG-silane-grafted Coverslips

Sample	% Composition				
	Si	O	C	N	Other atoms
Glass	23.5	58.0	15.6	–	2.8
PEG	14.7	44.9	37.0	2.8	0.7
1% biotin-PEG	16.4	48.1	32.7	2.4	0.5
10% biotin-PEG	15.0	46.6	35.3	2.6	0.6

TABLE II

High-resolution XPS C1s Composition of mPEG-silane/Biotin-PEG-silane-grafted Coverslips

Sample	% Composition		
	CH ₂	C—O	O—C(O)—NH
Glass	60	37	3
PEG	13	82	5
1% biotin-PEG	12	83	5
10% biotin-PEG	11	85	4

TABLE III

Estimation of Surface-immobilized Ligand Density via Fluorescence Labeling and Detection

	Ligand Density (pmol/cm ²)	
	IFN- γ	LPS
PEG:IFN- γ	0.2 ± 0.02 (6 ± 0.5)	–
PEG:LPS	–	0.04 ± 0.01 (0.8 ± 0.2)
PEG:IFN- γ /LPS	0.01 ± 0.003 (4 ± 0.8)	0.002 ± 0.0003 (0.4 ± 0.05)

Values within parentheses indicate total amount of ligand presented to macrophages per experiment (ng/well).