Characterization of Macrophage Behavior in the Human Immunoresponse and Bone Remodeling

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BRIEF. This study examined the relationship between macrophages and bacteria in chronic wounds and adapted a protocol studying osteoclast resorption.

ABSTRACT. Macrophages, common scavenger cells, play a versatile role in the human body, including the human immunoresponse and bone remodeling. This study focused on the response to external stimuli of two kinds of macrophages: osteoclasts and derivations of human monocytes. First, we aimed at determining if bacterial biofilms modify the immunoresponse of macrophages in wound healing. We treated macrophages with media preconditioned by bacteria (biofilm and planktonic) and mesenchymal stem cells. We triple stained the macrophages for phagocytosis and reactive oxygen species (ROS) production. Second, we adapted a protocol to examine osteoclasts' resorption of implant materials]. We filtered osteoclasts by size through a serum gradient and seeded onto the materials, then TRAP (tartrate resistant acid phosphatase) stained and imaged them to quantify cellular differentiation and material resorption. We did not observe any differences in ROS or phagocytic activity, save for an increase in phagocytic activity 16 hours after treatment. We recommend further testing of other variations of the conditioned media, due to the limited monocyte supply. Portions of the resorption protocol were adapted; in the future, we want to develop material testing.

INTRODUCTION.

Macrophages are important cells in humans that perform many vital jobs, especially within the immunoresponse and bones. In this study we examined the relationship between macrophages and bacteria in chronic wounds; second, we adapted a protocol for studying osteoclast resorption of implant materials.

In the immunoresponse (our immune system responding to foreign invaders) macrophages are among the first line of defense. Macrophages recognize invaders by using detectors (toll-like receptors) that identify ligands produced or present on the membrane of invaders [1]. Once detected, the macrophage destroys the invader by "eating", a process called phagocytosis. In phagocytosis, a macrophage engulfs the invader and injects it with reactive oxygen species (ROS), an oxidizing chemical which breaks down the invader and kills it. Then, the macrophage attaches bits of the invader onto its outer membrane, signaling a secondary line of defense[1]. In wound healing areas, macrophages also communicate with mesenchymal stem cells (MSCs) to direct wound healing [1]. MSCs are cells that could potentially turn into osteoblasts (bone-producing cells), chondrocytes (cartilage cells), and adipocytes (fat cells) [1]. This makes macrophages key to infection control.

In infected wounds, macrophages encounter two forms of bacteria: planktonic (free-floating) and biofilm. Biofilms harbor and protect the bacteria from the outside environment. Biofilms may produce signals that modify macrophages' normal activities. This could explain why chronic wounds resist healing [2]. Little research has been done on biofilms in chronic wounds; therefore, we sought to identify how bacteria, both biofilms and planktonic, signal with macrophages and/or MSCs to alter their normal activity. By conducting this research, we hope to draw closer to controlling infection in implant wounds, thus preventing costly medical operations and speeding up patient recovery time.

Macrophages also play a significant role in remodeling bone, where they differentiate to form large multi-nucleated osteoclasts. Osteoclasts use enzymes, such as TRAP, to break down and process existing bone – a process known as resorption. Osteoclasts resorb bone to make way for the formation of blood vessels (vascularization), remove diseased bone, and heal bones [3].

Osteoclasts' ability to resorb materials is also useful to break down implant materials for biomedical purposes. Biomedical implants are put in bone wounds to provide support and infection control, but must also dissipate to make room for natural bone. We developed a method to study osteoclast resorption of implant materials, an important consideration in material design [4]. We successfully adapted a protocol that can be used to examine osteoclast resorption of potential implant materials like polyurethane scaffolds.

In wounds osteoclasts need to use both the ability to fight off infection and to resorb bone to help moderate wound healing. This makes them a key player in wound sites. In this study we hope to further examine their potential role in helping implant materials.

MATERIALS AND METHODS.

Culture (Human Monocytes).

We cultured primary human monocytes (Reachbio, Seattle, WA) in Corning T25 flasks with 10 mL of modified Dulbecco's modified Eagle medium (DMEM) media and split them every 9 days. We changed media on Day 1, 5, and 7, according to Table 1. Penicillin streptomycin was used as the antibiotic.

Composition of Media Changes

Day	DMEM Media	FBS	Human Serum	Antibiotic
1	69%	20%	10%	1%
5	89%	0%	10%	1%
7	98%	0%	1%	1%

We split cells and seeded at a density of 7 x 10^4 cells/flask with 50 ng of macrophage colonizing-stimulating factor (M-CSF) for 9 days in an incubator at 37°C. We replenished M-CSF with media changes on Days 5 and 7. FBS was purchased from Fisher Scientific (Pittsburgh, PA) and human serum from Sigma Aldrich.

Conditioned Media.

The U.S. Army Institute of Surgical Research (ISR) collected conditioned media. To make this media, they extracted both biofilms and planktonic bacteria, varying in strength and invasiveness, from the nare and deep wounds and cultured the bacteria in media. ISR used the media from this to culture MSCs, and then sent the resulting media to our lab for testing

Seeding With LPS and Conditioned Media.

We cultured macrophages with lipopolysaccharide (LPS, Sigma Aldrich product #L2880), seeded the macrophages in 24 well plates at a density of 2.59 x 10^4 cells/well, and filled up to 0.5 mL with a 3.9% LPS in Day 5 Media solution. Cells incubated at 37°C for 3 hours and were then triple stained.

We also cultured macrophages with the conditioned media mentioned previously. We seeded cells in 48 well plates at 2.55 x 10^4 cells/well; after 24 hours the media was replaced with 0.25 mL of Day 5 media and 0.25 mL of the respective conditioned media. In this study, we used a Non-Invasive/Strong/Biofilm (34820 B/M) and Invasive/Weak/Biofilm (37370 B/M) conditioned media; additionally, previous research in the lab used an Invasive/Strong/Biofilm (107917 B/M) and Invasive/Strong/Planktonic (107917 P/M) conditioned media. We added a control well, consisting of 3.25 x 10^4 cells/well and 0.5 mL of Day 5 media.

We incubated plates at 37°C for 16 and 24 hours (107917 B/M, 107917 P/M) or for 72 hours (34820 B/M, 37370 B/M) and triple stained them.

Triple Stain.

After culturing, we simultaneously stained macrophages for activity markers of phagocytosis and ROS production, along with a stain for cell count.

For phagocytosis activity, we stained cells with 200 µL of fluorescent BioParticle suspension (Vybrant Phagocytosis Assay Kit (V-6694)) according the kit's instructions. Next, we washed the cells with 0.5 mL of warm PBS for 5 minutes at 37°C. We added 0.5 mL of a solution containing 100 ng/mL Hoechst (stain for cell nucleases; ThermoScientific) and 5 µM Dihydroethidium (DHE, stain for ROS; Sigma Aldrich) in PBS to each well and incubated for 15 minutes at 37°C. Afterwards, we washed the cells with 0.5 mL of warm PBS in the incubator.

We read the well plates *via* a fluorescent plate reader, using the software i-Control 1.7, and reading fluorophores one at a time, with customized excitation bandwidths and emission wavelengths (Phagocytosis: 480, 520; Hoechst: 355, 495; DHE: 340, 612). We manually adjusted the gain to 25 or 35 for Phagocytosis and 50 for Hoechst and DHE, normalizing the readings to the Hoechst stain.

Culture (RAW-OCs).

We cultured RAW264.7 (received from the Center of Bone Biology, Vanderbilt University) cells in Corning T75 flasks with 12 mL of Complete DMEM Media (89% DMEM media, 10% FBS, 1% Antibiotic) for 3-4 days. We split cells and seeded at a density of 1.13 x 10^7 cells/flask with Complete DMEM Media. Two hours after splitting, we cultured cells with 30 ng/mL of receptor activator of nuclear factor kappa-B ligand (RANKL, R&D Systems) for 4 days in an incubator at 37°C, replenishing RANKL and media every 2 days [4,5].

Serum Gradient.

After 4 days of culturing, we passed RAW-OCs through a serum gradient according to two previous publications [4,5]. Collagenase, trypsin, and EDTA were purchased from Sigma Aldrich (product number C0255, T1426, and 2854 respectively). Once the cells were filtered, we seeded them at a density of 1-2 x 10^5 cells/well onto polyurethane (PUR) films and Dentine chips in a 24 well plate. We sterilized biomaterial samples and soaked them in Complete DMEM Media a day prior to seeding. We incubated cells at 37°C for 15 minutes, and then filled the wells up to 1 mL Complete DMEM Media and incubated at 37°C for 24 hours [4,5].

TRAP Stain and Imaging.

We stained samples for TRAP using the Sigma TRAP Stain Kit (Sigma Aldrich 387A). We removed the old media in wells and fixated cells with 250 µL of Fixative Solution (25.5% Citrate Solution, 66.3% Acetone, 8.2% Formaldehyde (37%)) for 30 seconds; we removed the fixative solution washed samples twice with 300-400 µL of DI H2O. We removed the DI H2O and added 0.5 mL of staining solution to each well. The staining solution was composed of 90.9% pre-warmed DI H2O, 2% Fast Garnet GBC Solution (50% Fast garnet GBC Base Solution, 50% NaNO2), 1% Naphthol AS-BI Phosphate (N-AS-BI-P) Solution, 4% Acetate, and 2% Tartrate; we warmed the staining solution to 37°C before adding to samples. We removed the well plate after incubating at 37°C for 1 hour and washed samples with 300-400 µL DI H2O. We examined samples using an inverted microscope.

RESULTS.

Triple Stain.

We generated fluorescent readings (Fluorescence vs. nm) from a fluorescent plate reader to measure phagocytosis activity, ROS production, and cell number. We normalized phagocytosis and ROS measurements by the cell number to ensure differing amounts of cells in each well did not skew the measurements. We analyzed all treatments in the same manner.

We then normalized the results of this analysis to the controls in each series of experiments. Each experiment contained the same control conditions but a different cell count, so data sets could not be compared directly; thus, normalizing to the control enabled data sets to be directly compared. Results were expressed in 'relative fluorescence' (Figures 1 and 2); a value of 1 means relative fluorescence is equal to the control.

Phagocytosis.

Phagocytosis activity results are shown in Figure 1. At 16 hours, we found significant differences ($p < 0.05$) between all LPS, biofilm, and planktonic groups and the control. At 16 hours, we did not observe a significant difference between the biofilm and planktonic groups. We also noticed that the 16 hour treatment groups produced a stronger fluorescent signal than the 24 and 72 hour treatments.

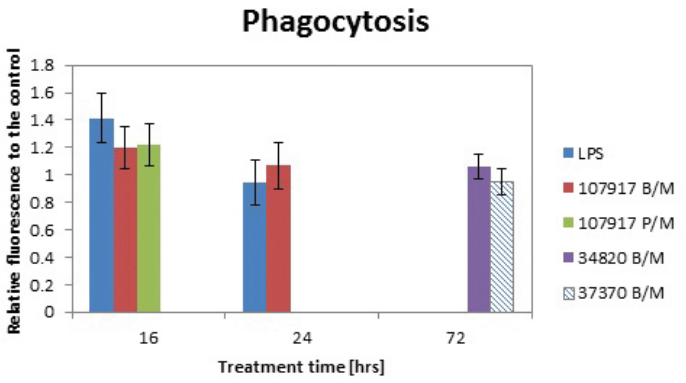

Figure 1. This graph shows the results of the phagocytosis activity marker. Error bars show standard deviation.

ROS Production.

Figure 2 shows ROS production results. None of the treatment groups displayed a significant difference from the control group. Also, none of the treatment times were significantly different from each other.

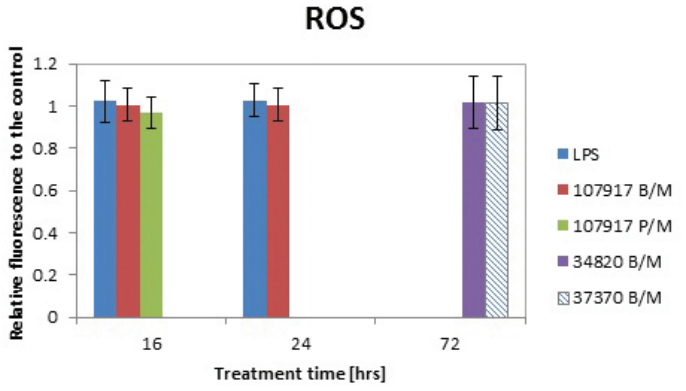

Figure 2. This graph shows the results of the phagocytosis activity marker. Error bars show the standard deviation of each data set.

Serum Gradient.

Figures 3A-D show images of a successful serum gradient at a scale of 100 μm.

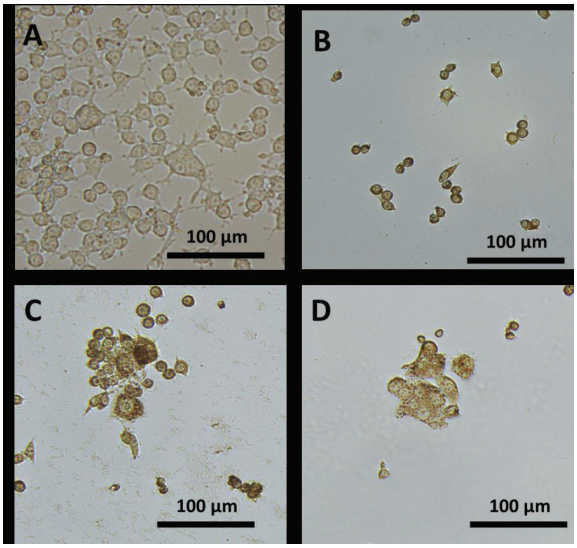

Figure 3. A. RAW-OCs before being passed through the Serum Gradient. **B.** Mononucleated RAW-OCs from the top layer of the Serum Gradient. **C.** Mono- and multi-nucleated RAW-OCs from the middle layer. **D.** Multi-nucleated RAW-OCs from the bottom layer.

DISCUSSION.

Triple Stain.

The phagocytosis stain results (Figure 1) suggest bacteria modify macrophage phagocytic activity at 16 hours. However, we observed no significant difference between planktonic and biofilm bacteria, suggesting both modify phagocytic activity in a similar manner. The fluorescent signal decreased from 16 to 72 hours, implying the conditioned media influences macrophage behavior at early (16 hours) instead of later time points (24 and 72 hours). This is important because it suggests macrophage phagocytic activity is modified early after exposure to bacteria present in the wound. To support this, we could analyze the stains in time increments between 0 and 16 hours, seeing as the macrophage response may occur even sooner.

The ROS stain results (Figure 2) show no difference in ROS production levels between treatment and control groups. This suggests that bacteria, both planktonic and biofilm, do not modify ROS production, contradicting the hypothesis posed previously. Prior research has found that bacteria have numerous motives and mechanisms to modify macrophage activity, ROS being one of them [1]. Bacterial effects on macrophages could vary by species and the bacterial specimens used in this study were not a purified strain.

Overall, the Triple Stain results do not strongly support the hypothesis that bacteria in infected wounds modify macrophage behavior. We did not find significant differences between the treatment and control groups, except in phagocytosis activity at 16 hours. However, we focused on only two forms of macrophage activity, phagocytosis and ROS production, meaning bacteria could interact with macrophages in other ways, such as by modifying their surface to avoid detection [1]. Additionally, we used bacteria taken from infected wounds, not pure bacteria species employed in other studies. One sample can contain several bacteria species, each of which may affect macrophage activity differently. Although isolated bacteria provide a clinically relevant study material, variability in the results could be eliminated with the utilization of pure strains.

Bone Remodeling.

We successfully replicated the serum gradient and separated RAW-OCs based on size (Figures 3A-D). With a resorption protocol in place, we can now investigate osteoclast degradation of implant materials. Continued degradation research is necessary because the natural degradation of biomaterials, hydrolysis, does not occur at a fast enough rate. Previous research has found that implant materials remain solid in wounds too long, blocking natural healing; in addition, most degradation is non-specific and cannot be controlled [4,8]. To solve this dilemma, we propose to include peptides specific to enzymes secreted by osteoclasts in the materials' formulation; this method should present a manageable way to control degradation location and rate [4]. Currently our lab has included Cathepsin K (Cath K) sensitive peptides in the backbone of biodegradable polyurethanes. We will use the adapted resorption protocol to characterize the osteoclast degradation of the developed polyurethanes. We expect to identify resorption pits created by the differentiated RAW-OC on the surface of the Cath K-sensitive materials and quantify the osteoclasts' effect by measuring the area and number of resorption pits.

CONCLUSION.

This study examined the behavior of macrophages in the human immunoresponse and bone remodeling. To characterize macrophage activity in bacterial-infected wounds, we cultured macrophages with conditioned media and measured for phagocytosis activity and ROS production. Based on these results, we conclude the 16 hour treatment is preferable to the 24 or 72 hour treatments; and that overall phagocytosis and ROS production activity markers did not significantly differ from the control. This implies the bacteria did not modify macrophages strongly; however, this is not conclusive because both the Hoechst and DHE stain target nucleic acids. For further testing, we must reevaluate the staining. Additionally, a small human monocyte supply due to the difficulty in culturing these cells limited testing; in the future, we would prefer to experiment with other variations of conditioned media on a larger supply of monocytes, in the hopes of a stronger conclusion.

To further the study of osteoclasts in bone remodeling, we adapted and implemented a protocol for seeding RAW-OCs onto implant materials. We differentiated RAW cells into RAW-OCs with RANKL and then through a serum gradient filtering by size. We then seeded RAW-OCs onto implant materials (PUR films and Dentine) and analyzed them for resorption. In the future, we want to utilize this protocol to study degradation of different biomaterials in a wound setting. For example, we are using the developed protocol to study osteoclast mediated degradation of biomaterials which incorporate enzyme-sensitive peptides produced by active osteoclasts.

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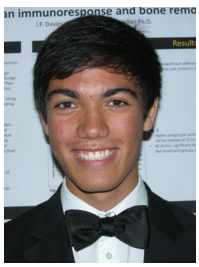

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