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A Macrophage Reporter Cell Assay to Examine Toll-Like Receptor-Mediated NF-kB/AP-1 Signaling on Adsorbed Protein Layers on Polymeric Surfaces

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Cover Letter

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Dr. Nandita Singh
Senior Science Editor: JoVE

Dear Dr. Singh,

Please find attached the manuscript we are submitting as an invited methods article to the Journal of Visualized Experiments (JoVE), entitled "A macrophage reporter cell assay to examine Toll-like receptor-mediated NF-kB/AP-1 signaling on adsorbed protein layers on polymeric surfaces". The attached manuscript was authored by Laura A. McKiel, Kimberly A Woodhouse and Lindsay E. Fitzpatrick (corresponding author) and is a methods paper that describes the use of a macrophage report cell line to investigate the contribution of damage-associated molecular patterns and Toll-like receptors on various polymer surfaces. The many of methods have previously been described in an original research article "McKiel LA & Fitzpatrick LE. Toll-like Receptor 2-Dependent NF-κB/AP-1 Activation by Damage-Associated Molecular Patterns Adsorbed on Polymeric Surfaces. *ACS Biomater. Sci. Eng.* 4, 3792–3801 (2018)." All authors have seen and approved the submission of this manuscript.

The performance of implanted biomedical devices is heavily dependent on the host response that occurs at the biomaterial-tissue interface. However, the molecular mechanisms that determine macrophage activation and inflammatory responses to biomaterials are not fully understood. Toll-like receptors (TLR) play a critical role in host defense by initiating sterile inflammatory responses to damage-associated molecular patterns (DAMPs), released by damaged and stressed tissues. While the role of TLR in sterile inflammatory host responses to solubilized polymer molecules, nanoparticles and phagocytosable microparticles is relatively well established, the current literature on the role of TLR signaling in macrophage responses to solid, non-phagocytosable biomaterials is limited. Therefore, we chose to examine TLR signaling in macrophages using an *in vitro* model for generating DAMP-containing adsorbed protein layers. This manuscript provides detailed methods on generating poly(methyl methacrylate), poly(dimethylsiloxane) and fluorinated poly(tetrafluoroethylene) surfaces, generation of 3T3 fibroblast lysate as an in vitro model of a cell-derived, complex DAMP-containing protein mixture, and cell culture protocols for indirectly measuring NF-κB/AP-1 activity in macrophages cultured on protein adsorbed surfaces using a colourimetric alkaline phosphatase activity assay. Representative results are provided as well. We believe the focus and scope of this manuscript is well suited for publication in JoVE.

We look forward to receiving your feedback about the suitability of this manuscript for publication. Should you require further information, please do not hesitate to contact me directly.

Yours sincerely,

Lindsay Fitzpatrick, Ph.D.
Assistant Professor
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TITLE:

A Macrophage Reporter Cell Assay to Examine Toll-Like Receptor-Mediated NF-kB/AP-1 Signaling on Adsorbed Protein Layers on Polymeric Surfaces

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KEYWORDS:

protein adsorption, foreign body reaction, macrophage, toll-like receptor, inflammation, polymer, biomaterial, transcription factor, damage-associated molecular patterns, cell-material interactions

SUMMARY:

This protocol provides researchers with a rapid, indirect method of measuring TLR-dependent NF-κB/AP-1 transcription factor activity in a murine macrophage cell line in response to a variety of polymeric surfaces and adsorbed protein layers that model the biomaterial implant microenvironment.

ABSTRACT:

The persistent inflammatory host response to an implanted biomaterial, known as the foreign body reaction, is a significant challenge in the development and implementation of biomedical devices and tissue engineering constructs. Macrophages, an innate immune cell, are key players in the foreign body reaction because they remain at the implant site for the lifetime of the device, and are commonly studied to gain an understanding of this detrimental host response. Many biomaterials researchers have shown that adsorbed protein layers on implanted materials influence macrophage behavior, and subsequently impact the host response. The methods in this paper describe an in vitro model using adsorbed protein layers containing cellular damage molecules on polymer biomaterial surfaces to assess macrophage responses. An NF-κB/AP-1 reporter macrophage cell line and the associated colorimetric alkaline phosphatase assay were used as a rapid method to indirectly examine NF-κB/AP-1 transcription factor activity in response to complex adsorbed protein layers containing blood proteins and damage-associated molecular patterns, as a model of the complex adsorbed protein layers formed on biomaterial surfaces in vivo.

INTRODUCTION:

The foreign body reaction (FBR) is a chronic host response that can negatively impact the performance of an implanted material or device (e.g., drug delivery devices, biosensors), through the persistent release of inflammatory mediators and by impeding integration between the implanted material and the surrounding tissue[1]. This innate immune response is initiated by the implantation procedure and is characterized by the long-term presence of innate immune cells and fibrous capsule formation around the implant[1]. Within the context of material host responses, macrophage-material interactions have a significant impact on the progression of the host response and development of a FBR[1]. Macrophages are a diverse innate immune cell population, recruited to the implant site either from tissue-resident macrophage populations or from the blood as monocyte-derived macrophages. They begin to accumulate at the implant site shortly after implantation, and within days become the predominant cell population in the implant microenvironment. Material-adherent macrophages, along with foreign body giant cells (FBGC) formed through macrophage fusion, can persist at the material surface for the lifetime of the implant[2,3]. Consequently, macrophages are considered to be key players in the foreign body response due to their roles orchestrating the characteristic steps of the FBR: acute inflammatory response, tissue remodeling, and formation of fibrotic tissue[1].

Toll-like receptors (TLRs) are a family of pattern recognition receptors that are expressed by many immune cells, including macrophages, and have been shown to play a significant role in inflammation and wound healing. In addition to pathogen-derived ligands, TLRs are able to bind endogenous molecules, known as damage-associated molecular patterns (DAMPs), which are released during cell necrosis and activate inflammatory signaling pathways resulting in the production of proinflammatory cytokines[4]. We and others have proposed that damage incurred during soft tissue biomaterial implantation procedures release DAMPs, which then adsorb to biomaterial surfaces in addition to blood proteins and modulate subsequent cell-material interactions[5,6]. When macrophages interact with the adsorbed protein layer on an implant, their surface TLRs may recognize adsorbed DAMPs and activate proinflammatory signaling cascades, leading to NF-κB and AP-1 transcription factor activation and production of proinflammatory cytokines. We have previously shown that murine macrophages have significantly increased NF-κB/AP-1 activity and tumor necrosis factor α (TNF-α, proinflammatory cytokine) secretion in response to DAMP-containing adsorbed protein layers on a variety of polymeric surfaces compared to surfaces with adsorbed serum or plasma only (i.e., no DAMPs present), and that this response is largely mediated by TLR2, while TLR4 plays a lesser role[5].

The NF-κB/AP-1 reporter macrophage cell line (**Table of Materials**) used in this protocol is a convenient method to measure relative NF-κB and AP-1 activity in macrophages[5,7,8]. In combination with TLR pathway inhibitors, this cell line is a useful tool for investigating TLR activation and its role in inflammation in response to a variety of stimuli[5,7,8]. The reporter cells are a modified mouse macrophage-like cell line that can stably produce secreted embryonic alkaline phosphatase (SEAP) upon NF-κB and AP-1 transcription factor activation[9]. The colorimetric enzymatic alkaline phosphatase assay (**Table of Materials**) can then be used to quantify relative amounts of SEAP expression as an indirect measure of NF-κB/AP-1 activity. As NF-κB and AP-1 are downstream of many cell signaling pathways, neutralizing antibodies and inhibitors targeting specific TLRs (e.g., TLR2) or TLR adaptor molecules (e.g., MyD88) can be used

to verify the role of a specific pathway. The methodology described in this article provides a simple and rapid approach for assessing the contribution of TLR signaling in murine macrophage responses to a variety of polymeric surfaces with adsorbed protein layers containing both blood proteins and DAMPs as an in vitro model of implanted biomaterials.

PROTOCOL:

1. Media and reagent preparation

1.1. Prepare fibroblast media. Combine 450 mL of Dulbecco's modified Eagle medium (DMEM), 50 mL of fetal bovine serum (FBS), and 5 mL of penicillin/streptomycin. Store at 4 °C for up to 3 months.

1.2. Prepare reporter macrophage growth media in 50 mL aliquots. Combine 45 mL of DMEM, 5 mL OF FBS, 5 µg/mL mycoplasma elimination reagent (**Table of Materials**), and 200 µg/mL phleomycin D1 (**Table of Materials**). Store at 4 °C for up to 3 months.

1.3. Prepare reporter macrophage assay media in 50 mL aliquots. Combine 45 mL of DMEM, 5 mL of heat inactivated FBS (HI-FBS), 5 µg/mL mycoplasma elimination reagent, and 200 µg/mL phleomycin D1. Store at 4 °C for up to 3 months.

2. Coating cell culture surfaces with poly(methyl methacrylate)

2.1. Dissolve poly(methyl methacrylate) (PMMA) in chloroform at 20 mg/mL (e.g., 100 mg of PMMA in 5 mL of chloroform) in a 20 mL glass scintillation vial. Place a magnetic stir bar in the vial and allow to stir for at least 2 h, until all solids are dissolved.

CAUTION: Chloroform is harmful if inhaled. Ensure to use solvent in a fume hood while wearing PVA gloves.

2.2. Pipette 400 µL of PMMA solution onto the center of a borosilicate glass microscope slide in a spin coater, and spin at 3000 rpm for 2 min. Prepare the number of slides required for the assay, as well as 3−5 extra for water contact angle measurement. Store slides in a clean box (sprayed and wiped with 70% ethanol) for future use.

NOTE: Spin coating is often used to deposit a thin, uniform coating on a flat surface. A spin coater rotates a substrate at high speeds, using centrifugal force to spread the coating solution over the surface.

2.2.1. Measure water contact angle at two random positions on the surface of extra coated slides (i.e., not the slides being used for cell culture) with a goniometer to ensure glass surface was completely coated with the polymer.

NOTE: Only water of the highest purity (e.g., glass triple distilled) should be used for water

contact angle measurements.

2.3. In a biological safety cabinet (BSC) attach 8-chamber sticky wells to PMMA coated-slides using sterile forceps and following aseptic technique. Press firmly on the top of the sticky wells to make sure they are strongly attached. Incubate the slides with attached sticky-wells at 37 °C overnight to secure the seal.

2.3.1. Test the seal of the sticky wells by adding 200 µL of cell culture grade (endotoxin-free) water to each well. Incubate at room temperature (RT) for 60 min and ensure no leakage before proceeding. Aspirate the water, being careful not to disturb the PMMA coating.

2.4. Perform endotoxin-free water washes by adding 300 µL of endotoxin-free water to each well and incubating for 1 h (three times), 12 h, and 24 h prior to use to remove any remaining solvent.

2.5. Test endotoxin concentration of the slides to be used for cell culture. Incubate 200 µL of endotoxin-free reagent water (**Table of Materials**) in one well of each slide for 1 h. Measure endotoxin concentration in the extract using an endpoint chromogenic endotoxin assay (**Table of Materials**).

NOTE: The following protocol is specific to the endotoxin assay kit listed in the **Table of Materials**. Use only water and consumables (i.e., pipette tips, microcentrifuge tubes and well plates) that are certified pyrogen-free (i.e., endotoxin-free) for this work. Also, any glassware used in the preparation of the polymer-coated surfaces should be depyrogenated using dry heat sterilization (250 °C for 30 min) prior to use[10]. Measuring endotoxin in the extract solution, as described here, can result in an underestimation of endotoxin on the material surface[11,12]. Consequently, it is recommended that when developing a polymer coating protocol, perform the endotoxin assay reaction (i.e., steps 2.5.4–2.5.6 for test samples [reagent water] or spike controls) directly within wells containing the coated sample to ensure no sources of endotoxin are inadvertently introduced into the system during the coating process.

2.5.1. Bring all test samples (i.e., extracts) and endotoxin assay reagents to RT. Reconstitute chromogenic reagent in assay buffer and endotoxin standard in reagent water, allow to dissolve for 5 min and gently swirl before using. Cover all bottles with paraffin film when not in use.

2.5.2. Create a 5–8 point standard dilution curve of endotoxin standard ranging from the lower to the upper limit of the assay by performing a serial dilution of the endotoxin standard in reagent water.

2.5.3. To control for enhancement or inhibition of the endotoxin assay in test samples, prepare a positive control (also called a spike control or spiked sample) by diluting a known amount of endotoxin in unused test sample solution.

NOTE: The concentration of the positive control should be the same concentration as a standard in the middle of the standard curve. If the recovered amount of the endotoxin spike (i.e.,

concentration of the positive control minus the concentration of the unspiked test sample) is within 50−200% of the nominal concentration of the endotoxin spike, the extraction solution can be considered to not significantly interfere with the assay.

2.5.4. Add 50 µL of standards, samples, or spike controls to each well of a 96-well plate in duplicate or triplicate. Use reagent water as a negative control.

2.5.5. Add 50 µL of chromogenic reagent to every well. Add reagent quickly to all wells. Use a timer to record the amount of time it takes to add reagent to all wells. Cover the plate with an adhesive seal and incubate at 37 °C (incubation time is lot-dependent and stated on Certificate of Analysis included in the chromogenic reagent kit). Alternatively, check on the plate every 15 min during incubation until color change is observed in all standard wells.

2.5.6. After incubation, add 25 µL of 50% acetic acid to each well (final concentration of 10% acetic acid per well) to stop the reaction. Add acetic acid in the same order as the chromogenic reagent was added. Read absorbance of the plate using a plate reader at 405 nm. Aspirate liquid and discard plate.

NOTE: Acetic acid addition should take the same length of time to add to each well as the chromogenic reagent took (± 30 s).

2.6. Ultraviolet (UV) sterilize the slides for 30 min prior to cell culture experiments.

3. Coating cell culture surfaces with polydimethylsiloxane

3.1. Mix polydimethylsiloxane (PDMS) elastomer in a 10:1 weight ratio (base:curing agent). In a biological safety cabinet, pipette approximately 10 mL of polydimethylsiloxane base into a sterile tube. Weigh the tube and slowly add curing agent until 10% has been added.

CAUTION: Use PDMS reagents in a well-ventilated area and avoid eye contact by wearing safety glasses.

3.2. Thoroughly mix the elastomer by stirring with a sterile serological pipette tip and by pipetting up and down. Add approximately 200 µL of the solution to each well of a 48-well plate. Tilt the well plate slowly to ensure complete coverage of wells with elastomer solution.

3.3. Place the well plate with elastomer into a vacuum oven set at 50 cmHg, 40 °C. Remove the lid and cover with a single-ply wipe to prevent other debris from falling into the wells. Allow to incubate for at least 48 h.

3.3.1. Confirm the wells are completely coated via visual inspection. Ensure the elastomer is fully cured by gently prodding with a sterile pipette tip before removing.

3.4. Add 300 µL of 70% ethanol (made with absolute ethanol and endotoxin-free water) and

incubate at RT for 1 h. Remove the ethanol and perform endotoxin-free water washes by adding 300 µL of endotoxin-free water to each well and incubating for 1 h (three times), 12 h, and 24 h prior to use to remove any remaining solvent.

3.4.1. Incubate 200 µL of endotoxin-free water in three wells of each plate for 1 h. Measure endotoxin concentration of the water extracts using an endpoint chromogenic endotoxin assay (steps 2.5.1−2.5.6).

4. Coating cell culture surfaces with fluorinated poly(tetrafluoroethylene)

4.1. Make a 1 mg/mL solution of fluorinated poly(tetrafluoroethylene) (fPTFE) (e.g., add 10 mg of fPTFE to 10 mL of fluorinated solvent [**Table of Materials**]) in a 20 mL glass scintillation vial. Place a magnetic stir bar in the vial and allow to stir for at least 24 h, until all solids are dissolved.

4.2. Add approximately 150 µL of the polymer solution to each well of a polystyrene 48-well plate (i.e., not tissue culture treated). Tilt the well plate slowly to ensure complete coverage of all wells with polymer solution. Replace lid.

4.2.1. To ensure effective fPTFE-coating of wells, glass coverslips should be coated in fPTFE and used for water contact angle measurement (step 4.3.1). Place coverslips inside the wells of a 24-well plate. Add approximately 400 µL of the polymer solution to each well containing a coverslip. Push the coverslips down using sterile forceps, ensuring they are completely covered in polymer solution, and cover the well plate with a lid.

4.3. Place the well plate with polymer solution and/or coverslips into a vacuum oven set at 50 cmHg, 40 °C. Remove the lid and cover with a single-ply wipe to prevent other debris from falling into the wells. Allow to incubate for at least 48 h.

4.3.1. Measure water contact angle of fPTFE-coated coverslips with a goniometer to ensure effective coating.

NOTE: Only water of the highest purity (e.g., glass triple distilled) should be used for water contact angle measurements.

4.4. Add 300 µL of 70% ethanol (made with absolute ethanol and endotoxin-free water) and incubate at RT for 1 h. Remove the ethanol and perform endotoxin-free water washes by adding 300 µL of endotoxin-free water to each well and incubating for 1 h (three times), 12 h, and 24 h prior to use to remove any remaining solvent.

4.4.1. Incubate 200 µL of endotoxin-free water in three wells of each plate for 1 h. Measure endotoxin concentration of water extracts using an endpoint chromogenic endotoxin assay (steps 2.5.1−2.5.6).

4.5. UV sterilize the well plates for 30 min prior to cell culture experiments.

5. Making lysate from 3T3 cells

5.1. Grow 3T3 cells in multiple T150 flasks to 70% confluence. To detach cells, aspirate media, wash surface with 5 mL of PBS, and aspirate PBS. Add 5 mL of animal origin-free, recombinant cell dissociation enzyme (**Table of Materials**) and incubate at 37 °C for 3–5 min.

5.2. Detach cells by gently tilting the flask back and forth. Add 5 mL of PBS to neutralize the recombinant enzyme used for cell dissociation. Transfer the detached cells from the flasks into a centrifuge tube and mix via pipetting. Perform a live cell count using a hemocytometer and cell viability dye.

NOTE: A cell dissociation enzyme that can be neutralized through dilution in PBS was selected to avoid the introduction of serum-based proteins in the lysate preparation. If trypsin is used to dissociate cells, it should be neutralized with a serum-containing solution, and an additional PBS wash should be performed to reduce the amount of serum proteins carried over into the lysate preparation.

5.3. Centrifuge the cells at 200 x *g* for 5 min. Aspirate the supernatant and resuspend cells in original volume (i.e., 10 mL x number of flasks) of PBS to wash off any remaining media. Repeat.

5.4. Centrifuge the cells again at 200 x *g* for 5 min and aspirate the supernatant. Add the volume of PBS required to achieve a final cell concentration of 1 x 10^6 cells/mL. Place the cell solution into a -80 °C freezer until sample is fully frozen (at least 2 h).

5.5. Thaw cell solution in a 37 °C water bath. Once completely thawed, place the solution back into the -80 °C freezer until totally frozen. Repeat for a total of 3 freeze-thaw cycles.

5.6. Perform a micro bicinchoninic acid (BCA) assay on the cell lysate at a variety of dilutions (e.g., 1/100, 1/200, 1/500, 1/1000) to determine the protein concentration. Dilute the cell lysate to a protein concentration of 468.75 µg/mL, aliquot, and store at -80 °C for future use.

NOTE: Final protein concentration in a 48-well plate is 125 µg/cm^2 (based on the surface area of one well, 0.75 cm^2).

5.7. Perform a Western blot to assess presence of DAMPs in lysate (e.g., heat shock protein 60 [HSP60], high mobility group box 1 [HMGB1]) by loading 40–60 µg of lysate protein in loading buffer onto a 1.5 mm thick 10% polyacrylamide gel and follow standard Western blot procedures.

6. Assessing effect of adsorbed protein layers and Toll-like receptors on NF-κB activity of macrophages

NOTE: For a schematic of the experimental workflow and plate layout, refer to **Figure 1A and Supplemental Figure 1**, respectively.

6.1. Grow reporter macrophages in an appropriately sized flask to 70% confluence. Aspirate media, wash surface with PBS, and aspirate PBS. Add the recombinant cell dissociation enzyme and incubate at 37 °C for 8 min.

6.2. Detach cells by firmly tapping the sides of the flask. Inactivate the recombinant cell dissociation enzyme by adding an equal volume of growth media (containing 10% FBS). Perform a live cell count using a hemocytometer and cell viability dye.

NOTE: Expected viability for the reporter macrophages following an 8 min incubation in the cell dissociation enzyme is 90%.

6.3. Centrifuge cells at 200 x *g* for 5 min. Aspirate supernatant and resuspend in original volume of PBS to wash cells. Centrifuge again and resuspend cells at 7.3 x 10^5 cells/mL in assay media (containing heat inactivated FBS).

6.4. Separate cell suspension into 3 different tubes: TLR4 inhibitor, anti-TLR2, and untreated. Incubate cells with 1 µg/mL TLR4 inhibitor for 60 min at RT or with 50 µg/mL anti-TLR2 for 30 min at RT.

6.5. Add 200 µL of lysate, 10% FBS, 10% commercial mouse plasma (**Table of Materials**), or a mixture of the protein solutions to a 48-well plate (or equivalent) and allow protein to adsorb at 37 °C for the desired amount of time (i.e., 30 min, 60 min, or 24 h). Aspirate protein solutions from wells, using a fresh Pasteur pipette for each protein solution, and wash surfaces with 250 µL of PBS for 5 min. Aspirate PBS. Repeat for a total of 3 washes.

NOTE: This step may need to be started earlier in the protocol depending on desired adsorption time. Adjust protocol accordingly.

6.6. After incubation period with the TLR4 inhibitor or anti-TLR2, pipette cells to resuspend. Add 200 µL of cell solution to each well.

6.7. For TLR2 positive control condition, add Pam3CSK4 to a final concentration of 150 ng/mL. For TLR4 positive control condition, add lipopolysaccharide (LPS) to a final concentration of 1.5 µg/mL. Incubate cells at 37 °C for 20 h.

6.8. Sample 20 µL of supernatant from each well and plate in duplicate into a 96-well plate. Include two wells of 20 µL assay media as a background control. Add 200 µL of SEAP reporter assay reagent to each well. Cover the plate with an adhesive seal and incubate for 2.5 h at 37 °C.

NOTE: The incubation time may vary depending on experimental conditions, and should be optimized for a strong difference in absorbance between positive and negative control wells.

6.8.1. Transfer the remainder of the supernatant to a 1.5 mL tube (per well). Centrifuge at 1,000

x *g* for 10 min to pellet any debris. Transfer supernatant to a new 1.5 mL tube and store at -80 °C. Analyze supernatant for the presence of proinflammatory cytokines (e.g., TNF-α, interleukin 6) via enzyme-linked immunosorbent assay (ELISA).

6.9. Remove adhesive plate seal. Read absorbance of the plate using a plate reader at 635 nm. Aspirate liquid and discard plate.

REPRESENTATIVE RESULTS:

Cleaning methods for the polymer-coated surfaces were tested to ensure there was no disruption of the coating, which would be seen as a change in the water contact angle to an uncoated glass coverslip (**Figure 2**). Soaking PMMA-coated microscope slides in 70% ethanol for 1 h was found to remove the PMMA coating (**Figure 2**, left panel), likely due to the solubility of PMMA in 80 wt% ethanol[13], therefore PMMA-coated surfaces were cleaned using 30 min of UV sterilization alone. The concentration of PMMA for coating was optimized previously[5]. A 1 h 70% ethanol soak was used to clean PDMS, and UV sterilization was neglected since UV light can cause chain scission and influence the surface wetting properties of PDMS[14]. Both 70% ethanol soak and UV sterilization did not influence the water contact angle of fPTFE-coated coverslips (**Figure 2**, right panel), therefore the two methods, in succession, were used to clean fPTFE coatings. The method of fPTFE coating was previously described by the Grainger group[15].

A Western blot was performed on the 3T3 lysate to ensure that DAMP species were present in the complex molecular mixture. The results showed that both HMGB1 and HSP60, two well-documented DAMPs[16,17], were present in the lysate (**Figure 1B**). The adsorption of TLR ligands from the lysate onto the polymer surfaces was confirmed by culturing reporter macrophages (untreated, TLR2 neutralized, or TLR4 inhibited) for 20 h on protein-adsorbed polymer surfaces (i.e., tissue culture-treated polystyrene [TCPS], PMMA, PDMS, fPTFE), and then indirectly assessing NF-κB/AP-1 activity based on SEAP production using an enzymatic assay (**Figure 1C** and **Figure 3**). Furthermore, the reporter macrophages had significantly increased NF-κB/AP-1 activity on adsorbed lysate compared to adsorbed FBS or plasma and no pre-adsorbed protein (media) (**Figure 4**). TLR ligands synthetic triacylated lipopeptide (Pam3CSK4, TLR2 ligand) and lipopolysaccharide (LPS, TLR4 ligand) were included as positive controls to confirm the antibody or inhibitor and the assay were working properly. TLR2 neutralization had a noticeably stronger reduction in NF-κB/AP-1 response of reporter macrophages to adsorbed lysate compared to TLR4 inhibition. As well, small amounts of lysate diluted in serum (based on total protein) induced significantly increased NF-κB/AP-1 response compared to serum alone, with the lowest effective dilution dependent on the polymer surface (**Figure 5**). These results demonstrate the potency of the adsorbed lysate-derived molecules on inducing TLR-dependent NF-κB/AP-1 activity in reporter macrophages on a variety of polymeric surfaces.

FIGURE LEGENDS:

Figure 1: Methods and results for the alkaline phosphatase assay of NF-κB/AP-1 reporter macrophages on TCPS, PMMA, PDMS, and fPTFE. (**A**) Diagram of the workflow for the reporter macrophage alkaline phosphatase assay. (**B**) Western blot of lysate confirming the presence of

DAMP species HMGB1 and HSP60, with β-actin as the loading control. (**C**) NF-κB/AP-1 activity (represented by absorbance) of reporter macrophages cultured on media (negative control), 10% FBS, lysate, and Pam3CSK4 (TLR2 ligand, positive control) for 20 h. Data shows the results of one experiment and is representative of results from at least 2 separate experiments, shown as mean ± standard deviation (SD). Each experiment used n = 3 separate wells per condition, and each well was plated in duplicate for the enzymatic assay. Analyzed using one-way ANOVA and Tukey post-hoc test. *** $p < 0.001$. This figure has been adapted with permission from McKiel and Fitzpatrick[5]. Copyright 2018 American Chemical Society.

Figure 2: Optimization of cleaning methods for PMMA- and fPTFE-coated surfaces, assessed using water contact angle (WCA). Measurements were taken on 2 separate spots of at least 3 coverslips. Data is shown as mean ± SD. Analyzed using one-way ANOVA and Tukey post-hoc test. * $p < 0.05$. This figure has been adapted with permission from McKiel and Fitzpatrick[5]. Copyright 2018 American Chemical Society.

Figure 3: TLR-mediated NF-κB/AP-1 activity (represented by absorbance) of reporter macrophages cultured on 10% FBS (control), lysate, and positive control for 20 h. (**A**) Influence of TLR2 neutralization on reporter macrophage responses to adsorbed lysate. Positive control is Pam (Pam3CSK4, TLR2 ligand). (**B**) Influence of TLR4 inhibition on reporter macrophage response to adsorbed lysate. Positive control is LPS (TLR4 ligand). Data shows the results of one experiment and is representative of results from at least 2 separate experiments, shown as mean ± SD. Each experiment used n = 3 separate wells per condition, and each well was plated in duplicate for the enzymatic assay. Analyzed using one-way ANOVA and Tukey post-hoc test. ** $p < 0.01$, *** $p < 0.001$. This figure has been adapted with permission from McKiel and Fitzpatrick[5]. Copyright 2018 American Chemical Society.

Figure 4: NF-κB/AP-1 activity (represented by absorbance) of reporter macrophages cultured on media (negative control), 30 min and 24 h adsorbed protein layers, and Pam3CSK4 (positive control) on TCPS for 20 h. Data is combined from 3 separate experiments and shown as mean ± SD. Each experiment used n = 3 separate wells per condition, and each well was plated in duplicate for the enzymatic assay (i.e., n = 9 non-independent cell culture wells and n = 18 non-independent enzymatic assay wells). Analyzed using one-way ANOVA and Tukey post-hoc test. *** $p < 0.001$.

Figure 5: Reporter macrophage NF-κB/AP-1 activity (represented by absorbance) after 20 h in response to dilutions of lysate in FBS (total protein = 280 μg/well) adsorbed to polymer surfaces for 30 min. (**A**) TCPS. (**B**) PMMA. (**C**) PDMS. (**D**) fPTFE. Data shows the results of one experiment and is representative of results from at least 2 separate experiments, shown as mean ± SD. Each experiment used n = 3 separate wells per condition, and each well was plated in duplicate for the enzymatic assay. Analyzed using one-way ANOVA and Tukey post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. This figure has been adapted with permission from McKiel and Fitzpatrick[5]. Copyright 2018 American Chemical Society.

Supplemental Figure 1: Example layouts used for NF-κB/AP-1 reporter macrophage cell culture

assay in 8-chamber and 48-well plate formats.

DISCUSSION:

A primary focus of our lab is the host response to solid biomaterial soft tissue implants, and in particular how the cellular damage incurred during the implantation procedure impacts the host response. The work presented here describes preliminary experiments using a reporter macrophage cell line and in vitro-generated DAMP-containing cellular lysate, to investigate the influence of molecules released during cellular damage (i.e., from the implant surgery) on macrophage responses to biomaterials. Fibroblast cell lysate was used to model the cellular damage and release of DAMPs due to biomaterial placement. Fibroblasts were chosen to create the lysate because of the prevalence of fibroblasts in soft tissue, as well as their ability to secrete a variety of extracellular matrix (ECM) proteins, including fibronectin[18]. Freeze-thaw cycling was chosen as the method of lysis to produce both intracellular and ECM-derived DAMPs, similar to what would be present in the implant environment. Protease inhibitors were not used to make this lysate. While uncontrolled cell lysis like freeze-thaw cycling can result in the release of proteases that may degrade DAMPs, these enzymes would also likely be present in the biomaterial implant environment when cells are damaged during the implantation procedure. The presence of DAMPs in the complex molecular mixture of the lysate was confirmed by Western blot (**Figure 1B**; HMGB1 and HSP60) and SEAP reporter assay (**Figure 1C**; NF-κB/AP-1 activity in response to adsorbed lysate). We have also performed assays where lysate was diluted in FBS based on total protein concentration and adsorbed onto cell culture surfaces (**Figure 5**) to better reflect the complexity of the implant environment, since it will contain an abundance of blood proteins as well as DAMPs[6]. Reporter macrophage NF-κB/AP-1 activity remained significantly increased on adsorbed layers from lysate diluted in FBS, and the lowest dilution to achieve significant activation was surface dependent, ranging from 0.1% (TCPS) to 10% (PDMS and fPTFE).

The polymers PMMA, PDMS, and PTFE were chosen for this work because they are nondegradable and have been used extensively in the literature to assess protein adsorption and macrophage response to biomaterials[19–25]. TCPS was also used for comparison since it is a common substrate used for in vitro macrophage and TLR signaling work[21,26–28]. The materials used in our work are representative examples of non-degradable, solid biomaterials. However, many other materials could be used with this model, provided the material can be coated onto cell culture plates or microscope slides and properly decontaminated. The NF-κB/AP-1 reporter macrophage cell line was selected for this in vitro model because it enables rapid, indirect measurement of NF-κB/AP-1 activity through the NF-κB/AP-1 inducible expression of SEAP. NF-κB/AP-1 reporter macrophages require the use of phleomycin D1 in the culture media as a selective antibiotic to ensure that only cells with the NF-κB/AP-1 inducible SEAP gene are present[29]. For the alkaline phosphatase assay, it is critical to use HI-FBS in the cell culture media to avoid potential false positive results generated by alkaline phosphatases present in serum. Our research to date suggests that FBS-adsorbed surfaces do not generate a detectable false positive result, likely because the serum molecules are strongly adsorbed to the culture surface and are not released into the supernatant. The culture time point for the reporter macrophages (20 h), assay incubation timepoint (2.5 h), and absorbance reading wavelength (635 nm) for the alkaline

phosphatase assay were optimized with this system to ensure robust and reproducible measurements for all conditions.

An initial protein adsorption timepoint of 30 min was chosen for this work due to its common use in protein adsorption literature (**Figure 1C**)[30–34]. However, we have also explored longer adsorption times (i.e., 60 min and 24 h, **Figure 4**) to better represent the adsorbed protein layer that macrophages would interact with in vivo, which is likely to occur 4−24 h following implantation[1]. It has been postulated that the majority of protein adsorption and exchange occurs in the first 60 min of exposure to a surface[26,35,36], therefore a 60 min adsorption time may be a more relevant timepoint. We have also moved from using FBS as a negative control for the presence of DAMPs in the adsorbed protein layer to commercial mouse plasma. The rationale for using plasma instead of serum is that plasma proteins are known to play significant roles in protein adsorption and macrophage response[1], and that plasma provides a better representation of the proteins in the wound environment. Plasma used in protein adsorption experiments is commonly prepared as a 1−10% dilution[26,36,37], which motivated our use of 10% plasma. Human plasma is commonly used[26,36], as it is easier to obtain in large quantities and more clinically relevant, compared to mouse plasma. However, we chose to use commercial mouse plasma for in this model to keep the species of the protein solutions consistent with that of the reporter cells.

The use of the reporter macrophage cell line introduced some limitations within the study. First, using a murine leukemic macrophage cell line has inherent limitations, as the phenotype and behavior may vary from primary macrophage cultures. While this limitation will be addressed in future work using primary macrophages, the parental macrophage cell line was shown to closely mimic mouse bone marrow-derived macrophages in terms of their cell surface receptors and response to microbial ligands for TLRs 2, 3 and 4[38]. Furthermore, the NF-κB/AP-1 reporter macrophages yielded similar results in response to HMGB1 and LPS stimulation when compared to peritoneal primary murine macrophages[39]. It should be noted that the NF-κB/AP-1 reporter macrophages, and their parental strain, do not express TLR5[40]. Researchers have demonstrated that HMGB1 was able to activate NF-κB transcription factors via the TLR5 signaling pathways in HEK-293 cells stably transfected with human TLR5[41]. Therefore, the contribution of HMGB1-TLR5 signaling to overall NF-κB activity on lysate-coated surfaces was neglected in this model. Additionally, the reporter macrophages and their parental strain do not express the ASC adaptor protein, and consequently do not form most types of inflammasomes and cannot process inactive IL-1β or inactive IL-18 to their mature forms[42]. Therefore, the model we have used does not account for the contribution of ASC-dependent inflammasome activity and subsequent autocrine IL-1β and IL-18 signaling in macrophage responses to lysate-adsorbed surfaces. Consequently, this assay is intended as a preliminary examination of TLR-dependent NF-κB activation, and subsequent research using primary macrophages is recommended to provide a more complete and representative understanding of macrophage activation and phenotype on material surfaces of interest.

The alkaline phosphatase assay indirectly measures the NF-κB/AP-1 activity of the reporter macrophages. However, there are many signaling pathways other than TLRs that involve NF-

κB/AP-1 (e.g., interleukin-1 receptor [IL-1R][43] and tumor necrosis factor receptor [TNFR][44]). Therefore, it was necessary to assess the contribution of TLR2 and TLR4 signaling in the increased NF-κB/AP-1 response to lysate-adsorbed surfaces using inhibition assays (**Figure 3**). The rationale for selecting these two surface TLRs was that at least 23 DAMPs that have been shown to signal through TLR2 and TLR4[45], including the well-characterized HMGB1, and both receptors are expressed on the cell surface and can interact directly with the biomaterial surface[6]. The TLR2 and TLR4 inhibition assays demonstrated that when TLR2 or TLR4 signaling were blocked, the NF-κB/AP-1 response of the reporter macrophages to adsorbed lysate was reduced, indicating that both pathways are involved. However, there was a noticeably larger reduction in NF-κB/AP-1 activity when TLR2 signaling was neutralized, suggesting that TLR2 may play a primary role in the response of reporter macrophages to adsorbed lysate. We recognize that there may be some off-target inhibition with the TLR signaling pathway neutralizing antibodies and inhibitors. A neutralizing antibody was used to inhibit the TLR2 pathway since there were not commercially available TLR2 inhibitor molecules at the time of this work.

The methods presented here use lysate, as a complex source of DAMPs, and NF-κB/AP-1 reporter macrophages as an in vitro model for macrophage responses to DAMPs and other proteins adsorbed to polymeric biomaterials (**Figure 1**). We anticipate our protocol can be used to quickly analyze NF-κB/AP-1 responses and upstream TLR signaling of reporter macrophages to a variety of materials (including degradable materials, porous scaffolds or hydrogels) and adsorbed protein layers (**Figure 3**). However, the use of porous materials and hydrogels will introduce complexity within the system, as it may be challenging to distinguish between adsorbed molecules and entrained molecules. We also anticipate that this protocol can be easily adapted to investigate the contribution of other signaling pathway upstream of NF-κB/AP-1 (e.g., C-type lectin receptors[46] and nucleotide-binding oligomerization domain (NOD)-like receptors[47]) with the appropriate inhibitors. Furthermore, the NF-κB/AP-1 response of reporter macrophages could be compared between different materials, provided responses are normalized to baseline cell activity (i.e., cells in media on each surface with no pre-adsorbed protein) and all materials have undetectable endotoxin levels.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

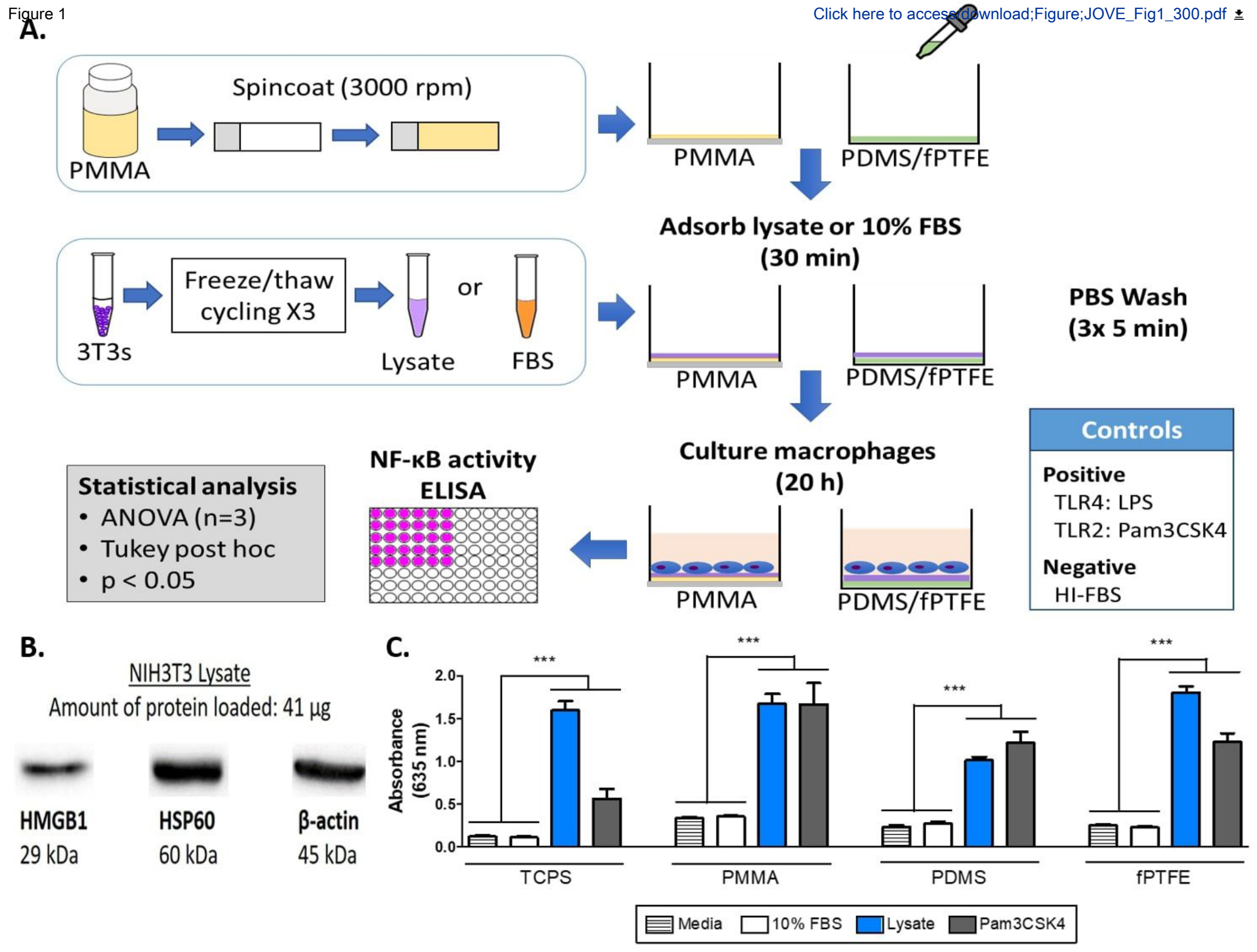


Figure 2

Click here to access/download;Figure;JOVE_Fig2_300.pdf

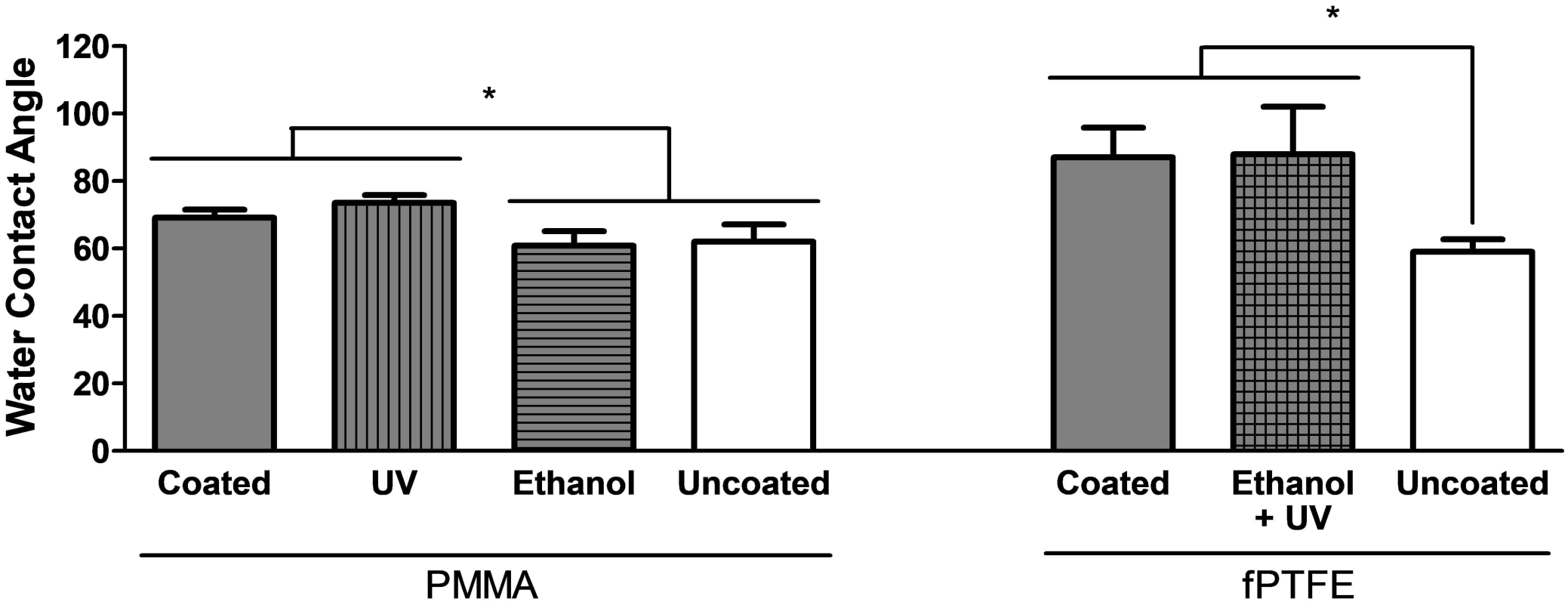


Figure 3

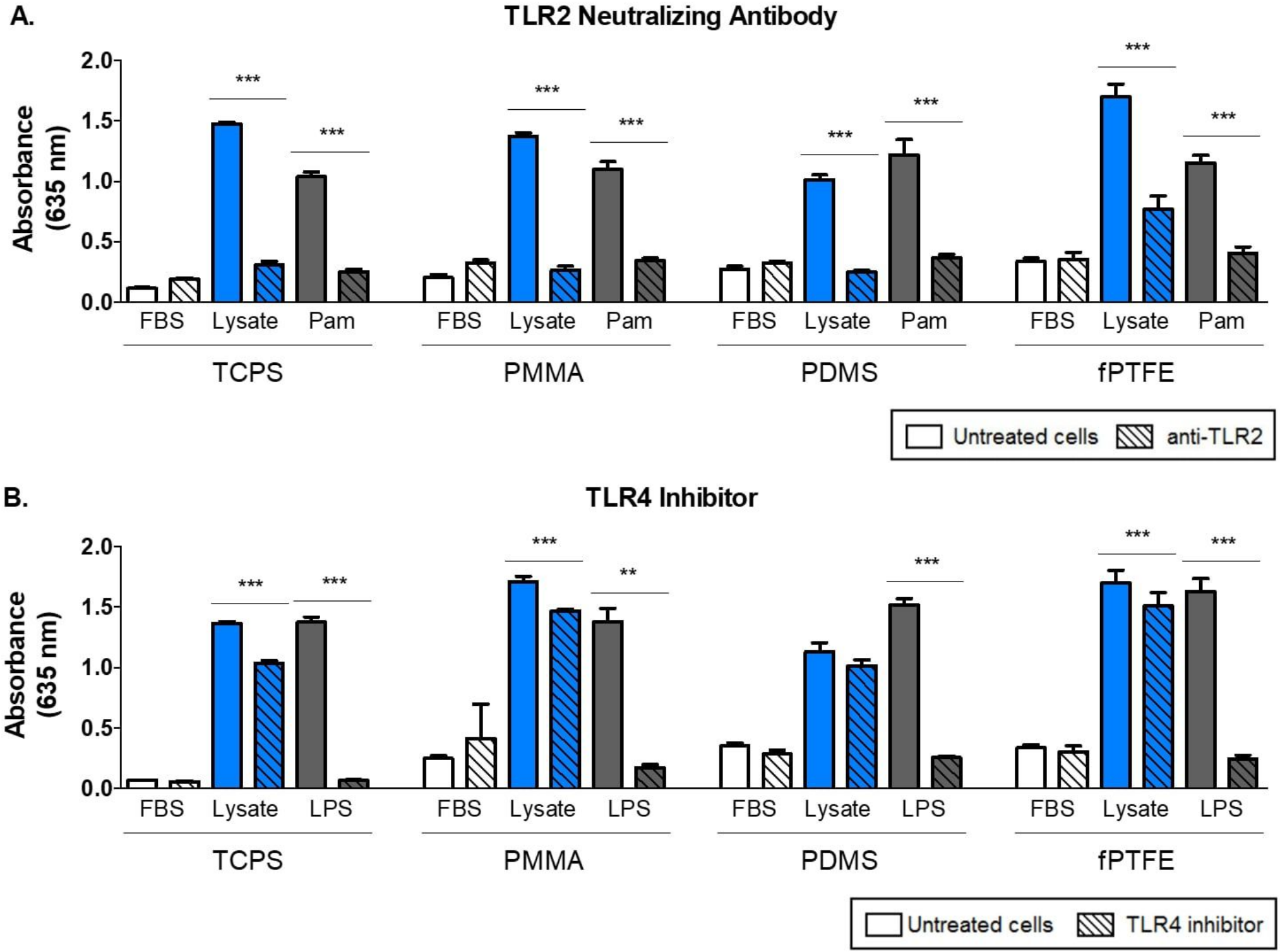


Figure 4

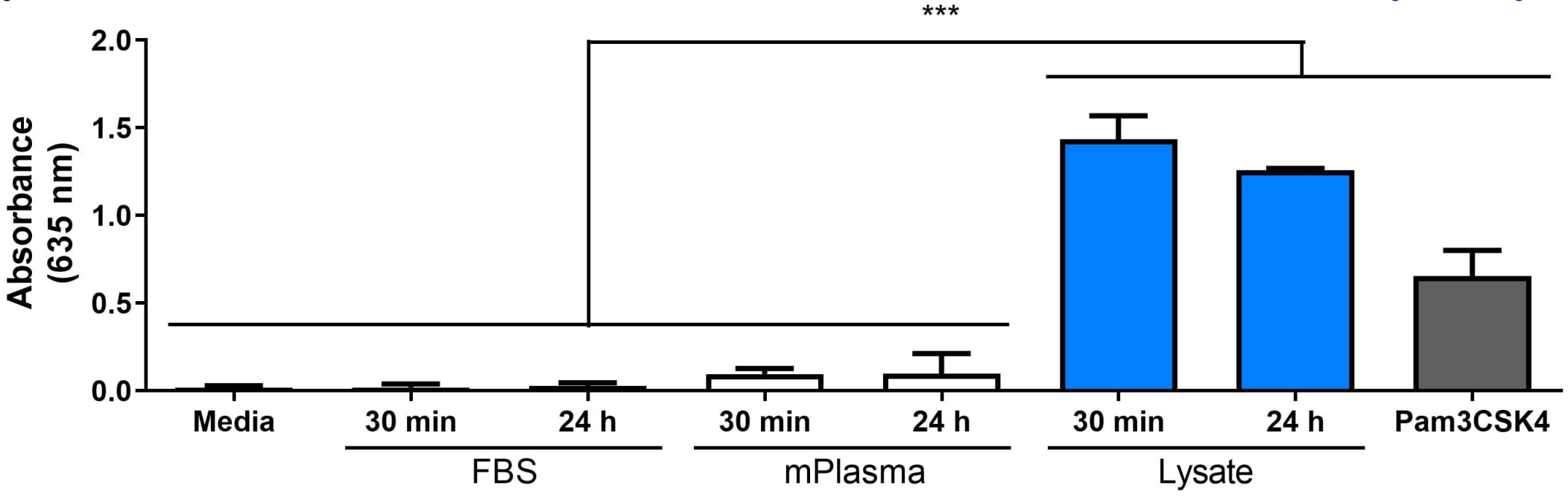
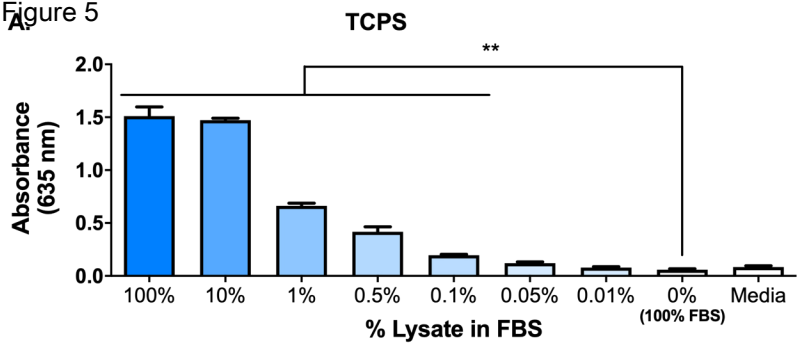
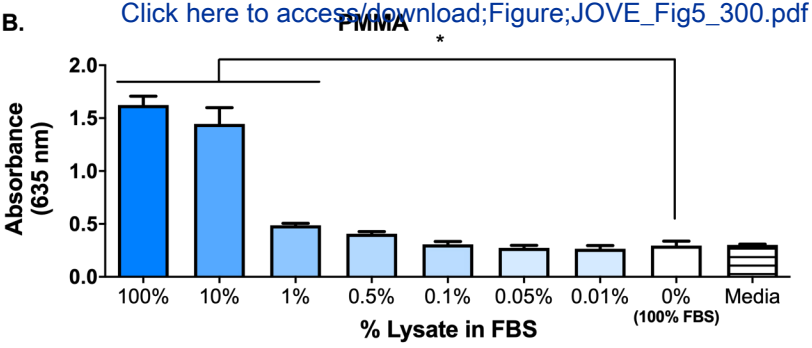
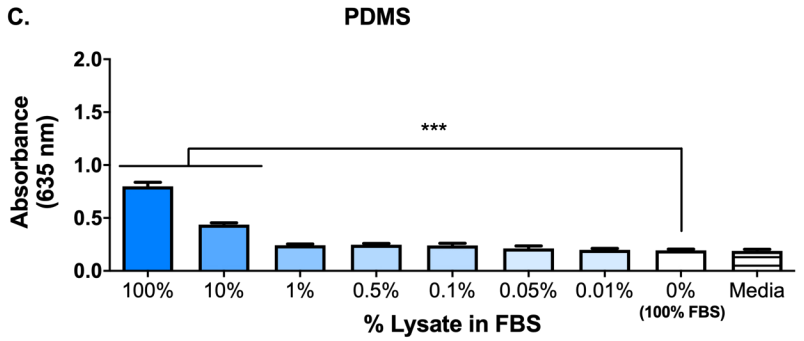
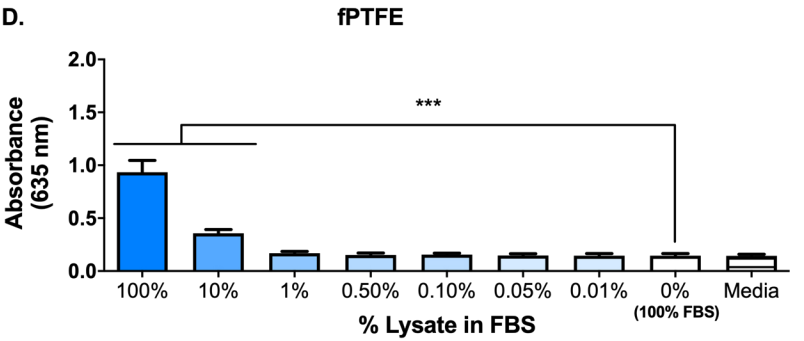


Figure 5









Cell culture reagents

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
anti-mouse/human CD282 (TLR2)	Biolegend	121802	
CLI-095 (TLR4 inhibitor)	Invivogen	TLRL-CLI95	
C57 complement plasma K2 EDTA 10ml, innovative grade US origin	InnovativeResearch	IGMSC57-K2 EDTA-Compl-10ml	Mouse plasma
Dulbecco's modified eagle medium (DMEM)	Sigma Aldrich	D6429-500ML	
Dulbecco's phosphate buffered saline (DPBS)	Fisher Scientific	14190250	No calcium, no magnesium
Fetal bovine serum (FBS), research grade	Wisent	98150	
LPS-EK	Invivogen	TLRL-EKLPS	Lipopolysaccharide from Escherichia coli K12
NIH/3T3 fibroblasts	ATCC	CRL-1658	
Pam3CSK4	Invivogen	tlrl-pms	Synthetic triacylated lipopeptide - TLR1/2 ligand
Penicillin/streptomycin	Sigma Aldrich	P4333-100ML	
Plasmocin	Invivogen	ANT-MPP	Mycoplasma elimination reagent
RAW-Blue cells	Invivogen	raw-sp	NF-κB/AP-1 reporter macrophage cell line
Trypan blue solution, 0.4%	Fisher Scientific	15250061	
TrypLE express enzyme (1X)	Fisher Scientific	12604021	animal origin-free recombinant cell dissociation enzyme
Zeocin	Invivogen	ANT-ZN-1	

Kits and assays

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
ELISA precoated plates, mouse IL-6	Biolegend	B213022	
ELISA precoated plates, mouse TNF-α	Biolegend	B220233	
Endotoxin (Escherichia coli) - Control standard endotoxin (CSE)	Associates of Cape Cope Inc.	E0005-5	Endotoxin for standard curve in chromogenic endotoxin assay
LAL water, 100 mL	Associates of Cape Cope Inc.	WP1001	Used with chromogenic endotoxin assay
Micro BCA protein assay	Fisher Scientific	PI23235	

Limulus amebocyte lysate (LAL) Pyrochrome endotoxin test kit	Associates of Cape Cope Inc.	C1500-5	Chromogenic endotoxin assay reagent
QUANTI-Blue alkaline phosphatase detection medium	Invivogen	rep-qb2	Alkaline phosphatase assay to indirectly measure NF-κB/AP-1 activity

Polymeric coating reagents

Name of Reagent/ Equipment	**Company**	**Catalog Number**	**Comments/Description**
Chloroform, anhydrous	Sigma Aldrich	288306-1L	
Ethyl alcohol anhydrous	Commercial Alcohols	P006EAAN	Sigma: Reagent alcohol, anhydrous, 676829-1L
Straight tapered fine tip forceps	Fisher Scientific	16-100-113	
Fluorinert FC-40 solvent	Sigma Aldrich	F9755-100ML	Fluorinated solvent for fPTFE
Cell culture grade water (endotoxin-free)	Fisher Scientific	SH30529LS	
Poly(methyl methacrylate) (PMMA)	Sigma Aldrich	182230-25G	
Sylgard 184 elastomer kit	Fisher Scientific	50822180	
Teflon-AF (fPTFE)	Sigma Aldrich	469610-1G	Poly[4,5-difluoro-2,2-bis(trifluoromethyl)-1,3-dioxole-co-tetrafluoroethylene]

Consumables

Name of Reagent/ Equipment	**Company**	**Catalog Number**	**Comments/Description**
Adhesive plate seals	Fisher Scientific	AB-0580	
Axygen microtubes, 1.5 mL	Fisher Scientific	14-222-155	
Borosilicate glass scintillation vials, with white polypropylene caps	Fisher Scientific	03-337-4	
Clear PS 48-well plate	Fisher Scientific	08-772-52	
Clear TCPS 96-well plate	Fisher Scientific	08-772-2C	
Clear TCPS 48-well plate	Fisher Scientific	08-772-1C	
Cover glasses, circles	Fisher Scientific	12-545-81	
Falcon tissue culture treated flasks, T25	Fisher Scientific	10-126-10	
sticky-Slide 8 Well	Ibidi	80828	

Superfrost microscope slides	Fisher Scientific	12-550-15
Tissue culture treated flasks, T150	Fisher Scientific	08-772-48

Journal: Journal of Visualized Experiments
Manuscript ID: JoVE60317
Title: "A macrophage reporter cell assay to examine Toll-like receptor-mediated NF-kB/AP-1 signaling on adsorbed protein layers on polymeric surfaces"
Author(s): Laura A McKiel, Kimberly A Woodhouse, Lindsay E Fitzpatrick

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Response to Reviewers:

Dear Dr. Cao,

We are pleased to submit our revised manuscript entitled "A macrophage reporter cell assay to examine Toll-like receptor-mediated NF-kB/AP-1 signaling on adsorbed protein layers on polymeric surfaces" in response to the revisions requested by the reviewers of our original manuscript submission (JoVE60317). A point-by-point response to editorial and reviewer comments is provided below. For clarity, the editorial and reviewers' comments are copied in black bolded text and our responses are provided in blue text.

My co-authors and I would like to thank both reviewers and the editorial team for the time you have dedicated to reviewing this manuscript, and hope you find the revised manuscript suitable for publication in JoVE.

Sincerely,

Lindsay Fitzpatrick
Assistant Professor
Department of Chemical Engineering
Queen's University

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The manuscript has been thoroughly proofread and we understand JoVE will not copy-edit the final manuscript.

2. Please revise lines 423-426 to avoid textual overlap with previously published work.

The text has been revised as requested.

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The text has been revised as requested.

5. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of " RAW-Blue" or "QUANTI-Blue" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.

The text has been revised as requested.

6. Figure 2: Please change the time units "hrs" to "h" and "mins" to "min". Please include a space between numbers and units (5 min). Please remove commercial language (RAW-Blues).

The figure has been revised as requested.

7. Figure 4: Please change the time unit "m" to "min". Please include a space between numbers and units (30 min, 24 h).

The figure has been revised as requested.

8. Table of Materials: Please remove any ™/®/© symbols. Please sort the materials alphabetically by material name.

The Table of Materials has been revised as requested.

9. References: Please do not abbreviate journal titles; use full journal name.

The text has been revised as requested, using JoVE reference formatting in Mendeley. Please note that changes weren't tracked for References because the bibliography is automatically updated through Mendeley.

Reviewers' comments:

Reviewer #1:

Major Concerns:

1.1. It's not clear to me what this manuscript adds to the literature beyond the authors recent publication on this topic. Most of the methods are pretty standard and the primary method is use of a commercially-available reporter cell line and an assay kit that is sold by the same company and recommended by that company for use with the reporter cells. Indeed, 4 of the 5 figures are adapted from the authors recent publication.

Thank you for your thorough review of our manuscript. Please note that ***novelty is not a requirement for publication*** *in JoVE, and this manuscript is intended as a detailed methods guide to improve the reproducibility of our experiments described in our recent publication, and for individuals new to the area of macrophage-material interactions. Consequently, we have not made any revisions to the manuscript in response to this concern.*

1.2. It's not clear to me how measuring the contact angle at 2 spots per cover slip ensures even coating with the polymer as stated in section 2.2.1

Thank you for your comment; we have amended the text to remove the word "even" as this may imply a consistent coating thickness, which was not our intention. Instead, we state that checking the contact angle at two random locations on the cover slip is done to "ensure glass surface was completely coated with the polymer". The glass coverslips used in this study are relatively small (14mm diameter), and can only fit 2 separate drops of water, which are positioned randomly on the cover slip. Also, as the polymer solution is pipetted directly onto the center of the coverslip prior to spinning, we assume the centre is covered. We respectfully argue that if both randomly positioned water droplets provide the expected water contact angle for the polymer coating of interest (as found in literature), it is reasonable to assume the cover slip is completely coated.

1.3. The endotoxin assays appear to have been done on water incubated in the wells to measure endotoxin that eluted from the polymers or the wells. This is not adequate as

endotoxin is very adherent to many surfaces, including polymers, and therefore may have resulted in false negatives (see PMID 16609962 and PMID 22359211). Moreover, details of the endotoxin assay need to be provided as that is a controversial subject (see PMID 22359211).

Thank you for your comment regarding the endotoxin assay. We are aware that performing the assay using water incubated within the coated well for 1 hr may underestimate the amount of endotoxin on the surface, compared to performing the endotoxin assay reaction in the presence of the biomaterial. Furthermore, we agree that this caution should be communicated within the article and have amended to text to reflect this, as described below.

However, unlike testing endotoxin on sample from a particle preparation (as described in the publications by highlighted by Reviewer 1), it is not possible to do perform the endotoxin assay directly within the wells being used for cell culture, as exposing the wells to the endotoxin assay kit reagents would preclude using those wells for subsequent experiments. While it would be technically feasible to reserve one well-plate from a batch of prepared plates, the cost of doing so would quickly become cost prohibitive and would not definitively conclude that the other plates from that batch were not contaminated with endotoxin. Indeed, the FDA endotoxin limits for medical devices are based on measuring the endotoxin within an extraction volume (as we describe here), in recognition that direct testing in the presence of a device is often infeasible. Furthermore, one advantage of using the RAW-Blue reporter cell line is that it is very sensitive to endotoxin through the TLR4 pathway. So, any endotoxin contamination on the prepared surfaces would yield an increased SEAP activity for the negative controls in the coated wells, compared to the SEAP activity obtained in uncoated well plates (used as provided from manufacturer), which has not been observed in our experiments.

Consequently, we test an incubated water sample from each plate used in the subsequent cell assays, according to the recommendations provided by the manufacturer of the endotoxin testing kit used in our lab (Pyrochrome Chromogenic LAL Endotoxin Test Kit from Cape Code Associates Inc) and published protocols [Fitzpatrick, Chan & Sefton, Biomaterials 2011 Dec;32(34):8957-67, doi: 10.1016/j.biomaterials.2011.08.021; Fitzpatrick, Lisovsky & Sefton, Biomaterials 2012 Jul;33(21):5297-307, doi; 10.1016/j.biomaterials.2012.04.008] . We have also verified that any potential substance released into the water during incubation neither inhibited nor enhanced the test using a spike control to confirm the recovery of a known amount of added endotoxin.

To address Reviewer #1 concerns, we have added further detail regarding the endotoxin test to the methods (steps 2.5.1 – 2.5.5) as requested, and included Notes (Methods; Step 2.5) that (1) state the limitation of testing endotoxin in this manner and (2) instruct researchers developing their own polymer coating method to perform initial endotoxin test directly within prepared wells to ensure no sources of endotoxin are inadvertently being introduced into the system during the coating process (either through contaminated reagents, water rinses etc). We also note that is it imperative to use endotoxin-free plastic consumables, endotoxin-free water for

any rinses and buffers used within the assay, as water is one of the main sources of endotoxin contamination, and to ensure all glassware is depyrogenated by dry heat sterilization prior to use (250^{o}C for 30 minutes) [Gorbet MB, Sefton MV, Biomaterials. 2005 Dec; 26(34):6811-7, doi: 10.1016/j.biomaterials.2005.04.063].

1.4. Statistics are not clear. In Figure 4, it is unclear whether the figure shows mean +/- SD of n=3 independent experiments, n=9 non-independent wells, or n=18 non-independent enzyme assays. The other Figures are representative of at least 2 separate experiments but the data in the figures appear to represent n=3 separate wells from a single experiment or n=6 separate enzyme assays from a single experiment.

Additional details were added to figure captions to clarify the statistical analysis.

1.5. A major limitation of the RAW macrophages is that they do not express ASC and therefore do not form most types of inflammasomes and cannot process inactive IL1b or inactive IL18 to the mature forms of those cytokines. RAW cells are therefore less useful for extended exposures to stimuli (such as 20-24 hours used in this study) where IL1b and/or IL18 are expected to enhance the macrophage response in an autocrine manner.

Thank you for your comment. We are aware and have acknowledged the limitations of the RAW-Blue cell line in the Discussion section of this manuscript (see lines 685-689), and have added an additional sentence to the Discussion to acknowledge the absence of ASC in RAWs. While we agree that the inflammasome is both present and important in TLR-mediated macrophage responses in vivo, the motivation for this protocol and rationale for selecting the RAW-Blue reporter cell line was to provide a rapid, quantitative tool for assessing the direct activation of the TLR pathway by adsorbed DAMPs. Consequently, the autocrine contribution of the inflammasome to enhance macrophage activation on biomaterial surfaces was beyond the scope of this protocol. The limitations of the RAW-Blues, including the absence of ASC and subsequent inflammasome activity, will be addressed in future work with primary bone marrow derived macrophages.

Minor Concerns:
1.6. The spin coater and it's use should be described.

Additional details about spin coating have been added to step 2.2.

1.7. Section 4: It needs to be clarified whether the fPTFE is used to coat the polystyrene plate or the coverslips.

Thank you for this suggestion. fPTFE was used to coat both the well plate (for cell culture) and the coverslips (used for water contact angle measurements). The text has been updated to include step 4.2.1 to clarify this point.

1.8. Section 5.2: PBS is not usually considered sufficient to neutralize cell dissociation enzymes.

The cell dissociation enzyme used in this step of the protocol is TrypLE Express Enzyme, and was selected because it can be neutralized by dilution in PBS (the product sheet states "dilution alone inactivates TrypLE™ Express, avoiding the need for trypsin inhibitors, such as FBS."). However, we acknowledge that this distinction may not be clear in the methodology, and have added a note for step 5.2.

1.9. Section 6.1: Trypsin is not usually considered useful to detach macrophages.

In our experience the RAW-Blue macrophage reporter line is not as strongly adherent as primary macrophages, and is effectively detached using the TrypLE Express Enzyme described in this manuscript. Trypan-blue exclusion viability counts for RAW-Blues following the 8-min dissociation time using this enzyme preparation is generally 90%. This is likely due to the purity of the enzyme preparation, and the reduced adherence of this cell line.

1.10. Source and preparation of the mouse plasma should be described.

We have added this information to the Table of Materials, and added specification in the manuscript that commercial mouse plasma was used.

Reviewer #2:
For the written section I have just two minor comments:
2.1. Reference 1 is a poor reference for the points the authors make in the first sentence, as well as being a bit old for this advancing field.

Reference 1 has been updated to [Anderson JM, Rodriguez A, Chang DT, Seminars in Immunology. 2008; 20(2):86-100, doi: 10.1016/j.smim.2007.11.004] which is regarded in our field as a seminal article detailing the foreign body response to biomaterials.

2.2. "Measure water contact angle of extra coated slides" -- The authors should indicate that only water of the highest purity (for example, glass triple distilled) should be used for this measurement.

The text had been added to the manuscript as requested.

Reviewer #3:
Minor Concerns:
3.1. at ~ln 75: make it more explicit that without the DAMP signals (i.e., protein alone) no macrophage activation is seen.

The text has been amended, as requested.

3.2. In general, it is suggested that you can use this to screen a variety of materials, with different materials showing different activation levels/readout levels from the assay that can be compared (e.g., here PDMS and fTPE showed lower activation than PMMA) but this is not explicitly stated. Further, I think the authors could make it clear that the materials used in here are representative examples and that any material could be used.

Thank you for this suggestion. We have addressed this point by including additional information in lines 890-893. To directly compare between materials, the SEAP activity would need to either be normalized to the baseline (activity in media control), which was not done in the Figures for this publication. Therefore, we did not make any statements regarding relative NF-kB activity among material groups.

3.3. I don't think the first sentence of the Discussion is reflective of the work in this paper (Perhaps the "project" refers to a larger body of work) but the statement seems loftier that what is described here

The first lines of Discussion have been revised to more accurately describe the aim of the protocol presented here.

3.4. There are a lot of groups and I think a plate layout with the various groups and controls would help the reader (especially during the reading of the methods, before the results help you piece it together)

A sample plate layout has been included as a supplemental figure (Figure S1).

3.5. Specify what "cell dissociation enzyme" was used for 3T3s.

The cell dissociation enzyme used was TryplE Express (Table of Materials) and has been described as an “animal origin-free, recombinant enzyme for cell dissociation” in the manuscript to avoid using commercial language.

3.6. The cell lysate concentration (468.75 ug/ml) and the fact that 200uL is used in 48 well to give 125ug/ml doesn't make sense to me. Please double-check the math or explain. The values chosen should also be based on literature reasoning.

Thank you for this comment. The concentration given in the manuscript is 125 ug/**cm^2**. Here is the unit analysis (using the area of one well of a Falcon TCPS 48-well plate, 0.75 cm^2) to demonstrate how the math works.

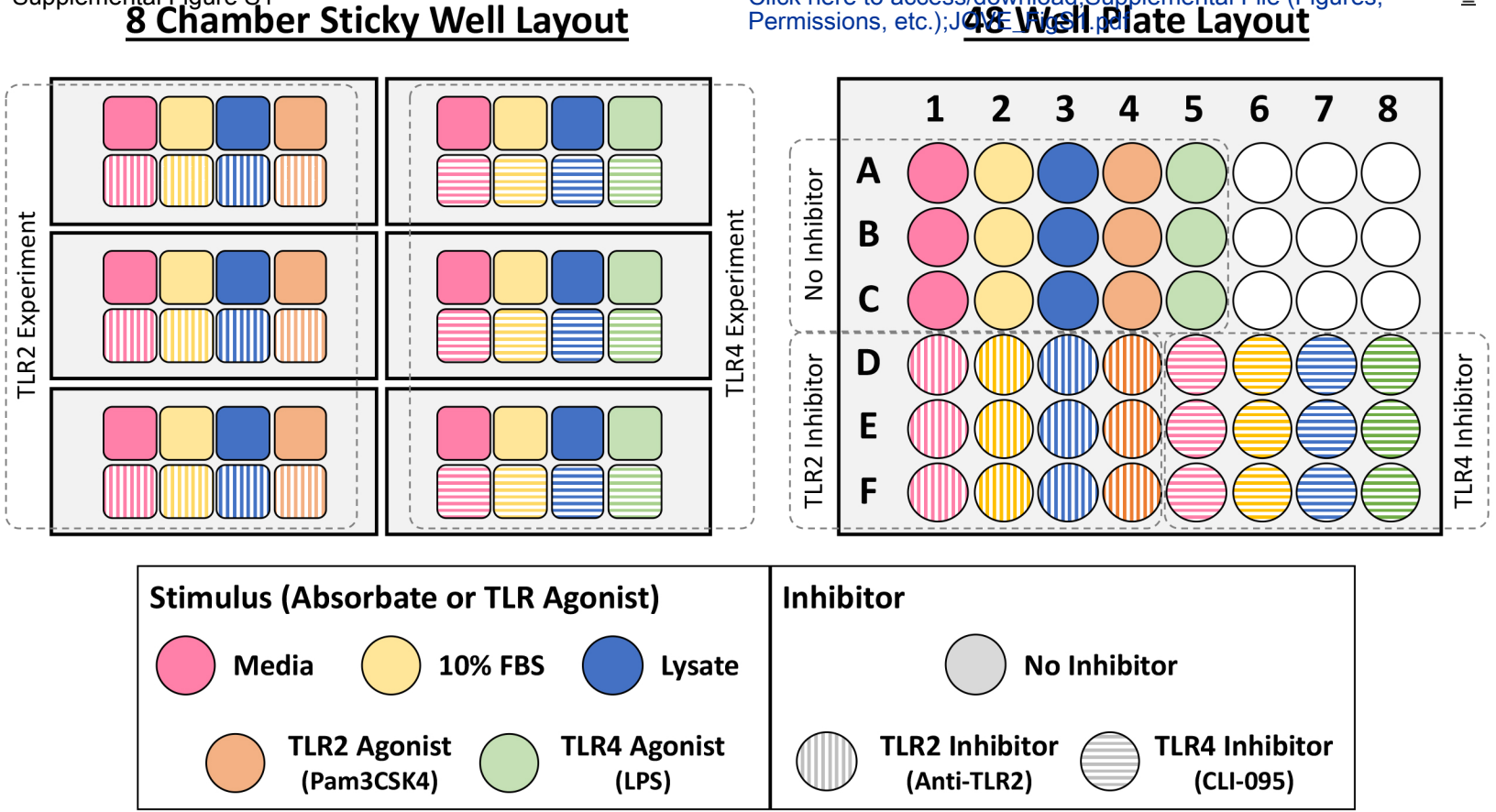
$$\frac{468.75\ \mu g}{mL} \times (0.2\ mL) \times \frac{1}{0.75\ cm^2} = 125\ \mu g/cm^2$$

The protein concentration value was chosen because it was the average protein concentration in our lysate when lysing fibroblasts suspended in PBS at $1x10^6$ cells/mL. To our knowledge there are no other groups using cell lysate adsorbates, therefore there is no literature value to use as a comparator for our protein concentration. Looking in the literature, early protein adsorption studies with albumin have used concentrations ranging from 200-450 ug/mL on polymeric biomaterials (surface area not stated), which corresponds to our chosen concentration [Gombotz WR, Guanghui W, Horbett TA, Hoffman AS, Journal of Biomedical Materials Research. 1991; 25:1547-1562, doi: 10.1002/jbm.820251211; Ishihara K et al, Journal of Biomedical Materials Research. 1991; 25:1397-1407, doi: 10.1002/jbm.820251107]. As well, time course studies of lysate adsorption on RAW-Blues demonstrates that the NF-κB/AP-1 activity of the cells is constant for protein adsorption times > 30 mins, which indicates to us that the surface is saturated and protein adsorption equilibrium has been reached with this concentration of lysate.

3.7. Can you speculate on the use of the assay for 3D materials (e.g., hydrogels, porous scaffolds)

Thank you for this question. While we have not performed the protein adsorption and TLR-inhibition assays on porous and/or degradable materials or hydrogels, we anticipate that the protocol can be adapted to these different materials (and have plans to do so in the future). However, these types of scaffolds would introduce more complexity to the system. For example, it would be challenging (if not impossible) to distinguish between adsorbed and entrained/sequestered DAMPs within the scaffolds. This speculation has been included at the end of the Discussion (lines 882-888).

Supplemental Figure S1



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Title: Toll-like Receptor 2-Dependent NF-κB/AP-1 Activation by Damage-Associated Molecular Patterns Adsorbed on Polymeric Surfaces

Author: Laura A. McKiel, Lindsay E. Fitzpatrick

Publication: ACS Biomaterials Science & Engineering

Publisher: American Chemical Society

Date: Nov 1, 2018

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