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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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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.

3. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

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4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: QUANTI-Blue™, RAW-Blue™, Kim wipe, Fluorinert, etc.

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).