Toll-like Receptors Contribute to the Foreign Body Response in a Biomaterial-dependent Manner

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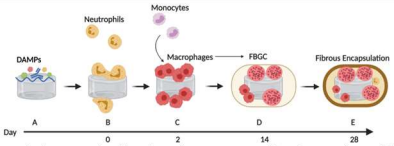
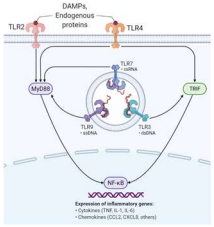
Background and Motivation



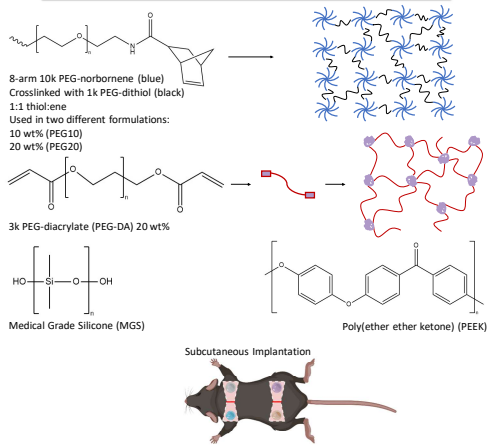
The **foreign body response (FBR)** is an innate immune response driven by macrophages. This response causes chronic inflammation that surrounds an implant and can lead to pain and implant loosening. Overtime, this response completely encapsulates essentially all non-biological implants with fibrous collagen. Immediately after implantation of a non-biological material in tissue, proteins adsorb to the surface (A). It is theorized that **damage associated molecular patterns (DAMPs)** adsorbed to and around the implant can trigger a pro-inflammatory signaling cascade that initiates the FBR. DAMPs are patterns in molecules that cells can recognize as being caused by damage to tissues, such as HMGB1, which is released when cells necrose. In addition, proteins unfolded on the surface of a material may reveal epitopes that are recognized as DAMPs and trigger an inflammatory response. Within hours of implantation neutrophils migrate to the implant (B) and begin recruiting monocyte-derived macrophages to the implant site (C). Eventually, the macrophages become frustrated because they cannot phagocytose the material and as a result, fuse into foreign body giant cells (FBGC) (D). Macrophages and FBGCs recruit fibroblasts, that create an avascular collagenous capsule around the implant (E). This capsule completely walls off the implant and prevents it from interacting with the rest of the body. This response negatively impacts biosensor implants and tissue engineering scaffolds that need to interact with the body long-term.



Toll like receptors (TLRs) are pattern recognition receptors that recognize molecular patterns associated with damage and pathogens. It is known that **TLR 2 and TLR 4** are capable of recognizing DAMPs that are derived from endogenous proteins. Upon activation, TLR 2 and TLR 4 lead to the activation of NF-κB which releases pro-inflammatory cytokines such as interleukin 6 and tumor necrosis factor alpha (TNF-α). This occurs through the MyD88 pathway that has been previously shown to mediate the FBR.[1]

The severity of the FBR varies depending on the material properties of the implant that effect protein adsorption on the surface, such as: surface roughness, chemistry, modulus, and mesh size. Thus, 5 commonly used biomaterials with a wide range of material properties was chosen to study the role of TLR 2 and TLR 4 in the FBR. The goal of this study was to investigate the role of TLR 2 and TLR 4 in the FBR to a wide range of commonly used biomaterials *in vivo*.

Materials and Methods



All hydrogels were formed using 0.05% photoinitiator (Irgacure 2959) in phosphate buffered saline. Each solution was sterile filtered and polymerized under 32 nm light at 6 mW/cm for 7 minutes. Sterile MGS was acquired from Invotec International, and PEEK was acquired from Victrex and autoclaved to sterilize.

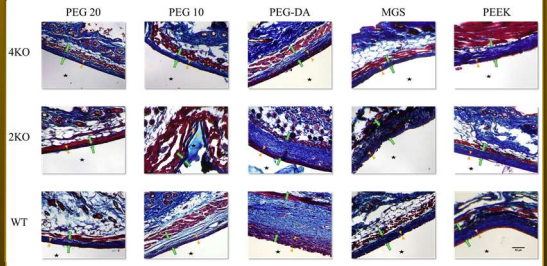
Each material was implanted subcutaneously in mice and the location of each material was randomly assigned. Each mouse received four implants, one above each shoulder and one above each hip. A blunt dissection technique was used to push the implant away from the initial incision to prevent any wound healing response interference. Two strains of mice were engineered, a TLR 2 knockout (2KO), and a TLR 4 knockout (4KO), and wildtype (WT) were used as a control. The implants were left in the mice 28 days in order to characterize the chronic stage of the FBR. Each material was punched at 5 mm in diameter and 1 mm thick. Compressive modulus was measured to 15% strain, and the hydrogels were measured fully swelled. Hydrophobicity was measured using water contact angle, and >65 degrees is considered hydrophobic. Protein adsorption was measured using bovine serum albumin (BSA) and a Pierce protein adsorption assay. Histology was performed on all samples and stained with Masson's trichrome stain to quantify the inflammatory cell layer and the fibrous capsule layer. Quantification was measured using ImageJ. Statistics were run using one factor Anova.

Results

Material Characterization

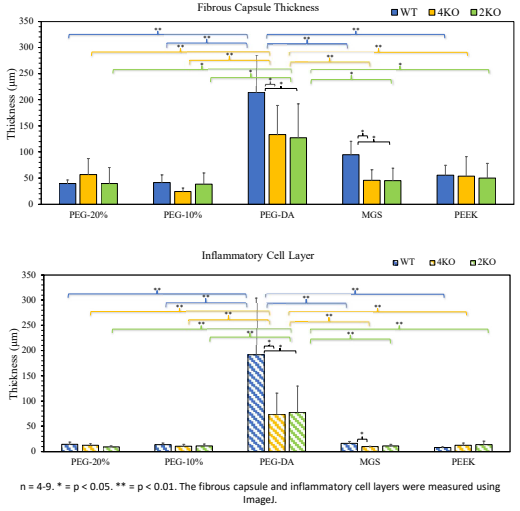
Material	Compressive Modulus	Contact Angle (degrees) n=3	Adsorption of BSA (ug/mm^2) n=3	Mass swelling ratio n=3-6
PEG-DA	500 ±70 kPa n=15	-	19 ±9.2	6.1 ±0.13
PEG-NB 10wt%	140 kPa n=3	-	31 ±7.2	9.9 ±1.5
PEG-NB 20wt%	250 kPa n=3	-	49±17	7.9 ±0.99
MGS	15 ±1 MPa n=4	93 ±7	25±1.5	-
PEEK	1.2 ±0.1 GPa n=3	65 ±13	25±4.9	-

Histological Assessment of the FBR



Sections were stained with Masson's Trichrome stain. Green arrows indicate the fibrous capsule, orange triangles indicate the inflammatory cell layer, and the black star indicates the location of the implant. All images are of the dorsal side of the implant and were taken at 10x. Scale bar is 50 μm.

Quantification of the FBR



n = 4-9. * = p < 0.05. ** = p < 0.01. The fibrous capsule and inflammatory cell layers were measured using ImageJ.

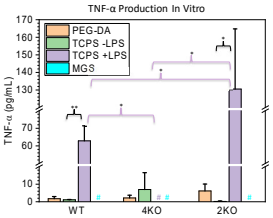
Of the materials investigated, the FBR is most severe to PEG-DA. The fibrous capsule thickness and the inflammatory cell layer thickness in response to PEG-DA and MGS was significantly reduced when either TLR 2 or TLR 4 is deleted.

Discussion

- Albumin is the most abundant protein in serum. BSA adsorbed to all five materials regardless of hydrophilicity. The two hydrophobic materials, MGS and PEEK, had similar protein adsorption. Protein adsorption to the different PEG hydrogels was not dependent on the modulus or the swelling ratio. These findings demonstrate that proteins readily adsorb to materials independent of their physical properties.
- PEG-DA caused the most severe FBR and surprisingly was much greater than the PEG-NB hydrogels. This finding suggests that the PEG chemistry may not be driving the FBR to PEG-DA. Several differences between PEG-DA and the two PEG-NB hydrogels that could have contributed to the more severe FBR include: a) a higher stiffness, b) the presence of unreacted acrylate groups that is more reactive than norbornene, and c) hydrophobic regions that result from the polyacrylate kinetic chains that may impact the types of protein adsorbed.
- DAMPs can signal by TLR2, TLR4, or both. The deletion of TLR 2 or TLR 4 partially reduces the FBR severity to PEG-DA and MGS, suggesting that there are DAMPs on the surface of these materials that exclusively signal by TLR2 and other that exclusively signal by TLR4.
- TLR2 or TLR4 were not required for the FBR to PEG-NB hydrogels and to PEEK. One possible explanation is that the DAMPs on these materials signal through both TLR2 and TLR4. Alternatively, other pattern recognition receptors (e.g., other TLRs) may be involved.
- Taken together, these findings show that TLR signaling in the FBR is biomaterial dependent. The way proteins unfold on the surfaces of a material could cause different TLR-mediated responses.

Future Work

- Characterize the hydrophobic regions of PEG-DA to better understand the protein adsorption.
- Determine the FBR to each of these materials in a TLR 2 and TLR 4 double knockout (DKO) mouse.
- *In vitro* study using bone marrow derived macrophages from each strain of mouse to determine the pro-inflammatory cytokine production to each material. This study would also allow us to analyze whether serum proteins unfolded on the surface of each material trigger an inflammatory response in mice. Mouse serum will be used in these studies instead of fetal bovine serum to eliminate any inflammation that may be caused by a difference of species. Each material will be soaked in serum before plating the cells to simulate protein adsorption and impure LPS will be used to activate TLR 2 and TLR 4. The pro-inflammatory cytokine expression and production will be measured using qPCR and ELISA's. Preliminary ELISA data is shown below, this study is ongoing.



# Indicates below detectable limit
n = 3. * = p < 0.05. ** = p < 0.01

Based on these preliminary results, neither PEG-DA nor MGS cause an inflammatory response when only serum proteins are adsorbed to the material, leading us to believe that serum proteins are not acting as DAMPs on the surface of PEG-DA and MGS. Further investigation is needed to determine if LPS is triggering TLR 2 in the absence of TLR 4, and thus it is unclear whether TLR 2 or TLR 4 is causing the release of TNF-α.

Acknowledgments

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References:

1. Amer, L. D., Saleh, L. S., Walker, C., Thomas, S., Janssen, W. J., Alper, S., & Bryant, S. J. (2019). Inflammation via myeloid differentiation primary response gene 88 signaling mediates the fibrotic response to implantable synthetic poly(ethylene glycol) hydrogels. *Acta Biomaterialia*, *100*, 105–117.