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The effect of CD47 modified polymer surfaces on inflammatory cell attachment and activation

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Abstract

CD47 is a transmembrane protein that is a marker of “self”. CD47 binding to its cognate receptor in leukocytes and macrophages, signal regulatory protein alpha (SIRP α), causes inhibition of inflammatory cell attachment. We hypothesized that immobilization of recombinant CD47 on polymeric surfaces would reduce inflammation. Recombinant CD47 was appended to polyvinyl chloride (PVC) or polyurethane (PU) surfaces via photoactivation chemistry. Cell culture studies showed that CD47 immobilization significantly reduced human neutrophil (HL-60) and human monocyte derived macrophage (MDM) (THP-1) attachment to PVC and PU respectively. A neutralizing antibody, directed against SIRP α , inhibited THP-1 and HL-60 binding to PU and PVC surfaces respectively. This antibody also increased the level of SIRP α tyrosine phosphorylation, thereby indicating a direct role for SIRP α mediated signaling in preventing inflammatory cell attachment. Studies using human blood in an *ex vivo* flow-loop showed that CD47 modified PVC tubing significantly reduced cell binding and neutrophil activation compared to unmodified tubing or poly-2-methoxy-ethylacrylate (PMEA) coated tubing. In ten-week rat subdermal implants, CD47 functionalized PU films showed a significant reduction in markers of MDM mediated oxidative degradation compared to unmodified PU. In conclusion, CD47 functionalized surfaces can resist inflammatory cell interactions both *in vitro* and *in vivo*.

1. Introduction

In the present studies we investigated inhibiting the inflammatory response to biomaterials by targeting cellular immune receptor proteins that negatively regulate the immune response via an amino acid consensus sequence known as the immunoreceptor tyrosine-based inhibitory motif (ITIM). Signal-regulatory protein alpha (SIRP α) is an ITIM containing transmembrane protein that is expressed in cells of myeloid origin [1]. SIRP α binding to the Ig extracellular domain of its cognate ligand, CD47, prevents CD47 expressing cells from

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being targeted by inflammatory cells [2-4]. This immune regulatory mechanism is both conserved and species specific, with sequence homology in the Ig domain differing by 38% between mice and humans [2-4]. In biological systems, hematopoietic stem cells and leukemia cells evade phagocytosis partly by upregulating CD47 expression [5]. In addition, myxoma viruses express a CD47 homologue that may facilitate evading phagocytosis [6]. Similarly, CD47-SIRP α interactions have been shown to inhibit phagocytosis of opsonized microbeads by regulating the phosphorylated state of myosin which in turn downregulates the subsequent engulfment of the microbeads [7].

Given the ability of CD47 to confer “self” status upon expressing cells [2, 7-9], we immobilized the extracellular domain of recombinant CD47 onto two widely used polymeric biomaterials, polyvinyl chloride (PVC) and polyurethane (PU), to test the hypothesis that surface immobilized CD47 can attenuate the inflammatory response to biomaterials due to its role as a “molecular marker of self” (Figure 1). PVC tubing is a major component of cardiopulmonary bypass (CPB) circuits, and other blood contacting medical device surfaces, and represents a model polymer to test the hypothesis with respect to the neutrophil mediated acute inflammatory response, which occurs as a result of blood-material interactions. Similarly PU is used in long-term implants, such as pacemaker lead insulation [10] and left ventricular assist device blood contacting surfaces [11, 12], and thus was used in these experiments as a model surface to examine the chronic inflammatory effects mediated by MDM.

The goals of this study were: 1. Formulate and characterize the surface modification chemistry to append molecular CD47 onto polymeric biomaterials. 2. Identify the contribution of the CD47-SIRP α pathway to inflammatory cell attachment to PVC and PU surfaces. 3. Demonstrate proof of concept, using established *ex vivo* and *in vivo* models, of the ability of CD47 modified surfaces to attenuate the inflammatory response to biomaterials.

2. Materials and Methods

2.1 Materials

The PU used was Tecothane TT1074A (Thermedics, Waltham, MA), a polyether polyurethane. Blood tubing modified with Poly(2-methoxyethylacrylate) (PMEA), and marketed as Terumo-XTM coated tubing, and unmodified polyvinyl chloride (PVC) tubing was acquired from Terumo Cardiovascular Systems (Ann Arbor MI). A mouse monoclonal antibody directed against human CD47 (B6H12) or was purchased from BD Pharmingen (Franklin Lakes, NJ). Rabbit polyclonal antibody directed against phospho tyrosine (PY350) and a mouse monoclonal antibody directed against human SIRP α (SE7C2) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). 4'-6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories (Burlingame, CA). Tween 20 and sodium dodecylsulfate-polyacrylamide electrophoresis gels were purchased from Bio-Rad (Hercules, CA). Protease Inhibitory Cocktail Tablets were acquired from Roche (Indianapolis, IN). Immunoaffinity agarose beads were purchased from Calbiochem (San Diego, CA). Sephadex and the enhanced chemiluminescence detection system is a product of Amersham, GE Healthcare (Piscataway, NJ). N-Succinimidyl 3-[2-pyridyldithio]-propionate (SPDP), avidin, 2-mercaptoethanol (2-ME), 12-myristate 13-acetate (PMA), and all other chemicals and solvents, unless otherwise specified, were purchased from Sigma (St. Louis, MO).

2.2. Biotinylated CD47

Plasmids encoding the extracellular domain of hCD47 or bCD47 (GenBank Accession Number NM_174708) were PCR amplified and ligated in frame with the vector pEF-BOS-XB [13] which formed the in frame fusion of CD4d3+4 biotin at the C-terminus of the extracellular domain of CD47. This construct was transfected into CHO (–K1) cells and the secreted CD47– CD4d3 + 4 was concentrated, biotinylated at the C-terminus, and dialyzed. The protein was affinity purified using monomeric avidin and dialyzed against PBS [7].

2.3. Cell Culture

All cell lines (human MDM THP-1, human promyelocytic HL-60, rat alveolar macrophage cell line NR8383) were acquired from American Type Culture Collection (Manassas, VA) and maintained as recommended. The human MDM cell line (THP-1) were grown in RPMI media supplemented with 5% fetal bovine serum and 0.05 μ M 2-ME. The human promyelocytic cell line (HL-60) were grown in Modified Dulbecco's Medium supplemented with 20% fetal bovine serum. The rat alveolar macrophage cell line, NR8383, were grown in Ham's F12K medium supplemented with 15% fetal bovine serum. Where indicated, cells were transduced with the addition of 1.6×10^{-7} M of PMA to the media.

2.4. Casting PVC and PU Films

PU or PVC was dissolved in dimethylacetamide and tetrahydrofuran respectively, and then solvent cast as films with thickness ranging between 159 and 220 μ m as used in prior studies [14, 15]. Films, both PU and PVC, were then cut into appropriate sizes for *in vitro* and *in vivo* analysis.

2.5. Appending CD47 to the PU and PVC Surfaces

We surface modified PU and PVC films (1cm²), and the luminal surfaces of PVC tubing (1/4 $\times$ 1/16 inch), by first soaking them in a solution of 0.1% hexadecylpyridinium chloride for 1.5 hours at room temperature. Films or tubing were rinsed in water and then soaked in a solution of 2-pyridyldithio,benzophenone (PDT-BzPh) [16](1 mg/ml) + KHCO₃ (0.67 mg/ml) in water, that was acidified with 15% KH₂PO₄ (150 μ l/3 ml of PDT-BzPh). The films and tubing were incubated in the acidified PDT-BzPh for 40 minutes and then rinsed with diluted (1:1000) acetic acid and both sides of the film were exposed to UV irradiation from a BioRAD UV Transilluminator 2000 (analytical mode), for 15 minutes per side. The entire length of tubing was rotated over a BioRAD UV Transilluminator 2000 (analytical mode), for thirty minutes. The modified surfaces were then soaked in KHCO₃ (20mg/ml) for 20 minutes and washed 5 times with reverse osmosis (RO) treated water.

Avidin (10mg/ml) was reacted with 0.58 mg of SPDP (dissolved in dimethylformamide) for 1 hour at room temperature. The SPDP treated avidin was passed through a Sephadex column. 2-pyridyldithio (PDT) groups, on the surfaces, were reduced to Thiol-groups by incubating for 5 minutes with a solution of 20 mg/ml of TCEP (1 ml/film) in degassed PBS. Films and tubing were then rinsed 5 times in degassed PBS and then reacted, overnight, with avidin that was prior treated with SPDP and filtered. The avidin-immobilized surfaces were then washed 5 times in dH₂O and biotinylated CD47 (see below for details) was added to each film.

2.6. Western Blot Analysis

Cultured HL-60 and THP-1 cells, cultured in the presence or absence of anti-SIRP α IgG antibodies as detailed below, were washed three times with PBS and scraped in ice-cold lysis buffer (100 mM pH 8.1 Tris-HCl, 10 mM EDTA, 1% Triton X-114, a proportioned amount of complete protease inhibitor cocktail, 0.2 mM orthovanadate). The cellular lysates

were passed through a 21-gauge needle, and the proteins were quantified using a micro BCA protein assay kit. Prior to immunoprecipitation, the cellular lysates were spun down at 10,000 g for 10 minutes. The collected supernatant was first incubated with 5 µg of anti-SIRPα antibody (SE7C2) for an hour and then, incubated with the corresponding amounts of immunoaffinity agarose beads on a rotator rack at 4°C overnight. The immunoprecipitated SIRPα complex proteins were resolved on a 4-15% gradient sodium dodecylsulfate-polyacrylamide electrophoresis gel using the method described by Laemmli [17], and the proteins were transferred to a 0.2 µm pore size polyvinylidene fluoride (PVDF) membrane (Invitrogen, Carlsbad, CA), followed by immunoblotting for the presence of phosphorylated tyrosine using anti-phospho tyrosine antibody (PY350) at manufacturers' recommended dilutions in 10 mM pH 7.5 Tris-HCl, 100 mM NaCl, and 0.1 % Tween 20 (TTBS) with 5% non-fat milk. The immune complexes were detected with the species-appropriate, horseradish peroxidase-conjugated secondary antibodies in recommended dilutions in TTBS with 5% non-fat milk and were visualized with an enhanced chemiluminescence detection system on X-ray films.

Where indicated, the autoradiographs were scanned, imported to the Quantity One version 4.6.9 (Biorad, Hercules, CA) program and individual bands were automatically separated using the band attributes function. To determine the relative intensity, the histogram function in Adobe Photoshop (Adobe, San Jose, CA) was used to measure the mean level of grey scale intensity. This value was subtracted from the background value and correlated to the loading control band of the same sample.

2.7. Cell Attachment Studies (in vitro)

THP-1, HL-60, or NR8383 cells were differentiated with the addition of PMA to their respective media and were cultured on PU or PVC films for a predetermined period of time. Additional studies examined the binding of activated HL-60 to PVC tubing or PVC tubing that was modified by surface immobilized CD47 (via avidin-biotin affinity) or avidin modified PVC tubing (avidin control). The tubes were capped at both ends and shaken for 3 hours at 37°C. At the conclusion of the protocol, films or tubing were washed and remaining cells were fixed with 4% paraformaldehyde and stained with the nuclei specific stain DAPI. Cell retention was quantified by staining with the fluorescent dye DAPI (blue color on fluorescent micrographs) and visualized using a fluorescent microscope with the appropriate filter set and separate 200X fields were counted. Where indicated cells were cultured in the presence of anti-SIRPα antibody (SE7C2) directed against the protein's extracellular domain, or IgG control.

2.8. Rat Subdermal Implants

Male Sprague Dawley rats, weighing between 300 and 350 grams were used for subdermal implant studies as previously described [18]. Rats were anesthetized with isoflurane and administered Flunixinamyl post surgery for analgesia. Each of the five rats per group received three 1 cm² PU-films that were composed of unmodified PU or PU surface modified with covalently appended human CD47 (hCD47) or bovine CD47 (bCD47). At the 70-day termination of the study, rats were euthanized by carbon dioxide asphyxiation and the subdermal implants were removed, rinsed briefly with saline and further processed as detailed below. All procedures and animal husbandry were in compliance with NIH standards pertaining to the care and use of laboratory animals as approved by the IACUC of the Children's Hospital of Philadelphia.

2.9. Assessment of hCD47 from explanted PU films

Following explantation, PU films were placed into 0.5 ml of a 1 % SDS solution and sonicated. The films were then transferred to a fresh solution of 1% SDS and sonicated. The

films were then transferred to a 0.5 ml of PBS and sonicated. All sonicates were pooled from each film and vacuum dried. The resulting pellet was resuspended in PBS and processed for Western blotting analysis as mentioned above using the human CD47 specific antibody (B6H12).

2.10. Environmental Scanning Electron Microscopy (ESEM)

Images were acquired from FEI XL30 field emission ESEM (FEI, Hillsboro, OR). Samples were cooled to 5°C using a Peltier cooling stage and pressure was maintained between 4-5 torr to achieve a relative humidity of approximately 70%.

2.11. Fourier transform infrared spectroscopy-attenuated total reflectance

Explanted PU films were processed for FTIR as previously described [18]. Briefly, explanted films were washed in a 1% Triton X-100 in PBS solution followed by a second series of washes in PBS. A final rinse in 70% ethanol was performed to remove residual detergent. The films were then scanned with Fourier transform infrared spectroscopy-attenuated total reflectance (FTIR-ATR) spectroscopy using a Nicolet 5-Protégé 460 spectrophotometer E.S.P. (Nicolet Madison, WI). All spectra were obtained under identical conditions from 200 scans collected at a resolution of 2 cm^{-1} , at a 45° angle of incidence and the atmospheric water vapor and carbon dioxide were accounted for by subtracting the appropriate reference spectrum using the Omnic software package. Deconvoluted peak measurements were acquired for the 1174 cm^{-1} peak and normalized to the measured 1590 cm^{-1} peak heights [14, 18, 19].

2.12. Statistical Analysis

Data were calculated as means $\pm$ standard error (SE). Student's *t*-test was used to determine the significance of differences. Statistical significance was noted with $p \leq 0.05$

3. Results

3.1. CD47 Surface Immobilization

The photo-crosslinker, PDT-BzPh [16], was used to functionalize the surface of PU and PVC films (Figure 2A). We used an avidin-biotin linking strategy to immobilize CD47 onto the PU or PVC surfaces. Thus, we reacted avidin with the bifunctional cross linker SPDP (Figure 2A). The SPDP treated avidin was then reacted with the thiolated synthetic surfaces. The immobilized avidin provided binding sites for the subsequent attachment of biotinylated CD47. The presence of immobilized CD47 on the synthetic surfaces was initially characterized using a FITC-conjugated mouse anti-human CD47 antibody directed against the extracellular domain of human CD47. Films qualitatively showed robust fluorescence (Supplemental Figure 1) in CD47 immobilized PU and PVC surfaces, with an absence of fluorescence in control films.

3.2. CD47 inhibition of inflammatory cell attachment in vitro

We used a human-derived macrophage cell line, THP-1, cultured on recombinant human CD47 (hCD47) immobilized PU surfaces, or unmodified control PU films to test, *in vitro*, inflammatory cell interactions with CD47 polymeric surfaces. Similarly, the human-derived neutrophil cell line, HL-60, was cultured on PVC films in the presence or absence of immobilized hCD47. Both cell types were activated with phorbol 12-myristate 13-acetate (PMA) and cultured for three days (THP-1 cells) or 4 hours (HL-60 cells). After washing the films with phosphate buffered saline (Figure 2B), THP-1 or HL-60 seeded on the unmodified surfaces were largely retained. In contrast, cells seeded on hCD47 functionalized surfaces were almost entirely displaced. These results strongly suggest that

the cellular attachment mechanics are altered on CD47 modified surfaces. Images of the cells prior to washing (Supplemental Data Figure 2) show that cells on CD47 modified surfaces appeared to be less spread, reflecting impaired attachment, compared to the cells on unmodified control surfaces.

To quantify cellular attachment to CD47 functionalized surfaces, additional *in vitro* studies were performed using activated THP-1 and HL-60 cells. The THP-1 cells were cultured on PU films, or PU films modified with avidin or CD47, for 3 days under static conditions. CD47 functionalized PU surfaces inhibited THP-1 attachment approximately 20-Fold compared to unmodified PU surfaces (Figure 2C). Compared to unmodified PU, surface bound avidin had no significant effect upon cell attachment. To ascertain the effects of surface immobilized CD47 on inflammatory cell attachment to polymeric blood conduits, HL-60 cells were cultured for 4 hours in PVC tubing, or PVC tubing surfaces modified with avidin or CD47, while shaking. HL-60 attachment to PVC was modestly, but not significantly inhibited by the presence of surface immobilized avidin. In contrast, the surface attachment of CD47 to the PVC tubing almost completely blocked HL-60 attachment to the PVC tubing, as documented by the quantitative data (Figure 2C).

We determined the effective surface concentration range of CD47 that inhibits THP-1 or HL-60 binding to either PU or PVC respectively. Recombinant CD47 was appended to the polymeric surfaces over a range of loadings as determined by FITC-conjugated anti CD47 antibody [2, 20]. As shown in Figure 3A, immobilized CD47 significantly ($p < 0.001$) reduced THP-1 and HL-60 attachment at surface concentrations as low as 4 ng CD47/cm². There was a further four-fold reduction in cellular attachment to CD47 modified surfaces at CD47 concentrations between 31.25 ng CD47/cm² – 125 ng CD47/cm². Interestingly HL-60 binding to CD47 modified PVC was 3-fold higher at the lower CD47 loading concentrations compared to THP-1 binding to PU. However, the binding profiles were comparable between the two cell types seeded on the two surfaces when the loading concentration of CD47 exceeded 16 ng CD47/cm². Cell attachment on either surface was virtually abolished at CD47 surface concentrations greater than 250 ng CD47/cm². Based upon these results, we modified all subsequent surfaces at a concentration of 250 ng CD47/cm².

We compared the efficacy of either immobilized CD47, of human (hCD47) or bovine (bCD47) origin, upon THP-1 binding to PU (Figure 3B) and HL-60 attachment to PVC (Figure 3C). Shown in Figure 3B, THP-1 cell attachment was significantly reduced on both bCD47 and hCD47 functionalized surfaces, compared to unmodified films. However, immobilized hCD47 was eight times more effective in reducing THP-1 attachment to the polymeric surface compared to bCD47 (Figures 3 B). This increased inhibition was reversed by preincubating the hCD47 immobilized films with 10µg/ml of anti hCD47, which increased THP-1 binding to PU films to levels comparable with bCD47. In contrast the antibody had no effect on cell binding to bCD47-immobilized films. Similar results were noted for HL-60 cell attachment to PVC (Figure 3C).

3.3 Contribution of SIRPα to cell attachment to PU and PVC surfaces

THP-1 attachment to PU surfaces (Figure 4A) was not affected by the presence of 10 µg/ml of non-specific IgG antibody added to the culture media. However, binding was significantly ($p < 0.001$) reduced in THP-1 cultures that had 2.5 µg or 5 µg of SIRPα blocking antibody added to the media. Similarly the presence of 10 µg of anti SIRPα antibody significantly reduced HL-60 binding to PVC surfaces (Supplemental Figure 3). These data demonstrate the contribution of the SIRPα signaling pathway to myeloid cell binding to synthetic surfaces.

To test the hypothesis that the blocking antibody is acting in the capacity of a SIRP α agonist, the phosphorylated state of SIRP α was compared in HL-60 cells cultured for one hour, in the presence of 10 μ g/ml anti-SIRP α blocking antibody or the same concentration of IgG control. Following incubation, cellular lysates were immunoprecipitated with anti-SIRP α antibody and analyzed for the presence of phosphorylated tyrosine. Western Blot analysis (Figure 4B) showed a clear increase in the phosphorylated state of SIRP α . These data confirm the fact that this antibody is indeed acting as an agonist of SIRP α by initiating the phosphorylation of tyrosine residues.

3.4 CD47 Functionalized Surfaces: Blood-material interactions

We assessed the potential of CD47 functionalized surfaces to mitigate the inflammatory response to clinically used polymeric blood conduits using an *ex vivo* model. The Chandler Loop Apparatus is a device that is used to examine modified surfaces with respect to blood-surface interactions [21-23] by flowing blood within a circular closed loop of tubing. In this study we used the Chandler Loop to analyze the effects of the CD47 surface modification upon cell attachment and neutrophil activation. We compared CD47 modified PVC tubing with unmodified PVC tubing and clinically used tubing, surface modified by the manufacturer with Poly(2-methoxyethylacrylate) (PMEA), with respect to cellular attachment to the blood contacting surface and neutrophil activation. Fluorescent microscopy of DAPI stained tubing following the procedure, and environmental scanning electron microscopy of tubing sections showed abundant cell attachment to the blood contacting surfaces of PMEA and unmodified tubing that was absent in CD47 modified tubing (Figure 5A). Quantitative analysis (Figure 5B) showed that immobilization of CD47 almost completely inhibited cell attachment on the tubing's blood contacting surface. In contrast PMEA modified CPB tubing and unmodified PVC tubing had similar levels of cell attachment that were approximately 30 times greater than that observed with CD47 modified surfaces. Flow cytometry was used to compare CD18, a marker of neutrophil activation [24, 25], surface expression in cells exposed to either PMEA, PVC-CD47, or unmodified PVC tubing. Shown in Figure 5C, CD47 immobilization on the PVC surface significantly reduced CD18, as determined by flow cytometry, compared to all other tubing tested. Surprisingly, the levels of CD18 expression were in fact significantly lower in blood samples acquired from CD47 functionalized tubing compared to freshly drawn blood samples not exposed to PVC tubing. These data confirm the anti-inflammatory effects of surface bound CD47 on PVC tubing.

3.5. Rat Subdermal Implant Studies

Based upon our earlier results (Figure 3 B and C) demonstrating that high concentrations (250 ng CD47/cm²) of CD47 can significantly inhibit cell attachment to synthetic surfaces irrespective of species, we hypothesized that bCD47 would inhibit inflammatory cell interactions with polyurethane films in a rat subdermal implant model. Our initial *in vitro* studies to explore this concept used the rat alveolar macrophage cell line, NR8383, to assess the interaction between rat cells and bCD47 functionalized surfaces. NR8383 attachment to bCD47 modified PU surfaces was significantly ($p \leq 0.001$) reduced with only 13.07 ± 4.9 cells/200 X field adhering to the bCD47 modified surfaces compared to 218 ± 34.9 cells/200 X field counted on the unmodified PU surfaces (See Supplemental Data Figure 4). These studies provided proof of principle that the bCD47 functionalized PU could hypothetically be resistant to the chronic inflammatory response in rats.

Unmodified PU films or films modified with avidin, hCD47, or bCD47 were inserted into subdermal dorsal pouches in rats and explanted after 10 weeks. The rats tolerated the implants, as there was no evidence of morbidity or infection. We used an antibody specific for human CD47 (B6H12), and unreactive with rat CD47 [2, 20], to determine if the

recombinant hCD47 remained on the surface of explanted hCD47 modified PU following 10 weeks *in vivo* implantation. Our previous results showed that the B6H12 antibody was not reactive with bCD47 (Figure 3B and C). Thus, the retention of bCD47 on explanted PU was not studied with Western Blot analysis. Shown in Figure 6A, Western blot analysis of extracted protein from PU films that were surface modified with hCD47 showed the presence of hCD47 following 10 weeks implantation, thus confirming the persistence of the CD47 modification. In support of the specificity of the antibody used to identify human CD47 on the explanted hCD47-modified PU films, no immunoreactive band was observed in the extracted proteins from unmodified PU films.

We quantified oxidative degradation of explanted films using established markers of oxidative degradation described as follows. FTIR analysis has shown that the oxidation of polyurethane elastomers corresponds with an increase in peak intensity at the 1174 cm^{-1} , assigned to the $\nu(\text{C-O-C})$ of branched ether, which has been identified as a marker of oxidative degradation [14, 18, 19, 26]. The extent of ether cross-linking is quantitatively determined by normalizing the 1174 cm^{-1} spectral peak intensity with the peak intensity of the 1590 cm^{-1} spectral peak, which corresponds to a non-oxidized aromatic ring [14, 18, 19, 26]. Shown in Figure 6B (Spectra shown in Supplemental Data Figure 5), the immobilization of either hCD47 or bCD47 significantly reduced the cross-linking of the polyurethane's ether groups. In contrast, the addition of Avidin to the PU films only marginally reduced oxidative degradation.

Scanning electron micrographs of the explanted films (Figure 6C) confirmed the FTIR results by showing that the most extensive surface cracking and large scale pitting was observed on the unmodified PU surface. In contrast, the CD47 modified PU surfaces showed only diffuse, submicron scale pitting as evident by the appearance of punctate circular irregularities, likely due to oxidative degradation. SEM assessment showed no appreciable differences between bCD47 and hCD47 modified films.

4. Discussion

CD47 functionalized polymeric surface

The use of functionalized CD47 surfaces as a localized anti-inflammatory strategy is unique in that it targets an ITIM expressing protein with the intention of reducing the inflammatory response to the material, by establishing a peptide based biomimetic surface. In support of the central role of CD47 in engineered immunoresistant biomaterials we showed the following: 1. Photoactivation chemistry, based upon PDT-BzPh polymers, can be used to functionalize polymeric surfaces. 2. Immobilized CD47 can reduce the inflammatory response *in vitro*, *ex vivo*, and *in vivo*. 3. CD47 functionalized surfaces are capable of maintaining their efficacy beyond 10 weeks *in vivo*. Thus, taken together, these data demonstrate that CD47 surface modification can inhibit the inflammatory response to biomaterials.

We demonstrated for the first time that the photoactive polymer, PDT-BzPh, can be applied to large surfaces to provide covalent attachment points to polymeric surfaces (PVC and PU). Results shown herein demonstrate that appended CD47 was uniformly immobilized on the tested surfaces and was effective in reducing the attachment of inflammatory cells in *in vitro* and *in vivo* models. Furthermore, this photochemistry can be applied to any hydrocarbon surface, thereby allowing CD47 to be immobilized on the vast array of biomaterials where the inflammatory response to biomaterials needs to be controlled.

Implantable Polymeric Biomaterials

PVC tubing is widely used as a blood conduit for devices such as CPB circuits and extracorporeal membrane oxygenators [27-29], and its ability to elicit an inflammatory response as a result of aberrant biocompatibility between the blood contacting surface and blood components is widely documented [30, 31]. Based on a strategy to reduce protein adsorption on the blood contacting surface, polymeric coatings such as PMEA, represent the most current advances to alleviate the inflammatory response to blood conduits [32, 33]. Using the Chandler Loop Apparatus, PMEA coated surfaces have demonstrated mixed results in their ability to reduce inflammatory markers in blood exposed to polymeric tubing [23, 34]. In the present studies we showed, in Chandler Loop Apparatus studies, that CD47 functionalized surfaces were superior to PMEA and control surfaces with respect to reducing cellular attachment and expression of CD18. The reduction in CD18 surface expression is important as CD18 expression has been associated with adverse outcomes following clinical procedures, such as CPB, where there is extensive interactions between blood and synthetic surfaces [24, 35]. It has been previously observed that CD47-SIRP interactions occur in the presence of opsonins [7, 36, 37], thus any effects of adsorbed proteins on CD47 modified surfaces should be minimal with respect to its anti-inflammatory capacity.

Previous work by our group and others has addressed the oxidative degradation of polyurethane with the addition of antioxidants [14, 18, 19, 26]. Although this strategy proved effective both *in vivo* and *in vitro* at inhibiting oxidative mediated damage to PU, the fact that the antioxidants are expended after reacting with oxygen radicals means that this approach is only a temporary solution to a chronic inflammatory issue. Thus, functionalizing biomaterial surfaces with recombinant CD47 is a potential new strategy to prevent the failure mechanisms associated with the chronic inflammation of implantable devices. Using a well established *in vivo* model of MDM mediated oxidative degradation, with well established endpoints of oxidation, we showed that CD47 modification on the PU surface significantly reduced oxidative degradation of PU. It is noteworthy that the CD47 modification was retained for at least 10-weeks *in vivo*. These data strongly support the use of CD47-functionalized surfaces as a therapeutic approach to reduce the chronic inflammation and its outcomes associated with long-term implantable devices.

CD47 and SIRP α Signaling

Investigations into CD47-SIRP α signaling mechanisms have been largely based on cell-cell interactions with no investigations into the role of CD47 in the cellular response to synthetic surfaces [7, 36, 37]. Recently we showed that CD47, through *cis* signaling mechanisms involving integrin $\alpha_v\beta_3$, performs an active role in the attachment of endothelial cells to modified polymeric surfaces [38]. In the present investigations, we characterized the ability of CD47 to establish a biomimetic synthetic surface through its putative interactions with SIRP α . To that end, we showed that CD47 functionalized surfaces inhibit THP-1 and HL-60 attachment *in vitro* and blood cell attachment *ex vivo*. Consistent with previous reports showing that CD47/SIRP α interactions alter cytoskeletal organization [7], we noted the different morphology of cells seeded upon CD47 surfaces compared to identical cells seeded on control surfaces.

SIRP α -CD47 interactions are species specific [7, 36, 37]. In support of that claim, we showed that focused concentrations of hCD47 was more effective than bCD47 in inhibiting attachment of the human cell lines, THP-1 and HL-60 cells, to their respective surfaces. Recently it was shown that at higher concentrations of CD47, the species specificity governing CD47-SIRP α interactions was abated [37]. We indeed found that bCD47 significantly reduced cell binding to the polymeric surfaces, compared to unmodified

surfaces. In addition, in our rat subdermal implant studies we showed that both bCD47 and hCD47 functionalized surface were significantly more effective at resisting oxidative degradation than unmodified PU surfaces. These results demonstrate the necessity for further investigations into CD47-SIRP α signaling with respect to synthetic surfaces.

Our results (Figure 4A) showing that the addition to the culture medium of antibodies to SIRP α decreases THP-1 cell attachment may appear counter-intuitive. However, the immunoprecipitation studies (Figure 4B) showed that there was indeed a two-fold increase in phosphorylated tyrosine in these HL-60 cell lysates. This indicates that the SIRP α antibody used in these present studies is initiating signaling mechanisms, via tyrosine phosphorylation, that regulate the cellular attachment to the synthetic surface. These findings demonstrate the need for further investigations into SIRP α mediated immunoinhibitory mechanisms and their potential for developing future therapeutic strategies to control inflammation.

5. Conclusions

This study demonstrated that functionalizing polymeric surfaces with recombinant CD47 is effective for reducing the acute and chronic inflammatory response to polymeric biomaterials. In addition to significantly reducing inflammatory cell attachment in *in vitro* and *ex vivo* models, CD47 also effectively reduced the expression of CD18, a marker of neutrophil activation. The CD47 surface modification was retained in long term implants and reduced the oxidative damage caused by MDM release of reactive oxygen species presumably by reducing the affinity for the MDMs for the modified surfaces. The species specificity of CD47-SIRP α interactions appears to be attenuated at higher concentrations of CD47. The use of CD47 to mitigate the inflammatory response to synthetic surfaces could be an effective therapeutic strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

The concept and molecular mechanism conferring anti-inflammatory properties to polymeric surfaces by surface immobilizing recombinant CD47. SIRP α expressing inflammatory cells will not adhere to CD47 functionalized surfaces. CD47 binding to SIRP α initiates SHP-1 mediated signaling through the ITIM motif of SIRP α . Downstream targets of SHP-1 are involved with the inhibition of myosin assembly and cell contraction, thereby preventing cellular attachment to the CD47 modified surfaces.

Fig. 2. Fabrication and in vitro characterization of CD47 functionalized synthetic surfaces. (A) Schematic diagram of immobilizing CD47 to polymeric surfaces using a polymeric photo cross linker (PDT-BzPh) composed of 2-pyridylthio groups (PDT) linked to the benzophenone (BzPh) photoreactive groups. PDT groups were reduced with tris(2-carboxyethyl)phosphine (TCEP). Avidin was reacted with the bifunctional crosslinker N-Succinimidyl 3-[2-pyridyldithio]-propionate (SPDP). (B) Representative photomicrographs of hCD47 polymeric films, or unmodified control films, after seeding, then washing with phosphate buffered saline (PBS). Films, surface modified with hCD47, exhibited little or no cellular retention following PBS washing. Original magnification is 200 X. (C) Quantitative analysis of THP-1 (black bar) and HL-60 (open bar) attachment to PU and PVC surfaces respectively. CD47 immobilization on the polymers surface significantly inhibited cellular attachment to the surfaces. Data points are the mean $\pm$ SEM from $n = 27$ separate fields * $p < 0.001$ compared to unmodified or avidin modified surfaces.

Fig. 3.

Dose response and specificity of CD47 immobilized polymeric surfaces in resisting inflammatory cell attachment. (A). THP-1 attachment to polyurethane films (closed squares) and HL-60 cell attachment to PVC films (open circles) were assessed over a range CD47 surface concentrations. CD47 reduced cellular attachment to both surfaces at all CD47 surface concentrations. At lower concentrations, HL-60 cells were less sensitive to CD47 inhibition of cellular attachment compared to THP-1 cells. * $p < 0.001$ compared to 0 ng CD47/cm² and THP-1 cell counts at identical surface concentration. (B and C) Recombinant CD47 of human (hCD47) or bovine (bCD47) origin was immobilized on polyurethane films (B) or PVC films (C) in the presence or absence of a function blocking antibody (ab) directed against human CD47. THP-1 cells (B) were then cultured on the modified PU films and assessed for cell retention following washing with phosphate buffered saline. * $p < 0.001$ in comparison with Ctrl. PU, *# $p < 0.001$ in comparison with hCD47 and Ctrl. PU. (C) HL-60 cells were then cultured on the modified, and unmodified control PVC films and assessed for cell retention following washing with phosphate buffered saline. Irrespective of species, CD47 immobilization significantly blocked cellular attachment on both polymers tested. Note the increase in cell attachment to hCD47 surfaces pretreated with anti CD47 antibody. *† $p < 0.001$ in comparison with Ctrl. PVC, *‡ $p < 0.001$ in comparison with hCD47 and Ctrl. In all experiments (A, B, and C) Data points are the mean $\pm$ SEM from $n = 27$ separate fields.

Fig. 4.

SIRP α mediates MDM attachment to PU surfaces. (A) Attachment of activated THP-1 cells to PU surfaces was blocked by anti-SIRP α antibody. Data points are the mean $\pm$ SEM from $n = 27$ separate fields * $p < 0.001$ compared to unmodified or avidin modified surfaces. * $p < 0.05$ in comparison with IgG control, 0 μ g of anti-SIRP α antibody, and 10 μ g of anti-SIRP α antibody. ** $p < 0.001$ in comparison with IgG and 0 μ g of anti-SIRP α antibody. (B) Representative Western Blot analysis of HL-60 lysates incubated with anti-SIRP α antibody and immunoprecipitated with anti-SIRP α confirming an increase in tyrosine phosphorylation as a results of antibody incubation. Gel-scans of the Western blots showed that the presence of SIRP α antibody increased tyrosine phosphorylation approximately two-fold, compared to incubating HL-60 cells with control IgG antibody. Data points are the mean $\pm$ SEM from $n = 3$ separate immunoprecipitation experiments.

Fig 5.

Human whole blood was exposed to flow conditions, using a Chandler Loop Apparatus with either unmodified PVC tubing, CD47 surface modified PVC tubing, or PMEA surface-modified tubing. (A) Representative DAPI staining (Original Magnification 200X) and ESEM micrographs of cell attachment to tested tubing surfaces. Bar = 100 μ m (250 X) and 20 μ m (1000X). (B) Cell counts with DAPI staining shows a significant (* $p < 0.001$ compared to all other surfaces) reduction in cell attachment in PVC-CD47 tubing compared to unmodified PVC or PMEA modified tubing. Data points are the mean $\pm$ SEM from $n = 45$ separate fields for each tubing modification and control. (C) Flow cytometry shows that CD47 immobilization significantly (* $p < 0.05$ compared to all other surfaces and reference) reduces CD18 expression. The reference is CD18 surface expression at time zero in unexposed blood. Data points are the mean $\pm$ SEM from $n = 5$ measurements.

Fig. 6.

Analysis of CD47 functionalized surfaces in long-term implants. (A) Representative Western Blot analysis showing retention of hCD47 modification after 10 weeks *in vivo*. A single band was observed only in extracted proteins from hCD47 functionalized surfaces. (B) Quantitative assessment of ether cross linking, as determined via FTIR analysis, shows that CD47 functionalized PU significantly inhibited oxidation-mediated ether crosslinking. Data points are the mean $\pm$ SEM from $n = 5$ explanted films * $p < 0.001$ compared to unmodified or avidin modified surfaces, * $p = 0.05$ versus unmodified. (C) Representative scanning electron micrographs (Magnification 1000X) showing surface cracking and pitting, as a result of oxidative degradation, on unmodified PU films explanted from rat subdermal pouches after 10 weeks. Little or no surface cracking, but submicron pitting was observed on hCD47 or bCD47 functionalized PU surfaces. Insets are representative films prior to implantation. Bar = 20 μ m.