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Intracellular signaling mechanisms associated with CD47 modified surfaces

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Abstract

We have previously established that recombinant CD47 can ameliorate the inflammatory response to synthetic polymeric surfaces. Here, we begin to profile, at the transcriptional, translational and cell signaling level, the inflammatory cell response when blood interacts with CD47 modified polyvinyl chloride (PVC) (CD47-PVC). We used qPCR arrays to compare transcriptional changes between human whole blood exposed to CD47-PVC or PVC. Transcription of IL1F5, IL1F10, IL17F, CCL3, CCL8, CCL28, CXCL12, and CXCL13 was upregulated in blood exposed to PVC, compared to CD47-PVC. The increase in CCL3 and CCL8 transcription correlated with an increase in the chemokines' presence in the plasma. Exposure of blood to CD47-PVC resulted in an increase, compared to PVC, in transcription of CCL2, CCL4, CCL20, CXCL1, TGF β 3, GDF3, GDF10, CD40LG, and TNFSF10. CD47-PVC exposure resulted in an increase of the following matrix metalloproteinase related genes: MMP1, MMP7, MMP13, and MMP16. Phosflow cytometry, and assays examining transcription factor binding, cell attachment, and genome wide chromatin association indicated that members of the JAK-STAT signaling pathway, particularly JAK2 and STAT5, mediate inflammatory cell interactions with CD47-PVC. Our data demonstrate that differential molecular responses to CD47 involve downregulation of cytokines, upregulation of MMPs, and JAK/STAT signaling mechanisms.

Keywords

Biocompatibility; Gene expression; Metalloproteinase; Cell Signaling

1. Introduction

Immune cell interactions with polymeric biomaterials result in an inflammatory response that can negatively affect medical device function or cause clinical complications [1-5]. Thus, developing an anti-inflammatory strategy to address this issue remains an unmet need in biomaterial science. To that end, numerous attempts have been made to enhance biocompatibility by functionalizing synthetic surfaces with bioactive or bioinert molecules

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[6-8]. Unfortunately, fewer efforts have been made to elucidate the intracellular signaling mechanisms that are initiated when cells come in contact with synthetic surfaces [9]. Such information would be invaluable in establishing a mechanism-based design strategy for future biomaterial development.

Under homeostatic conditions, the immune response to foreign stimuli is tightly regulated to ensure that the appropriate reaction is elicited. Downregulation of the immune response is partially maintained by a family of immune inhibitory receptors with a conserved amino acid motif known as the immunoreceptor tyrosine-based inhibitory motif (ITIM). Ligand induced signaling of ITIM receptors is mediated by tyrosine phosphorylation [10-12]. Downstream targets of ITIM receptors are often Src homology 2 (SH2) domain containing phosphatases, such as SHP-1 and SH2-containing inositol polyphosphate 5-phosphatase (SHIP) [10-12]. These proteins in turn target down stream effectors, through transphosphorylation events, that ultimately inhibit the immune response.

We previously described and characterized a biomaterial surface modification appending the recombinant extracellular Ig domain of human CD47 [13,14]. CD47 functions as a marker of self-recognition via its interaction with the ITIM expressing Signal Regulatory Protein alpha (SIRP). In a series of studies, we demonstrated decreased cellular attachment and activation of neutrophils, monocyte derived macrophages (MDM), and platelets on CD47 modified PVC and polyurethane surfaces [13,14]. Interestingly, these previous results showed that CD47 modified surfaces reduced the inflammatory response to synthetic surfaces beyond reducing inflammatory cell attachment. Specifically, we showed that markers of inflammatory cell activation were also reduced when cells were exposed to CD47 functionalized surfaces [13, 14]. These studies strongly suggested that the CD47-SIRP signaling pathway mediates a broader range of inflammatory cell physiological processes than currently identified.

It is known that the ITIM motif of SIRP inhibits phagocytosis and cellular attachment through Src homology region 2 domain containing phosphatase-1 (SHP-1) [15]. However, additional downstream effectors of SIRP are largely unknown. Recently, members of the JAK/STAT family signaling pathway have been identified as potential downstream mediators of SIRP signaling [16]. Among its many functions in cellular processes, the JAK/STAT signaling pathway has been implicated in inflammatory cell physiology [17-19]. Thus, examining the effects of CD47 modified surfaces upon JAK/STAT mediated signaling in inflammatory cells provided a unique opportunity to further examine the effect of SIRP upon the JAK/STAT pathway.

In these present studies, we use a molecular biology approach to test the hypothesis that CD47, immobilized on the surface of polymeric biomaterials, mediates inflammatory cell transcription across a range of genes. The goals of the study were as follows: 1. Identify differentially expressed proinflammatory genes, 2. Examine the effects of recombinant CD47 functionalized surfaces upon the JAK/STAT signal transduction pathway in inflammatory cells 3. Outline the signal transduction pathway responsible for the observed inhibition of the inflammatory response to the CD47 functionalized biomaterial surface. Information from these investigations can be applied to developing a bioactive material surface.

2. Materials and Methods

2.1. Materials

Standard clinical grade cardiopulmonary bypass tubing composed of polyvinyl chloride (PVC) was acquired from Terumo Cardiovascular Systems (Ann Arbor MI). 4 -6-

diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories (Burlingame, CA). Tween-20 was purchased from Bio-Rad (Hercules, CA). The enhanced chemiluminescence detection system is a product of GE Healthcare (Piscataway, NJ). Tetrahydrofuran, N-Succinimidyl 3-[2-pyridyldithio]-propionate (SPDP), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 2-mercaptoethanol (2-ME), and all other chemicals and solvents, unless otherwise specified, were purchased from Sigma (St. Louis, MO).

2.2. Recombinant CD47 Production and Purification

We have previously published the methodology to produce recombinant human CD47 [13]. Briefly, the extracellular domain of human CD47 (GenBank Accession Number NM_174708) was amplified from human cDNA using gene specific primers. The PCR product was ligated into a thymidine/adene vector containing the rat CD4 domains 3 and 4 (rCD4D3+4) coding sequence. A poly-lysine coding sequence was cloned into the vector at the three prime end of the rat CD4 sequence to aid in protein purification and appendage to the polymeric surfaces. The expression cassette (5' – hCD47-rCD4d3+4-Poly-Lysine – 3') was then subcloned into pcDNA5-FRT (Invitrogen, Carlsbad, CA). All vectors were sequenced by the Children's Hospital of Philadelphia Research Institute Nucleic Acid Facility and confirmed to be free of coding errors and in-frame for accurate protein production. The pcDNA5-FRT-hCD47-rCD4d3+4-Poly-Lysine (hCD47L) vector was co-transfected with pOG44, used to transiently provide the recombinase enzyme required for integration of the hCD47-rCD4d3+4 into the genomic DNA of the host cell, into CHO Flp-In cells to allow for genomic integration of the expression cassette. Recombinant protein was isolated from the cell culture medium, concentrated, desalted, and purified using an anti-CD47 amino-link protein purification column obtained from Thermo Fisher Scientific (Wilmington, DE). Protein concentration and purity were analyzed by the Bradford assay and SDS-PAGE Western blot analysis.

2.3. Casting of Polyvinyl Chloride Films

Clinical grade PVC was dissolved in tetrahydrofuran until completely solubilized. The polymer was then solvent cast to generate films with thickness ranging from 159 and 220 μm as used in prior studies [14]. Films were washed in 1 $\times$ PBS and subsequently used in cellular adhesion assays.

2.4. Attachment of CD47 to Polyvinyl Chloride

PVC surfaces were reacted with 2-pyridylthio-benzophenone (PDT-BzPh) and reduced with TCEP to obtain a thiol reactive surface, as previously described [13,14]. The poly-lysine tail of the recombinant CD47L protein was reacted with SMCC for one hour to form thiol-reactive groups. SMCC was removed via a desalting protein cut off column and the poly-lysine CD47 protein was incubated with the synthetic surface overnight at 4°C. Confirmation of recombinant protein attachment was determined by conjugation with a FITC-tagged antibody specific for human CD47 (BD Biosciences, Franklin Lakes, NJ).

2.5. Cell Culture

Whole blood was obtained aseptically from seven consenting healthy adults following the guidelines of the Children's Hospital of Philadelphia Institutional Review Board. Healthy females and males between 18 – 55 years of age who are not taking any medications were included in these studies. Whole blood was collected using venipuncture of the median cubital vein into a sterile 35 mL syringe containing 5 mL of acid citrate dextrose. Where indicated, the citrated whole blood was processed for plasma by centrifugation at 3000 rpm

for 10 minutes and the plasma samples were removed and stored, for further analysis, in 1 ml aliquots at -80°C.

THP-1 cells (American Type Culture Collections, Manassas, VA) were cultured in RPMI-1640 containing 10% fetal bovine serum, 0.05 mM 2-mercaptoethanol, and 1× penicillin-streptomycin. Differentiation of THP-1 cells from human acute monocytic leukemia cells to macrophage-like cells was accomplished using 1:1000 phorbol 12-myristate 13-acetate (PMA) in DMSO.

2.6. Chandler Loop Apparatus

The Chandler Loop apparatus was used to expose whole blood or cultured THP-1 cells to the luminal surfaces of unmodified commercially available clinical grade blood conduits (Terumo Cardiovascular Systems, Ann Arbor MI) or identical conduits modified with recombinant CD47 as detailed above and previously [13,14]. Briefly, 10 mls of whole blood or cultured THP-1 cells were perfused over the polymeric surfaces for three hours. At the end of the protocol, the blood samples or cultured cells were withdrawn and processed for their respective endpoint analysis.

2.7. qPCR Inflammatory Gene Expression Arrays

RNA was extracted and purified from whole blood using Trizol reagent (Life Technologies, Carlsbad, CA) as per manufacturer's instructions and quantified by Nanodrop spectrophotometry (Fisher Scientific, Wilmington, DE). Expression levels of 84 critical genes, which represent functional gene groups involved in specific cellular processes, were analyzed by quantitative PCR using the Human Inflammatory and the Human Extracellular Matrix and Adhesion Molecule RT² Profiler PCR array (SABiosciences, Valencia, CA), following the manufacturers protocol. Briefly, cDNA was prepared from 1 µg of total RNA using the RT² PCR array first strand kit (SABiosciences, Frederick, MD). qPCR reactions were conducted in a 25 µl mixture that included 12.5 µl of 2× qPCR master mix, 11.5 µl of nuclease free water and 1 µl of cDNA template. The thermal cycle profile consisted of an initial 10 minute step at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Real time quantitations were determined using the ABI 7500 software suite (Life Technologies, Carlsbad, CA). Data was analyzed using the SABiosciences qPCR macro for data analysis. Fold changes were calculated using the 2^{-C_t} method.

2.8. Western Blotting

Plasma samples were resolved on a 4–15% gradient sodium dodecylsulfate-polyacrylamide electrophoresis gel using the method described by Laemmli [20], and the proteins were transferred to a 0.2 µm pore size polyvinylidene fluoride (PVDF) membrane (Invitrogen, Carlsbad, CA). The following polyclonal antibodies (Santa Cruz Biotech, Santa Cruz CA) were diluted according to manufacturer's recommendations in 10 mM pH 7.5 Tris-HCl, 100 mM NaCl, and 0.1% Tween 20 (TTBS) with 5% non-fat milk, and used for immunoblotting of relevant proteins: goat anti-human CCL8 (C-17), goat anti-human CCL3 (C-16), goat anti-human CCL2 (C-17), and rabbit anti-human TGF- β_3 . The antibody-antigen complexes were detected with the species-appropriate, horseradish peroxidase-conjugated secondary antibodies in recommended dilutions in TBST with 5% non-fat milk and were visualized with an enhanced chemiluminescence detection system on X-ray films.

2.9. ELISAs

Commercial sandwich ELISA kits were purchased from AssayBiotech (Sunnyvale, CA) to detect the presence of CCL3, CCL8, CCL2, and TGF- β_3 in platelet poor plasma. In all cases, the procedure was done in accordance with the recommended manufacturer's protocol.

Absorbance was measured at 450 nm with a SpectraMax 190 Microplate Reader (Molecular Devices, Sunnyvale, CA).

2.10. Transcription Factor Binding Assays

To analyze changes in transcription factor binding, the TranSignal protein/DNA Array kit was used following the manufacturer's protocol (Affymetrix, Fremont, CA, USA). The nuclear extract was prepared using the nuclear extraction kit (Affymetrix). For quantitative results from the arrays, film images of the arrays at matching exposure times were scanned and analyzed for pixel number/array spot and mean intensity/array spot using ImageQuant Array Function (GE Biosciences, Piscataway, NJ) to find differences that were at least two fold (increase or decrease) between control and exposure to polymers or modified polymer surfaces.

2.11. PhosFlow Cytometry

THP-1 cells were harvested in FACS buffer, consisting of 1× PBS containing 1% BSA, blocked with goat serum and labeled with antibodies at 4°C for 45 min in the dark using Alexa Fluor-conjugated anti-pSTAT4 (pY693), PE-conjugated anti-pSTAT5 (pY694), PerCP Cy5.5-conjugated anti-pSTAT3 (pY705), and Alexa Fluor-conjugated anti-NF- κ B p65 (pS529) with the corresponding conjugated isotype control Igs (BD Biosciences, Franklin Lakes, NJ). All samples were analyzed using a BD LSR II or Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ). The results are reported as the percentage of the cells positive for the particular analyte. Cells are first subjected to forward-/side-scatter analysis to identify cell populations that correspond to viable cells and from this gated population, the cells that stain positive for pSTAT3, pSTAT4, and pSTAT5 are evaluated. The pSTAT3, pSTAT4, and pSTAT5 staining in each case is gated using isotype controls with the same fluorochrome.

2.12. Cellular Adhesion Assays

THP-1 cells were differentiated with the addition of PMA to their respective media and were cultured on PVC films for 72 hours. Where indicated, 50 μ M of pharmacological inhibitors to STAT5 (N-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide) or JAK 2 (1,2,3,4,5,6-Hexabromocyclohexane) were added to the culture. Films were washed in 1× PBS three times and remaining attached 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 200× fields were counted.

2.13. Multiplex Chromatin Immunoprecipitation Sequencing (ChIP-Seq)

THP-1 cells were exposed to standard PVC tubing or hCD47-modified tubing for three hours on the Chandler loop apparatus [13]. As a control, unexposed THP-1 cells were also used for the ChIP-Seq experiment. Following biomaterial exposure, the cells were fixed and proteins crosslinked to the genomic DNA using 1% proteomics-grade formaldehyde followed by quenching of the formaldehyde using 1× glycine. Cells were washed three times in ice cold 1× PBS with the final wash containing protease inhibitors. Cell pellets were flash-frozen and stored at -80°C. To digest the genomic DNA into mononucleosomes with minimal dinucleosomes, we used the EZ-Zyme (micrococcal nuclease) enzymatic chromatin prep kit from Millipore (Temecula, CA) following manufacturer instructions. Confirmation of mononucleosome chromatin preparation was completed by 2% agarose gel electrophoresis. Chromatin immunoprecipitation was completed using the Magna ChIP G Kit (Millipore, Temecula, CA) following manufacturer protocol. The anti-STAT5 antibody for ChIP was purchased from Santa Cruz Biotechnology (#SC-836X, Santa Cruz, CA). This

is a ChIP verified antibody previously used for ChIP-Seq [21]. The ChIP Seq DNA Sample Preparation guide (Illumina, San Diego, CA) was used to prepare the digested chromatin for multiplexed chromatin immunoprecipitation. Controls included no template (negative) and input library (positive) for each sample analyzed. After successful library preparation and verification of quality, samples were sequenced using the Illumina hiSeq 2000 sequencer (Illumina, San Diego, CA) with 50bp SR runs and 300 million raw reads per sample. Samples were multiplexed, which allows for up to 6 samples per lane. After run completion, reads were aligned to the genome using ELAND and areas of enrichment were determined by HOMER. Final analysis was completed using Partek genomic software suite for human genomic analysis (Partek, Saint Louis, MO).

2.14. Statistical Analysis

Statistical analysis of the difference between groups was assessed using ANOVA, followed by Tukey's test. $P = 0.05$ was taken as the significant level of difference. Assessment of statistical correlation was carried out using Pearson correlation analysis.

3. Results

3.1. CD47 Functionalized Polymeric Surfaces and Inflammatory Gene Expression

The effects of functionalized CD47 surfaces upon inflammatory cells have been well documented [13, 14]. However, a differential expression profile of pro-inflammatory genes was never performed. To that end, we analyzed mRNA from whole blood, of five different donors, exposed to PVC surfaces versus CD47 functionalized PVC surfaces for 3 hours using the Chandler Loop model [13,14]. The mRNA was converted to cDNA, which was then analyzed using the qPCR gene expression arrays. Table 1a is a list of all inflammatory genes, and their description that demonstrated a greater than 4-fold change in expression when blood cells are exposed to PVC modified surfaces compared to CD47 modified surfaces. Similarly, Table 1b is a list of inflammatory genes that demonstrate a greater than 4-fold increase in expression when cells are exposed to CD47 modified compared to unmodified PVC. As shown in Table 1a, exposure to unmodified PVC elicited an increase in pro-inflammatory cytokines and chemokines. Exposure to CD47 modified surfaces (Table 1b) increased the transcription of three chemokines (CCL2, CCL4, and CCL20). However, transcription of TGF β and several developmentally related genes, Growth Differentiation Factors 3 and 10, was also increased.

To begin to correlate transcriptional changes with protein expression, we measured the plasma levels of two clinically relevant chemokines that were upregulated when whole blood was exposed to unmodified PVC. CCL3 and CCL8, are chemokines involved in the recruitment and activation of inflammatory cells, and have both been shown to be upregulated when patients undergo cardiopulmonary bypass during cardiac surgery [22-24]. Figure 1A depicts representative Western Blot analysis and ELISA results for CCL3 expression. CCL3 levels were markedly, albeit not significantly, reduced when blood was exposed to CD47 modified surfaces compared to unmodified control surfaces. We also demonstrated that CCL8 levels (Figure 1B) were significantly ($p = 0.04$) decreased as a result of blood exposure to CD47 modified surfaces. Thus, the transcriptional up regulation of CCL8 and CCL3, observed in the microarray analysis when blood is exposed to unmodified PVC is also associated with an increase in CCL3 and CCL8 plasma levels.

CCL2 is a chemokine that is responsible for monocyte recruitment to areas of infection or tissue injury [25]. CCL2 gene expression was found to be increased when blood is exposed to CD47 modified surfaces. We examined the protein expression of CCL2 when blood is exposed to CD47 modified surfaces. Interestingly we found that the protein's expression was extremely variable across donors (Figure 2A). In addition, we also noted that overall CCL2

protein expression was undetectable when attempts were made to measure, via ELISA, its plasma concentration (data not shown). These data suggest that the CD47 SIRP pathway may not play a significant role in CCL2 expression.

Given the protein's significant role and cell signaling, we examined the protein expression profile of TGF β 3 as a function of blood exposure to CD47 modified surfaces. Shown in Figure 2B, TGF β 3 protein levels do not seem to fluctuate as a result of whole blood exposure to CD47 surfaces. Similarly, TGF β 3 protein expression was undetectable when attempts were made to measure, via ELISA, its plasma concentration (data not shown).

Although there were no discernable differences between TGF β 3 protein expression across test groups, the increase in TGF β 3 gene expression was compelling. Specifically, TGF β 3 is a potent mediator of extracellular matrix remodeling and cell adhesion [26,27]. To determine if CD47 exposure had any effects upon gene expression related to cell adhesion and matrix remodeling, we performed microarray analysis on whole blood exposed to CD47 modified and unmodified PVC tubing. Shown in Table 2, there was over a hundred-fold increase due to CD47 in specific genes associated with matrix remodeling and cell adhesion. Of particular note was the increased expression of several members of the matrix metalloproteinase (MMP) family. Specifically, expression of MMP-1, MMP-7, MMP-13, and MMP-16 was increased over 100-fold. In the case of MMP-7 and MMP-13, expression was increase 200-fold when blood was exposed to CD47 modified surfaces compared to unmodified PVC surfaces. These data strongly implicate a role for SIRP in ECM remodeling.

3.2. Changes in Transcription Factor Binding Activity on PVC Surfaces

Given the evidence that SIRP can signal through the JAK/STAT pathway [16], we were interested in examining the effects of CD47 exposure upon STAT activation. To measure the effect of CD47 upon inflammatory cell STAT activity, we measured the change in the transcription factor binding activity to double stranded DNA. This assay is superior to traditional measures of transcription factor activation because it measures binding activity rather than phosphorylation. Due to the large volume of cells required for this assay, we used a cell line model, THP-1 cells. THP-1 cells are a human monocytic-like cell line previously used by our laboratory and others to characterize the biocompatibility of various clinically used polymers [14,28]. In order to measure protein binding activity, nuclear lysates were prepared from untreated control THP-1 cells, THP-1 cells exposed to PVC for 2 hours, and THP-1 cells exposed to CD47 modified PVC for 2 hours using the Chandler Loop model. Transcription factors within the nuclear lysates were allowed to bind double stranded biotinylated oligonucleotides of 350 different transcription factor consensus sequences. Protein bound DNA was separated from unbound DNA and the protein bound biotinylated oligonucleotides were hybridized to an array containing complimentary sequences. This indirectly measures the protein-DNA binding capacity of 350 different characterized transcription factors. Figure 2 shows the decreased binding activity on STAT binding elements in cells that were exposed to PVC surfaces for 2 hours using the Chandler Loop model. These data demonstrate the possible involvement of STAT transcription factors in the gene regulation.

3.3. Exposure to PVC Surfaces and Transcription Factor Activity

We used phosflow cytometry to measure the intracellular phosphorylation status of the STAT transcription factors that were shown to be enriched in the transcription factor binding assay. THP-1 cells were exposed to PVC surfaces, CD47 modified PVC surfaces, or not exposed (control). At the indicated time point, cells were fixed and permeabilized for intracellular staining. Polychromatic phosflow cytometry was used to measure the

phosphorylation status of four different transcription factors simultaneously. Data indicate decreased phosphorylation of STAT5 when THP-1 cells were exposed to PVC surfaces (Figure 3). In contrast, THP-1 cells exposed to CD47 modified PVC and the control THP-1 cells maintained steady levels of STAT5 phosphorylation (Figure 3). These data confirm the findings of the transcription factor binding assay and suggest that decreased STAT5 activity may contribute to the change in inflammatory gene expression in response to polymeric biomaterials.

3.4. Cellular Attachment to PVC Films in the presence of JAK/STAT inhibitors

Since JAK2, which has been identified as a component of the SIRP signaling pathway, has been documented to activate STAT5 [29-31], we analyzed the effect of JAK2 and STAT5 inhibitors on cellular attachment to polymeric biomaterials. THP-1 cells were treated with PMA to induce differentiation and either pretreated with 50 μ M JAK2 (1,2,3,4,5,6-Hexabromocyclohexane) or 50 μ M STAT5 (N-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide) inhibitors for 30 minutes. Cells were plated on PVC films and allowed to attach over 72 hours. Cells pre-treated with the JAK2 or STAT5 inhibitors demonstrated decreased cellular attachment to the PVC surfaces (Figure 4). Since JAK2 and STAT5 inhibitors block cellular attachment to polymeric surfaces, it further reinforces their involvement in biocompatibility.

3.5. Genome Wide Analysis of STAT5 Binding Activity on In Vivo Promoters

We used chromatin immunoprecipitation for STAT5 coupled to next generation sequencing (ChIP-Seq) to profile, at the entire genome level, the changes in STAT5-DNA association. We compared THP-1 cells in culture (control) to THP-1 cells exposed to PVC tubing for 3 hours (PVC) and THP-1 cells exposed to CD47 modified PVC tubing for 3 hours (CD47). The list of genes that demonstrated differential STAT5 binding as a function of PVC surface exposure are outlined in Table 3, and are represented as fold-change over the no-antibody control. As shown, basal levels of STAT5 chromatin binding was rather limited with STAT-5 detected on the DNA sequences associated with only two genes. Interestingly STAT5-DNA associated of these two genes, AMZ2 and SHISA7, was also observed when THP-1 cells were exposed to unmodified PVC, but not CD47-modified PVC. Consistent with a role for STAT 5 in the inflammatory cell response to unmodified PVC, STAT 5 chromatin binding was identified with 25 genes following THP-1 exposure to unmodified PVC surfaces. In contrast, only two genes, SMAP2 and HSF2BP, were shown to have STAT5 chromatin association following exposure of THP-1 cells to CD47 modified PVC. Taken together, these results are consistent with a role of STAT5 in the regulation of inflammatory cells exposed to polymeric biomaterials.

4. Discussion

4.1. CD47 modified surfaces

The inflammatory response observed when blood is exposed to polymeric surfaces has been well documented, by others and us, and is associated with negative clinical outcomes [22-24]. Our previous work showed that recombinant CD47 can be appended to PVC surfaces with the overall goal of ameliorating the inflammatory response [13,14]. The studies herein were aimed at understanding, at the molecular level, how CD47 modified surfaces contribute to this observed enhanced biocompatibility. To that end, this work is amongst the first of its kind to employ a broad molecular approach to compare the differential inflammatory response to an unmodified, clinically relevant, polymer and the identical polymer modified with a bioactive anti-inflammatory surface. We showed herein that inflammatory gene expression is largely downregulated when whole blood is exposed to

CD47 functionalized surfaces, and that the changes in transcription appear to correspond to a reduction in plasma protein levels.

We also demonstrate that CD47 exposure differentially regulates STAT transcription factor binding and the phosphorylated status of STAT5. These findings are in accordance with previous work demonstrating that SIRP has the capacity to activate components of the JAK/STAT signaling pathway [16]. Specifically, these previous studies suggested that in macrophages, SIRP signaling stimulated macrophage responses, such as increased NO release and H₂O₂ signaling. These data are somewhat contrary to the results published by our laboratory, and others, showing that CD47-SIRP signaling pathway reduces pro-inflammatory events [13,14]. However, these previous results establish the JAK/STAT pathway as a potential cell signaling mechanism of interest in our ongoing investigations into how surface immobilized, recombinant CD47 confers anti-inflammatory properties to biomaterials [13,14]. Taken together, these results begin to explain the molecular mechanisms responsible for the observed anti-inflammatory capabilities of CD47 modified surfaces and indicate a possible involvement of the STAT transcription factors in the inflammatory response to polymeric biomaterials.

4.2. Gene expression in response to CD47 modification of polymeric biomaterials

Microarray analysis showed that eleven genes, associated with inflammatory processes, were upregulated when blood was exposed to unmodified PVC surfaces compared to CD47 functionalized surfaces. Further analysis of CCL3 and CCL8 gene expression showed that the increase in transcription correlated with an increase in CCL3 and CCL8 plasma protein levels. These data are consistent with previous clinical results that show an increase CCL8 gene transcription in patients undergoing cardiopulmonary bypass (CPB) [32]. In that study, it was demonstrated that protein coated CPB circuits had a reduced expression profile of inflammatory markers compared to heparin coated CPB circuits [32]. Unfortunately the study did not investigate the protein expression patterns of these pro-inflammatory markers.

Given the previously cited clinical results associated with CCL3 and CCL8, we examined if the changes in mRNA expression, observed with the microarray results, were consistent with changes in the expression of these two pro-inflammatory chemokines. As shown, both CCL3 and CCL8 protein expression was increased in blood exposed to unmodified surfaces. These data support the clinical results and suggest a role for both CCL3 and CCL8 in the inflammatory response to CPB.

These experiments showed increased transcription of several broadly defined families of genes as a result of CD47 exposure. Transcription of the chemokines CCL2, CCL4, and CCL20 was increased from 3 to a 6-fold when blood was exposed to CD47 modified surfaces compared to unmodified control PVC surfaces. However, the fold change in transcription of other chemokines was far greater (4 to 15-fold increase), compared to CD47 modified surfaces, when blood was exposed to non-modified PVC. CCL2 and CCL4 have been shown to be potent monocyte attractants [33,34]. This is an interesting observation as we have shown that CD47 modified surfaces to be noninflammatory as evident by a significant reduction in several markers of inflammatory cell activation [13,14]. Both CCL2 and CCL4 have been shown to be upregulated in the presence of endotoxin [25]. Thus the presence of endotoxin, as a result of the CD47 modification, cannot be discounted. Of note, is that the presence of CCL2 protein in the plasma did not appear to be associated with CD47 exposure. Whether this was due to relatively short window of time (three hours) that the blood was exposed to CD47 surfaces before appreciable levels of plasma CCL2 could be quantified, or that CCL2 is not directly regulated through the CD47-SIRP pathway, remains to be determined.

We also showed that CCL20 transcription was increased 5.87-fold when blood is exposed to CD47 functionalized surfaces. CCL20 has been shown to inhibit the production of reactive oxygen species in inflammatory cells [35]. These observations are consistent with our previous studies showing that CD47 modified polyurethane was significantly resistant to ROS mediated degradation compared to unmodified polyurethane. Understanding the role of differential chemokine transcription, as a function of CD47 exposure, would have been exhaustive and beyond the scope of these current experiments. However, these data do provide a foundation for additional studies into CD47-SIRP signaling.

Some of the highest levels of differential gene transcription observed in blood exposed to CD47 modified PVC came from genes responsible for growth and differentiation. Specifically, transcription of Growth Differentiation Factors (GDF) 3 and 10 and TGF β_3 were all upregulated 6-8 fold as a result of CD47 exposure. Due to its role in scar tissue reduction and extracellular matrix remodeling [27,36,37], we chose to further investigate the effects of immobilized CD47 exposure upon TGF β_3 protein expression and potential downstream signaling. TGF β_3 has been shown to inhibit fibrosis by increasing expression of matrix metalloproteinase (MMP) [26,38,39]. Indeed, recombinant TGF β_3 , known as Avotermin, has been examined by the pharmaceutical industry for just that purpose [40,41]. The results shown herein demonstrate that CD47 exposure can increase the transcription of TGF β_3 , as well as several members of the MMP family. Taken together, these data suggest that surface immobilized CD47 may reduce the fibrosis observed as a result of the inflammatory response to implanted biomaterials. In addition, these results also suggest a role for SIRP signaling mechanisms in scar reduction.

4.3. JAK/STAT pathway

These studies represent the first attempts to identify the molecular components that contribute to the anti-inflammatory properties of CD47 functionalized surfaces. To that end, we focused on the role of the JAK/STAT signaling pathway. This was driven largely by the role of the JAK/STAT signaling pathway in inflammation, and that others showed that SIRP was capable of activating JAK2 [16-19]. We used a transcription factor binding array to examine the differential response of members of the STAT family of transcription factors to exposure to CD47 modified PVC surfaces. The advantage to this technique was measurement of physical binding to DNA rather than changes in protein expression or phosphorylation, which at times are less likely to reveal functional changes. As we shown herein, the changes in the STAT pathway encompassed nearly the entire family of STAT proteins.

To further ascertain the effects of CD47 exposure to STAT physiology we examined the intracellular changes in the phosphorylation state, via phosflow cytometry, of members of the STAT family. We found significantly lower phosphorylation of STAT5 in cells exposed to PVC surfaces compared to THP-1 cells exposed to CD47 modified surfaces and those THP-1 control cells not exposed to the tubing. Interestingly, CD47 modified PVC surfaces had similar phosphorylation levels of STAT5 as the control group. These data implicated STAT5 regulation as a possible downstream target of SIRP signaling. It has been shown that JAK2 can regulate STAT5. Thus, the identification of STAT5 is of interest when considering that SIRP plays a role in JAK2 regulation.

The STAT5 chromatin binding studies, detailed in Table 3, did not reveal any obvious correlations with the microarray analysis. This may be due to the fact that the microarray results were from human whole blood and the STAT5 chromatin binding study were from the THP-1 cell line. However, the STAT5 chromatin binding studies demonstrated a large difference in STAT5 binding, as a result of THP-1 cells exposure to unmodified or CD47 modified surfaces, across a range of genes. Specifically, STAT 5 binding was observed to be

more prevalent when THP-1 cells were exposed to unmodified PVC. Of note, several pro-inflammatory genes were identified in this genome-wide screening. Bactericidal/permeability-increasing protein, Interleukin 31 receptor A, Archaelysin family metallopeptidases 2 are all associated with inflammatory cell activation.

In these studies we identified STAT5 chromatin binding to the genes encoding α 1 integrin and Septin 9 when THP-1 cells were exposed to unmodified PVC. Both of these proteins are associated with increased cellular adhesion and pseudopod extension [42-44]. These data are consistent with the THP-1 binding study performed herein that showed a significant decrease in THP-1 binding to PVC surfaces in the presence of JAK-STAT inhibitors. In addition, groups, including our own, have observed decreased cellular attachment and pseudopod extension when inflammatory cells are exposed to CD47 functionalized surfaces [13-15, 45]. These results begin to implicate JAK2 and STAT5 as molecular regulators in inflammatory cell interactions with synthetic surfaces.

It must be noted that the molecular components of the JAK/STAT signaling pathway, in the overall inflammatory response, are not entirely understood [19]. For example, STAT activation has been shown to elicit both anti-inflammatory and proinflammatory cellular responses [19]. In our studies we show that STAT 5 appears to be preferentially phosphorylated when cells are exposed to non-inflammatory, CD47 functionalized surface. However, the presence of JAK2 and STAT5 inhibitors reduces the overall inflammatory response, as measured by THP-1 cell attachment to PVC films (Figure 5). As these two results contradict each other, the model presented in Figure 6 provides a plausible explanation for our results. First, the proinflammatory response is a SIRP independent signaling pathways that elicits a Jak2 and STAT5 mediated proinflammatory response. However, this proinflammatory response can be overridden when SIRP is liganded with CD47. As STAT5 has been shown to occasionally function in the presence of cofactors [46], the presence of such additional intracellular proteins cannot be ruled out from our current studies.

5.0. Conclusions

CD47-SIRP signaling mechanisms mediate a more global anti-inflammatory response that extends beyond its canonical role as an inhibitor of phagocytosis. Within the context of acute whole blood exposure to CD47 modified and unmodified control blood conduits, CD47 mediated signaling regulates chemokine and cytokine transcription, increases MMP transcription, and reduces the plasma concentration of known pro-inflammatory chemokines that are associated with negative clinical outcomes. Results presented herein also indicate that JAK2 and STAT 5 are molecular components that contribute to the parameters that define biocompatibility. These data contribute to our understanding of the established anti-inflammatory environment conferred by recombinant CD47 upon synthetic surfaces. In addition, they also identify JAK2 and STAT5 as components of a molecular signaling pathway that may be further explored to decrease cellular attachment and activation on clinically used biomaterials.

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A. **B.** **Fig 1.**

Protein plasma expression of select chemokines showing increased translation following whole blood exposure to unmodified control blood conduits compared to CD47 modified conduits. (A) Representative Western blot images and ELISA analysis of CCL3 and Albumin (control for equal loading) protein expression, using plasma from human whole blood. Results show a decrease in CCL3 expression when blood is exposed to CD47 modified PVC. (B) Representative Western blot images and ELISA analysis of CCL3 and Albumin (control for equal loading) protein expression, using plasma from human whole blood. Results show a decrease in CCL3 expression when blood is exposed to CD47 modified PVC. Control (CTRL) Samples were not exposed to the Chandler Loop apparatus, exposed to PVC for 4 hours (PVC), or exposed to CD47-modified PVC for 4 hours (CD47).

ELISA results represent the average and standard error of the means of triplicate samples of n=7 individual donors.

Fig 2.

Western Blot results showing plasma expression of CCL2 and TGF β_3 following whole blood exposure to unmodified control blood conduits compared to CD47 modified conduits. (A) Western blot images from three separate donors demonstrating donor variability, during blood exposure to CD47 surfaces, in CCL2 plasma levels. (B) Representative Western blot analysis showing that TGF β_3 plasma expression in whole blood, exposed to CD47 modified or control unmodified PVC blood conduits, was unchanged. Control (CTRL) represents whole blood not exposed to the Chandler Loop protocol.

Fig 3.

Changes in Transcription Factor Binding Activity. Transcription factor binding activity was assessed for members of the STAT family of proteins. Exposure to CD47 modified PVC surfaces increased STAT transcription factor binding compared to unmodified PVC surfaces.

Fig. 4.

Differential STAT 5 phosphorylation in response to CD47 surfaces. THP-1 cells were exposed to PVC tubing (open square), CD47 modified PVC (closed circle) for 90 minutes as detailed in *Materials and Methods*. Control cultures (black triangle) were not exposed to PVC tubing. Phosphorylation of members of the STAT family of protein was assessed via PhosFlow analysis. STAT 5 phosphorylation was significantly reduced when cells were exposed to unmodified PVC surfaces. $*p = 0.005$ for each time point comparing PVC exposure with control or CD47 functionalized PVC.

Fig. 5. JAK2 and STAT5 Inhibitors Block Cellular Adhesion to Polymeric Biomaterials. (A) Representative fluorescent micrographs of DAPI staining of THP-1 cells cultured on PVC with standard media, as detailed in Materials and Methods, or in the presence of 50 μ M pharmacological inhibitors to JAK 2 (1,2,3,4,5,6-Hexabromocyclohexane) or STAT 5 (N - ((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide). (B) Quantitative analysis showing cells counts from DAPI stained fields (200 $\times$) demonstrating that treatment of JAK2 or STAT5 inhibitors significantly ($*p < 0.001$ compared with control) decreased cellular attachment to the PVC surfaces.

Fig. 6. Proposed Mechanisms for CD47 mediated JAK/STAT signaling. CD47 functionalized surfaces initiate an inhibitory response, via the Immune cell tyrosine inhibitory domain (ITIM) on SIRP α . SIRP α signaling mechanisms appear to involve members of the STAT, particularly STAT 5, family of transcription factors. In the absence of CD47, JAK2 and STAT5 appear to function in a pro-inflammatory capacity, facilitating the attachment of inflammatory cells to polymeric surfaces. The presence of an intracellular cofactor may mediate the CD47 dependent switch.

Table 1a

Genes upregulated on unmodified PVC surfaces compared to CD47 functionalized PVC.

Gene Symbol	Gene Name	Gene Function	Change Expression
IL1F5	Interleukin 1 Family member 5 (Delta)	Pro-inflammatory cytokine	8.35
IL1F10	Interleukin 1 Family member 10 (Theta)	Pro-inflammatory cytokine	11.32
IL17F	Interleukin 17F	Pro-inflammatory cytokine	13.9
INH1A	Inhibin Alpha	Pro-inflammatory cytokine	17.18
BMP2	Bone Morphogenetic Protein 2	Bone Formation	7.77
CCL3	Chemokine(C-C Motif) Ligand 3	Neutrophil Recruitment	6.54
CCL8	Chemokine(C-C Motif) Ligand 8	Pro-inflammatory chemokine	15.92
CCL28	Chemokine(C-C Motif) Ligand 28	Pro-inflammatory chemokine	5.15
CXCL12	Chemokine (C-X-C Motif) Ligand 12	Leukocyte recruitment	6.63
CXCL13	Chemokine (C-X-C Motif) Ligand 13	B lymphocyte recruitment	4.81
TNFSF15	Tumor Necrosis Factor (Ligand) Superfamily, Member 15	Pro-inflammatory mediator	5.23

Table 1b

Genes upregulated on CD47 functionalized PVC surfaces compared to unmodified PVC.

Gene Symbol	Gene Name	Gene Function	Change Expression
TNFSF10	Tumor Necrosis Factor (Ligand) Superfamily, Member 10	Pro-apoptotic cytokine	4.51
GDF3	Growth Differentiation Factor 3	Regulates TGF-b superfamily members	8.25
GDF10	Growth Differentiation Factor 10	Development	6.33
TGFB3	Transforming Growth Factor Beta 3	Reduce scar formation	9.98
CCL2	Chemokine(C-C Motif) Ligand 2	Pro-inflammatory cytokine	3.97
CCL4	Chemokine(C-C Motif) Ligand 4	Macrophage inflammatory protein	4.02
CCL20	Chemokine(C-C Motif) Ligand 20	Macrophage inflammatory protein	5.87
CXCL1	Chemokine (C-X-C Motif) Ligand 1	Neutrophil activating protein	6.11
CD40LG	CD40 Ligand (TNF Superfamily)	Expressed on activated T-cells	11.76

Table 2

Cell Adhesion related genes upregulated on CD47 Functionalized PVC surfaces compared to unmodified PVC.

Gene Symbol	Gene Name	Gene Function	Change Expression
CTNND2	Catenin delta 2	Cell adhesion	94.09
MMP1	Matrix Metalloproteinase-1	Extracellular Matrix Remodeling	138.94
MMP7	Matrix Metalloproteinase-7	Extracellular Matrix Remodeling	215.24
MMP13	Matrix Metalloproteinase-13	Extracellular Matrix Remodeling	201.58
MMP16	Matrix Metalloproteinase-16	Extracellular Matrix Remodeling	102.91
CNTN1	Contactin 1	Cell adhesion	120.63

Table 3
Differential STAT-5 chromatin binding in THP-1 cells exposed to CD47-modified or unmodified control PVC

<i>Basal STAT-5 Chromatin Binding</i>			
Symbol	Definition	Fold-Change	Accession No.
AMZ2	Archaealysin family metallopeptidase	105.60	2NM_16627
SHISA7	Protein shisa-1	56.39	NM_001145176
<i>STAT-5 Chromatin Binding in cells exposed unmodified PVC</i>			
Symbol	Definition	Fold-Change	Accession No.
SMAP2	Small ArfGAP2	10.6	NM_022733
SMAP2	Small ArfGAP2	10.6	NM_001198978
LOC100130557	Region of chromosome 1	4.84	NR_024567
NFYC	Nuclear transcription factor Y	4.84	NM_014223
NFYC	Nuclear transcription factor Y	4.84	NM_001142590
CASQ1	Calsequestrin 1	1.59	NM_001231
NOS1AP	Nitric oxide synthase 1	1.61	NM_014697
ITGB1	β_1 integrin	2.11	NM_133376
SPOP	Speckle-type POZ protein	7.6	NM_001007226
AMZ2	Archaealysin family metallopeptidase 2	105.60	NM_001033569
9-Sep	Septin 9	1	NM_001113491
SHISA7	Protein shisa-1	56.39	NM_001145176
C2orf67	KAT8 regulatory NSL complex subunit 1	6.05	NM_152519
TMEM169	Transmembrane protein 169	1	NM_138390
BPI	Bactericidal/permeability-increasing protein	8.50	NM_001725
HSF2BP	Heat shock transcription factor 2 binding protein	1	NM_007031
CACNA2D3	Calcium channel, voltage dependent, α_2/δ subunit 3	2.03	NM_018398
IL31RA	Interleukin 31 receptor A	1	NM_139017
PCBD2	Pterin-4- α carbinolamine dehydratase/dimerization cofactor	1.39	NM_032151
C7orf50	Chromosome 7 open reading frame	.87	NM_001134395
PTK2B	Protein kinase 2 beta	1.89	NM_173175
EPPK1	Epiplakin 1	2.23	NM_031308
PTPRD	Protein tyrosine phosphatase receptor D	2.28	NM_002839
GRIN1	Glutamate receptor, ionotropic, N-methyl D-aspartate	1	NM_021569
LOC442459	X chromosome non-coding region	2.08	NR_024608
<i>STAT-5 Chromatin Binding in cells exposed to CD47 modified PVC</i>			
Symbol	Definition	Fold-Change	Accession No.
SMAP2	Small ArfGAP2	2.59	NM_022733
SMAP2	Small ArfGAP2	10.60	NM_001198978
HSF2BP	Heat shock transcription factor 2 binding protein	1	NM_007031