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Biomaterials differentially regulate Src kinases and phosphoinositide 3-kinase- γ in polymorphonuclear leukocyte primary and tertiary granule release

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Abstract

In the foreign body response, infiltrating PMNs exocytose granule subsets to influence subsequent downstream inflammatory and wound healing events. In previous studies, we found that PMNs cultured on poly(ethylene glycol) (PEG)-containing hydrogels (i.e., PEG and gelatin + PEG hydrogels) had enhanced primary granule release, yet similar tertiary granule release compared with PMNs cultured on polydimethylsiloxane or tissue culture polystyrene. PMN primary granules contain microbicidal proteins and proteases, which can potentially injure bystander cells, degrade the extracellular matrix, and promote inflammation. Here, we sought to understand the mechanism of the enhanced primary granule release from PMNs on PEG hydrogels. We found that primary granule release from PMNs on PEG hydrogels was adhesion mediated and involved Src family kinases and PI3K- γ . The addition of gelatin to PEG hydrogels did not further enhance PMN primary granule release. Using stable-isotope dimethyl labeling-based shotgun proteomics, we identified many serum proteins – including Ig gamma constant chain region proteins and alpha-1-acid glycoprotein 1 – that were absorbed/adsorbed in higher quantities on PEG hydrogels than on TCPS, and may be involved in mediating PMN primary granule release. Ultimately, this mechanistic knowledge can be used to direct inflammation and wound healing following biomaterial implantation to promote a more favorable healing response.

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Conflicts of interest

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Keywords

Neutrophil; Acute inflammation; Degranulation; Poly(ethylene glycol); Gelatin; Protein adsorption

1. Introduction

Infiltrating PMNs play a critical role in the inflammatory response to biomaterials. PMNs contain nearly 300 granule proteins, which are compartmentalized into four granule subsets (i.e., primary granules, secondary granules, tertiary granules, and secretory vesicles) [1–3]. Of the four granule subsets, secretory vesicles are released first and most readily, upon PMN adhesion to the endothelium. PMN tertiary granules are released next, during PMN diapedesis, and contain extracellular matrix (ECM)-degrading enzymes such as matrix metalloproteinase-9 (MMP-9) [4]. PMN secondary and primary granules are released after PMN extravasation to the site of inflammation, and contain antimicrobial reagents and ECM-degrading enzymes such as myeloperoxidase (MPO), cathelicidin LL-37, human neutrophil peptides 1–3 (HNP1–3), cathepsin G, and elastase [4]. PMN primary and secondary granule proteins also facilitate monocyte (MC) chemotaxis and mediate MC/macrophage activation and function [4–15]. Selected PMN granule proteins and their functions are shown in Supplementary Table 1, demonstrating their importance in inflammatory and wound healing events.

Previous studies from our group showed that PMNs cultured on poly(ethylene glycol) (PEG)-containing hydrogels (i.e., PEG hydrogels and gelatin + PEG [GP] hydrogels) had significantly greater primary granule release than PMNs cultured on polydimethylsiloxane (PDMS) or tissue culture polystyrene (TCPS) [16,17]. To understand the underlying mechanisms of this enhanced primary granule release, we investigated the following:

1. The kinetics of PEG hydrogel-mediated PMN degranulation to confirm that primary granule release is a cell adhesion-mediated event.
2. The role of gelatin in promoting PMN degranulation. Other groups have demonstrated that ECM components such as collagen and RGD tripeptide can attenuate the pro-inflammatory foreign body response to synthetic materials [18–23].
3. The role of Src family kinase and phosphoinositide 3-kinase- γ (PI3K- γ) signaling in PMN degranulation in the presence or absence of bacterial peptide formyl-Met-Leu-Phe (fMLP). We previously found that Src family kinases were involved in primary granule release from PMNs cultured on PEG hydrogels, TCPS, and GP hydrogels and in tertiary granule release from PMNs cultured on PEG hydrogels, PDMS, TCPS, and GP hydrogels [17]. PI3K- γ is a signaling molecule that has been shown to be involved in early, adhesion-independent, fMLP-induced PMN activation [24–26].
4. The role of adsorbed proteins on PEG hydrogels and TCPS in PMN degranulation.

2. Materials and methods

2.1. Biomaterial substrate preparation

PEG diacrylate (molecular weight 3350) and cysteine/PEG-modified gelatin (gel-PEG-cys) were synthesized as previously described [27] and characterized by proton NMR (Varian Unity-Inova 400 MHz; Varian Inc., Palo Alto, CA). To prepare PEG hydrogels, we dissolved 10 weight percent PEG diacrylate (molecular weight 3350) and 0.1 weight percent 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Sigma-Aldrich, St. Louis, MO) in water with heat (37 °C). GP hydrogels were prepared by dissolving 10 weight percent PEG diacrylate (molecular weight 3350), 0.1 weight percent 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone and 10 weight percent gel-PEG-cys in water with heat (37 °C). The PEG and GP hydrogel solutions were injected into Teflon molds held between two pre-cleaned glass slides and polymerized under a UV light (UV CF1000 LED, $\lambda_{\text{max}} = 365$ nm; Clearstone Technologies, Minneapolis, MN) for 3 min per side. The PEG and GP hydrogel films were incubated in 70% ethanol (Decon Labs, Inc., King of Prussia, PA) for 45 min and equilibrated overnight in Dulbecco's phosphate-buffered saline (DPBS; Cellgro, Manassas, VA). We used biopsy punches (Miltex, Inc., York, PA) to cut the PEG and GP hydrogel films into 8-mm diameter disks. The hydrogel disks were placed into 48-well plates (Corning Inc., Corning, NY) and incubated in 70% ethanol for 45 min. Hydrogels containing varying compositions of PEG and gelatin were prepared by mixing PEG diacrylate and gelatin (type B, bloom 75, from bovine skin) at the indicated weight percentages in water containing 0.1 weight percent 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone. These hydrogel solutions were injected into Teflon molds held between two glass slides, polymerized under a UV light, and cut into 8-mm diameter disks as described above.

Gelatin hydrogels were prepared by dissolving 15 or 20 weight percent gelatin (type B, bloom 75, from bovine skin; Sigma-Aldrich, St. Louis, MO) in water with heat (37 °C). The gelatin solution was added to wells of a 48-well plate (200 μ l/well). The well plate was incubated at -20 °C for 5 min to allow the hydrogels to gel. The hydrogels were then crosslinked with 4% paraformaldehyde in PBS (Affymetrix, Inc., Cleveland, OH) for 20 min at room temperature. The 4% paraformaldehyde solution was removed and the gelatin hydrogels were washed three times with DPBS. The hydrogels were washed three more times with DPBS per day for another two days prior to seeding with PMNs.

PDMS sheets (0.762 mm thickness, non-reinforced vulcanized gloss/gloss; Specialty Manufacturing Inc., Saginaw, MI) were cut into 8-mm diameter disks with biopsy punches and sonicated in a 0.5 weight percent solution of sodium dodecyl sulfate (Amresco, Solon, OH) for 10 min. The PDMS disks were rinsed with deionized water, placed into 48-well plates, and incubated in 70% ethanol for 45 min. PEG hydrogels, GP hydrogels, hydrogels containing varying compositions of PEG and gelatin, and PDMS disks were washed three times with DPBS and equilibrated in RPMI-1640 basal medium (Cellgro, Manassas, VA) prior to seeding with cells. TCPS wells (Corning Inc., Corning, NY) were also washed three times with DPBS and equilibrated in RPMI-1640 medium prior to cell seeding.

2.2. Culture and analysis of human blood-derived PMNs

Peripheral whole blood was obtained from healthy adult donors in accordance with protocols approved by the University of Wisconsin–Madison Institutional Review Board. PMNs were isolated as previously described using a density gradient method [28]. Unless otherwise indicated, PMNs were adjusted to 1×10^6 cells/ml in RPMI-1640 medium supplemented with 10% autologous human serum (AHS) and were statically seeded onto the biomaterial substrates in 48-well plates (0.5 ml/well for a density of approximately 5.3×10^3 PMNs/mm²). The cells were incubated in a humidified atmosphere at 37 °C with 5% CO₂.

Metabolic capacity as an indicator of cell viability was measured using CellTiter-Blue[®] Reagent (Promega Corporation, Madison, WI). At pre-determined time points, the culture media was aspirated from PMN cultures and replaced with a solution containing 10% CellTiter-Blue[®] Reagent in RPMI-1640 medium supplemented with 10% AHS. PMN cultures containing the CellTiter-Blue[®] Reagent were incubated for 4 h in a humidified atmosphere at 37 °C with 5% CO₂. The culture media was then transferred to wells in a 96-well plate (100 µl/well; Corning Inc., Corning, NY) and the fluorescent signal (584-nm excitation, 620-nm emission) was measured using a plate reader (FLUOstar OPTIMA; BMG Labtech, Cary, NC). Fluorescent intensities (FI) of PMN samples were normalized to biomaterial control samples that did not contain cells.

Supernatants from PMN cultures on the biomaterials (seeded at a density of approximately 5.3×10^3 PMNs/mm²) were collected and centrifuged at $2500 \times g$ for 10 min at 4 °C to remove cells and cellular debris. MPO and MMP-9 in supernatants from PMN cultures were measured using a multi-analyte Luminex[®] screening assay kit (R&D Systems, Inc., Minneapolis, MN) as biomarkers for the release of primary and tertiary granule subsets, respectively. The assay was read using a Bio-Plex 100 Suspension Array System (Bio-Rad Laboratories, Hercules, CA). MPO and MMP-9 in the 10% AHS culture medium ranged from 3.8–10.3 ng/ml and 7.1–15.2 ng/ml, respectively, depending on donor.

2.3. PMN pre-treatment with Src family kinase inhibitor PP1 and/or PI3K-γ inhibitor AS604850

After isolation, PMNs were suspended at a concentration of 2×10^6 cells/ml in RPMI-1640 medium supplemented with 10% AHS and 10 µM Src family kinase inhibitor PP1 (4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo-d-3,4-pyrimidine; EMD Millipore, Billerica, MA), 1 µM PI3K-γ inhibitor AS604850 (Echelon Biosciences Inc., Salt Lake City, UT), or an equivalent volume of dimethyl sulfoxide (DMSO; Sigma–Aldrich, St. Louis, MO). These doses were selected based on previous reports [25,29,30]. The PMNs were statically seeded onto PEG hydrogel, PDMS, TCPS, GP hydrogel, and 15 weight percent gelatin hydrogel surfaces in 48-well plates (0.25 ml/well for a density of approximately 5.3×10^3 PMNs/mm²). The plates were incubated in a humidified atmosphere at 37 °C with 5% CO₂ for 10 min. After 10 min, an additional 250 µl of RPMI-1640 medium supplemented with 10% AHS or 10% AHS containing 200 nM fMLP was added to the wells, resulting in a final fMLP concentration of 100 nM for half of the wells. This concentration of fMLP is used to stimulate PMN degranulation [31]. The well plates were incubated in a humidified

atmosphere at 37 °C with 5% CO₂. After 2 h, cell viability, MPO, and MMP-9 were measured as described above.

2.4. Serum protein adsorption on PEG hydrogels and TCPS

Peripheral blood was drawn from fasted human donors into a container without anticoagulant. The blood was incubated in a 37 °C water bath for 2 h to allow for clotting. The clotted blood was centrifuged for 10 min at 1600 × g at room temperature. Serum was collected, filtered through a 0.22 µm filter, and stored at −20 °C. Serum was fractionated using PEG 4000 to reduce albumin [32,33]. This method has been validated previously in our group: removal of albumin had no effect on the relative concentration of total proteins adsorbed on PEG hydrogels or TCPS, as determined by Fourier transform infrared spectroscopy and bicinchoninic acid test [32]. Briefly, PEG (MW 4000; Sigma–Aldrich, St. Louis, MO) was added to the serum at a concentration of 1 g PEG per 10 ml serum (i.e., 3.5 g PEG to 35 ml serum). The solution was stirred in a beaker on ice. After 60 min, the solution was centrifuged at 800 × g for 30 min at 4 °C. The pellet was set aside as Fraction 1. The supernatant was added into a new beaker and the same amount of PEG as used previously (i.e., 3.5 g) was added to the beaker. The solution was stirred on ice for 30 min. The solution was centrifuged at 800 × g for 30 min at 4 °C. The pellet was set aside as Fraction 2 and the supernatant was removed and set aside as Fraction 3. Fractions 1 and 2 were combined and resuspended to the original volume of the serum (i.e., 35 ml) with DPBS. PEG hydrogel disks 74 mm in diameter were prepared as described above and placed into tissue culture dishes with an 80-mm internal diameter and 60.1 cm² growth area (TPP, Trasadingen, Switzerland). RPMI-1640 supplemented with 10% of serum fractions 1 and 2 was added to the dishes containing PEG hydrogels and to empty TCPS dishes (12 ml per dish) and the dishes were placed in a humidified atmosphere at 37 °C with 5% CO₂ for 2 h. Because PEG hydrogels are porous, both adsorption and absorption of proteins could occur, so we will use the term adsorption to refer to both adsorption and absorption.

2.5. On-surface enzymatic digestion, dimethyl labeling, and nanoLC-MS² analysis

All reagents were purchased from Fisher Chemical (Fair Lawn, NJ) unless otherwise indicated. After 2 h, the dishes containing PEG hydrogels and TCPS were removed from a humidified atmosphere at 37 °C with 5% CO₂ and washed 2 times with 12 ml DPBS for 10 min at 37 °C with gentle rocking. Proteins adsorbed on PEG hydrogels and TCPS were denatured and disulfide bonds were reduced by adding 5 ml per dish of a solution containing 5 mM dithiothreitol (DTT), 8 M urea, and 50 mM ammonium bicarbonate and rocking gently for 60 min at 37 °C. Cysteines were alkylated by adding 100 µl of 750 mM iodoacetamide (IAA) to each dish for a final concentration of 15 mM IAA and incubating the dishes in the dark for 30 min. Proteins on the materials were digested by adding approximately 17 µg trypsin/Lys C Mix (Promega Corporation, Madison, WI) in a solution containing 1 M urea and 50 mM ammonium bicarbonate pH 8 (3.3 ml per dish). The dishes were incubated at 37 °C overnight with gentle rocking. The digested peptide solutions were removed and the materials were washed with 0.5 M NaCl (1 ml per dish). The washes were added to the solutions of digested peptides and were acidified to pH 2 with 10% trifluoroacetic acid (TFA) to quench the digestion. Peptides were desalted using a Sep-Pak C18 solid phase extraction (SPE) cartridge (Waters, Milford, MA) and dried *in vacuo* by

Speed-Vac. Peptides were dissolved in 1 M urea, 50 mM ammonium bicarbonate pH 8 and subjected to a second in-solution digest with 3 µg trypsin/Lys C Mix at 37 °C overnight. The digestion was quenched by acidification to pH 2 with 10% TFA, and the peptide samples were cleaned with SCX SpinTips (Protea Biosciences, Morgantown, WV).

Stable-isotope dimethyl labeling was used to dimethylate peptide N-termini and lysine side-chains as described previously [34]. PEG hydrogel samples were labeled with light formaldehyde and TCPS samples were labeled with heavy formaldehyde-d₂. Peptides in 1% TFA were bound to Sep-Pak C18 SPE cartridges, washed with 0.1% formic acid (FA), and labeled with 6 ml light or heavy formaldehyde solutions (0.8% formaldehyde or formaldehyde-d₂, 0.12 M sodium cyanoborohydride, 50 mM sodium phosphate buffer pH 7.4). After washing again with 0.1% FA, labeled peptides were eluted with 60:40 acetonitrile (ACN):water and dried *in vacuo*.

Labeled samples were combined and analyzed by nanoflow reversed-phase liquid chromatography-tandem mass spectrometry (nanoLC-MS²) using a Waters nanoAcquity UPLC system (Milford, MA) coupled to a Thermo Scientific Orbitrap Elite mass spectrometer (San Jose, CA). Peptide samples in 3% ACN and 0.1% FA were injected onto a 15 cm microcapillary column fabricated with an integrated emitter tip and packed with 15 cm C18 particles (Bridged Ethylene Hybrid, 1.7 µm, 130 Å; Waters, Milford, MA). Mobile phase A was composed of water, 5% DMSO, and 0.1% FA; mobile phase B was composed of ACN, 5% DMSO, and 0.1% FA. Separation was performed using a gradient elution of 5%–35% mobile phase B over 90 min at a flow rate of 300 nl/min. Mass spectra of peptide precursors from 380 to 1600 *m/z* were acquired at a resolving power of 120 k (@ 400 *m/z*) and the top ten precursors were selected for data-dependent higher-energy collisional dissociation (HCD) tandem mass (MS²) acquisition at a resolving power of 15 k. Precursors were subject to dynamic exclusion for 15 s.

Identification and quantitation of labeled peptides was performed using Proteome Discoverer (version 1.4.0.288, Thermo Scientific, Waltham, MA). We searched raw files against UniProt *Homo sapiens* reference database using the Sequest HT algorithm with trypsin selected as the enzyme. Searches were performed with a precursor mass tolerance of 25 ppm and a fragment mass tolerance of 0.03 Da. Static modifications consisted of light or heavy dimethylation on peptide N-termini (C₂H₂: +28.0313; C₂D₂: +32.0542 Da) and carbamidomethylation of cysteine residues (+57.02146 Da). Dynamic modifications consisted of light or heavy dimethylation on peptide N-termini (C₂H₂: +28.0313; C₂D₂: +32.0542 Da) on lysine residues and oxidation of methionine residues (+15.99492 Da). Peptide spectral matches were validated based on q-values to 1% false discovery rate using Percolator algorithm. Quantitation of light and heavy precursor ion peaks was performed in Proteome Discoverer. We identified proteins that were adsorbed in 1.8-fold or greater quantities on PEG hydrogels compared with TCPS for all three donors, or that were only adsorbed on PEG hydrogels and not TCPS for all three donors. Differences are often selected using a fold-change cutoff between 1.5 and 2.0 [35,36].

2.6. Statistical analysis

Unless otherwise indicated, each experiment was repeated between three and seven times using a different blood donor for each trial. Results represent the mean $\pm$ standard deviation with each data point representing a different donor. Statistical analysis was performed using repeated measures one- or two-way analysis of variance (ANOVA) combined with Bonferroni's multiple comparison post tests (GraphPad Prism, San Diego, CA). Values of p 0.05 were considered statistically significant.

3. Results/discussion

3.1. Biomaterial-mediated PMN degranulation

Degranulation in response to soluble stimulators such as fMLP usually occurs within 10 min [37], whereas adhesion-mediated PMN activation and degranulation occurs after engagement of PMN integrins [25,38]. Depending on the surface, PMN adhesion can begin as early as 15 min after seeding and begins to decline after 2–4 h [17,39–41]. To determine if the enhanced primary granule release from PMNs on PEG hydrogels is indeed adhesion mediated, we cultured PMNs on PEG hydrogels and TCPS for 5, 15, 30, 60, 90, 120, and 240 min. PMNs cultured on PEG hydrogels and TCPS had similar levels of MPO release at 5 and 15 min of culture (Fig. 1A), suggesting that immediate contact with PEG hydrogels or with soluble mediators leached from PEG hydrogels does not induce PMN primary granule release. By 30 min, there was more MPO release from PMNs on PEG hydrogels than from PMNs on TCPS (not significant) (Fig. 1A). At 60, 90, and 120 min, PMNs cultured on PEG hydrogels released significantly more MPO than PMNs cultured on TCPS ($p < 0.05$). At 240 min (i.e., 4 h), there was a slight but not significant increase in MPO release from PMNs on PEG hydrogels compared with PMNs on TCPS. MPO release from PMNs on PEG hydrogels and TCPS decreased significantly between 2 and 4 h ($p < 0.0001$) (Fig. 1A). PMN release of MMP-9 was comparable between PEG hydrogels and TCPS at all time points and peaked between 60 and 120 min (Fig. 1B). These data suggest that the enhanced primary granule release from PMNs cultured on PEG hydrogels is adhesion mediated as it coincides with the time frame of cell adhesion [17,39–41]. The MPO concentrations released into the supernatant at 2 h correspond to 10–22% of total MPO content for PMNs cultured on PEG and GP hydrogels and 1–4% of total MPO content for PMNs cultured on PDMS and TCPS based on an MPO content of 30 pmol or 2.5 μg MPO per 10^6 PMNs [42]. Xu et al. demonstrated that PMNs cultured on wells coated with fibronectin, fibrinogen or albumin and stimulated with phorbol myristate acetate and Mn^{2+} released 15–20% of their MPO content, which is comparable to what we observed in these studies for PMNs cultured on PEG and GP hydrogels [43].

To determine how gelatin influences PMN degranulation, we cultured PMNs on 15 weight percent gelatin-only hydrogels for 2 h and measured MPO and MMP-9 release. PMNs cultured on gelatin hydrogels released significantly less MPO than PMNs cultured on PEG or GP hydrogels ($p < 0.0001$), but released comparable levels of MPO as PMNs cultured on PDMS and TCPS (Fig. 2A). MMP-9 release from PMNs cultured on gelatin hydrogels was significantly lower than that from PMNs cultured on PEG hydrogels, TCPS, or GP hydrogels ($p = 0.001$ – 0.05) and was also lower than that from PMNs cultured on PDMS

(Fig. 2B). MMP-9 degrades gelatin so it is likely that MMP-9 was actively cleaving the gelatin hydrogel substrate and therefore was not as freely available in the culture medium to be detected by immunoassay [17]. PMN cell viability was significantly lower on gelatin hydrogels than on PEG hydrogels or TCPS ($p < 0.01$) (Supplementary Fig. 1), suggesting that the decreased MPO and MMP-9 release from PMNs on gelatin hydrogels may be due in part to decreased PMN viability on gelatin hydrogels.

Based on the high primary granule release from PMNs cultured on GP hydrogels and the low primary granule release from PMNs cultured on gelatin-only hydrogels, we hypothesized that the PEG component of the GP hydrogel more strongly influences PMN degranulation than the gelatin component. Therefore, we cultured PMNs on hydrogels with varying compositions of gelatin and PEG and measured primary and tertiary granule release after 2 h. PMNs cultured on the gelatin-only hydrogels containing 15 and 20 weight percent gelatin released significantly less MPO and MMP-9 than PMNs cultured on nearly all the PEG-containing hydrogel formulations (Fig. 3). We did not observe a dose response in MPO or MMP-9 release or PMN viability with respect to increasing PEG or gelatin content in the PEG/gelatin hydrogels (Fig. 3 and Supplementary Fig. 2). In general, PMNs on all PEG-containing hydrogels released similar quantities of MPO and MMP-9 and had similar cell viability, regardless of the hydrogel gelatin content. However, there was lower PMN viability on the gelatin-only hydrogels (Supplementary Fig. 2). Overall, these data demonstrate that PEG is more influential than gelatin in promoting PMN primary granule release.

3.2. fMLP and Src family kinase coordination in PMN tertiary granule release

Pre-treatment with PP1, an Src family kinase inhibitor, significantly decreased the release of MPO from PMNs on PEG hydrogels, TCPS, and GP hydrogels ($p < 0.05$) and the release of MMP-9 from PMNs on PEG hydrogels, PDMS, TCPS, and GP hydrogels ($p < 0.01$) (Figs. 4 and 5). While treatment with fMLP did not greatly influence the effect of PP1 pre-treatment on MPO release (Supplementary Fig. 3), treatment with fMLP reversed the significant PP1-mediated decreases in MMP-9 release from PMNs on PEG hydrogels, PDMS, and GP hydrogels that were observed in the absence of fMLP (Fig. 6). This suggests that fMLP-mediated signaling pathways compensate for the inhibition of Src family kinases to promote tertiary granule release. The PP1-mediated decrease in MPO and MMP-9 release from PMNs on GP hydrogels may have been influenced in part by a decreased cell viability of PMNs on GP hydrogels pre-treated with PP1 (Supplementary Figs. 4 and 5). Pre-treatment with PP1 did not influence cell viability of PMNs on PEG hydrogels, PDMS, TCPS, or gelatin hydrogels (Supplementary Figs. 4 and 5).

3.3. PEG hydrogel-specific modulation of PI3K- γ in PMN primary and tertiary granule release

PI3K- γ has shown to be involved in early, adhesion-independent, fMLP-induced PMN activation, specifically reactive oxygen species (ROS) generation and Akt and ERK phosphorylation [24,25]. Fumagalli et al. demonstrated that 1 μ M AS604580 significantly attenuated fMLP-induced ROS generation in PMNs at early time points (from 0 to 2 min) but had no effect on fMLP-induced ROS generation in PMNs at later time points (from 5 to 30 min) [25]. Therefore, we expected that PMN pre-treatment with PI3K- γ would have a

limited effect on biomaterial-mediated PMN degranulation at 2 h, especially in the absence of fMLP. However, we observed that PMN pre-treatment with AS604850 decreased MPO and MMP-9 release from PMNs on PEG hydrogels at 2 h in both the absence and presence of fMLP (Figs. 4–6, Supplementary Fig. 3), suggesting that PI3K- γ mediates primary and tertiary granule release from PMNs on PEG hydrogels in an fMLP-independent pathway (Fig. 7). While both PEG and GP hydrogels enhance PMN primary granule release, PI3K- γ was only involved in primary granule release from PMNs on PEG hydrogels, suggesting that the addition of gelatin to the PEG hydrogel formulation diminished the role of PI3K- γ in regulating PMN primary granule release. In support of this theory, PI3K- γ was not involved in primary granule release from PMNs on gelatin-only hydrogels (Fig. 4E and Supplementary Fig. 3E).

The AS604850-mediated decreases in MPO and MMP-9 release from PMNs on PEG hydrogels were more significant in the absence of fMLP (Figs. 4–6, Supplementary Fig. 3), suggesting that fMLP may activate additional pathways that compensate for the AS604850-mediated decreases in MPO and MMP-9 from PMNs on PEG hydrogels. AS604850 did not influence cell viability based on metabolic capacity on PEG hydrogels (Supplementary Figs. 4A and 5A), indicating that the AS604850-mediated decreases in MPO and MMP-9 from PMNs on PEG hydrogels were not likely an artifact of changes in cell viability. Overall, there were minimal additive effects when PMNs on the materials were treated with both PP1 and AS604850, regardless of fMLP treatment (Figs. 4–6, Supplementary Figs. 3–5). These novel biomaterial-mediated signaling events involved in PMN degranulation are summarized in Fig. 7.

3.4. Serum protein adsorption on PEG hydrogels versus TCPS

To gain insights into what biomaterial-adsorbed serum proteins may be involved in regulating PMN degranulation, we used stable-isotope dimethyl labeling-based shotgun proteomics to quantify relative protein adsorption on PEG hydrogels versus TCPS. PEG hydrogels were used instead of GP hydrogels because PEG is more influential than gelatin in promoting PMN primary granule release (Fig. 3). Out of a total of 242 adsorbed protein groups (with 2 or more matched peptides) identified across the 3 donor serum samples, 188 were shared among all 3 donors. Many of the identified shared proteins were adsorbed in higher quantities on PEG hydrogels than on TCPS (Table 1). For example, many Ig gamma constant chain region proteins were identified in higher quantities on PEG hydrogels than on TCPS. The constant region of Ig gamma can bind to Fc gamma receptors on the surface of PMNs, and thereby could mediate degranulation [44]. Additionally, some proteins like alpha-1-acid glycoprotein 1 and zinc-alpha-2-glycoprotein were only identified on PEG hydrogels and not on TCPS. A previous mass spectrometry-based study of serum protein adsorption also showed that alpha-1-acid glycoprotein 1 was present on PEG hydrogels but not on TCPS [32]. Alpha-1-acid glycoprotein (AGP) induces rises in cytosolic Ca^{2+} in anti-I-selectin-treated PMNs via AGP binding to sialic acid binding immunoglobulin-like lectins and activation of Src family kinases and PI3 kinases [45]. Because we found that Src family kinases and PI3K- γ are also involved in PMN primary granule release, adsorbed AGP on PEG hydrogels may be involved in promoting PMN primary granule release. Many other proteins – including vitronectin and fibrinogen – were adsorbed in higher quantities on

TCPS than on PEG hydrogels. In agreement, our group has previously shown by ELISA that vitronectin and fibrinogen adsorb from serum in greater quantities on TCPS than on PEG hydrogels [32]. It is important to note that this mass spectrometry-based analysis of serum protein adsorption does not take into account protein orientation or distribution, factors which can also impact cell activation.

4. Conclusions

The presence of PEG in either single or dual component biomaterials (i.e., PEG or GP hydrogels) enhanced PMN primary granule release. For PEG hydrogels, this was found to be adhesion mediated and involve Src family kinases and PI3K- γ . The involvement of Src family kinases in PMN primary granule release was not unique to PEG hydrogels, whereas PI3K- γ involvement in PMN primary and tertiary granule release was unique to PEG hydrogels. Serum proteins like Ig gamma constant chain region proteins and alpha-1-acid glycoprotein 1 were identified in higher quantities on PEG hydrogels than on TCPS, and may be involved in mediating PMN primary granule release.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2015.01.050>

Fig. 1. MPO (A) and MMP-9 (B) release from PMNs cultured on PEG hydrogels and TCPS at time points between 5 and 240 min. Results represent the mean $\pm$ standard deviation with each data point representing a different donor, $n = 4-6$. *: significant difference between materials, $p < 0.05$.

Fig. 2. MPO (A) and MMP-9 (B) release from PMNs cultured on PEG hydrogels, PDMS, TCPS, GP hydrogels, and 15 weight percent gelatin hydrogels (Gel) for 2 h. Results represent the mean $\pm$ standard deviation with each data point representing a different donor, $n = 4-7$. ‡: significantly different from PDMS, TCPS, and gelatin hydrogels, $p < 0.001$; *: significant difference between materials, $p = 0.01-0.05$; **: significant difference between materials, $p = 0.001-0.01$.

Fig. 3. MPO (A) and MMP-9 (B) release from PMNs cultured for 2 h on hydrogels prepared with varying compositions of PEG and gelatin. P = PEG weight percent and G = gelatin weight percent. Gelatin-only hydrogels (G15 and G20) were crosslinked with 4% paraformaldehyde and other hydrogels were UV polymerized as described in Materials and Methods. Results represent the mean $\pm$ standard deviation with each data point representing a different donor, $n = 3-6$. ‡: significantly different from all PEG-containing hydrogels (i.e., P20, P10, G5P15, G10P10), $p < 0.05$; †: significantly different from P20, G5P15, G10P10, $p < 0.05$.

Fig. 4.

MPO concentrations in supernatants from PMN cultures pre-treated with 10 μ M PP1 and/or 1 μ M AS604850 on (A) PEG hydrogels, (B) PDMS, (C) TCPS, (D) GP hydrogels, and (E) 15 weight percent gelatin hydrogels at 2 h. Results represent the mean $\pm$ standard deviation with each data point representing a different donor, $n = 4-6$. *: significant difference from untreated ($-/-$), $p = 0.01-0.05$; ***: significant difference from untreated, $p = 0.0001-0.001$; ****: significant difference from untreated, $p < 0.0001$.

Fig. 5.

MMP-9 concentrations in supernatants from PMN cultures pre-treated with 10 μ M PP1 and/or 1 μ M AS604850 on (A) PEG hydrogels, (B) PDMS, (C) TCPS, (D) GP hydrogels, and (E) 15 weight percent gelatin hydrogels at 2 h. Results represent the mean $\pm$ standard deviation with each data point representing a different donor, $n = 4-6$. **: significant difference from untreated ($-/-$), $p = 0.001-0.01$; ***: significant difference from untreated, $p = 0.0001-0.001$; ****: significant difference from untreated, $p < 0.0001$.

Fig. 6.

MMP-9 concentrations in supernatants from PMN cultures pre-treated with 10 μ M PP1 and/or 1 μ M AS604850 and treated with 100 nM fMLP on (A) PEG hydrogels, (B) PDMS, (C) TCPS, (D) GP hydrogels, and (E) 15 weight percent gelatin hydrogels at 2 h. Results represent the mean $\pm$ standard deviation with each data point representing a different donor, $n = 4-6$. *: significant difference from untreated ($-/-$), $p = 0.01-0.05$; ***: significant difference from untreated, $p = 0.0001-0.001$; ****: significant difference from untreated, $p < 0.0001$.

Fig. 7.

PMN degranulation can occur via formyl peptide receptor (fMLP receptor)-mediated pathways and cell adhesion-mediated pathways. Integrin receptors such as CD11b/CD18 are involved in cell adhesion and can activate Src family kinases. The fMLP receptor also activates Src family kinases via the $G\alpha$ protein subunit [26]. Here, we showed that Src family kinases are involved in the release of primary granules from PMNs cultured on PEG hydrogels, TCPS, and GP hydrogels in the absence and presence of fMLP and in the release of tertiary granules from PMNs cultured on PEG hydrogels, PDMS, TCPS, and GP hydrogels in the absence of fMLP, and on TCPS in the presence of fMLP (right and middle red boxes). Additionally, Mocsai et al. showed that Src family kinases are involved in the release of secondary granules from PMNs cultured on TCPS [30]. Src family kinases also activate p38 MAPK and Vav1 [26,46]. fMLP-induced release of primary and secondary granules from PMNs in suspension is mediated by p38 MAPK activated by Src family kinases [46]. Vav1 activates the Rho GTPase Rac2 [47–49], which is involved in actin remodeling, NADPH oxidase activation, and the release of primary granules from PMNs in suspension [50–54]. PI3K- γ is activated via the $\beta\gamma$ subunit of the fMLP receptor and promotes the formation of phosphatidylinositol 3,4,5-triphosphate (PIP3), which activates Vav1 [55]. In this study, we found that PI3K- γ is involved in the release of primary and tertiary granules from PMNs cultured on PEG hydrogels, but not from PMNs cultured on PDMS, TCPS, or GP hydrogels (left red box). PI3K- γ was involved in primary and tertiary granule release from PMNs on PEG hydrogels in both the absence and presence of fMLP treatment. These results suggest that PEG hydrogels mediate primary and tertiary granule release from PMNs via an fMLP-independent, PI3K- γ -dependent pathway. (For interpretation of the references to colour in this figure caption, the reader is referred to the web version of this article.)

Table 1

Proteins adsorbed only on PEG hydrogels or in at least 1.8-fold greater quantities on PEG hydrogels than on TCPS for all three donors. Results are averaged from two LC-MS² replicates using serum from each of three donors.

Accession	Description	Ratio (PEG to TCPS) of protein adsorption			Mean	Standard deviation
		Donor 1	Donor 2	Donor 3		
P02768	Serum albumin	71.1	34.9	52.4	52.8	18.1
P43652	Afamin	77.7	31.1	49.2	52.7	23.5
P19652	Alpha-1-acid glycoprotein 2	65.1	62.1	29.2	52.1	19.9
P01857	Ig gamma-1 chain C region	91.0	17.6	40.8	49.8	37.5
P01781	Ig heavy chain V-III region GAL	83.6	7.9	40.7	44.0	37.9
P01624	Ig kappa chain V-III region POM	98.7	7.3	21.5	42.5	49.2
P02790	Hemopexin	52.2	45.5	20.7	39.5	16.6
P02787	Serotransferrin	44.3	32.6	41.1	39.3	6.0
P02774	Vitamin D-binding protein	43.4	33.2	37.8	38.1	5.1
Q5VY30	Retinol binding protein 4, plasma, isoform CRA_b	38.5	47.0	17.9	34.5	15.0
P04217	Alpha-1B-glycoprotein	61.4	2.1	37.9	33.8	29.9
P00450 and E9PFZ2	Ceruloplasmin	49.6	10.6	20.2	26.8	20.3
P02766	Transferrin	33.3	26.9	19.2	26.5	7.0
P01859	Ig gamma-2 chain C region	36.2	18.6	14.3	23.0	11.6
P68871	Hemoglobin subunit beta	27.8	3.1	33.5	21.5	16.1
P01617	Ig kappa chain V-II region TEW	43.4	8.1	10.7	20.7	19.7
P01834	Ig kappa chain C region	19.4	14.6	27.7	20.6	6.6
G3XAM2	Complement factor I light chain	28.4	20.6	12.2	20.4	8.1
P05543	Thyroxine-binding globulin	25.7	23.2	9.8	19.6	8.6
P36955	Pigment epithelium-derived factor	35.8	9.1	10.2	18.4	15.1
P01011	Alpha-1-antichymotrypsin	23.7	11.8	16.1	17.2	6.0
P80748	Ig lambda chain V-III region LOI	34.4	3.9	11.4	16.6	15.9
P01598	Ig kappa chain V-I region EU	24.6	15.0	9.2	16.3	7.8
P00747	Plasminogen	26.7	4.5	13.5	14.9	11.1
P69905	Hemoglobin subunit alpha	14.7	2.2	26.8	14.6	12.3
P01860	Ig gamma-3 chain C region	29.8	2.0	11.3	14.4	14.2

Accession	Description	Ratio (PEG to TCPS) of protein adsorption				Mean	Standard deviation
		Donor 1	Donor 2	Donor 3	Mean		
P01876	Ig alpha-1 chain C region	23.9	2.8	9.6	12.1	10.8	
P01009	Alpha-1-antitrypsin	21.0	3.2	11.2	11.8	8.9	
P02743	Serum amyloid P-component	25.1	3.8	6.3	11.7	11.7	
P01019	Angiotensinogen	5.1	7.4	19.5	10.7	7.7	
B9A064	Immunoglobulin lambda-like polypeptide 5	20.6	2.2	9.0	10.6	9.3	
P29622	Kallistatin	15.9	4.4	8.3	9.5	5.8	
Q96PD5	N-acetylneuramoyl-L-alanine amidase	18.0	3.7	6.3	9.3	7.6	
P05452 and E9PHK0	Tetranectin	8.3	14.0	4.1	8.8	4.9	
P07360	Complement component C8 gamma chain	15.5	2.1	7.8	8.5	6.7	
P01861	Ig gamma-4 chain C region	16.8	3.1	3.1	7.6	7.9	
P01742	Ig heavy chain V-I region	14.5	2.5	4.7	7.3	6.4	
H0YFH3	Complement C1r subcomponent	11.0	4.1	5.3	6.8	3.7	
P02775	Platelet basic protein	9.2	7.1	3.7	6.7	2.8	
P0CG05	Ig lambda-2 chain C regions	9.5	7.2	3.1	6.6	3.2	
P02656	Apolipoprotein C-III	9.9	5.8	2.8	6.2	3.6	
P02765	Alpha-2-HS-glycoprotein	8.7	4.0	2.8	5.2	3.1	
P05090	Apolipoprotein D	6.5	3.3	3.3	4.4	1.9	
Q9BYX2	TBC1 domain family member 2A	6.1	2.2	2.3	3.5	2.2	
P02749	Beta-2-glycoprotein 1	4.6	1.9	1.9	2.8	1.6	
P02763	Alpha-1-acid glycoprotein 1	Only on PEG hydrogels					
P25311	Zinc-alpha-2-glycoprotein	Only on PEG hydrogels					