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Extended interaction of $\beta 1$ integrin subunit-deficient cells (GD25) with surfaces modified with fibronectin-derived peptides: culture optimization, adhesion and cytokine panel studies

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Abstract

The modification of biomaterials with extracellular matrix-mimicking factors to influence the cellular response through mainly integrin-mediated signaling is a common technique. The inherent limitations of antibody-inhibition studies necessitate the use of complementary methods to block integrin function to confirm cell–surface interaction. In this study, we employed a $\beta 1$ integrin-deficient cell line, GD25, to investigate the role of $\beta 1$ subunit in cell adhesion and subsequent cytokine (granulocyte macrophage colony stimulating factor, interleukin (IL)-1 α , IL-1 β , IL-6, granulocyte macrophage colony stimulating factor -1, regulated upon activation, normal T-cell expressed, and secreted, tumor necrosis factor- α) release kinetics in the presence of tissue culture polystyrene (TCPS) and semi-interpenetrating polymer networks (sIPN) modified with fibronectin (FN)-mimic peptides (RGD, PHSRN). Culture conditions (i.e. seeding density, medium, serum supplementation) were optimized for long-term observation. Differences in cell adhesion, cell viability and cytokine release behavior were dependent on the presence of the $\beta 1$ integrin subunit, FN, sIPN cast method and peptide identity. By comparing two complementary techniques for assaying integrin function, we observed both similarities (i.e. decreased adhesion to FN-absorbed TCPS and increased IL-1 β release at 96 h) and differences (i.e. no difference in adhesion or IL-1 β release in the presence of sIPN surfaces) when the function of the $\beta 1$ subunit was blocked in cell adhesion and signaling in the presence of biomaterials.

Keywords

Biological response; Polymeric biomaterial; Tissue engineering; Cellular growth; Surface structure

1. Introduction

One of the main emphases of biomaterial research is to influence cell–substrate interactions. A frequently employed technique involves modifying the biomaterial with extracellular matrix (ECM) components or ECM protein-mimics to imitate the normal cell microenvironment [1, 2]. Integrin receptors are primary mediators of cell–ECM interactions. RGD, a common

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integrin-binding motif, is found in many ECM proteins, including collagen, vitronectin, laminin and fibronectin [3]. Unique to fibronectin is the synergy sequence, PHSRN, which is specific to the $\alpha 5 \beta 1$ integrin and can lead to an increase in cell adhesion and mobility, as well as enhance fibronectin matrix formation [4–6]. Several other integrin pairs are able to bind to fibronectin, including $\alpha 4 \beta 1$, $\alpha 3 \beta 1$, $\alpha v \beta 6$ and $\alpha v \beta 3$, the latter binding the RGD motif without employing the synergy sequence [5–7]. Depending on the integrin pair that complexes with fibronectin, a variety of downstream effects can occur within the cell through differential activation of outside-in signaling pathways.

Antibodies against individual integrin subunits are commonly used to elucidate the specific role of integrins in cell adhesion, signaling and other subsequent cell behaviors modulated by ECM-containing biomaterials. The inherent limitations of antibody inhibition studies, including the kinetic nature of antibody binding, non-specific antibody binding, steric interference of normal cell adherence and likely modified intracellular signaling events, call for the use of complementary methods to neutralize integrin function. Such alternative methods include the use of siRNA or cell lines with knocked-out genes of interest. In this study, we employed a $\beta 1$ integrin subunit-deficient fibroblastic cell line (GD25) derived from the embryonic stem cell line ES-D3 [8] to study the role of this subunit in regulating cell adhesion to a poly(ethylene glycol) (PEG)- and gelatin-based semi-interpenetrating polymer network (sIPN) and the subsequent release of common inflammatory cytokines. Through our extensive literature surveys and database searches, we found no other readily available $\beta 1$ integrin subunit knock-out human cell line. The binary in situ photopolymerizable sIPN system contains PEGylated immobilized peptides derived from fibronectin (FN), and previous work has demonstrated its use as a tissue scaffold and drug delivery vehicle [9–12].

Previous studies examining the behavior of human-derived primary monocytes pretreated with anti- $\beta 1$ or anti- $\beta 3$ integrin subunit antibodies in the presence of the aforementioned biomaterial as well as tissue culture polystyrene (TCPS) found variations in protease and cytokine release patterns, depending on antibody inhibition [13]. On sIPN surfaces, matrix metalloprotease-2 release was reduced by a greater amount when cells were treated with anti- $\beta 1$ as compared to anti- $\beta 3$ integrin subunit antibodies. This trend was reversed, however, in the case of interleukin (IL)-1 β release. On fibronectin-adsorbed TCPS (TCPS-FN), IL-1 β release from cells treated with anti- $\beta 1$ subunit antibodies increased by 96 h compared to the release from untreated cells. To complement these findings, this study investigated the release kinetics of seven common fibroblast-derived cytokines from the $\beta 1$ subunit-deficient cell line: granulocyte macrophage colony stimulating factor (GM-CSF), IL-1 α , IL-1 β , IL-6, monocyte chemoattractant protein 1 (MCP-1 or CCL2), RANTES (regulated upon activation, normal T-cell expressed, and secreted; CCL5) and tumor necrosis factor α (TNF- α). IL-1, IL-6 and TNF- α are proinflammatory cytokines expressed by a variety of cell types which encourage host responses typical of early stage wound healing [14,15]. GM-CSF has been shown to play a role in the local activation, recruitment and survival of macrophage lineage cells [15,16]. MCP-1 and RANTES are potent stimulators of monocyte and T-cell migration [16–18]. Overall, this panel of key inflammatory proteins should provide a representation of the $\beta 1$ subunits' role in inflammatory signaling.

The behavior of the GD25 cells in the presence of biomaterials was compared to that of GD25 cells expressing the $\beta 1A$ integrin (GD25 $\beta 1A$). The $\beta 1A$ integrin splice variant is ubiquitously expressed [19,20], has a broad distribution in vivo [21,22], is capable of activating focal adhesion kinase tyrosine phosphorylation and localizes to focal adhesions [23]. Previous studies employing the GD25 and GD25 $\beta 1A$ lines have not considered the reaction of the cells past 24 h of adhesion and have not examined how the loss of the $\beta 1$ -integrin affects the cellular response to biomaterials beyond the use of protein adsorbed to TCPS [6,24–34]. In this study, cytokine release from cells adhered to PEGylated-RGD-modified gelatin sIPNs (RGD-sIPN)

and PEGylated-PHSRN-modified gelatin sIPNs (PHSRN-sIPNs) were compared to cells adhered to TCPS, TCPS-FN and sIPN, with PEG hydrogel controls. To do so, we first optimize culture conditions to allow for 7 days of cell survival while minimizing the non-specific adhesion and subsequent cell response caused by the absorption of proteins present in fetal bovine serum (FBS) in the culture medium.

2. Materials and methods

2.1. Synthesis and characterization of sIPN-containing gelatin modified with PEGylated-peptide

RGD and PHSRN were synthesized using a solid phase synthesis scheme based on Fmoc resin cleavage and deprotection (EMD Biosciences, Darmstadt, Germany) [35]. Peptides were analyzed with a reverse-phase high performance liquid chromatography (HPLC) system (Gilson, 10–100% acetonitrile, 1 ml min⁻¹ flow rate for 30 min) coupled with ultraviolet/visible (UV/Vis) and evaporative light scattering detectors and MALDI or electrospray ionization in the positive ion mode. Samples analyzed by MALDI utilized dihydroxybutyric acid as the matrix. The method for the modification of gelatin with PEGylated-peptide has been previously published in detail [36]. All starting materials used were purchased from Aldrich unless otherwise specified. Briefly, the terminal alcohol groups of PEG-diol (2 kDa) were converted to ethylacetate and then to carboxylic acid to form bis-carboxyalate-PEG (bis-COOH-PEG). The final product was characterized using a reverse-phase HPLC system (Gilson, 10% to 100% acetonitrile, 1 ml min⁻¹ flow rate for 30 min) coupled with UV/Vis and evaporative light scattering detectors. *N*-Hydroxysuccinimide and *N,N'*-dicyclohexycarbodimide were then reacted with the carboxylic acid groups of bis-COOH-PEG to produce bis-*N*-hydroxysuccinimide-PEG (bis-NSu-PEG). The resulting mixture containing bis-NSu-PEG was characterized using the reverse-phase HPLC method described earlier. A total of 1.5 eq. mol GGG, RGD or PHSRN in combination with 1.5 eq. mol *N,N*-diisopropyl ethylamine was added to 1 eq. mol bis-NSu-PEG in order to graft the ligand onto one of the two PEG terminal groups to form NSu-PEG-peptide. Next, 1 eq. mol of NSu-PEG-peptide was mixed with 1% gelatin (Sigma, 300 bloom, porcine skin) in phosphate-buffered saline at pH 8.0 for 1 h in order to graft remaining terminal group of the PEG-peptide to the lysine groups in gelatin. A pressurized ultrafiltration system with a 30 kDa membrane filter was used to separate peptide-PEG-gelatin from the remaining bis-peptide-PEG and unreacted NSu-PEG-peptide. A similar protocol was used to produce methoxy-PEG-modified gelatin except 2 kDa monomethoxy-PEG was used in place of the 2 kDa PEG diol and no peptide was grafted to the PEG. The modified gelatin was characterized by an ultrahydrogel column in a GPC system (Waters, 80% 0.1M NaHO₃, 20% acetonitrile at a 0.7 ml min⁻¹ flow rate for 60 min) coupled with a refractive index detector. The percent modification of gelatin lysyl groups with PEG-peptide was determined using an established method based on trinitrobenzenesulfonic acid (TNBS) and spectrophotometry [37,38].

The following procedure for sIPN synthesis is adapted from previously established procedures [39,40]. Methoxy-PEGylated-modified, PEGylated-GGG-modified, PEGylated-RGD-modified, PEGylated-PHSRN-modified or unmodified gelatin was dissolved in deionized water at a concentration of 0.1 g ml⁻¹ at 60°C to synthesize methoxy-PEG-modified gelatin sIPNs (mPEG-sIPN), PEGylated-GGG-modified gelatin sIPNs (GGG-sIPN), RGD-sIPN, PHSRN-sIPN, and unmodified gelatin sIPNs (Unmod-sIPN or mold-cast Unmod-sIPN), respectively. The gelatin solution was mixed with poly(ethylene glycol) diacrylate (PEGdA) (575 Da) in a 4:6 gelatin:PEGdA weight ratio. A 20 µl volume of an initiator solution containing 2,2-dimethoxy-2-phenyl-acetophenone at 0.02 g in 0.5 g PEGdA was added to the mixture and vortexed. sIPNs were either cast directly into 48-well plates by placing 100 µl sIPN solution in each well (mPEG-sIPN, GGG-sIPN, RGD-sIPN, PHSRN-sIPN, Unmod-sIPN) or were

poured into Teflon® molds (8 mm dia. × 0.75 mm thick) (mold-cast Unmod-sIPN). Different surface topographies (Fig. 1) were anticipated to result from these separate casting methods in order to elucidate if variations in cellular response could be attributed to the sIPN casting method. The acrylate groups were cross-linked using UV light (maximal intensity, 365 nm, 21,700 $\mu\text{W cm}^{-2}$, UVP, model B 100AP) for 5 min. For sterility, sIPNs were γ -irradiated (1 Mrad) before cell culture. All sIPNs were equilibrated for 24 h in Dulbecco's modified Eagle's medium (DMEM) (Mediatech Inc., Herndon, VA) at 37°C and 5% CO_2 before cell seeding.

2.2. Synthesis of PEG hydrogels

PEGdA (3.4 kDa) was synthesized using a previously established procedure [41]. Briefly, PEG diol (3.4 kDa) was mixed with acryloyl chloride and triethylamine in a 1:5:6 molar ratio for 3 h. The solution was filtered and precipitated to obtain 3.4 kDa PEGdA. The final product was checked for purity using the reverse-phase HPLC method described earlier. To make the PEG hydrogels, the PEGdA was dissolved in dionized water to form a 10 wt.% solution. The photoinitiator Irgacure 2959 (Ciba, Basel, Switzerland) was also included at 0.1 wt.%. The solution was poured into Teflon® molds (8 mm dia. × 0.75 mm thick) and cross-linked using UV light (maximal intensity, 365 nm, 21,700 $\mu\text{W cm}^{-2}$, UVP, model B 100AP) for 5 min. The hydrogels were cold sterilized in 70% ethanol following well-accepted protocols [42] for approximately 40 min and equilibrated in DMEM at 37°C and 5% CO_2 prior to the addition of cells.

2.3. Preparation of fibronectin treated TCPS surfaces

Human serum fibronectin (Millipore, Billerica, MA) was adsorbed onto 48-well TCPS plates (BD Falcon™, San Jose, CA) at a concentration of 500 $\mu\text{g ml}^{-1}$ (66.7 $\mu\text{g cm}^{-2}$) in PBS for 24 h under sterile conditions. Wells were rinsed twice with Dulbecco's PBS (D-PBS) (Mediatech Inc., Herndon, VA) immediately before seeding cells.

2.4. Cells

The GD25 cells were a generously provided by Dr. Fassler (Department of Molecular Medicine, Max-Planck Institute of Biochemistry, Martinsried, Germany) and the GD25 β 1A cells were a kind gift from Dr. Mosher (Department of Medicine, University of Wisconsin–Madison School of Medicine, Madison, WI). Both cell types have been described previously in detail [6,8]. Briefly, GD25 are fibroblastic cells derived from β 1-null murine embryonic stem cells (line ES-D3). GD25 β 1A consist of GD25 cells transfected to express the β 1A integrin. Both cell types were maintained in DMEM containing L-glutamine, sodium pyruvate, 4500 mg ml^{-1} glucose and pyridoxine (Mediatech Inc., Herndon, VA), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C and 5% CO_2 . GD25 β 1A cells were kept under selection pressure with the addition of 10 $\mu\text{g ml}^{-1}$ puromycin. Cells were passaged using 2.5% trypsin solution (Lonza Bioscience Inc., Basel, Switzerland). Cells were at passage 9 or 10 when seeded onto experimental surfaces.

2.5. Cell adhesion assay – culture optimization study

Culture conditions were first optimized to maintain cell viability for 7 days while minimizing the non-specific adhesion and subsequent cell response caused by the absorption of proteins in FBS in the medium. GD25 and GD25 β 1A were seeded onto TCPS at a concentration of 10⁵ cell ml^{-1} (786 cells mm^{-2}) at 37°C and 5% CO_2 in DMEM or 1:1 DMEM:F12 (Mediatech Inc., Herndon, VA) supplemented with various concentrations (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75 and 1%) of FBS. In addition, different pre-incubation conditions were tested: DMEM with 10% FBS (no pre-incubation), 24 h incubated in DMEM without FBS, and 1:1 DMEM:F12 with 1% FBS (no pre-incubation). Cells were cultured up to 168 h and then stained with Wright's stain. Three different 0.36 mm^2 areas in each well were imaged and the adhered cell

densities were quantified. If cells reached confluence and therefore could not be individually resolved and counted, an approximated adherent density of 700 cells mm⁻² was used.

2.6. Cell adhesion, viability and cytokine release assay

GD25 and GD25β1A were seeded onto TCPS, TCPS-FN, PEG hydrogel and sIPN surfaces at a concentration of 10⁵ cell ml⁻¹ (786 cells mm⁻²) at 37°C and 5% CO₂ in DMEM supplemented with 0.4% FBS. No media changes were made for the entirety of the culture time. At 2, 24, 96 and 168 h, all surfaces were rinsed with D-PBS and stained with LIVE/DEAD (Invitrogen, Carlsbad, CA). For each sample, a minimum of five different areas totaling at least 0.77 mm² were imaged and both the live and dead cells were counted. Supernatants were collected for all surfaces except PEG hydrogel at 2, 24, 96 and 168 h. Levels of GM-CSF, IL-1α, IL-1β, IL-6, MCP-1, RANTES and TNF-α were quantified simultaneously using the Bio-Plex cytokine bead array system (Bio-Rad, Hercules, CA). The sensitivity of the assay varied between cytokines and different assay runs: GM-CSF (2.10 ± 70%–57,100 ± 5% pg ml⁻¹), IL-1α (1.95 ± 17%–9930 ± 35% pg ml⁻¹), IL-1β (1.18 ± 11%–22,000 ± 62% pg ml⁻¹), IL-6 (1.14 ± 36%–13,800 ± 37% pg ml⁻¹), MCP-1 (69.2 ± 61%–32,400 ± 20% pg ml⁻¹), RANTES (0.90 ± 62%–22,400 ± 49% pg ml⁻¹) and TNF-α (4.29 ± 21%–69,800 ± 23% pg ml⁻¹). The cytokine release rate was calculated by averaging all possible differences (*n*=9) in cumulative cytokine release between consecutive time points. As separate sets (*n*=3) of each type of surface were used to assay cumulative release at each time point, similar amounts of release over time were assumed for each set to calculate the release rate. Negative rate calculations were obtained when the cumulative release at the later time point was the lower of the two values; those results were stated as “0” for that time frame.

2.7. Statistical analysis

All data is shown as mean ± standard error of the mean. Cell density data (*n*=3), cell viability data (*n*=3), cytokine concentrations (*n*=3), cytokine release rate data (*n*=9), and TNBS data (*n*=3) were averaged. Cell density data, cell viability data, cytokine concentrations and cytokine release rate data were analyzed by unpaired Student's *t*-test. A value of *p* < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Modified gelatin characterization

RGD and PHSRN peptides were determined to be >90% pure as analyzed by HPLC. MALDI and ESI results showed the molecular weight of PHSRN and RGD to be 609.3 and 346.4 Da, respectively, which are consistent with the theoretical molecular weights of 609.6 and 346.3 Da, respectively. The four different modified-gelatin species showed decreased peak GPC elution times compared to the unmodified gelatin species, which indicates an increase in molecular weight (effective volume) with the addition of PEGylated-RGD, PEGylated-PHSRN, PEGylated-GGG or mPEG consistent with previously published work [36,39]. TNBS results indicated a percent modification of the lysyl groups in gelatin of 70±10% for PEGylated-RGD-gelatin, 87±3% for PEGylated-PHSRN-gelatin, 84±4% for PEGylated-GGG-gelatin and 87±3% for mPEG-gelatin (*n* =3). ¹H-NMR comparisons between the various peptides and peptide-modified gelatins demonstrated differing characteristic chemical shifts gelatin modification with PEGylated-peptides. Gelatin exhibited a broad doublet peak at 7.250 ppm, a sharp peak at 8.411 ppm, and broad, asymmetrical peaks from 0.885 to 4.803 ppm [43]. Prominent PEG peaks were present in all modified-gelatin samples at approximately 3.64 ppm [44]. The various PEGylated-peptide-gelatins also displayed additional peaks as follows: RGD-modified gelatin showed an overlapped doublet of triplet peaks at approximately 1.36 ppm, a broad group of peaks at 1.40 ppm, single peaks at 2.79, 2.87 and 3.03 ppm, and two overlapping triplet peaks at approximately 3.38 ppm; PHSRN showed broad peaks composed

of many overlapping small peaks at 1.7, 1.75, 1.92, 3.18, 4.19 and 4.2 ppm and two sharp peaks at 4.15 and 7.02 ppm; PEGylated-GGG-gelatin showed three singlets at 4.07, 4.12 and 4.17; and mPEG-gelatin showed sharp peaks at 3.2 and 3.7 ppm and a broad peak at 3.95 [13].

3.2. Cell adhesion

Cell culture optimization was conducted to maintain cell viability for 7 days, limit the effects that FBS has on cell behavior and allow for quantification through imaging by minimizing cell confluence. Optimization experiments were conducted on TCPS since other published work had shown that TCPS supports greater adhesion and faster growth than the sIPN surface [8, 13,39]. Seven different FBS concentrations (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75 and 1%) and three different growth media conditions (DMEM with 10% FBS, DMEM with 10% FBS with 24 h incubation without FBS, and 1:1 DMEM:F12 with 1% FBS) were tested for 1 week (Fig. 2). At a seeding density of 10^5 cell ml^{-1} (786 cells mm^{-2}), adherence of both GD25 and GD25 β 1A was well within the region of adherent cell density that can be accurately resolved when cultured with 0.4% FBS with or without pre-incubation without FBS.

3.2.1. PEG hydrogel surfaces—In general, PEG surfaces, including PEG hydrogels, are viewed in the literature as being unfavorable to cell adhesion due to the polymer's high hydrophilicity preventing protein absorption [1,2,45]. In this study, however, the percent viable cells and adherent density of GD25 on PEG hydrogels beyond 2 h of culture were comparable to TCPS and TCPS-FN surfaces. Additionally, the adherent density of viable GD25 β 1A on PEG hydrogels was significantly greater than zero (Table 1 and Table 2). For both cell types, the adherent density of viable cells remained relatively constant after 24 h and never reached confluency. Factors such as the molecular weight of the PEG, the amount of cross-linkers [46] and the PEG weight percentage, as well as the form in which the hydrogel is cast, may contribute to different PEG–cell interactions. No significant differences were seen in either adhesion density or percent viability between GD25 and GD25 β 1A adhered to PEG hydrogel surfaces (Table 1 and Table 2), suggesting that β 1A does not play a significant role in mediating adhesion onto the PEG hydrogel surface.

3.2.2. TCPS vs. FN-TCPS surfaces—While there were no significant differences between adherent cell density on TCPS and TCPS-FN for either cell type, the adherent density of GD25 β 1A to TCPS-FN was generally higher than that of GD25. The lack of difference in adherent density on TCPS vs. TCPS-FN may be attributed to the presence of the α v β 3 integrin whose ligand is the same RGD sequence on fibronectin to which α 5 β 1 integrin binds. Additionally, there is evidence that suggests α v β 3 is the primary receptor responsible for mediating adhesion to fibronectin [6]. The adherent density of viable GD25 β 1A was generally higher than that of GD25 on TCPS-FN surfaces at 2, 96 and 168 h (Table 1). This result reflects a similar trend which was seen for the previous antibody inhibition study [13]. The greater adherent density of viable GD25 β 1A on TCPS-FN at 168 h also coincides with an increase in viability which was seen not only at 168 h but also at 24 h on both TCPS-FN and TCPS surfaces (Table 2). Percent viability of GD25 β 1A adhered to TCPS and TCPS-FN surfaces were comparable and remained at approximately 90% over 7 days.

3.2.3. sIPN surfaces—The adherent density of viable cells (Table 1) was statistically greater for GD25 β 1A than GD25 on all direct-cast sIPN surfaces at 24 and 96 h. Although a lack of survival for GD25 was found on TCPS surfaces, the greater lack of viable cells on direct-cast sIPN surfaces suggests that β 1A may play an important role in mediating adherence and subsequent survival on the sIPN surface. While the lower adherence of cells without a functioning β 1 subunit on sIPN surfaces is consistent with the results of the antibody inhibition studies, the effect was primarily seen on sIPNs containing RGD or PHSRN ligands, indicating a possible inherent difference between the two models [13]. The adherent density of viable

GD25 β 1A on RGD-, PHSRN- and GGG-sIPN significantly increased from 2 to 24 h, but significantly decreased on those surfaces and mPEG-sIPN from 96 to 168 h. On Unmod-sIPN surfaces, however, GD25 β 1A maintained approximately the same viability with time, indicating that there are different characteristics, such as surface charge and other biophysical properties [46,47], between the sIPN surfaces that support adhesion or survival.

3.2.4. Mold-cast vs. direct-cast sIPN—In addition to the material formulation, the manner in which the hydrogel was cast also affected the adhesion of both cell types. While the adherent density of viable GD25 was similar on direct-cast and mold-cast Unmod-sIPN surfaces, a significantly higher adherent density of viable GD25 β 1A was found on the direct-cast Unmod-sIPN than on the mold-cast Unmod-sIPN between 24 and 168 h (Table 1). Between cell types, the adherent density was similar on mold-cast Unmod-sIPN surfaces but dissimilar for direct-cast Unmod-sIPN.

3.3. Cytokine release kinetics

The release kinetics of GM-CSF, IL-1 α , IL-1 β , IL-6, MCP-1, RANTES and TNF- α was quantified for the all surfaces as a cumulative amount released between cell seeding and 2, 24, 96 and 168 h (Table 3–Table 10). To further analyze the results, the rate of release between consecutive time points was calculated from the cumulative release data (Fig. 3–Fig. 5).

3.3.1. TCPS vs. FN surfaces—In general, cells adhered to TCPS and TCPS-FN surfaces displayed the greatest amount of cytokine release. On TCPS surfaces, GD25 β 1A showed a higher cumulative release than GD25 of MCP-1 (24 h), GM-CSF (96 h) and IL-6 (24 and 96 h). Conversely, GD25 showed a higher cumulative release than GD25 β 1A of RANTES (24, 96 and 168 h) (Table 3). These results suggest that the presence of β 1A mediated an earlier release of IL-6 and MCP-1 while also inhibiting the release of RANTES. With the addition of fibronectin to the TCPS surface, GD25 β 1A continued to have a higher cumulative release of MCP-1 (2 h) and IL-6 (2 and 24 h) (Table 4). The cumulative release of RANTES (24 h) as well as of IL-1 β and TNF- α (96 h) from GD25 β 1A, however, was lower than GD25. The shift in cumulative release behavior between GD25 and GD25 β 1A when in the presence of fibronectin suggests that β 1A or possibly its binding to fibronectin may stimulate an earlier release of MCP-1 and IL-6. The pattern of IL-1 β release also correlates with the antibody-inhibition study, where a similar increase at 96 h was seen when the β 1 subunit was not available [13]. Lastly, the cumulative release of IL-1 α was generally less than 5 pg ml⁻¹ and contained no significant differences depending on cell type or substrate (Table 3 and Table 4).

Overall, GD25 displayed the highest rate of release of IL-1 β , MCP-1, IL-6 and GM-CSF at a later time period than GD25 β 1A on TCPS (Fig. 3). These results suggest that β 1A may play a role in mediating a large amount of release of these four cytokines at an earlier time point. The rate of release of RANTES (2–168 h), however, was higher from GD25 than from GD25 β 1A (Fig. 3). On TCPS-FN, only the rate of release of IL-6 continued to follow the trend seen between cell types on TCPS. MCP-1 also continued to have a higher rate of release from GD25 β 1A than from GD25 early on (0–2 h), but a lower rate of release from GD25 β 1A was not seen at 168 h on TCPS-FN, unlike on TCPS. The release rate of RANTES (2–24 and 96–168 h) continued to be higher from GD25 than from GD25 β 1A. The time period of the most rapid release of IL-1 β differed between GD25 and GD25 β 1A and changed with the addition of fibronectin. Such results indicate that the release of IL-1 β may be inhibited by the presence of β 1A only when cells are exposed to fibronectin. On TCPS and TCPS-FN surfaces, distinct patterns of IL-6 and MCP-1 release were seen based on cell type (Table 5). While it is possible that the β 1A subunit directly mediates this differential release behavior, the lack of influence that fibronectin had on this system suggests that other factors were the primary mediators. Such

factors may include other differentially regulated cytokines and activation of alternative integrin heterodimers, in which case β 1A would act as a secondary signaling regulator.

3.3.2. sIPN surfaces—On RGD-sIPN surfaces, there were no significant differences in cumulative release of any cytokine between GD25 and GD25 β 1A cells (Table 6). MCP-1 release by GD25 β 1A on RGD-sIPN was generally lower than the other sIPN surfaces over time. The release of TNF- α from both cell types exposed to PHSRN-sIPN, GGG-sIPN and mPEG-sIPN was significantly greater than zero (Table 6–Table 9). For PHSRN-sIPN, GD25 β 1A release of TNF- α did not occur until 24 h, unlike GD25, which started to release it within the first 2 h. Since no significant release of TNF- α was seen for either cell type from Unmod-sIPN (Table 10), release was likely affected by both the presence of a PEGylated-ligand and the ligand identity. The cumulative release of both IL-6 and RANTES, while statistically significant, was less than 15 pg ml⁻¹ and generally similar between cell types on all direct-cast sIPN surfaces (Table 6–Table 11).

In general, when cumulative release of a cytokine was considered significant in the presence of direct-cast sIPN surfaces, most release was observed to occur within 2 h of seeding. From 2 to 96 h, there was a very little cytokine released compared to the rate at 0–2 h (Fig. 4). There were, however, a couple exceptions to this general tendency. For example, the rate of release from GD25 β 1A of IL-6, MCP-1 and RANTES from 2 to 24 h in the presence of Unmod-sIPN, while not as high as from 0 to 2 h, was greater than the rates from 24 to 168 h (Fig. 5). This trend was not seen for GD25, suggesting that the presence of the β 1A may play a role in extending the period of release of these three cytokines. Additionally, GD25 β 1A on PHSRN-sIPN surfaces demonstrated a higher rate of TNF- α release than when adhered to other sIPN surfaces from 24 to 96 h; GD25 cells on PHSRN-sIPN, however, released hardly any TNF- α during the same time period (Fig. 4).

These results are not completely in agreement with previous work within our laboratory using human-derived primary monocytes adhered to mold-cast sIPN surfaces as well as established trends in the literature when anti- β 1 integrin inhibitory antibodies were employed. Typically, the presence of RGD on a surface, whether adsorbed or tethered, increases cell adhesion [3, 13,39]. Additionally, when either the β 1 or β 3 integrin subunits are blocked with antibodies, cell adhesion decreases significantly, particularly when RGD is present on the surface, and IL-1 β release is significantly reduced in the presence of the sIPN [13]. Our data suggest that an up-regulation of other signaling pathways within GD25 due to the loss of the β 1 integrin, including a possible increase in the expression of other integrin pairs, may be primarily responsible for noted downstream effects. Thus, similar responses could be seen with or without the β 1A integrin due to alternative signaling systems becoming more prominent within GD25. The possible signal compensation may also be present when inhibitory antibodies are used but manifest in a different fashion. The general lack of differences in cytokine expression between GD25 and GD25 β 1A on direct-cast sIPN surfaces could also be due to a loss of cell viability over time, especially with GD25 cells. The small number of viable GD25 cells, however, also emphasizes the high amount of release seen for cytokines such as MCP-1.

3.3.3. Direct-cast vs. mold-cast sIPN surfaces—Cytokine release from direct-cast and mold-cast Unmod-sIPN surfaces differed mainly through the release of IL-6, MCP-1 and RANTES (Table 8 and Table 9). The cumulative release of MCP-1 and RANTES (96–168 h) from GD25 was higher in the presence of mold-cast Unmod-sIPN as compared to direct-cast Unmod-sIPN. The cumulative release of IL-6 (96–168 h), MCP-1 (24–168 h) and RANTES (24–168 h) from GD25 β 1A was also higher in the presence of mold-cast Unmod-sIPN. This increase in cytokine release is especially significant considering that there was a lower adherent density of viable GD25 β 1A on mold-cast Unmod-sIPN than on direct-cast Unmod-sIPN. In addition, cytokine release from mold-cast Unmod-sIPN demonstrated variations between the

two cell types that were not seen from direct-cast Unmod-sIPN. The cumulative release of IL-6 (24–168 h), MCP-1 (2–168 h) and RANTES (2–168 h) in the presence of mold-cast Unmod-sIPN was higher from GD25 β 1A than from GD25. The increase in cumulative release of these cytokines seen for GD25 β 1A cells can be attributed to a longer period (0–168 vs. 0–2 h) of significant release than seen for GD25 (Fig. 5). The differences in cell behavior based on different casting methods may be attributed to a variety of factors, including the air–polymer interface formed in direct casting affecting the surface chemical, and physical characteristics such as hydrophobicity and topography.

4. Conclusions

While competitive inhibition studies using antibodies are extensively employed, the application of knock-out cell lines is a valuable complementary means to investigate how specific ligand–receptor interactions affect the cellular response to biomaterials. This is especially important since both methods are likely to alter intracellular signaling pathways and phenotypic/functional expression. The results from this study demonstrated adhesion and cytokine release behavior, which both corresponded and differed to previous antibody inhibition study which examined the cellular response to identical biomaterials.

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Fig. 1. Diagram showing the approximated forms that could result from mold-casting and direct-casting the sIPN. The right side shows representative cross-sections from the center of the cast IPN. Degrees of curvature are exaggerated schematically for emphasis.

Fig. 2.

Results of culture condition optimization studies to determine the ideal FBS concentration that allows for seven day survival and non-confluent adhesion of GD25 (■) and GD25β1A (■). Three different pre-incubation media conditions were used: DMEM with 10% FBS (— ■ —), 24 h pre-incubation with DMEM without FBS (— ■ —), and 1:1 DMEM:F12 with 1% FBS (— ■ —). The point “700 cell mm⁻²” was assigned when cells reached confluence and could not be resolved individually for quantification.

Fig. 3.

Rate of GM-CSF, IL-1 β , IL-6, RANTES and MCP-1 release from GD25 and GD25 β 1A cells in the presence of TCPS and FN-TCPS between 0–2 h (□), 2–24 h (■), 24–96 h (▒) and 96–168 h (▤). Rates were calculated by averaging all possible differences in cytokine levels between consecutive time points and dividing this average by the time difference.

Fig. 4. Rate of IL-6, RANTES, TNF- α and MCP-1 release from GD25 and GD25 β 1A cells in the presence of direct-cast sIPN surfaces between 0–2 h (white), 2–24 h (black), 24–96 h (grey) and 96–168 h (dotted). Rates were calculated by averaging all possible differences in cytokine levels between consecutive time points and dividing this average by the time difference.

Fig. 5.

Rate of IL-6, RANTES and MCP-1 release from GD25 and GD25 β 1A cells in the presence of direct-cast and mold-cast Unmod-sIPN surfaces between 0–2 h (□), 2–24 h (■), 24–96 h (▒), and 96–168 h (▤). Rates were calculated by averaging all possible differences in cytokine levels between consecutive time points and dividing this average by the time difference.

Table 1
Adherent density of viable GD25 and GD25β1A cells adhered to various surfaces at 2, 24, 96 and 168 h

Cell type	Surface	Adherent viable cell density (cells mm ⁻²)				Time (h)			
		2	24	96	168	2	24	96	168
GD25	sIPN	§ 5	§ 7	§ 2	§ 2	1	1	1	1
		7	3	2	2	1	1	1	2
		§ 6	§ 3	1	1	1	1	1	1
		mm 14	§ 4	2	2	0	0	0	0
		27	§ 8	10	3	4	4	4	3
	TCPS	2	13	2	6	3	3	3	2
		165	i 361	41	i 366	105	105	105	36
		i 100	i 409	94	i 446	41	41	41	43
		1	227	82	i 244	32	32	32	15
GD25β1A	sIPN	47	176	44	180	21	21	21	1
		16	126	30	206	9	9	9	0
		63	175	22	414	107	107	107	2
		30	184	57	79	24	24	24	2
		12	194	47	412	148	148	148	33
	TCPS	mpu 2	rg 39	20	mpu 46	10	10	10	7
		174	821	317	mpu 909	132	132	132	262
		i 438	mpg 515	106	mk 747	219	219	219	71
		pkf 51	280	102	i 302	139	139	139	43

All values expressed as mean ± SE; n=3 and statistically analyzed by Student's t-test at p < 0.05.

§ Significantly different than GD25β1A.

α Significantly different than 2 h.

Δ Significantly different than 24 h.

† Significantly different than 96 h.

‡ Significantly different than RGD-sIPN.

§ Significantly different than PHSRN-sIPN.

¶ Significantly different than GGG-sIPN.

‡ Significantly different than mPEG-sIPN.

u Significantly different than Unmod-sIPN.

k Significantly different than mold-cast Unmod-sIPN.

^gSignificantly different than TCPS.

^hSignificantly different than TCPS-FN.

ⁱSignificantly different than all sIPN surfaces.

Table 2
Percentage of viable GD25 and GD25β1A cells adhered to various surfaces at 2, 24, 96 and 168 h

Cell type	Surface	Percent adherent live cells				Time (h)		
		2	24	96	168			
GD25	TCPS	94 ±	85 ±	75 ±	57 ±			
	TCPS-FN	98 ±	95 ±	90 ±	67 ±			
	PEG hydrogel	100 ±	95 ±	93 ±	80 ±			
GD25β1A	TCPS	97 ±	97 ±	93 ±	94 ±			
	TCPS-FN	96 ±	99 ±	92 ±	90 ±			
	PEG hydrogel	95 ±	98 ±	97 ±	85 ±			

All values expressed as mean ± SE; n=3 and statistically analyzed by Student's t-test at $p < 0.05$.

- § Significantly different than GD25β1A.
- ± Significantly different than 2 h.
- ∇ Significantly different than 24 h.
- ‡ Significantly different than 96 h.
- ′ Significantly different than TCPS.
- ƒ Significantly different than TCPS-FN.

Table 3
Cumulative release of seven inflammatory cytokines from GD25 and GD25β1A cells in the presence of TCPS

Cell type	Cytokine	Cumulative cytokine release (pg ml ⁻¹)			
		2	24	96	168
GD25	GM-CSF	< 4.44	5.28 ± 5.28	17.89 ± 1.90	31.79 ± 6.12
	IL-1α	2.12 ± 0.60	2.33 ± 1.16	0 ± 0	± 0.19
	IL-1β	3.02 ± 1.55	1.72 ± 1.72	6.10 ± 4.29	± 0.34
	IL-6	1.61 ± 0.09	4.55 ± 0.24	17.23 ± 1.19	44.55 ± 9.01
	MCP-1	233.83 ± 1	3681.79 ± 3	9769.45 ± 89	17448.27 ± 1005.5
	RANTES	1.12 ± 1.02	11.14 ± 0.75	83.16 ± 7.94	129.50 ± 9.27
GD25β1A	TNF-α	13.57 ± 4	31.70 ± 2.98	27.50 ± 5.70	12.77 ± 5.57
	GM-CSF	< 4.44	19.86 ± 0.83	39.69 ± 1.91	38.42 ± 0.56
	IL-1α	2.74 ± 0.75	2.95 ± 0.59	0.24 ± 0.24	± 0.19
	IL-1β	0.18 ± 0.18	3.02 ± 1.55	0.81 ± 0.26	± 0.52
	IL-6	2.39 ± 0.33	7.20 ± 0.18	61.54 ± 7.87	53.44 ± 18.29
	MCP-1	685.14 ± 30	5214.24 ± 3	11512.84 ± 896	13306.78 ± 2092.2
GD25β1A	RANTES	0.90 ± 0.25	8.40 ± 0.65	11.86 ± 2.02	13.74 ± 2.75
	TNF-α	18.73 ± 7.42	20.36 ± 3.10	22.69 ± 9.43	10.25 ± 3.57

All values expressed as mean ± SE; n=3 and statistically analyzed by Student's t-test at $p < 0.05$.

[§]Significantly different than GD25β1A

^αSignificantly different than 2 h.

^ΔSignificantly different than 24 h.

[†]Significantly different than 96 h.

^rSignificantly different than the respective data point on RGD-sIPN (Table 6).

^pSignificantly different than the respective data point on PHSRN-sIPN (Table 7).

^gSignificantly different than the respective data point on GGG-sIPN (Table 8).

- ^m Significantly different than the respective data point on mPEG-sIPN (Table 9).
- ⁿ Significantly different than the respective data point on Unmod-sIPN (Table 10).
- ^k Significantly different than the respective data point on mold-cast Unmod-sIPN (Table 11).
- ^f Significantly different than the respective data point on TCPs-FN (Table 4).
- ⁱ Significantly different than the respective data point on all sIPN surfaces (Table 6–Table 11).

Table 4
Cumulative release of seven inflammatory cytokines from GD25 and GD25β1A cells in the presence of TCPS-FN

Cell type	Cytokine	Cumulative cytokine release (pg ml ⁻¹)				
		Time (h)				
		2	24	96	168	
GD25	GM-CSF	<4.44	5.35	± 2.80	± 3.14	± 56.50
	IL-1α	<i>uk</i> 1.86	2.08	± 1.09	± 0	± 1.88
	IL-1β	0.82	2.98	± 1.66	± 1.61	± 1.24
	IL-6	<i>§</i> 1.68	<i>§it</i> 6.66 <i>it</i> 294.	± 0.54	± 4.34	± 31.30
	MCP-1	<i>§</i> 212.83	4908.97	± 88	± 8	± 4818.
	RANTES	1.63	<i>§it</i> 17.31	± 1.05	± 8.65	± 92
GD25β1A	TNF-α	23.23	20.83	± 6	± 5.51	± 24.92
	GM-CSF	2.72	12.41	± 6.68	± 13.32	± 4.19
	IL-1α	2.08	3.16	± 1.35	± 0	± 0.26
	IL-1β	2.12	<0.55	± 0.81	± 0.81	± 0.39
	IL-6	<i>m</i> 2.49 <i>rpmuk</i>	12.53	± 1.23	± 64.8	± 5.20
	MCP-1	706.40	<i>i</i> 6106.70	± 980.	± 2865.	± 1788.
GD25β1A	RANTES	<i>g</i> 1.77	<i>k</i> 7.37	± 2.01	± 35.68	± 76
	TNF-α	22.70	<i>rk</i> 28.11	± 7.48	± 72.12	± 1.90
					<i>g</i> 0.81 < 3.22	± 4.07

All values expressed as mean ± SE; n=3 and statistically analyzed by Student's t-test at $p < 0.05$.

§ Significantly different than GD25β1A.

uk Significantly different than 2 h.

Q Significantly different than 24 h.

† Significantly different than 96 h.

r Significantly different than the respective data point on RGD-sIPN (Table 6).

p Significantly different than the respective data point on PHSRN-sIPN (Table 7).

g Significantly different than the respective data point on GGG-sIPN (Table 8).

m Significantly different than the respective data point on mPEG-sIPN (Table 9).

u Significantly different than the respective data point on Unmod-sIPN (Table 10).

k Significantly different than the respective data point on mold-cast Unmod-sIPN (Table 11).

^fSignificantly different than the respective data point on TCPS (Table 3).

^gSignificantly different than the respective data point on all sIPN surfaces (Table 6–Table 11).

Table 5

Changes in the rate of release of IL-6 and MCP-1 over time from GD25 and GD25β1A cells in the presence of TCPS and TCPS-FN

(decrease in rate; increase in rate; : no change in rate)

Cytokine	Cell type	Surface	0-2 to 2-24	Time period (h) 2-24 to 24-96	24-96 to 96-168
IL-6	GD25	TCPS
		TCPS-FN
	GD25β1A	TCPS
		TCPS-FN
MCP-1	GD25	TCPS
		TCPS-FN
	GD25β1A	TCPS
		TCPS-FN

Table 6
Cumulative release of seven inflammatory cytokines from GD25 and GD25β1A cells in the presence of RGD-sIPN

Cell type	Cytokine	Cumulative cytokine release (pg ml ⁻¹)				
		2	24	96	168	
GD25	GM-CSF	13.99	3.65	16.78	kt < 3.22	0
	IL-1α	pt 0.05	m 0	0	0	0
	IL-1β	5.55	2.44	7.75	3.95	0.62
	IL-6	3.77	tf 2.11	tf 5.98	2.34	0.40
	MCP-1	80.57	guf 58.85	ktf 180.83	6	46.19
GD25β1A	RANTES	1.68	tf 0.83	ktf 2.07	1.09	0.61
	TNF-α	13.24	gm 1.07	24.33	12.7	0
	GM-CSF	3.65	t 3.65	t < 3.22	ktf 3.65	3.65
	IL-1α	gmt 0	pgm 0	0	0	0
	IL-1β	t 2.44	1.63	kα 0.45	pgti 2.44	0
GD25β1A	IL-6	1.71	k 5.02	kt 2.69	ktf 3.08	1.22
	MCP-1	149.05	gmif 28.4	pgmktf 70.0	pkif	
	RANTES	1.33	9	138.56	132.03	74.41
	TNF-α	gm 2.15	kt 1.60	ukt 1.65	ktf 2.50	0.94
			pgmuf < 3.22	g < 3.22	1.07	1.07

All values expressed as mean ± SE; n=3 and statistically analyzed by Student's *t*-test at *p* < 0.05.

^α Significantly different than 2 h.

[†] Significantly different than 96 h.

^p Significantly different than the respective data point on PHSRN-sIPN (Table 7).

^g Significantly different than the respective data point on GGG-sIPN (Table 8).

^m Significantly different than the respective data point on mPEG-sIPN (Table 9).

^u Significantly different than the respective data point on Unmod-sIPN (Table 10).

^k Significantly different than the respective data point on mold-cast Unmod-sIPN (Table 11).

^t Significantly different than the respective data point on TCPS (Table 3).

^f Significantly different than the respective data point on TCPS-FN (Table 4).

Table 7
Cumulative release of seven inflammatory cytokines from GD25 and GD25β1A cells in the presence of PHSRN-sIPN

Cell type	Cytokine	Cumulative cytokine release (pg ml ⁻¹)			
		2	24	96	168
GD25	GM-CSF	9.63 <i>§rguk</i>	5.0 8		
	IL-1α	1.08	0.0 1		
	IL-1β	2.03	0.9 9	<i>tf</i> < 0.67 <i>α</i> < 0.19	<i>kt</i> < 0.67 0.46 ± 0.46
	IL-6	1.89 <i>uk</i>	0.1 8	<i>f</i> < 1.28	<i>r</i> 0.74 ± 0.27
	MCP-1	250.48	16. 08	<i>tf</i> 4.19 ± 1.64 <i>kt</i> 2.06 ± 0.83	<i>ktf</i> 2.06 ± 0.83
	RANTES	8.377	0.9 3	<i>tf</i> 441.47 ± 55	242.69 ± 1
	TNF-α	8.29.96	7.5 6	<i>ktf</i> 1.87 ± 0.55	<i>ktf</i> 1.33 ± 0.84
GD25β1A	GM-CSF			<i>gtf</i> < 2.45	3.69 ± 3.69
	IL-1α	<i>gmi</i> 0	<i>t</i> 6.26 ± 3.21 <i>urkα</i> 2.05 ± 0.52	<i>t</i> < 0.67 0.65 ± 0.65	<i>ktf</i> < 0.67 0.89 ± 0.45
	IL-1β	1.99	< 0.55	0.74 ± 0.36	<i>r</i> 0.44 ± 0.23
	IL-6	2.78 <i>gmif</i>	<i>ktf</i> 1.89 ± 0.31 <i>rktfα</i> 71.8 ± 6	<i>kt</i> 2.57 ± 0.59 <i>rktfα</i> 88.9 ± 6	<i>ktf</i> 2.74 ± 0.44 <i>rktfα</i> 57.3 ± 7
	MCP-1	201.04	446.99 ± 0.4	569.48 ± 0.89	537.93 ± 0.67
	RANTES	8.84	<i>kt</i> 2.67 ± 0.53	<i>kt</i> 2.97 ± 12.9	<i>ktf</i> 2.54 ± 1.37
	TNF-α	<i>gm</i> 1.07	<i>krα</i> 25.68 ± 4.26	28.24 ± 1	<i>D</i> 2.40 ± 1.37

All values expressed as mean ± SE; *n*=3 and statistically analyzed by Student's *t*-test at *p* < 0.05.

§ Significantly different than GD25β1A.

α Significantly different than 2 h.

D Significantly different than 24 h.

r Significantly different than the respective data point on RGD-sIPN (Table 6).

g Significantly different than the respective data point on GGG-sIPN (Table 8).

m Significantly different than the respective data point on mPEG-sIPN (Table 9).

n Significantly different than the respective data point on Unmod-sIPN (Table 10).

^kSignificantly different than the respective data point on mold-cast Unmod-sIPN (Table 11).

^lSignificantly different than the respective data point on TCPS (Table 3).

^fSignificantly different than the respective data point on TCPS-FN (Table 4).

Table 8
Cumulative release of seven inflammatory cytokines from GD25 and GD25β1A cells in the presence of GGG-sIPN

Cell type	Cytokine	Cumulative cytokine release (pg ml ⁻¹)			
		2	24	96	168
GD25	GM-CSF	1.48	<i>pmu</i> 6.39	<i>tf</i> 4.18	<i>kt</i> 0.67
	IL-1α	<i>sp</i> 0.06	<i>ua</i> 3.69	<i>Q</i> 0.37	<i>Q</i> < 0.19
	IL-1β	1.65	0.82	<i>f</i> 1.75	<i>r</i> 0.72
	IL-6	1.59	<i>tf</i> 2.53	<i>tf</i> 3.17	<i>ukt</i> 2.01
		<i>muk</i>	<i>rif</i>	<i>tf</i>	<i>kff</i>
GD25β1A	MCP-1	271.21	444.89	438.53	262.08
	RANTES	<i>k</i> 2.31	<i>tf</i> 3.01	<i>kff</i> 2.21	<i>kff</i> 2.17
			<i>ruk</i>	<i>pukfa</i>	
GD25β1A	TNF-α	12.63	21.85	22.39	<i>Q</i> 4.35
	GM-CSF	<i>rpmuk</i>	<i>ut</i> 1.48	<i>t</i> 1.47	<i>kff</i> 1.23
	IL-1α	1.00	<i>ruk</i> 2.79	1.47	0.81
	IL-1β	<i>u</i> 0.18	0.14	1.20	0.68
GD25β1A	IL-6	1.99	0.18	<i>ukt</i> 1.66	0.50
		<i>rpuk</i>	<i>kff</i> 1.89	<i>rkff</i>	0.37
	MCP-1	542.16	110.2	108.1	73.1
	RANTES	<i>ptf</i> 3.44	616.14	355.04	6
			<i>kt</i> 2.18	<i>ukt</i> 3.18	0.35
GD25β1A	TNF-α	<i>tp</i> 21.09	<i>rk</i>	<i>rukff</i>	19.87
			27.54	20.63	3.86

All values expressed as mean ± SE; n=3 and statistically analyzed by Student's t-test at p < 0.05.

[§] Significantly different than GD25β1A.

[□] Significantly different than 2 h.

^Q Significantly different than 24 h.

[†] Significantly different than 96 h.

^r Significantly different than the respective data point on RGD-sIPN (Table 6).

^p Significantly different than the respective data point on PHSRN-sIPN (Table 7).

^m Significantly different than the respective data point on mPEG-sIPN (Table 9).

^u Significantly different than the respective data point on Unmod-sIPN (Table 10).

^k Significantly different than the respective data point on mold-cast Unmod-sIPN (Table 11).

^gSignificantly different than the respective data point on TCPS (Table 3).

^fSignificantly different than the respective data point on TCPS-FN (Table 4).

Table 9
Cumulative release of seven inflammatory cytokines from GD25 and GD25β1A cells in the presence of mPEG-sIPN

Cell type	Cytokine	Cumulative cytokine release (pg ml ⁻¹)				
		Time (h)				
		2	24	96	168	
GD25	GM-CSF	<4.44	± 4.44	± 2.94	± 1.23	± 1.23
	IL-1α	1.17	± 0.44	± 0.16	± 0.16	± 0.19
	IL-1β	<0.55	± 0.55	± 0.68	± 0.51	± 0.13
	IL-6	§ 1.41	± 0.15	± 0.68	± 4.33	± 1.51
	MCP-1	§guk	20.2	494.	± 111.	± 579.7
GD25β1A	RANTES	193.87	± 7	± 18	± 89	± 9
	TNF-α	k 2.07	± 0.49	± 4.08	± 0.83	± 1.88
	GM-CSF	12.12	± 3.99	± 3.35	± 25.54	± 0.82
	IL-1α	mpguk	± 4.44	± 4.77	± 0.67	± 0.67
	IL-1β	2.36	± 0.36	± 0.32	± 0.26	± 0.26
GD25β1A	IL-6	0.82	± 0.82	± 0.18	± 1.98	± 0.70
	MCP-1	f 1.93	± 0.10	± 0.67	± 3.86	± 1.81
	RANTES	mpkf	17.9	232.	± 84.3	± 466.7
	TNF-α	359.74	± 1	± 94	± 5	± 6
	GM-CSF	g 1.58	± 0.38	± 0.48	± 4.30	± 0.93
GD25β1A	IL-1α	rp	± 3.93	± 1.98	± 13.86	± 7.69
	IL-1β	16.73	± 4.77	± 4.77	± 0.67	± 0.67
	IL-6	ruk	± 1.23	± 0.32	± 0.19	± 0.19
	MCP-1	k 0.13	± 0.13	± 0.13	± 0.13	± 0.13
	RANTES	kt 2.46	± 0.45	± 0.45	± 0.45	± 0.45
GD25β1A	TNF-α	rkf	± 232.	± 84.3	± 466.7	± 1.81
	GM-CSF	498.47	± 5	± 5	± 6	± 6
	IL-1α	ukf 2.47	± 0.19	± 0.19	± 0.19	± 0.19
	IL-1β	Δ 5.65	± 5.65	± 5.65	± 5.65	± 5.65
	IL-6	Δ 5.65	± 5.65	± 5.65	± 5.65	± 5.65

All values expressed as mean ± SE; n=3 and statistically analyzed by Student's t-test at $p < 0.05$.

§ Significantly different than GD25β1A.

mpguk Significantly different than 2 h.

mpkf Significantly different than 24 h.

ruk Significantly different than the respective data point on RGD-sIPN (Table 6).

k Significantly different than the respective data point on PHSRN-sIPN (Table 7).

kt Significantly different than the respective data point on GGG-sIPN (Table 8).

ukf Significantly different than the respective data point on Unmod-sIPN (Table 10).

Δ Significantly different than the respective data point on mold-cast Unmod-sIPN (Table 11).

Δ Significantly different than the respective data point on TCPS (Table 3).

f Significantly different than the respective data point on TCPS-FN (Table 4).

Table 10
Cumulative release of seven inflammatory cytokines from GD25 and GD25β1A cells in the presence of Unmod-sIPN

Cell Type	Cytokine	Cumulative cytokine release (pg ml ⁻¹)				
		Time (h)				
		2	24	96	168	
GD25	GM-CSF	3.65	3.65	3.65	3.65	5.40
	IL-1α	0	0	0	0	0
	IL-1β	2.44	2.44	2.44	2.44	1.35
	IL-6	2.78	2.78	2.78	2.78	1.39
	MCP-1	46.19	46.19	46.19	46.19	50.96
	RANTES	1.04	1.04	1.04	1.04	0.98
GD25β1A	TNF-α	5.47	5.47	5.47	5.47	3.22
	GM-CSF	3.65	3.65	3.65	3.65	3.65
	IL-1α	0.03	0.03	0.03	0.03	0
	IL-1β	2.76	2.76	2.76	2.76	1.25
	IL-6	2.06	2.06	2.06	2.06	1.77
	MCP-1	163.88	163.88	163.88	163.88	87.18
	RANTES	1.23	1.23	1.23	1.23	0.66
	TNF-α	6.85	6.85	6.85	6.85	3.22

All values expressed as mean ± SE; n=3 and statistically analyzed by Student's *t*-test at *p* < 0.05.

[§]Significantly different than GD25β1A.

[¶]Significantly different than 2 h.

^ΔSignificantly different than 24 h.

[†]Significantly different than the respective data point on RGD-sIPN (Table 6).

[‡]Significantly different than the respective data point on PHSRN-sIPN (Table 7).

[§]Significantly different than the respective data point on GGG-sIPN (Table 8).

^{||}Significantly different than the respective data point on mPEG-sIPN (Table 9).

^kSignificantly different than the respective data point on mold-cast Unmod-sIPN (Table 11).

[†]Significantly different than the respective data point on TCPS (Table 3).

^fSignificantly different than the respective data point on TCPS-FN (Table 4).

Table 11
Cumulative release of seven inflammatory cytokines from GD25 and GD25β1A cells in the presence of mold-cast Unmod-sIPN

Cell type	Cytokine	Cumulative cytokine release (pg ml ⁻¹)			
		2	24	96	168
GD25	GM-CSF	3.65 ± 3.65	< 3.22	<i>f</i> 10.80 ± 5.40	<i>§</i> <i>rpgmu</i> 25.51 ± 2.42
	IL-1α	<i>pf</i> 0 ± 0	0 ± 0	0 ± 0	0 ± 0
	IL-1β	2.44 ± 0	< 1.35	<i>f</i> 1.44 ± 1.44	3.31 ± 2.21
	IL-6	2.11 ± 0.40	<i>f</i> <i>§</i> 2.43 ± 1.12	<i>tf</i> <i>§</i> 5.10 ± 0.59	<i>§</i> <i>rpgu</i> 8.17 ± 0.69
	MCP-1	<i>§</i> <i>rpgmf</i> 0 ± 0	<i>tf</i> <i>§</i> 223.80 ± 0	<i>§</i> <i>rpgmu</i> 486.51 ± 23.3	<i>§</i> <i>rpgmu</i> 886.04 ± 128.6
	RANTES	<i>§</i> <i>rpgm</i> < 1.07	<i>tf</i> <i>§</i> 2.10 ± 1.03	<i>§</i> <i>rpgmu</i> 11.22 ± 2.27	<i>§</i> <i>rpgmu</i> 15.46 ± 1.42
	TNF-α	<i>p</i> 4.50 ± 3.04	<i>gmi</i> < 3.22	<i>gf</i> 3.43 ± 3.43	9.19 ± 9.19
GD25β1A	GM-CSF	4.62 ± 4.62	<i>t</i> 3.65 ± 3.65	<i>t</i> 12.06 ± 6.13	<i>rpgmu</i> 48.41 ± 4.56
	IL-1α	<i>gmi</i> 0 ± 0	<i>p</i> <i>gmi</i> 0 ± 0	0 ± 0	0 ± 0
	IL-1β	4.12 ± 1.68	1.63 ± 0.81	<i>rmi</i> 2.08 ± 0.36	1.63 ± 0.81
	IL-6	2.11 ± 0.40	<i>rpgmi</i> 25.13 ± 4.96	<i>rpgmu</i> 71.82 ± 3.37	85.61 ± 10.29
	MCP-1	<i>gmi</i> 30.1 ± 2	<i>rpgmu</i> 2280.73 ± 236	<i>rpgmu</i> 280 ± 280	<i>rpgmu</i> 730.2 ± 730.2
	RANTES	185.60 ± 2	<i>rpgmu</i> 2280.73 ± 47	5107.32 ± 98	6697.54 ± 3
	TNF-α	2.53 ± 0.74	35.65 ± 4.56	<i>rpgmu</i> 46.47 ± 0.99	<i>rpgmu</i> 47.91 ± 0.04
		8.45 ± 6.90	<i>p</i> <i>gmi</i> 3.43 ± 3.43	<i>g</i> 1.07 ± 1.07	2.15 ± 1.07

All values expressed as mean ± SE; *n*=3 and statistically analyzed by Student's *t*-test at *p* < 0.05.

§ Significantly different than GD25β1A.

α Significantly different than 2 h.

Δ Significantly different than 24 h.

† Significantly different than 96 h.

‡ Significantly different than the respective data point on RGD-sIPN (Table 6).

§ Significantly different than the respective data point on PHSRN-sIPN (Table 7).

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