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Engineering endogenous inflammatory cells as delivery vehicles

Weiyuan John Kao[a,b,*], Yiping Liu[a], Rathna Gundloori[a], Jing Li[b], Damian Lee[c], Nicole Einerson[b], Jeanine Burmania[b], Kelly Stevens[b]

[a]*Division of Pharmaceutical Sciences of the School of Pharmacy, University of Wisconsin — Madison, Madison, WI 53706, USA*
[b]*Department of Biomedical Engineering of the College of Engineering, University of Wisconsin — Madison, Madison, WI 53706, USA*
[c]*Department of Biochemistry of the College of Agricultural and Life Sciences, University of Wisconsin — Madison, Madison, WI 53706, USA*

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Abstract

Leukocytes are central in directing host inflammatory and immune processes; therefore, leukocyte response to biomaterials is extremely important. Although several leukocyte-derived molecules are used clinically, the long-term efficacy of treatments involving the systemic administration of these bioactive agents has yet to be demonstrated. Hence, the localized delivery of selected cytokines and growth factors produced by endogenous leukocytes is desirable and may have potential therapeutic values in the fundamental processes of tissue healing, growth regulation, and biocompatibility. The specificity and diversity of ligand–receptor interactions offer an attractive method in manipulating cellular behavior. Therefore, a more detailed understanding of the interplay between ligands and cell membrane receptors must be obtained. We designed interleukin-1-derived biomimetic agonists and antagonists to study and modulate leukocyte function in vitro. Selected agonists increased GM-CSF release by adherent human blood-derived macrophages in the presence of the natural IL1β antagonist, namely IL1ra. Furthermore, IL1-derived biomimetic antagonists neutralized the ability of IL1β in increasing the release of GM-CSF by adherent macrophages. We employed similar methodologies to elucidate the molecular mechanisms of integrin and extracellular matrix interaction in regulating leukocyte function. Oligopeptides were designed based on the functional structure of fibronectin and grafted on to a polymer network containing polyethyleneglycols. Macrophage adhesion was independent of the peptide identity that contained sequence RGD, PHSRN, PRRARV, or combinations thereof in an integrin-dependent fashion in vitro. However, integrin-dependent FBGC formation in vitro was highly dependent on both RGD and PHSRN in a single peptide formulation and with a specific orientation. From our intracellular signaling studies in vitro, protein tyrosine and serine/threonine kinases were found important in integrin signaling leading to macrophage adhesion mediated by fibronectin–integrin association. Furthermore, RGD and PHSRN appear to be significant in mediating this receptor–ligand association resulting in the necessary signaling characteristic for macrophage adhesion and the subsequent development. Our in vivo results showed that peptide identity played a minimal role in modulating the host inflammatory response and adherent macrophage density. RGD-containing peptides mediated rapid FBGC formation by 4 days of implantation by significantly increasing both the number of macrophages that participate in the cell fusion process and the rate of cell fusion. Both RGD and PHSRN domains were important in mediating FBGC formation at later implantation periods. These findings represent a mechanistic correlation between the role of protein functional architectures

*Corresponding author. Tel.: +1-608-263-2998; fax: +1-608-262-3397.
E-mail address: wjkao@pharmacy.wisc.edu (W.J. Kao).

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in ligand–receptor recognition and the post-ligation signaling events that control cellular behavior in vitro and in vivo.

Keywords: Interleukin-1; Extracellular matrix; Fibronectin; Leukocytes; Foreign body reaction; Biomimetics

1. The role of leukocytes in the foreign body reaction to biomaterials

The host inflammatory reaction is a normal response to injury and the presence of foreign objects. The magnitude and duration of the inflammatory process have a direct impact on biomaterial biostability and biocompatibility, hence affecting the efficacy of biomedical devices such as drug delivery vehicles [1,2]. Leukocytes such as polymorphonuclear leukocytes (PMN), monocytes, macrophages, macrophage-derived multinucleated foreign body giant cells (FBGC), and lymphocytes are central in directing host inflammatory and immune processes; therefore, leukocyte response to biomaterials is extremely important in understanding material-mediated host reaction. Several characteristic leukocyte functions are identified as critical events in the material–host interaction. First, leukocytes recognize adsorbed proteins on the biomaterial surface and may adhere on to the surface via several adhesion ligand–receptor superfamilies. Cell adhesion mediated by ligand–receptor complexation may be modulated by the presence of cytokines, growth factors, and other biologically active molecules [3–8]. The interplay among material physicochemical properties, bioactive factors, and various cells is complex and dynamic. Second, the process of adherent macrophage activation and fusion to form FBGCs is unique to the macrophage phenotype. The presence of FBGCs is used as a histopathology marker for chronic inflammation and the host foreign body reaction [2]. FBGCs have been demonstrated on biomaterials in vivo and the rate of material degradation underneath the giant cells has been shown to be markedly increased contributing to incidences of environmental stress cracking and device failure [9]. However, the molecular mechanisms involved in FBGC formation remain unclear. Third, activated leukocytes may release cytokines, growth factors, and other bioactive agents to modulate the function of other cell types such as fibroblasts and endothelial cells in the inflammatory environment [3,6,7,10].

2. Modulation of leukocyte functions for potential pharmaceutical applications

Several leukocyte-derived cytokines and growth factors are used on a limited basis to alter the inflammatory response and promote the healing process. For example, macrophage-derived interleukin-1beta (IL1β) and granulocyte/macrophage-colony stimulating factor (GM-CSF) are given to injured patients and bone marrow transplant recipients to assist the recovery of hematopoiesis [11–14]. Pharmaceutics based on the property, function, and structure of tumor necrosis factor-alpha (TNFα) are administered to patients suffering from chronic inflammation in order to enhance the wound healing process and attenuate inflammation [15,16]. However, the long-term clinical efficacy of treatments involving the systemic administration of these bioactive agents has yet to be demonstrated due to high cost, low bioavailability, and deleterious side-effects associated with the required high dosage. Hence, the localized delivery of selected cytokines and growth factors produced by endogenous inflammatory cells is desirable and may have potential therapeutic values in the fundamental processes of tissue healing, growth regulation, and biocompatibility. The specificity and diversity of ligand–receptor interactions offer an attractive means to manipulate precise cellular functions. Such design strategies are fundamental in the emerging field of ‘cellular engineering’. However, before the ability to control specific cellular function via biomaterials can be realized, a more detailed understanding of the interplay between material-bound ligands and receptors on the cell surface must be obtained. Furthermore, the redundancies that exist between receptors and target proteins must also be addressed. For instance, the tripeptide RGD sequence is found in several extracellular matrix proteins such as fibronectin, vitronectin, and fibrinogen. Several cell membrane receptors on different type of cells have been shown to complex with the RGD cell-adhesion motif of

these matrix proteins [17,18]. For example, platelet surface glycoprotein IIb/IIIa recognizes the RGD sequence of fibrinogen, and integrin $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ receptors on macrophages also recognize the RGD motif of fibronectin. Other integrin receptors on a variety of cells types that recognize the RGD cell-binding motif are $\alpha_{(2,3,4,5,7,8,v)}\beta_1$ and $\alpha_v\beta_{(1,3,5,6,8)}$. Some of the current biomaterial development and research use the RGD sequence to provide bioactive site for cell adhesion. However, it is apparent that such a design strategy to elicit a specific cellular function is limited by the redundancies that exist between receptors and target proteins. In the development of biofunctional materials by mimicking ligand–receptor interactions, a clear understanding of the function–structural relationship between target proteins and cell membrane receptors is crucial. The ultimate challenge of eliciting specific cellular function by using biofunctional materials lies within the normal host foreign body response, which might overcome the bioactive functionalities in the material intended to modulate cellular function.

3. Modulation of macrophage cytokine release in vitro by biomimetic molecules derived from interleukin-1 family proteins

In the design of biofunctional molecules to study and modulate host cell behavior, we had chosen a family of active proteins (i.e. interleukin-1, IL1) as a model in the design of biofunctional molecules. IL1β is a potent proinflammatory cytokine that upregulates cellular function upon ligation with IL1 receptor type I (IL1RI) on the extracellular membrane [19]. IL1β-activated macrophages may release high levels of IL1β, TNFα, and/or GM-CSF which all have important roles in the healing process [6,12]. Circulating IL1 receptor antagonist (IL1ra) is the natural antagonist for IL1β. IL1β ligates with Domain 1, 2, and 3 of IL1RI and initiates signaling pathways to upregulate cellular functions; whereas, IL1ra only binds with Domain 1 and 2 of IL1RI resulting in no post-ligation signal transduction [20]. It has been proposed that IL1β complexes with Domain 3 of IL1RI by a strong electrostatic interaction. Based on the fundamental understanding of IL1 proteins, receptors, and functional mechanisms, we designed macromolecules based on the functional architecture of IL1β and IL1ra. Antagonists were designed from known IL1β and IL1ra amino acid residues showing strong avidity towards Domain 1 and 2 of IL1RI [20]. These residues are $R^{(4)}$ $L^{(6)}$ $F^{(46)}$ $I^{(56)}$ $K^{(103)}$ $E^{(105)}$ of IL1β [21,22] and $W^{(17)}$ $Q^{(21)}$ $Y^{(35)}$ $Q^{(37)}$ $Y^{(148)}$ of IL1ra [23]. Strong evidence suggests that these amino acids are essential in ligand–receptor interaction. The tertiary structures of the native IL1β and IL1ra molecule in solution were used as a guide in determining the spatial interrelationship between each residue [20–23]. The minimum distance between each residue was approximated using the structural coordinates archived in the SwissProt Database®. Based on the measurement, a poly-glycine sequence of approximately the same length was used to link each amino acid at all possible orientations. We further hypothesized that the resulting antagonist sequences, which target Domains 1 and 2 of IL1RI, can be converted to agonists by coupling a strong electrostatic moiety (i.e. poly-lysine) to the terminal amino acid of the antagonist sequence, thus allowing the complexation between the peptide macromolecule and Domains 1, 2, and 3 of IL1RI to occur. To formulate agonists, a trimeric glycine linker was used to join the antagonist domain with the poly-lysine domain. The trimeric glycine linker was designed to introduce spatial flexibility between the poly-lysine sequence and the antagonist oligopeptide domain since chain mobility may impact the dynamics of ligand–receptor association. Consequently, a library of linear oligopeptides were formulated and synthesized using solid-resin methods with standard 9-fluorenylmethyloxycarbonyl chemistry [24]. Peptides thus formulated were designated as follows. Antagonists included that modeled after the native IL1β molecule: (IL1iant) RGGLGGFGGIGKGGEG; (IL1iiant) FGRGGLGGGIGKGGEG; (IL1iiiant) LGGRGFGGIGKGGEG; and that modeled after the native IL1ra molecule: (IL1ivant) WGGGQGGYGGQGGGYG; (IL1vant) WGGYGGQGGYGGQG; and that modeled after antagonists developed via combinatorial chemistry [25]: (IL1viant) YWQPYALPL. Agonists included that modeled after the IL1β molecule: (IL1i) KKKGGGRGGLGGFGGIGKGGEG; (IL1ii) KKKGGGFGRGGLGGGIGKGGEG; (IL1iii) KKKGGGLGGRGFGGIGKGGEG; and that

modeled after the IL1ra molecule: (IL1iv) KKKGGGWGGGQGGYGGQGGGYG; (IL1v) KKKGGGWGGYGGQGGYGGQG; and that modeled after antagonists developed via combinatorial chemistry [25]: (IL1vi) KKKGGGYWQPYALPL; (IL1vii) YWQPYALPLGGGKKK. Human blood monocyte-derived macrophages were allowed to adhere on tissue-culture polystyrene (TCPS) for 2 h in the presence of autologous serum in the culture medium. After 2 h, nonadherent cells were removed and adherent cells were challenged with free peptides at an optimal concentration of 50 pmol/ml that was determined previously. Simultaneously, adherent cells were also challenged with or without recombinant human IL1β or IL1ra at 25 pmol/ml. Peptides with scrambled amino acid sequences were also employed as peptide controls. Cells were cultured thereafter in the presence of autologous serum and supernatants were collected at various time points for assay. Our results demonstrated the following [26,27]. At 4 h after the peptide challenge, no differences in GM-CSF release by adherent macrophages were observed among all test samples and controls. At 18 h after the free peptide challenge, IL1β treated adherent macrophages on TCPS showed a higher GM-CSF release than that without treatment controls (Fig. 1a). Adherent macrophages treated with agonist IL1v showed a higher GM-CSF release than that treated with IL1β, IL1ra, none, and other agonists [26,27]. It is known that IL1β upregulates GM-CSF release by human macrophages via the induction of AP-1 and NFκB gene expression factors [20]. The result suggests that IL1v activates these gene expression factors resulting in an increased GM-CSF production. The GM-CSF level of cells treated with IL1v remained comparable when IL1ra was added simultaneously as the IL1v peptide indicating that the effect of IL1v in increasing GM-CSF release by adherent macrophages was not neutralized in the presence of the natural IL1β antagonist, namely IL1ra. No significant differences in the GM-CSF release were observed between macrophages without treatment and that treated with both IL1β and one of the antagonists [26,27] (Fig. 1b). These results indicate that the IL1-derived biomimetic antagonists neutralized the ability of IL1β in increasing the release of GM-CSF by adherent macrophages. Recombinant IL1β and IL1ra

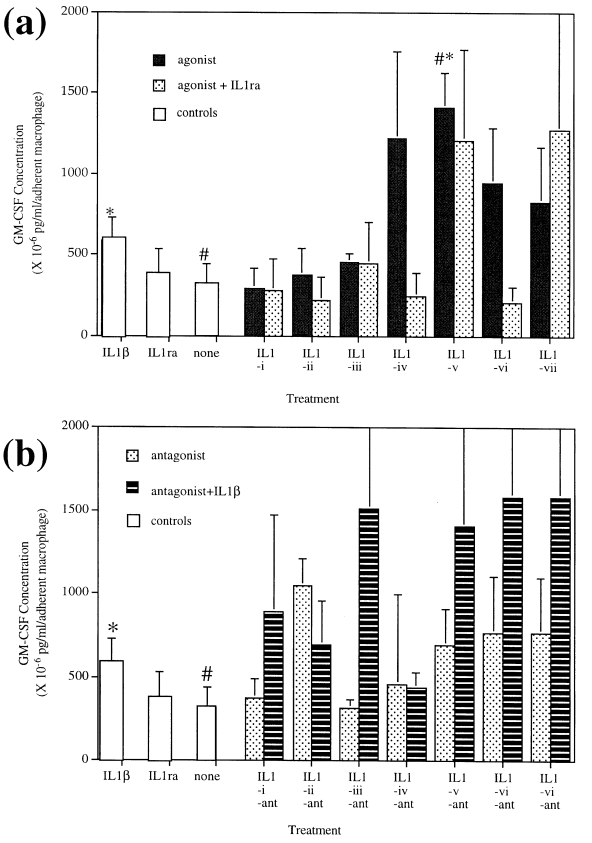

Fig. 1. The release of GM-CSF by adherent human blood-derived macrophages (normalized to adherent macrophage density) on tissue-culture polystyrene after 18 h incubated with: (a) biomimetic agonists derived from IL1-family proteins with or without recombinant human IL1ra. $*p<0.05$ versus values of 'none' control; $^{\#}p<0.05$ versus values of IL1β control; IL1v values were larger than that of IL1i, IL1ii, IL1iii, IL1vi, IL1vii at $p<0.05$; or (b) biomimetic antagonists derived from IL1-family proteins with or without recombinant human IL1β. $*p<0.05$ versus values of 'none' control; $^{\#}p<0.05$ versus values of IL1β control; no differences at $p>0.95$ were found among each test group indicating that the GM-CSF release mediated by antagonists was not neutralized by the presence of IL1β. All values in (mean±S.E.M., $n=4$).

proteins have been investigated for their potential therapeutic values. For example, IL1β augments hematopoiesis and increases wound healing; whereas, IL1ra attenuates host inflammatory reaction [11,12,19]. However, the clinical value of these regimens has yet to be demonstrated due to high cost, low bioavailability, deleterious side effects of the high dosage that is often required, and the

presence of natural agonists, antagonists, or serum proteases. Hence, IL1-derived agonists and antagonists are currently under extensive investigation by pharmaceutical companies and basic science research laboratories to improve the therapeutic value of native IL1β and IL1ra moieties. IL1-derived agonists have demonstrated potential in increasing hematopoietic stem cell proliferation, increasing B-cell proliferation, enhancing local wound healing, and modulating collagenase production. IL1-derived antagonists have shown promise in decreasing the extent of acute and chronic inflammation in diseases such as rheumatoid arthritis, treating septic shock, suppressing chronic leukemia, and modulating bone resorption by osteoclasts. We demonstrated the uniqueness of the biomimetic approach to develop synthetic molecules derived from naturally occurring precursors such as IL1-family proteins. These biomimetic molecules demonstrate enhanced biochemical and physical properties that can be employed to study biological systems, modulate cellular behavior, and potentially improve clinical medicine.

4. Modulation of macrophage adhesion and giant cell formation in vitro via the study of integrin–fibronectin association

Some of the current drug delivery, tissue engineering, and biomaterial development and research utilize the RGD amino acid sequence as bioactive sites to promote cell interaction. However, it is apparent that such a design strategy to elicit a specific cellular function is limited by the redundancies that exist between receptors and target proteins. For instance, the tripeptide RGD sequence is found in several extracellular matrix proteins that are recognized by several cell membrane receptors on different type of cells [17,18]. Hence, in the development of biofunctional materials by mimicking ligand–receptor interactions, a clear understanding of the function–structural relationship between target proteins and cell membrane receptors is crucial.

Several methodologies were used to examine the interrelationship between material chemistry and cellular behavior at protein and cellular levels under in vitro and in vivo environments. Based on these findings, we devised strategies to probe the molecular mechanism of receptor–ligand recognition and post-ligation cellular function. In one of the studies, we designed and employed biomimetic oligopeptides to probe the effect of primary and tertiary structures of a model protein (i.e. fibronectin) in ligand–receptor interaction. Synthetic peptides were formulated based on the primary and tertiary structures of human plasma fibronectin [28,29]. Specifically, the amino acid sequences PHSRN [30] and RGD [31], which are located in the FIII-9 and FIII-10 modules, respectively, of the central cell-binding domain and the sequence PRRARV [28,29,32,33] of the C-terminal heparin-binding domain of fibronectin were chosen for exploration. RGD and PHSRN are present on adjacent loops of two connecting FIII modules and bind synergistically to the same integrin receptor [34–36]. The heparin-binding domain of fibronectin in which the sequence PRRARV resides also binds directly with integrin receptors [32,33]; however, the precise mechanisms involved in this association remain unclear. The tertiary structure of fibronectin [35] was used as a guide in the design of peptides that included RGD and PHSRN. The distance between these sequences was approximated using the structural coordinates archived in the SwissProt Database® (sequence FINC_HUMAN P02751). Based on the measurement, a hexamer of glycine (G_6) of approximately the same length was used to link the two bioactive sequences in both possible orientations. The combination of RGD and PRRARV was studied using a G_6 linker; although in this case the G_6 linker was not selected based on any structural considerations. A terminal trimeric glycine domain (G_3) was employed as a spacer in all peptides. Oligopeptides were synthesized using solid-resin methods with standard 9-fluorenylmethyloxycarbonyl chemistry [24]. The following oligopeptides were synthesized: G_3RGDG, G_3PHSRNG, G_3PRRARVG, G_3RGDG$_6$PHSRNG, G_3PHSRNG$_6$RGDG, G_3RGDG$_6$PRRARVG, and G_3PRRARVG$_6$RGDG. A cyclic RGD peptide with an amino acid sequence of LNQEQVSPD(cRGDGRN) was used for comparison [37]. LNQEQVSPD is a leader sequence and the cyclical RGDGRN sequence has been shown to bind with $\alpha_v\beta_3$ integrin with high specificity and affinity. Peptides were immobilized on to a polymer network terpolymerized with monomethoxy poly-

ethyleneglycol monoacrylate (mPEGmA), acrylic acid (Ac), and trimethylolpropanetriacrylate (TMPTA) [26,38–40]. The network is a random copolymer and is hydrophilic, nonionic, low swelling, glassy, optically transparent, and colorless [40]. Differential scanning calorimetry analysis showed that these materials are completely amorphous and the mPEGmA component is completely phase-mixed in the crosslinked TMPTA matrix [40]. We demonstrated previously that networks without grafted peptides or with inactive peptides mediated nominal levels of human and murine macrophage, human dermal fibroblast, and human umbilical vein endothelial cell adhesion in the presence of serum in vitro for a long duration of time [38,39]. Bioactive oligopeptides were grafted on to mPEGmA-co-Ac-co-TMPTA networks and the resulting surface mediated cell adhesion in a receptor-peptide specific manner [38,39]. Macrophage adhesion and FBGC formation in vitro assays [41] were performed. Under the FBGC culture condition described, FBGCs containing up to 50 nuclei/cell formed consistently on TCPS control surfaces. Briefly, freshly isolated human blood-derived monocytes were incubated with samples in RPMI containing 20% autologous serum. At 96 and 168 h, the medium was changed to RPMI with heat-inactivated autologous serum+10 ng/ml of recombinant human interleukin-4+5 ng/ml of recombinant human GM-CSF. Each adherent cell with three or more nuclei per cell was defined as a FBGC. Competitive inhibition studies using soluble free peptides and neutralizing antibodies were performed to ascertain ligand–receptor specificity and identification.

Based on the results [26,27,39], we found that serum fibronectin modulated macrophage adhesion and the extent (i.e. size) of FBGC formation on TCPS control surfaces in the presence of serum proteins. Macrophages adhered to all peptide-grafted mPEGmA-co-Ac-co-TMPTA networks with relatively subtle differences between adhesion mediated by peptides containing sequence RGD, PHSRN, PRRARV, or combinations thereof. The β_1 integrin subunit was essential in macrophage adhesion to peptide-grafted networks in a receptor-peptide specific manner; whereas, β_3 integrin subunit was less important. Macrophage adhesion to surfaces grafted with PRRARV-containing peptides was mediated primarily by the direct interaction with integrins. Networks grafted with peptides that contain RGD or PHSRN alone did not provide an adequate substrate for macrophage fusion to form FBGCs. However, the PHSRN synergistic site and the RGD site in a single oligopeptide provided a substrate for FBGC formation that was statistically comparable to that on the TCPS positive control material in the presence of serum proteins. This response was highly dependent upon the relative orientation between RGD and PHSRN. Surfaces grafted with peptides that contain PRRARV alone or in tandem with RGD in a single peptide formulation did not support FBGC formation. Neutralizing antibody was used in the FBGC culture assay to partly determine the role of integrins and fibronectin-derived oligopeptides in modulating the function of adherent macrophages to fuse and form FBGCs. Antibody isotype negative controls were also used for confirmation. Results showed that no-antibody and anti-β_3 neutralizing antibody treated groups had a comparable FBGC density on TCPS. On most of the peptide-grafted network, no FBGC formation was observed in the anti-β_3 antibody treated group. One notable exception was the peptide that contained the PHSRN and the RGD domain in the optimal orientation, namely G_3PHSRNG_6RGDG, on which anti-integrin β_3 reduced FBGC formation by about 70%. When anti-β_1 neutralizing antibody was used, no FBGC formation was observed on all samples. Macrophages and FBGCs express α_2, α_4, β_1, and β_3 integrin subunits. It is also known that the first 160 amino acids of integrin β_1, specifically the α-helix formed by residues 141–160, are critical in integrin-ligand recognition. The above findings suggest that the association between this region of the integrin β_1 receptor and G_3PHSRNG_6RGDG, but not G_3RGDG$_6$PHSRNG, results in the necessary binding characteristic that determine the subsequent cellular event leading to FBGC formation. Activated integrin β_1 or β_3 intracellular domains stimulate cell migration, modulate proliferation and gene expression, induce the assembly of F-actin cytoskeleton, and localize the activity of focal adhesion kinase $pp125^{FAK}$. These cellular events may contribute to the process of FBGC formation; however, the exact interrelationship between ligand–receptor architec-

ture and association in activating intracellular signaling events resulting in the control of cellular behavior remains unclear.

5. Modulation of host foreign body reaction, macrophage adhesion, phenotypic development, and cytokine release in vivo via biomimetic fibronectin-derived peptides

From the above study, we demonstrated that the RGD and PHSRN domain of fibronectin and the spatial orientation between the motifs were crucial in regulating macrophage function in vitro. To extend this finding further, we probed the role of RGD and PHSRN in modulating the host inflammatory response and macrophage behavior in vivo. Oligopeptides containing RGD and/or PHSRN domains (i.e. G_3RGDG, G_3PHSRNG, G_3RGDG$_6$PHSRNG, G_3PHSRNG$_6$RGDG, and G_3RDGG negative controls) were grafted on to mPEGmA-co-Ac-co-TMPTA networks and subcutaneously cage-implanted into rats. The well-established subcutaneous cage-implant system [42–45] was used to study the effect of implanted materials on the host foreign body reaction. Briefly, the polymer samples were then inserted under sterile conditions into an autoclaved cylindrical cage measuring 3.5 cm long, 1 cm in diameter, and constructed from medical grade stainless steel wire mesh. Cages containing various polymer samples were subcutaneously implanted at the back of 3-month-old female Sprague–Dawley rats. Empty cages were employed and implanted as controls. The inflammatory exudate that collects in the cage was withdrawn at 4, 7, 10, 14, and 21 days post-implantation and analyzed for the quantitative evaluation of cellular and humoral response to the test material using standard hematology techniques. Specifically, the distribution of lymphocyte, monocyte, and PMN subpopulations in the exudate was determined. The presence of a high concentration of PMNs in the inflammatory exudate indicates an acute inflammatory response, which occurs from the onset of implantation and attenuates with time [2,42]. This is followed by the chronic inflammatory response, which is characterized by the presence of a high concentration of monocytes and lymphocytes in the exudate [2,42]. Hence, the cage implant system allows us to observe the host inflammatory reaction to the test sample as a function of time and material property. A drop of each exudate sample was also cultured on brain–heart infusion agar plates to check for the incidence of infection. No infection was observed at any retrieval time for any sample. At 4, 7, 14, 21, 35, and 70 days post-implantation, test polymer samples were retrieved and the adherent cell morphology and density were quantified.

Our results [46] showed that peptide identity played a minimal role in modulating the host inflammatory response and adherent macrophage density (Table 1). FBGC densities and average FBGC size (Table 2) for all test samples were comparable

Table 1
Adherent macrophage density on cage-implanted mPEGmA-co-Ac-co-TMPTA networks grafted with various fibronectin-derived oligopeptides

Peptide	Adherent macrophage density ($\times$10 macrophages/mm^2) at various post-implantation times (days)					
	4	7	14	21	35	70
G_3RGDG	138±22[a]	85±12[ab]	33±12[ab]	15±3[b]	14±2[b]	4±2[b]
G_3PHSRNG	124±12[a]	57±10[ab]	31±11[ab]	10±0[b]	9±1[b]	4±2[b]
G_3RGDG$_6$PHSRNG	126±8[a]	58±12[ab]	23±4[ab]	14±4[b]	6±5[b]	0±0[b]
G_3PHSRNG$_6$RGDG	183±27[a]	69±6[ab]	30±5[ab]	16±4[b]	12±5[b]	3±1[b]
G_3RDGG	75±16	36±5[b]	15±3[b]	15±6[b]	9±3[b]	3±2[b]
No grafted peptide	74±26	37±4[b]	14±2[b]	19±3[b]	6±3[b]	1±1[b]

All values expressed as mean±S.E.M., $n=3$.
[a] $p<0.05$ versus respective values of 'no grafted peptide' controls.
[b] $p<0.05$ versus respective values at Day 4 of the same sample type.

Table 2
Adherent FBGC density and average FBGC size on cage-implanted mPEGmA-co-Ac-co-TMPTA networks grafted with various fibronectin-derived oligopeptides

Peptide	Adherent FBGC density (FBGC/mm^2) and (average FBGC size ($\times$1000 μm^2/cell with values in (parentheses)) at various post-implantation times (days)					
	4	7	14	21	35	70
G_3RGDG	12±9[a]	10±3[a]	11±4[a]	11±0[a]	12±1[a]	9±3[a]
	(3±3)	(13±4[b])	(32±5[ab])	(31±6[ab])	(33±5[ab])	(75±7[ab])
G_3PHSRNG	0±0	7±1[ab]	13±4[ab]	11±1[ab]	13±1[ab]	9±2[ab]
	(0±0)	(12±5[b])	(24±3[ab])	(43±5[ab])	(47±3[ab])	(89±10[ab])
G_3RGDG$_6$PHSRNG	1±1	4±1[a]	7±1[ab]	10±2[ab]	9±1[ab]	12±2[ab]
	(1±1)	(8±2[b])	(23±4[ab])	(43±10[ab])	(84±14[ab])	(82±5[ab])
G_3PHSRNG$_6$RGDG	5±3[a]	8±4[a]	8±1[a]	7±1[a]	11±2[a]	9±2[a]
	(1±1)	(11±4[b])	(21±4[ab])	(46±9[ab])	(40±6[ab])	(71±4[ab])
G_3RDGG	0±0	1±1	3±2	4±1[b]	5±2[b]	3±0[b]
	(0±0)	(17±14)	(10±5[b])	(19±8[b])	(21±1[b])	(38±19[b])
No grafted peptide	0±0	1±1	2±1	3±1[b]	3±1[b]	3±2[b]
	(1±1)	(11±11)	(12±2[b])	(10±3[b])	(22±3[b])	(39±6[b])

All values are expressed as mean±S.E.M., n=3.
[a] $p<0.05$ versus respective values of 'no grafted peptide' controls.
[b] $p<0.05$ versus respective values at Day 4 of the same sample type.

and were higher than respective values of G_3RDGG or 'no grafted peptide' controls at each retrieval time from 14 to 70 days post-implantation. The percentage of FBGC coverage (total FBGC area per total sample area) for surfaces grafted with G_3RGDG, G_3PHSRNG, G_3RGDG$_6$PHSRNG, G_3PHSRNG$_6$RGDG, G_3RDGG nonspecific controls, and 'no grafted peptide' negative controls, was (38±10, 61±17), (59±2, 79±6), (69±1, 93±5), (46±14, 65±7), (18±8, 28±18), and (13±8, 22±12) for (35 and 70 days post-implantation), respectively. These results showed that networks grafted with fibronectin-derived peptides mediated extensive FBGC coverage that increased with increasing implantation time. Specifically, surfaces grafted with G_3RGDG$_6$PHSRNG showed the highest FBGC coverage at about 90% of the total sample area when compared with other sample types and controls by 70 days of implantation. These in vivo findings indicate that the RGD motif, specifically in the configuration of G_3RGDG or G_3PHSRNG$_6$RGDG but not G_3RGDG$_6$PHSRNG, modulates a rapid macrophage fusion to form FBGCs that is observed at the early stage of implantation (i.e. within 4 days of implantation). Both the RGD and PHSRN motifs were important in mediating FBGC formation at the later implantation time (i.e. from 14 to 70 days of implantation). Furthermore, the PHSRN motif, specifically in the configuration of G_3RGDG$_6$PHSRNG but neither in G_3PHSRNG nor G_3PHSRNG$_6$RGDG, was more important in modulating the extent of FBGC formation (i.e. percent coverage) at the later implantation time. Our previous results obtained via the utilization of an established in vitro FBGC assay showed that mPEGmA-co-Ac-co-TMPTA networks grafted with G_3PHSRNG$_6$RGDG mediated a comparable level of FBGC density as TCPS positive surface controls and both were significantly higher than that on networks grafted with G_3RGDG$_6$PHSRNG, G_3PHSRNG, G_3RGDG, or peptide and surface controls by 10 days of culture [41]. Although the in vitro FBGC assay system does not fully mimic the complex and dynamic host foreign body response in terms of FBGC formation, both in vivo and in vitro results point to the important spatial relationship between RGD and PHSRN domains in mediating FBGC development as a function of time.

A previously developed mathematical model describing the in vivo kinetics of macrophage fusion to form FBGCs on biomaterials was employed to provide insights into the effect of peptide identity on the kinetics of FBGC formation [43–45]. Specifically, two kinetic parameters were calculated based on

results of the FBGC size-distribution at day 4, 7, 14, and 21 of implantation: (a) the density adherent macrophages that participate in the FBGC formation and (b) the rate constant of cell fusion. Calculations showed that the density of adherent macrophages that participate in the FBGC formation was 127 ± 10, 100 ± 5, 62 ± 10, 123 ± 30, 53 ± 12, and 46 ± 10 macrophages/mm^2 for networks grafted with G_3RGDG, G_3PHSRNG, G_3RGDG$_6$PHSRNG, G_3PHSRNG$_6$RGDG, G_3RDGG, or no grafted peptide, respectively. Our results showed that the density of adherent macrophages that participate in the FBGC formation was significantly higher ($p<0.05$) for mPEGmA-co-Ac-co-TMPTA networks grafted with G_3RGDG, G_3PHSRNG, or G_3PHSRNG$_6$RGDG than that for networks grafted with G_3RDGG nonspecific controls and networks without peptide grafting. The rate constant of cell fusion ranged from 2.4×10^{-3} to 5.6×10^{-3} mm^2/cell per week with a medium value of 4.0×10^{-3}. The rate constant of cell fusion results indicates that the cell fusion rate on surfaces grafted with G_3PHSRNG, G_3RGDG$_6$PHSRNG, G_3PHSRNG$_6$RGDG, or G_3RDGG was comparable but was slower than that on G_3RGDG and faster than that on 'no grafted peptide' controls. By coupling the results of measured FBGC density and the kinetic analysis, our findings indicate that the presence of grafted G_3RGDG or G_3PHSRNG$_6$RGDG on mPEGmA-co-Ac-co-TMPTA networks modulate a high level of FBGC formation by 4 days of implantation by significantly increasing both the number of macrophages that participate in the cell fusion process and the rate of cell fusion. Since adherent macrophage densities on networks grafted with G_3RGDG, G_3PHSRNG, G_3RGDG$_6$PHSRNG, or G_3PHSRNG$_6$RGDG were comparable at each given implantation time, kinetic analysis results suggest that the presence of these grafted peptides did not affect other events which must occur prior to cell fusion, namely, monocyte adhesion. Our in vivo investigations ascertained the importance of RGD and PHSRN amino acid sequences in modulating macrophage function in a time- and orientation-dependent fashion under physiologic conditions.

The ligation of fibronectin and integrin receptors had been demonstrated to modulate the expression of cytokine receptors on leukocytes (Fig. 2) [47–50].

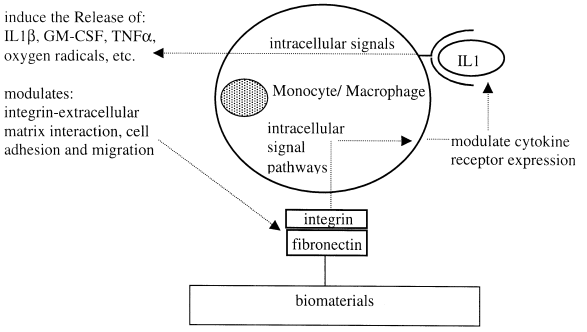

Fig. 2. Interrelationship between the complexation of integrin receptors and extracellular matrix protein fibronectin and the expression of IL1 receptors and IL1 on macrophages.

For example, fibronectin modulates the expression of IL1β and its receptor antagonist in human mononuclear cells. Interleukin-1-signaling pathways such as the NFκB-family factors are modulated by integrin binding to fibronectin and actin reorganization. Conversely, IL1β and other macrophage-derived cytokines and growth factors increase macrophage adhesion and integrin expression to promote interaction with extracellular matrix proteins such as fibronectin. To elucidate the role of grafted RGD and/or PHSRN domains on mPEGmA-co-Ac-co-TMPTA networks in modulating macrophage IL1β release upon ligation with integrin receptors, exudate IL1β concentrations were assayed using enzyme-linked immunosorbent assay at various implantation times. The results showed the exudate IL1β concentration decreased rapidly between 4 and 7 days of implantation for all samples (Table 3). No differences in IL1β concentration was observed for empty cage controls, no grafted peptide network controls, and networks grafted with G_3RDGG for all implantation time suggesting that the presence of the network with or without biologically inactive peptide modulated similar macrophage IL1β release. Grafted G_3PHSRN significantly lowered IL1β concentration at 14 and 21 days post-implantation; whereas, G_3RGDG and G_3RGDG$_6$PHSRNG showed a lower IL1β release at 14 days of implantation (Table 3). We demonstrated that peptide identity did not affect exudate total and macrophage concentrations and adherent macrophage density. The current IL1β release results suggest the differential regulation of macrophage cytokine production by grafted fibronectin-derived peptides. Spe-

Table 3
Release of IL1β by adherent macrophages on cage-implanted mPEGmA-co-Ac-co-TMPTA networks grafted with various fibronectin-derived oligopeptides

Peptide	Exudate IL1β concentration (pg/ml) at various post-implantation times (days)			
	4	7	14	21
G_3RGDG	260±23	85±31	31±10[ab]	27±18
G_3PHSRNG	253±89	73±5	26±8[ab]	4±1[ab]
G_3RGDG_6PHSRNG	152±37	240±197	16±1[ab]	47±30
G_3PHSRNG_6RGDG	282±9	86±31	69±57	24±21
G_3RDGG	220±21	49±16	83±12	33±32
No grafted peptide	343±44	80±19	114±43	54±25
Empty cage controls	376±188	46±19	101±26	99±42

All values expressed as mean±S.E.M., $n=3$. All values for days 7, 14, and 21 for a given peptide identity are less ($p<0.05$) versus respective values of day 4.

[a] Differences ($p<0.05$) versus respective value of empty cage controls.

[b] Differences ($p<0.05$) versus respective value of no grafted peptide controls.

cifically, the presence of PHSRN domain lowered IL1β release in a sequence orientation-dependent manner.

IL1ra and IL1ra-derived antagonists have shown promise in decreasing the extent of acute and chronic inflammation in diseases such as rheumatoid arthritis, treating septic shock, suppressing chronic leukemia, and modulating bone resorption by osteoclasts. We demonstrated the uniqueness of biomimetic approach to develop synthetic molecules derived from naturally occurring precursors (i.e. fibronectin). These biomimetic molecules demonstrate enhanced biochemical and physical properties that can be employed to study biological systems, modulate cellular behavior, and potentially improve clinical medicine. It is clear that cytokines play an important role in mediating cellular interaction with extracellular matrix proteins, and vice versa, resulting in the modulation of cellular behavior and development. This complex interrelationship between IL1 and extracellular matrix/plasma protein fibronectin (Fig. 2) offers a working model of the dynamic interaction involved in the modulation of macrophage behavior under the complex physiologic condition. By exploiting the mechanisms involved in these interactions, biomaterial researchers may gain insights into the design of materials with specific bioactivity to modulate the behavior of macrophages and other cell types.

6. Direct mechanistic correlation between ligand functional architecture and ligand–receptor association in initiating distinct intracellular signaling cascade to control macrophage behavior

To yield insights into the mechanisms coordinated by the interaction between integrins and fibronectin in mediating macrophage adhesion and FBGC formation, we used the aforementioned biomimetic fibronectin-derived oligopeptides to probe the structure–functional characteristic and signaling pathways of fibronectin–integrin association in modulating cellular function. Specifically, the key role played by RGD, PHSRN, and the spacing and orientation between the peptide sequences in modulating macrophage and FBGC behavior was investigated. Freshly isolated human blood-derived monocytes were preincubated with inhibitors of various selected signaling molecules to screen candidate signaling cascades in regulating macrophage behavior mediated by fibronectin and fibronectin-derived biomimetic oligopeptides. The signaling events and the corresponding inhibitor chosen for exploration included activated protein tyrosine kinases (PTK) inhibitor AG82, Src-family kinases inhibitor Lavendustin A, activated protein serine/threonine kinases (PSK) inhibitor H-7, protein kinase-A (PKA) inhibitor 14-22 amide, protein kinase-C (PKC) inhibitor EGF-R

fragment, PI-3K inhibitor wortmannin and MAPK inhibitor PD98059. Activated integrin receptors have been shown to up-regulate these selected signaling molecules under a variety of ligand–receptor associations (Fig. 3). For example, Src is involved in integrin signaling upon ligation with extracellular matrix proteins, such as fibronectin, fibrinogen, or vitronectin, leading to macrophage adhesion and focal adhesion kinase formation [51]. Treated human blood-derived monocytes were incubated with TCPS or mPEGmA-co-Ac-co-TMPTA networks pre-adsorbed or immobilized with recombinant human fibronectin or fibronectin-derived biomimetic oligopeptides containing RGD and/or PHSRN domains in culture medium supplemented with autologous serum. Surfaces without pre-adsorption or immobilization were employed as surface controls. Inhibitor vehicle was also used as additional inhibitor controls and G_3RDGG was employed as nonspecific controls for peptides. From the macrophage adhesion assay (Table 4), the results showed the following [27,52]. On TCPS or networks pre-adsorbed with fibronectin, macrophage adhesion was found to be dependent on PTK but not Src and PSK but not PKA at 24 and 48 h. However, on networks grafted with fibronectin, macrophage adhesion was found to be independent of PTK or PSK at 48 h. Furthermore, macrophage adhesion on TCPS with G_3RGDG but not networks was found to be dependent on PTK or PSK. Specifically, we found that macrophage adhesion on fibronectin pre-adsorbed-TCPS or networks, but not on fibronectin-grafted networks, was depen-

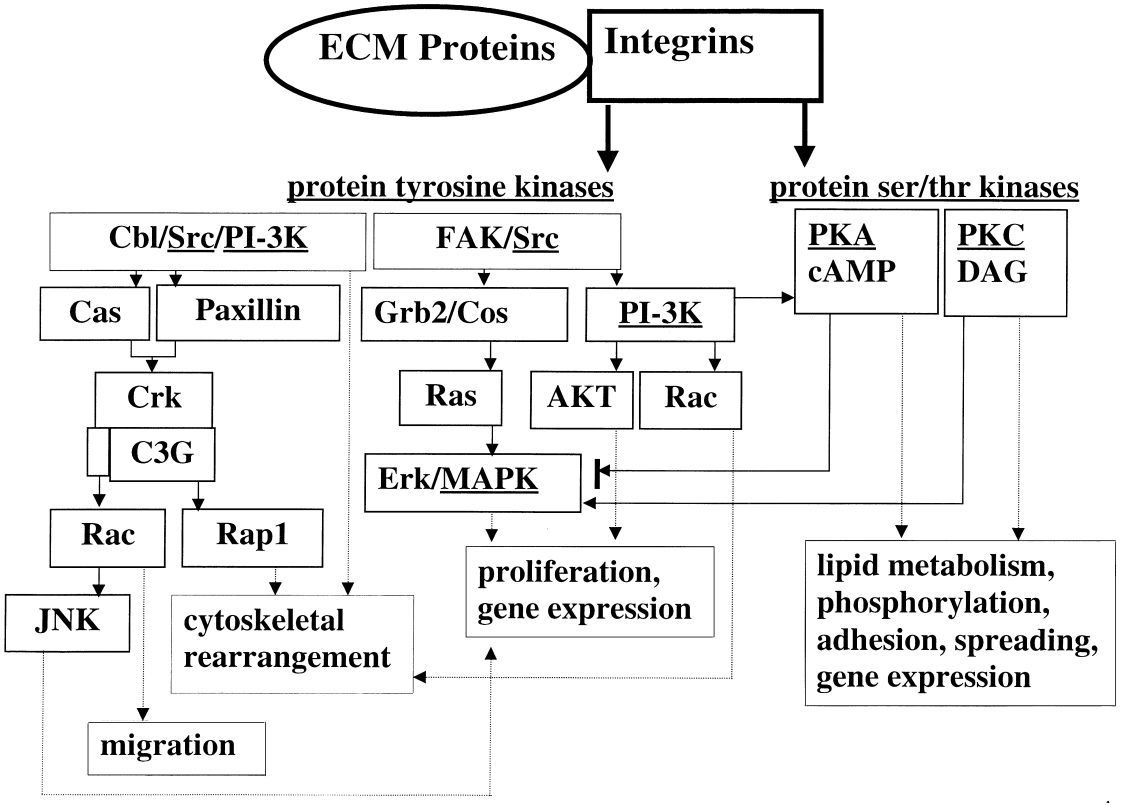

Fig. 3. Intracellular signal transduction cascades initiated by the association of extracellular membrane integrin receptors and extracellular matrix proteins (ECM) such as fibronectin resulting in the modulation of leukocyte function and behavior. Signaling molecules that are underlined are those investigated in our studies.

Table 4
Summary of the effect of intracellular signaling inhibitor on adherent macrophage density with 24- and 48-h culture times

	Inhibitor of								
	PTK		PSK		Src	PKA	PKC	PI3 K	MAPK K
	24 h	48 h	24 h	48 h	24 h	24 h	24 h	24 h	24 h
Pre-adsorbed on TCPS									
Fibronectin	↓	↓	↓	↓	↔	↔	↓	↓	↓
G_3RGDG	↓	↓	↓	↓	↔	↔	↔	↔	↔
$G_3RGDG_6PHSRNG$	↔	↔	↔	↔	↔	↔	↓	↓	↓
Pre-adsorbed on networks									
Fibronectin	↓	↓	↓	↓	↔	↔	↓	↓	↓
G_3RGDG	↔	↔	↔	↔	↔	↔	↔	↔	↔
$G_3RGDG_6PHSRNG$	↔	↔	↓	↓	↔	↔	↔	↔	↔
Grafted on networks									
Fibronectin	↔	↔	↓	↔	↔	↔	↔	↔	↔
G_3RGDG	↔	↔	↔	↔	↔	↔	↔	↓	↔
$G_3RGDG_6PHSRNG$	↔	↔	↔	↔	↔	↔	↑	↓	↑

Each comparison indicates that the level of adherent macrophage density of cells treated with respective intracellular signaling molecule inhibitor remained unchanged (↔), increased (↑), or decreased (↓) when compared with the level of adherent macrophage density of cells treated with respective vehicle controls (data not shown) at 95% ($p<0.05$) confidence level.

dent on PKC, PI3 kinase or MAPK kinase. Furthermore, TCPS pre-adsorbed with $G_3RGDG_6PHSRNG$ mediated a similar PKC-, PI3 kinase- and MAPK-dependency for macrophage adhesion as TCPS pre-adsorbed with fibronectin. However, the adherent macrophage density on networks grafted with $G_3RGDG_6PHSRNG$ increased with the presence of PKC or MAPK kinase inhibitor, but decreased with the presence of PI3 kinase inhibitor. In addition, the activation of PKC by PMA did not compensate PTK inhibition in mediating macrophage adhesion on fibronectin that was immobilized on to networks or TCPS, thus suggesting that various PKC isoforms may be involved. Therefore, our data indicate that a specific substratum-dependent adhesion signaling is present with fibronectin or $G_3RGDG_6PHSRNG$ modification.

In vitro FBGC assays demonstrated the following [53] (Table 5). The inhibition of Src, but not PTK, decreased FBGC formation on TCPS with pre-adsorbed fibronectin, which was similarly observed on TCPS with pre-adsorbed G_3RGDG or $G_3RGDG_6PHSRNG$. Conversely, the inhibition of Src increased FBGC formation on networks with pre-adsorbed or covalently grafted fibronectin, which was similarly observed on networks pre-adsorbed with G_3RGDG or grafted with $G_3PHSRNG_6RGDG$ or $G_3RGDG_6PHSRNG$. PKA inhibition increased FBGC formation on fibronectin or $G_3RGDG_6PHSRNG$ pre-adsorbed TCPS; whereas PKC inhibition decreased FBGC formation on TCPS or networks pre-adsorbed or grafted with fibronectin, which was similarly seen on TCPS pre-adsorbed with G_3RGDG or networks grafted with G_3RGDG, $G_3PHSRNG_6RGDG$ or $G_3RGDG_6PHSRNG$. The presence of PI3 kinase inhibitor decreased FBGC formation on networks pre-adsorbed with fibronectin, G_3RGDG, $G_3PHSRNG_6RGDG$ or $G_3RGDG_6PHSRNG$. However, the presence of MAPK kinase inhibitor conversely increased FBGC formation on networks grafted with fibronectin. Therefore, the interrelationship among substrates, fibronectin-derived ligands, and ligand immobilization methods modulates differential signaling cascades for FBGC formation. The specific orientation of the RGD and the PHSRN in a single peptide formulation mimicked fibronectin in modulating similar intracellular signaling events of FBGC formation. However, this relationship is highly dependent upon the substrate and the immobilization

Table 5
Summary of the effect of intracellular signaling inhibitor on adherent FBGC density

	PTK	Src	PKA	PKC	PI3 K	MAPK K
Pre-adsorbed on TCPS						
Fibronectin	↔	↓	↑	↓	↔	↔
G_3RGDG	↔	↓	↔	↓	↔	↔
$G_3PHSRNG_6RGDG$	↔	↔	↔	↑	↓	↔
$G_3RGDG_6PHSRNG$	↔	↓	↑	↔	↓	↔
Pre-adsorbed on networks						
Fibronectin	↔	↑	↔	↓	↓	↔
G_3RGDG	↔	↑	↔	↔	↓	↔
$G_3PHSRNG_6RGDG$	↔	↔	↔	↔	↓	↔
$G_3RGDG_6PHSRNG$	↔	↔	↔	↔	↓	↔
Grafted on networks						
Fibronectin	↔	↑	↔	↓	↔	↑
G_3RGDG	↔	↔	↔	↓	↓	↔
$G_3PHSRNG_6RGDG$	↔	↑	↔	↓	↓	↔
$G_3RGDG_6PHSRNG$	↔	↑	↔	↓	↔	↔

Each comparison indicates that the level of adherent FBGC density of cells treated with respective intracellular signaling molecule inhibitor remained unchanged (↔), increased (↑), or decreased (↓) when compared with the level of adherent FBGC density of cells treated with respective vehicle controls (data not shown) at 95% ($p<0.05$) confidence level.

method. Taking the results of the above investigations together, we demonstrated the role of selected kinases in integrin signaling leading to macrophage adhesion and FBGC formation mediated by fibronectin–integrin association. Furthermore, RGD and PHSRN appear to be significant in mediating this receptor–ligand association resulting in the necessary signaling characteristic for macrophage adhesion and the subsequent development. Specifically, Src-PI3K-PKC cascade showed a significant role in modulating FBGC formation mediated by fibronectin, most importantly, RGD and PHSRN domains of fibronectin. We showed the important role of RGD and PHSRN domains of fibronectin, and specifically the interpositional spacing between the motifs, in the complexation with integrin receptors to regulate selected kinases in mediating macrophage adhesion and FBGC formation. These findings represent a mechanistic correlation between the role of protein functional architectures in ligand–receptor recognition and the post-ligation signaling events that control cellular behavior. The fundamental understanding of these complex phenomena provides future researchers with the necessary tools in the development of unique biomimetic enabling technologies that are vital for the advancement of cellular engineering and tissue engineered devices for the development of novel drug delivery methods and vehicles.

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