

The Spontaneous Adhesion of BMMC onto Self-Assembled Peptide Nanoscaffold without Activation Inhibits Its IgE-Mediated Degranulation

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Mast cells play a distinct role in the innate immune response. Engineered microenvironments for the express purpose of influencing mast cell activity will provide a novel means of designing biomaterials, as well as a means to systematically investigate mast cell biology in a 3D setting. Here, the effect of nanoscaffolds composed of self-assembling peptides, namely (RADA)₄, on bone-marrow-derived murine mast cell (BMMC) activity is reported. Unlike most studies that stimulate mast cells to induce adhesion, this results show that BMMCs spontaneously adhere to the artificial nanoscaffold without initiating their activation. It is observed that the classical immunoglobulin (IgE) antigen-mediated degranulation of adhered BMMC is inhibited by the nanoscaffold, while non-IgE (A23187)-induced degranulation is unaffected. The inhibition of IgE-antigen-mediated degranulation is likely a result of inhibited molecular diffusion within the matrix; antigen diffusion, IgE-FcεRI complex shuttling, and/or formation of multiple IgE-FcεRI clusters may be physically hindered in the presence of the polyvalent nanofiber network. Moreover, the IgE/antigen-induced inflammatory cytokine tumor necrosis factor α release from adherent BMMCs is significantly reduced likely due to interaction with the nanofiber matrix. This work is considered the first step in quantifying mast cell activity in artificial matrices composed of self-assembling peptides.

cell surface induces the initiation of both a rapid degranulation response and the long-term expression of many inflammatory mediators.^[1] Mast cell degranulation is characterized by a rapid release of a myriad of proinflammatory mediators that can initiate an immune response toward a specific antigen. These inflammatory effector molecules include histamine, heparin, and serine proteases. The release of cytokines and chemokines is the long-term response mounted by activated mast cells; some of these expressed cytokines and growth factors, such as tumor necrosis factor alpha (TNF-α) or vascular endothelial growth factor, may also be found in granules and be a part of the rapid response of activated mast cells.^[3,4]

Generally, mast cell progenitors circulate in the blood and upon migration into tissues; they complete differentiation into mature mast cells.^[5] Both mast cell progenitors and mature mast cell express the stem cell factor (SCF) receptor c-kit, the survival factor for all mast cell subtypes. The tissue microenvironment strongly influences the mast cell differentiation

1. Introduction

Mast cells play an important role in wound healing, angiogenesis, and the inflammatory process.^[1,2] Antigen-induced crosslinking of the high-affinity IgE (FcεRI) receptors on the

such that mast cells are divided into at least two main subtypes, tryptase-positive (MC_T) and tryptase/chymase positive (MT_{TC}), and display distinct phenotypes. Each mast cell subtype has distinct interaction with its extracellular matrix (ECM) through the tightly regulated processes of cell adhesion,^[6] migration,^[6,7] proliferation,^[8] and cytokine production.^[9] Despite this requirement for an ECM during maturation and function, the majority of in vitro mast cell investigations are performed in a suspension culture, in the absence of a “scaffold” or adhesion matrix. Some studies of mast cell activation have used fibronectin (FN), laminin, vitronectin, collagen, or Matrigel (protein matrix extracted from a tumor-rich ECM) as a 2D coating upon which mast cells are allowed to adhere and show that adhesion to these proteins are important in eliciting mast cell activation.^[10] Moreover, these adhesion studies are done in the presence of stimulators: inflammatory cytokines and stem cell factors, thrombin, or even IgE alone.^[6,8,11–14] The adhesion of mast cells, without these stimulators, to natural or artificial ECM is rarely reported.

Self-assembled peptides are promising materials for regenerative medicine and tissue engineering applications.^[15,16]

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 The ORCID identification number(s) for the author(s) of this article can be found under <https://doi.org/10.1002/adhm.201700334>.

DOI: 10.1002/adhm.201700334

The self-assembling peptide, (RADA)₄, is composed of alternating hydrophobic and hydrophilic amino acids and has been shown to form a β -sheet nanofiber-based 3D hydrogel matrix in physiologically relevant solutions.^[17–22] Transmission electron microscopy (TEM) and microrheological analysis showed that 0.5% wt (RADA)₄ formed a more-rigid hydrogel compared to the same concentration of collagen I and Matrigel due to the self-assembling longer nanofiber and more crosslinks.^[17,23] These types of self-assembling peptides have been employed in various in vitro and in vivo studies, including 3D cell culture,^[16] wound healing,^[24] and tissue repair.^[25] Due to the fact that these self-assembling peptides can be programmed at the molecular level with biochemical information (i.e., bioactive peptides and lipids), they may be particularly suited for developing an ECM mimic for fundamental cell-biology studies. Thus, an understanding of how the (RADA)₄ matrix, itself, interacts with mast cells is the first step in developing an ECM mimic that may be able to modulate mast cell activity: inhibition, amplification, or regulation. Furthermore, this ECM mimic may also directly translate to clinical applications due to it being purely synthetic and not derived from animal sources. In particular, (RADA)₄ nanoscaffolds are of direct interest for several reasons. First, (RADA)₄ nanoscaffolds are already an important biomaterial for investigating cellular responses in a 3D microenvironment, where exquisite control over the presentation of bioactive moieties can be incorporated, and there are currently very limited number of 3D cell culture experimental platforms being used for mast cell studies. Due to the relative biocompatibility already shown for (RADA)₄ nanoscaffolds, it is also of interest to further understand the initial innate immune response to these materials, in general, so as to justify further development of these classes of materials for tissue engineering applications where it is well known that mast cells are widely distributed (i.e., connective tissues). In this study, we characterized the adhesion of bone-marrow-derived mast cells (BMMCs) on 0.5% (w/v), (RADA)₄ nanoscaffolds and their response to either IgE/antigen or A23187 (Ca²⁺ ionophore) stimulation. Degranulation and inflammatory mediator release from both adherent and suspended BMMCs were evaluated. It was observed that BMMCs can directly adhere to (RADA)₄ matrix with or without IgE sensitization. The IgE-mediated degranulation of adherent BMMCs was inhibited, compared to solution-free BMMCs, BMMCs in suspension with the nanoscaffold, and the non-IgE-mediated degranulation. Furthermore, quantifying inflammatory cytokines (TNF- α , IL-6, and IL-13) expressed upon IgE/antigen activation had to account for the fact that the matrix

itself may absorb these molecules. This work paves the way for further design of bioactive motif inclusion into the nanoscaffold for understanding the key parameters in directing mast cell activity, in both a 2D and a potentially 3D culture system.

2. Results

2.1. BMMCs Viability and Adhesion to (RADA)₄ Matrix

The effect of the (RADA)₄ matrix on mast cell viability was evaluated using a standard 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) viability assay. Both unsensitized and IgE-sensitized BMMCs (0.5×10^6 cells mL⁻¹) were incubated with phosphate buffered saline control (PBS) or 0.5% w/v (RADA)₄ matrix for 0.5, 18, 28, or 48 h (Figure 1a). The IgE sensitization significantly enhanced the cell survival of BMMCs and result in cell proliferation during incubation; however, there is no significant difference among the treatments with PBS and matrix. Results indicate that the (RADA)₄ matrix was not cytotoxic to BMMCs and had no statistically significant effect on BMMC proliferation after these time points. For the following experiments, we choose 18 h incubation for convenience.

The adherence of sensitized (+IgE) or unsensitized (–IgE) BMMC to the matrix was observed (Figure 1b). Approximately 30% of the total unsensitized cells adhered to the matrix, and this value did not significantly change from 0.5 to 18 h of incubation. Upon sensitization of the BMMCs, the total number of cells adhered to the matrix after 0.5 h was similar to unsensitized cells ($27.7 \pm 1.2\%$). However, after 18 h incubation, the amount of adhered cells significantly ($p < 0.05$) increased ($45.6 \pm 0.7\%$) compared to either the sensitized BMMC at 0.5 h or the unsensitized BMMC at 18 h.

BMMC adhesion to the (RADA)₄ nanoscaffold matrix and resultant morphology, with and without presensitization with IgE, was further characterized using light microscopy (Figure 2). Compared to almost no adhesion on a 96-well plate surface (polystyrene), a considerable amount of BMMCs adhered to the matrix after 18 h incubation. Moreover, from these results it seems that most of the adherent cells were located within the same plane (Figure 2d,f). However, there were still some fuzzy or smaller cell-like shapes outside of this focal plane, suggesting that these cells may have become embedded into the matrix. All adherent mast cells retained their original, spherical, morphology.

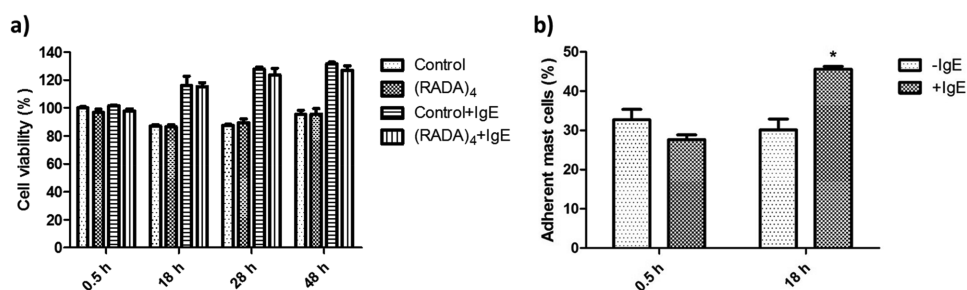


Figure 1. Effect of 0.5% w/v (RADA)₄ matrix on a) mast cell viability and b) adherence. Adherent cells were determined by counting the suspended cells (in supernatant and washed buffer) over total cells. Data represent mean $\pm$ 1 SEM, for $n \geq 3$ repeats.

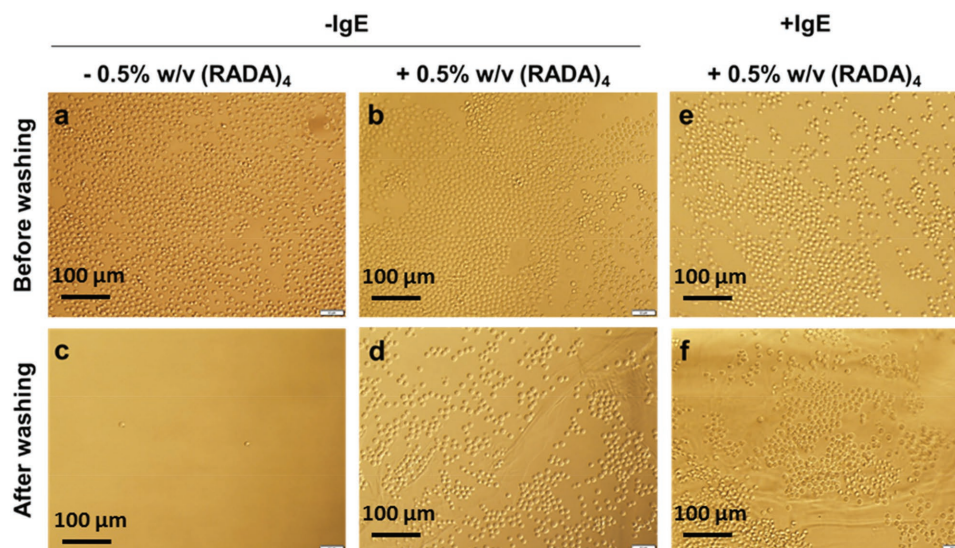


Figure 2. Light microscopy images of nonsensitized BMMCs at 20 $\times$ magnification. BMMCs in media (50 μ L, 1.0×10^6 cell mL^{-1}) were cultured with 50 μ L PBS in a,c) a 96-well plate (polystyrene) or b,d) 0.5% w/v (RADA) $_4$ for 18 h, the supernatant was removed and the matrix was rinsed prior to image capture. Presensitized BMMCs (50 μ L, 1.0×10^6 cell mL^{-1}) were cultured with e,f) 50 μ L of 0.5% w/v (RADA) $_4$ for 18 h; the supernatant was removed and the matrix was rinsed prior to image capture.

The adhesion process of live cell to (RADA) $_4$ matrix was observed by time lapse microscopy. The attached and non-attached (or weakly bound) BMMCs can be easily divided. The nonattached BMMCs are observed as dark ring objects

moving rapidly and randomly throughout the imaging area (Brownian motion), which finally gathered on the matrix surface during 24 h incubation (**Figure 3**, black arrowed cells). When a BMMC attaches to the (RADA) $_4$ matrix, it progressively

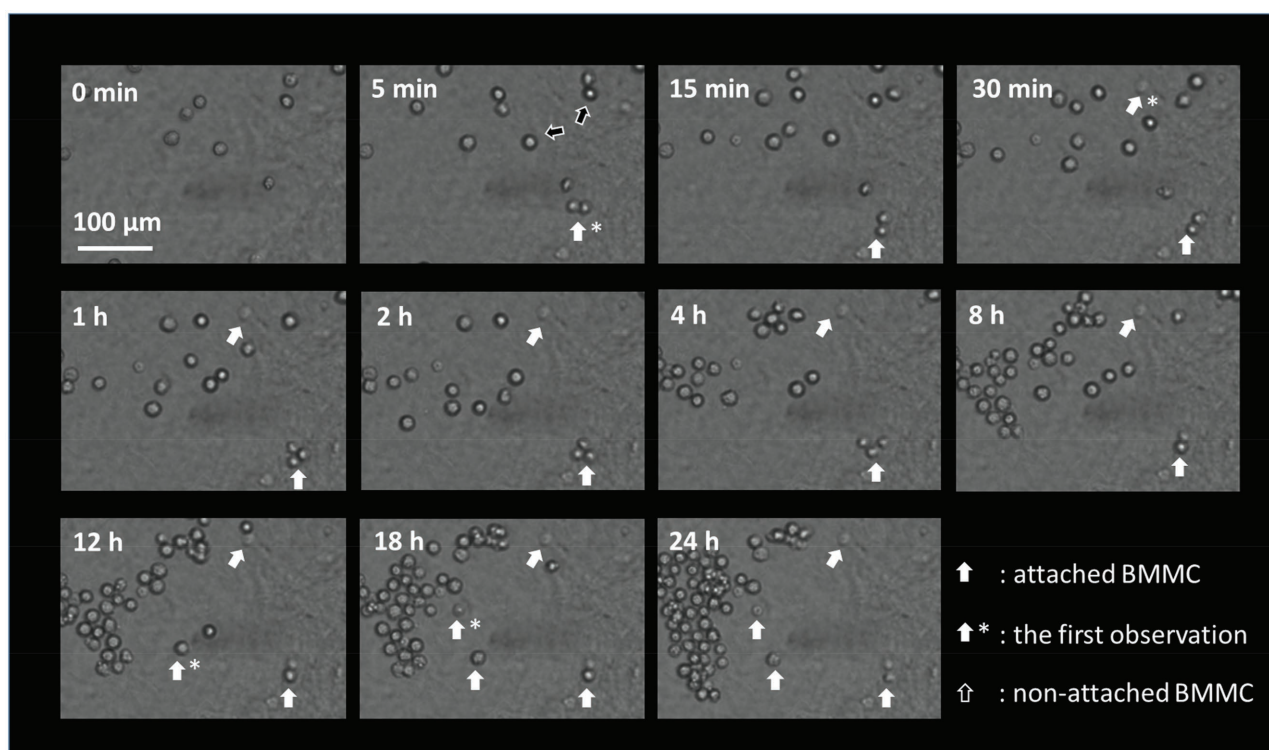


Figure 3. The BMMC adhesion process observed by time lapse microscopy. Nonattached BMMCs in suspension (two examples are marked by the black arrows in “5 min”) are observed as dark patterns on the matrix surface which move fast and randomly during incubation. The attached BMMCs that likely have started their adhesion process (examples are marked by the white arrows, and the position of the first time been observed are marked as “*”) appear to have a less sharpness as they immersing into the matrix, and followed with a random but limited movement.

loses its large scale Brownian motion as immersing in to the matrix (Figure 3, white arrowed cells). The position shifting of attached or adhered BMMCs still exists but only in a limited space ($<50\ \mu\text{m}$) as a result of molecular interactions and/or nanofiber network restraint between the cell and the matrix. During incubation, the attached cells gradually display as light colored pattern with less contrast. This phenomenon may due to at least two reasons: (1) the change in distance to the image sensor (the focus is at the surface of matrix);^[26] (2) the refractive index of the peptide-based nanoscaffold matrix is more close to the cell itself than culture media. The result indicates that the attached cells tend to immerge into the nanoscaffold matrix (see Movie S1 provided in the Supporting Information).

2.2. Effect of Nanoscaffold Matrix on Presensitized Mast Cell Degranulation

The effect of the (RADA)₄ nanoscaffold matrix on mast cell activation was further evaluated (Figure 4). After 18 h incubation, the BMMCs that remained in suspension were carefully separated from those that had adhered to the matrix. Control BMMCs were obtained from those cultured in PBS. IgE- and non-IgE-mediated β -hexosaminidase (β -hex) release from these cell populations was evaluated, where the release of β -hex from granules is considered a rapid response mechanism which is indicative of mast cell activation. Control systems used the same cells without the presence of the matrix, where the positive control included 2,4-dinitrophenol (DNP) (DNP+, Figure 4a) or A23187 (A23187+, Figure 4b) and the negative controls lack these compounds. For IgE-sensitized BMMCs, degranulation was induced through the crosslinking of high-affinity IgE (Fc ϵ RI) receptors on the mast cell surface by DNP-bovine serum albumin (BSA). IgE/DNP activated the release of β -hex from both the positive control (DNP+) and the suspended cells to a similar degree: $33.4 \pm 1.0\%$ and $31.4 \pm 2.5\%$ of the total β -hex in cell lysate, respectively. Alternatively, these cells that adhered to the matrix showed a significantly lower β -hex release ($p < 0.001$) of only $12.6 \pm 1.1\%$ (Figure 4a). Non-IgE-related degranulation was investigated using compound A23187, a common Ca²⁺ ionophore that increases intracellular Ca²⁺ concentration and induces mast cell activation, circumventing the IgE/Fc ϵ RI signaling pathway (Figure 4b).^[24] A23187 activated statistically similar levels of β -hex release for all systems studied, which was a statistically significant increase relative to the negative control ($p < 0.001$). Therefore, nanoscaffold matrix inhibition of BMMC activation is specific to Fc ϵ RI-mediated activation.

Due to the fact that the adherent cell population exhibited an inhibited IgE-mediated activation, coupled to the fact that the images suggest that perhaps the cells are intimately interacting with the matrix to the point of potentially being embedded within, the effect of increasing peptide density on mast cell activation was examined (Figure 4c,d). To determine the effect of the nanofiber density on DNP-BSA/IgE-dependent degranulation, (RADA)₄ peptide solutions (12.5 , 25 , 50 , 100 , 200 , 400 , $800 \times 10^{-6}\ \text{M}$) in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer ($10 \times 10^{-3}\ \text{M}$, with 0.4% BSA, pH 7.4) were used to resuspend BMMCs before DNP-BSA or compound

A23187 stimulation (the (RADA)₄ peptide solutions have no significant effect on BMMC viability; see Figure S1 (Supporting Information)). For BMMCs that were activated with IgE/DNP-BSA, there was a significant decrease ($p < 0.001$) from the control ($29.4 \pm 1.0\%$) in β -hex release as the concentration of (RADA)₄ peptide increased beyond $50 \times 10^{-6}\ \text{M}$ ($19.0 \pm 1.0\%$) (Figure 4c). As the peptide concentration increased to $800 \times 10^{-6}\ \text{M}$, the β -hex released ($4.9 \pm 0.2\%$) was similar to the negative control (DNP-, $4.8 \pm 0.1\%$). When BMMCs were stimulated with A23187, a significant decrease in activation was still observed. As peptide concentration increased over $100 \times 10^{-6}\ \text{M}$, β -hex release significantly ($p < 0.05$) decreased to $32.8 \pm 0.8\%$ compared to the control of $36.7 \pm 1.2\%$. The ultimate decrease in β -hex release at $800 \times 10^{-6}\ \text{M}$, for A23187 stimulation was not as great as that observed for the corresponding DNP system.

Moreover, baseline BMMC degranulation for presensitized cells in peptide solutions without any stimuli was determined as a negative control (Figure 4e). The result showed that β -hex release was constant in all peptide solutions, suggesting that the peptide did not contribute to the degranulation progress nor did it sequester β -hex and result in a false decrease in β -hex release in the assays. To determine whether the peptide influenced the β -hex found in the granules, BMMC cell lysis (same number of cells by sonication) was incubated with the indicated concentration of peptide, and the cells were lysed to represent artificial degranulation (Figure 4f). The results showed a very constant β -hex release, indicating that (RADA)₄ peptides do not affect β -hex release or compartmentalization into granules.

2.3. The Morphology of Adherent Mast Cell in Nanoscaffold Matrix

In order to imaging the morphology of both unsensitized and IgE-sensitized mast cells that attached to (RADA)₄ nanoscaffold matrix, the cell F-actin and nuclei were stained by phalloidin conjugated fluorescein isothiocyanate (FITC) and 4',6-diamidino-2-phenylindole (DAPI), respectively, and z-plane slices of each sample were captured using a laser scanning confocal microscope. Reconstructed images (Figure 5a,b) illustrate the representative cell distribution throughout the matrix. Similar to the light microscopy results, mast cells were found to be distributed over the matrix (Figure 5a,b, up). From the horizontal view (Figure 5a,b, middle), it was apparent that the majority of cells were located within a $60\text{--}100\ \mu\text{m}$ layer. This is a significant depth, as mast cells size are $\approx 8\text{--}10\ \mu\text{m}$ in diameter^[23] (also shown in Figure 5e,f), meaning that adherent cells are likely found within the matrix, as opposed to that in a 2D plane on the matrix surface. The 2D images show that IgE-sensitized BMMCs with a matrix generally keep the similar round-shape-like unsensitized BMMCs (Figure 5e), but the IgE sensitization leads to enhancement of the cortical F-actin ring (Figure 5f). Upon A23187-induced activation, a significant disassembly of the F-actin ring structure and reduction in total F-actin of nonsensitized BMMCs can be observed (Figure 5g), which is a sign of mast cell degranulation.^[27,28] Meanwhile, the Fc ϵ RI crosslinking induced by DNP-BSA seems to affect the structure and shape of IgE-sensitized

BMMCs in matrix; however, the enhanced F-actin ring still exists (Figure 5h).

2.4. Artifact in Characterizing TNF- α Release Observed

In addition to the rapid release of granule contents, the sustained activation of mast cells can result in the expression of

a host of proteins, including inflammatory cytokines. BMMCs were cultured for 18 h and then induced with DNP-BSA for 24 h to assess the effect of the nanoscaffolds on BMMC expression of TNF- α , IL-13, and IL-6 (Figure 6). The TNF- α release from matrix adherent BMMCs decreased significantly ($p < 0.001$; Figure 6a) compared to controls. However, there was no difference in the production of IL-13 and IL-6 between the (RADA) $_4$ -exposed and -unexposed BMMC (Figure 6b,c).

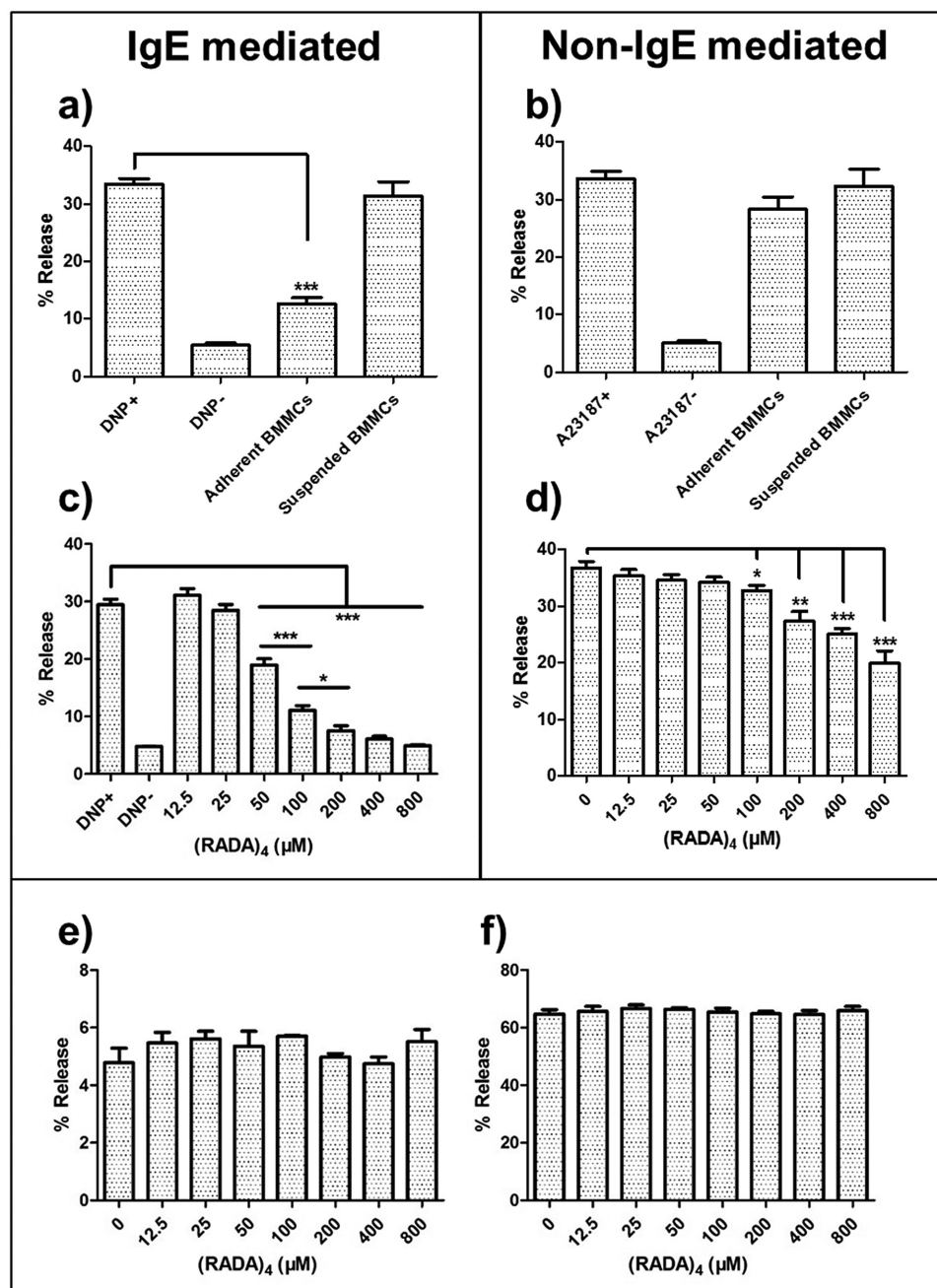


Figure 4. The effect of (RADA) $_4$ peptide on BMMC degranulation. a) β -hex release for adherent and suspended IgE-sensitized BMMC after activating with DNP-BSA. b) β -hex release for adherent and suspended BMMC after activating with compound A23187; control is BMMC with and without A23187 treatment. c) IgE-sensitized BMMC β -hex release with (RADA) $_4$ peptide after DNP-BSA activation. d) β -hex release of BMMC with (RADA) $_4$ peptide after A23187 activation. e) β -hex release of BMMC with (RADA) $_4$ peptide without DNP-BSA. f) β -hex release test of BMMC cell lysis (same number of cells by sonication) with indicated concentration of (RADA) $_4$ peptide without activation. Data represent mean $\pm$ 1 SEM, for $n \geq 6$ repeats.

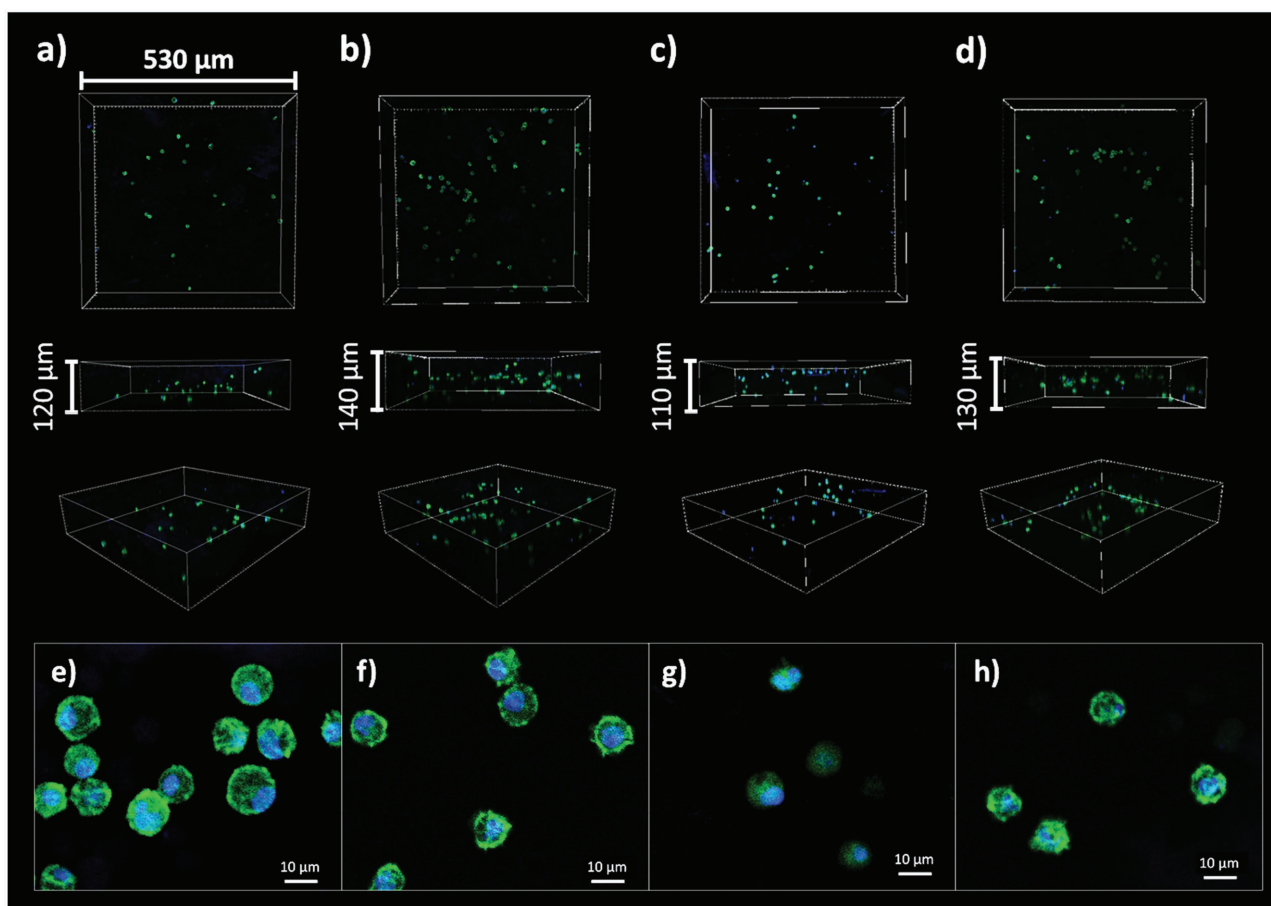


Figure 5. Laser confocal scanning microscopy images of adherent BMMCs in 0.5% w/v (RADA)₄ matrix, F-actin (green), and nuclei (blue). a,e) Unsensitized BMMCs; b,f) IgE-sensitized BMMCs; c,g) unsensitized BMMCs + A23187; d,h) IgE-sensitized BMMCs + DNP-BSA. (a–d) Vertical view (up), horizontal view (middle), and 3D distribution (down). (e–h) 2D images of BMMCs. The z-dimension scale was determined by the first and the last visible cell.

Since (RADA)₄ seemingly decreased TNF- α but had no effect on the production of the other proteins studied, it is suggested that perhaps the matrix was interacting with TNF- α . Thus, the absorption of these cytokines from solution to the (RADA)₄ matrix was evaluated so as to understand if the

peptide matrix itself sequestered these proteins from solution, resulting in a false enzyme-linked immunosorbent assay (ELISA) result. It was found that all cytokines significantly absorbed to the nanofiber matrix, in a peptide-concentration-dependent manner (Figure 7a–c), within an period of 18 h.

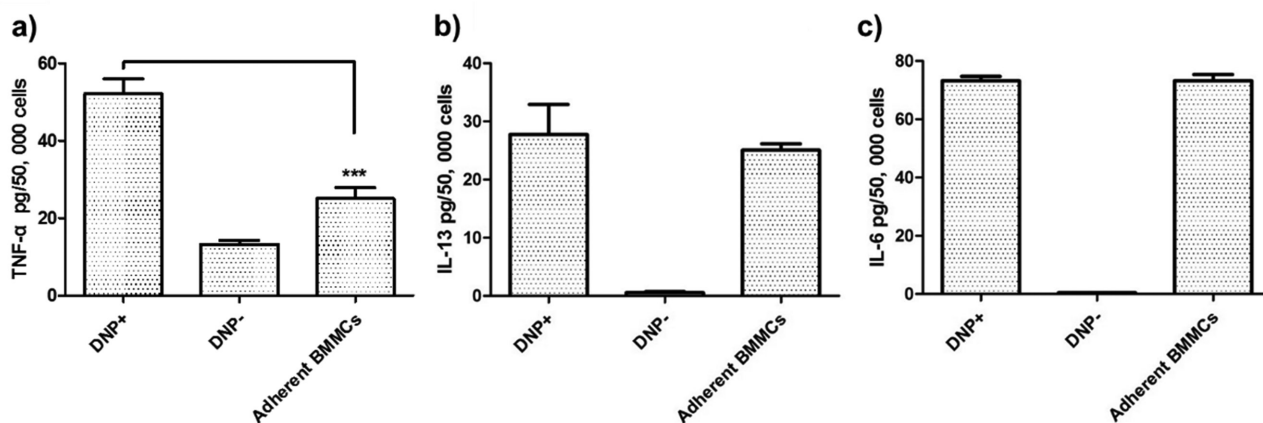


Figure 6. (RADA)₄ matrix effect on TNF- α , IL-13, and IL-6 production. a) TNF- α , b) IL-13, and c) IL-6 production/secretion was measured using ELISA. Data represent mean $\pm$ 1 SEM, for $n \geq 5$ repeats.

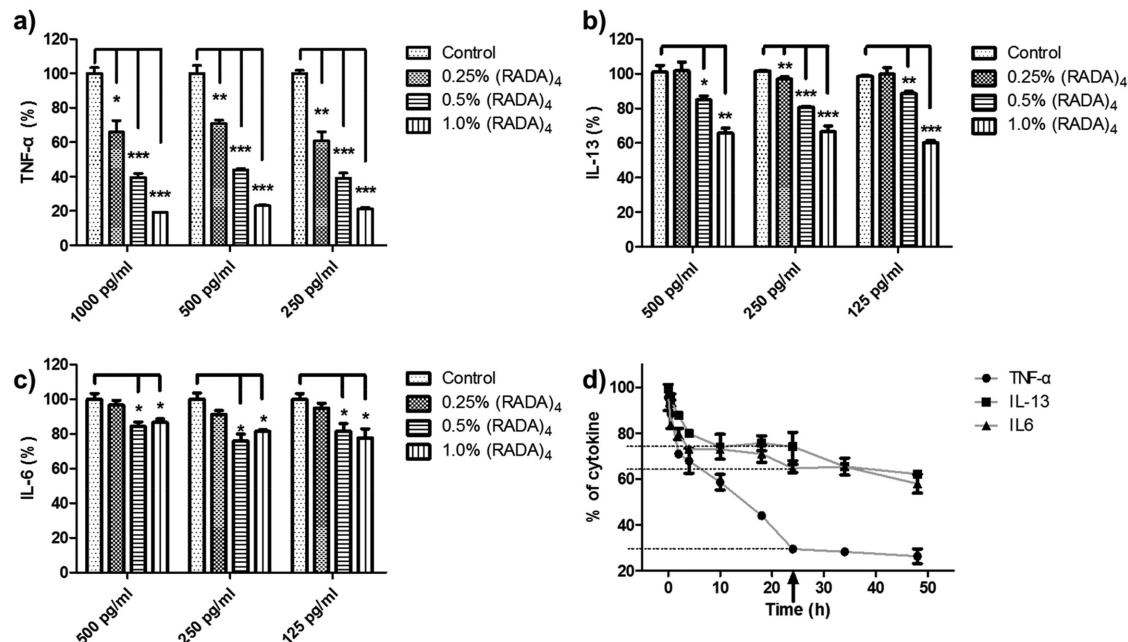


Figure 7. The absorption of inflammatory cytokines into (RADA)₄ matrix. Different initial concentrations of a) TNF-α (1000, 500, 250 pg mL⁻¹), b) IL-13 (500, 250, 125 pg mL⁻¹), and c) IL-6 (500, 250, 125 pg mL⁻¹) in media were incubated with different concentrations of (RADA)₄ hydrogel (0.25%, 0.5%, and 1.0% w/v; PBS was used as control) for 18 h. After 18 h, the concentration of cytokines in hydrogel-free supernatants was measured by using ELISA. d) The cytokines absorption in 0.5% w/v (RADA)₄ hydrogel with time. Initial concentrations are TNF-α: 500 pg mL⁻¹; IL-13, and IL-6: 250 pg mL⁻¹. The arrow in part (d) points to 24 h. Data represent mean ± 1 SEM, for *n* ≥ 3 repeats.

Also, TNF-α had a much stronger interaction with (RADA)₄ matrix compared to either IL-13 or IL-6, where ≈50% of TNF-α in the media was absorbed to 0.5% w/v (RADA)₄ matrix. The absorption kinetics for these cytokines were also characterized (Figure 7d), showing that TNF-α, IL-13, and IL-6 reached equilibrium after 24, 10, and 4 h, respectively. After a 24 h incubation, the absorbed amounts of TNF-α, IL-13, and IL-6 were ≈71%, 26%, and 35%, respectively.

3. Discussion

Immature mast cells are recruited through the circulation and become mature in connective tissues, particularly the connective tissues that are close to external environments (i.e., skin, lung, and intestine).^[29] The local microenvironments in which these cells reside can directly affect their properties and function.^[30] The adhesion of mast cell progenitors to ECM proteins in connective tissue, such as FN, is thought to play a critical role in mast cell migration, distribution, proliferation, differentiation, activation, and secretion.^[31] In this work, to our knowledge for the first time, it was shown that mast cells could both adhere to and integrate with a purely synthetic peptide nanoscaffold, without the requirement for any prestimulation.

The BMSCs remained viable within the (RADA)₄ nanoscaffold matrix, although the IgE-sensitization significantly enhanced the BMSC's survival and promoted cell proliferation in 48 h;^[32] however, the nanoscaffold itself has no significant effect on cell viability, which means that cell proliferation was neither inhibited nor promoted. Namely, the total cell number in each treatment group was constant with or without

the (RADA)₄ nanoscaffold matrix. Although it was found that ≈30% of the total cultured BMSCs strongly interacted with the (RADA)₄ nanoscaffold matrix without sensitization, 45.6 ± 0.7% of IgE-sensitized cells interacted with the matrix directly. In neither case were these cells found to have altered morphology or activated in a significant way. As previously reported, various stimuli including thrombin, phorbol myristate acetate, ionophore, or antigen/IgE stimulation have been reported to induce mast cell adhesion to FN or laminin after mast cell activation.^[14,33] Additionally, SCF^[12] or IgE alone^[13] can also induce mast cell adhesion to ECM proteins without activation. However, the adhesion of unsensitized BMSCs to ECM coating is usually negligible, being less than ≈10%.^[14,33] Mast cell adhesion to natural ECM proteins is primarily attributed to the expression of integrins, which couple to the ECM through established peptide motifs (RGD or IKVAV).^[34] Although these domains are not conserved on the (RADA)₄ peptide, the RAD peptide may facilitate integrin binding; the difference between RAD and RGD for other cell-binding assays has shown that both sequences yielded statistically similar binding.^[35] Not only this could explain the direct, rapid attachment of mast cells without IgE sensitization, but it could also explain why IgE sensitization enhanced this attachment: IgE alone can stimulate expression of integrins yielding increased adhesion of BMSCs to FN.^[13] Moreover, we have done a simple adhesion test with on 96 wells coated with (RADA)₄ peptide with different concentration. Interestingly, the adhesion follows a concentration-dependent manner, and only the wells incubated with 0.1% wt (RADA)₄ solution (≈0.6 × 10⁻³ M) have significant (*p* < 0.001; Figure S2, Supporting Information) adhesion effect (≈25–30%). It has been reported that the critical aggregation concentration

of (RADA)₄ peptide is about $(0.144 \pm 0.003) \times 10^{-3} \text{ M}$,^[22] which means the solution with lower concentration (0.01% and 0.001%) could not only contain less peptide but also lack of nanofiber structure. The result implies that the β -sheet secondary structure and nanofiber structure play a crucial role in BMNC adhesion.

In addition to cell attachment to the matrix, all of the results seem to suggest that these adherent cells are gradually integrated into the matrix. It is likely that BMNCs were not only attached to the matrix, but probably also embedded within the matrix. To prove this hypothesis, the time lapse microscopy has been used to measure dynamic adhesion process of live BMNCs. It is easy to distinguish attached BMNC from non-attached (or weakly bound) BMNC (Figure 3) by position and contrast. The interaction between cell and nanofiber network results in a limited movement, while the nonattached cells move rapidly on the matrix surface. During incubation, the attached cells have a tendency to immerge into the matrix. The 3D distribution of adherent BMNCs was also shown in confocal microscopy images (Figure 5a,b); the cells are located near the surface of matrix with a variation of the depth in a 60–100 μm layer, which corresponds to the time lapse microscopy observation.

The IgE-mediated activation was triggered by the interaction between antigen (DNP-BSA) and IgE molecules already bound to Fc ϵ RI. Antigen binding by multiple IgE-Fc ϵ RI complexes leads to their crosslinking, which initiates degranulation and subsequent signaling events. Both degranulation tests and confocal images show the matrix effect on IgE-mediated, but not non-IgE mediated (A23187), activation of adherent BMNCs. It has been reported that Fc ϵ RI-induced activation of BMNC leads to the cytoskeletal rearrangements, and the F-actin disassembly was dependent on intracellular Ca^{2+} .^[28] The Ca^{2+} ionophore A23187, which can directly increase the intercellular Ca^{2+} of cells, and apparently the matrix have almost no effect on A23187 but strongly affect DNP-BSA/IgE-related molecular interactions.

The observed inhibition of IgE-mediated degranulation is likely attributed to the particular structure of the (RADA)₄ matrix. It is relatively well established that (RADA)₄ self-assembles into a β -sheet-rich solution of nanofibers that are $\approx 10 \text{ nm}$ in diameter (Figure S3a,b, Supporting Information), with a pore size ranging between 5 and 200 nm (reported by literatures^[16,36]). This nanoscale morphology inherently forms a natural network that may impose a steric barrier to molecular diffusion^[19,20] and a large surface area that may affect protein-nanofiber interactions^[21] For example, protein diffusion through the nanofiber matrix can be directly affected by the nanofiber density and the hydrodynamic radius of the moving molecules.^[20] As described, the IgE-mediated activation involves the motion of multiple molecules and molecular complexes, all of which may be hindered by the presence of nanofibers at or near the cell membrane. This nanofiber matrix may then play an important role in inhibiting antigen (DNP-BSA) diffusion, IgE-Fc ϵ RI complex shuttling and crosslinking. As has been calculated previously, different proteins from small to large, including lysozyme, trypsin inhibitor, BSA, and IgG, have significantly different diffusion constants in (RADA)₄ matrix.^[20] Therefore, the relationship between the diffusion

constant and hydrodynamic radius (r_h) of each molecule can be concluded (Figure S4, Supporting Information). From this tendency, we can roughly estimate the diffusion constants of compound A23187, DNP-BSA, and IgE. More importantly, the crosslinking of multiple IgE-Fc ϵ RI complexes that anchored on the cell membrane by antigen (DNP-BSA) will form much larger receptor clusters and finally aggregate into micrometer-sized receptor patches of $\approx 206 \text{ nm}$ diameter.^[37] As has been described, the average pore size of the (RADA)₄ matrix is around 5–200 nm, and the receptor cluster could be easily trapped by the matrix network and hinder this rearrangement of clusters on the cell surface; this effect may be more significant than merely inhibiting the diffusion of DNP-BSA to the IgE-Fc ϵ RI receptors. Those nanoscale molecular movements, including antigen diffusion and IgE-Fc ϵ RI cluster aggregation during incubation, are essential processes for allergic reaction, which may be restricted by nanoscale nanofiber networks. This hypothesis also explains why the matrix network had a minimal effect on activation using A23187, and the strong inhibition of the IgE-mediate activation of adherent, but not suspended BMNCs by DNP-BSA and IgE (Figure 8). Additionally, as can be seen from Figure 4c,d, this inhibitory effect was further confirmed as it was shown to be dependent on the concentration of (RADA)₄, or namely the nanofiber density.

Cytokine expression by activated mast cells plays an important role in inflammation. Despite some issues quantifying the amounts of expressed cytokines, it was observed that the nanoscaffold used throughout this study did not alter the amount of cytokines expressed compared to the controls. This may be because cytokine production is a result of gene expression, which shares only the membrane-proximal portion of the degranulation signaling pathways.^[38] Moreover, the required degree of IgE receptor crosslinking for degranulation and cytokine express has been reported to be different. It has been proved that the IgE-dependent degranulation progress requires a higher concentration of antigen than IgE-dependent cytokine secretion.^[4,39] Also, incubation time for cytokine express is 24 h; however, the degradation test is 30 min. The different incubation time represents short- and long-term stimulation. That said, the nanoscaffold matrix did not really affect the activation of cytokine expression; however, the adsorption of these proteins to the matrix led to a false-negative response in the production of TNF- α . This preferred interaction between the nanoscaffold and TNF- α is not without its merits, however. Alternatively, the TNF- α present during a mast cell response is promising for regulating mast-cell-mediated inflammation. For example, hydrogels functionalized with TNF- α antibody-derived peptides can be used to protect encapsulated cells from host immune cells.^[40]

The TNF- α inhibition effect was shown to be directly related to its absorption by the peptide matrix. In fact, the amount of absorbed TNF- α was over 20 $\times$ greater than that detected in solution. For example, there was a 3–4 times difference in TNF- α absorption between 0.25% and 1.0% w/v (RADA)₄ matrix. This may due to the higher density of nanofibers in the matrix, providing a greater surface area for cytokine-nanofiber interactions. The absorption of cytokines is also a slow process; the absorption rate increased during incubation; and this may be due to molecular diffusion. Other than TNF- α , IL-13 also

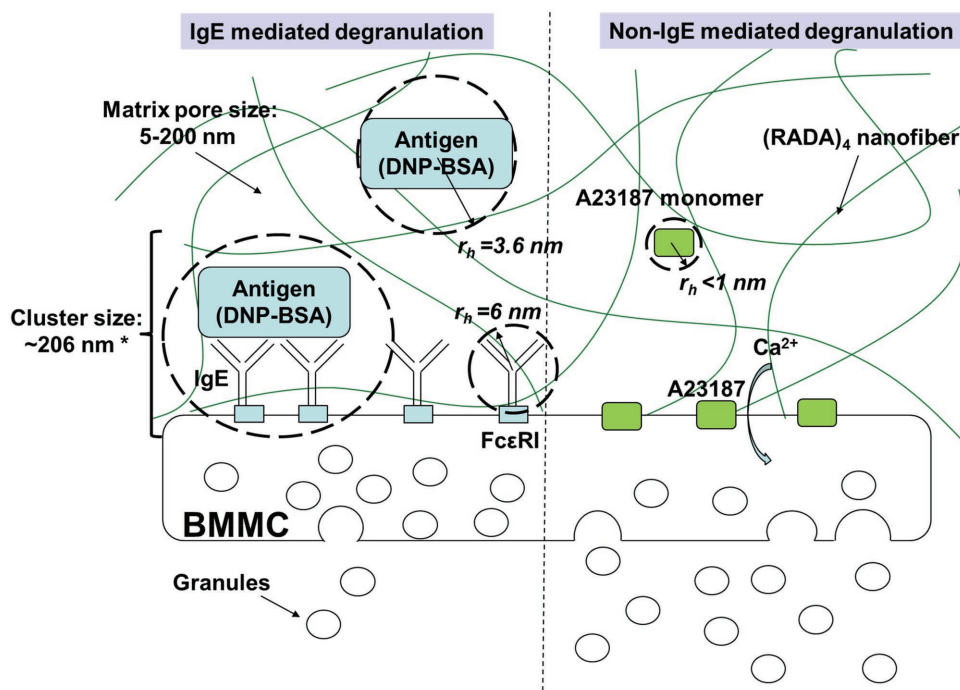


Figure 8. Schematic representation of both IgE-mediated (left) and non-IgE-mediated (right) mast cell degranulation in (RADA)₄ matrix. The dimension of each molecule or nanostructure is described as shown. The IgE-mediated degranulation is inhibited by the nanofibers; however, non-IgE-mediated degranulation is not inhibited. *: The average size of IgE–FcεRI cluster was described in literature.^[37]

demonstrated a smaller absorption effect. However, the mechanism of the cytokine absorption is not clear. As can be seen in Table 1, these molecules have a similar molecular dimension at ≈ 2 nm in r_h , much less than larger proteins such as BSA, IgG, and IgE. The diffusion differences due to molecular size cannot explain such a difference in absorption. Thus, we suspect that the absorption may be due to direct interactions between the proteins and the matrix.

4. Conclusion

BMMCs were shown to adhere to (RADA)₄ nanoscaffolds in significant amounts, without any presensitization or cellular

activation. The matrix itself inhibited IgE-mediated, but not non-IgE-mediated, degranulation for conditions used during cell culture. The inhibitory effect of matrix may be explained by the nanostructure of β -sheet nanofibers that hinder the diffusion and/or shuttling of IgE-mediated activation-related critical macromolecules such as antigen and IgE–FcεRI, which prevented the crosslinking progress of the IgE–FcεRI complex on the cell surface. The expression of inflammatory cytokines IL-13 and IL-6 was not significantly affected by the matrix. However, TNF- α released from adherent mast cells seemed to be affected as it adsorbed strongly to the nanofiber matrix. This study showed that (RADA)₄ matrix could be amenable as a matrix for mast cell research, such as 3D culture, but may itself be a platform where some peptide engineering could be employed to regulate mast-cell-mediated inflammation.

Table 1. The chemical and physical properties of proteins and compounds.

Protein or compound	Mol mass [kDa]	r_h [nm]
IgE	≈ 190	6.0 ^[41]
DNP–BSA	69	3.6 (BSA) ^[42]
A23187	0.52362	1.0 (dimer) ^[43]
TNF- α	17 (monomer); 51 (trimer)	1.9 (monomer); 2.3 (trimer) ^[44]
IL-13	11.5	$\approx 1.6^a$
IL-6	21.7	$\approx 2.0^{[45]}$

^a) The size was estimated from empirical results of several model proteins and enzymes based on amino acid residue number by online tool: http://www.calctool.org/CALC/prof/bio/protein_size.

5. Experimental Section

Materials: The (RADA)₄ peptide (purity > 95%, high-performance liquid chromatography–HPLC; about 10–15% wt of the total peptide is Trifluoroacetic acid - TFA salt) was purchased from RS Synthesis (Louisville, KY, USA), checked for purity using mass spectroscopy, and used without further purification. Unless stated explicitly, all reagents purchased were used as received without further purification. Nucleus fluorescent stain DAPI, anti-DNP IgE, DNP–BSA, and compound A23187 were purchased from Sigma (USA).

Endotoxin levels of all reagents were tested using ToxinSensor Chromogenic LAL Endotoxin Assay Kit from GenScript (Piscataway, NJ, USA). (RADA)₄ peptide contained <0.1 EU mg^{−1} endotoxin (e.g., 50 μ L of 0.5% w/v nanoscaffold contained <0.05 EU endotoxin), and the endotoxin levels of anti-DNP IgE, DNP–BSA, and compound A23187

at working concentration were less than 0.01 EU mL^{-1} (sensitivity: 0.005 EU mL^{-1} , $R^2 = 0.9952$).

Nanoscaffold Preparation: (RADA)₄ stock solutions (0.5%, 1.0%, and 2.0% w/v) were prepared by dissolving peptide powder in syringe filtered (0.2 μm) Milli-Q water. Peptide stock solutions were sonicated for 30 min to avoid bulk aggregates and reduce viscosity. Peptide solution was mixed with $2\times$ PBS (pH 7.4) at a ratio of 1:1 (v/v) to get nanoscaffolds with peptide concentrations of 0.25%, 0.5%, and 1.0% (w/v), respectively. About 50 μL of the mixture was placed at the bottom of a 96-well plate, overnight at 4°C and washed with $1\times$ PBS (pH 7.4) buffer three times.

BMMC Culture: Male BALB/c mice (6–8 weeks of age, 20–25 g) were obtained from Charles River Laboratories, and were given food and water ad libitum. All protocols for animal use were approved by the University of Alberta Health Sciences Laboratory Animal Ethics Committee. Approval was taken to use bone marrow from mice at the facility.

BMMCs, derived from mouse femoral bone marrow, were incubated in RPMI 1640 media supplemented with L-glutamine ($4 \times 10^{-3} \text{ M}$), sodium pyruvate ($1 \times 10^{-3} \text{ M}$), penicillin (100 U mL^{-1}), streptomycin ($100 \mu\text{g mL}^{-1}$), β -mercaptoethanol ($50 \times 10^{-6} \text{ M}$), nonessential amino acids ($0.1 \times 10^{-3} \text{ M}$), fetal bovine serum (10%), HEPES ($25 \times 10^{-3} \text{ M}$, Thermo Fisher Scientific, Burlington, ON, Canada), and mouse recombinant IL-3 (10 or 30 ng mL^{-1} ; Peprotech, Inc., Rocky Hill, NJ, USA). BMMCs were collected via centrifugation ($200 \times g$, 5 min at room temperature), and the fresh media were replaced every 3 to 5 days. Mast cell phenotype was confirmed by the expression of c-Kit and Fc ϵ R1 α and analyzed by flow cytometry using a PE-conjugated anti-mouse CD117 (c-Kit) (Biolegend, San Diego, CA, USA), and a phycoerythrin (PE) conjugated antimouse Fc ϵ R1 α antibody (eBiosciences, Santa Clara, CA, USA); see Figure S5 in the Supporting Information. Flow cytometry and toluidine blue staining were shown to be 99% positive mast cells by week 4 of culture.

Cell Viability Analysis Using XTT Assay: BMMCs were suspended in media ($50 \mu\text{L}$, $1.0 \times 10^6 \text{ cells mL}^{-1}$) and placed on top of $50 \mu\text{L}$ of nanoscaffold material. After incubation, cell viability of each sample was measured using a XTT proliferation kit (Roche Molecular Biochemicals, Indianapolis, IN, USA), according to the manufacturer's supplied instructions with one exception; to facilitate a uniform distribution of color throughout the nanoscaffold, dimethyl sulfoxide (DMSO, $50 \mu\text{L}$) was added into each well prior to analysis.

To determine the effect of (RADA)₄ peptide solutions on BMMC viability, the cells were incubated with a series of concentration of (RADA)₄ peptide in culture media, then cell viability was measured as shown in Figure S1 (Supporting Information).

Light Microscopy: After 18 h incubation, with or without nanoscaffold, nonadherent BMMCs were collected and counted, and washed with PBS for three times. BMMCs remaining in the plate well, with or without nanoscaffold, were observed under $200\times$ using a light microscope (IX-81 motorized inverted research microscope, Olympus, Japan).

Time Lapse Microscopy of Live Cell Adhesion: BMMCs were suspended in media ($50 \mu\text{L}$, $1.0 \times 10^6 \text{ cells mL}^{-1}$) and placed on top of $50 \mu\text{L}$ of nanoscaffold material in a glass bottomed 96-well plate. The plate was placed in an incubator (Omega temperature control device, Washington, PA) with 5% v/v CO₂ supply and humidity control on an Zeiss microscope (AxioImagerZ, AxioPlan IIM or Axiovert 200M, Germany) with differential interference contrast optics, mounted on a vibration-free base. Images were captured with a cooled charge-coupled device camera (CCD, Sensicam from Optikon, Cascade II EMCCD or CoolSnapHQ (Photometrics)).

Evaluation of Degranulation Using the β -Hex Release Assay: BMMCs were sensitized with $0.5 \mu\text{g mL}^{-1}$ mouse anti-DNP IgE (Sigma-Aldrich) in media for 24 h. For each well, 0.5×10^5 cells were washed and resuspended in HEPES buffer ($90 \mu\text{L}$, $10 \times 10^{-3} \text{ M}$, with 0.4% BSA, pH 7.4), and activated by adding DNP-BSA ($10 \mu\text{L}$; Sigma-Aldrich, final concentration of $0.1 \mu\text{g mL}^{-1}$) in HEPES buffer ($10 \times 10^{-3} \text{ M}$, with 0.4% BSA, pH 7.4) for 30 min at 37°C . β -hex release was quantified through analysis of the hydrolysis of p-nitrophenyl-N-acetyl-b-D-glucosamide (Sigma-Aldrich, Oakville, ON, Canada) in sodium citrate buffer (0.1 M ,

pH 4.5) for both the supernatant and in total cell lysates solubilized with 0.01% Triton X-100 for 90 min at 37°C . The reaction was stopped by adding glycine buffer (pH 10.7). BMMCs were also activated using A23187 ($1 \times 10^{-6} \text{ M}$) for 30 min, and β -hex release was measured as described above.

In some case, BMMCs were pretreated with low concentration (RADA)₄ solutions (12.5 , 25 , 50 , 100 , 200 , 400 , $800 \times 10^{-6} \text{ M}$ in HEPES + BSA buffer, pH 7.4) to evaluate the effect of (RADA)₄ on the BMMCs without or with minimal nanofiber formation for 30 min before activation. To evaluate the effect of the (RADA)₄ matrix on IgE-mediated degranulation, adherent BMMCs incubated with $50 \mu\text{L}$ of matrix were carefully washed with HEPES buffer (with 0.4% BSA, pH 7.4) three times, and the nonadherent cells collected, activated, and the β -hex release was measured as described above. Adherent BMMCs, incubated in $50 \mu\text{L}$ nanoscaffold matrix, were activated by adding HEPES buffer ($50 \mu\text{L}$, $10 \times 10^{-3} \text{ M}$, with 0.4% BSA, pH 7.4) with DNP-BSA. After 30 min incubation, cell-free supernatant and cells in the matrix were collected for total cell lysate analysis. The latter was isolated by solubilizing matrix with 0.01% Triton X-100, followed by centrifugation ($300 \times g$, 5 min).

Confocal Microscopy: After 18 h incubation, adherent BMMCs were washed with warm PBS for three times. For the activation groups, cells were treated with A23187 or DNP-BSA, as described above, for 30 min. These 3D matrices, with adherent BMMCs, were fixed using paraformaldehyde in PBS (4%, pH 7.4) for 20 min. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min, washed in PBS for three times, then followed by 3% BSA PBS solution blocking for 30 min and three times washing in PBS. To stain F-actin, samples were incubated with phalloidin-FITC ($0.5 \times 10^{-6} \text{ M}$, Sigma-Aldrich, USA) for 30 min followed by three times washing in PBS. Cell nuclei were stained with DAPI ($1 \mu\text{g mL}^{-1}$, Sigma-Aldrich, USA) in PBS for 30 min. After three times washing in PBS, Z-slice images of the matrix, with adhered BMMCs, were collected using laser scanning confocal microscopy (LSM710, Carl Zeiss AG, Oberkochen, Germany) with an inverted $10\times$ objective.

Measurement of Cytokine Production Using ELISA: IgE-sensitized BMMCs ($50 \mu\text{L}$, $1.0 \times 10^6 \text{ cells mL}^{-1}$) were layered on $50 \mu\text{L}$ of nanoscaffold. After 18 h incubation, the media were aspirated, washed with PBS, and fresh media with DNP-BSA ($50 \mu\text{L}$, final concentration of $0.1 \mu\text{g mL}^{-1}$) was allowed to incubate with the system for 24 h at 37°C . Cell-free supernatants were isolated by centrifugation ($300 \times g$, 5 min), and the production of TNF- α , IL-6, and IL-13 was measured using commercially available ELISA kits: mouse TNF- α , mouse IL-6, and mouse IL-13 ELISA Ready-SET-Go kit (eBioscience, San Diego, CA). The minimum detection limits for these assays were reported to be 8 pg mL^{-1} for TNF- α , 4 pg mL^{-1} for IL-6, and 4 pg mL^{-1} for IL-13, respectively. Released cytokine amounts (normalized for 50 000 total cells) were calculated based on adherent cell numbers.

Cytokine absorption to the nanoscaffold matrix was analyzed by incubating TNF- α (1000 , 500 , 250 pg mL^{-1}), IL-13 (500 , 250 , 125 pg mL^{-1}), and IL-6 (500 , 250 , 125 pg mL^{-1}) at 37°C with 0.25%, 0.5%, and 1.0% w/v matrix and analysis conducted at 0.5, 2, 4, 10, 18, 24, 34, and 48 h. Supernatant solutions were collected and likewise analyzed for cytokine production using ELISA, as described above.

Evaluation of BMMC Adhesion on (RADA)₄ Peptide Coating: The 96-well costar high-binding plates were coated with (RADA)₄ water solutions ($100 \mu\text{L}$) overnight at 4°C , then washed with PBS for three times. Then wells were blocked by 3% BSA PBS solution and washed with PBS for three times. About $100 \mu\text{L}$ of BMMCs ($0.5 \times 10^5 \text{ cells}$) in the media was added to each well and then incubated for 18 h. After incubation, the media were disposed and the well was carefully washed by media for three times to remove weakly binding cells, and then replaced with the fresh media ($100 \mu\text{L}$). The number of adhered cell was estimated by using XTT assay. The standard curve was determined by total cells incubated without coating. The result is shown in Figure S2 (Supporting Information).

Statistical Analysis: All data were conducted in triplicate with independent repeats using BMMCs from three donors. The results were presented as average $\pm$ standard error of the mean (SEM,

$n \geq 3$). The statistical significance of differences between mean values was determined using one-way ANOVA followed by Student's *t*-test for analysis of variance, where the significance was evaluated for $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This work was supported by NSERC (Canada), NINT-NRC. L.L. is kindly supported by a grant from the China Scholarship Council (CSC). The authors gratefully acknowledge Fei Qian and Dr. Brett Duguay for useful and valuable suggestions during the work.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

degranulation, IgE, mast cells, self-assembling peptides, TNF- α

Received: March 14, 2017

Revised: May 18, 2017

Published online:

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Supporting Information

for *Adv. Healthcare Mater.*, DOI: 10.1002/adhm.201700334

The Spontaneous Adhesion of BMMC onto Self-Assembled Peptide Nanoscaffold without Activation Inhibits Its IgE-Mediated Degranulation

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Supporting Information:

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nanoscaffold without activation inhibits its IgE-mediated degranulation**

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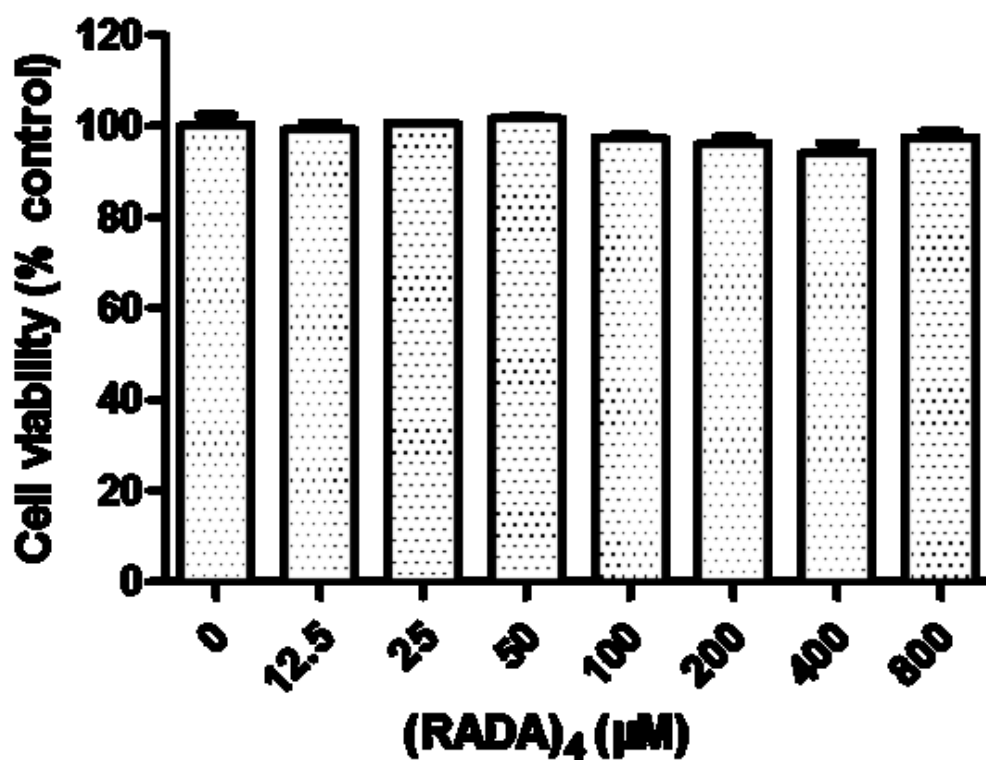


Figure S1. (RADA)₄ peptide has no effect on mast cell viability. BMMCs (0.5×10^5 cells) were incubated with (RADA)₄ peptide in culture media for 1 h, then the cell viability was determined by XTT assay.

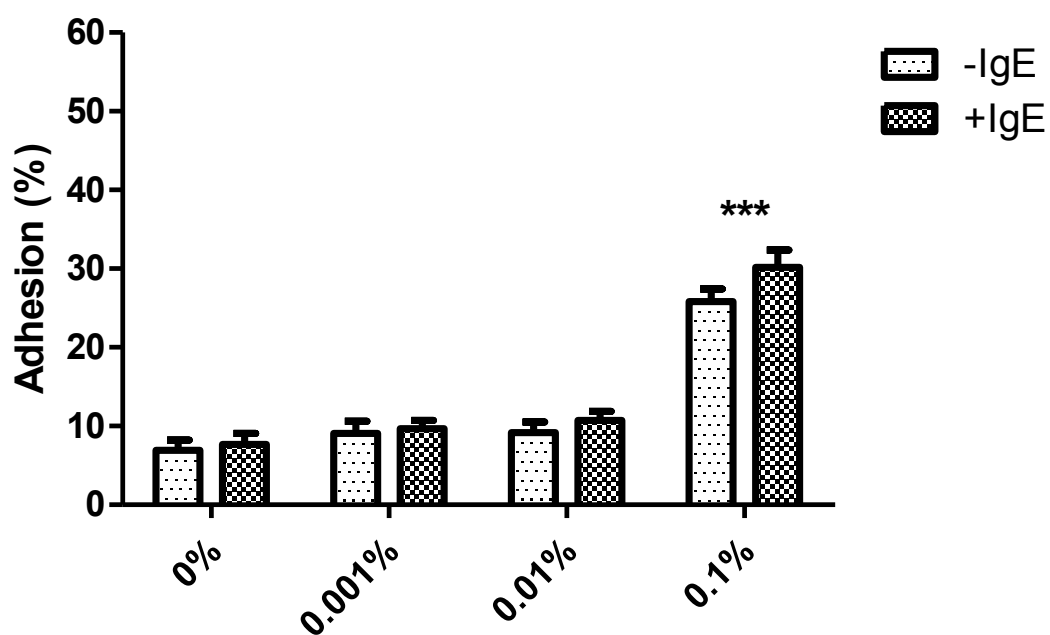


Figure S2. The adhesion of BMMCs to (RADA)₄ peptide coating in a concentration dependent manner. The relative cell number was determined by using XTT assay.

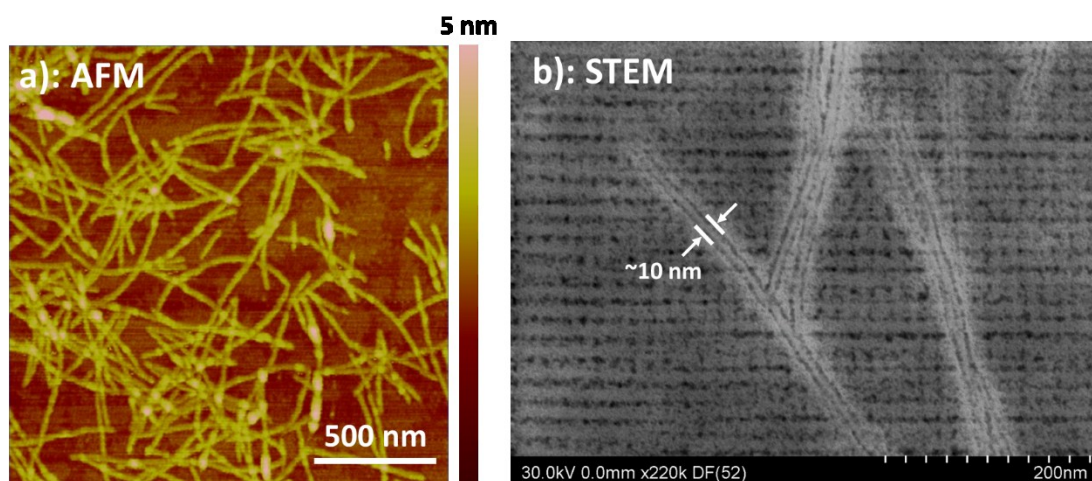


Figure S3. The dimension of (RADA)₄ nanofiber. a): Atomic Force Microscopy (AFM, Veeco Bruker DI Dimension 3100, USA) image of 500 times dilution of 0.5% w/v (RADA)₄ matrix on mica. b): Scanning transmission electron microscopy (STEM, Hitachi S-5500, Japan) image of untreated (RADA)₄ nanofiber on carbon grid.

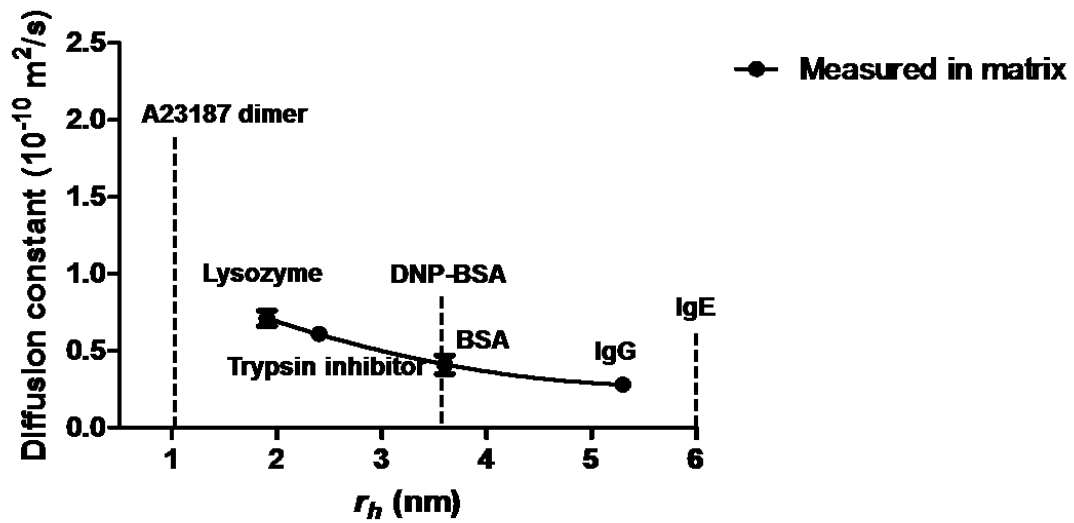


Figure S4. The relation between different constant and hydrodynamic radius (r_h), concluded from the data of previous study ^[19]. The black line represent the tendency that conclude form the measured diffusion constant of lysozyme, trypsin inhibitor, BSA and IgG in matrix. The dash line point to the molecular radius (r_h) of compound A23187, DNP-BSA and IgE.

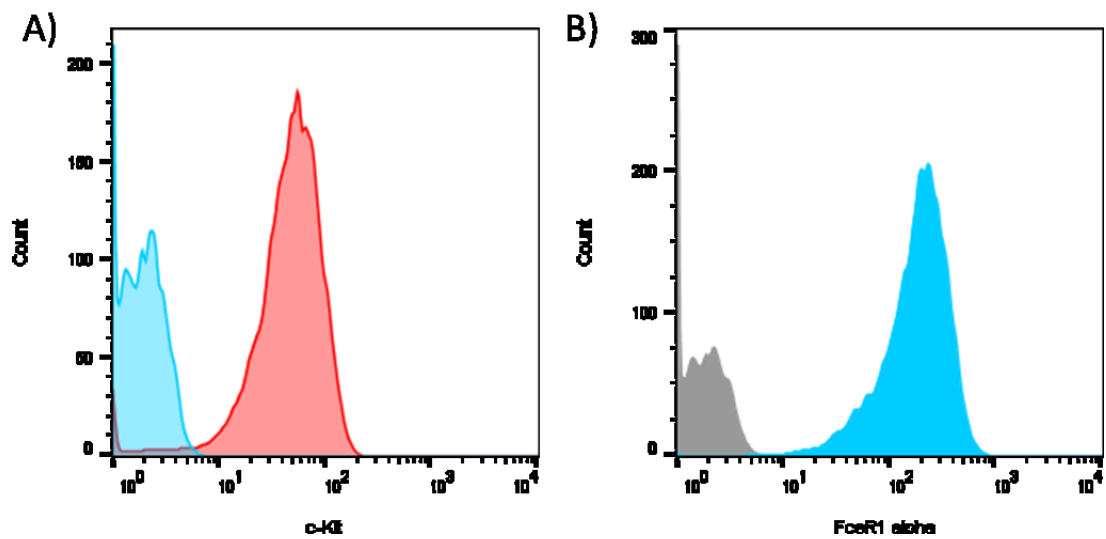


Figure S5. Flow cytometry analysis. BMMC purity was evaluated at the fourth week in culture, by the expression of c-Kit and FceR1 alpha and analyzed by flow

cytometry. A) BMMC stained with a PE-conjugated anti-mouse CD117 (c-Kit) antibody (red histogram) and a PE conjugated isotype control (blue histogram). B) BMMC stained with a PE-conjugated anti-mouse FcεR1 alpha antibody (blue histogram), and a PE-conjugated isotype control (grey histogram).