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The effect of surface modification of mesoporous silica micro-rod scaffold on immune cell activation and infiltration

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Abstract

Biomaterial scaffold based vaccines show significant potential in generating potent antigen-specific immunity. However, the role of the scaffold surface chemistry in initiating and modulating the immune response is not well understood. In this study, a mesoporous silica micro-rod (MSR) scaffold was modified with PEG, PEG-RGD and PEG-RDG groups. PEG modification significantly enhanced BMDC activation marker up-regulation and IL-1 β production *in vitro*, and innate immune cell infiltration *in vivo*. PEG-RGD MSRs and PEG-RDG MSRs displayed decreased inflammation compared to PEG MSRs, and the effect was not RGD specific. Finally, the Nlrp3 inflammasome was found to be necessary for MSR stimulated IL-1 β production *in vitro* and played a key role in regulating immune cell infiltration *in vivo*. These findings suggest that simply modulating the surface chemistry of a scaffold can regulate its immune cell infiltration profile and have implications for the design and development of new material based vaccines.

Keywords

Mesoporous silica; Poly(ethylene glycol); RGD; acute inflammation; inflammasome

1. Introduction

Various immunotherapies have shown significant preclinical and clinical success, and, for example, is a new promising approach for cancer treatment and management [1].

Biomaterials have shown substantial potential to integrate synergistically with current vaccine strategies and enhance their effectiveness [2, 3]. Biomaterial scaffold vaccines have

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been proposed to locally recruit and program host immune cells to induce effective adaptive immunity. In these vaccines, dendritic cells (DCs), professional antigen presenting cells (APCs) capable of bridging innate and adaptive immunity, are recruited to the scaffold where they interact with the immune-modulatory cues delivered from the scaffold. Using this approach, an implantable poly(lactide-co-glycolide) (PLGA) based scaffold cancer vaccine was shown to induce complete melanoma regression in subset of animals [4–6]. We recently proposed a new approach of injectable, *in situ* spontaneously assembling scaffolds to modulate host immune cells. In this system, we demonstrated that mesoporous silica micro-rods (MSRs) of high aspect ratio could spontaneously assemble *in situ* to form a 3D cellular microenvironment *in vivo* [7].

Beyond serving as residents for immune cells and delivery vehicles for immune-stimulatory drugs, the surface chemistry of the scaffolds also likely modulates immune cell trafficking and activation. Surface hydroxyl group content on aluminum oxyhydroxide nanoparticles was shown to influence maturation of human dendritic cells and monocytes [8]. Surface modifications of amorphous silica particles with -COOH, -NH₂, -SO₃H, or -CHO groups reduced endosome rupture, IL-1 β and reactive oxygen species (ROS) production by human monocytes as compared to unmodified particles [9]. The effect of surface chemistry of mesoporous silica particles on immune cell function remains largely unexplored. However, uptake of unmodified mesoporous silica nanoparticles by macrophages resulted in generation of reactive oxygen species, increased levels of nitric oxide (NO) and production of pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α [10]. Additionally, mice vaccinated with the MSR scaffold loaded with ovalbumin (OVA) showed high titers of anti-OVA IgG1 antibody, suggesting that the MSR scaffold itself has adjuvant properties [7].

The production of pro-inflammatory cytokines by immune cells is the result of stimulating pattern recognition receptors (PRRs) [11, 12]. Phagocytosis of particulates such as crystalline silica particulates (SiO₂), calcium phosphate and PLGA was shown to result in the activation of the NOD-like receptor, Nlrp3, an intracellular PRR [11, 13, 14]. This crystal-induced Nlrp3 activation was shown to be a result of lysosomal perturbation rather than direct sensing of the crystal structure [13]. Upon activation, Nlrp3 forms an intracellular multi-protein complex that regulates the production of highly inflammatory cytokines such as IL-1 β and IL-18, which leads to the recruitment of innate immune cells to the affected tissue to assist in clearing of the particulate materials [13].

In this study, we investigated whether modifying the surface groups of the MSR scaffold can modulate its inflammatory properties and the profile of immune cell infiltration. Poly(ethylene-glycol) (PEG), which is typically considered by many to be nontoxic and nonimmunogenic, is among the most widely used polymers to decrease immune recognition and minimize nonspecific uptake [15]. Modifying materials with the integrin-binding ligand Arg-Gly-Asp (RGD) is widely used to enhance cell adhesion and previous work demonstrate that DCs express several integrins that bind to RGD [16]. Therefore, we hypothesized that functionalizing the MSR scaffold with PEG would reduce its immunogenicity and thus decrease immune cell infiltration, whereas modifying the MSR scaffold with PEG-RGD would enhance immune cell adhesion and infiltration. We also investigated the role of the Nlrp3 inflammasome in immune cell infiltration and activation.

2. Materials and Methods

2.1 Mice

Unless otherwise noted, all *in vivo* studies were carried out using female C57BL/6J mice (Jackson Laboratories) between 6 to 10 weeks old at the beginning of the experiment. Nlrp3^{-/-} mice were obtained from Millennial Pharmaceuticals. The generation of Nlrp3^{-/-} mice was previously described [17]; the mice were backcrossed onto the C57BL/6 genetic background for at least four generations. The Nlrp3^{-/-} mouse colony was maintained at Harvard University. All animal studies were performed in accordance with NIH guidelines, under approval of Harvard University's Institutional Animal Care and Use Committee.

2.2 Synthesis and surface modification of MSRs

MSRs were fabricated as described previously [7]. Briefly, 4g of P123 surfactant (average Mn ~5800, Sigma-Aldrich) were dissolved in 150 g of 1.6 M HCl solution and were stirred with 8.6 g of tetraethyl orthosilicate (TEOS, 98%, Sigma-Aldrich) at 40 °C for 20 h, followed by aging at 100 °C for 24 hours. TEO S was extracted in 1% EtOH in HCl at 80 °C for 18 hours. Pristine MSRs were used for surface modification (Fig. 1). MSRs were first functionalized with (3-Aminopropyl)triethoxysilane (APTES) (Sigma Aldrich) by stirring 15ug of APTES with 100mg of pristine MSR in ethanol for 18 hours at room temperature. Particles were washed extensively in ethanol and dried overnight at 70 °C. Extent of modification was measured using a zeta potential analyzer (Malvern Zen3600) and surface amines were quantified using fluorescamine and Lavapep (GelCompany) according to manufacturer's manual.

To PEGylate the MSRs, 100mg of APTES modified MSRs were reacted with 100 μmol NHS-MS(PEG)₁₂ (Thermo Scientific). Reaction was allowed to proceed for 2 h and particles were washed extensively with PBS. 100 mg APTES-modified MSRs were then reacted with 100 μmol PEGylated bis(sulfosuccinimidyl)suberate BS(PEG)₉ (Life Technologies) and subsequently with 3 molar excess of GGGGRGDSP/GGGGRDGP (Peptide 2.0). MSRs were then washed extensively with and PEGylation and PEG-RGD/RDG reaction efficiencies were quantified by assessing residual surface amines using Lavapep. RGD and RDG peptide content was assayed using micro-BCA (Thermo Scientific). All materials were tested to be endotoxin free using an LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific).

2.3 Transmission electron microscopy

MSRs were diluted to 0.1mg/ml in 200-proof ethanol (Sigma Aldrich) and dripped on Formvar/Carbon TEM grids (Ted Pella) and air-dried for 1–2 hours at room temperature. The MSRs were then imaged using the JEOL 2100 transmission electron microscope.

2.4 Nitrogen adsorption and desorption analysis

To remove adsorbed water from the surface, MSRs were first dried under low pressure (~50mmHg) and at 150 °C for 8 hours. Nitrogen sorption isotherms of MSRs were then measured on a Micromeritics ASAP 2000 apparatus at liquid nitrogen temperature (77 K). Surface area was determined by Brunauer-Emmett-Teller (BET) method. Pore size

distribution was calculated from the adsorption branch of the isotherm using the Barret–Joyner–Hallenda (BJH) model.

2.5 Bone marrow derived dendritic cells (BMDC) isolation and culture

Bone marrow cells were isolated from female C57Bl/6J mice between 6 and 15 weeks old. Cells were cultured in RPMI based media (Sigma Aldrich) supplemented with essential amino acids, 10% heat inactivated FBS (Sigma-Aldrich), 1% penicillin/streptomycin, 50 μ M β -mercaptoethanol and 20 ng/ml murine GM-CSF (Peprotech) and cultured at 37C and 5% CO₂ [18]. Non-adherent dendritic cells were harvested and used for experiments between day 7 and 8 of differentiation. Differentiation was confirmed using the CD11c, CD11b and MHC II surface markers.

2.6 In vitro stimulation of BMDCs with MSR particles

On day 7 or 8 of BMDC differentiation, non-adherent cells were collected, counted and seeded in tissue culture plastic wells at 2.5x10⁶/ml. BMDCs were allowed to attach for 1 hour and MSRs were added directly to the culture for an additional 18 hours. Cells were stained with PE anti-CD86 (eBioscience) to measure their activation. For experiments measuring IL-1b production, BMDCs were primed with 20ng/ml LPS (Sigma-Aldrich) immediately before adding the MSRs. Cells were cultured for 18 hours and supernatant was collected for cytokine analysis.

2.8 Surface marker staining and flow cytometry analysis

After single cell suspensions were made, cells were stained with a live/dead dye followed by fluorophore-conjugated antibodies for 15 minutes on ice. Cells were washed 3 times in cold staining buffer containing 1% BSA and 0.1% sodium azide and analysed using multi-colored flow cytometry (BD). APC-conjugated CD11c, and FITC-conjugated CD11b stains were conducted for DC and leukocyte recruitment analysis. APC-conjugated CD11c, FITC-conjugated MHC II, and PE-conjugated CD86 stains were conducted for DC maturation analysis. Myeloid cells/neutrophils were stained using Pacific Blue conjugated Ly6C, FITC conjugated Ly6G and PE-CY7 conjugated CD11b. Macrophages were stained using PE conjugated F4/80 and FITC conjugated CD11b. All antibodies were purchased from eBiosciences and used according to the manufacturer's protocols. Live cells were determined using a fixable live/dead dye (eBiosciences) or 7-AAD (Biolegend). Live cells were first gated on forward and side scatter followed by the live/dead channel. Fluorophore gating was then performed on isotype control.

2.9 In vivo MSR scaffold injection

Five (5) mg of sterile MSRs were lyophilized, resuspended in sterile PBS (150ul) and injected into the left flank, midpoint between the inguinal lymph node and the axillary lymph node, of C57BL/6J mice.

2.10 Scaffold harvesting and cell infiltration analysis

Scaffolds were excised and tissues were processed through mechanical disruption, washed and suspended in PBS. The resulting cell suspension was then filtered through a 40 μ m cell

strainer. Cells and small remaining MSR particles were pelleted and counted. The portion of cells in the mixture of cells and small silica particles was assessed using side scatter, forward scatter and a live/dead dye in flow cytometry (BD LSR II). Number of live cells in the scaffold was calculated as the count of cell and particle mixture multiplied by the percentage of live cells determined using flow cytometry.

2.11 In vivo tissue processing for cytokine analysis

Tissue samples between 1 mm and 3 mm from the scaffold were extracted from the animals and digested using the Tissue Protein Extraction (T-Per) Reagent (Pierce), and homogenized via brief sonication. Cell debris was pelleted with centrifugation. The supernatant was analyzed using Bio-Plex Pro™ mouse cytokine 23-plex immunoassay (Bio-Rad) and ELISA (eBioscience), according to the manufacturer's instructions.

3. Results

3.1 Surface modification and characterization

MSRs were first functionalized with primary amine groups using APTES according to established procedures (Fig 1). Modification was confirmed using fluorescecamine and the resulting material had 0.13 μmol of APTES per mg of MSR. Modification with PEG and PEG-RGD was performed on APTES modified MSRs and was quantified as a reduction of primary amine groups. The extent of modification was 76.2 $\pm$ 0.3% and 78.1 $\pm$ 0.6%, respectively (Fig 2A). No change in the bulk structure and lengths of the MSRs after modification was observed. Nitrogen adsorption/desorption analysis revealed that the modified materials possessed a typical type IV isotherm with a well-defined presence of mesopores (Fig 2B). The PEG and PEG-RGD modified MSRs had a slightly decreased and narrow BJH pore size distribution (around 10 nm) and significantly decreased total surface area and pore volume compared to APTES-only modified MSRs (Fig 2C, Table S1). The decrease in surface area and pore volume was likely due to space occupied by the PEG polymers and peptides. Additionally, TEM confirmed an aligned and open pore structure in the modified MSRs (Fig 2D).

3.2 In vitro studies

DC activation in the scaffold has been shown to be crucial to induce a potent antigen specific immune response. Therefore, the effect of the modified MSRs on DC maturation and activation *in vitro* was analyzed. Bone marrow derived DCs (BMDCs) were harvested and stimulated with 20 $\mu\text{g}/\text{ml}$ of unmodified and surface modified MSRs. After 18 hours, BMDCs were collected and co-stained with the DC marker CD11c and activation marker CD86 (primary flow cytometry plots in Fig S1). As expected, both unmodified and APTES modified MSRs led to significant CD86 up-regulation (Fig 3a) compared to the PBS control. PEG modified MSRs led to even higher CD86 expression (Fig 3a). Incorporating RGD in PEG modified MSRs reduced CD86 expression as compared to PEG modified MSRs (Fig 3a). In a separate experiment, the effect was found to be not sequence specific, as no significant difference was observed between PEG-RGD modified and PEG-RDG modified MSRs (Fig 3b).

The effect of the surface modified MSRs on DC cytokine production was also examined as activated DCs also secrete pro-inflammatory cytokines. BMDCs were first primed with 20ng/ml of LPS and stimulated with the surface modified MSRs. A panel of inflammation related cytokines from DCs stimulated with unmodified and PEG modified MSRs was first analyzed (Fig S2). Among the 18 cytokines analyzed, IL-1 α and IL-1 β were significantly increased in PEG modified MSRs compared to unmodified MSRs, with IL-1 β secreted at higher quantities. Secretion of IL-1 β by DCs after stimulation with various concentrations of PEG modified, PEG-RGD modified and PEG-RDG modified MSRs was then measured with ELISA. Consistent with what was observed from the DC activation study, PEG modified MSR significantly enhanced IL-1 β production in a dose dependent manner, while PEG-RGD modified and PEG-RDG modified MSRs decreased the effect as compared to PEG-MSRs (Fig 3c).

One of the most important regulators of IL-1 β production is the Nlrp3 inflammasome, which is a member of the NLR family capable of sensing danger-associated molecular patterns (DAMPs). To investigate whether the Nlrp3 inflammasome was responsible for the enhanced IL-1 β production after stimulation by the MSRs, BMDCs from Nlrp3^{-/-} mice were stimulated with 5 ug/ml unmodified and surface modified MSRs, and no secretion of IL-1 β was found after stimulation regardless of the surface groups.

3.3 In vivo studies

The inflammatory response to the MSRs was next analyzed *in vivo*. Unmodified MSRs, PEG MSRs, PEG-RGD MSRs and PEG-RDG MSRs were injected subcutaneously into the flank of mice. As demonstrated previously, the MSRs spontaneously assembled after injection, forming a 3D scaffold that allowed for cell infiltration (Fig 3a). On day 5, the subcutaneous nodule formed by PEG modified MSRs was noticeably larger than that formed by unmodified, PEG-RGD MSRs and PEG-RDG MSRs (Fig 3b, PEG-RDG MSR image not shown). Analysis of the cell content revealed that nearly 10 times more cells infiltrated the PEG MSR scaffold than the unmodified MSR scaffold. Although both PEG-RGD MSR and PEG-RDG MSR scaffolds also contained significantly more cells than the unmodified MSR scaffold, nearly 4 times fewer cells were found than in the PEG MSR scaffold (Fig 3c). No significant difference was observed between PEG-RGD modified and PEG-RDG modified scaffolds.

The types of immune cells that infiltrated the scaffolds were next analyzed. Specifically, DCs, macrophages and myeloid cells/neutrophils were quantified (flow cytometry primary plots in Fig. S3). DC and macrophage infiltration has been shown to be important for eliciting a potent adaptive immune response, and myeloid cells/neutrophils are among the first responders to a foreign material at sites of inflammation. Interestingly, PEG scaffolds contained a significantly lower percentage of DCs than unmodified scaffolds (<1% of total infiltrating cells), and PEG-RGD scaffolds and PEG-RDG scaffolds contained more DCs than PEG scaffolds, but lower numbers than unmodified scaffolds (Fig 4d). Nevertheless, the total number of DCs was not significantly different among the scaffold conditions (Fig S4a). This trend was also observed for activated DCs and macrophages in the scaffolds (Fig S4b-c). Myeloid cells/neutrophils made up more than 50% of immune cells in all scaffold

conditions at this time point. In contrast to what was observed with DCs and macrophages, PEG scaffolds had a significantly higher percentage and total number of myeloid cell/neutrophils compared to unmodified, PEG-RGD modified and PEG-RDG modified scaffolds (Fig 4e and Fig S4d).

In addition to immune cell infiltration, the development of a fibrous capsule around the scaffold was also analyzed. Scaffolds were explanted, and the weight of the fibrous tissue around the scaffold was measured. The fibrous capsule around the PEG scaffold was significantly heavier and noticeably thicker than that around unmodified and PEG-RGD scaffolds (Fig 4f). The fibrous capsule around the PEG scaffold also contained significantly higher IL-1 β levels than unmodified and PEG-RGD scaffold fibrous capsules (Fig 4g). Analysis of a panel of inflammation related cytokines revealed that IL-1 β was one of the highest secreted inflammatory cytokines in unmodified scaffolds (Fig S5).

Finally, the role of the Nlrp3 inflammasome on immune cell infiltration *in vivo* was examined. Unmodified and modified MSRs were injected subcutaneously into the flanks of wild type B16 mice and Nlrp3^{-/-} mice. Scaffolds in Nlrp3^{-/-} mice showed significantly reduced total cell infiltration from all surface modifications as compared to wild type mice (Fig 5a). However, similar to the previous observations in wild type mice, PEG scaffolds in Nlrp3^{-/-} mice exhibited significantly increased cell infiltration as compared to the other surface chemistries. In both wild type and Nlrp3^{-/-} mice, the primary cells that infiltrated the scaffolds were again myeloid cells/neutrophils, and there was no significant difference between wild type and Nlrp3^{-/-} mice (Fig 5b). Interestingly, both unmodified and PEG modified scaffolds contained increased percentage of DCs when placed in Nlrp3^{-/-} mice compared to wild type mice (Fig 5c). However, unmodified scaffolds in Nlrp3^{-/-} mice contained fewer activated DCs as compared to scaffolds in wild type mice, suggesting most of the DCs infiltrating the scaffold in Nlrp3^{-/-} mice may exhibit an immature phenotype. Finally, and consistent with the previous observations, in both wild type and Nlrp3^{-/-} mice, PEG modified scaffolds had a reduced DC population and were enriched in the myeloid cell/neutrophil population.

4. Discussion

PEG modified MSRs displayed substantially higher inflammatory responses compared to unmodified, PEG-RGD modified and PEG-RDG modified MSRs. PEG MSRs stimulated increased production of the pro-inflammatory cytokine IL-1 β , up-regulation of DC maturation markers *in vitro*, and increased immune cell infiltration into the scaffolds *in vivo*. PEG is typically considered a “stealth” polymer and, by extension, has been shown to decrease serum protein adsorption and reduce host response [15, 19–21]. However, recent evidence has suggested that treatment with some PEGylated drugs can result in immune responses against the PEG, which could limit the drug efficacy [22–25]. For example, clinical studies of PEG-uricase [24] and PEG-asparaginase [26] show substantial increase of serum anti-PEG antibodies that were associated with decreased drug efficacy. Likewise, a number of studies have also suggested that PEG containing materials can elicit pro-inflammatory responses under specific contexts [22, 27–32]. For instance, human monocytes seeded on PEG diacrylate hydrogels secreted more IL-1 β , TNF- α and GM-CSF compared to

those seeded on tissue culture plastic [27]. More recently, a series of studies show that implantation of PEG hydrogels *in vivo* resulted in a more robust inflammatory response, characterized by a thicker fibrous capsule around the hydrogel, compared to PEG-RGD hydrogels [28]. Murine macrophages seeded on PEG hydrogels showed higher expression of IL-1 β and TNF- α [28, 29] and PEG hydrogels adsorbed more serum proteins related to acute inflammation than PEG-RGD hydrogels [31].

Several reasons for the immunogenicity from PEGylation have been proposed, including the direct interaction between PEG itself and serum proteins and through the immunogenicity of the anchoring material or drug [22]. It has been shown that PEG hydrogels can activate serum complement proteins, leading to subsequent immune cell recognition [31]. Monodispersed and highly concentrated PEG polymers ranging from 2K to 11K [33], PEG₅₀₀₀ stabilized carbon-nanotubes [34] and lipid-PLGA-PEG nanoparticles [35] were shown to generate complement activation when incubated with human serum. Though the exact mechanism by which PEG may activate complement proteins remains unclear, the effect likely depends on surface PEG modification density [23, 33, 36]. For example, 10% PEG-PLA nanocapsules increased complement activation over PLA nanocapsules, but 30% PEG-PLA decreased the effect [36]. In this study, relatively short PEG polymers (PEG₁₂ and PEG₉ for PEG MSR and PEG-RGD/RDG MSR, respectively) were used. Since the MSRs have large surface area to volume ratio, it is likely that PEG covered only a fraction of the total surface and increasing PEG coverage may alter the inflammatory response. PEG was anchored onto the MSRs through stable amide bonds. Mesoporous silica nanoparticles have been shown to be stable in serum *in vitro* over a period of 60 days [37, 38], and PEGylation was shown to decrease the biodegradation rate of silicon-based materials [39]. However, the degradation of the MSRs could be significantly accelerated *in vivo* due to the presence of proteases and reactive oxygen species in the inflammatory microenvironment, and the local concentration of the degradation products may contribute to the immunogenicity. Finally, the immunogenicity of PEG may depend on the immunogenicity of the anchoring material. For example, immunization with PEG-OVA given in Freund's complete adjuvant resulted in increased anti-PEG serum antibody, but not after immunization with PEG-OVA alone [23]. Since the MSRs are inherently inflammatory, as shown by increased activation markers and pro-inflammatory cytokine secretion in BMDCs, it is possible that the immunogenicity of the MSRs amplified the immune response towards PEG. Overall, future studies are required to investigate the effect of PEG surface chain density, length and conformation.

PEG-RGD modified and PEG-RDG modified MSRs showed decreased inflammatory responses compared to PEG MSRs. The effect was not specific to the integrin-binding domain, as no difference was observed between the behaviors of PEG-RGD and PEG-RDG MSRs *in vitro* and *in vivo*. In this context, it is more likely that incorporating the small peptide to PEG hindered the interaction between the PEG chain, or its terminal group, and components in the serum, reducing immune cell recognition. A number of studies have shown that terminal groups, such as hydroxyl groups, can selectively activate proteins in the complement pathway [40–42]. The present study utilized PEG polymers carrying a methoxy group, which has been suggested to limit complement activity in some contexts [35, 41, 43,

44]. However, the effect of the terminal group in this system on complement protein activation and serum protein adsorption needs to be evaluated.

The Nlrp3 inflammasome was found to be necessary for IL-1 β production after stimulation with the MSRs in vitro, and significantly impacted the degree of immune cell trafficking in vivo. Previous works showed that uptake of amorphous and crystalline silica particulates by macrophages can induce intracellular membrane damage and the production of reactive oxygen species [9, 13, 45, 46]. DAMPs released by this process are sensed by the Nlrp3 inflammasome, leading to enhanced IL-1 β and IL-18 secretion. The MSRs likely acted through a pathway similar to the previously studied amorphous silica particulates in vitro, but the strength and duration of pathway activation remains to be elucidated. Abolishing Nlrp3 also significantly reduced total immune cell infiltration in vivo. Myeloid cells/neutrophils have been shown to exhibit high expression of Nlrp3, but there was no reduction in the percentage of myeloid cells/neutrophils in scaffolds from Nlrp3^{-/-} mice [47, 48]. Rather, scaffolds in Nlrp3^{-/-} mice enriched for CD11c⁺CD11b⁺ myeloid DCs, and these DCs exhibited a more naïve phenotype in the unmodified scaffolds.

PEG modification of MSRs could potentially make the resulting scaffold a stronger adjuvant. The PEG MSRs could increase the response to the vaccine by acting directly on infiltrated immune cells through NLRs and TLRs. On day 5 and 7 post injection, PEG MSRs were highly enriched in CD11b⁺Gr.1⁺ cells but exhibited a reduced percentage of APCs. It is possible that APCs infiltrated the scaffold at an earlier time point, were activated upon interacting with PEG MSRs and emigrated from the scaffold, as it has been demonstrated that activated APCs up-regulate CCR7, leading them to home to draining lymph nodes. While biomaterial design has traditionally avoided immune-activation to minimize rejection, adjuvant properties of the scaffold could be exploited and may act synergistically with other adjuvants such as CpG or poly(I:C) to induce potent adaptive immune responses [43, 49, 50]. For instance, aluminum salt adjuvants were shown to activate immune cells through Nlrp3 by a mechanism of lysosomal disruption. However, as CD11b⁺Gr.1⁺ myeloid cells may inhibit DC and NK cell function and mediate T cell suppression [51–53], it will be important investigate the functional interaction between CD11b⁺Gr.1⁺ myeloid cells, DCs and macrophages in the scaffolds to better understand their functional roles.

5. Conclusion

This study showed that modulating surface chemistry on the MSR scaffold significantly impacted its inherent inflammatory response in vitro and in vivo. PEG MSRs enhanced pro-inflammatory cytokine production in vitro and immune cell, especially CD11b⁺Gr.1⁺ myeloid cell, infiltration into the scaffold in vivo. Conversely, PEG-RGD modified and PEG-RDG modified MSRs displayed decreased inflammatory effects as compared to PEG MSRs, but the effect was not specific to the integrin-binding domain RGD. Finally, the Nlrp3 inflammasome was necessary for MSR stimulated IL-1 β production and played a key role in regulating immune cell infiltration and cell phenotype in the MSR scaffolds. Overall, the findings from this study suggest that changing the surface chemistry on a scaffold could alter its immune cell infiltration profile. These findings contribute to the understanding of

the interaction between biomaterial scaffold surface and immune responses, and may, for example, aid the design of new materials-based vaccines for a variety of applications.

Supplementary Material

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Acknowledgments

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Figure 1. Schematics of MSR surface modification

MSRs were functionalized with APTES, and the amine groups were then modified with methoxyl terminated PEG₁₂ (NHS-MS(mPEG)₁₂) to achieve PEG modified MSRs, or methoxyl terminated PEG₉ (BS(mPEG)₉) followed by RGD/RDG peptides for PEG-RGD/RDG modified MSRS.

Figure 2. Characterization of MSRs after modification

(a) Percentage of PEG and PEG-RGD coverage of $-\text{NH}_2$ groups from APTES functionalized MSRs (mean and s.d, $n=3$ or 4). (b) Nitrogen sorption isotherms of modified MSRs and (c) BET pore size distribution from adsorption branch of modified MSRs. (d) TEM images of modified MSRs (scale bar = 20nm).

Figure 3. In vitro BMDC activation and cytokine production after stimulation with surface modified MSRs

(a) and (b) Flow cytometry analysis of CD11c and CD86 expression on BMDCs after 18 hours of stimulation with surface modified MSRs. Data expressed as percentage of BMDCs double positive for CD11c and CD86 (data are shown as mean and s.d with n=3). (c) ELISA analysis of IL-1 β concentration in BMDC supernatant after 18 hours of stimulation with various concentration of surface modified MSRs and (d) ELISA analysis of IL-1 β concentration in Nlrp3^{-/-} or WT BMDC with 5 μ g/ml MSR (mean and s.d, n=3 or 4). * indicates $p < 0.05$. ns indicates no significance.

Figure 4. In vivo immune cell infiltration, fibrous tissue formation and cytokine production in surface modified MSR scaffolds

(a) schematic of experimental set-up. (b) Images of subcutaneous nodules formed by the unmodified and surface modified MSR scaffolds after 5 days. Scale bar = 5 mm. (c) Total cell count in the explanted MSR scaffolds. (d) Flow cytometry analysis of CD11c and CD11b expression in cells isolated from the scaffolds. Cells double positive for CD11c and CD11b were quantified. (e) Flow cytometry analysis of Ly6c and Ly6g expression in cells isolated from the scaffolds. Cells expressing high levels of Ly6g and medium levels of Ly6c were quantified. (f) Weight of fibrous capsule around the scaffolds. (g) ELISA analysis of

IL-1 β concentration in the fibrous capsule. Data are shown as mean and s.d. with n = 4. * indicates p < 0.05. ns indicates no significance.

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Figure 5. Involvement of the Nlrp3 inflammasome in immune cell infiltration into unmodified and surface modified MSR scaffolds in vivo

(a) Total cell counts in the MSR scaffolds. (b) Flow cytometry analysis of Ly6c and Ly6g expression in cells from scaffolds injected in Nlrp3^{-/-} and WT mice. Cells expressing high levels of Ly6g and medium levels of Ly6c were quantified. (c) Flow cytometry analysis of CD11c and CD11b expression in cells from scaffolds injected in Nlrp3^{-/-} and WT mice. Cells double positive for CD11c and CD11b were quantified. (d) Flow cytometry analysis of CD86 expression. Results expressed as percent of CD86⁺ cells within the CD11c⁺ population. Data are shown as mean and sd with n = 4. * indicates p < 0.05. ns indicates no significance.