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Modification of Biomaterials with a Self Protein Inhibits the Macrophage Response

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Abstract

A biomaterial that inhibits the host immune response by displaying an endogenously expressed immunomodulatory molecule, CD200. Immobilization of CD200 onto biomaterial surfaces effectively suppresses macrophage activation and reduces inflammatory response to subcutaneously implanted materials.

Keywords

biomaterial; host response; immunomodulation; biocompatibility

The foreign body response to biomaterial implants has been a major challenge in translating many medical devices into the clinic.[1, 2] Although some inflammation may be desirable to

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mediate the healing process, a persistent inflammatory response around the implanted device prevents its functional interaction with the surrounding tissue, and will eventually lead to device failure.[2] This biomaterial-induced host response is a cascade of events that is initiated by the injury from surgical implantation, followed by protein adsorption on the biomaterial surface, and infiltration and activation of inflammatory cells (neutrophils, and monocytes/macrophages) in the tissue surrounding the implant.[3, 4] Extensive efforts to reduce foreign body response have been largely focused on preventing non-specific protein adsorption, based on the idea that if protein adsorption is prevented, the ensuing interaction with inflammatory cells, and their activation, will consequently be minimized.[5, 6] However, materials such as poly(ethylene glycol) (PEG), which has been widely used as an anti-fouling coating material,[5–7] have only had moderate success at reducing inflammatory response *in vivo*.[8, 9] Interestingly, studies have shown that the incorporation of the adhesive peptide RGD (L-arginine, glycine, L-aspartic acid) to PEG in fact attenuated inflammatory response when compared to unmodified PEG hydrogels.[8, 9] These data suggest that simply preventing protein adsorption and macrophage adhesion is not sufficient to reduce the foreign body response, and that promoting specific interactions with immune cells may in fact improve the overall host response. In this work, we demonstrate that modifying a biomaterial with an immunomodulatory protein which interacts with surface receptors expressed by immune cells, effectively inhibits the macrophage inflammatory response to foreign materials.

A key feature of the immune system is the ability to distinguish dangerous from non-dangerous entities, in order to specifically target and eliminate invading pathogens or apoptotic cells while preventing damage to healthy host tissue. Immune cells recognize molecular patterns displayed on the surface of pathogens by their specific receptors and become activated. For example, lipopolysaccharide on the bacterial cell wall is recognized by CD14 and toll-like receptor 4 (TLR4) expressed by macrophages. In contrast, host cells express surface proteins that are specifically recognized by immune cells, leading to inhibition of inappropriate inflammatory immune activation and prevention of spurious activation on self tissue. Such immunoregulatory molecules include CD200 (OX-2), CD47, and CD55 (decay accelerating factor), as well as carbohydrates such as sialic acids, many of which bind to receptors expressed on the surface of myeloid cells (CD200R, SIRP-α (signal regulatory protein-α), siglecs) and inhibit their activation.[10, 11] These immunomodulatory molecules are required for maintaining homeostasis and preventing immune hyperactivity, and defects in their expression have been shown to lead to chronic inflammation, autoimmunity, or allergy.[12]

CD200 is a broadly expressed endogenous immunomodulatory protein[13] that ligates to its receptor CD200R, which is expressed on the surface of myeloid cells including neutrophils, macrophages, microglia, and dendritic cells. Disrupting CD200 interaction with CD200R using CD200-knockout mice or blocking antibodies causes increased numbers of infiltrating macrophages and susceptibility to autoimmune diseases, including collagen-induced arthritis and experimental allergic encephalomyelitis.[10, 14] The finding that CD200 delivers a strong inhibitory signal to macrophages through binding with CD200R[15] has created a significant interest in manipulating this interaction therapeutically. Administration of CD200-Fc fusion

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protein suppresses macrophage activation in a number of inflammatory diseases including arthritis,[16, 17] multiple sclerosis,[18] influenza infection,[19] and organ transplantation[20]. These data suggest that delivery of CD200 may provide a sufficient inhibitory signal to prevent macrophage activation and chronic inflammation. This work led us to hypothesize that modifying biomaterials with CD200 could potentially reduce the local macrophage response to implanted materials (Scheme 1).

Here, we show that immobilization of CD200, a potent ligand for active immunomodulation, onto a model biomaterial surface effectively inhibits the macrophage inflammatory response using established *in vitro* and *in vivo* models. We first generated soluble recombinant CD200 protein from mammalian CHO-K.1 (Chinese Hamster Ovary) cells transfected with plasmid DNA containing the extracellular region of CD200, AviTag sequence at the COOH terminus for site-specific biotinylation, and 6x His sequence for purification (Figure 1a). Supernatant containing the secreted protein product was concentrated and purified, and then subjected to SDS- and native polyacrylamide gel electrophoresis (PAGE) for characterization. A single band was observed in the Coomassie-stained gel at approximately 50 kDa (Figure 1a), which was expected for the heavily glycosylated 25 kDa protein.[21, 22] The presence of protein was further confirmed by Western blot.

The purified CD200 protein product was enzymatically biotinylated and then immobilized onto streptavidin-coated polystyrene surfaces. The binding capacity of the surface was characterized by incubating varying amounts of CD200-biotin and evaluating by using a fluorescently labeled CD200 antibody. Measured fluorescence intensity linearly increased from 0.001 to 0.5 μg (0.02 to 10 pmol) of CD200 added to each well, and the surface was saturated with approximately 0.5–1 μg (10–20 pmol) of CD200 added to each well (Figure 1b). The stability of the CD200-immobilized surfaces was assessed in phosphate buffered saline (PBS) or serum-containing media for two weeks after initial coating. For surfaces incubated in PBS, we observed no significant decrease in fluorescence intensity, indicating that the immobilized CD200 remained stable for at least 2 weeks (Figure S1a). However, for surfaces incubated in DMEM cell culture media containing 10% fetal bovine serum (FBS), approximately 60% of the initial protein concentration remained after two weeks, suggesting that some of the CD200 protein was degraded or displaced with serum proteins (Figure S1b).

The immune-inhibitory properties of CD200-modified surfaces were evaluated *in vitro* by examining the response of bone marrow derived macrophages (BMDM) seeded on the CD200-coated or plain polystyrene surface, followed by 18 hour stimulation with interferon-γ (IFN-γ) and/or lipopolysaccharide (LPS), potent stimulators of inflammatory immune response. We found that modification of surfaces with CD200 caused the BMDM to have a more rounded morphology compared to cells on polystyrene, and that cells clustered together when IFN-γ was present (Figure 2a). However, cells on the polystyrene surface were spread in all conditions, and dramatic differences were not observed between stimulated and unstimulated cells due to the confluency of the culture. The rounded morphology observed in cells cultured on CD200-modified surface suggests reduced macrophage inflammatory activation as previously described.[23, 24]

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To assess macrophage activation, the release of pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), was examined by enzyme-linked immunosorbent assay (ELISA). BMDM were seeded on CD200-coated, streptavidin-coated, and uncoated surfaces as controls, and stimulated with LPS and/or IFN-γ. We found that cells seeded on CD200-coated wells exhibited lower secretion levels of both TNF-α and IL-6 (Figure 2b), when compared to cells on control surfaces. In addition, IFN-γ and/or LPS induced secretion of TNF-α and IL-6 was also inhibited by the CD200-coated surface. These differences were not caused by changes in cell viability, since cells on CD200-coated and uncoated surfaces were all viable (Figure S2). The minimum concentration of CD200 coating density necessary to efficiently inhibit macrophage activation was 1 pmol (0.05 μg)/well (Figure S3). Moreover, the orientation of protein was important for the maximal inhibition of macrophage inflammatory response. Cells cultured on CD200 surfaces that were generated by a non-site specific reaction between exposed amine groups on the protein and a maleic-anhydride activated polystyrene surface exhibited greater levels of inflammatory cytokine secretion when compared to cells cultured on CD200-coated surface created using the biotin-streptavidin site-specific interaction (Figure S4).

In order to confirm that the suppression of macrophage activation was indeed due to CD200/CD200R binding, we used an antibody targeted to CD200 to block this interaction. To ensure that the Fc portion of the antibody was not triggering an immunogenic response, cells were pre-treated with an antibody targeted to CD16/32 (Fc blocker). BMDM cells were seeded on CD200-coated surfaces, CD200-blocked surfaces, streptavidin-coated, and uncoated surfaces, and then stimulated with LPS and/or IFN-γ. TNF-α secretion level was reduced on the CD200-coated surfaces more than 70% compared to polystyrene surfaces, whereas BMDM cells on the CD200-blocked surfaces showed higher TNF-α secretion when compared to cells on CD200-coated surfaces (Figure S5). This result clearly demonstrated that the suppression of macrophages resulted from the specific interaction of CD200 on the material surface and CD200R on macrophages.

The immunomodulatory effect of CD200 coating was confirmed using 20 μm diameter polystyrene microbeads as a base material, because this system could easily be tested in *in vivo* experiments. In addition, microbeads larger than 12 μm diameter were known to be not internalized by macrophages.[25] We first assessed the materials *in vitro* by seeding BMDM on tissue culture polystyrene, and then adding CD200-coated, streptavidin-conjugated, or plain polystyrene microbeads to the cell culture medium, along with IFN-γ and/or LPS. Macrophages were the most highly activated in response to plain polystyrene beads, and activation was reduced by approximately 40% by coating the microbead surface with streptavidin. CD200-coated microbeads significantly inhibited macrophage activation, and reduced the level of secreted pro-inflammatory cytokines by greater than 70% in all conditions tested (Figure 2c). Coating beads with bovine serum albumin led to a similar macrophage response as coating with streptavidin (data not shown). This result demonstrated that the ligation of immobilized CD200 to CD200R on macrophages significantly suppressed their activation, while streptavidin only partially reduced macrophage activation.

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We next examined the effect of CD200 on the inflammatory response to materials subcutaneously injected into mice. CD200-coated, streptavidin-conjugated, and plain polystyrene microbeads were injected into the subcutaneous region on the dorsal side of C57BL/6 mice. Twenty-four hours after implantation, skin tissue containing the injected microbeads were retrieved, and stained with hematoxylin and eosin (H&E). Many infiltrated cells were observed in the tissue surrounding the plain polystyrene microbeads, whereas a moderate amount of infiltrated cells were found near streptavidin-conjugated microbeads, and even fewer cells were present near CD200-coated microbeads (Figure 3a). These data were well-correlated with levels of inflammatory cytokines released by macrophages after incubation with CD200-coated microbeads *in vitro* (Figure 2c). Infiltrated cells were further characterized by immunohistochemical staining using myeloperoxidase (MPO) and F4-80 antibodies as markers of neutrohils and macrophages, respectively. Interestingly, we found that infiltrated cells surrounding polystyrene beads included both neutrophils and macrophages, but cells surrounding streptavidin-conjugated and CD200-coated beads were primarily neutrophils, not macrophages. (Figure S6).

To assess the inflammatory response in a more quantitative manner, we examined the release of reactive oxygen species (ROS) at the site of material implantation by bioluminescence imaging, as has been previously demonstrated.[26, 27] ROS are released by activated phagocytes to further recruit and activate inflammatory cells during the foreign body response, and thus ROS generation has been used as an indicator of inflammatory response to implanted biomaterials.[26, 28] In this study, luminol and lucigenin were used as complementary probes to detect reactive oxygen species released by neutrophils and macrophages. Twenty-four hours after microbead injection, bioluminescent agents were administered and animals were imaged approximately 10–20 minutes afterwards. Quantification of luminescence, or flux of photons, revealed that plain polystyrene microbeads induced the highest level of ROS, and CD200-coated microbeads elicited a significantly lower signal (Figure 3b and c). Modification with streptavidin, again, only moderately reduced ROS levels. These data further confirm the *in vitro* cytokine production and *in vivo* histological observations, and suggest that CD200 indeed lowers the level of inflammatory response when compared to uncoated or streptavidin-coated control microbeads.

This study provides a critical proof-of-concept demonstration that the immunomodulatory molecule, CD200, suppresses macrophage activation to biomaterials *in vitro* and prevents macrophage infiltration *in vivo*. This work may potentially lead to a new paradigm for materials used in biomedical implants, where materials are designed to actively modulate local immune response through specific molecular interactions with surface receptors expressed by immune cells. This strategy is also currently being explored for the delivery of nanotherapeutics.[29] We demonstrate that site-specific conjugation is important for effective immunomodulation, but translation of this technology will likely be enhanced by the discovery of small immunomodulatory molecules, for example a peptide fragment of CD200, which may have a similar inhibitory function to the full protein, but enhance the stability of the surface.[30, 31] Ultimately, the general strategy of modifying biomaterials

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with immunomodulatory molecules to evade immune response to implanted materials may improve the efficacy of numerous medical devices.

Experimental

Generation of recombinant mCD200 protein

CD200-AviTag recombinant protein was constructed in the following order (N- to C-ter mini): the extracellular domain of mouse CD200 (GenBank Accession Number AAB93980; amino acids 1–232), a 15-amino acid stiff linker sequence (SLSTPPTPSPSTPPT), an AviTag amino acid sequence (GLNDIFEAQKIEWHE) for biotinylation, and a 6x His sequence for purification. The stiff linker-AviTag fragment was generated by high-temperature annealing, followed by primer extension. Plasmids encoding the extracellular domain of mCD200 (cDNA sequence 187–882) were PCR amplified using primers and PCR condition described in Supplementary Information. The constructed gene encoding (*Hind*III)-mCD200-(*Bam*HI)-AviTag-6x His-stop-(*Xba*I) was transferred to a pEE14 expression vector, kindly provided by A. Yap (The University of Queensland, Brisbane, Australia). The final sequence of the gene was confirmed by DNA sequencing.

CHO-K.1 cells (Chinese Hamster Ovary cells, ATCC, Manassas, VA) were cultured according to published protocols[32] and described in Supplemental Information. The cells were stably transfected with plasmid DNA of pEE14 vector containing mCD200-AviTag-6x His gene and the glutamine synthase minigene as a selectable marker, using Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions. Cells containing the transfected plasmid were selected in a glutamine-free culture media with 25–50 μM L-methionine sulfoximine (Sigma).[32] Among ~10–15 colonies, a transfected CHO-K.1 cell line secreting the largest amount of mCD200-AviTag-6x His was determined by Western blot. Cells from the selected cell line were grown for 8 days in serum-free condition using chemically defined Pro CHO-AT media (Lonza, Verviers, Belgium) containing 1% HT supplement (Gibco). Supernatant containing CD200 protein was collected, filtered through a polyethylenesulfone 0.22-μm filter, and concentrated using a Pellicon XL Device and Labscale Tangential Flow Filtration system (Millipore). Concentrated supernatant was purified by applying to a HisTrap Ni column (GE healthcare, Uppsala, Sweden) at 4°C. Purified mCD200-AviTag protein was detected by Western blot after running the protein on native-PAGE, and further biotinylated by incubating with BirA enzyme at 30°C for 2 h following the manufacturer's instructions (Avidity, Aurora, CO). Protein concentration was determined using μBCA Protein Assay Kit (Thermo Scientific, Rockford, IL). One liter of medium yielded approximately 1 mg of biotinylated mCD200. Purified protein contained less than 30 EU/mg protein, as determined by the *Limulus* Amebocyte Lysate (LAL) gel clot endotoxin assay kit (GenScript, Piscataway, NJ).

Preparation of CD200-coated surfaces

Biotinylated CD200 (0–5 μg/well) was incubated in a streptavidin-coated polystyrene 96-well microplate (Thermo Scientific) at room temperature for 2 h under shaking, washed three times with Tris buffer containing 0.1% BSA and 0.05% Tween-20. The density of CD200 coated on the well was measured by incubating with a saturating concentration of a

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PE-conjugated antibody directed against the extracellular domain of CD200 (BioLegend) for 30 min at room temperature under agitation. Plates were washed thoroughly and incubated in PBS. The fluorescence intensity at 590 nm emission with 544 nm excitation was measured by a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA).

To generate CD200-coated microspheres, 20 μm-diameter carboxyl-functionalized polystyrene microspheres were purchased from Bangs Laboratories (Fishers, IN), and streptavidin (Sigma) was conjugated onto microspheres using NHS and EDC coupling chemistry. 100 mg of carboxylated microspheres were sterilized in 70% ethanol, washed by centrifugation, and resuspended in activation buffer (100 mM MES, pH 5.5). 30 mg of 1-ethyl-aminopropyl-carbodiimide (EDC, Thermo Scientific) and 45 mg of *N*-hydroxysuccinimide (NHS, Thermo Scientific) were introduced simultaneously, and incubated for 15 min at room temperature with continuous mixing. Unreacted EDC and NHS were removed by several PBS washes, and the NHS-ester-modified microspheres were combined with 0.5 mg of streptavidin dissolved in PBS and mixed for 2 h at room temperature. Remaining active NHS esters were deactivated using 30 mM ethanolamine with 0.05% BSA, and streptavidin-coated microspheres were blocked with Superblock solution (Thermo Scientific) after several washes. Finally, biotinylated CD200 solution was added to streptavidin-conjugated microspheres.

Macrophage response in vitro

Mouse C57BL/6 macrophage cells derived from bone marrow were cultured in DMEM (Gibco, Carlsbad, CA) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 10% M-CSF, 100 U/mL penicillin, and 100 μg/mL streptomycin. On day 7 of growth, cells were dislodged using cell-dissociation buffer (Gibco) after washing twice with Hank's Balanced Salt Solution (HBSS, Gibco). 1 μg of CD200-biotin per well was incubated in a streptavidin coated polystyrene microplate for 2 h at room temperature under shaking, followed by washing thoroughly with sterile PBS (Lonza). Endotoxin level released from CD200-modified surface after incubation in PBS overnight was less than 0.25 EU/mL, measured by LAL gel clot endotoxin assay. Macrophage response was examined by seeding 1×10^5 of bone marrow derived macrophages (BMDM) in culture media described above on CD200-modified, streptavidin-coated, and plain polystyrene surfaces.

For incubation with CD200-coated microbeads, 1×10^5 macrophages in culture media were seeded into each well of a 96-well polystyrene tissue culture plate, and 8×10^4 plain polystyrene, streptavidin-conjugated, or CD200-coated microspheres were added 2 h later. Number of cells and beads was counted by using a Countess® Automated Cell Counter (Invitrogen). After an additional 2 h, cells were stimulated with 0.5 ng/mL recombinant murine IFN-γ (R&D systems, Minneapolis, MN), and/or 0.05 ng/mL *E.coli* LPS (Sigma) for 18 h. Phase contrast images were acquired with an inverted microscope (Nikon Eclipse TE300) with a 20x objective to observe the cell morphology. Cell culture supernatants were collected and analyzed for secretion of pro-inflammatory cytokines, IL-6 and TNF-α, by enzyme-linked immunoabsorbent assay (ELISA) following the manufacturer's instructions (BioLegend, San Diego, CA).

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All data were normalized against cells treated with LPS and IFN-γ, unless otherwise noted. For experiments with microspheres, the background levels of cytokine secretion caused by the polystyrene tissue culture plate were relatively small (less than 20%) when compared to activation of cells by microspheres, but nonetheless the average absolute amount of secreted levels was subtracted from the overall cytokine secretion levels in order to specifically represent the activation by microspheres. Data was analyzed by using two-way ANOVA with Tukey's *post hoc* test for multiple comparisons in each condition, and $p < 0.05$ was considered statistically significant.

Biomaterial implantation, in vivo imaging and histology

All procedures involving animals were performed in accordance with UC Irvine Institute for Animal Care and Use Committee (IACUC) approved protocols. 6–8 week-old female C57BL/6 mice (Jackson Laboratories, Sacramento, CA) were anesthetized by 2–3% isoflurane inhalation. CD200-coated microbeads, streptavidin-conjugated, and plain polystyrene microbeads were injected subcutaneously on the dorsal side of mice after removal of hair and disinfecting the skin surface. Each injection contained 100 μL of 20% (w/v) particle suspension in PBS. For tissue harvest and histology processing, mice were euthanized and skin samples containing the injected microparticles were excised and fixed in 10% formalin solution, embedded in paraffin blocks, sectioned and stained with hematoxylin and eosin (H&E) by AML Laboratories (Baltimore, MD). The histology slides were imaged using a microscope (Nikon Eclipse E800) equipped with a 40X objective and an Olympus camera.

For bioluminescence imaging, luminol (5-amino-2,3-dihydro-1,4-phthlazinedione, purchased from Alfa Aesar, Ward Hill, MA) and lucigenin (bis-N-methylacridinium nitrate, Sigma) were used as complementary probes to detect reactive oxygen species released by neutrophils and macrophages. 50 mg/mL of luminol and 5 mg/mL of lucigenin stock solutions were prepared in sterile PBS prior to injection. Solutions (100 μL) were i.p. injected into mice, and animals were imaged using an IVIS imaging system (Caliper Life Sciences, Hopkinton, MA) every 5 minutes after luminol/lucigenin injection with a 1 min exposure time. Approximately 10–20 minutes after injection was determined to be the time-point for peak luminescence[26]. The images were analyzed with Living Image software (Caliper Life Sciences) and the total photon flux was quantified for each bead type. One-way ANOVA was used, followed by Tukey's *post hoc* test to compare bioluminescence generated at each injection site, and $p < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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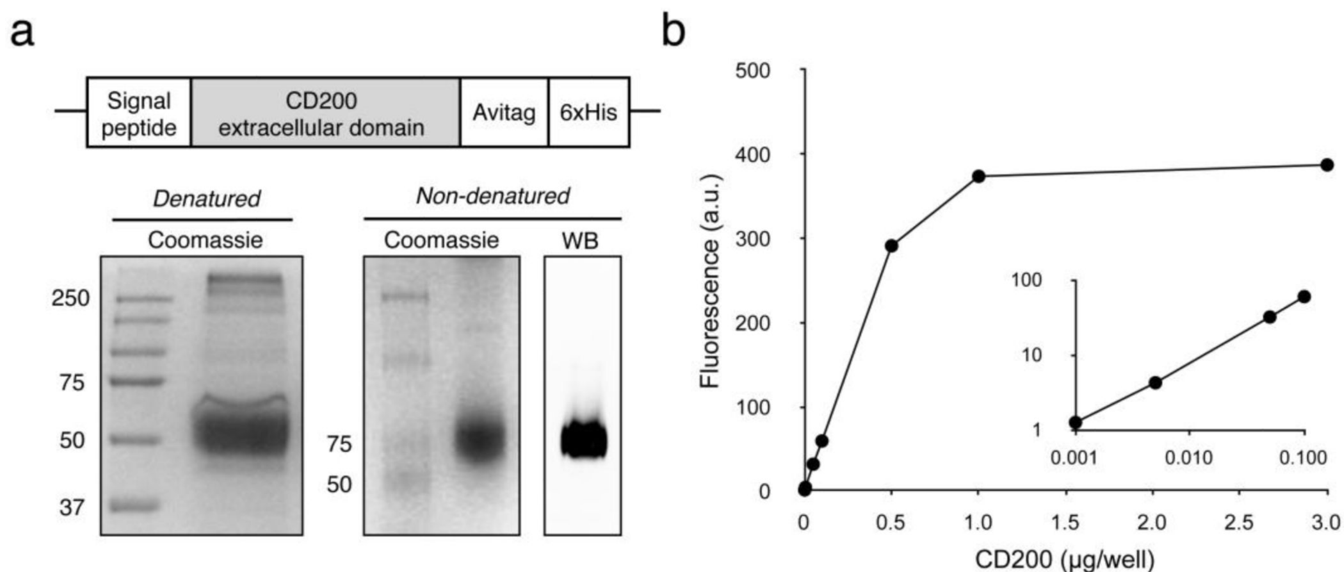

Figure 1.

Generation and characterization of CD200-modified surfaces. (a) Schematic of construct used for soluble expression of extracellular CD200 protein (top). Coomassie-blue stained SDS-PAGE (left) and native PAGE (middle) gels, and Western blot analysis (right) of purified recombinant CD200 product (bottom). (b) Binding profile of CD200 immobilized on the streptavidin-coated polystyrene plate, measured with PE-labeled anti-mCD200.

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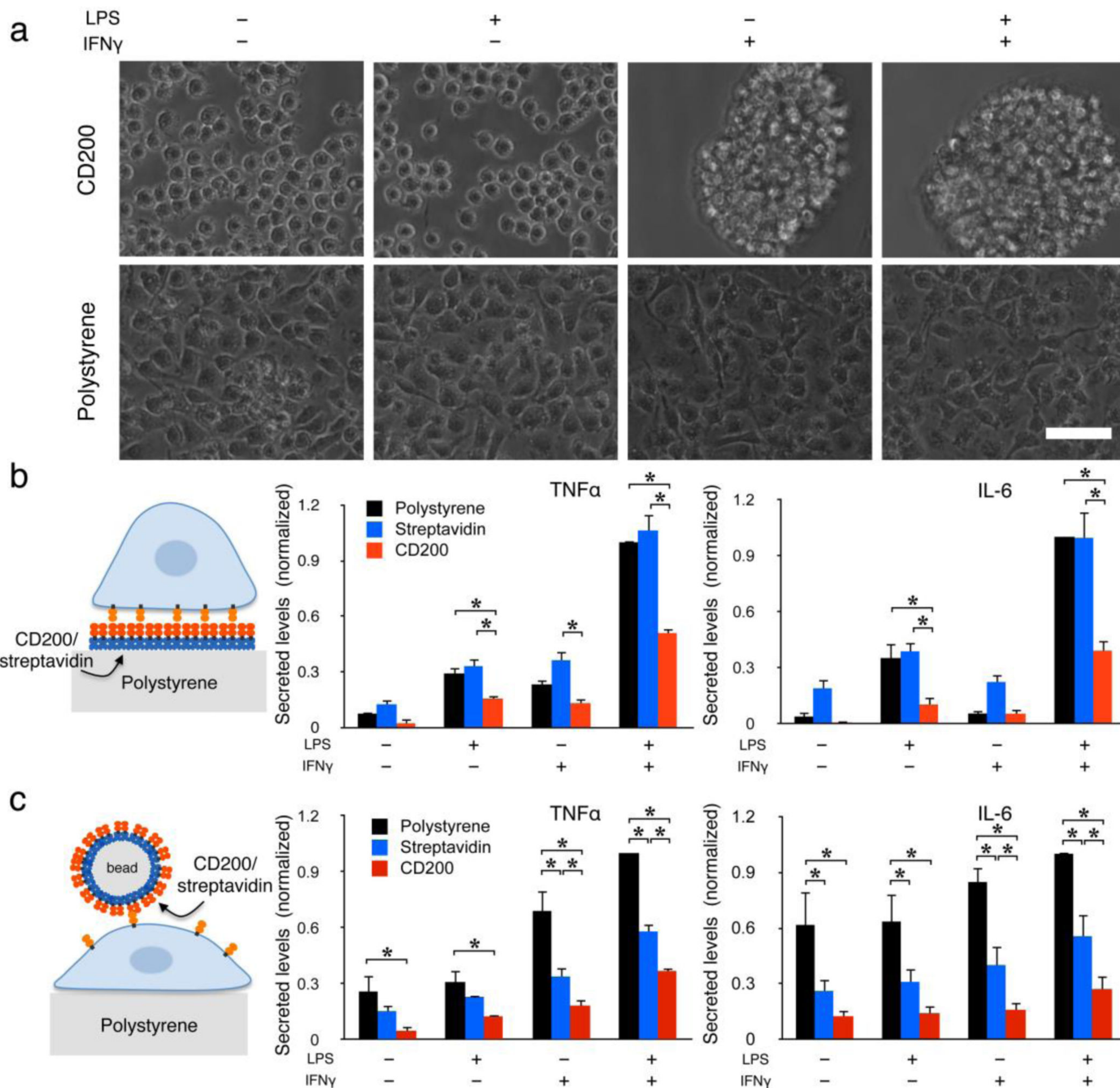

Figure 2.

In vitro characterization of the inflammatory response to CD200-modified surfaces. (a) Representative phase contrast images of bone marrow derived macrophages (BMDM) seeded on mCD200-modified surfaces, or plain polystyrene surfaces, followed by stimulation with IFN-γ (0.5 ng/mL) and/or LPS (0.05 ng/mL). Scale bar 20 μm. (b) Quantitative analysis of secreted pro-inflammatory cytokines (TNF-α and IL-6) by ELISA. CD200 immobilization on the polystyrene surface significantly inhibited macrophage activation with reduced level of pro-inflammatory cytokines. Secreted amounts were normalized to BMDM seeded on polystyrene and stimulated with IFN-γ and LPS. (c) Quantitative analysis of secreted pro-inflammatory cytokines (TNF-α and IL-6) of BMDMs

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incubated with CD200-coated, streptavidin-conjugated, and plain polystyrene 20-μm microbeads, and stimulated with IFN-γ and/or LPS. Error bars indicate standard error of the mean across three independent experiments. * $p < 0.05$ as determined by two-way ANOVA with Tukey's *post hoc* test for multiple comparisons in each condition.

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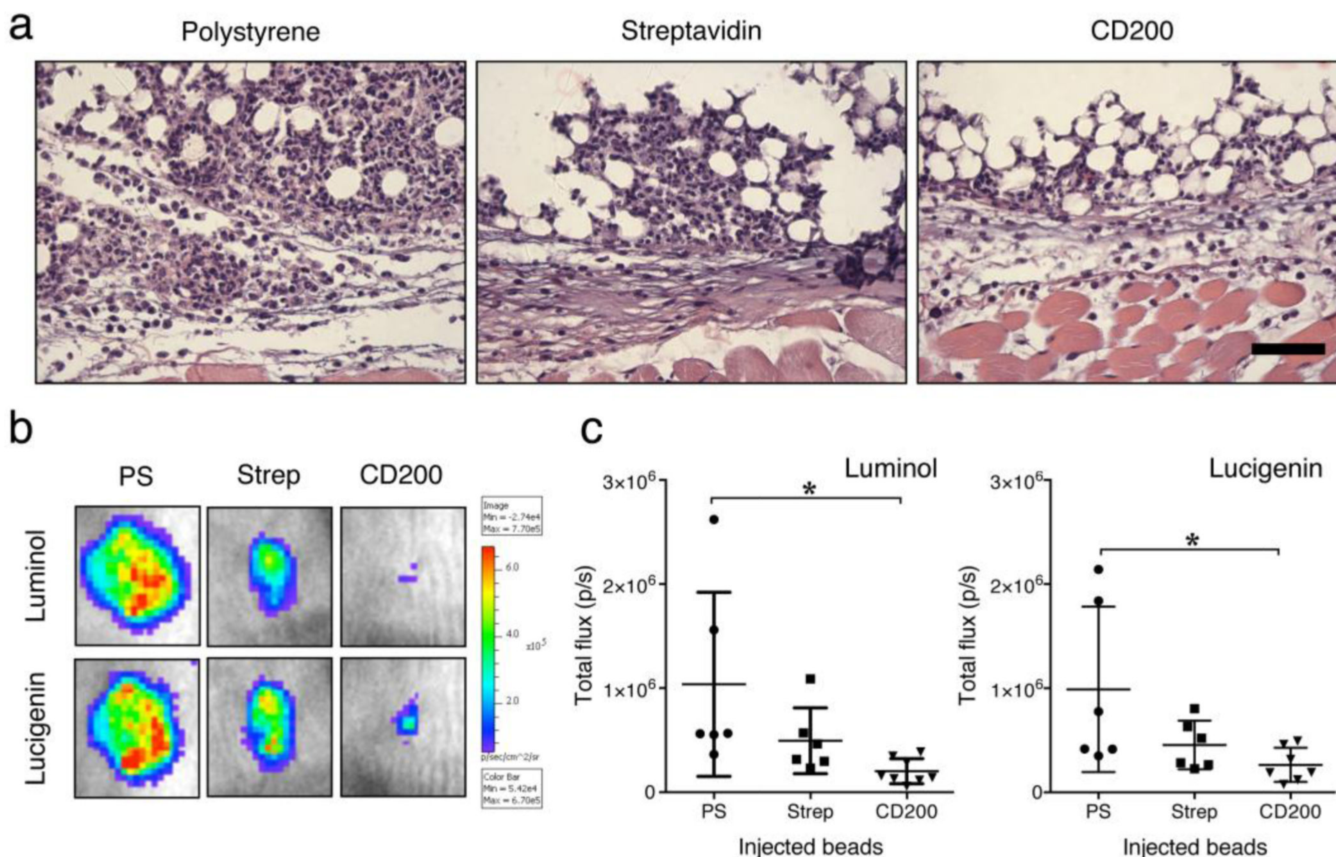

Figure 3.

In vivo characterization of the inflammatory response to CD200-modified surfaces. (a) Representative sections of skin tissue containing subcutaneously injected CD200-coated, streptavidin-conjugated and plain polystyrene microbeads, stained with H&E. Scale bar 50 μm. (b) Representative images of luminol and lucigenin bioluminescence generated at each injection site 1 day after microsphere implantation. (c) Quantification of total photon flux in at least 5 animals for each bead type. * $p < 0.05$ as determined by one-way ANOVA with Tukey's *post hoc* test.

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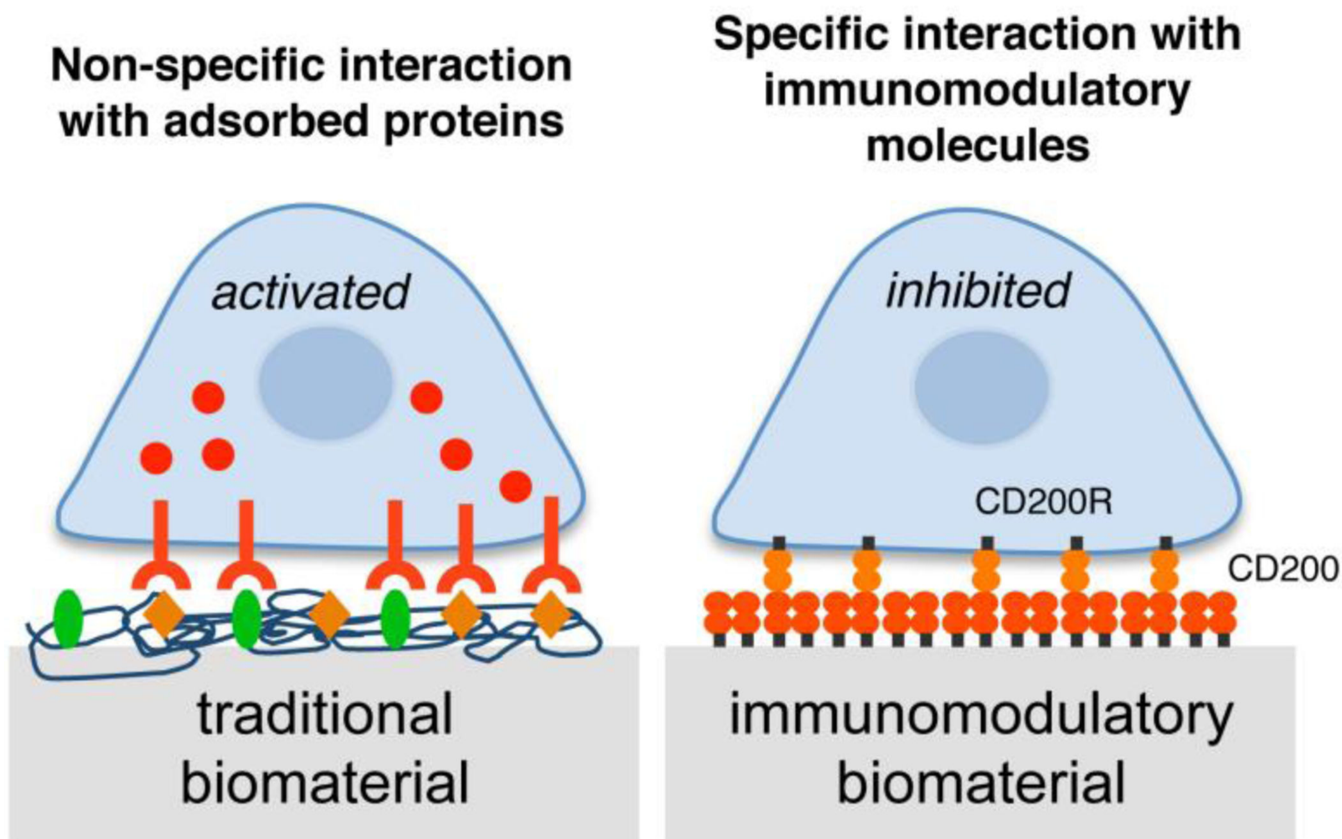

Scheme 1.

The concept of conferring immune-inhibitory properties using CD200-coated surfaces. Traditional biomaterials non-specifically adsorb proteins, which activate immune cells. Immunomodulatory biomaterials displaying CD200 will bind to CD200R and activate inhibitory pathways to prevent immune cell activation.