Foreign Body Giant Cell Formation Is Preceded by Lamellipodia Formation and Can Be Attenuated by Inhibition of Rac1 Activation

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Macrophages that are recruited to the site of implanted biomaterials undergo fusion to form surface-damaging foreign body giant cells. Exposure of peripheral blood monocytes to interleukin-4 can recapitulate the fusion process *in vitro*. In this study, we used interleukin-4 to induce multinucleation of murine bone marrow-derived macrophages and observed changes in cell shape, including elongation and lamellipodia formation, before fusion. Because cytoskeletal rearrangements are regulated by small GTPases, we examined the effects of inhibitors of Rho kinase (Y-32885) and Rac activation (NSC23766) on fusion. Y-32885 did not prevent cytoskeletal changes or fusion but limited the extent of multinucleation. NSC23766, on the other hand, inhibited lamellipodia formation and fusion in a dose-dependent manner. In addition, we found that in control cells, these changes were preceded by Rac1 activation. However, NSC23766 did not block the uptake of polystyrene microspheres. Likewise, short interfering RNA knockdown of Rac1 limited fusion without limiting phagocytosis. Thus, phagocytosis and fusion can be partially decoupled based on their susceptibility to NSC23766. Furthermore, poly(ethylene-co-vinyl acetate) scaffolds containing NSC23766 attenuated foreign body giant cell formation *in vivo*. These observations suggest that targeting Rac1 activation could protect biomaterials without compromising the ability of macrophages to perform beneficial phagocytic functions at implantation sites. (*Am J Pathol* 2007, 171:632–640; DOI: 10.2353/ajpath.2007.061213)

body response, and is especially prevalent where phagocytosis is an insufficient primary mechanism of material degradation.^{1,2} These giant cells are the product of macrophage-macrophage fusion, a process that remains mechanistically undefined.^{3,4} Because cell to cell fusion is a critical event in several biological processes such as fertilization, bone remodeling, muscle development, and stem cell differentiation, the topic has received significant interest.^{5,6} Biomaterial-induced FBGC formation and the associated fibrous encapsulation result in a physical walling off effect that enhances material degradation and prevents appropriate molecular transport and vascularization.^{1,7,8}

We have recently described a requirement for the chemokine CCL2/MCP-1 in the formation of FBGCs.⁹ Specifically, we have shown limited FBGC formation *in vivo* in MCP-1-null mice and after the addition of MCP-1 blocking antibody and peptides to interleukin (IL)-4-treated human peripheral blood monocytes *in vitro*. However, we were unable to determine whether MCP-1-null macrophages were inherently deficient in fusion. In the present study, we describe the preparation of murine bone marrow-derived macrophages and the establishment of the IL-4 fusion assay using these cells. Furthermore, we confirmed the relevance of the assay by demonstrating an inherent fusion defect in MCP-1-null macrophages.

Previous studies using human peripheral blood monocytes have shown that these cells undergo significant cytoskeletal rearrangement during IL-13-induced fusion.¹⁰ Specifically, it was shown that, in comparison to untreated macrophages, more F-actin was located in the immediate proximity of the ventral plasma membrane of FBGCs. Formation of podosome-like structures has also been reported in the murine macrophage cell line 264.7

Supported by the National Institutes of Health (grant GM 072194-01 to T.R.K.).

Accepted for publication April 25, 2007.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

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Foreign body giant cell (FBGC) formation is a hallmark of the host response to biomaterials, known as the foreign

exhibiting FBGC formation on highly polished pure titanium.² Limited fusion has also been reported in the murine macrophage cell line IC-21.¹¹ However, these and several other macrophage cell lines were found to be poorly fusogenic in response to IL-4 (S.M.J., E.S., and T.R.K., manuscript in preparation). Changes in macrophage shape and formation of cell protrusions have also been reported *in vivo* after the implantation of cellophane films in rats.¹²

Although the critical signaling events leading to FBGC formation via macrophage-macrophage fusion are unknown, there are shared features with phagocytosis.¹³ Specifically, the involvement of the phagocytic cellular machinery, including vacuolar-type ATPase and calcium-independent phospholipase A2, has recently been shown to be critical for fusion.¹³ In phagocytosis, macrophages use opsonic (Fc γ R, C3) and nonopsonic (C-type lectins) receptors to phagocytose microbes and particles.^{14,15} Engulfment of particles through the C3 receptor occurs through a direct sinking of the plasma membrane and is morphologically distinct from Fc γ R-mediated phagocytosis, which involves pseudopod extension. Interestingly, IL-4-induced FBGC formation has been shown to involve pseudopod formation as well as direct sinking of the plasma membrane of one cell to another.^{13,16} In addition, similar to phagocytosis, actin polymerization and reorganization are observed during macrophage fusion and, when inhibited, prevent fusion and subsequent FBGC formation.¹⁷

In the present study, we expanded on these observations by identifying lamellipodia formation as a key feature of the IL-4-induced cytoskeletal changes in macrophages. Consistent with the role of Rac1 in this process, we detected IL-4-induced Rac1 activation before changes in cell shape. By using pharmacological inhibitors of small GTPases and a Rac1-specific short interfering RNA (siRNA), we show that inhibition of Rac activation or Rac1 knockdown can prevent lamellipodia formation and fusion without interfering with the ability of macrophages to uptake polystyrene microspheres. Previous studies have shown the efficient knockdown of Rac1 via siRNA.¹⁸ In addition, the specificity and efficacy of NSC23766 in preventing the activation of Rac1 *in vitro* and *in vivo* has been established.^{19,20} Here, we also demonstrated that localized release of NSC23766 attenuated FBGC formation *in vivo*.

Materials and Methods

Cell Lines and Primary Macrophages

Mouse macrophages were obtained from the bone marrow of C57BL6 mice (The Jackson Laboratories, Bar Harbor, ME). Selected studies were performed with macrophages derived from mice lacking MCP-1 (C57BL6). Marrow was flushed from the femurs of mice and collected in Iscove's modified Dulbecco's medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum and penicillin/streptomycin/fungizone. Cells were layered over Lympholyte-M (Accurate

Chemical, Westbury, NY) and centrifuged according to the supplier's instructions. The fraction containing mononuclear cells was collected and plated (2.5×10^6 cells/ml) in expansion medium [Iscove's modified Dulbecco's medium supplemented with 20% fetal bovine serum, L-glutamine, penicillin/streptomycin/fungizone, and 1.5 ng/ml huM-CSF (R&D Systems, Minneapolis, MN), and 100 ng/ml flt-3 ligand (R&D Systems)]. Cultures were fed on day 5, and cells were collected by scraping on day 10 and used for analysis. In general, three mice per genotype were used in each isolation procedure.

In Vitro Fusion Assay, Rac1 Activation, and Small GTPase Inhibition

Expanded monocytes/macrophages were plated at 1×10^6 cells per well in nontissue culture-treated polystyrene 24-well plates in expansion media lacking M-CSF and flt-3 ligand and supplemented with 10 ng/ml recombinant mouse GM-CSF (R&D Systems) and 10 ng/ml recombinant mouse IL-4 (R&D Systems). Media were changed at days 3 and 5, and fusion was analyzed on day 7. Three parameters of fusion were quantified including percent fusion, number of FBGCs per high-power field, and number of nuclei per FBGC. Three 24-wells per group were analyzed and the experiments were performed at least in triplicate. Each experiment was repeated with a newly expanded monocyte preparation. Three to six images per well were collected and analyzed allowing for the analysis of a total of 25 to 50 images per group as indicated in the figure legends.

Rac1 activity was determined in protein lysates using the luminescence-based Rac1 G-LISA activation assay (cytoskeleton) according to the manufacturer's instructions. Rac1 inhibitor NSC23766 (Calbiochem, La Jolla, CA) was added to wells at a concentrations varying from 50 to 200 μ mol/L, at the initiation of fusion and during scheduled feedings. A concentration of 100 μ mol/L was found to be optimal for inhibition of Rac1 activation. Rho kinase inhibitor, Y-32885 also known as Y-compound (Sigma, St. Louis, MO), was added to wells at concentrations varying from 5 to 50 μ mol/L, at the initiation of fusion and during scheduled feedings. A concentration of 10 to 30 μ mol/L was found to be optimal for Rho kinase inhibition. Higher doses had a deleterious effect on cells. siRNA transfections of expanded monocytes were performed using Lipofectamine (Invitrogen, Carlsbad, CA) and 50 or 100 nmol/L *mRac1* (5'-CAGACAGACGUGUUCUAAUUUGCU-3') or a 100 nmol/L scrambled control siRNA with no known target sequence (both from Invitrogen). Knockdown efficiency was determined 24 hours after transfection by Western blot using anti-Rac1 antibody (clone 23A8; Upstate Technology, Lake Placid, NY).

Phagocytosis and Rac1 Inhibition

Phagocytosis was induced by the addition of Fluoresbrite YG microspheres with a 3- μ m diameter (Polysciences, Warrington, PA) at sphere:cell ratios of 1:1, and 10:1, as

noted. Some cultures were treated with IL-4 and exposed to beads in the presence or absence of 10 $\mu\text{mol/L}$ NSC23766 to evaluate the effect of the inhibitor on phagocytosis and fusion. To evaluate whether there was a higher threshold for inhibition of phagocytosis by NSC23766, the inhibitor was added to wells at various concentrations as described above. Uptake was evaluated at 1 to 3 hours by phase microscopy or at 7 days by fluorescence microscopy. To facilitate comparisons, the results are expressed as number of microspheres per nucleus by dividing the number of beads by the number of nuclei in multinucleated cells.

Cell Staining

For counting and morphological analysis, cells were double-stained with May-Grunwald stain (Sigma) and Wright-Giemsa stain (Sigma) according to standard protocols. All experiments were done in triplicate, and a total of 25 to 50 high-power fields were analyzed for each group. For visualization of the acting cytoskeleton and nuclei, cells were fixed in 4% paraformaldehyde (J.T. Baker, Phillipsburg, NJ) for 20 minutes at room temperature and stained with rhodamine-phalloidin and 4,6-diamidino-2-phenylindole according to standard protocols. All wells were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and examined with the aid of an Axiovert 200M microscope (Carl Zeiss, Thornwood, NY) equipped with fluorescent optics. To confirm the uptake of polystyrene spheres in the presence of the Rac1 inhibitor, 0.9- μm -thick optical sections were obtained and analyzed. To analyze the effect of Rac inhibition on the uptake of microspheres, the percentage of cells containing microspheres per high-power field and the number of microspheres per cell were counted.

Fabrication of NSC23766-Loaded EVAc Scaffolds

Poly(ethylene-co-vinyl acetate) (EVAc) scaffolds were prepared via a solvent evaporation method, as described previously.²¹ In brief, 92 mg of Ficoll (M_n , 70,000) were mixed with 8 mg of NSC23766 in DI H_2O and lyophilized into a powder. The resultant powder was ground via mortar and pestle to facilitate homogenous distribution into the EVAc solution. A 100-mg/ml solution of EVAc in methylene chloride was prepared, of which 1 ml was mixed extensively with the fine powder, with the resulting suspension being poured into a chilled Teflon mold and cooled at -80°C for 30 minutes. The mold was then further chilled at -20°C for 48 hours and dried under a vacuum at 0°C for an additional 48 hours to enable solvent evaporation. The dried EVAc was then cut into small disks ($\sim 3\text{-mm}$ diameter, $\sim 2\text{-mm}$ thickness, 11.19 ± 1.07 mg) with a cork borer and sterilized under UV light for 20 minutes. Control disks were made with 100 mg of Ficoll.

Figure 1. Bone marrow-derived murine macrophages display efficient fusion. Representative phase-contrast images of fusion in C57BL/6 wild-type (A) and MCP-1-null (B) primary macrophages are shown. A: Primary wild-type macrophages displayed robust fusion by forming numerous and large multinucleated cells (arrows). B: MCP-1-null macrophages displayed a reduction in fusion and formed smaller multinucleated cells (arrows). Quantification of fusion shows reduced overall fusion in MCP-1-null cells (C, measured as percentage of cells with three or more nuclei), reduced number of FBGCs (D), and reduced number of nuclei per FBGC (E). $n = 50$; mean $\pm$ SD; * P value ≤ 0.05 . Original magnifications, $\times 100$.

Implantation of Biomaterials

All procedures were performed in accordance with the regulations adopted by the National Institutes of Health and approved by the Animal Care and Use Committee of Yale University. Subcutaneous implantations were performed essentially as described previously.⁹ A total of six control mice (C57BL/6), age 3 to 4 months, were used for implantations. Each mouse received one control and one experimental implant that were placed at least 2 cm apart, allowing for six implants per group. Implants were excised *en bloc*, at 2 weeks after implantation, as described previously and processed for histological and immunohistochemical analysis.⁹

Results

IL-4 and GM-CSF Induce Fusion in Marrow-Derived Murine Macrophages

Macrophages were expanded from the bone marrow of wild-type mice and induced to fuse into multinucleated giant cells through concurrent addition of GM-CSF and IL-4. Under baseline conditions, fusion was determined as the percentage of nuclei that were part of multinucleated cells and was found to be $75.5 \pm 16.7\%$. To validate the use of marrow-derived macrophages in the fusion assay system we prepared and examined the fusion of cells derived from mice that lack MCP-1. Previously, we have shown that FBGC formation *in vivo* was compromised in these mice and that inhibition of MCP-1 activity limited the fusion of human monocytes.⁹ Consistent with our previous findings, the fusion of MCP-1-null macrophages was reduced in comparison to cells prepared from littermate control mice (Figure 1). Overall fusion was estimated to be 78.9 ± 12.4 in

Figure 2. Cell elongation and lamellipodia formation precede fusion. Mouse macrophages were cultured in the presence (**B** and **C**) or absence (**A** and **D**) of a fusogenic cocktail (IL-4, GM-CSF) and monitored for changes in cell shape. **B:** At 48 hours, microscopic analysis of cells by phase contrast indicated the presence of numerous elongated cells in the treatment group (**arrows**). **C:** Staining of the actin cytoskeleton (phalloidin) and nuclei (4,6-diamidino-2-phenylindole) revealed the presence of peripheral actin in untreated cells. **D:** Induction of fusion caused a significant rearrangement of the actin cytoskeleton with loss of actin rings, accumulation of punctate actin at the edges of cells (**arrows**), and extensive formation of lamellipodia (**inset** shows enlargement of area denoted by square). Original magnifications, $\times 400$.

control macrophages and $31.8 \pm 7.36\%$ in MCP-1-null macrophages ($P \leq 0.05$). In addition to a reduction in overall fusion, MCP-1-null macrophages formed less and smaller FBGCs containing a reduced number of nuclei (Figure 1).

Cell-Shape Changes and Lamellipodia Formation Are Early Fusion-Associated Events

Changes in cell shape in macrophages that were induced to fuse were evident as early as 24 hours and became prominent at 48 hours after addition of the fusion cocktail (Figure 2B). Visualization of the actin cytoskeleton with rhodamine-phalloidin confirmed the loss of peripheral actin rings that were present in control cells (Figure 2, C and D). Dramatic elongation was characteristic of cells that appeared in the process of fusion (Figure 2D). In addition, assembly of punctate actin in a polarized manner was observed in multinucleated cells (Figure 2D). These results are consistent with reported observations on the fusion of human monocytes.¹⁰ Mouse macrophages also displayed extensive lamellipodia formation at 48 hours, which was predominantly a feature of single cells in the vicinity of FBGCs, even though occasionally FBGCs were seen to have such structures (Figure 2D).

Fusion and Small GTPases

Small GTPases have a well-established role in regulating cytoskeletal rearrangements.²² Consistent with this function, we detected an increase in activation of Rac1 after exposure to the fusogenic stimulus (Figure 3A). Furthermore, application of the small molecular weight and highly specific inhibitor of Rac activation (NSC23766) for 7 days limited the fusion of macrophages (Figure 3). All three parameters of fusion, including percent fusion (Figure 3E), the number of FBGCs (Figure 3F), and the num-

ber of nuclei per FBGC (Figure 3G) were decreased by treatment with $50 \mu\text{mol/L}$ NSC23766. Visualization of the cell cytoskeleton by rhodamine-phalloidin at earlier time points, ie, 2 and 4 days, indicated that cells treated with NSC23766 underwent minimal elongation and did not form lamellipodia (Figure 3, B and D). In addition, the activation of Rac1 was decreased below baseline levels (Figure 3A). The response to the inhibitor seemed to be dose-dependent because cells treated with $50 \mu\text{mol/L}$ NSC23766 exhibited more fusion than cells treated with $100 \mu\text{mol/L}$ (Figure 3, E–G). Treatment of cells with $200 \mu\text{mol/L}$ NSC23766 did not reduce fusion further (data not shown).

To confirm the specificity of the requirement for Rac1 activation in the fusion process, we repeated the experiments in the presence of Y-32885, a specific inhibitor of the Rho kinase. A dose-response experiment indicated that this reagent was effective in the 5 to $30 \mu\text{mol/L}$ range. At $50 \mu\text{mol/L}$, we observed morphological abnormalities in cells consistent with cell death and a reduction in the number of cells (not shown). Macrophage fusion was slightly reduced ($59.9 \pm 8.8\%$ in $5 \mu\text{mol/L}$ and $56.7 \pm 7.1\%$ in $10 \mu\text{mol/L}$) but was much higher in comparison to cultures treated with NSC23766. Interestingly, at 10 to $30 \mu\text{mol/L}$ Y-32885 FBGC formation was altered, evidenced by the formation of more but smaller FBGCs (Figure 4). Cell elongation was observed in all IL-4-induced cells treated with Y-32885, suggesting that the process is not dependent on the function of Rho kinase.

Inhibition of Rac1 Does Not Prevent the Uptake of Microspheres

Because lamellipodia formation has been shown to play a role in some aspects of phagocytosis,²³ we examined whether NSC23766 could inhibit both processes. Application of NSC23766 to macrophage cultures that were induced to fuse in the presence of polystyrene micro-

Figure 3. Knockdown and inhibition of Rac1 activation limits fusion. Mouse macrophages were cultured for 7 days in the presence of fusogenic cocktail (IL-4, GM-CSF) and in the presence (**B** and **D**) or absence (**C**) of 50 $\mu\text{mol/L}$ Rac1 inhibitor (NSC23766). **A:** Rac1 activation was determined by G-LISA at 20 minutes after treatment and found to be induced by the fusogenic cocktail and inhibited by NSC23766. **B:** A phase-contrast representative image shows the presence of fewer and smaller FBGCs in the presence of the inhibitor (**arrow**). Visualization of the actin cytoskeleton with rhodamine-phalloidin revealed the presence of actin rings in cells treated with the inhibitor (**D, arrow**) and the presence of elongated cells in control wells (**C, arrowheads**). Quantification of fusion was performed by analysis of images double-stained with May-Grunwald and Wright-Giemsa and indicated reduced fusion (**E**), reduced number of FBGCs (**F**), and reduced number of nuclei per FBGC (**G**) in a dose-dependent manner ($n = 25$; mean $\pm$ SD; $*P$ value ≤ 0.05). Original magnifications: $\times 100$ (**B**); $\times 400$ (**C** and **D**).

spheres indicated that even though fusion was limited, the uptake of spheres was not compromised (Figure 5). Examination of cultures shortly after addition of microspheres (1 to 3 hours) revealed similar uptake in control and NSC23766-treated cells (Figure 5, A and B). At 7 days, in the absence of the Rac1 inhibitor, giant cell formation and uptake of beads were evident (Figure 5C). As expected, limited lamellipodia formation was observed in cells treated with NSC23766, but it did not have an effect on the uptake of microspheres (Figure 5D). To confirm the intracellular location of the microspheres in cultures treated with NSC23766, we obtained 0.9- μm -thick optical sections that included both nuclei and spheres confirming the intracellular location of the latter (Figure 5E). To exclude the possibility that the lack of an effect on phagocytosis was attributable to a difference in dose sensitivity, we repeated the experiment in the pres-

ence of varying concentrations of NSC23766. No differences in microsphere uptake, measured as percentage of cells containing microspheres and number of microspheres per nucleus, were observed (Figure 5, E and F). Consistent with the notion that fusion and phagocytosis are distinct processes, we observed that MCP-1-null macrophages were not compromised in their ability to uptake microspheres (Figure 5F). Taken together, these observations suggest that monocyte fusion and the uptake of microspheres are mediated by distinct intracellular mechanisms.

siRNA Knockdown of Rac1 Attenuates Fusion

To confirm further the involvement of Rac1 in macrophage fusion, we used a Rac1-specific siRNA approach.

Figure 4. Y-compound, inhibitor of Rho kinase, limits FBGC formation. Mouse macrophages were cultured for 7 days in the presence of fusogenic cocktail (IL-4, GM-CSF) and in the presence of 5 (**A**), 10 (**B**), or 30 (**C**) $\mu\text{mol/L}$ Rho kinase inhibitor (Y-32885). May-Grunwald- and Wright-Giemsa-stained representative images show the presence of FBGCs (**A–C, arrows**). Cell elongation could be observed at all three concentrations (**A–C, arrowheads**). **D** and **E:** Y-32885 limits formation of large FBGCs. **E** and **F:** Quantification of fusion, measured as the number of cells with three or more nuclei, was performed by analysis of digital images and showed that the Y-compound limited the expansion of FBGCs, indicated by the formation of numerous cells with a small number of nuclei. $n = 45$. $*P$ value ≤ 0.05 . Original magnifications, $\times 100$.

Figure 5. Inhibition of Rac1 activation during fusion does not prevent phagocytosis. Wild-type (A–E) and MCP1-null (F) macrophages were cultured for 7 days in the presence of fusogenic cocktail (IL-4, GM-CSF) and in the presence (B, D, and E) or absence (A, C) of 50 µmol/L Rac1 inhibitor (NSC23766) and fluorescein isothiocyanate-polystyrene microspheres at a 1:1 (A–D) or 10:1 (E and F) sphere/cell ratio. Representative phase-contrast images at 3 hours of culture (A and B) and images double-stained with May-Grunwald and Wright-Giemsa (F) or rhodamine-phalloidin and 4,6-diamidino-2-phenylindole (C–E) at 7 days are shown. Normal uptake of microspheres was observed at 3 hours (A and B, arrows) even though phagocytosis was not complete (arrowheads point to free microspheres). At 7 days, uptake of microspheres in single cells (D) and FBGCs (C) was evident in all. E: To confirm the uptake of microspheres by cells in the presence of the inhibitor, 0.8-µm-thick optical sections at the level of the nucleus were obtained. F: Uptake of microspheres was complete in MCP1-null macrophages. G and H: NSC23766 does not prevent phagocytosis. Microspheres were added to macrophages at a ratio of 1:1 in varying concentrations of NSC23766, and uptake was visualized in cultures double-stained with May-Grunwald and Wright-Giemsa. Quantification of microsphere uptake by macrophages, measured as percentage of cells containing microspheres (E) and the number of microspheres per nucleus (F), indicated similar levels in all concentrations of NSC23766 ($n = 50$, mean $\pm$ SD). Original magnifications: $\times 200$ (A, B, and F); $\times 400$ (C, D, and E).

Treatment of cells with 50 or 100 nmol/L mRac1 siRNA resulted in significant reduction in total Rac1 in comparison to cells treated with 100 nmol/L scrambled control siRNA (Figure 6A). Addition of IL-4 induced robust fusion in control-treated cells, whereas fusion was limited in cells treated with mRac1 siRNA (Figure 6, B and C). Analysis of fusion indicated a reduction in all three parameters of fusion in both 50- and 100-nmol/L-treated cells (Figure 6, E–G). Overall, fusion was limited to an extent that was similar to that observed in cultures treated with NSC23766. To determine whether Rac1 knockdown influenced phagocytosis, we exposed mRac1 siRNA-treated cultures to microspheres and monitored their uptake from 1 hour to 7 days. Consistent with our findings with NSC23766, we found that phagocytosis was not compromised in mRac1 siRNA-treated cells (Figure 6D).

Inhibition of FBGC Formation in Vivo by Prolonged Release of NSC23766

NSC23766-eluting EVAc scaffolds were prepared and the release of the drug was evaluated *in vitro* and determined to be in the range of 5 µg/mg of scaffold and was sustained for at least 18 days (Supplemental Figure S1 at <http://ajp.amjpathol.org>). EVAc scaffolds with or without NSC23766 were implanted subcutaneously in wild-type mice for 2 weeks and the formation of FBGCs was evaluated. A significant reduction in the number of FBGCs surrounding NSC23766-eluting implants in comparison to control implants was observed. Macrophage recruitment, assessed by immunohistochemistry with Mac3 antibody (Figure 7, C and D), and the overall foreign body response, assessed by H&E staining (Figure 7, A and B),

Figure 6. siRNA knockdown of Rac1 limits fusion. Macrophages were transfected with either scrambled (control) or Rac1-specific siRNA for 24 hours. **A:** Western blot analysis revealed reduced total Rac1 in protein lysates from cells treated with 50 or 100 nmol/L mRac1 siRNA. Cultures treated with either control (**B**) or mRac1 siRNA (**C**) were induced to fuse for 7 days and visualized by May-Grunwald and Wright-Giemsa stain. **C:** Numerous round cells (**arrows**) and limited fusion were observed in mRac1 siRNA-treated cultures. Quantification confirmed reduction in fusion (**E**), number of FBGCs (**F**), and number of nuclei per FBGC (**G**) ($n = 50$; mean $\pm$ SD; * P value ≤ 0.05). **D:** mRac1 siRNA did not prevent the uptake of microspheres (**arrows** point to cells containing microspheres). Original magnifications: $\times 100$ (**B** and **C**); $\times 400$ (**D**).

was similar between the two groups. The lack of an association between FBGC formation and the foreign body response is consistent with our previous observations in MCP-1-null mice in which we observed a reduced number of FBGCs but normal biomaterial encapsulation.⁹

Discussion

IL-4-induced formation of multinucleated giant cells at the surface of biomaterials *in vitro* has been extensively investigated in human monocytes. However, similar induction in macrophage cell lines and mouse cells has been limited.^{24–26} In the present study, we characterized fusion in mouse bone marrow-derived macrophages. These cells displayed robust fusion at a rate that exceeded that previously reported for human peripheral blood monocytes.^{9,27} Perhaps this reflects the more fusogenic state of the mouse cells; a relationship between monocyte and macrophage maturation and fusion has been shown.²⁸ The use of mouse bone marrow cells in the IL-4-induced fusion assay was reported previously, but fusion was low ($\sim 20\%$), and the majority of the cells were shown to have less than five nuclei.²⁵ We believe that the high level of fusion we observe in our cultures is attributable to the fact that we isolated and expanded an enriched monocyte population before inducing fusion. More importantly, the establishment of a murine macrophage fusion assay should allow for the examination of cells derived from genetically modified animals. Such studies can be performed without the need for molecular

Figure 7. Localized inhibition of Rac1 activation limits FBGC formation *in vivo*. Sections of EVAc scaffolds with (**B** and **D**) and without (**A** and **C**) NSC23766 implanted subcutaneously in wild-type mice for 2 weeks were stained with H&E (**A** and **B**) and Mac3 Ab (**C** and **D**) and visualized with the peroxidase reaction (brown color). **Arrows** indicate the presence of FBGCs in mice that received control scaffold (**A** and **C**). Despite the paucity of FBGCs in mice that received drug-eluting scaffolds, the recruitment of inflammatory cells (**B**, **arrows**), including macrophages (**D**, **arrows**) was not affected. Nuclei are visualized with methyl green counterstain (**C** and **D**). **E:** NSC23766 causes reduction in FBGC formation *in vivo*. The number of FBGCs (cells with three or more nuclei) per unit length of scaffold was estimated by examination of H&E- and Mac3-stained sections ($n = 30$; mean $\pm$ SD; * P value ≤ 0.05). **F:** Normal encapsulation of EVAc scaffolds. Capsule thickness was measured at various points around the scaffolds from calibrated digital images ($n = 30$, mean $\pm$ SD). Original magnifications, $\times 400$.

or pharmacological inhibitors and can lead to the elucidation of additional genetic and molecular determinants of fusion. To verify the utility of the assay, we present evidence for an inherent fusion deficiency in macrophages derived from MCP-1-null mice, previously shown to display reduced FBGC formation *in vivo*.⁹ Because multinucleated FBGCs and osteoclasts are formed from the fusion of monocyte-derived cells, it will also be possible and informative to test cells from mice with known osteoclast defects for their ability to form FBGCs. Such an approach has identified a critical role for dendritic cell-specific transmembrane protein (DC-STAMP) in the formation of both cell types.²⁹

Previous studies investigating fusion have noted the occurrence of cytoskeletal changes similar to those observed in phagocytosis.^{13,30} Although the rearrangement of the cell's cytoskeleton is an important component of both phagocytosis and fusion, the role of lamellipodia formation in fusion has not been previously reported. Consistent with the vital role of Rac1 in lamellipodia formation,³¹ we observed Rac1 activation in IL-4-treated cells before changes in cell shape. Furthermore, reduction of total Rac1 via siRNA or the pharmacological inhibition of its activation led to a dose-dependant reduction in fusion, suggesting that lamellipodia formation is critical

for this process. Inhibition of Rho kinase, on the other hand, did not have a profound effect on fusion. In addition, there was no effect on cell elongation and no apparent delay in the appearance of fused cells. Thus, we conclude that the cellular changes that precede fusion are not dependent on Rho-mediated processes. Interestingly, inclusion of Y-32885 limited the formation of larger FBGCs suggesting a role for Rho in the growth of FBGCs. We speculate that this might be attributable to a negative effect of the inhibitor on the recruitment of single cells to multinucleated cells.

Because of the profound effect of siRNA-mediated knockdown and NSC23766 treatment on fusion and the fact that Rac1 has been shown to be required for certain phagocytic processes,³² we investigated the effect of Rac1 down-regulation or inhibition on the phagocytosis of microspheres. Our primary goal was to examine whether fusion and phagocytosis proceeded through the activation of the same cellular machinery. We considered this to be a critical issue because we believe that strategies that aim to limit FBGC formation should not compromise other macrophage functions that might be beneficial, such as clearance of microscopic debris from implantation sites. Neither NSC23766 nor Rac1 knockdown could block the uptake of microspheres, suggesting that the process is independent of Rac1. We examined the cell cytoskeleton during the uptake of microspheres, and we did not observe pseudopod extensions, implicating a mechanism involving the direct sinking of the particles into cells. The fact that Rac1 activity is not required for this uptake mechanism provides further support for our conclusion.³³ To confirm further the involvement of distinct signaling pathways involving phagocytosis and fusion, we observed that MCP-1-null macrophages, which are inherently deficient in fusion, displayed normal engulfment of microspheres.

To expand on these findings and to examine the biological relevance of our *in vitro* observations, we used a strategy to deliver NSC23766 *in vivo* during FBGC formation. Specifically, we prepared the drug-loaded polymer EVAc, which has been previously shown to serve as a vehicle for long-term drug release *in vivo*.^{34,35} We hypothesized that the polymer itself would induce FBGC formation and allow us to examine the effect of Rac1 inhibition on this process as well as the overall foreign body response. We found that localized release of NSC23766 *in vivo* resulted in a significant reduction of FBGCs, confirming the importance of Rac1 activation in the formation of FBGCs. Attenuation of FBGC formation did not have an effect on the overall encapsulation of the EVAc scaffold suggesting that the tissue remodeling around biomaterials is independent of FBGCs. However, we have previously shown that reduction of FBGCs is associated with reduced degradation and damage to biodegradable biomaterials.⁹

We have demonstrated that mouse marrow-derived macrophages can be used efficiently in an *in vitro* fusion assay. Furthermore, we found that fusion is dependent on early cytoskeletal remodeling, specifically formation of lamellipodia, and can be attenuated by

down-regulation or inhibition of Rac1 activation. Prevention of lamellipodia formation did not compromise the ability of macrophages to uptake microparticles. Thus, the processes of fusion and phagocytosis can be partially decoupled. In addition, localized delivery of the Rac1 inhibitor limited FBGC formation *in vivo*. Therefore, strategies can be developed that aim to prevent damage to biomaterials while preserving phagocytic activity in macrophages.

Acknowledgments

We thank Drs. Jordan Pober, Mark Saltzman, William Sessa, and Michelle Lin for helpful discussion and critical reading of the manuscript; Dr. Barrett Rollins for the MCP-1-null mice; and Jianmin Zeng for assistance with biomaterial implantation.

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