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PHENOTYPIC EXPRESSION IN HUMAN MONOCYTE-DERIVED INTERLEUKIN-4-INDUCED FOREIGN BODY GIANT CELLS AND MACROPHAGES *IN VITRO*: DEPENDENCE ON MATERIAL SURFACE PROPERTIES

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Abstract

The effects of different material surfaces on phenotypic expression in macrophages and foreign body giant cells (FBGC) were addressed using our *in vitro* system of interleukin (IL)-4-induced macrophage fusion and FBGC formation. Arginine-glycine-aspartate (RGD)-, vitronectin (VN)-, and chitosan (CH)-adsorbed cell culture polystyrene, carboxylated (C, negatively-charged) polystyrene, and unmodified (PS, non-cell culture treated) polystyrene were compared for their abilities to support monocyte/macrophage adhesion and IL-4-induced macrophage fusion. Pooled whole cell lysates from 4 different donors were evaluated by immunoblotting for expression of selected components in monocytes, macrophages, and FBGC. In addition to RGD and VN as previously shown, we find that CH supports macrophage adhesion and FBGC formation, whereas C or PS support macrophage adhesion but do not permit macrophage fusion under otherwise identical conditions of IL-4 stimulation. Likewise, components related to macrophage fusion (CD206, CD98, CD147, CD13) are strongly expressed on RGD-, VN-, and CH-adsorbed surfaces but are greatly diminished or not detected on C or PS. Importantly, material surfaces also influence the FBGC phenotype itself, as demonstrated by strong differences in patterns of expression of HLA-DR, B7-2, B7-H1, and toll-like receptor (TLR)-2 on RGD, VN, and CH despite morphologic similarities between FBGC on these surfaces. Likewise, we observe differences in the expression of B7-2, α 2-macroglobulin (α 2Mac), TLR-2, and fascin-1 between mononuclear macrophages on C and PS. Collectively, these findings reveal the extent to which material surface chemistry influences macrophage/FBGC phenotype beyond evident morphological similarities or differences and identify CH as an FBGC-supportive substrate.

INTRODUCTION

The presence of foreign body-type multinucleated giant cells (FBGC) on and around implanted biomaterials is regarded as evidence of a chronic inflammatory host tissue response to these materials. The widely believed roles for these multinucleated cells in the degradation of certain polymers stems from early studies demonstrating pits or cracks in retrieved material surfaces underneath FBGC (1). It was thus inferred that polymer

degradation could be caused by the concentration of phagocytic oxidative activities at the FBGC/biomaterial interface.

However, of the three macrophage fusion factors we have subsequently identified, i.e. the two so-called anti-inflammatory cytokines interleukin (IL)-4 and IL-13 and the antioxidant molecule α -tocopherol (2–4), each is known to strongly down-regulate inflammation, promote wound healing, and exhibit host tissue protective effects (5–7). Thus, an alternative scenario includes the initial expression of a pro-inflammatory phenotype by biomaterial-adherent monocyte-derived macrophages. This degradative phenotype is more likely to be responsible for oxidative damage to polymers, and it may be subsequently down-modulated as IL-4 and/or IL-13 act to promote macrophage multinucleation or fusion into FBGC (8,9).

Material surface properties have been demonstrated to influence monocyte-derived macrophage adhesion and fusion to form FBGC, cytokine and chemokine production, and apoptosis (10–17). Questions remain, however, as to the underlying functional significance of these giant multinucleated macrophages at sites of biomaterial implantation and the degree of influence of biomaterial surface properties on the FBGC phenotype itself. To begin to address these unknowns from within a controlled *in vitro* environment, we have employed our established system of human monocyte-derived macrophage development and IL-4-induced macrophage fusion to form FBGC (2) and a panel of distinct material surfaces.

Our work with an RGD polymer adsorbed to polystyrene or glass culture materials has consistently provided an optimal, positive control surface for FBGC formation in our previous studies (18). Vitronectin (VN) provides a biologically relevant surface, as it is known to adsorb readily to many biomaterials (19), and the propensity for VN adsorption may in turn govern FBGC formation on biomaterials (12). Chitosan (CH) is a bioresorbable polymer derivative of chitin (20) that is regarded as a new material with potential for a variety of applications (21,22). It has been shown to influence the production of cytokines by macrophages (23) and the expression of extracellular matrix components and adhesion behavior in other cell types (24,25). Therefore, we chose to evaluate this material for its ability to support or restrict FBGC formation. We also included two commercially available polystyrene materials, i.e. a carboxylated, and therefore negatively charged, polystyrene (C) and an unmodified non-cell culture treated polystyrene (PS).

For the present material-dependent phenotypic analyses, we selected functional groups of molecules of which several members have been pursued on the RGD surface in our previous studies, including components that are involved in or potentially participate in the mechanism of macrophage fusion, lymphocyte co-stimulatory molecules, widely-employed macrophage markers, and toll-like receptors (TLRs).

We report that adsorbed CH supports the formation of very large FBGC that are morphologically indistinguishable from FBGC on RGD- and VN-adsorbed surfaces. However, these multinucleated giant cells do exhibit phenotypic differences from the FBGC that form on RGD or VN. Moreover, under otherwise identical culture conditions, we observe differences in the phenotypic expression of selected components in both macrophages and FBGCs on different surfaces. Collectively, these findings indicate for the

first time that the potential physiological significance of FBGC on different biomaterials can vary with material surface properties; morphologic likenesses do not necessarily extend to functional similarities.

MATERIALS AND METHODS

Material surfaces

All solutions were filter-sterilized (0.2 μ m) prior to adsorptions in 24-well cell culture-treated polystyrene culture plates (Becton-Dickinson, Franklin Lakes, NJ), and all procedures were carried out under sterile conditions. RGD (arginine-glycine-aspartate) polymer reagent (fibronectin-like protein polymer, Sigma-Aldrich, St. Louis, MO) was dissolved in phosphate-buffered saline containing calcium and magnesium (PBS⁺⁺, Invitrogen, Grand Island, NY) and adsorbed at 25 μ g per ml for 30 minutes at room temperature as previously (18,26). Human plasma vitronectin (VN, EMD Biosciences, San Diego, CA) was dissolved in PBS⁺⁺ and adsorbed at 14 μ g per ml for 30 minutes at 37°C (12). Chitosan (CH, Sigma-Aldrich, high purity MW 110,000–150,000) was initially dissolved as a 1% solution in 0.2M acetic acid (20) in cell culture grade endotoxin-screened distilled water (Invitrogen) and then further diluted in PBS⁺⁺ for adsorption at 25 μ g per ml for 30 minutes at 37°C. Adsorptions were followed by two washes with sterile PBS⁺⁺. Carboxylated polystyrene (C, negatively charged, Becton-Dickinson PureCoat Carboxyl) and unmodified polystyrene (PS, Becton-Dickinson Non-Tissue Culture Treated) were not adsorbed.

Monocyte/macrophage culture and FBGC formation

Human monocytes were obtained and isolated (18), suspended in serum-free medium for macrophages (SFM, Invitrogen) supplemented with 20% autologous serum and antibiotics/antimycotic (PenStrepFungizone, Invitrogen), and added to prepared wells at 5×10^5 monocytes per well. After 1.5 hr at 37°C in a humidified atmosphere of 5% CO₂ and 95% air, non-adherent cells were removed by washing with pre-warmed (37°C) PBS⁺⁺. At this point, wells designated for the Day 0 time point were processed for immunoblotting, i.e. lysates were prepared (see below). The remaining cultures were replenished with pre-warmed SFM without serum and incubated until Day 3. On Day 3, cultures were either processed for immunoblotting or the culture medium was changed to pre-warmed SFM containing 5% heat-treated (56°C for 45 minutes) autologous serum and without or with 15 ng per ml of recombinant human IL-4 (R and D Systems, Minneapolis, MN) to induce macrophage fusion (2). On Day 7, the cultures were processed and terminated. For each time point of each experiment on each material, culture wells were designated for May-Grünwald/Giemsa staining, adherent cell counting, and determinations of percent fusion as previously described (12, 18, 26). Representative images of stained cultures used for immunoblotting analyses are shown.

Preparation of whole cell lysates for immunoblotting

Cultures were washed twice with warm (37°C) PBS⁺⁺, briefly cooled on ice, and then solubilized in RIPA solution with Halt protease inhibitors (Thermo Scientific, Rockford, IL) for 30 minutes on an orbital shaker at 4°C. Lysates were transferred to microcentrifuge tubes

and centrifuged at $10,000 \times g$ for 30 minutes at 4°C. Supernatants were stored at -80°C. Prior to analysis, lysate pools were prepared by combining equal volumes of supernatant from 4 donors for each material and time point: Day 0, Day 3, Day 7 and Day 7+IL-4.

Immunoblotting

Lysate pools for each time point were diluted 1:1 with Laemmli sample buffer containing 2-mercaptoethanol (Bio-Rad Laboratories, Hercules, CA). Equal volumes of lysates (25µl) were loaded onto Tris-HCl Ready Gels, 4–15% acrylamide (Bio-Rad) in parallel with positive control cell lysates or recombinant proteins (Table 1), and subjected to SDS-PAGE under standard conditions. Proteins were transferred to polyvinylidene difluoride membranes at 100 v for 45 minutes using standard protocols. All remaining steps were carried out on a rocking platform at room temperature. Membranes were incubated in tris-buffered saline with 0.05% Tween-20 (TTBS) for 5 minutes, and non-specific sites were blocked with 5% Blotto (Santa Cruz Biotechnology, Santa Cruz, CA) in TTBS for 2 hr at room temperature. Primary antibodies (Table 1) were applied overnight in 1% Blotto/TTBS, and membranes were washed thrice in TTBS for 10 minutes each. Secondary antibody conjugates, either goat anti-mouse IgG or donkey anti-goat IgG conjugated to horseradish peroxidase (Bio-Rad) were diluted 1:25,000 in 1% Blotto/TTBS and applied for 1 hr. Membranes were then washed thrice with TTBS and once with TBS for 15 minutes each and then developed with a substrate kit (Immobilon Western Chemiluminescent HRP, Millipore Corporation, Billerica, MA). Representative images are shown. Positive control cell lysates or recombinant proteins were employed in all cases to confirm detection, and actin and GAPDH were employed as lysate loading controls (Table 1) as for our previous studies (27).

RESULTS

Material surface dependency of macrophage fusion/FBGC formation

Figure 1A contains representative photomicrographic images that depict the morphologies of adherent monocytes (Day 0), short-term cultured macrophages (Day 3), long-term cultured macrophages (Day7), and fusing macrophages and FBGC (Day 7+IL-4) on the five materials employed for comparison in this study. On Day 0, all materials demonstrate good monocyte adhesion, which, as expected, is decreased by Day 3 of culture on all materials. At Day 7 without IL-4, macrophage development continues to proceed in terms of cytoplasmic spreading on all surfaces. At Day 7+IL-4 and as we previously demonstrated (12,18), RGD and VN are materials that support robust IL-4-induced macrophage fusion leading to FBGC formation with high degrees of multinucleation and extensive cytoplasmic spreading.

Moreover, CH is revealed in this study to be yet another material that supports this striking morphologic progression. As shown, CH supports a high degree of FBGC formation comparable to RGD and VN. In contrast to RGD, VN, and CH, and yet under identical conditions of IL-4 stimulation, it can be seen in Figure 1A that C and PS do not support macrophage fusion or FBGC formation. These materials support macrophage adhesion and development but completely restrict the process of IL-4-induced macrophage fusion leading to FBGC formation.

Figure 1B, which features larger images of the Day 7+IL-4 fusing macrophages and FBGC on CH compared to the Day 7+IL-4 non-fusing macrophages on C, further emphasizes the striking morphologic disparities resulting from the interactions of monocytes/macrophages with these distinct surfaces under otherwise identical culture conditions.

In Table 2, monocyte/macrophage adhesion and fusion data are presented; these also reflect the similarities between RGD, VN, and CH, with fusion rates each within the range of 70–78% in the multinucleated giant cell cultures on these materials. C and PS, in contrast, exhibit negligible fusion rates.

Of note, our initial experiments also included a nitrogenated polystyrene (Becton-Dickinson PureCoat Amine). In contrast to the other surfaces employed in this study, it was found to support neither macrophage adhesion nor development. By Day 7 of culture and without or with IL-4, no adherent cells remained on this material. Therefore, while this was an interesting observation, the preparation of adherent cell lysates was not possible, and this polystyrene was necessarily excluded from further experiments of this type.

Components related to macrophage fusion

Our earlier studies revealed a role for activity of the mannose receptor, CD206, in IL-4-induced macrophage fusion and FBGC formation (28). Consistent with this, the mannose receptor is strongly expressed in FBGC on RGD-adsorbed materials (3). The present data confirm and extend mannose receptor expression to fusing macrophages/FBGC on VN and CH, which also exhibit strong evidence of CD206 (Figure 2). On C or PS, however, CD206 expression is relatively weak.

CD98, a marker of lymphocyte activation which has also been implicated in cell fusion through an interaction with CD147 (29), is expressed in FBGC on RGD-adsorbed culture surfaces (27). Here we find that the CD98 expression pattern nearly mimics that of CD206 on RGD, VN, and CH. Further analyses for CD147 as well as for the aminopeptidase CD13 demonstrate similar expression phenomena in that the presence of these molecules is detectable only in cultures of fusing macrophages/FBGC; they are essentially absent from C or PS.

The ubiquitous endoplasmic reticulum protein calnexin localizes to fusion interfaces, suggesting a role for the endoplasmic reticulum in the extensive degrees of fusion that occur on FBGC-supportive surfaces (30). Here we demonstrate strong calnexin expression in cultures of fusing macrophages on RGD, VN, and CH compared to non-fusing macrophages on C and PS. Likewise, the cross-linking protein transglutaminase (TGase) has been implicated in macrophage fusion (31). Similarly to calnexin expression, the present data indicate robust TGase expression in lysates from IL-4-induced FBGC compared to those from IL-4-induced but non-fusing macrophages on C or PS. Furthermore, galectin-3, which is cross-linked by TGase during phagocytosis (32), is strongly expressed in IL-4-induced FBGC on RGD-adsorbed surfaces (our unpublished observation). Here, we demonstrate consistent galectin-3 expression on all five test surfaces.

Lymphocyte co-stimulatory molecules

We have reported selective expression of lymphocyte co-stimulatory molecules on fusing macrophages and FBGC interacting with RGD-adsorbed surfaces (27). These findings are now extended to include two additional FBGC-supportive surfaces as well as two surfaces that do not support FBGC formation under identical conditions of IL-4 stimulation.

In Figure 3, it can be seen that HLA-DR is strongly evident in lysates from RGD as well as in those from VN, with ample expression also present on C and PS, which do not support FBGC formation. In sharp contrast, however, HLA-DR is absent from lysates of fusing macrophages/FBGC that form on CH, although the multinucleated giant cells on RGD, VN, and CH are not morphologically distinguishable.

As previously noted on RGD, B7-1 is also absent from cultures on VN, CH, C, and PS. B7-2, however, is evident not only on RGD, but also even more strongly apparent on VN and PS. On C, expression is comparable to RGD. On CH, however, B7-1 expression is poor.

Expression of B7-H1 on the additional FBGC-supportive surfaces, i.e. VN and CH, is consistent with our earlier results on RGD, although expression on VN or CH occurs less robustly in direct comparison to that on RGD. B7-H1, however, is absent or very poorly expressed in non-fusing macrophages on C and PS. In interesting contrast, B7-H2 appears only on the latter two surfaces, i.e. on surfaces that do not support IL-4-induced FBGC formation.

Macrophage markers

Figure 4 represents data that further extends our prior phenotypic analysis of IL-4-induced fusing macrophages/FBGC on RGD and includes four additional culture surfaces. The macrophage marker CD14 is poorly expressed on all surfaces; its weak expression on RGD at Day 7+IL-4 continues to decline and was shown to be absent after 10 days (27). Conversely, the macrophage marker CD68 appears to be more consistently expressed, although it is most strongly evident on RGD, VN, and CH. CD44 exhibits a strong pattern of material-dependent expression and is evident only on the latter surfaces, i.e. those that support IL-4-induced macrophage fusion leading to FBGC formation. Ferritin HC (heavy chain) has been proposed to be a new marker for macrophages (33). However, it also demonstrates a distinctive material-dependent expression pattern similar to that of CD44. In interesting contrast, α_2 -macroglobulin (α_2 Mac) is strikingly present in lysates from C, whereas it is absent on PS and nearly absent on RGD, VN, or CH.

TLRs, immunoglobulin receptors (IgR), and other functional markers

We further exploited these test surfaces to explore the expression of TLRs and discovered variable expression of TLR-2, with strong to moderate expression on RGD > PS > VN = CH (Figure 5). In contrast, on C, TLR-2 was nearly undetectable by comparison. Neither TLR-4 nor TLR-5 is detectable in Day 7+IL-4 lysates on any test surface.

In terms of receptors for IgG, the CD32B inhibitory isoform of IgGR II known to be associated with down-regulation of the immune response (34) is readily detectable in cultures of IL-4-induced fusing macrophages/FBGC but is not apparent on C or PS. We also

note that neither CD64 (IgGRI) nor CD16 (IgGRIII) is detectable in Day 7+IL-4 lysates on our five materials, and neither the polymeric IgA receptor, IgARI (CD89), nor IgERII (CD23) is detectable in fusing macrophages/FBGC on RGD (data not shown).

Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) expression was discovered in lysates from RGD-adsorbed surfaces in our earlier study (27). Therefore, we continued to explore the expression of this dendritic cell- and macrophage-specific molecule in the present investigation. Although we observe the most robust expression of DC-SIGN on RGD, it is nevertheless consistently and strongly observed on all materials.

The actin-bundling protein fascin-1 was found to be present in IL-4-induced FBGC on adsorbed RGD (35). Consistent with this, fascin-1 is also present in fusing macrophages/FBGC on VN and CH. Further, fascin-1 expression is equally evident in IL-4-stimulated non-fusing macrophages on PS. In sharp contrast, however, it is greatly diminished on C under the same conditions of IL-4 stimulation.

Time- and/or material-dependent expression of selected molecules

We also explored the time-dependent expression of multiple components on each test surface from adherent monocytes on Day 0, short-term cultured macrophages on Day 3, and long-term cultured macrophages on Day 7 without IL-4 for comparison with Day 7+IL-4 lysate results. Data are presented from four different components that illustrate the influences of both time and culture material surface properties on the phenotypic expression of monocytes, macrophages, and fusing macrophages/FBGC (Figure 6).

The widely employed macrophage marker and lipopolysaccharide receptor CD14 is strongly expressed on Day 0 and Day 3. On Day 7 in otherwise unstimulated macrophages, however, CD14 is somewhat diminished on VN, C, and PS. As also shown in Figure 4, CD14 expression is largely lost at Day 7+IL-4 on all surfaces.

CD206 is not present on blood monocytes but is up-regulated during macrophage development and is particularly robustly induced by IL-4 (36). This is also the case for cells cultured on RGD, VN, and CH in the presence of IL-4. In contrast, macrophage development on C or PS is not accompanied by CD206 up-regulation; the weak detection of CD206 on Day 3 disappears by Day 7 on these surfaces. Even the presence of IL-4 cannot induce CD206 expression to levels observed on RGD, VN, or CH.

Another widely-employed macrophage marker, CD68, is weakly present on Day 0 but is very strongly and consistently expressed beginning on Day 3 of culture on all material surfaces. Its expression does not appear to be enhanced or diminished by IL-4 stimulation, although the data suggest that on C and PS, CD68 is less strongly apparent than it is observed to be on RGD, VN, or CH.

The time course of expression for ferritin HC follows a similar pattern as for CD206. Expression develops on all surfaces by Day 3 of culture, and there is sustained expression at Day 7 in otherwise unstimulated macrophages on all materials except PS. Very strong ferritin HC expression continues to be observed in lysates from Day 7+IL-4 fusing

macrophages/FBGC on RGD, VN, or CH, as compared with its notable absence in lysates obtained from C or PS under these otherwise identical culture conditions.

DISCUSSION

In this study, we asked how different material surfaces affect the expression phenotypes of macrophages and IL-4-induced FBGC. Of the five different surfaces employed as probes for expression analyses, three support IL-4-induced FBGC formation and two do not. We find that, under otherwise identical culture conditions, the patterns of expression for multiple markers are variably altered in a surface-dependent manner, including several proteins related to macrophage fusion, lymphocyte co-stimulatory molecules, an actin-bundling protein, and receptors for pathogen-associated molecular patterns (PAMPs). Expression patterns frequently, but not exclusively, correlate with FBGC formation. Variations in expression are also observed between morphologically indistinguishable non-fusing macrophages or fusing macrophages/FBGC on different surfaces, clearly revealing that morphology is not a reliable indicator of functional significance.

Adsorbed CH is discovered to be a novel substrate in our established *in vitro* system. We report that, by light microscopic morphologic criteria, the giant multinucleated cells that form on this polysaccharide derivative of chitin are not distinguishable from those that form on adsorbed RGD or VN. CH has potential applications a variety of areas, including tissue engineering, wound healing, and drug delivery (21,22). Interestingly, CH itself adsorbs VN from serum over a wide range of branching (37). Thus, the adsorption of VN to this material may provide the actual FBGC-supportive environment (12), although this possibility was not addressed in the present study.

Our combined results from previous studies have demonstrated that IL-4-induced macrophage fusion leading to FBGC formation is a phagocytic process that depends upon mannose receptor (CD206) activity (28, 30). The identification of calnexin at macrophage fusion interfaces indicated participation of the endoplasmic reticulum in the extensive degrees of fusion that bring about the formation of these giant cells (9,30). Others have implicated interactions between CD98 and CD147 in macrophage fusion (29,38). CD13 (aminopeptidase N) is of interest as it is induced by IL-4, mediates homotypic interactions between macrophages (39,40), and co-localizes with CD206 in IL-4-induced fusing macrophages on RGD (our unpublished observations). Our collective results demonstrate relatively strong expression patterns of CD206, CD98, CD147, CD13, and calnexin on FBGC-supportive surfaces compared to undetectable or low levels of detection on surfaces that do not permit FBGC formation, thereby confirming and extending earlier data.

Immunoblotting analyses for TGase-2 and galectin-3 each demonstrate broader expression on all surfaces, with the most robust expression of TGase-2 appearing on FBGC-supportive surfaces. TGases are known to play roles in cell adhesion and migration (41,42). Of note, TGase participates in phagocytosis (43) and was implicated in early studies of macrophage fusion (31). Consistent with this, we have observed TGase-2 co-localizing with CD206 and also with galectin-3 in IL-4-induced fusing macrophages (unpublished observations).

Galectin-3, which is induced by IL-4 (44), is said to be part of the macrophage 'phagosome' (45). This lectin is cross-linked by TGase during phagocytosis (32), binds and cross-links CD98 (46), and perhaps most intriguingly, can interact directly with membrane lipids and traverse lipid bilayers (47). These collective activities raise the possibility that both TGase-2 and galectin-3 may participate in or facilitate macrophage membrane fusion events.

Regarding lymphocyte co-stimulatory molecules, HLA-DR, in particular, exhibits a very interesting pattern of material-dependent expression. IL-4 is known to up-regulate HLA-DR in macrophages (6), and we find this to be the case on both RGD and VN. On CH, however, i.e. in IL-4-induced fusing macrophages/FBGC that are morphologically indistinguishable from those on RGD or VN, HLA-DR is nearly undetectable by comparison, suggesting that these CH/FBGC lack the HLA-DR-mediated antigen-presenting ability exhibited by RGD/FBGC and VN/FBGC. Conversely, on both C and PS, HLA-DR is readily observable in non-fusing macrophages.

Further extending our previous work on RGD-adsorbed culture surfaces, the lymphocyte co-stimulatory molecule B7-2 exhibits a similar pattern of strikingly diminished expression on CH. B7-H1 and B7-H2, as well as the IgG receptor CD32B, however, further support the recent perspective that IL-4-induced macrophage fusion leading to FBGC formation includes the acquisition of a lymphocyte down-modulatory phenotype (27, Table 1), which is not present in mononuclear macrophages under the same conditions.

In terms of macrophage markers, the lipopolysaccharide receptor CD14 is widely used in macrophage detection. Our data demonstrate that CD14 is strongly present at Day 0 and Day 3. On Day 7, a material-dependent pattern of expression evolves: RGD > CH > VN = C $\gg$ PS. Furthermore, Day 7+IL-4 data indicate that CD14 is not a reliable marker for FBGC or even IL-4-stimulated non-fusing macrophages on C or PS, as CD14 detection is greatly diminished on all surfaces compared to earlier time points.

CD68 is a lysosomal membrane protein that is also broadly employed as a macrophage identifier in the clinical setting, although it is known not to be strictly macrophage-specific (48,49). Ablation of CD68 results in osteoclasts which cannot efficiently resorb bone (50). We find CD68 to be abundantly expressed on all five test surfaces from Day 3 through Day 7 without or with IL-4, albeit most robustly on FBGC-supportive surfaces, indicating that it is a more reliable macrophage marker than CD14, which is nevertheless useful to identify adherent monocytes.

A more recently proposed marker for macrophages is ferritin HC (33). Our current data, however, do not completely support this assignment and indicate that ferritin HC expression is substrate- and time-dependent. Although ferritin HC is present on all materials at Day 3, expression is lost on PS at Day 7. It is abundantly expressed in Day 7+IL-4 fusing macrophages and FBGC on RGD, VN, and CH, but it is undetectable in non-fusing macrophages on C and PS. Expression of ferritin HC in IL-4-induced FBGC but not in IL-4-treated macrophages on fusion-restrictive materials is intriguing and further suggests that this component of iron metabolism plays an important role in macrophage fusion events

and/or in the resultant functions of FBGC following those events, for example in regulation of angiogenesis during wound healing (51).

CD44 is an intercellular adhesion molecule and ubiquitous component of extracellular matrices, mediating both cell-cell and cell-matrix interactions as well as leukocyte recruitment to inflammatory sites (52). CD44 also plays critical roles in resolution of the inflammatory response; the binding of its ligand hyaluronan is markedly affected by IL-4 or IL-13 (53). In the present study, the pattern of CD44 expression mimics that of CD147, CD13, and ferritin HC, all of which appear only on FBGC-supportive surfaces. Thus, CD44 expression is consistent with potential cell-cell interactions prior to or during macrophage membrane fusion events.

The pan-proteinase inhibitor α_2 Mac is produced by monocytes (54), is associated with mannose receptor-mediated phagocytic activity (55), and has been linked to oxidative stress cracking of polyurethane biomaterial (56). Like HLA-DR, α_2 Mac demonstrates a unique material-dependent expression pattern, but in this case there is selective expression on the carboxylated C surface at Day 7+IL4 in the absence of a strong signal on the other four materials, again illustrating the influence of surface properties on adherent macrophage phenotype.

TLR-2, -4, and -5 on monocytes/macrophages recognize microorganisms with PAMPs as follows: TLR-2 (Gram+, yeast), TLR-4 (Gram-, LPS), and TLR-5 (bacterial flagellin) (57). TLR-4 and -5 are not detectable in any Day 7+IL-4 lysates. However, we observe another unique and material-dependent pattern of expression by TLR-2, as it is readily detectable on all surfaces with the notable exception of the carboxylated C material.

DC-SIGN functions as an intercellular adhesion molecule and also binds to high-mannose carbohydrates and therefore, like CD206 and TLRs, recognizes PAMPs (58). These interactions bring about phagocytosis of the bound pathogen. Despite its name, DC-SIGN expression is not dendritic cell-specific; it is also present on macrophages, and its expression is IL-4-dependent (59). We reported a robust signal for DC-SIGN in IL-4-induced FBGC on RGD in a previous study (27). Here, we extend that finding to strong expression on all five test surfaces, with RGD being the surface on which the highest relative expression level is observed.

Fascin-1 is an actin bundling protein found to be expressed in IL-4-induced fusing macrophages and FBGC on the RGD surface (35). Fascin-1 plays roles in cell adhesion, mobility, invasiveness, and cell-cell contact and communication (60). We find that fascin-1 follows a material-dependent pattern of expression opposite that of α_2 Mac, but similar to that of TLR-2, in which strong expression is seen on all surfaces except the carboxylated C material.

Collectively, components related to macrophage fusion (CD206, CD98, CD147, CD13) or potentially involved in macrophage fusion or FBGC functions (HCAM, ferritin HC) are strongly expressed on RGD-, VN-, and CH-adsorbed surfaces but are poorly expressed or not detected on C or PS. Interestingly, however, material surfaces also influence the FBGC phenotype itself, as demonstrated by differences in patterns of expression of HLA-DR,

B7-2, B7-H1, and TLR-2 on RGD, VN, and CH in spite of morphologic similarities between FBGC on these surfaces. Furthermore, we observe differences in the expression of B7-2, α 2Mac, TLR-2, and fascin-1 between mononuclear macrophages on C and PS. These findings clearly indicate that morphologic similarities between macrophages and FBGC cannot be extended to predictions of functional outcome.

In summary, our results reveal the striking extent to which material surface chemistry influences macrophage/FBGC phenotype beyond the obvious morphological similarities or differences as derived from histological analyses at sites of biomedical material implantation. In order to more fully appreciate and accurately evaluate the host response to different materials, more comprehensive analyses of macrophage/FBGC phenotypes on a variety of biomaterial surfaces are thus warranted. Phenotypic characterizations must be carried out on a per material basis both *in vitro* and *in vivo* within the framework of, rather than with complete reliance on, macrophage or giant cell morphology or macrophage multinucleation. These studies identify adsorbed CH as a novel FBGC-supportive substrate and provide a greater level of perspective on the complexities of the inflammatory response to implanted biomedical materials.

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Figure 1.

(A) Material surface dependency of IL-4-induced FBGC formation. Human monocytes were cultured on various surfaces for the indicated times: day 0, adherent monocytes; day 3, short-term cultured macrophages; day 7, long-term cultured otherwise unstimulated macrophages; and day 7+IL-4, long-term cultured and IL-4-treated macrophages as described in Methods. Representative images from 4 monocyte donors are shown. (B) Larger images of Day 7+IL-4 fusing macrophages/FBGC or non-fusing macrophages on CH or C, respectively. May-Grünwald/Giemsa, 20X

Figure 2. Phenotypic expression related to macrophage fusion. Pooled whole cell lysates obtained following a 7-day culture period with IL-4 stimulation from days 3 to 7 as described in Methods. Lysates were analyzed by immunoblotting for the indicated markers. n=4 monocyte donors

Figure 3. Expression of lymphocyte co-stimulatory molecules. Pooled whole cell lysates obtained following a 7-day culture period with IL-4 stimulation from days 3 to 7 as described in Methods. Lysates were analyzed by immunoblotting for the indicated markers. n=4 monocyte donors

Figure 4.

Expression of macrophage markers. Pooled whole cell lysates obtained following a 7-day culture period with IL-4 stimulation from days 3 to 7 as described in Methods. Lysates were analyzed by immunoblotting for the indicated markers. n=4 monocyte donors

Figure 5. Expression of toll-like receptors and other functional markers. Pooled whole cell lysates obtained following a 7-day culture period with IL-4 stimulation from days 3 to 7 as described in Methods. Lysates were analyzed by immunoblotting for the indicated markers. n=4 monocyte donors

Figure 6. Time course of expression of selected markers. Pooled lysates obtained at the indicated culture time points were analyzed by immunoblotting for selected components. n=4 monocyte donors

Table 1

Antibodies and control cell lysates or proteins for immunoblotting

Antigen	Function	Antibody Source	Control (+)
CD206	mannose receptor, macrophage fusion	Santa Cruz sc-367108	mouse spleen
CD98	fusion regulatory protein	Santa Cruz sc-7095	HL-60
CD147	cell-cell interactions	Santa Cruz sc-374101	HeLa
CD13	aminopeptidase, cell-cell interactions	Santa Cruz sc-166105	CD13:293T
Calnexin	ER protein present at fusion interfaces	Santa Cruz sc-46669	HeLa
TGase-2	protein crosslinking	Santa Cruz sc-73612	HeLa
Galectin-3	macrophage 'phagosome' component	Santa Cruz sc-225279	HeLa
HLA-DR	antigen-presenting molecule	Santa Cruz sc-69673	BJAB
B7-1	lymphocyte co-stimulator	Santa Cruz sc-1633	B7-1:293T
B7-2	lymphocyte co-stimulator	Santa Cruz sc-19617	B7-2:293T
B7-H1	lymphocyte co-stimulator, down-regulatory	RnD AF-156	Daudi
B7-H2	lymphocyte co-stimulator, up-regulatory	RnD AF-165	BJAB
CD14	LPS receptor, macrophage marker	Santa Cruz sc-58951	CD14:293T
CD68	macrophage marker, lysosomal protein	Santa Cruz sc-17832	THP-1
CD44	cell-cell interactions	Santa Cruz sc-7297	HL-60
Ferritin HC	ferritin heavy chain, iron metabolism	Santa Cruz sc-376594	HeLa
α_2 Mac	associated with phagocytic activity	Santa Cruz sc-390544	α_2 M:293T
TLR-2	PAMP recognition, Gram+, yeast	RnD AF-2616	TLR-2:293T
TLR-4	PAMP recognition, Gram-, LPS	RnD MAB-1478	rhTLR-4
TLR-5	PAMP recognition, bacterial flagellin	Santa Cruz sc-16482	rhTLR-5
CD32B	IgGRII, B isoform, immune down-regulatory	Santa Cruz sc-12815	CD32:293T
DC-SIGN	PAMP recognition, mannose carbohydrates	Santa Cruz sc-74589	DC-SIGN:293T
Fascin-1	actin bundling	Santa Cruz sc-71100	fascin-1:293T
Actin	cytoskeletal protein, lysate loading control	Santa Cruz sc-1616	
GADPH	'housekeeping' protein, lysate loading control	Santa Cruz sc-166545	

α_2 Mac, α_2 -macroglobulin; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; ER, endoplasmic reticulum; GADPH, glyceraldehyde 3-phosphate dehydrogenase; HLA, human leukocyte antigen; LPS, lipopolysaccharide; PAMP, pathogen-associated molecular pattern; rh, recombinant human, TGase, transglutaminase; TLR, toll-like receptor; 293T, transfected fibroblasts

Table 2

Monocyte/macrophage adhesion and fusion on different surfaces

	Nuclei in mononuclear macrophages (Nuclei in FBGC)				
	RGD	VN	CH	C	PS
Day 0	397±39 (0)	264±9 (0)	264±54 (0)	334±17 (0)	305±35 (0)
Day 3	189±47 (0)	169±2 (0)	158±33 (0)	132±78 (0)	147±14 (0)
Day 7	97±11 (7±1)	98±7 (10±9)	94±10 (19±9)	87±5 (2±1)	107±13 (9±6)
% Fusion	6±1	3±3	16±8	2±1	9±6
Day 7+IL-4	33±5 (79±5)	22±5 (79±12)	30±3 (68±6)	88±11 (2±2)	97±17 (9±3)
% Fusion	72±2	78±6	70±3	2±2	8±2

Nuclei counted in 0.1192 mm², mean ± SEM, n=4 monocyte donors. % Fusion is the ratio of nuclei present in FBGC compared to total nuclei counted. FBGC = cells with >2 nuclei.