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FOREIGN BODY-TYPE MULTINUCLEATED GIANT CELL FORMATION REQUIRES PROTEIN KINASE C β , δ , and ζ

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Abstract

Multinucleated giant cells are a classic cellular feature of chronic inflammation, although the mechanism of macrophage fusion leading to their formation is not well understood. Here, we investigate the participation of protein kinase C (PKC) in the interleukin (IL)-4-induced fusion of human monocyte-derived macrophages and foreign body giant cell (FBGC) formation *in vitro*. The PKC inhibitors H-7 and calphostin C attenuated macrophage fusion, whereas H-8, which is more selective for PKA and PKG, did not. Macrophage fusion was also prevented by the phospholipase C inhibitor, Et-18-OCH₃, the PKC isoform inhibitors GO6983 or rottlerin and by peptide inhibitors for PKC (20-28), PKC β , or PKC ζ but not by HBDDE or peptide inhibitors for PKC ϵ or PKA. In cultures of fusing macrophages/FBGC, we detected only PKC α , β , δ , and ζ by immunoprecipitation and immunoblotting, and we also observed strong expression of these isoforms by immunocytochemistry. Our collective results suggest that the γ , ϵ , η , μ , θ , or ι PKC isoforms are not required in the mechanism of IL-4-induced macrophage fusion; whether PKC α is required is unclear. However, new evidence is provided that FBGC formation is supported by PKC β , PKC δ , and PKC ζ in combined diacylglycerol-dependent (PKC β and PKC δ) and - independent (PKC ζ) signaling pathways.

Keywords

diacylglycerol; foreign body giant cell; interleukin-4; monocyte; macrophage; fusion; multinucleated giant cell; protein kinase C

INTRODUCTION

Multinucleated giant cells are formed from blood monocyte-derived macrophage fusion at sites of chronic inflammation resulting from persistent microbial infection, otherwise unknown causes, e.g. sarcoidosis, rheumatoid arthritis, and various neoplasias, or the presence of foreign bodies (Chambers 1982, Anderson 2000). So-called foreign body-type giant cells (FBGC) form extensively on implanted biomedical devices, covering up to 25% of implant surface area, and mediate biomaterial degradation (Zhao 1991, Wiggins 2001) leading to implant failure and retrieval (Anderson 1988). Although multinucleated giant cells were documented well over a century ago in lesions of tuberculosis (Langhans 1868) and have long been regarded as hallmark

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histological features of chronic inflammation, little is known about the signals that drive their formation, the molecular mechanism(s) by which macrophage fusion occurs, or their physiological significance at sites of chronic inflammation.

Toward these unknowns, we devised correlative *in vivo* and *in vitro* systems for the study of FBGC formation. *In vitro*, we have demonstrated that the lymphokines IL-4 (McNally 1995) or IL-13 (DeFife 1997) induce macrophage fusion and the formation of FBGC that are morphologically indistinguishable from the IL-4-induced FBGC that form on implanted materials *in vivo* (Zhao 1991, Kao 1995). IL-4-induced macrophage fusion is dependent on the activity of a classic endocytic/phagocytic receptor, the mannose receptor (McNally 1996), and exhibits multiple other features of a phagocytic process (McNally 2005) that is supported by cell/substrate adhesive interactions between select $\beta 1$ and $\beta 2$ integrins and vitronectin (McNally 2002, McNally 2007a, McNally 2007b). Interestingly, we found that α -tocopherol is also a potent macrophage fusion factor, and that diacylglycerol kinase activity is required for both α -tocopherol- and IL-4-induced FBGC formation (McNally 2003).

Earlier studies also alluded to a role for diacylglycerol, generated by the action of phospholipase C on phosphatidylinositol, and the diacylglycerol-mediated activation of protein kinase C (PKC) in macrophage fusion. Addition of the synthetic diacylglycerol analog and PKC activator phorbol myristate acetate to 3 week-cultured macrophages resulted in macrophage fusion which was prevented by H-7, a non-selective inhibitor of PKC activity (Hassan 1989). In another study, the induction of Langhans-type multinucleated macrophages with a lymphokine-containing supernatant was also prevented by H-7 (Orentas 1992).

More recently, roles for PKC and its isoforms have been further defined. PKC isoforms fall into three classes: conventional (α , βI , βII , γ), novel (δ , ϵ , θ , η , μ), and atypical (ζ , ν/λ); conventional isoforms respond to diacylglycerol and calcium activation, novel isoforms respond only to diacylglycerol, and atypical PKCs do not respond to either of these mediators (Newton 2003). Multiple monocyte/macrophage activities that are now associated with PKC activation are also essential for FBGC formation, which requires initial monocyte adhesion followed by monocyte-to-macrophage morphological development and close apposition of macrophages to one another or to multinucleated giant cells (McNally 1994, 1995). These include PKC α in cell spreading, integrin interactions, and migration (Larsson 2006), PKC β in cytoplasmic spreading (Teslenko 1997), adhesion (Xie 1998), and chemotaxis (Carnevale 2003), and PKC δ in macrophage differentiation (Wang 1997).

Similarly, our recent demonstration that IL-4-induced FBGC formation is a phagocytic process (McNally 2005) suggests PKC involvement, as multiple reports further emphasize the importance of PKC in phagocytosis or IL-4 signaling. These include PKC α in Fc γ receptor-mediated phagocytosis (Breton 2000) and in the IgG-stimulated respiratory burst (Larsen 2000), PKC β in the phagocytosis of latex particles (Dieter 2000), and PKC β and PKC δ in the phagocytosis of *L. monocytogenes* and translocation of PKC β to early endosomes during bacterial escape from phagocytic vesicles (Wadsworth 2002). Novel PKCs δ and ϵ have also been linked to IgG-stimulated phagocytosis (Larsen 2000), and the phagocytosis of apoptotic cells by monocytes/macrophages requires PKC activity (Liu 2005). IL-4 induction of CD36 is blocked by PKC inhibition (Feng 2000), and IL-4 or IL-13 induces the activation of PKC δ and PKC ζ (Ikizawa 2001).

In this study, we further pursue the potential participation of PKC activities in IL-4-induced FBGC formation by first focusing on a requirement for PKC activity in macrophage fusion and FBGC formation followed by the identification of specific PKC isoforms in these remarkable multinucleated cells. We exploit pharmacological inhibition, immunoprecipitation with immunoblotting analyses, and immunocytochemistry in our established *in vitro* system

of FBGC formation from human blood monocyte-derived macrophages to address these questions.

MATERIALS AND METHODS

Monocyte/macrophage culture and induction of FBGC formation

Human monocytes were isolated and cultured as previously described (McNally 1995). For preparation of cell lysates for immunoprecipitation and immunoblotting, monocytes were plated in 6-well plates modified with RGD (Smartplastic, MP Biomedicals, Aurora, OH) at 3×10^6 cells per well in 2 ml per well of serum-free medium for macrophages (SFM, InVitrogen, Grand Island, NY) supplemented with 20% autologous serum and antibiotic/antimycotic mixture (InVitrogen). For inhibition experiments, monocytes were plated in 96-well RGD-modified plates (Smartplastic) at 2×10^5 per well in 0.1 ml of supplemented medium. For confocal microscopy, monocytes were similarly plated in 8-well glass chamber slides (Lab Tek II, Nalge-Nunc, Naperville, IL) at 5×10^5 monocytes per well in 0.25 ml. After 1.5 hr, nonadherent cells were removed by washing with phosphate-buffered saline containing Ca^{++} and Mg^{++} (PBS^{++} , GIBCO) at 37°C , recovered with 4.0, 0.2 or 0.5 ml of otherwise unsupplemented SFM, and incubated for 3 days at 37°C in a humidified atmosphere of 95% air and 5% CO_2 . On day 3, the medium was replaced with the same volumes of SFM supplemented with 5% heat-treated (56°C for 1 hr) autologous serum, and 15 ng per ml of IL-4 (R & D Systems, Minneapolis, MN) was added to induce macrophage fusion. Where indicated in figure legends, inhibitors, peptides (Table 1, Biomol, Plymouth Meeting, PA), or diluent controls were also added at this time. The macrophages were then incubated until day 7, and the wells were washed twice with PBS^{++} at 37°C . Cells in 96-well plates were fixed for 5 min with methanol and stained with May-Grünwald/Giemsa. Cells in chamber slides were fixed and permeabilized with acetone at -20°C for 2 min and stored at 4°C until immunocytochemical staining for confocal microscopy. Lysates were prepared from 6-well plates as described below; in each of these experiments, one well was also stained with May-Grünwald/Giemsa for evaluation of macrophage morphology and confirmation of FBGC formation.

Macrophage/FBGC morphology, % adhesion, and % fusion

Adhesion was evaluated for each sample by counting the average number of cells in two representative low power (20X) microscopic fields (~ 250 cells). Results with inhibitors or other reagents are expressed as a percentage of adherent cell numbers in control wells with no other additive or the appropriate diluent, i.e. % Adhesion. The overall extent of FBGC formation was evaluated by determining % Fusion, i.e. the fraction of nuclei within multinucleated giant cells (> 2 nuclei) in the same fields. Results represent the mean $\pm$ SEM for at least three different monocyte donors. Statistical significance was assigned at the 95% confidence level using the unpaired Student's *t* test. Photomicrograph images of representative samples were obtained using a Nikon DXM-1200 digital camera with ACT-1 software in single image acquisition mode. This is an established culture system in which 15 ng/ml of IL-4 typically induces $>75\%$ fusion of day 3 human monocyte-derived macrophages into FBGC on RGD-modified surfaces within 4-7 days (McNally 1995, McNally 2001).

Preparation of cell lysates

At each time point, cell cultures in 6-well plates were washed twice with 3 ml PBS^{++} at 37°C and incubated with 0.5 ml of RIPA buffer containing protease inhibitors (Pierce, Rockford, IL) for 30 min at 4°C on an orbital shaker (Lab Line Instruments, Inc., Melrose Park, IL). Lysate samples were then subjected to centrifugation at $10,000 \times g$ for 30 min at 4°C and the supernatant samples were stored at -80°C before evaluation.

Immunoprecipitation and immunoblotting

Cell lysates from three different donors were pooled and pre-cleared for 30 min at 4°C using agarose-conjugated mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) at 50 µl per ml of lysate. Mouse anti-panPKC (Santa Cruz, sc-17804) or normal mouse IgG was added to pre-cleared lysate samples at 10 µg per ml and incubated on a rotator at 4°C for one hr. Protein A/G PLUS agarose (Santa Cruz) was then added at 50 µl per ml of lysate sample and the incubation continued for one hr. PKC-antibody-agarose complexes were then collected by centrifugation, and washed three times with ice-cold 50% RIPA in PBS⁺⁺.

Immunoprecipitates were solubilized in Laemmli sample buffer, 200 µl per ml of lysate pool starting material (Biorad, Hercules, CA). All samples were boiled for 2 min before loading onto 7.5% acrylamide Tris-HCl mini-gels (Biorad) and subjected to SDS-PAGE, followed by transfer to polyvinylidene difluoride membranes (Biorad) at 100 V for 45 min. Immunoblotting steps were carried out at room temperature on a rotating platform. Membranes were equilibrated for 5 min in Tris-buffered saline (TBS) and blocked in 5% blotting grade non-fat dry milk (Blotto, Santa Cruz) in TBS containing 0.05% Tween-20 (TTBS) for 1 hr, washed for 5 min in TTBS, and incubated overnight with rabbit primary antibodies to PKC isoforms (Santa Cruz) diluted to 3 µg per ml in 1% Blotto/TTBS. Membranes were then washed thrice with TTBS for 8 min each, incubated for 1 hr with alkaline phosphatase-conjugated goat anti-rabbit IgG (Biorad) diluted 1:10,000 in 1% Blotto/TTBS, and washed thrice with TTBS and once with TBS for 8 min each. The blots were developed using an alkaline phosphatase substrate kit (Biorad) according to the manufacturer's instructions.

Immunocytochemical detection of PKCs

Acetone-fixed and permeabilized cells were treated for 1.5 hr at 37°C with 1 µg per ml RNase A (EMD Biosciences, La Jolla, CA) to degrade cytoplasmic nucleic acids, washed 3 times with PBS⁺⁺, and then blocked with 10% donkey serum (Sigma-Aldrich, St. Louis, MO) for 1 hr at 37°C. Primary detecting mouse (BD Biosciences, San Diego, CA, for PKCβ) or rabbit (Santa Cruz, for all others) antibodies to PKCs were applied at 20 µg per ml in 5% donkey serum for 2 hr at 37°C followed by four 5 min washes. The samples were then treated for 1 hr at 37°C with AlexaFluor 594-conjugated donkey anti-mouse or anti-rabbit IgG (reconstituted as directed and applied at a final concentration of 1/100, Molecular Probes, Eugene, OR) plus 0.1 µM YO-YO-1 (Molecular Probes) to stain nuclei. Finally, the samples were washed four times for 5 min each, mounted under glass cover slips, and viewed using an MRC-600 confocal laser scanning microscope (Bio-Rad, Richmond, CA) with settings adjusted to blacken any residual background fluorescence from the corresponding normal mouse or rabbit IgG control. Shown images are slices taken at the cell/culture material interface.

RESULTS

In our initial studies to explore PKC involvement in FBGC formation, we found that the widely-used but relatively nonspecific PKC inhibitor H-7 blocks macrophage fusion in our culture system without reducing adhesion (Figure 1). The more selective PKC inhibitors calphostin C (Figure 1) and GF109203X (not shown) also demonstrate potent concentration-dependent inhibition of fusion that is distinguishable from lesser effects on adhesion. Figure 2A shows typical morphology of IL-4-induced fusing macrophages/FBGC in the absence of any inhibitor. Note the remarkable degrees of cytoplasmic spreading and the prominent pseudopodial extensions that characterize these multinucleated cells. This morphology is altered by the PKC inhibitor H-7 (Figure 2B), which appears to permit extension of pseudopods and degrees of cytoplasmic spreading, but blocks macrophage fusion. In contrast, H-8, which exhibits selectivity for other kinases (PKA and PKG) over PKC at 1 µM, does not prevent FBGC formation at this concentration (Figure 2C). In addition, Figure 2D displays the effects of

another inhibitor, Et-18-OCH₃, on FBGC morphology. Et-18-OCH₃ does not directly inhibit PKC, but it prevents diacylglycerol production and subsequent activation of PKC by inhibiting phosphatidylinositol-phospholipase C. It can be seen that Et-18-OCH₃ also completely attenuates IL-4-induced macrophage fusion leading to FBGC formation; macrophage cytoplasmic spreading occurs without prominent pseudopodial extensions or fusion.

Further studies with isoform-specific PKC inhibitors were performed, and these results are presented in Figure 3. GO6983, which inhibits several conventional (α , β , γ), novel (δ), and atypical PKC (ζ) isoform activities but does not affect PKC μ , inhibits macrophage fusion but not adhesion in a concentration-dependent manner with complete inhibition at 10 μ M. HBDDE is another very useful reagent because it inhibits PKC α and γ but not PKC δ , β I, or β II. Notably, we found that HBDDE does not affect macrophage adhesion or fusion over an extended concentration range of up to 100 μ M, thus providing evidence against the participation of PKC α or PKC γ in the mechanism of macrophage fusion. In contrast, rottlerin, which is regarded as a selective PKC δ inhibitor, completely blocks macrophage fusion at only 3 μ M. Figure 4 depicts representative morphologies under the influences of GO6983, HBDDE, or rottlerin. Relative to no inhibitor (in Figure 2A), GO6983 completely restricts IL-4-induced macrophage fusion and FBGC formation and appears to diminish cytoplasmic spreading (Figure 4A). However, HBDDE does not alter morphology of fusing macrophages/FBGC (Figure 4B). Further, although 0.5 μ M rottlerin does not disrupt normal morphology of fusing macrophages/FBGC (Figure 4C), at 3 μ M, this reagent completely abrogates FBGC formation yet does not prevent macrophage cytoplasmic spreading and pseudopodial extensions (Figure 4D).

We then tested a panel of cell-permeable (myristoylated) pseudosubstrate peptides designed to target either all PKCs or specific PKC isoforms. The PKC (20-28) peptide serves as a panPKC-specific inhibitor, as it is identical to the pseudosubstrate sequence present in all PKCs. We also employed PKC β (inhibits α , β I, and β II, but not δ or ϵ), PKC ϵ (inhibits ϵ but not α , β I, or δ), and PKC ζ (inhibits atypical PKCs, i.e. ζ , ι/λ) peptides. Our results, which are shown in Figure 5, indicate that the PKC (20-28) inhibitor peptide does not affect macrophage adhesion, but it is able to completely prevent IL-4-induced macrophage fusion. Similarly, the PKC β or PKC ζ peptides each completely prevent macrophage fusion but not adhesion in a concentration-dependent manner. In contrast, the PKC ϵ peptide has no effect on either adhesion or fusion at concentrations up to 100 μ M. As a further control for specificity relating to PKC, a PKA (14-22) inhibitor peptide also was tested without effect (not shown), which is also consistent with experiments using the inhibitor H-8 (Figure 2C).

Collectively, these inhibitor data suggest that the α , γ , ϵ , and μ PKC isoforms do not participate in the mechanism of IL-4-induced macrophage fusion or FBGC formation. However, strong evidence is provided for the importance of PKC β , PKC δ , and an atypical PKC, i.e. ζ and/or ι/λ . In Figure 6, we further demonstrate attenuation of IL-4-induced FBGC formation by peptide inhibitors specific for PKC β and PKC δ but not for PKC ϵ . This figure also reveals that the PKC ζ inhibitor peptide exerts the most disruptive effect on macrophage morphology. Whereas the PKC β inhibitor peptide completely prevents macrophage fusion, it does not appear to interfere with macrophage development related to cytoplasmic spreading or the ability to form pseudopodial extensions. These macrophages appear to be arrested immediately prior to the point of macrophage-macrophage fusion. Cultures treated with the PKC ζ peptide, however, exhibit a nearly complete absence of cytoplasmic spreading with diminished pseudopodial extensions. Adherent macrophages are numerous, but appear as compact cells with low cytoplasmic:nuclear ratios more consistent with that of blood monocytes than developed macrophages. Revisiting Figure 4A, macrophages that were treated with GO6983, which inhibits PKC ζ as well as conventional PKCs and PKC δ , also appear to display diminished cytoplasmic spreading relative to macrophages treated with inhibitors that do not target PKC ζ .

To identify the PKC isoforms in IL-4-induced fusing macrophages/FBGC, cell lysates of these cultures (as shown in Figure 2A) were pooled and subjected to immunoprecipitation with a panPKC antibody followed by immunoblotting with PKC isoform-specific antibodies. Results of these experiments are represented in Figure 7, which demonstrates that the conventional PKC α and β I isoforms are readily detectable in IL-4-induced macrophages/FBGC, compared to a relatively much weaker signal for PKC β II. In addition, we detect both novel PKC δ and atypical PKC ζ isoforms. No signal is evident for PKC γ PKC ϵ PKC η , PKC μ or PKC ι (or PKC θ , not shown) in the same lysates.

We further investigated the identified PKC isoforms with immunocytochemistry and confocal microscopy to confirm and localize their expression in IL-4-induced fusing macrophages and FBGC. Figure 8 contains representative images from cultures that were double-stained for PKC isoforms (green) and for nuclei (blue) to mark macrophages/FBGC. By day 7, PKC β , δ , and ζ are readily detectable throughout fusing macrophages and FBGC, although there is very little signal for the PKC β II isoform. PKC α is also detectable with this technique (not shown). Expression of both PKC β and PKC δ appears to be particularly concentrated in areas where macrophages can be seen to undergo fusion. PKC ζ is distributed more uniformly in macrophages and FBGC. Prior to the IL-4 induction of fusion on day 3, however, these PKCs are not visible in macrophages by this method (not shown), suggesting that they are up-regulated by IL-4 during the induction of FBGC formation.

During the course of these studies, we also attempted to determine the effects of small interfering (si) RNAs for selected PKC isoforms. However, due to considerable technical obstacles related to this particular culture system, including toxicity of siRNAs in these long-term cultures (7 to 10 days), this approach has been unrewarding.

DISCUSSION

The present findings reveal that select conventional, novel, and atypical PKC isoforms participate in events leading to IL-4-induced macrophage fusion and formation of foreign body-type multinucleated giant cells. Namely, we demonstrate that PKC α , PKC β , PKC δ , and PKC ζ are present and that PKC β , PKC δ , and PKC ζ are required during this remarkable macrophage morphological transformation, which is a hallmark cellular feature of chronic inflammation. Using our established culture system, which exhibits high degrees of IL-4-induced FBGC formation from human blood monocyte-derived macrophages, the present results validate early reports that PKC appeared to be involved in multinucleated giant cell formation (Hassan 1989, Orentas 1992). Extending this, and based on a variety of pharmacological inhibitors as well as immunoblotting and immunocytochemistry, our findings further identify three PKC isoforms with functional roles in FBGC formation.

As illustrated in Figure 9, the combined results from these approaches indicate that IL-4-induced FBGC formation requires activation of at least three PKC isoforms, thus implicating diacylglycerol-dependent and -independent signaling pathways that appear to affect distinguishable stages in this morphological transformation. All three isoforms are apparently essential, as reagents that target either PKC β , PKC δ , or PKC ζ each completely abrogate IL-4-induced FBGC formation.

A diacylglycerol-dependent pathway is apparently initiated by the phosphatidylinositol-specific phospholipase C-mediated (i.e. prevented by Et-18-OCH₃) generation of diacylglycerol which activates conventional PKC β and novel PKC δ . Although the most ubiquitously expressed PKC isoform, PKC α , is also readily detectable by immunoblotting and immunocytochemistry, the HBDDE inhibitor that targets PKC α has no effect on macrophage fusion or FBGC formation. However, it is possible that PKC α is inhibited by the PKC β peptide

inhibitor. Therefore, whether PKC α , which has also been implicated in cell IgG-mediated phagocytosis (Breton 2000, Larson 2000), cell spreading, migration, integrin signaling (Larsson 2006), and calcium signaling (Reither 2006), participates in the events outlined in Figure 9 remains unclear. Alternatively, PKC α may play post-fusion roles in as yet undescribed FBGC activities at sites of chronic inflammation.

We note that although rottlerin is selective for PKC δ among other PKC isoforms, this reagent has also been reported to affect activities in addition to PKC δ (Davies 2000). Nevertheless, complete inhibition of FBGC formation by rottlerin at 3 μ M as well as detectable signals for PKC δ in our cultures by both immunoblotting and immunocytochemistry provide support for the participation of this IL-4-activatable isoform (Izika 2001) in macrophage fusion leading to FBGC formation.

Inhibition of either PKC β or PKC δ appears to block FBGC formation at the stage of macrophage fusion. This may be related to reports that PKC β is necessary for chemotaxis (Carnevale) and/or that PKC β (Dieter 2000, Wadsworth 2002) and PKC δ (Larson 2000) are each required for phagocytosis. If taken together with our demonstration that macrophage fusion exhibits multiple features of phagocytosis (McNally 2005), these reports suggest that PKC β and PKC δ may each facilitate phagocytosis signaling (Lennartz 1999, Greenberg 2002) during macrophage fusion. It is noteworthy that a diacylglycerol analog, phorbol myristate acetate, promotes a strong increase in PKC δ in monocytic cells (Schwende 1996). Moreover, IL-4 promotes translocation of both the diacylglycerol-dependent PKC δ and the diacylglycerol-independent PKC ζ to the membrane fraction (Izika 2001).

Accordingly, our results indicate that IL-4-induced FBGC formation also features a diacylglycerol-independent signaling pathway that activates PKC ζ . In contrast to PKC β and PKC δ , inhibition of PKC ζ appears to arrest the macrophage developmental response to IL-4 in terms of cytoplasmic spreading and/or migration. This is consistent with a report implicating PKC ζ in the promotion of macrophage differentiation (Lui 1998). Therefore, activation of PKC ζ may provide critical early stage signaling for macrophage development and/or cytoplasmic spreading during the pre-fusion stages in this macrophage morphological transformation. This could explain why phorbol ester alone (Hassan 1989, McNally 1994) is unable to promote FBGC morphology identical to that obtainable with IL-4 *in vivo* and *in vitro* (Zhao 1991, Kao 1995, McNally 1995). Whether PKC ζ also operates to facilitate macrophage fusion events cannot be determined from the present data. It is intriguing, however, that PKC ζ is required for complement receptor type-3-mediated phagocytosis (Lutz 2003), which quite closely resembles the morphology of macrophage-macrophage fusion during which fusing macrophages appear to “sink” into the cytoplasm of adjacent macrophages and/or FBGCs (McNally 1994, Aderem 1999, McNally 2005, and e.g. Figures 8 and 9 in this manuscript).

In conclusion, we find that the IL-4 induction of combined PKC signaling pathways fosters cellular events that initiate and support macrophage morphological development and the sustained degrees of macrophage fusion that lead to these remarkable multinucleated macrophages. Because IL-4 signaling mobilizes both PKC δ and PKC ζ (Izika 2001), the selective activation of these PKC isoforms may be characteristic of IL-4-mediated alternative macrophage activation. IL-4 induction of the mannose receptor, which is required for IL-4-induced FBGC formation (McNally 1996), is also regarded as a key feature of alternative macrophage activation (Stein 1992, Goerdts 1999, Mosser 2003). Thus, we suggest that the identified PKC isoforms may be additional characteristics of an alternative macrophage activation phenotype that supports FBGC formation. Further studies of the molecular events in IL-4-induced macrophage fusion will foster greater understanding of the underlying phenotypic and functional significance of macrophage multinucleation and FBGC formation

within the pathological context of chronic inflammation. This increased perspective will facilitate the development of opportunities for clinical modulation of these mechanisms where desirable.

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Figure 1.

Effects of the PKC inhibitors H-7 or calphostin C on percent macrophage adhesion (circles) and fusion (squares) during the IL-4 induction of FBGC formation. Day 3 macrophages were treated with 15 ng per ml of IL-4 to induce fusion and the indicated concentrations of inhibitors. Cultures were terminated on day 7 and stained with May-Grünwald/Giemsa. $n = 3$ monocyte donors. *Significantly different from the control value with no inhibitor ($P < 0.05$).

Figure 2. Effects of inhibitors on IL-4-induced FBGC formation. Day 3 macrophages were treated with 15 ng per ml of IL-4 to induce fusion and either (A) no inhibitor, (B) 25 μ M H-7, (C) 1 μ M H-8, or (D) 0.5 μ M Et-18-OCH₃. Cultures were terminated on day 7 and stained with May-Grünwald/Giemsa. Scale bar, 50 μ m.

Figure 3.

Effects of isoform-selective PKC inhibitors GO6983, HBDDE, or rottlerin on percent macrophage adhesion (circles) and fusion (squares) during the IL-4-induction of FBGC formation. Day 3 macrophages were treated with 15 ng per ml of IL-4 to induce fusion and the indicated concentrations of inhibitors. Cultures were terminated on day 7 and stained with May-Grünwald/Giemsa. $n=3$ monocyte donors. *Significantly different from the control value with no inhibitor ($P < 0.05$).

Figure 4. Effects of isoform-selective PKC inhibitors on IL-4-induced FBGC formation. Day 3 macrophages were treated with 15 ng per ml of IL-4 to induce fusion and either (A) 10 μ M GO6983, (B) 100 μ M HBDDE, (C) rottlerin at 0.5 μ M, or (D) rottlerin at 3 μ M. Cultures were terminated on day 7 and stained with May-Grünwald/Giemsa. Scale bar, 50 μ m.

Figure 5.

Effects of the panPKC (20-28) peptide inhibitor or PKC isoform sequence-specific peptide inhibitors for PKC β , PKC ϵ , and PKC ζ on macrophage adhesion (circles) and fusion (squares) during the IL-4 induction of FBGC formation. Day 3 macrophages were treated with 15 ng per ml of IL-4 to induce fusion and the indicated concentrations of peptide inhibitors. Cultures were terminated on day 7 and stained with May-Grünwald/Giemsa. $n = 3$ monocyte donors.

*Significantly different from the control value with no inhibitor ($P < 0.05$).

Figure 6. Effects of PKC isoform peptide inhibitors on IL-4-induced FBGC formation. Day 3 macrophages were treated with 15 ng per ml of IL-4 to induce fusion and 50 μ M of peptide inhibitors to either (A) PKC β , (B) PKC ϵ , or (C) PKC ζ . Cultures were terminated on day 7 and stained with May-Grünwald/Giemsa. Scale bar, 50 μ m.

Figure 7.

Immunoprecipitation and immunoblotting for PKC isoforms (conventional, novel, and atypical) in fusing macrophages/FBGC. Whole cell lysates were pooled from cultures of fusing macrophages/FBGC (as shown in Figure 2A), $n = 3$ monocyte donors. Lysates were subjected to immunoprecipitation with mouse anti-panPKC followed by immunoblotting with rabbit PKC isoform-specific antibodies. Bands are approximately 80 kD. Normal mouse IgG immunoprecipitation control and anti-PKC θ are negative at this molecular weight (not shown).

Figure 8. Expression of identified PKC isoforms by immunocytochemistry and confocal microscopic detection in day 7 fusing macrophages/FBGC. Day 3 macrophages were treated with 15 ng per ml of IL-4 to induce fusion. Cultures were terminated on day 7 and stained for nuclei (blue) and either (A) PKC β , (B) PKC β II, (C) PKC δ , or (D) PKC ζ (green). Scale bar, 50 μ m.

Figure 9.

IL-4-induced FBGC formation *in vitro*. The morphological transformation of human monocyte-derived macrophages into foreign body-type multinucleated giant cells (FBGC) is represented relative to culture time and stages of morphological progression. Adherent monocytes are cultured for three days. Day 3 monocytes/macrophages are treated with IL-4 to promote macrophage development and sustained degrees of macrophage fusion leading to FBGC formation between days 7 and 10. Inhibition of diacylglycerol-independent $PKC\zeta$ arrests this progression at the early stage of macrophage development, whereas inhibition of diacylglycerol-dependent $PKC\beta$ or $PKC\delta$ interferes with subsequent macrophage-macrophage fusion.

Table 1
Inhibitors for studies of IL-4-induced FBGC formation

Inhibitor	Targets	IC ₅₀ (μM) *
H-7	PKA, PKG, PKC	3.0, 3.8, 6.0
H-8	PKA, PKG, PKC	0.48, 1.2, 15
Calphostin C	PKC	0.05
GF109203X	PKC	0.02
Et-18-OCH ₃	PI-PLC	0.4-9.6
GO6983	PKCα, β, γ, δ, ζ (not μ)	0.007-0.06
HBDDE	PKCα, γ (not βI, βII, or δ)	43-50
Rottlerin	PKCδ	3-6 (20)
PKA (14-22) inhibitor, myr	PKA	5-25
PKC (20-28) inhibitor, myr	PKC, pan	5-25
PKCβ inhibitor, myr	PKCα, βI, βII, γ (not δ or ε)	5-25
PKCε inhibitor, myr	PKCε (not α, βI, or δ)	5-25
PKCζ inhibitor, myr	PKCζ, PKCι	5-25

DG, diacyl glycerol; HBDDE, 2,2'3,3',4,4'-hexahydroxy-1,1'-biphenyl-6,6'-dimethanol dimethyl ether; myr, myristoylated; PK, protein kinase; PI, phosphatidylinositol; PL, phospholipase.

* Where available from the manufacturer, IC₅₀ values are listed for assays using intact cells. All reagents are from Biomol, Plymouth Meeting, PA.