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The type of interaction with FcγR in human monocytes determines the efficiency of the generation of oxidative burst

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SUMMARY

Receptors for the Fc fragment of IgG (FcγR) have a well-documented role in the generation of oxidative burst. It is tempting to speculate that the type of interaction with FcγR could be a mechanism of regulation of this process. Here we report on a comparative study of the induction of oxidative burst in human monocytes activated by means of different types of interaction with FcγR. We studied non-primed monocytes obtained by centrifugal elutriation from healthy donors. These cells were submitted to FcγR interactions following two distinct models: one, using particulate material (IgG–SRBC leading to phagocytosis or rosetting), and another using soluble reagents followed by cross-linking of the receptors (monoclonal antibodies against FcγRI and FcγRII and natural ligands, namely several isotypes of murine and human IgG). Phagocytosis and oxidative burst were studied simultaneously in the monocytes, following the methodology described recently. Human non-primed monocytes were able to generate a very obvious oxidative burst response after activation of FcγR by particulate material. The same response was observed when FcγRII was blocked by monoclonal antibodies. Ingestion was not necessary for activation of the oxidative burst, since the model of rosetting induced a level of burst generation similar to the one obtained in the phagocytic process. Cross-linking of FcγRI by soluble reagents induced production of reactive oxidative intermediates (ROI) only when the ligand-binding site of the receptor was involved. These data lead to the conclusion that FcγR interaction with soluble or particulate material induces oxidative burst in non-primed human monocytes only when the binding site of natural ligands is involved. The type of interaction also determines the efficiency of the generation of ROI.This fact could represent a regulatory mechanism.

INTRODUCTION

Different types of receptors for the Fc fragment of IgG have been described showing differential expression in cells of the phagocytic system.[1] Human non-activated monocytes express mainly FcγRI and FcγRII,[2] both receptors showing different grades of polymorphism, as described recently.[1] These receptors have been documented as functionally active molecules, the generation of oxidative burst being one of the cellular events described after FcγR ligation. Several authors have observed the need for cytokine-priming of human monocytes for the induction of this type of reactive product after cross-linking of FcγR by monoclonal antibodies.[3–5] Since most of these reagents do not recognize epitopes related to the binding site of the receptor, it seems necessary to address the issue of ligation of these molecules by the site of union of the natural ligand.[6–8]

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Abbreviations: DCFH-DA, 2′,7′dichlorofluoresceindiacetate; FcγR, receptor for Fc fragment of IgG; ROI, reactive oxygen intermediates.

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Given the significance of this type of biological response as a defensive mechanism,[9] the organism must have a system to regulate its activation. Extracellular leakage would have deleterious effects on different tissues, being possibly involved in the pathogenesis of some inflammatory diseases.[10–12]

A mechanism of control of this process could consist of different types of interaction of Fc–FcγR. Using a methodology described recently,[13] we have studied the oxidative burst response (intracellular H_2O_2 production) after ligation of FcγR in non-activated human monocytes. We have compared a model of phagocytosis of IgG–sheep red blood cells (SRBC) or rosetting of the same material with the cross-linking of FcγR by monoclonal antibodies or by different isotypes of human and murine IgG.

We describe here differences in the induction of oxidative burst in non-primed monocytes according to the type of FcγR interaction (particulate or soluble) and whether the binding site of the ligand was involved or not.

MATERIALS AND METHODS

Materials

2′,7′ dichlorofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR); the density gradient

Lymphoprep was purchased from Nycomed Pharma (Oslo, Norway); and RPMI-1640 medium was obtained from Gibco (Paisley, UK). SRBC and all chemicals of reagent grade quality were purchased from local vendors.

Monoclonal antibodies against FcγRI (197, 32·2 and 22)[14,15] and FcγRII (IV.3)[16] were purchased from Medarex Inc. (West Lebanon, NH). Purified mouse myeloma immunoglobulins (IgG2a and IgG1) and goat $F(ab')_2$ fragment anti-mouse IgG (H + L) were obtained from Zymed Lab. Inc. (San Francisco, CA). Affinity-purified human IgG was purchased from Dako (Glostrup, Denmark), and goat anti-human IgG (γ-chain specific) was obtained from Sigma Chemical Co. (St Louis, MO). Purified rabbit IgG anti-SRBC was obtained from Nordic Immunology (Tilburg, the Netherlands).

Isolation and labelling of monocytes

Monocytes were isolated by centrifugal elutriation, as previously described.[13] The purity of the monocyte population obtained was 85 ± 10% (labelling of CD14), and the viability as assessed by trypan blue exclusion test was >90%.

In order to label the isolated monocytes with the probe DCFH-DA, cells were washed and resuspended in phosphate-buffered saline (PBS) Ca^{2+}- and Mg^{2+}-free containing 5 mM glucose and 0·1% gelatin at 1×10^6 cells/ml. The cells were exposed to 5 μM of DCFH-DA in absolute ethanol for 15 min at 37° with occasional shaking.[17,18] Thereafter, the cells were washed once and suspended in RPMI-1640. The labelling did not affect monocyte phagocytosis.

Phagocytosis and rosetting assays

DCFH-DA-loaded monocytes (1×10^6) were mixed and incubated with 20×10^6 SRBC sensitized with a suboptimal agglutinating dilution of IgG anti-SRBC (1/400; 6 μM IgG) in RPMI-1640. The mixture, after gentle centrifugation, was maintained for 40 min at 37°.[19] After that, the suspension was submitted to conditions of hypotonic lysis to eliminate extracellular SRBC. In order to document the specificity of the FcγR interaction, we performed phagocytosis after the incubation of monocytes with monoclonal antibody anti-FcγRII (IV-3) at 1·2 μg/10^6 cells in 100 μl of PBS, for 30 min at 4°, to block FcγRII. After washing in PBS the cells were submitted to the process of phagocytosis as above. In some experiments 10 or 20 mM EDTA was added to the incubating medium to facilitate the formation of rosettes without ingestion of SRBC–IgG. The formation of rosettes was checked by light microscopy. After elimination of extracellular SRBC by means of hypotonic treatment, the ingestion of SRBC was excluded in all cases.

After the completion of the assays described, cells were resuspended in PBS and analysed by flow cytometry to quantify the oxidative burst, measured as oxidation of DCFH-DA. As a control, DCFH-DA-labelled monocytes incubated with non-sensitized SRBC from each donor were run in parallel.

Cross-linking of FcγR by soluble reagents

DCFH-DA-loaded monocytes (1×10^6) were incubated with mAb anti-FcγRI (197, 32·2, and 22) and anti-FcγRII (IV.3) and with murine subclasses of IgG (IgG2a and IgG1) and human IgG. These reagents were used at two different concentrations: 1·2 and 6 μg/10^6 cells in 100 μl of PBS. Cells were incubated for 30 min at room temperature. After washing twice in PBS, cells were incubated in RPMI-1640 for 40 min at 37° with 3 and 10 μg/10^6 cells in 100 μl of PBS of the second reagent [goat IgG $F(ab')_2$ fragment anti-mouse IgG and goat IgG anti-human IgG), to facilitate the cross-linking of the receptor. We utilized two different concentrations of these reagents, to saturate the receptors and to possibly establish two levels of cross-linking. After this second step, cells were washed and resuspended in PBS, to be analysed by flow cytometry for quantification of oxidative burst. Again, cells from each donor were run in parallel as a control, omitting the incubation with the first reagent.

Quantification of oxidative burst by flow cytometry

Cells submitted to phagocytosis, rosetting or cross-linking by soluble reagents, were thereafter studied by flow cytometry (Epics Profile II; Coulter Electronics, Hialeah, FL,USA), in order to quantify the oxidative burst measured as oxidation of DCFH-DA. Monocytes were discriminated based on the characteristics of forward and 90° light scatter, this population being selected for study by means of electronic gating. The fluorescence was measured using logarithmic amplification on a linear scale of 1024 channels, and in all experiments a constant setting of the cytometer was maintained. For each histogram >5000 monocytes were analysed and data were reported as the peak of fluorescence intensity. To avoid possible individual variations in the load of DCFH-DA, the measurement of oxidative burst was calculated in all experiments by subtracting the peak channel fluorescence of the appropriate control from the peak channel fluorescence of the stimulated cell population.[18] Negative values were obtained when the fluorescence of an activated population was below the respective control. At the same time we quantified, when appropriate, the grade of phagocytosis by subtracting the peak channel of forward scatter of the negative control SRBC from the peak channel forward scatter of the phagocytic population (SRBC–IgG) on a linear scale of 1024 channels, with linear amplification.

Statistical analysis

Results are reported as mean ± SD, unless otherwise stated. Differences between the results of various experiments were evaluated by means of the Mann–Whitney *U*-test or Wilcoxon matched-pairs test.

RESULTS

Generation of oxidative burst in human monocytes through particulate interaction (SRBC–IgG) with FcγR

We studied two different models of particulate interaction with FcγR using SRBC–IgG as the interacting material: phagocytosis, and interaction with FcγR without ingestion by depleting the incubating medium of bivalent cations. Figure 1 shows that the induction of phagocytosis produced an important generation of oxidative burst (Fig. 1f) compared with a control interaction with SRBC without previous opsonization with IgG anti-SRBC (Fig. 1e). In the same figure, it is interesting to notice the changes in the mean channel forward scatter (Fig. 1b) as an index of phagocytosis performed by monocytes (see ref. 13 for further comments). When resting human monocytes were submitted to the formation of rosettes with SRBC–IgG without subsequent ingestion of this material, we could quantify a generation of oxidative burst of similar proportions to that performed by phagocytic cells (Fig. 1g, h). However, as

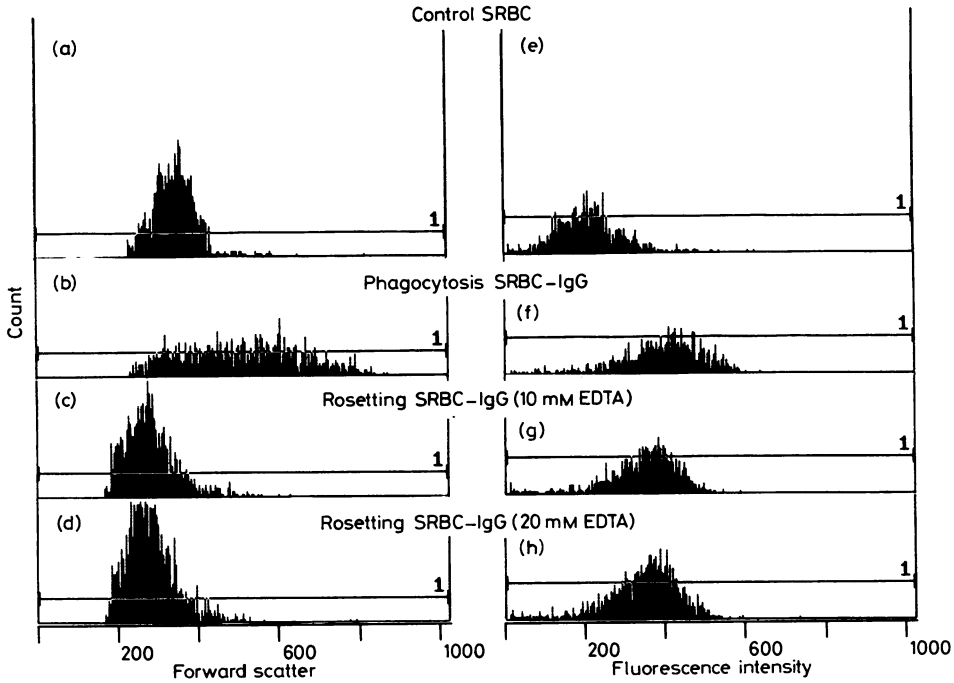

Figure 1. Oxidative burst induction in phagocytosis and rosetting: one representative case out of 10 experiments performed in identical conditions. Phagocytosis and oxidative burst of DCFH-DA-loaded monocytes incubated with SRBC–IgG were assessed by flow cytometry. (b) and (f) show the changes observed in monocytes submitted to phagocytosis, compared to the respective control in (a) and (e). (c, d, g, h) represent the changes in the same parameters when rosetting of monocytes at different concentrations of EDTA was performed.

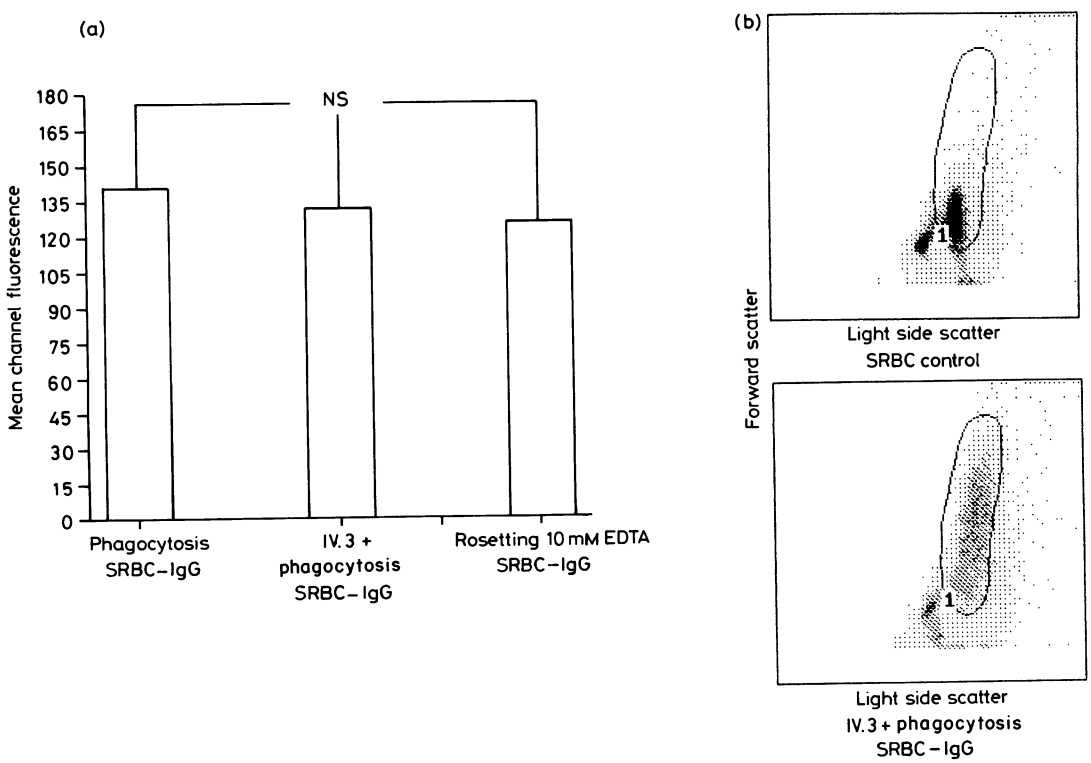

Figure 2. Quantitative representation of oxidative burst (fluorescence intensity) in conditions of phagocytosis and rosetting, in 10 experiments as described in Fig. 1. Previous incubation of monocytes with anti-FcγRII did not affect neither phagocytosis (b) nor oxidative burst response (a).

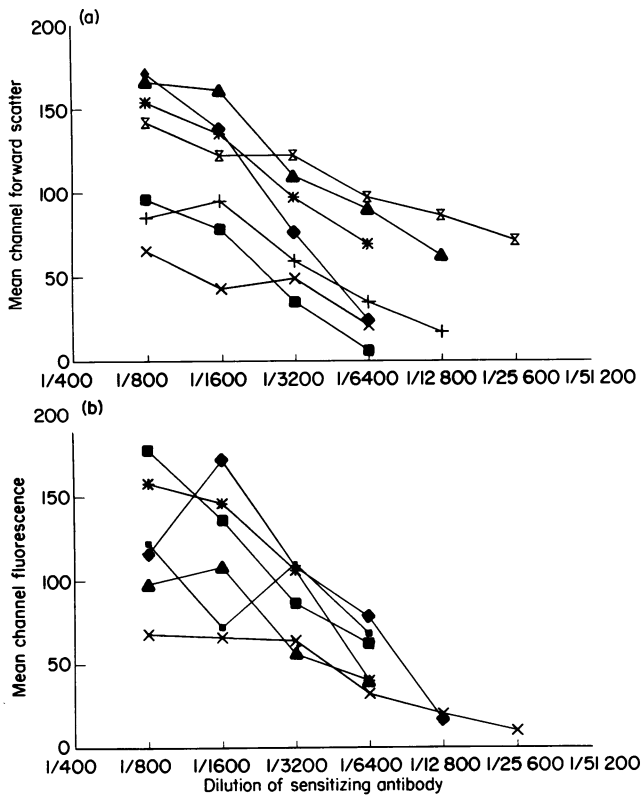

Figure 3. Effect of the dilution of the anti-SRBC antibody on the degree of phagocytosis (a) and oxidative burst (b) in different donors. Parameters shown on the *y*-axis represent the channel increase over the respective controls.

expected, we were not able to document changes in forward scatter because of the lack of ingestion of the material (Fig. 1c, d). Figure 2a is a quantitative representation of the data of 10 donors showing that no statistical differences in the generation of oxidative burst in the models of phagocytosis and rosetting were observed. These data illustrate that the depletion of extracellular bivalents cations did not interfere with the generation of oxidative burst in our model.

At the same time, Fig. 2a shows that the blocking of FcγRII with monoclonal antibody (IV-3) followed by FcγR-mediated phagocytosis, produced the same degree of oxidative burst compared with experiments in which this receptor was not previously blocked. Figure 2b shows that phagocytosis took place in spite of FcγR blocking. Phagocytosis (Fig. 3a) and oxidative burst (Fig. 3b) showed a good correlation in the degree of sensitization of SRBC, in every donor. It is interesting to notice that, in spite of low amounts of antibody bound to SRBC (dilution 1/3200; 0·7 μM IgG), it was still possible to generate a considerable amount of oxidative burst (increase of 60–120 channels in fluorescence intensity; Fig. 3b).

Generation of oxidative burst in human monocytes through FcγR cross-linking by soluble reagents

We studied the generation of oxidative burst in monocytes after cross-linking of FcγR by several anti-FcγRI and FcγRII monoclonal antibodies and natural ligands of these receptors (murine IgG subclasses and human monomeric IgG). The monoclonal antibodies utilized interacted with different epitopes than the binding-site of natural ligands. We were able to verify the presence of cross-linking by fluorescence microscopy (data not shown). As described in the Materials and Methods, we utilized different amounts of reagents in the assay. Figure 4a shows that the cross-linking of FcγR through different ligands did not induce the generation of oxidative burst when the first level of reagents was used (1·2 and 3 μg/1 × 10^6 cells for the first and second step, respectively). When a supposed higher degree of cross-linking was achieved (by increasing the amount of the reagents to 6 and 10 μg/1 × 10^6 cells), it was possible to induce the oxidative burst only when the natural ligand human IgG was used ($P < 0{\cdot}05$; Fig. 4b). The murine ligand IgG2a (fig. 4b) showed an increase in the production of oxidative burst that was not statistically significant. However, the difference observed in the degree of oxidative burst induced by this isotype at the two levels of cross-linking was statistically significant ($P < 0{\cdot}05$) (Fig.4a, b). It is noteworthy that the level of production of reactive oxygen intermediates (ROI) by

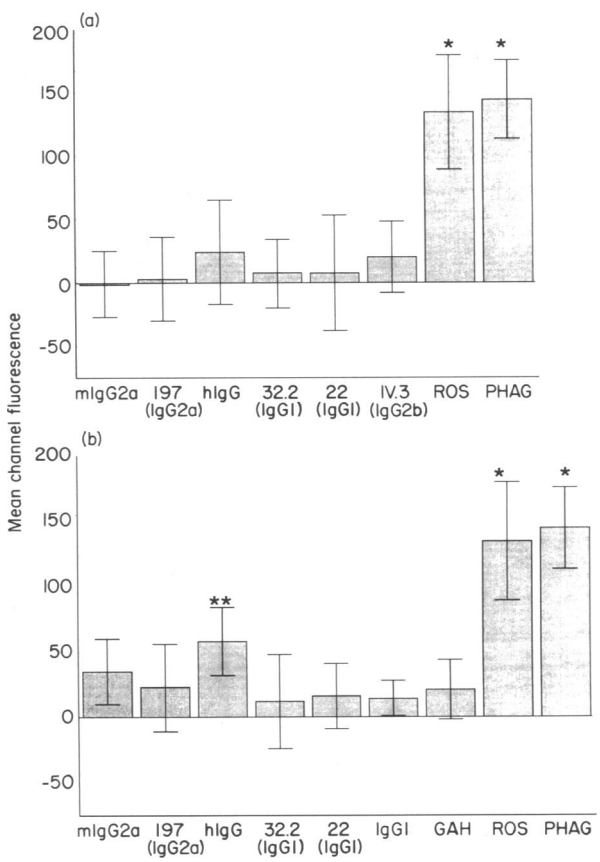

Figure 4. Oxidative burst (mean channel fluorescence) induced by cross-linking of FcγR, with different reagents detailed on *x*-axis. The number of cases varied between 6 and 14. (a) Represents the first level of cross-linking performed, and (b) the second one (see the Materials and Methods). The IgG subclass of each monoclonal antibody is indicated in parentheses. Mouse IgG1 and goat anti-human IgG (GAH) were used as control. Results of phagocytosis (PHAG) and rosetting (ROS) from other experiments are included as a comparative reference. *$P < 0{\cdot}001$ versus cross-linking; **$P < 0{\cdot}05$ versus cross-linking, except mIgG2a.

human IgG was much lower than the level observed with the particulate material (33% approximately; Fig. 4b).

DISCUSSION

ROI, such as superoxide, hydrogen peroxide and hydroxyl groups, are produced by phagocytes when exposed to certain stimuli. Engagement of Fcγ receptors, complement receptors, receptors for mannose terminal glycoproteins, or exposure to the phorbol ester phorbol myristate acetate (PMA), can stimulate an oxidative burst response.[20,21] A physiological role for these receptors is logical if one considers the contribution of phagocytic cells to the homeostasis of the organism. The possibility of leakage of these reactives and the consequent tissue damage is considered in some diseases to be the main pathological mechanism.[10–12]

For all these reasons, the existence of regulatory mechanisms for this process of cellular activation seems quite logical. One of the possibilities could be the type of interaction involved in the generation of these reactive products. We have hypothesized in this paper that different models of interaction with FcγR could lead to a different state of cellular activation and to a different level of production of ROI.

The models that we compared included the interaction of particulate material (SRBC–IgG) with two different approaches (phagocytosis and extracellular interaction through the formation of rosettes). The other possibility was the cross-linking of the receptor by several reagents, some of which were natural ligands of the receptors, while others recognized other epitopes of the same receptors.

Most of the published reports of the role of FcR in the generation of ROI in monocytes have used the model of soluble interactions and cross-linking by monoclonal antibodies.[3–5,14] Data from these reports led to the conclusion that the induction of ROI through FcR in resting monocytes is not possible, showing that only cells previously activated by cytokines were able to generate ROI. This occurred despite the fact that the methods used for the isolation of monocytes in these reports probably induced activation of the cell.[22,23]

The method of phagocytosis of SRBC–IgG as a means of FcγR interaction, is a well-established fact in the literature.[16,24] On the other hand, the studies performed in our laboratory, as well as by other investigators, concerning the blocking of FcγRII prior to phagocytosis with monoclonal antibodies which recognize the binding site of natural ligands (IV.3), are not conclusive because of the possibility of displacement of the blocking antibody by particulate material of higher affinity.[25] This possibility is even more likely with the utilization of non-blocking monoclonal antibodies (i.e. most of the antibodies recognizing FcγRI). The use of diluted purified rabbit IgG anti-SRBC eliminates, in our opinion, the possible implication of other receptors in this process (i.e. complement receptors).

We think that this model of FcR interaction with SRBC–IgG in resting monocytes has not been exhaustively explored in the literature regarding the induction of ROI. This model probably offers a useful approach to many situations that take place in physiological conditions (i.e. opsonized bacteria). On the other hand, the methodological strategy that we have developed recently[13] offered us an efficient tool to get adequate information. In this sense, the use of a flow cytometry technique that simultaneously quantifies phagocytosis and generation of ROI allowed us to get overall information and to draw more accurate conclusions.

When monocytes from healthy donors were submitted to phagocytosis of SRBC–IgG, we found that it was possible to induce generation of ROI after phagocytosis. Moreover, the two processes showed a quantitative correlation in every single donor. The generation of ROI, however, was not linked to the process of ingestion, because the extracellular interaction of particulate material without ingestion (rosetting) was enough to generate the same level of ROI. From these experiments it is also possible to conclude that the depletion of extracellular bivalent cations does not affect the generation of ROI.[26–28] Given the role of Ca^{2+} (mainly intracellular) in phagocytosis,[29] we have no clear explanation for the abolition of phagocytosis we observed with the depletion of extracellular Ca^{2+}.

Our data offer information on the level of ROI production in healthy donors and the possibility of comparative studies with different pathological states in which this mechanism could be impaired.[30,31] At the same time, given that the induction of ROI in this model was mediated through FcγR, it should be possible to study the efficiency of production of ROI through FcγR in diseases in which a defect in the process of signalling through FcγR is known or suspected (work in progress in our laboratory).[32,33]

Cross-linking of FcγR with monoclonal antibodies in resting monocytes, through epitopes not related to the binding site of natural ligands, did not induce appreciable generation of ROI in our model. However, when epitopes that recognize natural ligands were involved we were able to generate ROI, but at a reduced level compared with the model of particulate interaction. Both human IgG and murine IgG2a induced ROI, although we observed statistical significance only for human IgG, having no clear explanations for the difference between the two ligands. Perhaps the use of whole human IgG and the polymorphism of FcγR could help to explain these differences. The failure of murine IgG1 (the ligand for FcγRII) in the generation of ROI could be explained by the low affinity of this receptor for monomeric ligand. These data underline the importance of the engagement of FcγR through the active binding site in order to generate of ROI.[6–8]

When comparing the two models studied in this paper, it is interesting to notice the quantitative differences observed in the generation of ROI. One possible explanation could be the higher degree of cross-linking of the receptors that, conceivably, we would get with the particulate material. We do not think that this is the reason for these differences, because when we studied particulate interaction at very high dilutions of the opsonizing antibody, we still observed a level of ROI similar to the one obtained with the soluble model in positive cases. Besides, the particulate material is likely to interact simultaneously with FcγRI and FcγRII, while the soluble reagents are directed only to one type of receptor. Another possibility could be based on unknown additional ligand–receptor interactions.[20,21,34–36] These additional interactions could undoubtedly have a role in the particulate model (SRBC), being absent in the model of soluble ligand. It is possible to attribute a co-signalling function to these molecules, leading to a higher degree of cellular activation and, subsequently, to a higher level of ROI production. In summary, we show in this paper the possibility of ROI generation through FcγR in resting monocytes. By using a model that probably mimics a physiological situation,

we were able to demonstrate differences in cellular activation by varying the type of interaction with the receptor. This could represent a mechanism of regulation of the system, of evident physiological relevance. The control of the activation of oxidative burst could play a significant role in the pathogenesis of inflammation.

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