Expression of Fc Gamma Receptors on Peripheral Blood Monocytes and Evaluation of their Role in Phagocytosis Using a Novel Method.

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Abstract

Fc receptors play an important role in the immune system by providing a link between the B cell antibody production and the effector functions. FcγR bind the Fc region of the IgG molecule which cause receptor clustering and the phosphorylation of intracellular tyrosine signalling motifs. This in-turn triggers biochemical cascades which activate or inhibit the cells effector action. In humans there are four classes of IgG and eight genes encoding the Fc gamma receptors. Recent studies have suggested that different combinations of FcγR isotypes and IgG subclasses may trigger different immune responses.

The aim of this study was to investigate the percentage of FcγR positive monocytes within a random, healthy cohort and to examine their cells surface expression. Using FACS analysis we have looked at the percentage of cells expressing the different FcγRs and characterised their expression within individuals. We also wanted to study through which FcγR, IgG subclass specific immune complexes are bound and phagocytose. To study immune complex binding we used a rosetting assay and for phagocytosis we used a newly described method. This method involved using fluorescent dyed IgG1 or IgG3 opsonised human red blood cells which are phagocytosed into peripheral blood monocytes and can be measured by FACS analysis. We also wanted to study different methods of monocyte purification.

The results show that, on average, 71% of monocytes express FcγRI, 24% express FcγRIIb, 36% express FcγRIII while FcγRIIa is constitutively expressed. Each receptor is expressed at similar cell surface concentrations between individuals. FcγRI and FcγRIIb are expressed at low concentrations, while FcγRIIa is expressed at high concentrations. FcγRIII has varying cell surface expression. The rosetting studies showed that the cell surface concentration of FcγRI and FcγRIII contributes to immune complex binding while the percentage of cells expressing FcγRIIa was important for phagocytosis. This confirms that different immune functions can be activated depending on the FcγR isotype expression patterns and the IgG subclass of the immune complex.
List of Abbreviations

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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Ab</td>
<td>antibody</td>
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<tr>
<td>EDTA</td>
<td>ethylenediamineetraacetic acid</td>
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<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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<td>FcγR</td>
<td>Fc gamma receptor</td>
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<td>FCS</td>
<td>foetal calf serum</td>
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<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>IC</td>
<td>immune complex</td>
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<td>IFNγ</td>
<td>interferon gamma</td>
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<td>IgG</td>
<td>immunoglobulin G</td>
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<td>IL-6</td>
<td>interleukine 6</td>
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<td>ITAM</td>
<td>intra-cellular tyrosine-based activating motif</td>
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<td>intra-cellular tyrosine-based inhibitory motif</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>mAb</td>
<td>monoclonal antibody</td>
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<td>MFI</td>
<td>mean fluorescence intensity</td>
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<td>PBM</td>
<td>peripheral blood monocytes</td>
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<td>PBS</td>
<td>phosphatase buffered saline</td>
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<td>PE</td>
<td>phycoerythrin</td>
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<td>PI</td>
<td>propidium iodide</td>
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<td>RA</td>
<td>rheumatoid arthritis</td>
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<td>RBC</td>
<td>red blood cells</td>
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<td>Rh D</td>
<td>rhesus D protein</td>
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<td>TGFβ</td>
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<tr>
<td>TNFα</td>
<td>tumour necrosis factor α</td>
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Introduction

Fc gamma receptors (FcγR) are receptors for the antibody (Ab) immunoglobulin G (IgG). IgG is produced in response to foreign antigen (Ag) induced interferon γ (IFNγ) production which leads to B-cell activation and Ab class switching. IgG is composed of four chains, two heavy chains with four Ig domains and two light chains with two Ig domains. The Ab molecule can be separated into two functional regions, the Fab region which binds to the Ag and the Fc region which mediates the functional response. The Fc region of the IgG molecule activates an immune response by binding an FcγR on immune cells and activating downstream pathways within the cell. There are four subclasses of IgG in humans; IgG1, IgG2, IgG3 and IgG4. The corresponding IgG subclasses in mice are; IgG1, IgG2a, IgG2b, and IgG3. Each subclass has a different Fc fragment and interacts with the FcγR with different affinities.

FcγRs play a crucial role in the B-cell mediated immune response by providing a link between the activation of humeral immunity and the effector function. FcγRs bind to either an IgG molecule or an Ag which has been opsonised by IgG molecules, called an IgG immune complex (IC). The binding of an IgG molecule or IgG IC to the FcγR can activate several functions including phagocytosis, de-granulation, the production of cytokines, and Ab-dependent cellular cytotoxicity (ADCC). In humans there are three different classes of FcγR; FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). FcγRI and II can further be divided into A, B and C subclasses and FcγRIII has an A and B form (figure 1). In humans all eight FcγR genes are located on one gene locus located on chromosome 1. All FcγR consist of a
glycoprotein α-chain with 2 (FcγRII and FcγRIII) or 3 (FcγRI) Ig domains in the extracellular region.  

The FcγRs can be divided into two functional groups, activating and inhibitory.  

FcγRI is the high affinity activating receptor and can bind to monomeric IgG as well as IgG IC, while FcγRIIa and FcγRIII bind with low-affinity and only to polymeric IgG.  

The α-chain of both FcγRI and FcγRIII associate with a γ-chain which have an intra-cellular tyrosine-based activating motif (ITAM). Unlike FcγRI and FcγRIII, FcγRIIa does not associate with a γ-chain but rather has an intra-cellular ITAM on its α-chain.  

FcγRIIb is the only inhibitory FcγR. It has a 92% homology to FcγRIIa and differs only in the intra-cellular signalling motif. FcγRIIb has an intra-cellular tyrosine-based inhibitory motif (ITIM) on the intra-cellular domain of its α-chain.  

Different immune cells express different FcγR isotypes on their cell surface. The most common FcγR positive cells in blood are monocytes, neutrophils and B cells. Monocytes express three classes of FcγRs; FcγRI, FcγRIIa, FcγRIIb and FcγRIIIa. Neutrophils express FcγRI, FcγRII, FcγRIIb and FcγRIIIb. B cells have only the inhibitory FcγRIIb.  

Several studies have shown that there are many factors which can influence the phagocytosis and clearance of IgG opsonised Ag. It has been noted that the different FcγR isotypes have diverse affinities for individual IgG subclasses. FcγRI preferentially binds IgG1 and IgG3, FcγRII preferentially binds IgG3 and to a lesser extent IgG1 and IgG2. FcγRIII binds to IgG1 and IgG3 with a lower affinity than FcγRII. These observations have lead to further investigations into how the interaction between IgG subclass specific IC binding to particular FcγR subclasses leads to the activation of different cellular functions. It has been suggested that IgG1 IC are preferentially phagocytosed via FcγRI but are not bound in rosettes and that IgG3 IC could be easily bound but were not phagocytosed.  

Another factor which influences the ability of FcγR positive phagocytic cells to phagocytose an IgG opsonised Ag are the FcγR polymorphisms. There are several known polymorphisms for the FcγRs, for example FcγRIIIa- V158. It has been shown that RBC opsonised with specific IgG subclasses will be cleared with
different efficiencies depending on the FcγR polymorphism expressed on the cell and that the FcγR polymorphism can effect effector functions.

FcγRs bind IgG with a 1:1 stoichiometry which is why the Ag needs to be opsonised by many IgG molecules in order to activate an immune response. The binding of FcγRs to an IgG opsonised particle leads to the clustering of the FcγRs and the phosphorylation of intra-cellular tyrosine signalling motifs, ITAM or ITIM, by Src kinases bound to the cytosolic-side of the phospholipid layer. The phosphorylated tyrosine on the activating FcγR recruits many proteins including SH2 adaptor proteins which then trigger biochemical cascades leading to the activation of different immune functions. In phagocytosis it is necessary for the ITAM to recruit and activate the SH2 protein, p72Syk protein. Instead of recruiting SH2 proteins, FcγRIIB recruits inositol phosphatases which activate biochemical cascades leading to the down regulation of immune responses.

The precise role played by the different IgG-FcγR binding complexes in the activation of the immune responses is yet to be determined. Its importance is suggested by the observation that the expression of the FcγR differs significantly between healthy and disease states. Previously it has been shown that circulating monocytes are activated before they enter the joints in rheumatoid arthritis (RA) patients. Unpublished data from this research group has shown that there is an increase in FcγRI, FcγRII and FcγRIII in synovial biopsies from RA patients when compared to healthy controls (Sofia Magnusson, unpublished data). In this study, we have looked at the FcγR expression on peripheral blood monocytes (PBM) from healthy donors in order to compare it, in the future, to PBM FcγR expression from RA patients. We hope this will lead to a more complete picture of the role FcγRs play in RA.

In this study several aims were investigated; 1) to determine the variation in the FcγR expression on PBM from healthy donors 2) to investigate and compare different methods of PBM purification and 3) to investigate the role of FcγR in IgG specific IC binding and phagocytosis. Our findings suggest that there is broad expression of the FcγRI and FcγRIII within the general population and point to FcγRIIa as a contributor to the phagocytosis of subclass specific IgG IC. Our finding also show that the method of purification can influence the FcγR expression and the cell function.
Methods and Reagents

Purification of blood monocytes
Buffy coats were collected from healthy donors who were Rhesus (Rh) D protein positive. In this cohort there were 9 male and 7 female participants (Blodcentralen, Akademiska Sjukhuset, Uppsala, Sweden). The buffy coat was diluted one time with phosphatase buffered saline (PBS) with 0.2 mM ethylenediaminetetraacetic acid (EDTA). The mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) following the manufacturers instructions. The PBM were then isolated using one of three methods; plastic adherence, CD14 positive magnetic cell sorting (MACS, Miltany Biotec, Bergish, Germany) or CD14 negative MACS (Militany Biotec). For the data analysis of the functional assays (rosetting and phagocytosis) buffy coats were excluded if their donor had taken non-steroid anti-inflammatory drugs within the previous 10 days before blood collection.

Plastic adherence
Mononuclear cells were washed three times in PBS with 0.2 mM EDTA and resuspended in RPMI 1640 medium (SIGMA, Saint Louis, USA) with 1% heat inactivated foetal calf serum (FCS) (SIGMA), 100 U/ml penicillin, 100 µg/ml streptomycin and 1% glutamax (SIGMA) (complete medium) to a concentration of 1-2 x 10^6 cells/ml. The cells were incubated in polystyrene culturing flasks (BD Falcon, Franklin Lakes, U.S.A) for 30 minutes at 37°C with 5% CO2 to allow the monocytes to adhere to the plastic. After incubation the flasks were washed 3x with PBS and the adherent peripheral blood monocytes (PBM) were removed by gently scraping the plastic with a rubber policeman. The purified monocytes were then resuspended in the complete medium, counted using a Bürker chamber and diluted to a final concentration of 1-2 x 10^6 cells/ml. The cells were kept at 37°C, 5% CO2.

CD14 positive MACS
Positive MACS was performed using CD14 microbeads following the manufacturer’s instructions. Mononuclear cells were centrifuged at 1000 rpm for 10 minutes in PBS and the supernatant completely removed. The cells were then resuspended in
degassed MACS buffer to a concentration of $10^7$ cells per 80 µl buffer. Twenty µl of CD14 microbeads (Milteny Biotec) were added per $10^7$ cells and incubated for 20 minutes at 4°C. The cells were then washed in MACS buffer at 1000 rpm for 10 minutes, resuspended in 500 µl MACS buffer and added to a prepared MACS LS column (Milteny Biotec). The column was prepared by placing it in a magnetic field (VarioMACS, Milteny Biotec) and rinsing it with 3 ml MACS buffer. The CD14 negative cells were washed from the column by rinsing it with 3x 3 ml MACS buffer. The CD14 positive adhered cells were then eluted from the column by removing the column from the magnetic field and pushing 3 ml MACS buffer through the column with a rubber plunger. All steps were performed at 4°C. The cells were then centrifuged in MACS buffer at 1000 rpm for 10 minutes and resuspended in complete medium. They were counted using a Bürker chamber and diluted in complete medium to a final concentration of 1-2 x $10^6$ cells/ ml and kept at 37°C, 5% CO₂.

**CD14 negative MACS**

Negative MACS was performed using the Monocyte Isolation Kit II (Milteny Biotec) following the manufacturer’s instructions. Mononuclear cells were centrifuged at 1000 rpm for 10 minutes in PBS and the supernatant completely removed. The cells were then resuspended in 30 µl degassed MACS buffer (PBS pH 7.2, 0.5% BSA, 2 mM EDTA) per $10^7$ cells. MACS blocking reagent and MACS biotin antibody cocktail (Milteny Biotec) were added to the cell suspension, 10 µl of each per $10^7$ cells. The solution was then incubated for 15 minutes at 4°C. After incubation, 30 µl MACS buffer was then added per $10^7$ cells and 20 µl MACS anti-biotin microbead (Milteny Biotec) was added per $10^7$ cells. The cells were incubated for 20 minutes at 4°C. They were then washed in 1-2 ml MACS buffer by centrifugation at 1000 rpm for 10 minutes and the cells resuspended in 500 µl MACS buffer. An LS column was prepared by flushing it with 3 ml MACS buffer and the cell suspension added. The CD14 positive cells were washed through the column by eluting it with 3x 3 ml MACS buffer and the effluent, representing the enriched monocytes, was collected. The cells were then centrifuged at 1000 rpm for 10 minutes, and resuspended in complete medium. The cells were counted using a Bürker chamber, diluted in medium to a final concentration of 1-2 x $10^6$ cells/ ml and kept at 37°C, 5% CO₂.
Cell lines
Two monocyte-like cell lines were used in this study, MonoMac-6 and U-937 (kindly donated by Lars Hellman, Uppsala University Sweden). The MonoMac-6 cell line has phenotypic and biological characteristics of mature monocytes and is leukaemia derived. U-937 is a monocyte myelomonocytic cell line. Both cell lines were thawed from -70°C and expanded in RPMI 1640 medium with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% glutamax and kept at 37°C, 5% CO₂.

Antibodies
Phycoerythrin (PE) conjugated monoclonal (mAb) mouse anti-human CD14 (clone TüK4, IgG2a isotype, DakoCytomation, Glostrup, Denmark), matched isotype control PE conjugated mouse IgG2a (clone DAK-GO5, DakoCytomation). Fluorescein isothiocyanate (FITC) conjugated mAb mouse anti-human CD64 (clone 10.1, IgG1, BD Biosciences, Erembodegem, Belgium), FITC conjugated mAb mouse anti-human CD32 (clone KB61, IgG1) (DakoCytomation), FITC conjugated mAb mouse anti-human CD16 (clone DJ30c, IgG1, DakoCytomation) and matched isotype control FITC conjugated mouse IgG1 (clone DAK-GO1, DakoCytomation). Unconjugated anti-human FcγRIIa (clone IV.3, IgG2b isotype, kindly donated by Johan Rönnelid, Unit of Clinical Immunology, Uppsala University, Uppsala, Sweden), isotype control unconjugated mouse IgG2b (clone MOPC-141, SIGMA), unconjugated anti-human FcγRIIb (clone GB3, IgG1 isotype, kindly donated by Uwe Jacob, Max-Planck institute für biochemie, Abteilung Strukturforschung, Martinsreid, Germany) or isotype control unconjugated mouse IgG1 (clone MOPC-21, Sigma). PE conjugated polyclonal rabbit anti-mouse IgG (R-0439, DakoCyomation) was used as a secondary Ab.

PBM purity and FcγR expression determined by FACS analysis
The purity of the enriched monocyte cultures was determined by FACS analysis within 16 hours of the buffy coat collection. CD14 was used as a monocyte marker. CD14 is a lipopolysaccharide (LPS) receptor which binds LPS when complexed to an LPS-binding protein. The PBMs were washed twice in FACS buffer (1% BSA, 0.01% NaN₃) and resuspended at a concentration of 0.5 x 10⁶ cells/ 100 µl. The cells were then stained with anti-CD14 Ab and IgG2a isotype control. The cells were
incubated for 30 minutes in the dark. After incubation the unbound Ab was removed by centrifuging the cells at 1500 rpm at 4°C in 3 ml FACS buffer twice. The cells were then re-suspended in 500 µl FACS buffer. Before analysing the CD14 expression 0.5 µg propidium iodide (PI) (Sigma-Aldrich, Stockholm, Sweden) was added to the cells. PI stains DNA and is used to eliminate dead cells which can fluoresce un-specifically. PE fluorescence was measured in the FL-2 channel of a FACS machine (FACScan, BD biosciences) and measured on a logarithmic scale using CellQuest software (BD biosciences). 10,000 cells were analysed for each sample.

Double staining FACS analysis was performed on both the PBM and the two cell lines to determine the expression of FcγRI, FcγRII and FcγRIII within 16 hours of PBM purification. The PBMs were stained as described above with anti-CD14 and either anti-FcγRI, anti-FcγRII anti-FcγRIII or isotype controls. The cells were then washed and analysed as described above.

Single staining FACS analysis was performed on both the PBM and the cell lines to determine the FcγRIIa and FcγRIIb expression. Cells were stained as described above with either anti-FcγRIIa, anti-FcγRIIb or isotype controls. The cells were washed one time and stained as described above with the secondary Ab. The cells were then washed and analysed as described above.

IC binding by FcγR determined by rosetting

Preparation of IC

The ICs were prepared as previously described. Red blood cells (RBC) were isolated from the buffy coat through density gradient centrifugation using Ficoll-Paque Plus and kept in PBS at 4°C until needed. Initially for 12 samples 50 µl compact RBC was used to make the IC, however during the experiment it was noticed that halving the IC gave better results therefore only 25 µl compact RBC was used for future experiments. The packed RBCs were washed by centrifuging them three times in PBS at 2000 rpm for 7 minutes. Three different IC were made; 1) 7 µl of the compact RBC pellet was added to 125 µl PBS, 2 and 3) 7 µl of the RBC pellet was added to 117 µl PBS and mixed with either 8 µg anti-Rh D IgG1 Ab (BIRMA D6, International Blood Group Reference Laboratory, Bristol, United Kingdom) or 12 µg Anti-Rh D IgG3 Ab (BRAD 3, International Blood Group Reference Laboratory)
(please note the previous numbers are for the revised protocol with half the number of IC). The RBCs and Abs were incubated at 37°C for two hours. After two hours the unbound Abs were removed by centrifuging the IC in PBS at 2000 rpm for 7 minutes. The ICs were then diluted in 700 µl PBS.

**Rosetting assay**

The rosetting assay was performed on both the purified PBM and the cell lines. PBM were counted using a Bürker chamber and 0.5 x 10⁶ cells per sample were collected and centrifuged twice in PBS at 1000 rpm for 10 minutes. The cells were then resuspended in PBS to a concentration of 0.5 x 10⁶ cells per 100 µl. One hundred µl of the cell suspension was mixed with 175 µl of IC. For each type of IC, four replicates were made. The samples were centrifuged at 4°C, 1600 rpm for 1 minute to obtain maximum contact between the PBM and IC. The samples were then incubated at 37°C for 30 minutes. After incubation approximately 70 µl of the supernatant was removed and one drop of Sedistain (BD biosciences) was added. Sedistain consists of two dyes, crystal violet and safranin which are taken up by leukocytes, staining their nucleus and cytoplasmic granuals purple. The samples were resuspended by gently vortexing, taking care not to dislodge the bound IC from the PBM. The samples were numbered by an independent party and analysed blindly. Sixty µl of each sample was added onto a glass slide and the sample assessed under a microscope (Olympus BX60, Japan) at 40x magnification for the number of rosettes compared to unbound PBM. One hundred cells were counted for each sample and a rosette was defined and recorded if 3 or more RBC had bound to the PBM. The percentage of rosettes per total number of monocytes was calculated and the average percentage for the replicates was calculated for each type of IC.

**Phagocytosis of IgG subclass specific IC**

*Preparation of IC*

IC were prepared as previously described⁵ with the exception that 50 µl compact RBC was stained using PKH26 red fluorescence cell linker kit (SIGMA) following the manufacturers instructions before IC formation. A 2 µl PKH26 in dilutant C solution was made immediately prior to the staining procedure and the compact RBC were diluted in the same volume of dilutant C. Equal volumes of the RBC and PKH-26
solutions were mixed and incubated at 25°C for 5 minutes with occasional inversion. After the 5 minutes an equal volume of FCS was added to the solution and the reaction was allowed to stop for one minute. An equal volume of RPMI-1640 medium with 10% FCS, 100 µg/ml streptomycin and 1% glutamax was added and the cells were centrifuged at 2000 rpm for 7 minutes. To remove all unbound dye the cells were washed a second time in 10 ml RPMI 1640 medium and a final wash in PBS. PKH26 stains lipids in cell membranes. During the experiment it was noticed that halving the number of IC gave clearer results and so the number of stained RBC was halved to 25 µl. The control and IgG1 and IgG3 ICs were made with the stained RBC as described above.

**Phagocytosis assay**

The phagocytosis assay was performed on both PBM and the cell lines. PBM were purified as described above with the exception that they were resuspended in PBS to a concentration of 1 x 10^6 cells per 100 µl. For each type of IC, four replicates were made. A control was made which contained 100 µl cell suspension and 175 µl PBS. The PBMs and ICs were mixed and pelleted (as described above) and were incubated at 37°C for 1.5 hours. After the incubation the un-phagocytosed RBC were lysed using 1 ml ACK-lysis buffer (0.15 M NH₄Cl, 1.0 M KHCO₃, 0.1 mM EDTA, pH 7.2-7.4) for 1 minute. The reaction was stopped by adding 2 ml PBS. The samples were centrifuged at 1500 rpm for five minutes. The cells were then washed one time in 3 ml FACS buffer and resuspended in 500 µl FACS buffer. The PKH26 fluorescence was then read in the flow-cytometer in the FL-2 channel on a log scale using CellQuest software. Ten to twenty thousand cells were analysed from each sample.

**Blocking experiment**

FcγRIIa on PBM were blocked to investigate how it mediates phagocytosis. PBM were counted and prepared as above. Blocking was performed as previously described. Anti-FcγRIIa mAb (1.5 µg/ml, clone IV.3) was added to the cells and then incubated at 4°C for 30 minutes. The cells were then washed one time in PBS at 1500 rpm for 5 minutes at 4°C. Phagocytosis was performed as described above using the FcγRIIa blocked PBM.
Cytokine activation of cell-lines
To investigate if the FcγR expression on the MonoMac-6 and U-937 cell-lines could be able to up regulated we added LPS or different cytokines to the cell culturing medium. Cells were cultured for 72 hours at 37°C, 5% CO₂ with either; LPS 1 ng/ml, 1 ng/ml IL-6 (Pharma Genetic Engineering, CA, USA), 1 or 10 ng/ml TNFα (SIGMA), and 1 or 10 ng/ml TGFβ (SIGMA). The expression of FcγRs on the stimulated cells was determined by FACS analysis (see above for details).

Statistical analysis
ANOVA and Paired t-tests were performed using Graphpad Prism 4 software. A correlation co-efficient (r) values greater than 0.5 was considered significant and a P value less than 0.05 was considered significant.

Results

Plastic adherence, positive MACS and negative MACS gave the same purity of PBM
In this study PBM were purified from buffy coats using three methods: plastic adherence (n=14), positive MACS (n=2) and negative MACS (n=3). We were interested in seeing which gave the highest purity of PBM. PBM purity was assessed using CD14 FACS analysis. Plastic adherence and negative MACS gave an average purification of 25% and 26% respectively, while positive MACS gave an average purity of 42% (figure 2). We also used two purifying methods on one buffy coat; plastic adherence followed by negative MACS. This gave a PBM purity of 48% which was increased from 32% for just plastic adherence alone (data not shown). Thus positive MACS gives the best purity
of PBM and plastic adherence and negative MACS gives identical PBM purity. Using two methods of purification on the same buffy coat could increase the PBM purity.

**FcγR expression varied between healthy individuals**

In this study we looked at the expression of the different FcγR in healthy individuals. The FcγR expression was assessed by FACS analysis. Within the cohort studied there was anywhere from 24% to 94% of FcγRI positive monocytes with an average of 71% (figure 3A). The FcγRI geometric mean fluorescence intensity (MFI) was low with an average of 34 (figure 3B). The majority of PBM expressed FcγRII, the mean equalled 95%. In addition, to distinguish the two isotypes of FcγRII, FcγRIIa and FcγRIIb was investigated. FcγRIIa was expressed on a large proportion of the PBM, and had an average of 92% and a high MFI, average of 892 (figure 3A and C). In the cohort studied there was very low expression of FcγRIIb with the average proportion of FcγRIIb positive PBM at 24% (figure average MFI for FcγRIIb expression was 61

![Figure 3](image-url)
Figure 4 The expression of (A) FcγRI, (B) FcγRII, (C) FcγRIII, (D) FcγRIIa and (E) FcγRIIb were measured using FACS analysis on CD14 positive cells. In all dot plots the dead cells were gated out and for histograms the monocytes population was gated from the forward-scatter verses side-scatter plot. Filled line represents the isotype control and the un-filled line represents the PBM sample. The M1 region represents the background and the M2 represent the positive staining.

(figure 3B). FcγRIII had varied expression levels in this sample group, ranging from 7 to 86% (figure 3A). There was a tendency towards an overall low population of FcγRIII positive PBM with the average being 36%. The concentration of FcγRIII on the FcγRIII positive PBM also varied and the average MFI was 54 (figure 3). These results show that in this cohort of healthy individuals FcγRI and FcγRIIa are expressed on the majority of PBM while FcγRIIb and FcγRIII has varying expression.

The different FcγRs had differing expression patterns within an individual that were consistent throughout the whole cohort. FcγRI, FcγRII, FcγRIIa and FcγRIIb tended to
be expressed at constant concentrations on the PBM within an individual and formed a tight fluorescence peak (figure 4A, B, D and E). This contrasted with the expression of FcγRIII which tended to be varied and formed a smeared fluorescence peak (figure 4C). This is consistent with previous studies which have identified different populations of CD14 positive cells with different levels of FcγRIII expression.\(^{21, 25, 26}\)

**The FcγR expression is up regulated by plastic adherence**

Prior studies have suggested that purifying PBM by plastic adherence may activate them\(^{24}\) which could lead to the up-regulation of the FcγR. In this study FcγR FACS analysis was performed on one donor sample which had been purified by plastic adherence within 16 hours of blood collection and again 48 hours after. All the rosetting and phagocytosis experiments on the purified PBM were completed within 48 hours after PBM purification. Our results show that within 48 hours after PBM purification by plastic adherence, FcγRIII and FcγRIIb are up regulated (figure 5C and E). For FcγRIII the number of PBM expressing the receptor remained constant.

![Figure 5](image)

**Figure 5**  Cells were purified by plastic adherence and their FcγR expression was assessed by FACS analysis 16 hours and again 48 hours after purification. FcγRI (A), FcγRII (B), FcγRIII (C), FcγRIIa (D) and FcγRIIb (E) expression was measured by FACS analysis for both time points. In all samples gates were used to identify only the PBM. The filled line represents the isotype, the un-dotted line represents the FcγR expression 16 hours after and the dotted line represents the FcγR expression 48 hours after purification by plastic adherence.
but there was an increase in the receptor cell surface concentration (seen in the MFI) (figure 5C). The results also suggest that the activation of the cells during plastic adherence leads to a decrease in the amount of FcγRI (from 80% to 40%) and FcγRIIa (from 95% to 44%) positive PBM. For FcγRIIa there was also a decrease in the number of receptors on each cell (figure 5D). This was not seen for FcγRI who’s MFI remained constant (figure 5A). Statistical analysis of 16 hours and 48 hours after monocyte purification using the ANOVA test showed that the difference between the percentage of PBM expression of all the different FcγRs was not significant (p=0.314). There was a significant difference between the two time points in MFI up-regulation and down-regulation of all the FcγRs (p=0.007). This experiment was done on only one buffy coat, however, in addition a second buffy coat was purified first by plastic adherence then an aliquot of those cells were purified by negative MACS and the FcγR expression of the two cell samples were assessed. The results showed no difference in the FcγRI or FcγRII expression on the cells but there was an increase in FcγRIII positive PBM (from 12% to 19%). These results clearly suggest that the FcγR expression is affected by the method of purification.

**Qualitative analysis of phagocytosis using IgG1 and IgG3 IC**

Previously it has been shown that phagocytosis can be analysed by FACS using IC made of PKH26 dyed sheep red blood cells opsonised with IgG. In this study we show that PKH26 dyed human RhD positive RBC can be used in a similar way.

![Figure 6](image-url)  
**Figure 6** PBM purified by plastic adherence were incubated with PKH-26 dyed RBC (B), IgG1-RBC (C) or IgG3-RBC (D). Each sample was gated for monocytes (A) and fluorescence was looked for only in those cells.
During the FACS analysis samples were gated for monocytes (figure 6A) and the amount of PKH26 fluorescence in the identified monocyte population measured. PKH26-labelled RBC without any anti-RhD protein Ab was used as a negative control and positive phagocytosis was calculated as the total percentage of IgG1 or IgG3 IC phagocytosis minus the amount of phagocytosed control RBC (background). PKH26 fluorescence was seen as a positive smear suggesting that more than one IC could be phagocytosed (figure 6B-D). All PBM analysed for phagocytosis were purified by plastic adherence.

*Similar phagocytosis with either IgG1 or IgG3 IC*

In this study we wanted to see if there was a difference in IC phagocytosis when using specific types of IgG IC, IgG1 or IgG3. The results demonstrated that there no difference in the average percentage of phagocytosis of IgG1 (17%, n=12) or IgG3 (16%, n=12) IC (figure 7). We also wanted to investigate if there was a difference in IgG1 or IgG3 IC phagocytosis in men and women, but no significant difference (IgG1, p= 0.09 and IgG3, p= 0.37) was seen (data not shown).

We then wanted to investigate whether this positive phagocytosis correlated with FcγR expression. Using statistical analysis, the correlation coefficient (r) was calculated for the percentage of cells expressing the various FcγR and the percentage of phagocytosis with the different type of IC. There was no significant correlation between the expression of FcγRI and IgG1 nor IgG3 IC phagocytosis (r=0.01, p=0.43 and r=0.08, p=0.40 respectively). The expression of FcγRIII did not correlate to the amount of IgG1 nor IgG3 IC phagocytosis (r=0.11, p=0.37 and r=0.07, 0.42 respectively). However, there was a correlation between the FcγRII and the phagocytosis of both the IgG1 and IgG3 IC (r=0.68, p=0.01 and r=0.58, p=0.03 respectively). We also looked at the two isotypes of FcγRII, FcγRIIa and FcγRIIb. When FcγRIIa (n=6) was investigated, there was a trend indicating a correlation with both IgG1 and IgG3 IC phagocytosis but this was not significant (r=0.63, p=0.09).
and $r=0.58$, $p=0.11$ respectively). No correlation was found for the percentage of FcγRIIb positive PBM and phagocytosis of IgG1 or IgG3 IC. ($r=0.5$, $p=0.15$ and $r=0.08$, $p=0.44$, respectively). When investigating the FcγR expression at the cell surface level (MFI) there was no significant correlation with IgG1 or IgG3 IC phagocytosis (data not shown).

**Phagocytosis and purification**

One of the aims of this study was to investigate the effects different methods of purification had on the PBM phagocytosis. One buffy coat was purified by both plastic adherence and negative MACS and phagocytosis analysis was then performed for both cell populations using the same batch of IC. The average positive phagocytosis for plastic adhered PBM with IgG1 and IgG3 IC was 34% and 36% respectively. For the PBM purified by negative MACS the average percentage of positive phagocytosis with IgG1 IC was 0.4% and for IgG3 IC was 6%. This was a large difference and point to a difference between the phagocytic capabilities of PBM purified by plastic adherence and MACS methods.

PBM were purified from one buffy coat by both positive and negative MACS and the cells were assessed for phagocytosis. The average positive phagocytosis of the IgG1 IC was lower in the negative MACS purified sample (mean=19%) than for positive MACS purified PBM (mean=33%). There was no difference in the positive phagocytosis between the positive or negative MACS purified PBM phagocytosis of IgG3 IC (mean=27 and mean=29, respectively). There were too few results to assess whether these differences were significant. The results do not suggest a difference in the phagocytic function of PBM purified by positive or negative MACS.

**Blocking of FcγRs**

As we found a correlation between FcγRIIa and phagocytosis we wanted to investigate what happened when FcγRIIa was blocked. FcγRIIa was blocked on PBM from three separate donors. For two of the samples no phagocytosis was seen in either the un-blocked or the blocked sample. However, in the third sample phagocytosis was seen and we found that there was a decrease in the IgG1 IC phagocytosis in the blocked cells (un-blocked=42%, blocked=32%). There was no difference seen for the phagocytosis of the IgG3 IC (un-blocked=53%, blocked=52%).
Comparison of IC binding using IgG1 and IgG3 IC

Previous studies have shown that different FcγRs bind the IgG isotypes with different affinities,\textsuperscript{3, 4} suggesting that different immune functions could be activated through the binding of particular combinations of FcγR and IgG isotypes. We saw a difference in the average percentage of positive rosetting between IgG1 IC (average=5%) and IgG3 IC (average=10%) though this was not significant (p= 0.1, n= 7) (figure 8A). We also wanted to see if there was a difference in receptor binding between male and female buffy coats. For the binding assay there was a significant difference between male (n=5) and female (n=2) IgG3 IC binding (p= 0.03) but no significant difference in the IgG1 IC binding (p= 0.15) (figure 8B). The average binding of IgG3 IC in females was 23% compared to men which was 10% (figure 8B).

Furthermore we wanted to investigate if the binding was influenced by the number of FcγRs positive PBM. Our results do not show any significant correlation between any of the expressed FcγRs and the rosetting (data not shown). A correlation between rosetting and the two FcγRII isotypes, FcγRIIa and FcγRIIb, could not be assessed, as there were too few samples for statistical analysis (n=3). However, there was a trend

![Figure 8](image)

\textbf{Figure 8} Purified PBM were incubated with IgG subclass specific IC and rosetting was performed. To calculate IC binding the number of rosettes per 100 PBM was visually counted. (A) The average of IgG1 IC binding was 5% and the IgG3 IC binding 10%. (B) The samples were separated into sex groups. The average rosetting for male (M) with IgG1 IC was 3% and for IgG3 IC it was 6%. For females (F) the average IgG1 and IgG3 IC binding was 10% and 23% respectively.
of correlation between the cell surface expression (MFI) of the FcγRI and FcγRIII expressions and the IgG3 binding but it was not significant (r=0.57, p=0.09 and r=0.52, p=0.12 respectively, n=7).

**Characterisation of the cell-lines, MonoMac-6 and U-937, for FcγR expression and function**

MonoMac-6 and U-937 have been described as monocyte-like hybridoma cell-lines. FcγR characterisation for each cell line was performed. MonoMac expressed low levels of FcγRI and high amounts of FcγRIIa. U-937 expressed only FcγRIIa. No FcγRIII was detected for either cell-lines (figure 9A). Functional assays to detect the level of isotype specific IC binding and phagocytosis were performed on both cell lines (figure 9B). MonoMac-6bound IgG3 (9%) but not IgG1 IC and no positive phagocytosis was seen for IgG1 IC and only a little for IgG3 (3%). In contrast, U-937 could bind IgG1 IC (12%) but not IgG3 IC. We saw low levels of positive phagocytosis for the IgG3 IC (1%) but not for IgG1 IC.

*FcγR could not be up-regulated with LPS or cytokines*

Previous studies have used cytokines to up-regulate the FcγR expression on the cell surface of both MonoMac-6 and U-937 cell-lines. We tested LPS and three cytokines, IL-6, TNFα and TGFβ, to see if we could up-regulate expression of the

![Figure 9](image)

**Figure 9** (A) FcgRI, FcgRII, FcgRIII, FcgRIIa, and FcgRIIb expression was measured on the cell-lines MonoMac-6 (red) and U-937 (blue) using FACS analysis. MonoMac-6 had low expression of FcgRI and high expression of FcgRIIa. U-937 expressed only FcgRIIa. (B) Functional analysis of binding (Ros.) and phagocytosis (Phag.) was performed on the two cell-lines. MonoMac-6 had positive rosetting with IgG3 IC, but none with IgG1 IC and no phagocytosis. U-937 had positive binding only with IgG1 IC and no binding with IgG3 IC. It had a small amount of phagocytosis with IgG3 IC and none with IgG1 IC.
different FcγR. No increased expression of the receptors was seen for stimulation with LPS or any of the cytokines (data not shown). In some cases we found the opposite, TNFα actually down-regulated the FcγR expression (data not shown).

Discussion

The importance of FcγRs in autoimmunity has been well established by using different animal models and patient group studies. As we gain a better understanding of the FcγR isotypes, their mechanisms and functions, it has become apparent that a better understanding of the specific interactions with the different IgG subclasses is needed. In particular how the different FcγR:IgG complexes are translated into the different functional responses. In this study we have investigated the relationship between FcγR isotype expression and IC binding and phagocytosis functions. PBM were studied purified from healthy donors by three different methods. Additionally, two monocyte cells lines, MonoMac-6 and U-937 were investigated.

Previous studies in this research group have shown that there is an up regulation of FcγRI, FcγRII and FcγRIII in inflamed synovia of RA patients (unpublished data). In this study the FcγRI expression was particularly interesting as it was not seen to be expressed at all in healthy synovia but was highly expressed in the RA patients. FcγRII and FcγRIII were expressed in healthy synovia and were up-regulated in the RA patients. To continue this story we wanted to characterise the expression of the FcγRs on PBM in healthy individuals for comparison to RA patients in the future. We found that FcγRIIa was constitutively expressed at high levels on PBM. FcγRI was not constitutively expressed on PBM, however there tended to be a high proportion of FcγRI positive PBM with consistently low cell surface concentrations. Previous studies have shown that about 90% of PBM in an individual expresses FcγRI. We saw much more varied percentage of FcγRI positive PBM with a average, of 71%. As FcγRI is the high affinity activating receptor we would expect its expression to be tightly regulated. The previous reported finding from this research group demonstrated that FcγRI was up-regulated from almost no expression to a high expression level in the synovia of RA patients. The percentage of FcγRI positive monocytes may be an indicator of recent infections in the donor. This may explain why in some of the buffy coats tested there were lower levels (down to 24%) of FcγRI.
positive cells observed. These donors may not have had a recent infection, while the highest (94%) may have recently recovered.

FcγRIII has been implicated as a major contributor to autoimmune diseases. In the cohort studied here there was a wide range in the percentage of FcγRIII positive PBM population size but with quite a homologous cell surface expression level (MFI). There has been a lot of recent research into the different populations of FcγRIII positive PBM. These studies have shown that there are sub-populations of FcγRIII positive cells which can be defined by their CD14 and CD16 expression. These sub-populations account for the lack of a definitive peak seen in the FcγRIII expression within individuals. The role of FcγRIII on PBM has not been clearly defined but it has been suggested that FcγRIII positive cells are more mature than the FcγRIII negative cells.

FcγRIIib is the only inhibitory FcγR. This receptor was expressed only a small population of PBM and had a medium cell surface concentration. This expression pattern is consistent with the inhibitory role played by FcγRIIib. It should only be expressed on the surface of cells which have been activated as a down regulatory mechanism.

Previous findings have shown that different subclasses of IgG bind to different FcγR isotypes with different affinities. These observations have lead to the speculation that the different FcγR-IgG complexes could lead to the activation of different immune functions. Previous studies have suggested that IgG1 IC are phagocytosed via the FcγRI but are not bound in rosettes, while IgG3 IC are bound but not phagocytosed. Our results did not concur with this study rather we saw rosetting and phagocytosis with both the IgG1 and IgG3 IC. For phagocytosis the IgG1 and IgG3 IC gave equivalent positive phagocytosis while the rosetting assay showed that IgG3 IC binds better than IgG1 IC. The results show a correlation between phagocytosis and the FcγR expression with FcγRII (for both IgG IC subclasses). When the two FcγRII isotypes, a and b, were investigated, only FcγRIIa was found to correlate which was not found to be significant, however this may become significant when more samples are investigated. Binding correlated to the level (MFI) of FcγRI and FcγRIII. It is interesting that positive phagocytosis correlated with the percentage of FcγRIIa positive cells while binding correlated most with FcγRI and FcγRIII cell surface expression levels. This shows that for receptor
binding the cell surface expression of the receptor is most important while for phagocytosis it is the number of cells expressing the receptor.

Blocking the FcγRIIa impeded the ability of the cells to phagocytose IgG1 IC but not IgG3 IC. This suggests that FcγRIIa is involved in IgG1 IC phagocytosis but not IgG3 IC. FcγRIIa was the only receptor found to correlate to phagocytosis. Within the cohort studied here there was a wide range in the percentage of PBM expressing FcγRI and FcγRIII. The contribution of these receptors to phagocytosis may be proportionally greater for their population size than FcγRIIa. FcγRIIa is constitutively expressed at high cell surface concentrations, because of this its contribution may overshadow that of the other receptors. To assess this further more blocking studies should be performed. Blocking experiments would provide definitive proof for the role of different FcγR and IgG subclass specific IC combinations on phagocytosis. Working with, and improving, the protocol tested here will hopefully provide answers to these questions.

It was interesting that we saw an increase in the binding in female donors when compared to males for both IgG1 and IgG3 IC. Due to fact that there were only two female donors tested the significance could not be calculated. These results suggest that there is a large difference in binding with IgG1 and particularly IgG3 IC. It has been noted that in autoimmunity there is a marked prevalence in females when compared to males and that FcγRs are important in RA. One reason why we see this difference between the male and female donors could be that the male receptors could be more saturated than the females, possibly due to the hormonal environment. This could be tested if this trend continues.

There are two factors which could effect the binding and phagocytosis results. The first is the low amount of samples. Only twelve samples were investigated for phagocytosis function and seven for rosetting. Adding to this is the fact that the FcγRIIa and FcγRIIb detection Ab were only introduced into the experiment halfway through. This meant that their expression was only analysed for six of the samples. With higher cohort numbers the correlation between the FcγRs could become significant in particular for the FcγRIIa. The second factor is the change in the FcγR by the purification method, plastic adherence. As the MACS methods did not appear to work in this laboratory, all functional assays analysed used PBM purified by the plastic adherence method. This means that at the time that the phagocytosis assays
were performed the FcγR expression may have changed from that measured 16 hours after purification. If the FcγR expression had changed sufficiently, any correlation between the FcγR expression and the IgG IC phagocytosis may not be seen.

Unlike plastic adherence no study has noted an aberrant effect on the cells using either the beads or the magnet on the PBM or their functions. We compared phagocytosis with PBM purified by plastic adherence and negative MACS. Plastic adherence gave much higher levels of phagocytosis. Due to the un-reliability of the MACS purifications PBM from only one buffy coat were compared however the differences were dramatic (IgG1 IC; from 34% to 0.4% and IgG3; 36% to 6%). It is hard to say why there was this difference between the two purified samples, whether it was due to the MACS beads or magnet or because of the activation of the PBM when they adhered to the plastic, but it has been observed before that the adhesion of PBM to plastic leads to the activation of PBM. It would seem logical that if the PBM were activated by the plastic adherence method, they would be able to phagocytose IC easier than cells which have not been stimulated.

For functional analysis of a single healthy population, as done here, the priming of the PBM for phagocytosis and the up-regulation of the FcγRs may not have a negative effect and indeed may be beneficial. The activation and up-regulation of the PBM was consistent throughout the controls and tested populations meaning that this effect was consisted throughout all the experiments. This may be beneficial as the difference between the investigated samples and the controls may be exaggerated making them easier to see. The activation of the PBM may have negative implications for its use when studying diseased and healthy controls. It could in fact minimise the differences between healthy and disease states. Diseased samples will already be primed and have augmented FcγR expression and, unlike the healthy control samples, may not be able to be additionally activated by plastic adherence. This could mean that the effect that the plastic adherence has on the activity of the monocytes may not be equal between diseased and control groups.

In theory MACS purification should obtain a consistently higher purity than that seen in plastic adherence and it has not previously been shown to up regulate the FcγRs or affect the PBM function (although this should be tested in the lab). These factors would suggest that both positive or negative MACS should be better at purifying PBM than plastic adherence. Our results did not point to a greater purity
with the MACS methods but the up regulation of the FcγR with plastic adherence cannot be ignored. While the positive MACS seemed to give higher purities, these results are deceptive. Only two buffy coats were purified by this method and while one gave a high purity, 54%, the other gave low purity, 32%. If the problems with MACS can be resolved it should prove to be a better method for PBM purification.

Cell-lines are often used as a control cell population as they have predictable and reproducible levels of receptor expression. Due to the variability in FcγR expression on PBM we included two monocyte-like cell-lines, MonoMac-6 and U-937, in this study. We characterised their FcγR expression and found that MonoMac expresses FcγRIIa and low levels of FcγRI and U-937 expresses only FcγRIIa. We were unsuccessful in up regulating any of the FcγR by cytokine activation. Previous studies have suggested that IFNγ activation up regulates FcγRI on MonoMac. Another study has shown that both FcγRI and FcγRIII could be up regulated on U-937 after activation with IFNγ. In the future IFNγ should be tested to activate and up regulate the FcγRs on these cell-lines.

Both functional assays were tested on the MonoMac and U-937 cell-lines. MonoMac could not bind IgG1 IC but could bind IgG3 IC, while U-937 could bind IgG1 but not IgG3 IC. In terms of the FcγR expression the only difference between them is the expression of FcγRI on MonoMac-6. A possible future experiment would be to block the FcγRI expression on the MonoMac and see if this alters its ability to bind IgG3 IC. Another possible experiment is to activate the cells with IFNγ and see if there is an up regulation of the FcγRs and whether an activation with cytokines, even if they do not up regulate the receptors, increases the amount of phagocytosis or binding.

CONCLUSIONS

There is wide variation in the FcγR expression of PBM in the general population. This is important to keep in mind when comparisons are made between diseased and healthy states. In this report we have provided preliminary data on the FcγR expression in a random healthy cohort showing that there are predictable patterns of cell surface FcγR expression in a healthy individual; FcγRI and FcγRIIb
are expressed at low concentrations on all receptor positive PBMs, FcγRIIa is expressed at high concentrations, and FcγRIII is expressed at variable cell surface concentrations. There was no predictability in the percentage of PBMs expressing FcγRI, FcγRIIb or FcγRIII but FcγRIIa was constitutively expressed.

We have investigated a newly described method and its applicability to correlating the influence FcγR isotype and IgG subclass specific complexes have on phagocytosis. Our results suggest FcγRIIa as the major contributor to phagocytosis and that the amount of monocytes expressing this receptor is important. For binding it is the cell surface concentration of the FcγRI and FcγRIII which are important. We have made many suggestions to improve the method which will help future experiments in this laboratory.

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