Cytokine Profile of Fcγ Receptor-Stimulated Monocytes by Human IgG1- and IgG3-Immune Complexes

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Degree project in Biology
Examensarbete i biologi, 20p, höst 2006
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<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody (antibodies)</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen-induced arthritis</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorter</td>
</tr>
<tr>
<td>FcR</td>
<td>Fc receptor(s)</td>
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<tr>
<td>FcγR</td>
<td>Fc receptor(s) for IgG</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>HAGG</td>
<td>Heat-aggregated gammaglobulin</td>
</tr>
<tr>
<td>IC</td>
<td>Immune complex(es)</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ITAM</td>
<td>Intracellular tyrosine-based activation motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>Intracellular tyrosine-based inhibitory motif</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic cell sorting</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody (antibodies)</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell(s)</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell(s)</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid factor(s)</td>
</tr>
<tr>
<td>RPA</td>
<td>RNase protection assay</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
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</table>
Abstract

Fcγ receptors (FcγR) are expressed on immune cells such as monocytes, macrophages, mast cells, neutrophils, NK cells and lymphocytes. FcγR connect the humoral and cellular immunity by recognising the Fc portion of the IgG molecule, thereby capable of triggering and regulating immune responses. There are three classes of FcγR identified in humans: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). They are further divided as activating or inhibitory FcγR. An impaired regulation of the FcγR system may lead to the development of autoimmune diseases. Thus, the balance of activating versus inhibitory FcγR expression can be modulated by the cytokine milieu. In animal models for rheumatoid arthritis (RA), the activating FcγRIII trigger the disease while the only inhibitory FcγRIIb suppresses the immune complex (IC)-mediated inflammation.

In this thesis, we wanted to investigate if monocytes from healthy individuals could be activated with IC via FcγR to mimic monocytes of RA patients which are capable of producing high levels of pro-inflammatory cytokines. Monocytes were first purified from peripheral blood of healthy donors and analysed for their phenotypic FcγR expression using fluorescence activated cell sorting (FACS). IC of various types and subclasses were prepared: particulate IC, heat-aggregated gammaglobulin (HAGG) and immobilised IgG1 or IgG3 antibodies. Monocytes were stimulated with the different IC and total RNA was then isolated from them. The RNA was analysed using RNase Protection Assay (RPA) to detect cytokine gene expression. We could show that monocytes can be activated using immobilised antibodies to cross-link their FcγR and induce production of TNFα. We also showed that TNFα induction was mediated through FcγRI and/or FcγRIII. Other cytokines detected included TGFβ1, IL-1β and IL-1ra, but these were probably induced in a FcγR-independent pathway. These observations suggest that IC of specific IgG subclasses and activation of certain FcγR may regulate different cytokine pathways.
Introduction

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a severe, chronic disease characterized by synovial inflammation in the joints and progressive destruction of cartilage and bone. It affects around 1% of the world population, especially women. In RA, there is infiltration of T cells, B cells, macrophages, and neutrophils in the synovial membrane and fluid, leading to pannus formation which is a tissue enriched in macrophages and synovial fibroblasts. These cells produce pro-inflammatory cytokines and matrix metalloproteinases, promoting the degradation of the connective tissue [1 – 3]. Seventy percent of RA patients are positive for the presence of rheumatoid factors (RF). These are autoantibodies against the Fc fragment of self IgG molecules, which will form immune complexes (IC). Elevated levels of RF are associated with the severity of RA. RF can also be found in other autoimmune disease or even in healthy donors but at lower levels [4, 5]. Recently, IgG autoantibodies against citrullinated proteins have emerged as a more specific bio-marker for early diagnosis of RA [6].

Antibodies and immune complexes

Antibodies (Ab), or immunoglobulins (Ig), are glycoproteins produced in membrane-bound form or secreted form by B cells. They are antigen-binding molecules and capable of eliminating foreign substances in our bodies. An antibody molecule is made up of two identical heavy chains and two identical light chains, held together by disulphide bonds in a “Y”-shape (Figure 1A). There are 5 classes of Ab, IgA, IgD, IgE, IgG, and IgM. The IgG isotype can further be divided into different subclasses, IgG1, IgG2, IgG3, and IgG4 in humans [7]. IC are formed when antigens are bound by Ab and the size of IC can vary since
many antigens are multivalent (Figure 1B). Distribution of subclass specificity of autoantibodies in RA are predominantly IgG1 and IgG3 [8 – 10].

![Figure 1: Schematic diagram of an antibody molecule and immune complex.](image)

(A) Two parallel light and heavy chains, consisting of constant and variable regions, make up the antibody molecule. The variable light chain (V<sub>L</sub>) and variable heavy chain (V<sub>H</sub>) recognise and bind antigens, whereas the constant heavy chain (C<sub>H</sub>) can interact with Fc receptor or complement to mediate effector functions. (B) Antibody molecules binding to an antigen, forming an immune complex.

**Fc Receptors**

Fc receptors (FcR) are expressed on cell surface of haematopoietic cells and specifically bind the Fc fragments of Ig molecules. There are different FcRs for the different antibody classes: FcαR binds IgA, FcδR binds IgD, FcεR binds IgE, FcγR and neonatal FcR (FcRn) bind IgG, and FcμR binds IgM [11]. Fc receptors provide a link between the humoral and cellular responses. The FcR system is constituted by activating and inhibitory receptors and have been implicated in inflammatory and autoimmune diseases such as RA [12, 13].

**Fcγ Receptors (FcγR)**

Triggering of FcγR can initiate phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC), transcription of cytokine genes and release of inflammatory mediators such as cytokines, reactive oxidants and proteases. FcγR are also involved in IC clearance and
degradation, which later enhance antigen presentation (cited in [13]). Recent structural studies have provided insights that FcγR and IgG bind in 1:1 stoichiometry [14]. Perhaps this is why a monovalent binding of IgG does not trigger any signal, but clustering of FcγR with multivalent binding of IgG will trigger signaling pathways [13, 15].

Figure 2: Schematic diagram of human FcγR. FcγRI, IIa and IIIa are activating receptors, which contain ITAM in their cytoplasmic tails of accessory subunits or the ligand binding chain itself. FcγRIIib is an inhibitory receptor, containing an ITIM. FcγRIIib is linked to the plasma membrane via GPI anchor and does not transduce any signal by itself, only in conjunction with other signalling receptors. TM = transmembrane.

There are three classes of FcγR in humans, FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) (Figure 2), and they differ in cell distribution, function and affinity for IgG subclasses. FcγRI is classified as a high-affinity receptor ($K_a \approx 10^8$ M$^{-1}$), due to a third Ig-like domain, and binds monomeric IgG preferentially. FcγRII and FcγRIII have two Ig-like domains and are low-affinity receptors ($K_a < 10^7$ M$^{-1}$), which bind IgG in the form of IC. In humans, FcγR are further divided into three subclasses for FcγRI (Ia, Ib, and Ic) and FcγRII (IIa, IIb, and IIc); and two subclasses for FcγRIII (IIIa and IIIb) [12, 15]. This thesis will focus on FcγRIa and IIIa (written as FcγRI and III respectively unless otherwise stated), and also on FcγRIIa and IIb. The distribution of some FcγR in humans is summarised in Table 1.
Table 1: Expression of human FcγR and their affinity for IgG [13].

<table>
<thead>
<tr>
<th>Fc receptor</th>
<th>Expression</th>
<th>Isotype preference</th>
</tr>
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<tbody>
<tr>
<td>FcγRIa</td>
<td>Monocytes, macrophages, neutrophils, eosinophils</td>
<td>IgG1 ≥ 3 &gt; 4</td>
</tr>
<tr>
<td>FcγRIIA</td>
<td>Monocytes, macrophages, dendritic cells, neutrophils, platelets</td>
<td>IgG3 ≥ 1, 2</td>
</tr>
<tr>
<td>FcγRIIB</td>
<td>B cells, monocytes, macrophages, mast cells, neutrophils</td>
<td>IgG3 ≥ 1 &gt; 4 &gt; 2</td>
</tr>
<tr>
<td>FcγRIIIa</td>
<td>NK cells, macrophages, activated monocytes, neutrophils</td>
<td>IgG1, 3 &gt;&gt; 2, 4</td>
</tr>
<tr>
<td>FcγRIIIb</td>
<td>Neutrophils</td>
<td>IgG1, 3 &gt;&gt; 2, 4</td>
</tr>
</tbody>
</table>

Stimulatory FcγR

FcγRI, IIa, and IIIa are considered activating receptors because they contain the immunoreceptor tyrosine-based activation motif (ITAM) and are capable of triggering cellular activation. Multi-chain receptors, FcγRI and IIIa, have their α-chains associated with homodimeric γ-chains which contain the ITAM. In NK cells, FcγRIIIa can associate with the ITAM-containing γ-ζ heterodimeric chains or ζ-ζ homodimeric chains [7]. FcγRIIB is a single-chain FcR which have the ITAM in its cytoplasmic domain. Cross-linking of stimulatory FcγR results in phosphorylation of tyrosine residues in the ITAM by Src family protein kinases (Figure 3A). These phospho-tyrosines serve as docking sites for SH2 domain of Syk, a protein tyrosine kinase. This leads to the activation of phosphotidylinositol 3-kinase (PI-3K) and phospholipase Cγ (PLCγ), leading to calcium influx, activation of the MAPK pathways and cytoskeletal reorganisation [16].

Inhibitory FcγR

FcγRIIIB is the only inhibitory FcγR and does not contain ITAM, like its isoform counterparts. Instead, this single-chain receptor has an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain, therefore it is known as the inhibitory FcγR. FcγRIIIB has been shown to inhibit FcγRIIa-mediated phagocytosis [17]. FcγRIIIB is co-expressed with
activating FcγR and negatively regulates the latter by cross-linking with them upon engagement of IgG (Figure 3B). If FcγRIIb co-ligates with another FcγRIIb, it stays inert.

**Figure 3: Cross-linking of FcγR with immune complex leading to biochemical responses in a monocytes/macrophage.** (A) Positive regulation of FcγR (I or III) and activation of ITAM. (B) Negative regulation of stimulatory FcγR by inhibitory FcγRIIb. (C) MAPK signaling cascades can activate transcription factors, driving cytokine gene expression.

Upon co-cross-linking of FcγRIIb and stimulatory FcγR, Src family kinase, Lyn, phosphorylates tyrosine residues in ITIM of FcγRIIb. This is followed by the recruitment of inositol polyphosphate 5’ phosphatase (SHIP) via its SH2 domains. SHIP hydrolyses phosphatidylinositol-3, 4, 5- triphosphate (PIP₃) and thereby inhibits the generation of inositol.
triphosphate (IP$_3$) and diacylglycerol (DAG), i.e. blocks calcium influxes and therefore attenuating effector functions [16].

*Role of FcγR in arthritis*

Implication of FcγR in the pathogenesis of RA has been well-studied in murine models, such as collagen-induced arthritis (CIA). CIA can be induced in susceptible DBA/1 mice by immunising them with collagen type II (CII) in Freund’s complete adjuvant, and the onset of CIA is due to anti-CII antibody response [12]. Deficiency of the FcR γ-chain in DBA/1 mice protects them from CIA. In contrast, DBA/1 mice that are FcγRIIb-deficient are highly susceptible to the disease, with elevated anti-CII Ab production [18]. FcγRIII$^{-/-}$ mice are also found to be almost resistant to CIA, indicating that FcγRIII is the main activating FcγR responsible for disease induction [19]. IgG1 and IgG2b anti-CII monoclonal antibodies (mAb) are found to be arthritogenic as they are capable of triggering CIA by passive transfer to naïve mice [20]. There are polymorphisms in FcγR which influence the ability of the receptor to bind certain IgG subclasses. FcγRIII variants have been linked to arthritis susceptibility in both mice and humans [21, 22]. IgG1-IC is believed to be the main trigger of FcγRIII activation in CIA [23], while IgG subclass-IC dependency in FcγR activation in humans has yet to be shown.

**Cytokines**

Cytokines are small proteins that mediate growth, activation, differentiation, immune and inflammatory reactions in many cell types. They are produced transiently and rapidly secreted. Their effects are often pleiotrophic and redundant. Excessive cytokine synthesis brings about pathological consequences.
Cytokines in RA

IgG and RF IC are believed to contribute to the severity of RA by stimulating prolonged synthesis of pro-inflammatory cytokines (Figure 3). High levels of TNFα and IL-1 have been found in rheumatoid joints. Both TNF and IL-1 promote the production of IL-6 and IL-8. In addition, anti-inflammatory cytokines like IL-10, IL-1 receptor antagonist (IL-1Ra) and soluble TNF receptor (sTNF-R) have also been detected in RA joints [24].

The role of monocytes and macrophages in RA have been well-established [25, 26] as these are the main producers of TNFα and IL-1. It is believed that circulating blood monocytes in RA patients are activated and producing cytokines or metalloproteinases [27]. Also their expression of adhesion molecules is increased which allows them to migrate to the site of inflammation and differentiate into macrophages [26, 28, 29].

Cytokines regulates FcγR expression

Expression of FcγR can be differentially regulated by various cytokines or even induce FcγR expression in certain cell types, e.g. FcγRI can be induced with IFNγ in polymorphonuclear cells [30]. Higher expression of FcγRII and FcγRIII on monocytes were observed in RA patients compared to healthy controls and this is associated to cytokine influence on FcγR [31]. FcγRI and IIa are up-regulated by IFNγ and IL-10; FcγRIIb expression is decreased by IFNγ and TNF, and up-regulated by IL-4 and IL-10; TGFβ and IL-10 up-regulate FcγRIIIa [31 – 33]. Furthermore, expression of FcγRIII on monocytes from RA patients can be enhanced with TGFβ1, M-CSF, and IL-10 [28]. There has been great emphasis in cytokine equilibrium as it affects the balance of activating versus inhibiting FcγR that could swing the monocytes into an activation mode and determine one’s susceptibility to developing RA.
Figure 4: Schematic representation of RPA procedure. (A) Probe synthesis. T7 RNA polymerase transcribes from the DNA template, incorporating $^{32}$P-labeled UTP and producing anti-sense probe. (B) Hybridisation of probe and total RNA in solution. The probe hybridises to the target RNA where it is complementary. (C) RNase degradation of single stranded RNA and separation of dsRNA on denaturing gel.

**RNase Protection Assay (RPA)**

RPA allows one to map, detect and quantify specific mRNA species in total cellular RNA. The major advantage of RPA over other techniques such as S1 nuclease mapping technique, Northern blots and even RT-PCR, is that multiple gene expressions can be examined simultaneously in a sample. It is also sensitive and can detect as little as $10^5$ copies of particular mRNA species in a RNA sample [34 – 36]. The disadvantages are that it is labour-intensive, requires technical skills and involves radioactivity$^1$.

RPA is based on the principle of hybridising cellular RNA with a single-stranded, labeled RNA probe. Commercial kits are available which allows analysis of 20 samples in a single assay, and 10 – 15 cytokine genes per samples in tow. T7 RNA polymerase is used to direct in

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$^1$ A bio-chemiluminescent RPA kit is available from BD Pharmingen as well, that might cost slightly more. Biotinylated probes are used and there are additional steps involved like gel-transfer onto a nylon membrane and chemiluminescent detection.
vitro transcription to yield a set of radiolabeled anti-sense RNA probes that is complementary to the target mRNA. The probe set is hybridized with the RNA sample in solution, forming partial double-stranded RNA (dsRNA). The dsRNA hybrid is then treated with RNase to degrade unhybridised RNA. Only dsRNA fragments will be protected from RNase digestion and the fragments can be recovered by ethanol precipitation and analysed by electrophoresis on a denaturing polyacrylamide gel, followed by autoradiography and/or exposed to phosphorimage screen (Figure 4).

**Aim of the study**

Many studies have been done on the involvement of FcγR and cytokines in RA. In the CIA mouse model, it has been found that certain IgG subclasses of mAb or IC, can promote arthritogenicity and that via FcγRIII. But little is known if this is the case in humans. Here we wanted to investigate if monocytes from healthy human donors can be stimulated via their FcγR, either by particulate IC or immobilised Ab of IgG1 or IgG3 subclasses. Since the expression of FcγR varies between individuals, the FcγR expression was first analysed using fluorescence-activated cell sorting (FACS) on each individual’s monocytes. We wanted to examine the cytokine expression at mRNA level, using RPA, to see if the cytokine profiles between the individual donors and correlated to their FcγR expressions. And finally, through these cytokine profiles, we wanted to define which of the IgG subclasses is more potent in activating the FcγR in healthy monocytes. This will provide further insights on the underlying mechanisms of how monocytes become activated in RA patients.
Materials and Methods

Isolation of human peripheral blood monocytes

Buffy coats obtained from healthy donors (Blodcentralen, Akademiska Sjukhuset, Uppsala, Sweden) were diluted in phosphate buffered saline (PBS) containing 0.2 mM ethylenediamine-tetraacetic acid (EDTA) and peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). After isolation, the PBMC were washed three times in PBS with 0.2 mM EDTA and resuspended in complete medium: RPMI 1640 medium (Sigma, St. Louis, USA) supplemented with 1% fetal calf serum (FCS) (Sigma), 100 U/ml penicillin, 100μg/ml streptomycin and 1% glutamax (Sigma). Monocytes were further purified from the PBMCs using one of the following methods: plastic adherence or magnetic cell sorting (MACS).

Plastic adherence

PBMCs were transferred into polystyrene culture flask (BD Falcon, Franklin Lakes, USA) and incubated for 30 minutes in a 37°C, 5% CO₂ incubator. Non-adherent cells were washed three times with PBS. Adhered monocytes were gently harvested using a cell scraper and resuspended in complete medium to a concentration of 1 – 2 x 10^6 cells/ml and maintained in a humidified atmosphere with 5% CO₂ at 37°C.

MACS

CD14-labeled microbeads (Miltenyi Biotec) were used for the positive selection of monocytes from PBMCs, following manufacturer’s instructions. Magnetically labeled CD14⁺ cells were retained on a LS magnetic column that was placed in a strong magnetic field. The LS column
was washed three times. The magnetic field was then removed and a plunger was used to
flush out the CD14\(^+\) cells, and the cells were subsequently resuspended in complete medium
to a concentration of 1 – 2 \(\times 10^6\) cells/ml and maintained in a humidified atmosphere with 5% CO\(_2\) at 37\(^\circ\)C.

**Phenotypic analysis by flow cytometry**

Monocytes purified from PBMC were resuspended in PBS containing 1% bovine serum albumin (BSA) and 0.02% sodium azide to a concentration of on 2.5 \(\times 10^5\) cells per 100\(\mu\)l. Double staining were performed on the cells to analyse their purity and expression of Fc\(\gamma\)R. Cells were stained with 90 ng of phycoerythrin (PE)-conjugated anti-CD14 monoclonal antibody (mAb) (clone Tü4; Dako, Glostrup, Denmark) together with 2 \(\mu\)g of fluorescein isothiocyanate (FITC)-conjugated anti-Fc\(\gamma\)RI (clone 10.1; BD PharMingen, Erembodegem, Belgium), 0.15 \(\mu\)g of FITC-conjugated anti-Fc\(\gamma\)RII (clone KB61; Dako), or 0.56 \(\mu\)g of FITC-conjugated anti-Fc\(\gamma\)RIII (clone DJ130c; Dako) and incubated in the dark for 30 minutes at 4\(^\circ\)C. Isotype-matched controls were used and 0.1 \(\mu\)g of PE-conjugated mouse IgG2a (clone DAK-GO5; Dako) or 0.15\(\mu\)g of FITC-conjugated mouse IgG1 (clone DAK-GO1; Dako) were added to the cells.

The isoforms Fc\(\gamma\)IIa and Fc\(\gamma\)IIb are extracellularly homologous but differ in their cytoplasmic domains. Both isoforms are recognised by the CD32 monoclonal antibody KB61. In order to distinguish between the two isoforms, cells were stained with 0.45 \(\mu\)g of anti-Fc\(\gamma\)RIIa mAb (clone IV.3; kindly donated by Johan Rönnelid, Unit of Clinical Immunology, Uppsala University, Uppsala, Sweden), or 0.48 \(\mu\)g of anti-Fc\(\gamma\)RIIb mAb (clone GB3; kindly donated by Uwe Jacob, Max-Planck Institute für Biochemie, Abteilung Strukturforschung, Martinsreid, Germany). Control stainings were performed with 0.4 \(\mu\)g of mouse IgG1 (clone...
MOPC-21; Sigma), or 0.46 μg of mouse IgG2b (0.46 μg; MOPC-141, Sigma) for 30 minutes at 4°C. Cells were then washed and incubated with 2.5 μl of PE-conjugated polyclonal rabbit anti-mouse immunoglobulin (clone R 0439; Dako) or 5 μl of PE-conjugated polyclonal rabbit anti-mouse IgG (clone OBT0134R; BioSite, Täby, Sweden) for 30 minutes at 4°C.

The CD16 monoclonal antibody DJ130c does not discriminate between FcγRIIIa and FcγRIIIb, however, FcγRIIIb is expressed predominantly on neutrophils.

Flow cytometric data were obtained on a FACScan cytometer (Becton Dickinson) and analysed using CellQuest software (BD Biosciences). Prior to analysis, propidium iodide (Sigma-Aldrich, Stockholm, Sweden) was added and a total of 10 000 events were analysed for each sample. Propidium iodide stains the DNA and eliminates unspecific fluorescence that may be emitted by dead cells. PE fluorescence was measured in the FL2 channel. For analysis of monocytes, a live gate was set for viable monocytes based on their forward/sideways light-scatter patterns. In addition cells were gated for CD14-PE expression and percentages of FcγRI, IIa, IIb and III expression were determined.

**Cell Culture**

Monocytic cell lines are often used as *in vitro* models to study differentiation and function of human monocytes. We included U937 and Mono-Mac 6 cell lines in our stimulation studies to see if they have similar cytokine profiles as human monocytes. Previously, U937 and Mono-Mac 6 cells have shown to produce pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6 and TNFα when stimulated by LPS [37, 38]. There are several advantages in using cell lines: they are a single population, free from contaminating cells, and they do not express FcγRIII which is good for studying functional effects mediated by FcγRIII.
U937 cells (kindly donated by Lars Hellman, Uppsala University, Uppsala, Sweden) were grown in RPMI 1640 medium supplemented with 5% FCS, 100 U/ml penicillin, 100μg/ml streptomycin and 1% glutamax and maintained in a humidified atmosphere with 5% CO₂ at 37°C. U937 cell line was derived from malignant lymphoma cells and represents the early stages in monocytic development and express FcγRI and FcγRIIa (previously shown in our lab).

Mono-Mac 6 cells (kindly donated by Lars Hellman, Uppsala University, Uppsala, Sweden) were grown in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100μg/ml streptomycin and 1% glutamax and maintained in a humidified atmosphere with 5% CO₂ at 37°C. Mono-Mac 6 cell line was established from peripheral blood of leukaemia patient and has the functional and phenotypic characteristics of mature monocytes. Mono-Mac 6 cells also constitutively express CD14 [39].

**In vitro activation of peripheral blood monocytes, U937 or Mono-Mac 6**

*IgG-opsonised human red blood cells (RBC) IC preparation and stimulation of monocytes and Mono-Mac 6*

To create large immune complexes (IC), human RBC were opsonised with IgG1- or IgG3 mAb as described in [40]. RBC were isolated from Rhesus D (RhD)-positive buffy coats using Ficoll-Paque density gradient centrifugation and washed three times in PBS. Four μl of packed RBC were placed in PBS, either alone, or with 8 μg of human IgG1 anti-RhD mAb (BIRMA D6; International Blood Group Reference Laboratory (IBGRL), Bristol, UK), or human IgG3 anti-RhD mAb (BRAD 3; IBGRL) for 2 hours at 37°C. The IC was washed three times in PBS to remove any unbound Ab and was resuspended in PBS.
One million PBMC-derived monocytes or Mono-Mac 6 cells in 100 μl PBS were incubated with un-opsonised RBC, IgG1-RBC, IgG3-RBC, or 10 ng/ml LPS and were pelleted by low speed centrifugation to increase the contact between the IC and the cells. Cells in PBS only or cells incubated with un-opsonised RBC served as background controls. Cells incubated with LPS were included as positive controls (LPS is a well-known inducer of TNFα in monocytes/macrophages). Samples were incubated in a total of 1 hour at 37°C in a water bath. Cells were pelleted by low speed centrifugation before RNA extraction by TriPure Isolation Reagent (Roche, Indianapolis, IN, USA) according to the manufacturer’s instructions.

**Stimulation of monocytes, U937 and Mono-Mac 6 with heat-aggregated IgG (HAGG)**

One hundred μg of κ light chain-purified, human myeloma proteins (The Binding Site, Birmingham, UK) of the subclasses IgG1 or IgG3, were heat-aggregated for 30 minutes at 63°C and centrifuged at 12 000 rpm for 10 minutes [41], to prepare HAGG1 or HAGG3 specific subclasses respectively,. The protein concentration of the supernatant was measured. Monocytes, U937 or Mono-Mac 6 were plated in 24-well tissue culture dish at the density of 2 x 10^6 cells/ml. Thirty ng of the HAGG1 or -3 supernatant was added to the cells and incubated for 20 hours in a humidified atmosphere with 5% CO2 at 37°C. Cells were harvested and pelleted by centrifugation prior to RNA extraction by TriPure Isolation Reagent.

**FcγR cross-linking and stimulation of monocytes, U937 and Mono-Mac 6**

FcγR on cells can be cross-linked by immobilised IgG Ab. A 96-well MaxiSorp plate (Nunc, Möladal, Sweden) was coated 50 μl per well with 1 μg of purified human IgG1 or IgG3 myeloma proteins (The Binding Site) in PBS, and was left overnight at 4°C. As controls, 1 μg of ovalbumin (Sigma) was coated per well as a non-specific protein. In addition, uncoated
wells were used as negative controls. A total of 4 x 10⁵ cells in 200 μl serum-free RPMI media were plated per well and incubated for 4 hours in a humidified atmosphere with 5% CO₂ at 37°C. Cells stimulated with 10 ng/ml LPS was used as a positive control. After stimulation, the cells were harvested on ice in PBS containing 0.2 mM EDTA and pelleted by centrifugation prior to RNA extraction by TriPure Isolation Reagent.

**Blocking of FcγRIIa on monocytes**

One million monocytes were incubated with anti-FcγRIIa mAb (10 μg/ml) for 30 minutes on ice [42]. Cells were washed in PBS and resuspended in serum-free RPMI media and were then dispensed at 5 x 10⁵ cells per well, for further FcγR cross-linking with immobilised IgG1 or IgG3.

**RNase Protection Assay (RPA)**

Assays were performed with RiboQuant Multi-Probe RPA system (BD Biosciences, San Diego, CA) according to the manufacturer's instructions, using human cytokine multi-probe template sets, hCK-2b and hCK-3 (BD Biosciences) (Figure 5). In brief, 2 μg of the isolated RNA was hybridized with ³²P-labeled probes overnight at 56°C and then subjected to RNase treatments. The hybridised RNA-probes were purified and separated with high resolution on a 4.75% acrylamide-urea sequencing gel. The gel was transferred onto Whatman 3mm-filter paper, which was dried and later exposed to a x-ray film as well as on a phosphorimaging device.

Quantitation of cytokine mRNA expression was determined using a phosphorimaging device (PhosphorImager; Molecular Dynamics) and the level of each gene transcript was quantified.
by Image Gauge V3.45 (Fuji Photo Film Co., Ltd, Japan). This was further calculated by taking the ratio of the signal from the cytokine mRNA divided by the signal from the housekeeping genes, L32 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). L32 is a ribosomal protein that is a component of the 60S subunit, while GAPDH is an enzyme of the citric acid cycle and is expressed ubiquitously in all cells and its mRNA levels do not fluctuate significantly between cells of resting and growing states.

Figure 5: Autoradiograms of protection assays utilizing hCK-2b (A), and hCK-3 (B) Multi-Probe Template Sets with RNA samples from various cell populations.

**Statistical analysis**

Unpaired t-test was used for monocytes phenotypic analysis, while Mann-Whitney U-test was used for RPA analysis. A $P$ value $\leq 0.05$ was denoted significant.
Results

Here we have investigated if healthy monocytes can be stimulated using different types of IC: particulate IC, HAGG or immobilised Ab, and monitored the induction of cytokines using RPA. The procedures undertaken could be summarised and represented in a flow-chart. (Figure 6).

Monocytes purified from PBMC using MACS gave higher percentage of CD14\(^+\) cells

A pure population of monocytes is needed in order to study their specific cytokine profiles. The MACS method was therefore compared with the plastic adherence method to see which of the two methods could yield the most pure monocyte population. The purity of monocytes were assessed by using anti-CD14 PE-conjugated Ab and FACS analysis. CD14 is a known antigen marker of monocytes. The plastic adherence method was used on 8 buffy coats and the purity of monocytes obtained varied between 9\% and 67\%, with a mean of 39\%. The
MACS method was used on 6 buffy coats and proved to be a better method as higher percentages of CD14\(^+\) cells, between 49 to 74%, with a mean of 63\(\%\) \((P = 0.0127,\) compared with plastic adherence\), were obtained (Figure 7).

![Figure 7: Purity of monocytes isolated from PBMC by plastic adherence \((n = 8)\) or MACS \((n = 6)\). Horizontal lines shows the median for each method used.](image)

**Monocytes purified by plastic adherence had higher Fc\(\gamma\)RIIb and III expression**

The purified monocytes were further examined for their Fc\(\gamma\)R expression. Double staining was done using anti-CD64, anti-CD32, or anti-CD16 FITC-conjugated Ab and CD14-PE mAb. Unconjugated anti-Fc\(\gamma\)IIa or IIb mAb were also used. These mAb were followed by staining with a secondary polyclonal IgG-PE conjugated antibody. FACS analysis was done on viable monocytes based on their forward and side scatter.

**Table 2: Percentage of monocytes expressing the different Fc\(\gamma\)R in healthy donors \((\text{mean} \pm \text{SD})\).**

<table>
<thead>
<tr>
<th>Purification method</th>
<th>CD14(^+) Monocytes</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Fc(\gamma)I</td>
</tr>
<tr>
<td>Plastic adherence ((n = 4))</td>
<td>73.8 ± 24.9</td>
</tr>
<tr>
<td>MACS ((n = 4))</td>
<td>81.5 ± 13.3</td>
</tr>
</tbody>
</table>

All monocytes expressed Fc\(\gamma\)RI and IIa, some III and very few IIb (Table 2). No significant difference was found in the number of Fc\(\gamma\)RI\(^+\), IIa\(^+\) and IIb\(^+\), monocytes that had been purified
by plastic adherence or MACS. A higher percentage of monocytes expressing FcγRIII was found when plastic adherence was used, compare with MACS purification ($P = 0.04$). For cells that were stained with the pan-CD32-FITC antibody, the FcγRII overall expression was found to be $99.3 \pm 0.7\%$ for monocytes purified by plastic adherence, and $99.4 \pm 0.7\%$ for MACS-purified monocytes (data not shown).

**FcγR expression on monocytes in RA blood**

A small sample of blood was obtained from a RA patient and PBMC were isolated over Ficoll-Paque centrifugation. We wanted to see what percentage of monocytes is found in RA blood and if their FcγR expression differs from healthy monocytes. FACS analysis was performed on these PBMC and $56.1\%$ of the cells were CD14$^+$ (Figure 8A).

FcγRI$^+$, II$^+$ or III$^+$ monocytes were found to be $91.1\%$, $99.2\%$ and $43.3\%$ respectively (Figures 8B – D). Most of the FcγRII$^+$ cells were of the activating FcγRIIa isoform as $53.8\%$ of the monocytes were FcγRIIa$^+$ (Figure 8E), while only $9.2\%$ were FcγRIIb$^+$ (Figure 8F). The number of FcγRII$^+$ monocytes in RA did not differ much from the average number found in healthy monocytes purified by MACS (Table 2), whereas the percentages of FcγRI$^+$, IIb$^+$ and III$^+$ RA monocytes were higher. This indicates that monocytes in RA blood could be in a more activated state compared to healthy monocytes. However, this was based on only one individual and more samples should be analysed in order to obtain reliable statistics.
Figure 8: Dot plots of FcγR expression on monocytes in a RA patient’s blood. PBMC were stained with CD14-PE alone (A); or double-stained with CD64-FITC (B), pan CD32-FITC (C) or CD16-FITC (D). (E and F) Sequential staining was performed with CD32a or CD32b Ab, followed by IgG-PE. Cells were gated for viable monocytes based on their forward/side scatter and the percentages of positive cells are indicated in the respective quadrants.
Cytokine expression in IC-stimulated monocytes

RNA from plastic adherence-purified monocytes incubated alone (in PBS) or with IgG1-IC, IgG3-IC, unopsonised RBC or LPS, are isolated and used in RPA, using the hCK-2b and hCK-3 template. The cytokines, IL-10, IL-1α, IL-1β, IL-1ra, IL-6, TNFα and TGF-β1 were found to be expressed (Figure 9A and B). The IL-1β/GAPDH mRNA ratio had the highest value, more than 10 times of the other cytokine ratios, while IL-10 had the lowest ratio (Figure 9C). IgG1- or IgG3-IC induced a high IL-1β mRNA expression of 1.5 to 2 folds compared to the PBS control. As expected, LPS induced the highest cytokine mRNA levels including TNFα. It was hard to say if the little increase in TNFα mRNA production seen in IgG1-IC triggered monocytes was significant due to the high background of RBC stimulus. The TNFα mRNA production induced by IgG3-IC was not different from the controls, PBS or RBC stimuli.

Little or no effect when monocytes or the cell lines, Mono-Mac 6 and U937, were stimulated with HAGG IC

As the cells stimulated with unopsonised RBC gave a very high cytokine background, it was difficult to distinguish if IgG-opsonised RBC truly had an effect on cytokine mRNA production in monocytes. HAGG has previously been used as a model for IC stimulation and is known to be capable of stimulating cytokine secretion [41, 43] and this method was tried in order to eliminate the problems of high background with RBC. HAGG of IgG1 (HAGG1) and IgG3 (HAGG3) were prepared and used to stimulate monocytes and one RPA analysis was performed. However, the signal from the LPS positive control was so strong that it masked the bands of the HAGG1 or HAGG3 samples (results not shown). Mono-Mac 6 and U937 cell lines were also stimulated with HAGG1 or HAGG3, and little or no cytokine expression could be detected (results not shown). One possible reason for this was that I had used minute
amounts of HAGG1 or HAGG3 (30 ng) that may have been insufficient to activate the cells. Since this method did not work as well as we had anticipated in the cell lines, it was not worthwhile for us to repeat this experiment with monocytes.

Figure 9: Cytokine mRNA expression in monocytes after stimulation with or without particulate IC. RPA using multiprobe set hCK-2b (A) and hCK-3 (B). (C) Cytokines:GAPDH mRNA ratios.

**Immobilised IgG1 or IgG3 can cross-link FcγR on monocytes and induce cytokine production**

To improve the method to activate monocytes, this time, we used immobilised Ab on a single plane to cross-link FcγR on the monocytes (Figure 10A). As it was difficult to obtain enough CD14+ cells from a single buffy coat for both FACS analysis and stimulation experiments,
any available RNA obtained was only enough for performing one RPA. We chose the hCK-3 template, which detects TNFα mRNA. However, in one of the experiments we were lucky to obtain enough RNA which allowed us to use both the hCK-2b and hCK-3 templates. TNFα and TGFβ1 mRNA signals were detected in monocytes, with or without stimulation with immobilised IgG1 or IgG3 (Figure 10B).

![Diagram](image)

**Figure 10: Cross-linking of FcγR with immobilised IgG1 or IgG3 on monocytes.** (A) Diagram of immobilised Ab and their Fc components engaging and cross-linking FcγR on monocytes in a 96-well plate. MO = monocytes. (B) RPA using multiprobe hCK-3, for one experiment. (C) RPA analysis of TNFα or TGFβ1/GAPDH mRNA ratios. (Mean ± SEM of five experiments; * P < 0.05 and ** P < 0.01 TNFα/GAPDH ratios, compared with OVA stimulus).

The means of cytokine:GAPDH ratios from 5 experiments were calculated and presented as a bar chart (Figure 10C). The levels of TGFβ1 mRNA were similar among the different stimuli, compared to negative control. However, the TNFα mRNA production in Ab-stimulated monocytes was higher by 2.3 folds with IgG1 (P = 0.008) and 2.5 folds with IgG3 (P =
0.008), compared to stimulation by OVA. The positive LPS control induced 2.2 folds higher TNFα mRNA levels than OVA ($P = 0.03$). This demonstrates that the TNFα mRNA production can be induced by IgG1 or IgG3 cross-linking of FcγR. However, no difference in the magnitude of cytokine production between IgG1 or IgG3 stimuli was observed. In addition, TNFα/GAPDH ratio of monocytes in uncoated wells was slightly elevated, even though this was not statistically significant compared to OVA stimuli. This could explain how plastic adherence-purified monocytes can be activated to produce cytokines while adhering to plastic, and thereby up-regulating their FcγR expression.

*Blocking of FcγRIIa have no effect on TNFα mRNA induction*

In an earlier study on FcγR-mediated phagocytosis by a co-worker, FcγRIIa expression on monocytes was correlated with IgG1- and IgG3-IC binding. Furthermore when FcγRIIa was blocked, a slight reduction in the phagocytosis of IgG1-IC was seen [44]. We were interested to see if there would be a reduction in the cytokine expression when FcγRIIa on monocytes were blocked with the IV.3 mAb, which specifically recognize FcγRIIa. Monocytes were incubated with IV.3 mAb prior to the cross-linking experiment. RPA, using hCK-3 template, was done on the FcγRIIa-blocked monocytes in parallel with unblocked monocytes and mRNA levels of TNFα and TGFβ1 were detected. TNFα mRNA levels did not differ between unblocked or FcγRIIa-blocked monocytes, stimulated by the various stimuli (Figure 11A). Interestingly, a slight increase was observed for uncoated, OVA and LPS stimulated cells blocked with IV.3 mAb. It is possible that IV.3 mAb might, instead of blocking FcγRIIa, have stimulated the receptor. As there was little or no effect of IV.3 mAb on IgG1- or IgG3-stimulated monocytes, FcγRIIa was maybe not required for the Ab to induce TNFα mRNA production. Probably, TNFα induction was due to FcγRI and/or III cross-linking. However,
there was a reduction in TGFβ1 in IV.3 mAb-blocked monocytes stimulated with IgG1, IgG3 or LPS (Figure 11B). But this was insignificant as the standard deviation was high.

**Figure 11:** RPA analysis of unblocked or FcγRIIa-blocked monocytes stimulated with immobilised IgG. (A) TNFα mRNA expression. (B) TGFβ1 mRNA expression. (mean ± SEM of n =3).

_FcγRIIa cross-linking by IgG1 or IgG3 mediate different effects in inducing IL-1β and IL-1ra_

It is known that TNFα can regulate IL-1β production which in turn can up-regulate TNFα [24]. In one cross-linking experiment we hybridised the RNA from stimulated monocytes with the hCK-2b template, which detects IL-1β, in addition to the hCK-3 template. IL-1β and IL-1ra were readily detected and there were faint bands of IL-1α too (results not shown). Unexpectedly, some IFNγ mRNA was also detected (results not shown) which was surprising since IFNγ is produced mainly by T cells or NK cells. Thus, it should be taken into account that some mRNA may have been derived from other cell populations as the purity of
monocytes in this experiment was only 60%. When analysing IL-1β and IL-1ra mRNA ratios, both cytokines exhibited similar expression patterns when stimulated with the various parameters (Figure 12A and B). It seemed that there was little or no induction of IL-1β and IL-1ra by IgG1 or IgG3, when compared to negative controls (uncoated or OVA). But this does not mean that the cells were not activated by the IgG as TNFα mRNA induction had been seen when using the hCK-3 template (Figure 11).

A

\[\text{IL-1β mRNA ratio} \]

- Unblocked
- FcγRIIa-blocked

B

\[\text{IL-1ra mRNA ratio} \]

- Unblocked
- FcγRIIa-blocked

Figure 12: RPA analysis with hCK-2b template on a set of monocytes that had been FcγRIIa-blocked or unblocked and stimulated with immobilised IgG. (A) IL-1β mRNA expression. (B) IL-1ra mRNA expression.

The induction of IL-1β and IL-1ra mRNA could though be activated through FcγR-independent mechanism as LPS could induce IL-1β mRNA levels higher than the negative controls. When we blocked FcγRIIa and stimulated the cells with IgG1, we observed that there was a slight increment in IL-1β mRNA levels compared to unblocked monocytes.
It was vice versa when monocytes were stimulated with IgG3, i.e. FcγRIIa-blocked cells had reduced IL-1β levels, compared to its unblocked counterpart. Since FcγRIIa preferentially binds IgG3, FcγRIIa contributes to basal levels of IL-1β pathway only when stimulated by IgG3. In the case of IgG1 stimulation, blocking of FcγRIIa may have allowed more IgG1 molecules to be bound by FcγRI or III to activate IL-1β pathway to maintain a basal level of expression. It is also possible that an IgG1-FcγRIIa interaction could antagonise effects mediated by an IgG1-activated FcγRI and/or III. Likewise, this finding was similar for IL-1ra mRNA production, which was not surprising since IL-1ra is connected to IL-1β synthesis (Figure 12B). These results were based on one set of experiments and should be repeated to see if this phenomenon is seen again.

No TNFα detected in U937 cells stimulated by cross-linking with IgG

We were interested to see if monocytic cell lines could also be activated by immobilised IgG to induce cytokines. In one experiment U937 cells were stimulated with immobilised IgG1 or IgG3, no TNFα was detected and only very little TGFβ1 was observed which was not significant compared to the negative controls (Figure 13). As no TNFα was detected after stimulation, there was no point in doing further studies on this immature cell line.

**Figure 13: Detection of TGFβ1 mRNA in U937 cells after cross-linking with IgG.** (A) RPA with multiprobe hCK-3 template. (B) Analysis of TGFβ1:GAPDH mRNA ratio.
Mono-Mac 6 cells stimulated with particulate IC and blocking studies of FcγRIIa

Mono-Mac 6 cells were stimulated with or without particulate IgG1- or IgG3-IC, and RPA was performed on them using the hCK-2b and hCK-3 templates. Mono-Mac 6 cells expressed IL-12p35, IL-1β, IL-1ra, IL-6, TNFα and TGFβ1 (Figure 14 & 15). Mono-Mac 6 cells were also expected to be stimulated by LPS as they express CD14, the ligand for LPS. However, LPS induced extremely low levels of TNFα in the Mono-Mac 6 cells. When this experiment was performed, the Mono-Mac 6 cells had already been passaged for many months. Thus, FACS analysis was performed on them once again and it was found that the Mono-Mac 6 cells express very low levels of CD14. This could have attributed to the poor cytokine response by Mono-Mac 6 cells towards LPS. The highest amount of TNFα mRNA was detected in unstimulated Mono-Mac 6 cells (PBS control), followed by IgG1-IC, IgG3-IC and RBC. Mono-Mac 6 cells incubated in PBS also had the highest IL-1β, IL-1ra and TGFβ1 mRNA production. This implied that Mono-Mac 6 cells were easily activated by such cell-cell interactions in very close proximity. Their activation states were lowered when they were incubated with the large particulate IC which could have spaced out the Mono-Mac 6 cells.

Mono-Mac 6 cells express FcγRI, Ila and IIb but no FcγRIII, previously shown in our lab. FcγRIIa on Mono-Mac 6 cells were blocked with IV.3 mAb and the cells were stimulated with particulate IgG1- or IgG3-IC. RPA was performed with the hCK-3 template but only TNFα and TGFβ1 mRNA could be detected (Figure 15). All treatments induced TNFα expression although LPS stimulus induced very low TNFα mRNA levels. TNFα expression was also greatly reduced when FcγRIIa was blocked on the Mono-Mac 6 cells, except for cells stimulated with IgG1-IC. The TGFβ1 expression was down-regulated by FcγRIIa blocking in cells stimulated with PBS, IgG3 and LPS, but there was a slight increase in TGFβ1 in IgG1-IC and RBC-stimulated cells. This indicates that the TNFα mRNA basal
production in Mono-Mac 6 cells, was initiated mainly by FcγRIIa activation. TNFα activation pathway could though also be mediated through FcγRI activation by IgG1-IC. TGFβ1 mRNA production, on the other hand, could be induced by various pathways which were not FcγRIIa-dependent.

**Figure 14:** RPA analysis with the hCK-2b template on Mono-Mac 6 cells stimulated with particulate IgG-IC.

**Figure 15:** Stimulation of unblocked or FcγRIIa-blocked Mono-Mac 6 cells, with or without particulate IgG-IC. (A) TNF mRNA expression. (B) TGFβ1 mRNA expression.
IgG cross-linking of FcγR on Mono-Mac 6 cells

Mono-Mac 6 cells were also stimulated using the cross-linking method with immobilised IgG and RPA was performed. Again, only TNFα and TGFβ1 were detected using the hCK-3 template. There was no up-regulation of TNFα when stimulated with immobilised-IgG1, -IgG3, or LPS, compared with uncoated or OVA-coated wells, and IgG1 stimulus had the lowest induction of TNFα (Figure 16A). When FcγRIIa was blocked, no TNFα could be detected, only some TGFβ1 (Figure 16B). The TGFβ1 mRNA production was not FcγR-mediated but its mRNA levels were affected by blockage of FcγRIIa. It is possible that FcγRIIa have a role in basal expression of TGFβ1. FcγRI may not be involved in TGFβ1 synthesis as it did not maintain TGFβ1 levels when FcγRIIa was blocked. But that should be re-affirmed in blocking studies with FcγRI, which would elucidate the role of FcγRI in the cytokine production in Mono-Mac 6 cells. All experiments, which had explored the different stimulation methods and blocking of FcγRIIa on Mono-Mac 6 cells, were only done once and therefore, any conclusions cannot be made until these experiments have been repeated.

Figure 16: Unblocked or FcγRIIa-blocked Mono-Mac 6 cells stimulated by cross-linking with IgG. (A) TNF/GAPDH mRNA ratio in unblocked cells. (B) TGFβ1/GAPDH mRNA ratio in unblocked and FcγRIIa-blocked cells.


**Discussion**

In our studies, we have analysed the FcγR expression of monocytes from healthy donors and activated these monocytes using different types of IC: particulate IC, HAGG or immobilised Ab; and monitored cytokine production at mRNA level. Looking at mRNA levels may not necessary correlate to the protein expression, but it allows us to observe any changes in cytokine gene expression. The cytokine production induced by IgG IC can be influenced by several factors, for example, expression and polymorphism of FcγR on the individuals’ monocytes; the method of monocyte purification; the purity of monocytes obtained; and method of IC stimulation.

Since we did not obtain a high purity (< 90%) using either plastic adherence method or MACS, we have to take into consideration the possible presence of other cells, such as B and T lymphocytes, platelets and neutrophils, that could influence the activation state of monocytes through cell-cell interactions before challenged with IgG1- or IgG3-IC. Therefore, we set up controls that were not stimulated, so that we could deduct background cytokine levels from these cells. The purity of monocytes is important as we would like to solely elucidate the cytokine production to monocytes only. It is possible to perform a two-step purification, first with plastic adherence, followed by MACS. However, this would risk the monocytes differentiating into macrophages during the plastic adherence step. A key factor prior to MACS purification is the quality of the PBMC preparation and there should not be much dead cells or cell aggregates as this might affect the monocyte enrichment negatively in the MACS column. In addition, the presence of other cell types can affect the activation state of monocytes. Also the purification method used, can have activating effects. We have found that purifying monocytes by plastic adherence can up-regulate their FcγRIIb and III
expressions, which had been shown in our lab previously [44]. Thus, monocytes isolated by plastic adherence risk lymphocyte contamination, and transient activation [cited in 45]. Monocytes constitutively express certain chemotactic and/or adhesion molecules under non-inflammatory conditions so that they can migrate into tissues to become macrophages [29]. Therefore, monocytes are also capable of adhering to plastic and produce cytokines in a paracrine manner such that expression of adhesion molecules is increased [26] and FcγR expressions are modulated depending on the type of cytokines [32, 33, 46]. In addition, the drawback of not having pure CD14+ cells is that there is always contribution of cytokine mRNA from other cell populations. Small amounts of IFNγ were detected but these were dismissed as mRNA from contaminating T cells or NK cells, which are the main IFNγ producers. Although some IFNγ can be produced by macrophages, there is no evidence for this in blood monocytes [47].

It is known that blood monocytes from RA patients are activated and have a higher expression of FcγRI, IIa, and III [31]. When we analysed PBMC from a RA patient, the percentages of monocytes expressing FcγRI, IIb and III were considerably higher compared to healthy donors. This is somewhat different from other studies reporting that there is no difference in FcγRIIb expression between healthy and RA monocytes [46]. However, we were able to use a new mAb against FcγRIIb specifically that may have improved the evaluation of FcγRIIb on monocytes. However this was based on one RA blood sample and therefore not conclusive.

Obtaining enough blood from one RA patient is a problem, as most of the blood drawn is for diagnosis and testing, not much blood can be collected for research purposes. Therefore, we explored various methods of IC stimulation on healthy monocytes and activate them in such a way that they could simulate RA monocytes. It was possible to induce pro-inflammatory and
anti-inflammatory cytokines in healthy monocytes. However the type of IC stimulation used, influence the outcome of cytokine production. Particulate IC were found to be a more potent stimuli than the cross-linking method with the immobilised Ab, while HAGG did not work in our hands due to too little amounts of Ab for heat-aggregation. It was not economically viable to up-scale these type of IC as we were using specific IgG1 or IgG3 subclasses, and not poly IgG. In addition to the cross-linking method, various concentrations of subclass-specific IgG, used to coat the ELISA plate, should be tested in order to find the optimal amount to induce cytokine mRNA.

The quality and quantity of RNA is important as this will affect the efficiency of hybridisation in RPA. It is hard to isolate sufficient RNA from small populations of cells, therefore glycogen should be added as a carrier molecule to facilitate the RNA preparation. In addition, it is best to have at least 5 μg of RNA starting material for hybridisation, as the RNA molecules help to stabilise each other, even though RPA should be sensitive to detect as little as 100 femtograms of mRNA species per 2 μg total RNA [35]. The use of computer software to quantify the phosphorimaging data can be subjective when defining the bands as the software sometimes did not auto-detect certain bands that was obviously seen on the gel.

When monocytes were stimulated with particulate IC, mRNA from cytokines like TNFα, IL-1α, IL-1β, IL-1ra, IL-6, IL-10, and TGFβ1 were detected. In contrast, monocytes stimulated by the cross-linking method, could only induce TNFα, IL-1β, IL-1ra, and TGFβ1. There are two possible reasons for this: the monocytes stimulated by particulate IC had been purified using plastic adherence, thus these cells could have already been primed and activated, and then further activated by the particulate IC; or other ligands on the RBC might interact with scavenger or integrin receptors on the monocytes and synergistically enhance the activation,
thus the high background. The roles of TNFα and IL-1β in RA have been well-documented [24], and the IL-1β gene is found to be over-expressed in RA monocytes, compared to normal controls [27]. Here these cytokines were used as markers for monocytes activation in our experiments. It was hard to conclude if IgG1- or IgG3-opsonised RBC were activating the monocytes via FcγR, compared to unopsonised RBC, as the TNFα mRNA expression did not vary that much, although some differences was observed for IL-1β expression. In fact, the induction of IL-1β was found to be the highest, among all the cytokines expressed.

To avoid high background we decided to focus on the cross-linking method to stimulate monocytes and we used monocytes purified by MACS. IgG1 or IgG3 was immobilised on a single plane in attempt to mimic cell-bound IC at the cartilage, where anti-collagen Ab are bound to the cartilage with their Fc fragments sticking out. It has previously been shown that monocytes can be activated by cross-linking to produce TNFα [46]. In agreement with our earlier findings where FcγR on monocytes were found up-regulated by plastic adherence, TNFα mRNA levels were also found to be slightly higher when monocytes were plated in uncoated wells, compared to OVA-coated wells. However, we have also found that there is a significant up-regulation of TNFα when stimulated by IgG1 or IgG3, compared to the non-specific protein stimulus OVA. This shows that the activation of monocytes was mediated through FcγR. Both IgG1 and IgG3 stimuli elicited similar TNFα response in our experiments, thus it was hard to distinguish which of the two subclasses was more potent and would potentially be more arthritogenic. The lack of IL-1α, IL-6 or IL-10 mRNA production when monocytes were activated by IgG cross-linking may be due to that the 4-hour cross-linking stimulation is not sufficient to generate enough TNFα protein needed for the production of other cytokines, such as IL-1α, IL-6 or IL-10. This should be tested in a kinetic
assay, where monocytes are stimulated for different times, in order to obtain optimal activation and mRNA induction, as well as information on the half-lives of cytokines mRNA.

Because FcγR (and its polymorphism) have different binding affinities for the IgG subclasses, it would be interesting to see if their effector functions executed are dependent on the type of IgG employed. FcγRI can phagocytose particulate IgG1-IC, but with IgG3-IC it forms rosettes and rarely phagocytose [40]. This could be explained in structural studies with IgG3, which has a longer hinge region that allows it to form rosettes more efficiently than the shorter hinge in IgG1 [14]. FcγRIII has a higher affinity for IgG1 and binds to the latter’s middle hinge region, and disruptions of this middle region affects IgG1 binding and ADCC activity [48]. Carbohydrates moieties of IgG can also influence the interactions between FcγR and Ab.

FcγRIIa has been correlated with particulate IgG1- and IgG3-IC binding in an earlier study in our group. In a phagocytosis assay, particulate IgG1-IC was slightly affected when FcγRIIa was blocked (unpublished data). Here we blocked FcγRIIa prior cross-linking with IgG1 or IgG3 and found that it did not affect TNFα mRNA level. This indicates that TNFα is most likely induced via FcγRI or III, and not by FcgRIIa. However, more blocking studies should be done to determine which receptor is involved. Interestingly, Abrahams et. al. [49] have shown that FcγRIII, and not FcγRI or IIa, is responsible for TNFα production in monocytes. Furthermore, increased expression of FcγRIII on synovial macrophages has been correlated to increased TNFα expression in RA synovia [50]. This highlights the possible role of FcγRIII in RA and how the up-regulated FcγRIII might facilitate TNFα production. Conflicting studies exist though, reporting that TNFα production, as well as IL-1β and IL-8, is mediated through
FcγRIIa and not FcγRIII [51] and that cross-linking of FcγRI and desialylated FcγRII induce TNFα secretion in human monocytes [52].

The role of TGFβ in RA can be both anti-inflammatory and/or pro-inflammatory [24]. TGFβ is a chemoattractant for monocytes and is reported to be abundantly expressed in the synovium of RA patients [53, 54]. On the other hand, it can also suppress T cell functions, inhibit fibroblast expansion and inhibit production of metalloproteinases [55] and can also suppress FcγRI and III effects by the down-regulating γ-subunit expression [56]. TGFβ1 mRNA was detected in our cross-linking experiments but their levels were low compared to the TNFα mRNA produced. This was not surprising as TGFβ is known as a late cytokine and in CIA, it is expressed at chronic stages of disease [57] and increased TGFβ1 mRNA is associated with remission of CIA [58]. When FcγRII was blocked, the TGFβ1 expression was affected in cells stimulated with IgG1, IgG3 and LPS stimuli, though not significant. It seems that, by unknown mechanism, activated monocytes synergise with FcγRIIa to drive TGFβ1 expression.

As we could show that the TNFα mRNA production was FcγR-mediated in our cross-linking experiments, we wanted to see if this was also same for IL-1. We detected IL-1β and IL-1ra mRNA and their levels were higher than TNFα. However, the induction of IL-1β or IL-1ra did not seem to be FcγR-mediated as there was no increase in IL-1β or IL-1ra mRNA when the cells were stimulated with IgG1 or IgG3. Blom et. al. [50] could not find any relationship between FcγR and IL-1 production in macrophages from RA patients either. LPS stimulation of the monocytes up-regulated IL-1β expression, as shown previously [59]. It is possible that the presence of higher basal expression of IL-1β mRNA can be induced by cytokine-independent pathways, such as direct contact with T cells, denatured matrix proteins or
hormones [60], or toll-like receptors (TLR) ligands could have also triggered gene expression and synthesis of IL-1β precursor [61]. Apparently, in a FcγR-independent pathway, ligation of CD38, an orphan receptor, on resting monocytes could also lead to release of IL-1β [66].

Interestingly, when we blocked FcγRIIa on monocytes, IL-1β and IL-1ra expression was increased when cross-linked by IgG1, compared to unblocked monocytes, whereas IL-1β and IL-1ra mRNA levels were decreased in FcγRIIa-blocked monocytes when stimulated with IgG3. Activation of the appropriate FcγR may contribute to the basal expressions of IL-1β and IL-1ra to a certain extent. And it seems that different effector functions could be mediated by FcγR when activated by IgG1 or IgG3. Since FcγRIIa binds IgG3 predominantly, therefore when blocked, it was unable to mediate its effects when stimulated by IgG3, resulting in the decreased IL-1β and IL-1ra mRNA levels. It is possible that IgG1 mainly cross-linked FcγRIII when FcγRIIa was blocked and ligation of FcγRIII has shown to induce IL-1 production, in the presence of TNFα [49], possibly explaining our results. Recently, another group showed that the IL-1ra protein production by PBMC was highest when cross-linked with IgG3 and IgG1, but IL-1ra can also be produced when higher concentrations of IgG2 or IgG4 were used [63]. As our detection of IL-1β and IL-1ra was only based on a set of data, it should be repeated to see if this phenomenon happens again.

When U937 cells were stimulated by cross-linking method, latent TGFβ1 mRNA was found expressed regardless of the stimuli, while TNFα was completely absent, even when stimulated with LPS. U937 cells represent immature monocytes and have to be differentiated with calcitriol or IFNγ in order to achieve similar functional properties of monocytes, such as induction of CD14 expression [37]. This might explain why no TNFα was expressed, as CD14 is the receptor for LPS which transfers LPS to TLR4 and signals to activate the NFκB.
pathway, leading to TNFα production [59]. Therefore, we decided to use the more mature Mono-Mac 6 cells, which constitutively express CD14 and exhibit characteristics of monocyte functions such as phagocytosis [64, 65]. When Mono-Mac 6 cells were stimulated with particulate IC, IL-12p35, IL-1β, IL-1ra, IL-6, TNFα and TGFβ1 were expressed. Unexpectedly there was only little TNFα inducted by LPS. FACS analysis of the Mono-Mac 6 cells indicated little or no CD14 expression on the cell surface and this is probably due to Mono-Mac 6 cells being passaged over a long period and might have transformed into a constitutively active state, not expressing CD14. Despite employing two methods of stimulation, particulate IC or cross-linking, TNFα and TGFβ1 mRNA induction in Mono-Mac 6 cells was not found to be FcγR-mediated. Although there have been a slight contribution from pathways signaled by FcγRIIa as mRNA levels were decreased when FcγRIIa was blocked. The Mono-Mac 6 cells were only stimulated once and the experiment should be repeated to reconfirm our data. It is also possible that the stimulation period might have been too short to activate the cytokine genes. Perhaps the poor response in TNFα induction was due to that Mono-Mac 6 cells lack FcγRIII, because when using blood monocytes TNFα induction was associated with cross-linking of FcγRI and/or FcγRIII, which points to that cross-linking of FcγRIII leads to TNFα production. This should be proved by more blocking experiments of FcγR on monocytes and Mono-Mac 6 cells, transfecting and expressing FcγRIII in Mono-Mac 6 cells, or over-expressing FcγR in other cell lines that do not normally express FcγR.

The aetiology of RA remains elusive due to the complicated interplay of the different cells, types of IC, glycan profile of Ab, FcγR expressions and cytokine profiles. This study demonstrates that monocytes can be activated in vitro by cross-linking their FcγR with IgG1 or IgG3, to induce pro-inflammatory cytokines such as TNFα. As most of our data was based on a small cohort or a single experiment, the experiments need to be repeated. However, our
emphasis was on fine-tuning the methods used and to generate preliminary data so we could put forward a good hypothesis and improve the experimental design in order simulate *in vivo* scenarios in our future work. Not only should the experiments be optimised and repeated, it should also be extended to studies on monocytes from RA patients and cytokine protein expression should be analysed to see if they correlate with the mRNA detected. This will allow us to define the molecular mechanisms of IgG subclass activation pathways via FcγR on monocytes as specific targets for drug development in treating RA and other autoimmune diseases.
Acknowledgements

I would like to thank several people for all their help, support, kindness and lovely company filled with humour and contagious laughter:

My supervisor, Sandra Kleinau, for her guidance; giving me the opportunity to learn and gain invaluable experiences and techniques; and most importantly time, so that I could appreciate and enjoy the process. Sofia Magnusson, my kaffekompis, for showing me the ropes around the lab, discussions, and thoroughly-proof-reading my thesis. Kajsa Nilsson for ever being so helpful, reading my thesis as an opponent, her restaurant recommendations and especially lending me her TV set. Camilla Holt for her help in the lab. Former member, Maria Andrén, for taking time off her busy schedule to teach me how to quantify my autoradiograph data on the ImageGauge program.

Maike Gallwitz, for her technical expertise and advice, always ready to drop her things to help me, idea-exchanges, running tips and small talks with me. Maria Aveskogh, for her technical expertise as well, teaching me Små grodorna, introducing stensöta and keeping everyone in high spirits. Mattias Enoksson on the day shift and how we would day-dream of having a plumbing cum conference organizers business. Mattias Andersson on the night shift and his freshly brewed coffees. Jenny Reimer, for letting me use her computer, and the bacterial mass-culturist Niclas Olsson. Good company from other members of the immunology department, Lars Hellman, Sayran A. Muhammadamin, Madeleine Krieg, and Marie-Louise Pichl.

Folks from other departments of Institutionen för cell och molekylärbiologi: Shiyung Wu and Klas Udekwu for showing me how to use the PhosphorImager scanner as well as teaching me how to quantify my data using ImageQuant program. Cecilia Unoson for rushing my radioisotope orders. Chenhui Huang for showing me the use of the scintillation counter and teaching me Mandarin proverbs. Ikue Shiroyama for her friendliness and giving good tips on making Japanese food, and many other people who had made my stay here so enjoyable.

My husband, Daniel Högberg, for his support and fantastic cooking. The Högberg and Tan families, and friends (Rachel Nong, Elsa Ng, Evonne Say, Delina Swee, Sufen Ang & Charis Ng) from Singapore and Sweden for all their support and encouragement.
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