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The influence of IgG Density and Macrophage Fc (gamma) Receptor Cross-linking on Phagocytosis and IL-10 Production

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Summary

We have previously demonstrated that the addition of immune complexes (IC) to stimulated macrophages could profoundly influence cytokine production. In the present work we sought to determine the density of IgG on immune complexes necessary to mediate phagocytosis, inhibit IL-12 production and induce IL-10 production from stimulated macrophages. We developed immune complexes with predictable average densities of surface-bound immunoglobulin. We show that a threshold amount of IgG was necessary to mediate attachment of IC to macrophages. At progressively higher densities of IgG, Fc receptor-mediated phagocytosis resulted in an inhibition of IL-12 production and then an induction of IL-10. The reciprocal alterations in these two cytokines occurred when as little as one optimally-opsonized SRBC was bound per macrophage. Macrophage IL-10 induction by immune complexes was associated with the activation of the MAP Kinase, ERK, which was progressively increased as a function of IgG density. We conclude that signal transduction through the macrophage Fc gamma receptors vary as a function of signal strength. At moderate IgG densities, especially in the presence of complement, efficient phagocytosis occurs in the absence of cytokine alterations. At slightly higher IgG densities IL-12 production is shut off and eventually IL-10 induction occurs. Thus, the myriad events emanating from FcγR ligation depends on the density of immune complexes, allowing the Fc receptors to fine-tune cellular responses depending on the extent of receptor cross-linking.

Keywords

Macrophages; IL-10; immune complexes; phagocytosis; inflammation

2. Introduction

Macrophages have been designated “professional phagocytes” because they express receptors for the two major serum opsonins, antibody and complement. These receptors play a prominent role in macrophage receptor-mediated phagocytosis. The receptors for the Fc portion of IgG (FcγRs) are the best characterized class of leukocyte phagocytic receptors, and the intracellular signals emanating from these receptors has been thoroughly studied [1-4]. These receptors are expressed on virtually all leukocytes and bind the Fc portion of immunoglobulin (Ig) with varying affinities [1,4]. There are three types of FcγR in humans

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and four in mice. FcγRI is the high affinity receptor that binds monomeric IgG2a in mice and IgG1 and IgG3 in humans [1]. FcγRII and FcγRIII are low affinity receptors and require multivalent immune complexes (IC) in order to sufficiently increase avidity to promote binding [1]. FcγRIV is found in mice. It shares 60% amino acid identity with FcγRIII, and it binds to IgGs with affinities similar to FcγRII and III [2]. The sequence identity between the extracellular domains of the FcγR's is relatively high, especially when compared to the significant differences in their cytoplasmic domains [5]. FcγR I, III and IV signal through an ITAM-containing γ chain that is associated with the small cytoplasmic domain of the receptors [3,6,7]. The recruitment of the tyrosine kinase Syk to the γ chain ITAMs represents the initial step in cellular responses [8]. Syk is a multifunctional kinase that phosphorylates several substrates and recruits many adaptor molecules allowing FcγR signaling to initiate a complex variety of divergent cellular responses [1,9].

Macrophages are prodigious secretory cells that when appropriately stimulated can produce a variety of cytokines, chemokines, and lipid mediators [10,11]. These macrophage secretory products can have a profound effect on host immune and inflammatory responses. Stimuli typically activate innate pattern recognition receptors on macrophages whose engagement signals the activation of NF-κB, MAPKs, and other signaling pathways involved in mediator transcription, translation, and secretion [12]. The Fcγ receptors participate in the clearance of immune complexes (IC) [13,14] but this in itself is generally not an efficient signal for macrophage secretory responses. FcγR-mediated phagocytosis by macrophages typically results in minimal cytokine release, unless the target particle to which the antibody is directed also engages pattern recognition receptors. Although FcγR cross-linking alone is not an efficient inducer of cytokine production, this process can have a profound effect on cytokines produced in response to pattern recognition receptor activation. We and others have demonstrated that FcγR cross-linking can dramatically influence the production of cytokines by stimulated macrophages [15-18]. We have demonstrated that macrophage stimulation in the presence of immune complexes (IC) stop producing IL-12 [15] and induced the production of high levels of antiinflammatory IL-10 [16]. The induction of IL-10 by IC required the activation of the MAPK, ERK, which led to covalent modifications of chromatin at the *il-10* promoter [19]. Thus, FcγR cross-linking can change the phenotype of macrophages converting them into potent antiinflammatory cells producing high levels of IL-10 [20].

We considered this alteration in cytokine production to be somewhat of a paradox, because it suggested that IC would actually inhibit immune responses, by virtue of this reciprocal alteration in macrophage cytokine production. Our data implied that antibodies could actually diminish immune responses, due to macrophage FcγR cross-linking. This would not be consistent with the well-established role of IgG in host defense. Therefore, in the present work we examined cytokine production from macrophages exposed to immune complexes, with the idea that the density of IgG on IC would influence the secretory response of macrophages. We examined the production of IL-10 and IL-12 and the efficiency of receptor-mediated endocytosis as a function of IgG density. We demonstrate that at moderately low densities of IgG, IC efficiently mediated the clearance of antigen with little effect on IL-10 production. This was especially true when complement was present. High IgG densities, and therefore extensive FcγR cross-linking, were needed to reprogram macrophages to produce high levels of IL-10. These high-density immune complexes activated the MAPK, ERK, resulting in IL-10 production. Thus, the reprogramming of inflammatory macrophages by high-density IC may represent a mechanism to terminate immune responses.

3. Materials and Methods

Mice

Six- to eight-week-old BALB/c mice were purchased from Taconic Farms. All mice were maintained in HEPA-filtered Thoren units (Thoren Caging Systems) at the University of Maryland (College Park, MD). Mice were used at 6-10 weeks of age as a source of bone marrow-derived macrophages. All procedures were reviewed and approved by the University of Maryland Institutional Animal Care and Use Committee.

Bone Marrow Derived Macrophages

Bone marrow-derived macrophages were prepared as previously described [21]. Briefly, bone marrow was flushed from the femurs and tibias of mice and cells were plated in petri dishes in DMEM/F12 supplemented with 10% FBS, penicillin/streptomycin, glutamine, and 20% conditioned medium from the supernatant of M-CSF secreting L929 (LCM) fibroblasts. Cells were fed on day 2, and complete medium was replaced on day 6. Cells were used at 7-10 days for experiments.

Immune complexes

Insoluble IC were made using washed 10% sheep red blood cells (SRBC) from Lampire Biological Laboratories (cat: 7249008). SRBC were used within two weeks of receiving them. SRBC were opsonized with anti-SRBC IgG from Cappel MP Biomedicals (cat: Z-25360). Different concentrations of antibody to SRBC were added to a constant number of SRBC (2×10^8 SRBC in total 0.5 ml). IgG was added to RPMI culture media at the highest concentration and then serially diluted for indicated number of dilutions. The IgG-SRBC solution was gently rotated 40-50 minutes at room temperature. A constant number of opsonized SRBC (2.0×10^6) were added to macrophage monolayers for 1 hr at 37°C.

Flow cytometry

A fluorescent labeled F(ab) fragment of antibody to rabbit IgG (Zenon Rabbit IgG Labeling kit, Molecular Probes, Eugene, OR) was used to quantitate IgG on the surface of SRBC by flow cytometry according to manufacturer's specifications. After labeling IC were spun at $470 \times g$ for 5 minutes, supernatants were removed, and resuspended in 200 μl 5% paraformaldehyde for analysis by flow cytometry. To confirm Zenon labeling, IC were also measured using secondary FITC-conjugated antibodies (data not shown). For these studies supernatants were removed and cells were resuspended in 200 μl Flow Blocking Buffer (PBS, 5% FBS, 1% 0.5M EDTA), and stained with FITC-conjugated antibody to rabbit IgG for 30 minutes at room temperature. Fluorescence was measured on a *BD FACSCalibur* (BD Biosciences, San Jose) and results analyzed using FlowJo software (Tree Star).

ELISA

An Enzyme-linked Immunosorbent Assay (ELISA) was used to quantitate IgG on each IC. IC were generated as described above. Cells were centrifuged at $2100 \times g$ for 10 minutes. Supernatants containing free IgG were removed and saved. SRBCs were resuspended in ACK lysing buffer (Gibco: Invitrogen formula no. 06-0005DG), pipetted vigorously, and neutralized with RPMI culture media. IgG ELISAs were performed on both free IgG fraction and SRBC bound IgG fraction (after lysis of SRBC). ELISA was performed using Rabbit IgG ELISA Quantitation Kit (Bethyl Laboratories, catalog no. E120-111). Kit was used to manufacturer's specifications.

Cytokine Analysis

Bone marrow derived macrophages were prepared as described above. For cytokine analysis, 5×10^5 macrophages per well were seeded overnight in a 24 well plate in DMEM/20% LCM. Cells were washed and activated with either 10ng/ml LPS alone or in combination with 1:20 ratio of macrophage to IgG-SRBC IC. Supernatants were harvested ~20hrs later. Cytokines were measured by sandwich ELISA using the following antibody pairs from BD Pharmingen according to manufacturer's instructions: IL-12p40, C15.6 and C17.8; IL-10, JESS-2A5 and JES5-16E3.

SRBC phagocytosis

Bone marrow derived macrophages were prepared as described above. For phagocytosis analysis, 2.5×10^5 macrophages per well were seeded on cover slides overnight in a 24 well plate in DMEM/20% LCM. Cells were washed and activated with either 10ng/ml LPS alone or in combination with 1:20 ratio of macrophage to IgG-SRBC IC. Macrophages were washed of free SRBCs. Macrophages were then fixed and made permeable with Cytofix/Cytoperm Kit. SRBCs were opsonized again with primary anti-SRBC. SRBCs were then labeled with FITC conjugated secondary antibody. Macrophages were labeled with PI. Cover slides were removed, dried, and mounted on slides with Mowiol anti-fade. Slides were incubated overnight at 4°. Cells were visualized by fluorescence microscopy.

To quantitate SRBC phagocytosis by colorimetry, 1×10^5 bone marrow derived macrophages per well were seeded overnight in a 96 well plate in DMEM/20% LCM. Cells were washed and activated with either 10ng/ml LPS alone or in combination with 1:20 ratio of macrophage to IgG-SRBC IC. Cells were incubated for ~50 minutes. Macrophages were washed of free SRBCs three times with RPMI culture media. All cells were lysed using 0.5% SDS solution. Standard was made using known number of SRBCs and lysing with 0.5% SDS. Samples were transferred to 96-well flat bottom plate (Falcon or Costar) for measuring. The pseudo enzymatic activity of hemoglobin was used to measure SRBCs using 2,7-diaminofluorene (DAF) substrate. DAF stock solution was made by dissolving 100 mg of DAF (Product number: D17106; Sigma-Aldrich) into 10 ml of 99% glacial acetic acid with vigorous vortexing at room temperature. DAF working solution was made by adding 1 ml of DAF stock solution and 0.1 ml of 30% hydrogen peroxide (Cat. no. H323-500; Fisher Scientific) into 10 ml 0.2M Tris-HCl (pH 7.4) containing 6M urea. 100μl of DAF working solution was added to lysed cells. Wells were measured for color change and compared to color change of standard. Phagocytosis experiments were done with and without C5 deficient serum (5% by volume). C5 deficient serum mimics the opsonization function of complement without lysing the SRBC used as antigen.

Western Blotting

A total of 2×10^6 BMMΦ per well were plated overnight in six-well plates. Cells were treated with a 1:20 ratio of macrophage to IgG-SRBC immune complex in a final volume of 2 ml of DMEM without L929 conditioned medium. Cells were lysed in ice-cold lysis buffer (100 mM Tris (pH8), 2 mM EDTA, 100 mM NaCl, 1% Triton X-100 containing complete EDTA-free protease inhibitors from Roche Diagnostics, which included 5 mM sodium vanadate, 10 mM sodium fluoride, 10 mM β-glycerophosphate sodium, and 5 mM sodium phosphate). Equal amounts of protein were loaded onto 10% SDS-polyacrylamide gels, and then transferred to polyvinylidene difluoride membranes. Membranes were incubated with primary Abs overnight at 4°C, washed, and incubated with secondary Ab with HRP conjugates. The specific protein bands were visualized by using Lumi-Light™PLUS chemiluminescent substrate (Roche Diagnostics).

4. Results

Generating immune complexes with increasing density of IgG

Immune complexes (IC) were made by incubating sheep erythrocytes (SRBCs) with increasing concentrations of rabbit anti-SRBC, ranging from 4 ng/ml up to 10 μg/ml. To measure the degree of IgG opsonization, SRBC were washed and incubated with FITC-labeled anti-IgG F(ab) fragments and processed for flow cytometry. A progressive increase in fluorescence, reflecting an increase in the number of IgG molecules on the surface of erythrocytes was detected (Figure 1A). IgG opsonization in this *in vitro* system occurred after adding as little as 156 ng IgG/ml and increased progressively thereafter. At IgG concentrations above 2.5 μg/ml agglutination of SRBC began to occur, and at IgG concentrations above 10 μg/ml the agglutination was sufficient enough to hinder analysis by flow cytometry. This experiment was performed several times under identical conditions and there was always a similar progressive dose-dependent increase in IgG opsonization resulting in erythrocytes opsonized with increasing densities of IgG per SRBC.

We also quantified the average number of IgG molecules attached per red blood cell as a function of IgG input (Figure 1B). IC were washed to remove unbound IgG from SRBC and then SRBCs were lysed with mild detergent releasing IgG which was then quantified by ELISA. At the lowest IgG input concentrations bound IgG was below the limits of detection. IC that were made using higher input concentrations of IgG retained more IgG on the surface of SRBC, as expected (Figure 1B). Although less sensitive than flow cytometry, the IgG ELISA confirms the flow cytometry data in Figure 1A and demonstrates that an increase in IgG density occurs under these opsonizing conditions. The ELISA also provides an estimate for the average number of IgG molecules per SRBC.

Phagocytosis as a function of the IgG density on SRBC-immune complexes

We compared the number of molecules per SRBC with the ability of macrophages to phagocytose SRBCs. The pseudo enzymatic activity of hemoglobin was used to quantify opsonized SRBC uptake by macrophages. Hemoglobin reacts with 2,7-diaminofluorene (DAF) to produce a dark solution that can be used to measure the amount of hemoglobin by colorimetry. This was compared to a standard dose of increasing numbers of SRBC. Macrophages were stimulated with low levels of LPS alone (10ng/ml), or in combination with IC containing increasing densities of IgG/SRBC. Macrophages were washed free of unbound SRBCs after 50 minutes, and then lysed using 0.5% SDS solution. DAF substrate was added to lysed cells and the OD change was measured. Increasing concentrations of IgG resulted in progressive increases in the number of SRBC taken up by macrophage monolayers (Figure 2).

Figure 2 demonstrates that at low IgG input concentrations, subtle increases in IgG opsonization resulted in marked increases in SRBC phagocytosis. Thus, when one plots data from phagocytosis and compares it with average number of IgG molecules per SRBC, one sees that increases in IgG density correspond to increases in SRBC uptake by bone marrow macrophages. We confirmed our results by visualizing phagocytosis at the light microscopic level. Macrophages were seeded on cover slides and stimulated with low levels of LPS, prior to the addition of opsonized SRBC. Macrophages were washed to remove unbound SRBC after 50 minutes, leaving only bound and phagocytized SRBC. Two hundred macrophages were counted. The number of macrophages with attached SRBCs and the average number of SRBCs on each macrophage were quantitated (Table 1). Increasing IgG concentrations correlated with increasing amounts of phagocytosis, reflected by both an increase in the percentage of macrophages positive for RBC and an increase in the average number of RBC per macrophage (Table 1). The percentage of macrophages with attached

SRBCs began to decline at the highest IgG concentrations, presumably due to SRBC agglutination.

Macrophage cytokine production

Macrophages were stimulated with low levels of LPS (10ng/ml) alone or in combination with SRBC-IC. IL-10 and IL-12/23p40 were measured in supernatants by ELISA. Unopsonized SRBC did not bind to macrophages nor did they have any effect on LPS-induced cytokine production, as expected. In response to LPS stimulation (Figure 3A), macrophages produced high levels of IL-12/23p40, modest levels of IL-10, and high levels of TNF-α. In the presence of immune complex, IL-12 decreases and IL-10 increases but TNF-α remains unchanged. This led us to focus on alterations in IL-10 and IL-12 as a function of IgG density. At low IgG input concentrations, the production of these cytokines remains relatively unchanged (Figure 3B). However, as the density of IgG on IC increases, the levels of IL-12/23p40 production progressively decline to near undetectable levels (Figure 3B, black lines). As the IgG densities increase further, the production of IL-10 begins to increase progressively as a function of IgG density (Figure 3B, red lines). These data indicate that at low densities, IgG can efficiently mediate particle clearance without altering cytokine production. At higher densities, IC can give rise to a population of macrophages with a regulatory phenotype that produce high levels of IL-10 but little to no IL-12/23p40. TNF-α production is not altered by the addition of IC, indicating that the alterations in IL-12 and IL-10 production by IC is relatively specific to these two cytokines, as previously suggested [22].

To determine the number of SRBC required to induce this alteration in cytokine production, SRBC were opsonized with an optimal density of anti-SRBC antibody (100 μg/ml). Increasing numbers of opsonized SRBC/macrophages were added to monolayers and cytokine production was measured. Figure 4 demonstrates that in the absence of SRBC, soluble anti-SRBC at a concentration of 100μg/ml had no effect on cytokine production by stimulated macrophages. These cells produce relatively high levels of IL-12/23p40 and low levels of IL-10, as described above. The addition of a 1:1 ratio of SRBC to macrophage caused a dramatic (~50%) decrease in IL-12/23p40 production and a similar increase in IL-10 (Figure 4). At a 5:1 ratio (SRBC:macrophage) the cytokine switch was virtually complete. At this SRBC input, macrophages have phagocytized an average of only 1-2 SRBC/cell (data not shown). Therefore, macrophages need not encounter a large number of opsonized SRBC, but the SRBC that they encounter must be opsonized with a relatively high density of IgG to extensively cross-link FcγR.

ERK Activation

We and others have previously demonstrated that signaling through the macrophage Fcγ receptor activates the MAP Kinase ERK [19]. To investigate whether ERK activation also responded to increasing IgG densities, opsonized SRBC with increasing amounts of IgG were added to macrophage monolayers and ERK activation was analyzed by western blotting using an antibody specific for the phosphorylated forms of ERK 1 and 2 (Figure 5). Increasing the density of IgG on sheep erythrocytes induced a progressive increase in the phosphorylation of ERK. At low levels of opsonization, where no alterations in cytokine production were evident, there was little or no detectable activation of ERK. ERK activation occurred at higher IgG inputs corresponding to the densities of IgG that caused an increase in IL-10 production. Thus, both the induction of IL-10 and the activation of ERK occur at relatively high densities of IgG on immune complexes.

Phagocytosis and cytokine production

We performed a quantitative analysis of cytokine production and phagocytosis of opsonized SRBC as a function of IgG densities. We compared cytokine data with the uptake of opsonized SRBC by macrophages in the presence or absence of C5 deficient serum, as a source of opsonic complement. Figure 6 demonstrates that SRBC phagocytosis required a minimal degree of opsonization, below which no phagocytosis nor cytokine modulation occurred. At slightly higher IgG concentrations, SRBC phagocytosis occurs in the absence of any alterations in IL-10 production. This is especially evident when C5 deficient serum is added to the assay to mimic the synergistic opsonization between complement and immunoglobulin. In the presence of complement, there is a clear range of opsonization conditions where low levels of IgG mediate efficient SRBC uptake, without inducing IL-10 production (Figure 6). Thus, complement cooperates with IgG to promote the efficient ingestion of opsonized particles without inducing anti-inflammatory cytokine production by macrophages. At higher IgG concentrations, IL-10 is rapidly induced from macrophages.

Using data from figure 6 we present a “strength of signal” model: Very low densities of IgG (Figure 6, <0.009μg/ml starting concentration) on SRBCs do not induce phagocytosis, do not activate ERK, and do not induce changes in cytokine production by stimulated macrophages. Medium to low densities of IC (Figure 6, 0.009μg/ml to 0.15μg/ml starting concentration) induce SRBC phagocytosis but no induction of IL-10 secretion from macrophages. At higher IgG concentrations, however (> 1.17 μg/ml starting concentration), the phagocytosis of opsonized SRBC is associated with the activation of ERK and the production of high levels of IL-10 by macrophages.

5. Discussion

The humoral immune response results in the production of antibodies, which can mediate the clearance of immune complexes (IC) through receptor-mediated phagocytosis [13,14]. In addition to phagocytosis, signals emanating through the FcγRs on leukocytes result in myriad cellular alterations, including changes to the cellular cytoskeleton, the mobilization of cellular organelles, activation of the respiratory burst, as well as changes in maturation status of some cells. We have shown that FcγR cross-linking is also associated with dramatic alterations in cytokine production by stimulated macrophages [15,16]. These alterations in cytokine production can result in a phenotypic switch to a cell that has potent anti-inflammatory activity due to the over-production of IL-10 [23]. We have previously demonstrated that stimulation of macrophages in the presence of IC is associated with the downstream activation of the Mitogen-Activated Protein Kinase (MAPK), ERK. This ERK activation leads to modifications in histones associated with the *il-10* promoter [19] resulting in dramatic increases in macrophage IL-10 production [16].

In the present work, we examined the amount of IgG on immune complexes necessary to mediate alterations in macrophage cytokine production. For these studies we added progressively more IgG to a constant number (1×10^8) of sheep red blood cells (SRBC) in order to get particles with increasing densities of IgG (Figure 1). We used relatively high amounts of SRBC so we could identify concentrations of IgG below which opsonization did not occur, and then progressively higher concentrations of antibody at which phagocytosis and then cytokine alterations occurred. By flow cytometry we were able to obtain an average estimate of IgG density per particle, and by ELISA we could quantitate the average number of antibody molecules associated with each erythrocyte. We demonstrate that there is a threshold of IgG below which phagocytosis does not occur. At moderate IgG densities efficient phagocytosis of IC occurs with little effect on IL-10. At higher IgG densities, however, immune complexes induce the production of IL-10, an anti-inflammatory cytokine with the potential to terminate immune responses. The observation that the density of IgG on

IC can influence macrophage cytokine production may address an area of confusion regarding IC. Immune complex diseases are clearly associated with inflammatory pathology [24-26], and yet both in vitro [19] and in vivo [27] studies have indicated that IC can inhibit inflammation by inducing macrophage IL-10 production. It may be that the density of IgG on IC may determine the inflammatory character of the IC. Furthermore, the size of IC can be inversely proportional to their pathogenicity during Type III hypersensitivity [28]. This is because larger complexes formed by excess antibodiy and can be more easily eliminated by phagocytosis, whereas smaller IC can continuously stimulate inflammation. Here we show that IC composed of high amounts of IgG produce an antiinflammatory profile, and that this may represent another mechanism in the reduced pathogenicity of IC during type III hypersensitivity.

The fact that IC have both inflammatory and regulatory functions is largely due to the heterogeneity of FcγRs and the variety of cells that express them. For example, human FcγRIIb is known to dampen TLR-4 induced immune responses [29], whereas blood monocytes, signaling through human FcγRIIa, produce pro-inflammatory TNF, IL-1β, and IL-8 [30]. IC can also influence dendritic cell maturation [31] and cytokine production. Similar to macrophages, stimulation of DC in the presence of IC can down regulate IL-12 production, however unlike macrophages, IC do not induce IL-10 production from DC [31,32]. Neutrophil immune functions can also be down-regulated by IC [33]. Even platelets are responsive to IC, aggregating in response to FcγRIIa cross-linking [34]. Some data even suggests that antibody-receptor binding itself is regulated by the strength of signal in FcγRs [35]. Recognizing this property, some investigators have developed a substrate surface with “tunable” IgG density for use as a model for immune complex diseases [36]. Therefore, it becomes necessary to quantitatively analyze the spectrum of responses that can occur from various cells following the ligation of FcγR.

We propose a ‘strength of signal’ hypothesis to explain this response, similar to what has been proposed for the production of IL-10 in T cells [37]. IL-10 production in response to high-density IgG makes teleological sense because it suggests that when enough IgG is present on IC, macrophages up-regulate IL-10 to dampen immune responses before pathologically high levels of IgG accumulate. Our investigation suggests that macrophages contain mechanism(s) to recognize the density of IgG on IC and we demonstrate that as the density of IgG increases so does the activation of ERK. The correlation between the extent of receptor cross-linking and ERK activation appears to be important. Our observations are corroborated by research showing the activation of ERK following FcγR cross-linking on T cells [37], and endothelial cells [38].

The differential regulation of macrophage cellular functions by IgG density may be particularly important when considering possible therapeutic uses of IgG. For example, high dose IVIG has been used as a non-specific anti-inflammatory therapeutic [39,40]. It may be that extensive FcγR crosslinking and the resulting IL-10 production are responsible for some of the anti-inflammatory benefits of high dose IVIG. Furthermore, it has been suggested that targeting antigen to antigen presenting cells could be a promising way to improve vaccines [41,42]. However, in order for vaccine development to be successful we must first understand the ratio of antigen to antibody and under what circumstances the FcγRs are stimulatory or inhibitory.

In these studies we demonstrate that high density IC were necessary to induce IL-10 production from macrophages. We also observed that IC inhibited macrophage IL-12 production. These two alterations in cytokine production were not strictly reciprocal, because the induction of IL-10 occurred only at high density IgG, whereas the reduction of IL-12 appeared to occur as a function of receptor-mediated phagocytosis. Thus, although

these two cytokines are often inversely regulated, we conclude that the induction of IL-10 and the inhibition of IL-12 occur by distinct mechanisms. IL-10 induction is associated with ERK activation via high-density IC, implying a “strength of signal” mechanism. IL-12 inhibition, on the other hand, occurs following most receptor-mediated endocytic events and this is not dependent on signal strength.

In summary, this study identifies a regulatory role for immunoglobulin that occurs at the level of the macrophage, and is dependent on FcγR cross-linking. We are continuing to investigate the signaling events and the specific role that ERK plays in this induction. This study provides a foundation for future investigations into the changes that occur within macrophages stimulated through the FcγR.

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Abbreviations Page

C5	The fifth component of the complement pathway
DAF	Diaminofluorene, compound used to react with hemoglobin
DC	Dendritic Cell
ELISA	Enzyme-linked Immunosorbant Assay
ERK	Extracellular signal-regulated kinase, a MAP kinase
FcγR	Fc gamma Receptor, receptor for Fc portion of immunoglobulin
IC	Immune complex, and antigen-antibody complex
ITAM	Immuno-receptor tyrosine-based activation motif
IVIG	Intravenous immunoglobulin
MAPK	Mitogen-activated protein kinase
SRBC	Sheep red blood cell, sheep erythrocyte
TLR	Toll-like Receptor
TNF	Tumor necrosis factor alpha

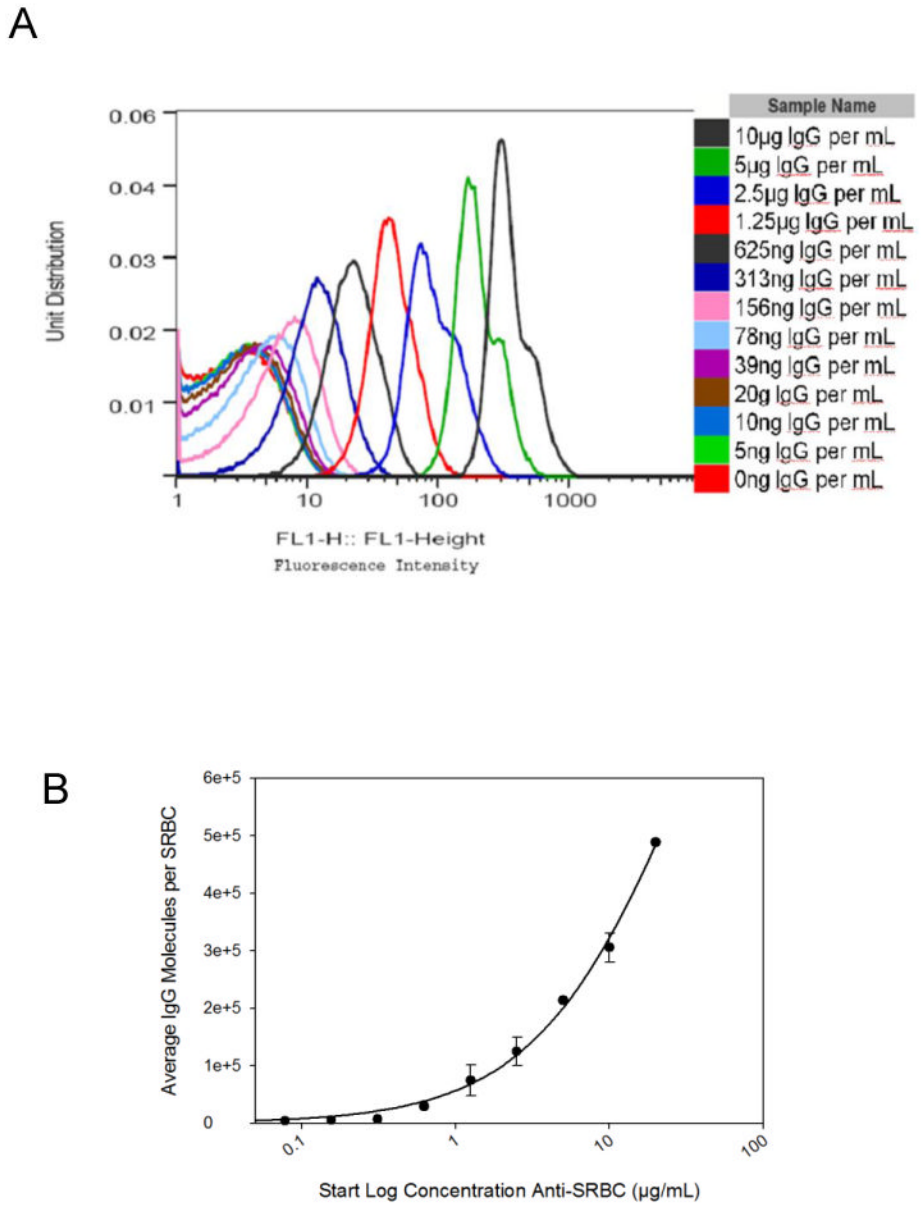

Figure 1.

Quantitation of IgG opsonization of SRBC. **A.** Immune complexes were made by incubating a constant number (1×10^8) SRBCs with increasing concentrations of rabbit anti-SRBC (right axis). IgG bound to SRBC was detected by an IgG fluorescent labeling kit *Zenon*. SRBC-IgG complexes were analyzed by flow cytometry. **B.** IgG opsonization of SRBC was determined by ELISA. The amount of bound IgG on washed SRBC was determined by lysing SRBC and measuring the amount of IgG in the lysates by ELISA.

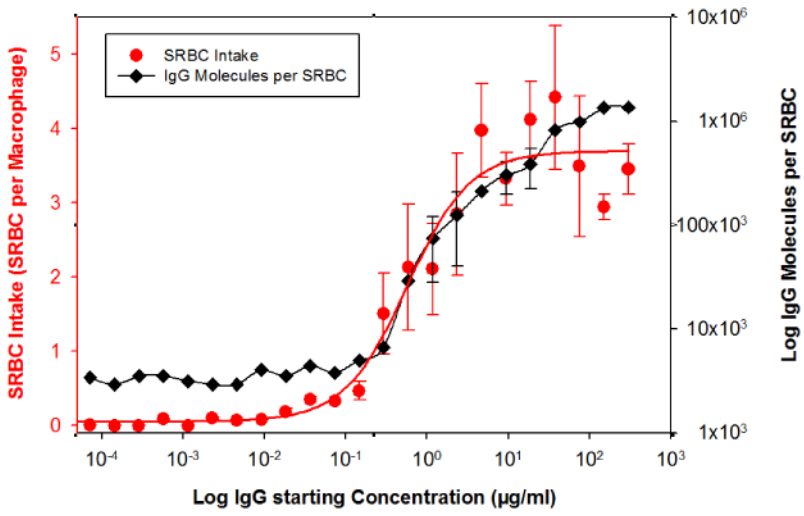

Figure 2.

The phagocytosis of IgG-opsonized SRBC as a function of IgG input. SRBC uptake by macrophages (circle) was compared to the average number of IgG molecules per SRBC (diamond). Mouse bone marrow derived macrophages were stimulated using LPS alone (10ng/ml) or in combination with IC. A 1:20 macrophage to SRBC ratio was used. Cells were incubated for 50 minutes. Macrophages were washed free of unbound SRBCs and then lysed with ACK lyse buffer containing a 0.5% SDS solution. DAF substrate was added to lysed SRBC and wells were measured for color change resulting from the pseudo enzymatic activity of hemoglobin.

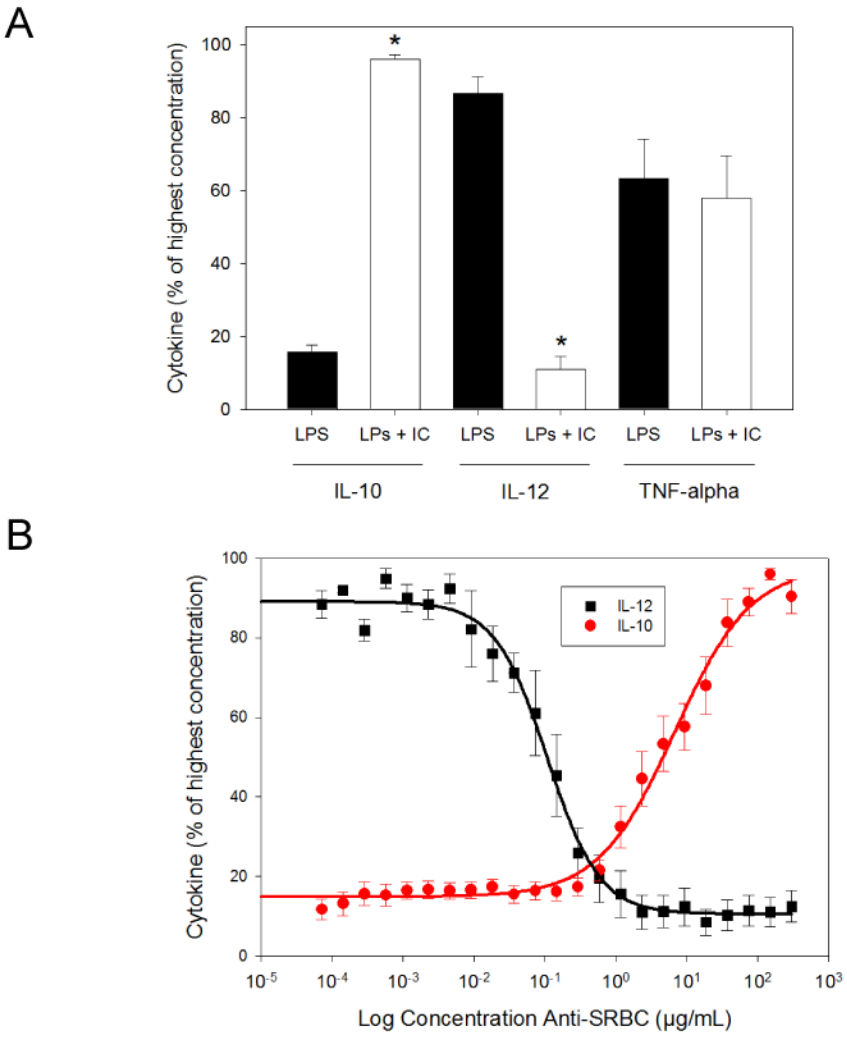

Figure 3.

Cytokine production by macrophages in the presence of IC. Mouse bone marrow-derived macrophages were stimulated with LPS alone (10ng/ml) or in combination with IC added at a 20:1 SRBC:macrophage ratio. **A.** The production of IL-10, IL-12, and TNF-α was measured by ELISA in macrophage supernatants after 20 hr incubation. **B.** Cytokine production by LPS stimulated macrophages in the presence of IC containing progressively higher densities of IgG expressed as the log concentration of IgG in µg/ml added to the original SRBC. In order to normalize data from these experiments the data point with the highest concentration of cytokine was set to 100 percent and the percentages for the other values were then averaged. These data are the mean from seven independent experiments ± SEM.

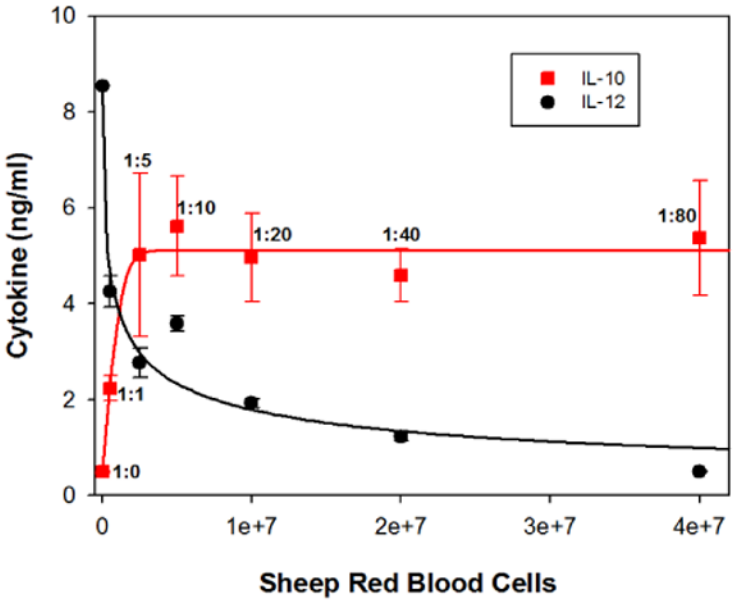

Figure 4.

Cytokine production by LPS-stimulated macrophages in combination with optimal IC. IgG-SRBC was made with 100μg/ml IgG, previously determined to be optimal for cytokine alterations. Increasing numbers of opsonized SRBC (X axis) were added to LPS-stimulated macrophages for 20 hrs. The ratio of macrophage vs. SRBC is above the symbols. IL-10 in the supernatants was measured by ELISA after a 20 hr incubation.

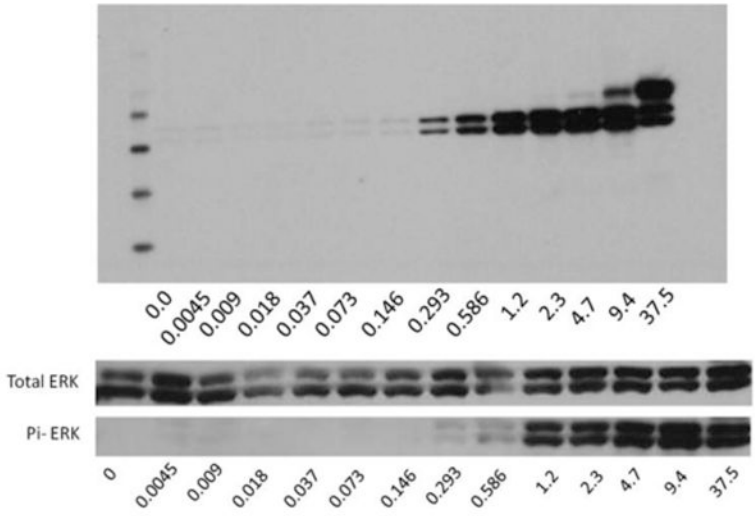

Figure 5.

Western blotting to show ERK phosphorylation in macrophages stimulated for 10 minutes with LPS alone or in combination with increasing concentrations of IC. Concentrations are expressed in μg/ml. Equal amounts of protein were loaded onto 10% SDS-polyacrylamide gels, and then transferred to polyvinylidene difluoride membranes. PhosphoERK1 and 2 were identified using primary antibody incubated at 4°C overnight and then incubated with a secondary HRP-conjugated antibody. Protein bands were visualized by using Lumi-Light™PLUS chemilumiescent substrate. Equal amounts of protein from cell lysates were subjected to Western blotting according to manufacturer's instructions.

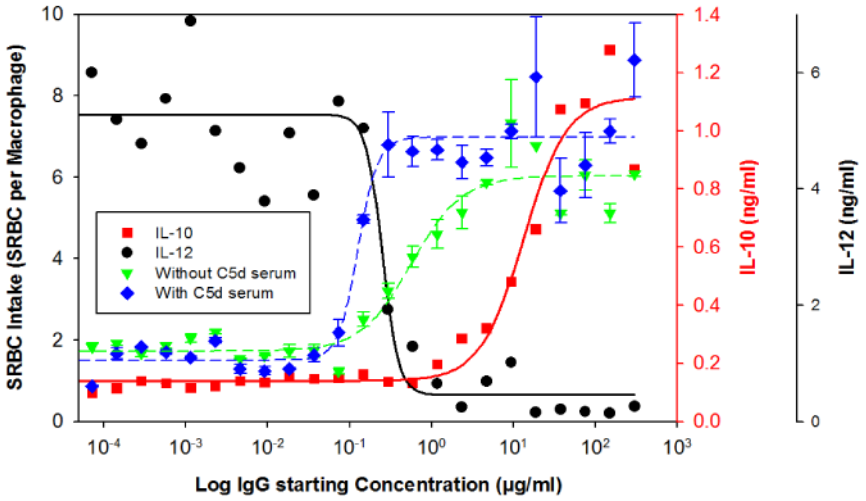

Figure 6.

Phagocytosis versus cytokine production in response to LPS + IC. SRBC intake by macrophages (dotted lines) was compared to cytokine production (solid lines). Mouse bone marrow derived macrophages were activated using LPS alone (10ng/ml) or in combination with IC. A 1:20 macrophage to SRBC ratio was used for all the above data. Phagocytosis was assayed with or without C5 deficient serum (5% by volume). SRBC were added to macrophages for 50 minutes. Macrophages were washed of free SRBCs and then lysed using 0.5% SDS solution. DAF substrate working solution was added to lysed cells and wells were measured for color change using the pseudo enzymatic activity of hemoglobulin to determine the number of SRBC/monolayer.

Table 1

SRBC Phagocytosis

Anti-SRBC μg/ml	Percentage with attached SRBC	Average number of SRBC per SRBC-positive Macrophage	Average number of SRBC per Macrophage
42.5	**36%**	**2.6**	**0.94**
5.3	**92%**	**2.1**	**1.9**
0.66	**89%**	**1.9**	**1.7**
0.33	**62%**	**1.6**	**1.01**
0.08	**18.5%**	**1.1**	**0.2**
0	**14%**	**1**	**0.14**

To analyze phagocytosis, 5×10^5 macrophages per well were seeded on cover slides overnight in a 24 well plate in DMEM/20% LCM. Cells were washed and activated with either 10ng/ml LPS alone or in combination with 1:20 ratio of macrophage to IgG-SRBC IC. Macrophages were washed of free SRBCs. Macrophages were then fixed and made permeable with Cytofix/Cytoperm (with Golgi plug). SRBCs were opsonized again with primary anti-SRBC. SRBCs were then labeled with FITC conjugated secondary antibody. Macrophages were labeled with PI. Cover slides were removed, dried, and mounted on slides with Mowiol glue. Slides were incubated overnight at 4°. Cells were visualized by microscopy and 200 Macrophages were counted.