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*J Immunol* 1999; 162:6217-6225;
http://www.jimmunol.org/content/162/10/6217

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Regulation of Monocyte Survival In Vitro by Deposited IgG: Role of Macrophage Colony-Stimulating Factor

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IgG deposition at tissue sites characteristically leads to macrophage accumulation and organ injury. Although the mechanism by which deposited IgG induces tissue injury is not known, we have recently demonstrated that deposited IgG stimulates the release of IL-8 and monocyte chemoattractant protein-1 from normal human monocytes, which may drive inflammation. Since IgG also induces macrophage accumulation in these diseases, we hypothesized that deposited IgG protects monocytes from apoptosis. As an in vitro model of the effect of deposited IgG on monocyte survival, monocyte apoptosis was studied after FcγR cross-linking. Monocytes cultured on immobilized IgG, which induces FcγR cross-linking, were protected from apoptosis, whereas monocytes cultured with equivalent concentrations of F(ab')2 IgG or 50 times higher concentrations of soluble IgG, neither of which induces FcγR cross-linking, were not protected. Moreover, this protection was transferable, as supernatants from immobilized IgG-stimulated monocytes protected freshly isolated monocytes from apoptosis and contained functional M-CSF, a known monocyte survival factor. M-CSF mediated the monocyte survival induced by FcγR cross-linking, as neutralizing anti-human M-CSF Abs blocked the monocyte protection provided by either immobilized IgG or IgG-stimulated monocyte supernatants. These findings demonstrate a novel mechanism by which deposited IgG targets tissue macrophage accumulation through FcγR-mediated M-CSF release. This pathway may play an important role in promoting and potentiating IgG-mediated tissue injury. The Journal of Immunology, 1999, 162: 6217–6225.

Targeted organ injury in immune complex-associated diseases is associated with deposits of IgG and expanded numbers of young, activated macrophages (1–4). Macrophages derived from bone marrow produce monocytes, which have a half-life of 24–48 h (5, 6). However, at sites of inflammation, monocyte/macrophage populations are frequently expanded (7–10). Since monocyte/macrophage replication is limited, and the monocyte/macrophage populations are frequently expanded (7–10). Since monocyte/macrophage replication is limited, and the monocyte life span short, tissue macrophage expansion must be controlled by prevention of apoptosis. However, the mechanisms that protect monocytes from undergoing apoptosis are only recently becoming understood. In this regard, macrophage populations are often expanded at sites of immune complex deposition, like the joint in rheumatoid arthritis or the alveoli in idiopathic pulmonary fibrosis (7–10). These compartmentalized macrophages may potentiate the tissue injury through the elaboration of cytokines and chemokines (11–14). We have recently found that monocyte FcγR cross-linking on immobilized human IgG, mimicking the effects of immune complexes or deposited IgG on these cells, induces monocytes to release biologically active IL-8 and monocyte chemoattractant protein-1 (MCP-1) (15–17). These chemokines mediate the regulated recruitment of neutrophils and monocytes in the response to opsonized bacteria and the unregulated influx of these inflammatory leukocytes in immune complex-associated diseases.

Localized MCP-1 recruits monocytes to tissues targeted by IgG deposition (15). Regulated monocyte recruitment and survival in response to IgG-opsonized bacteria may be beneficial in resolving localized bacterial infection (18), but unregulated recruitment and survival of monocytes in immune complex-related diseases may lead to tissue injury (1, 10, 19, 20). However, MCP-1 does not produce monocyte survival (21), suggesting that additional monocyte survival factors must be present. Thus, the goal of this study was to characterize the mechanisms that relate IgG deposition to macrophage accumulation to provide new targets to reverse tissue injury in Ab-mediated diseases.

Materials and Methods

Reagents

RPMI 1640 was obtained from BioWhittaker (Walkersville, MD). FCS was obtained from HyClone (Logan, UT). Polymyxin B was obtained from Rohrer Pharmaceuticals (New York, NY). Pooled human IgG (Sandoglobulin) was purchased from Sandoz (Basel, Switzerland). Human serum albumin was obtained from Sigma (St. Louis, MO). Immulon IV tissue culture plates were obtained from Dynatech (Chantilly, VA). Recombinant human M-CSF, murine anti-M-CSF mAbs (IgG2a isotype), and M-CSF ELISAs were obtained from R&D Systems (Minneapolis, MN). F(ab')2 of IgG (Fc domains removed by the manufacturer) and monoclonal IgG2a isotype Abs were obtained from The Jackson Laboratory (Bar Harbor, ME). Papain used to generate Fab of IgG Abs was obtained from Pierce (Rockford, IL). Wizard Miniprep DNA binding columns were obtained from Promega (Madison, WI). Charged membranes used to transfer DNA

Abbreviation used in this paper: MCP-1, monocyte chemoattractant protein-1.
and the ECL detection system were observed from Amersham (Hybond, Aylesbury, U.K.). Nick labeling of small m.w. DNA was modulated by Nick Translation Systems (Life Technologies, Gaithersburg, MD). TdT immunoassay kits were obtained from Oncogene Research Products (Cambridge, MA). Slides for immunohistochemical studies were obtained from Lab-Tek (Arlington Heights, IL).

### Cellular preparation and cell culture

Monocytes (66 ± 2.1% CD14+) were isolated from the heparinized blood of normal human volunteers as previously described (16) and resuspended at 2 × 10^6 monocytes/condition in RPMI 1640, 5% inactivated FCS, and polyoxymylin B (10 μg/ml). The presence of polyoxymylin B did not alter the ability of unstimulated cells to undergo apoptosis. Using light microscopy and Diff-Quik (Dade Diagnostics, Aguada, Puerto Rico) staining, there were an average of 65% monocytes, 34% lymphocytes, and <1% neutrophils in the preparations.

The monocytes were enriched by first diluting whole blood 1/1 with PBS and layering the blood/saline mixture onto 20 ml of Histopaque. The mixture was centrifuged at 2000 rpm at 18°C for 20 min without braking. The mononuclear layer was pooled and washed once with RPMI at 14,000 rpm for 10 min at 4°C with the brake on and an additional two times at 1,050 rpm. The cells were counted on a hemocytometer, resuspended to 5 × 10^5 cells/ml with RPMI containing 10% FBS in a polypyrrole tube, and rotated for 1 h at 4°C. The cells were layered onto cold FBS at a 1/1 ratio for 20 min to allow the monocytes to fall to the bottom of the tube. The mononuclear layer was removed, washed twice with RPMI at 4°C, and resuspended.

The monocytes were suspended in RPMI/5% FBS (2 × 10^6 cells/condition) for these studies. These monocytes were cultured with endotoxin-free pooled human IgG that had been either 1) immobilized to tissue culture plates overnight followed by washing of the plates twice with sterile PBS/polyoxymylin B the following day, or 2) added solubly to monocytes in RPMI/5% FBS at 37°C in 5% CO_2. Additionally, serial concentrations of recombinant human M-CSF (0.01–100 ng/ml) were added to monocytes (10^6/ml) and incubated for 24 h at 37°C in 5% CO_2. The IgG (25 μg/0.25-mm^2 well) was immobilized by random binding overnight at 4°C, and nonadherent IgG was washed off the following day. By PAGE and Western blotting, the maximum of approximately 0.5 μg/well of IgG bound. Apoptotic changes were quantitatively measured using densitometry of cytosolic oligonucleosomal DNA fragments.

After monocytes had been enriched from PBMC, lymphocyte subsets were further enriched by incubating them on nylon wool columns at 37°C for 45 min and eluting nonadherent cells from the columns using RPMI 1640 medium as previously described (15). The lymphocytes were 98.5 ± 1.5% CD14^−4% ± 4% CD3, 13 ± 3% CD56^+, and 0.5 ± 0.7% CD56 / CD3^+. After purification, the lymphocytes were washed, counted, and resuspended at 10^5/ml in FCS, RPMI, and polyoxymylin B. Subsequently, the lymphocytes were cultured on either immobilized IgG (0.5 μg/well) or adherent to plastic plates for an additional 24 h. The cells were then recovered and assayed for apoptosis by evaluating the cytosolic DNA fragment content as described above.

In other experiments, monocytes (2 × 10^6/ml) were incubated on equal concentrations of immobilized pooled human IgG (2), F(ab')_2, or IgG, human serum albumin (all at 0.1 ng/ml). After 24 h, cell-free supernatants were removed, and the cells were fixed. Apoptotic cells were identified using a TdT detection kit (TdT DNA fragmentation detection kit) to add biotinylated nucleotides onto 3'-hydroxy DNA fragments, as instructed by the manufacturer (Oncogene Research Products). Diaminobenzidine was used to generate an insoluble brown substrate at the site of DNA fragmentation. The sample was counterstained with methyl green to aid in the morphological evaluation and characterization of normal and apoptotic cells.

### M-CSF antigenic quantification

After 24 h, cell-free supernatants from monocytes that had been incubated on immobilized or soluble IgG were recovered, and M-CSF was assayed using a commercially available ELISA. Samples were measured in duplicate for four dilutions.

### M-CSF biologic function assay

To determine whether the antigenic M-CSF was also biologically active, a methylcellulose CFU assay was performed. This assay measured monocyte-inducing activity from blasts using freshly isolated human bone marrow (through Division of Bone Marrow Transplantation, Ohio State University). To determine CFU activity, supernatants from monocytes incubated on 1) immobilized IgG, 2) F(ab')_2, IgG, or 3) plastic alone were incubated with bone marrow. These supernatants were compared with supernatant from monocytes incubated with F(ab')_2. M-CSF activity was evaluated using an anti-M-CSF Ab (R&D Systems) or an isotype IgG2a control were used (both at 1 μg/ml) to block the effect.

### Statistical analysis

For ELISA measurements and M-CSF biological assays, ANOVA with post-hoc testing was performed using MINITAB software (State College, PA). To quantitate apoptosis, DNA fragments were assessed by densitometry, and statistical comparisons were performed between experimental conditions using ANOVA with post-hoc testing. Similarly, quantification of annexin V staining of apoptotic cells was evaluated by flow cytometry, and statistical analysis was determined by ANOVA with post-hoc testing. All results are reported as the mean ± SEM, and statistical significance was defined as p < 0.05.

### Results

**Deposited IgG induces monocyte survival through FcγR cross-linking**

To determine whether FcγR cross-linking induced by immobilized IgG prevents monocyte apoptosis, we plated equivalent concentrations of monocytes on immobilized IgG or equivalent concentrations of F(ab')_2 fragments on IgG, human serum albumin, or...
plastic. After 24-h incubations, monocytes incubated on immobilized IgG were morphologically different from those incubated on equivalent concentrations of immobilized F(ab')2 of IgG, human serum albumin, or plastic as assessed by light microscopy (Fig. 1A). Moreover, there was no difference in the total numbers of cells present in the different experimental conditions (IgG, 296 ± 613 cells/high powered field; F(ab')2 IgG, 287 ± 20 cells/high powered field; human serum albumin, 296 ± 19 cells/high powered field; plastic, 260 ± 18 cells/high powered field; p = 0.52). However, IgG stimulated monocyte adherence significantly better than the other conditions (p < 0.0001 vs all other conditions; Fig. 1B). The immobilized IgG-treated monocytes were protected from apoptosis as measured by the lack of cytosolic DNA fragmentation (p < 0.01 vs other conditions; Fig. 2) and the lack of TdT staining (Fig. 9). In contrast, cells incubated on immobilized F(ab')2 of IgG or on uncoated plastic culture plates, which do not induce FcγR cross-linking, underwent apoptosis as demonstrated by cytosolic DNA oligonucleosomal fragmentation (Fig. 2). Immobilized IgG protected monocytes from apoptosis in a dose-dependent manner (Fig. 3). By densitometry, only the 100 μg/ml plating dose of IgG suppressed oligonucleosomal DNA fragmentation statistically more than that in unstimulated cells (control; p = 0.025). To exclude the possibility that small numbers of lymphocytes contaminating enriched monocyte preparations were responsible for the DNA fragmentation, purified lymphocyte preparations demonstrated only minimal evidence of DNA fragmentation at 24 h regardless of incubation on IgG or plastic alone (p = 0.68; Fig. 4).

**IgG presentation determines monocyte protection and M-CSF production**

Immobilizing IgG to culture plates reduced IgG concentrations 50-fold (from 100 to 2 μg/ml; Fig. 5A). Despite the clear reduction in IgG concentration after immobilization, only immobilized IgG

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Immobilized IgG protects monocytes from apoptosis. A. Light microscope view of monocytes incubated for 24 h on equivalent concentrations of immobilized human IgG (IgG), immobilized F(ab')2 of IgG (Fab), immobilized human serum albumin (HSA), or adherence to plastic (0). This is representative of three separate experiments. B. Graph of total number of monocytes in each condition and the number of monocytes adherent to IgG, F(ab')2 IgG, human serum albumin, or plastic after 24-h incubation. Immobilized IgG induced the adherence of more cells vs other conditions (*, p < 0.0001). These results represent a mean of adherent vs total cells per three high-powered fields for two separate experiments ± SEM.

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Monocyte FcγR cross-linking induces monocyte survival. Shown is a representative cytosolic DNA fragmentation gel of monocytes incubated on equivalent concentrations of immobilized IgG, F(ab')2 of IgG, human serum albumin, or adherence to plastic for 24 h. Immobilized IgG protected cells from apoptotic changes, while other conditions did not (*, p < 0.01). These data represent the mean ± SEM for four experiments.
protected monocytes from apoptosis, assayed by the lack of oligonucleosomal DNA fragmentation (Fig. 5B).

**IgG stimulation induces biologically active M-CSF release from monocytes**

Immobilized IgG induced the release of 7.3 ± 1.6 ng/ml of M-CSF (Fig. 6A). In contrast, immobilized F(ab′)2 of IgG, immobilized human serum albumin, or plastic plates, which do not stimulate FcγR cross-linking, induced <0.03, 0.4 ± 0.3, or 0.2 ± 0.2 ng/ml, respectively (p < 0.0001 compared with immobilized IgG; Fig. 6A). Moreover, deposited IgG was approximately 10,000-fold more potent than soluble IgG in inducing M-CSF (Fig. 6B). FcγR-mediated M-CSF was biologically active, as evidenced by the ability to induce CFU activity in cells from human bone marrow. This effect was inhibited by a neutralizing monoclonal anti-human M-CSF Ab, but was not inhibited by an isotype control Ab (Fig. 6C). Importantly, in the absence of IgG, recombinant human M-CSF was able to protect monocytes from cytosolic DNA fragmentation in a dose-dependent manner (Fig. 7). M-CSF concentrations of 1 and 10 ng/ml induced monocyte survival more than unstimulated cells (p < 0.05; Fig. 7).

**FcγR-stimulated monocyte survival is mediated by M-CSF**

To determine whether M-CSF was the mediator of the FcγR-mediated survival, monocytes were incubated on 1) immobilized IgG alone, 2) immobilized IgG with Fab of a neutralizing anti-human M-CSF Ab, 3) immobilized IgG with Fab fragments of an isotype control Ab, or 4) plastic culture plates alone. Only the neutralizing anti-human M-CSF Ab reversed the protection against monocyte death.
monocyte survival are specifically mediated by IgG-induced FcγR-mediated activation and M-CSF release. Deposition of IgG protects monocytes by inducing M-CSF in both an autocrine and a paracrine manner, which may amplify monocyte survival at the site of immobilized IgG. M-CSF induction and monocyte survival are specifically mediated by IgG-induced FcγR cross-linking, as deposited IgG promotes monocyte survival while 50-fold higher concentrations of soluble IgG or equivalent concentrations of immobilized F(ab′)2 of IgG, which do not induce monocyte FcγR cross-linking, do not. Moreover, immobilized IgG is at least 10,000-fold more potent at inducing M-CSF release by monocytes than soluble IgG. The released M-CSF is biologically active as measured by monocyte CFU activity. As evidence of M-CSF’s role in the IgG-induced monocyte survival, neutralizing anti-M-CSF IgG, but not isotype control IgG, reverse the autocrine protection induced by deposited IgG. Furthermore, in the absence of IgG, recombinant human M-CSF prevents the monocyte apoptosis. M-CSF released by the monocytes also acts in a paracrine manner, as cell-free supernatants from monocytes incubated on immobilized IgG transfer protection to freshly isolated monocytes. Moreover, this protection can be removed with anti-M-CSF, but not isotype control, Abs.

The novel finding that deposits of IgG induce monocyte FcγR cross-linking to protect monocytes from apoptosis has relevance to human disease. This relevance is underscored by finding that tissue compartments injured in response to IgG deposition, like joints in rheumatoid arthritis or lungs in pulmonary fibrosis, have expanded numbers of macrophages (4, 7, 8, 10). We have previously demonstrated that monocyte FcγR cross-linking induces monocytes to produce MCP-1 production (15), a powerful monocyte chemottractant. In host defense, regulated monocyte recruitment and MCP-1 production are critical to clearing opsonized bacteria from target organs and promoting host survival in compartmentalized infection (18). However, MCP-1 does not protect monocytes from apoptosis; thus, newly recruited monocytes could not survive under the influence of MCP-1 alone (21). In contrast to the benefit of macrophage targeting in infectious diseases, unregulated macrophage-directed chemokine production and macrophage accumulation are hallmarks of joint injury in rheumatoid arthritis (2, 7, 13), renal injury in glomerulonephritis (1, 3, 14), and lung injury in pulmonary fibrosis (4, 10–12). Thus, the factors that affect monocyte recruitment and survival are important to host homeostasis. Since MCP-1 does not protect newly recruited monocytes from apoptosis (21), we hypothesized that a separate FcγR-mediated pathway was responsible for directing newly recruited monocytes to become macrophages.

We found that FcγR-stimulated M-CSF release controlled monocyte survival. These observations have in vivo relevance, as homozygous M-CSF-deficient osteopetrosis (op/op) mice are deficient in absolute numbers of circulating monocytes and tissue macrophages, including a deficiency in the number of osteoclasts resulting in dysfunctional bone remodeling (23, 24). M-CSF is responsible for this absolute macrophage and osteoclast deficiency, as injections of recombinant M-CSF reverse the problem (25–27). Studies involving op/op mice demonstrate that M-CSF and macrophages are important determinants of IgG-associated human diseases, including models of rheumatoid arthritis and glomerulonephritis (3, 20, 28–30). Similarly, FcγR-deficient mice are protected from targeted organ injury induced by localized deposits of IgG (29), suggesting a critical role for FcγR in immune complex-mediated inflammation. We bridge these independent observations by finding that monocyte FcγR cross-linking by deposited IgG induces biologically active M-CSF production. We speculate that once monocytes are recruited to the tissue compartment via FcγR-stimulated MCP-1 production (15), FcγR-stimulated M-CSF release induces their survival. This pathway focuses on a new target to reduce targeted tissue inflammation in human immune complex-mediated diseases and supports the concept that M-CSF is a critical monocyte survival factor that allows macrophage accumulation (31). This concept is supported by the observation that enforced expression of the antiapoptotic protein Bcl-2 in monocytes of op/op animals corrects much of the underlying bone
FIGURE 6. Immobilized IgG induces biologically active M-CSF release, which is capable of protecting monocytes from apoptosis. A, Antigenic M-CSF detection. Immobilized human IgG (IgG) induced more M-CSF release from monocytes than did equivalent concentrations of immobilized F(ab')2 of IgG (F(ab')2), immobilized human serum albumin (HSA), or plastic (p < 0.0001). Results are the mean ± SEM for six experiments. B, Effect of IgG presentation on M-CSF release. M-CSF release by monocytes incubated on either immobilized or soluble IgG was measured by ELISA and expressed as a percentage of maximal M-CSF production for two separate experiments. C, Functional M-CSF detection. Monocyte CFU activity from cell-free supernatants from monocytes incubated on immobilized IgG (0.5 μg/well) or with recombinant human M-CSF (10 ng/ml) for 24 h is shown. As a control, supernatants from monocytes stimulated with F(ab')2 of IgG were also assessed for monocyte CFU activity. A neutralizing anti-M-CSF (hatched bar) or an isotype control Ab (open bar) was used to determine the specificity of the M-CFU as M-CSF. Results are the mean ± SEM for three experiments.

FIGURE 7. Recombinant M-CSF induces monocyte survival. Recombinant human M-CSF (10, 0.01, and 0.0001 ng/ml) induces monocyte survival in the absence of IgG in a dose-dependent manner. The 10 and 1 ng/ml doses of M-CSF protected monocyte from apoptosis (p < 0.05 vs unstimulated control). These data represent the mean ± SEM of five experiments.
problems and macrophage deficiency, suggesting that accelerated apoptosis of monocyte precursors is a fundamental problem in this M-CSF-deficient animal (31).

Of interest, other CSFs, including GM-CSF and G-CSF, are powerful survival factors for neutrophils (32, 33). However, GM-CSF does not augment monocyte survival in the $op^+/op^-$ mouse (34), suggesting that the specificity of these growth factors does not include monocyte survival. While other authors have shown that M-CSF augments monocyte survival (35), the seminal observations in this paper are that monocyte FcR cross-linking protects monocytes from apoptosis and that this protection is mediated through the production and release of M-CSF.

FIGURE 8. Immobilized IgG protects monocytes from apoptosis through M-CSF release. Monocytes ($3 \times 10^6$/ml) were incubated on immobilized human IgG alone (100 $\mu$g/ml plating dose; IgG), on equivalent concentrations of IgG in the presence of Fab of neutralizing anti-human M-CSF IgG (1 $\mu$g/ml; M-CSF), on IgG in the presence of a neutralizing isotype control Ab (1 $\mu$g/ml; isotype), or on tissue culture plates in the absence of IgG (control) for 24 h. Shown is a representative DNA fragmentation gel and cumulative densitometry measurements demonstrating that αM-CSF, but not isotype IgG, removes IgG-stimulated cell survival (*, $p < 0.025$ for IgG or IgG plus isotype vs IgG plus αM-CSF). The results represent the mean ± SEM for three experiments.

FIGURE 9. Immunohistochemical detection of IgG-induced monocyte protection and blockade with anti-M-CSF Abs. TdT immunohistochemical localization of DNA fragments (brown color) in monocytes incubated on equivalent concentrations of immobilized human IgG, immobilized Fab', of IgG (Fab), immobilized human serum albumin (HSA), or plastic (0) for 24 h. Of note, anti-M-CSF Abs, but not isotype control Abs, removed IgG’s protective effects. These data are representative of two separate experiments.
Previous investigators determined that inflammatory cytokines, including IL-1β, TNF-α, IFN-γ, and GM-CSF, can promote monocyte survival (21). In addition, LPS can induce monocyte survival (36). Although these studies added important insights into the regulation of monocyte survival, they did not address the role of deposited IgG as a potential mediator of monocyte survival. This paper extends their findings to make the observation that deposits of IgG can also promote monocyte survival through the production of M-CSF.

In summary, we show that immobilized IgG protects monocytes from apoptosis through FcγR cross-linking, as assayed by cell morphology, cytosolic DNA fragmentation, annexin V staining, and TdT immunohistochemical analysis. The ability of IgG to mediate monocyte protection appears dependent on cross-linking monocyte FcγR. This property is illustrated by the finding that immobilized IgG prevents monocyte apoptosis, while soluble IgG or F(ab′)2 IgG, do not. Moreover, immobilized IgG is 10,000-fold more potent in stimulating monocyte M-CSF release than is soluble IgG. Interestingly, apoptosis prevention conferred by monocyte FcγR cross-linking is mediated through the production and secretion of FcγR-mediated M-CSF, suggesting that immobilized IgG may regulate monocyte survival through both autocrine and paracrine effects. These novel observations provide a potential mechanism to explain targeted organ injury caused by macrophages in immune complex diseases.

Acknowledgments

We thank Drs. Pascal J. Goldschmidt and Michael Caligiuri for helpful suggestions and review of the manuscript and Dr. Clark L. Anderson for helpful suggestions for the Fcγ receptor studies.

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