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Immunology

Monomeric IgG2a promotes maturation of bone-marrow macrophages and expression of the mannose receptor

(bone-marrow macrophage differentiation/macrophage mannose receptor/immunoglobulins)

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ABSTRACT **The macrophage mannose receptor, a 172-kDa lineage-specific glycoprotein, partakes in nonopsonin-mediated phagocytosis by recognition of terminal mannose residues on targeted particles. Because appearance of the receptor progresses with monocyte/macrophage differentiation, its expression is indicative of the maturational state of the cell. Monomeric IgG2a and IgG2b up-regulate mannose-receptor surface expression and biosynthesis by murine bone-marrow macrophage precursors as much as 7- to 12-fold in a dose-dependent manner. IgG2a accelerates macrophage mannose-receptor expression by several days during *in vitro* bone-marrow differentiation; however, treated and control cells ultimately express equivalent levels of receptor. Moreover, the effect is independent of cell cycle or ambient levels of colony-stimulating factor 1. The coinduction of another maturation-dependent lineage-specific antigen, F4/80, and the fact that macrophage precursors respond to IgG2a only within the first day of culture, indicate that the targeted cell is an early myelomonocytic precursor, responsive only during a short, early developmental window. The effect is specific for immunoglobulin molecules of the IgG2a and IgG2b subclasses and probably involves an Fc_γ-receptor signal-transduction pathway but not macrophage priming or activation. Most importantly, a paracrine mechanism of immunoglobulin-mediated bone-marrow macrophage differentiation is suggested by experiments in which basal levels of mannose-receptor expression are reduced by continual removal of B-cell-generated IgG from marrow cultures. Thus, IgG2a and IgG2b prompt mannose-receptor synthesis and bone-marrow macrophage differentiation and may, therefore, play a role in the regulation of macrophage differentiation in host defense.**

Bone-marrow macrophage precursors (BMMPs), as they differentiate, progressively develop phenotypic and functional characteristics pivotal to host defense, such as the expression of the macrophage mannose receptor (MMR), a lineage-specific 172-kDa cell-surface glycoprotein (1–3) thought involved in nonopsonin-mediated phagocytosis of targeted particles, such as bacterial glycoproteins (2, 3). As BMMPs mature *in vitro*, either through progressive culture under control conditions (4–8) or with agents such as 1,25-dihydroxy vitamin D_3 (4) or prostaglandin E (6), MMR expression increases. Thus, similar to another lineage-specific marker, the F4/80 antigen and the development of plastic adherence, MMR capacity serves as a hallmark of murine monocytic differentiation (4, 6, 8–10).

IgG is a glycoprotein capable of regulating various cellular events, a number involving B-cell functions. Different IgG subclasses are, in the context of host-defense reactions, selectively expressed (11). For example, IgG2a and IgG2b are elicited in reactions against pathogens, such as *Listeria monocytogenes*, and host defense against these pathogens also involves macrophages (12–14).

This communication focuses on the regulatory impact of immunoglobulins on macrophage maturation. By investigating two parameters of monocytic differentiation, namely MMR and F4/80 expression, we show that monomeric IgG2a and IgG2b specifically accelerate the rate at which BMMPs mature. The targeted cell appears to be an early progenitor, and this Fc-receptor-mediated event is independent of colony stimulating factor 1 (CSF-1) levels and cell cycle. Most importantly, our data suggest that IgG2a and IgG2b, secreted by resident bone-marrow B cells, provide a paracrine regulatory mechanism linking B lymphocytes to macrophage differentiation in host defense.

METHODS

Unless otherwise specified, all chemicals and media were obtained from Sigma. Horse and fetal calf sera were purchased from Hazelton Dutchland Research Products (Denver, PA) or GIBCO, heat inactivated, and depleted of IgG by protein G affinity chromatography. Mannosylated BSA (42 mol of sugar per mol of protein) was purchased from E-Y Laboratories. α-thrombin was from J. Fenton (New York State Department of Health, Albany), bovine IgG preparations were from A. Kulczycki (Washington University). Acrylamide beads coated with polyclonal rabbit anti-mouse IgG antibodies were purchased from Bio-Rad. Transwell dishes (diffusion chambers) were purchased from Costar.

Preparation of Stage I CSF-1. CSF-1 was prepared by modification of the method described by Stanley (15). Serum-free conditioned medium from L929 cells was partially purified (stage I) by the batch calcium phosphate method. CSF-1 activity was determined with use of a bioassay of cellular proliferation against known standards as described (4). Total protein concentration was assessed with the method of Lowry *et al.* (16). When necessary, CSF-1 was purified by the method of Stanley to stage V (17). All experiments were duplicated with highly purified stage V CSF-1, without any observable difference in the results obtained.

Marrow Cells. Nonadherent cells were obtained from bone-marrow cultures of 9- to 12-week-old male A/J mice (The Jackson Laboratory) or C3H mice (Jewish Hospital of

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Abbreviations: BMMP, bone-marrow macrophage precursor; BSA, bovine serum albumin; CSF-1, colony-stimulating factor 1; ManBSA, mannosylated BSA; ^{125}I-ManBSA, ^{125}I-iodinated mannosylated BSA; MMR, macrophage mannose receptor; PMA, phorbol 12-myristate 13-acetate.

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St. Louis) and prepared as described (6, 18). The initially adherent cells were discarded after overnight culture, and the nonadherent cells were designated marrow cells (6). These cells include both precursor cells as well as bone-marrow lymphocytes. When these marrow cells were cultured in the presence of CSF-1 at 500 units per ml for 7 days, >99% of colony-forming units [CFU-(c)s] exhibited macrophage morphology, plastic adherence, and expressed the monocyte-specific enzyme, α-naphthyl acetate esterase, as well as F4/80 (10).

Adherent Bone-Marrow-Derived Mononuclear Phagocytes. Marrow cells (150,000/ml) were cultured in 24-well plates (Nunc) in 2 ml of complete medium per well or in 50 ml of medium in tissue culture dishes (Falcon) (6). At designated intervals, adherent cells were used for binding studies or immunoprecipitation.

Monomeric IgG and MMR Expression. At various times, purified IgG subclass proteins from various myeloma cell lines (Sigma or The Binding Site, San Diego) were added to the marrow-cell cultures at a final concentration of 15 μg/ml (unless otherwise indicated). Appropriate controls included nanomolar amounts of Tris, which was contained in the IgG myeloma protein preparations. For cleavage into Fc and F(ab')2 fragments, papain or pepsin digestion (Pierce) was used, respectively. Cleavage products were separated via protein A affinity chromatography or by size (Sephadex G50-150, Pharmacia).

Aggregated IgG and MMR Expression. Bovine IgG (10 mg/ml) was incubated at 63°C until slight opalescence appeared and then was centrifuged at 1,600 × *g* for 10 min to remove larger aggregates (19). The supernatant was refiltered (0.2 μm) and dialyzed against α modification of minimal Eagle's medium (α-MEM). The amount of heat-aggregated IgG was assessed using the method of Lowry *et al.* (16). Various concentrations of aggregated bovine IgG were added to marrow-cell cultures 24 hr after plating. Forty-eight hours later MMR surface expression was determined by radioligand binding.

Superoxide Release. Superoxide (O_2^-) generation was assessed as described (20). In brief, marrow-cell cultures were exposed to aggregated IgG 36 hr after plating. Twelve hours later O_2^- release was stimulated by phorbol 12-myristate 13-acetate (PMA) (1 hr, 20 nmol in α-MEM). Superoxide-specific ferritocytochrome C oxidation was determined by comparison with controls in the presence of superoxide dismutase (540 nm).

^{125}I-Labeled Mannosylated BSA-Binding Assay. Assessment of surface-binding sites on adherent bone-marrow mononuclear phagocytes with ^{125}I-labeled mannosylated BSA (^{125}I-ManBSA) was modified from described techniques (21). Binding assays were done in duplicate at 4°C with a nonspecific binding representing <10% of total cell-associated counts.

Biosynthetic Radiolabeling and Immunoprecipitation of MMR. Marrow cells (5×10^7) were incubated for 3 days with or without IgG2a at 15 μg/ml. Immunoprecipitation was done as described (6, 22).

α-Thrombin-Binding Assay. ^{125}Iodination of α-thrombin and an α-thrombin-binding assay were done as described (6). Binding kinetics were analyzed by methods of Scatchard (23).

F4/80 Binding Assay. After incubation in mouse IgG (to block Fc_γ receptors) F4/80 surface-antigen expression was assessed by using a rat monoclonal antibody (Serotec) at a dilution of 1:10. ^{125}I-labeled sheep anti-rat IgG (Amersham) was used as a second-step reagent at a dilution of 1:10. Nonspecific binding was assessed by using rat IgG instead of anti-F4/80.

RESULTS

Effects of Monomeric IgG2a and IgG2b on ^{125}I-ManBSA Binding. Monomeric IgG2a or IgG2b from different murine myeloma protein sources added to marrow-cell cultures at plating, enhance binding of ^{125}I-ManBSA in a dose-dependent manner (Fig. 1*A*). IgG2a, which promotes as much as a 12-fold increase in ligand binding is more potent than IgG2b. Moreover, the effect is similar whether ^{125}I-ManBSA binding is calculated on a per cell or protein-associated basis (Fig. 1*B*), indicating that changes in volume and surface area are not responsible. The event is independent of substrate attachment as shown by culture in Teflon beakers (data not shown). IgG1, IgG3, IgM, and IgA myeloma immunoglobins, on the other hand, do not alter MMR expression, indicating that the phenomenon is idiotype- and subclass-specific. Scatchard analysis of ^{125}I-ManBSA binding of IgG2a (16 μg/ml)-treated and control cells reveals no differences in the dissociation constants (K_d = 3–5 × 10^{-9} M^{-1}) (Fig. 2), documenting that the

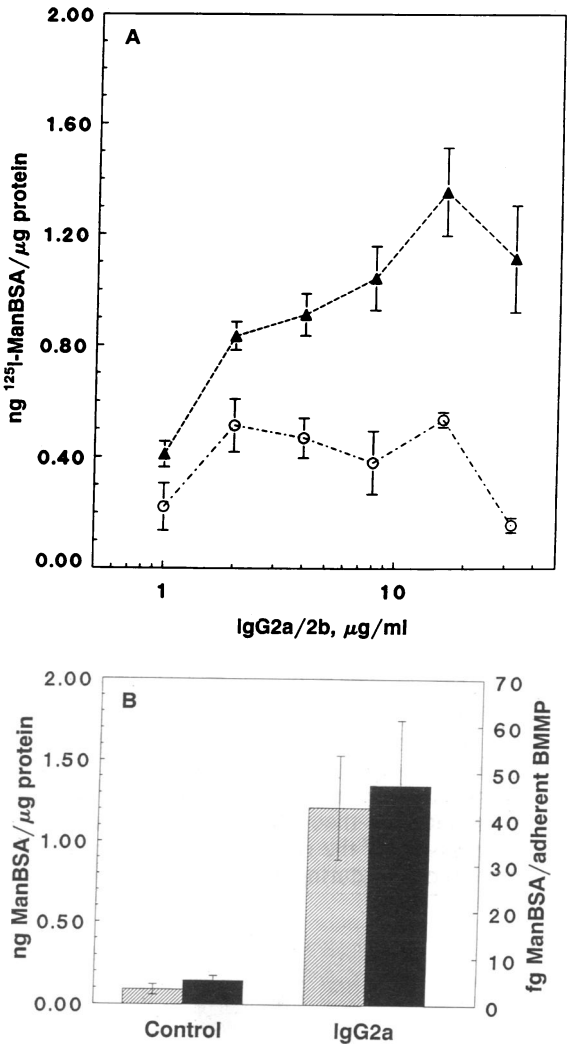

FIG. 1. Monomeric IgG2a and IgG2b up-regulate MMR expression in a dose-dependent manner. BMMPs were cultured in CSF-1 at 500 units/ml. (*A*) IgG2a and IgG2b were added to marrow-cell cultures at plating. MMR plasma membrane expression by adherent macrophages was assayed by ^{125}I-ManBSA binding 72 hr later. Both IgG2a (▲) and IgG2b (○) up-regulate the MMR in a dose-dependent manner (n = 3) as compared with control cells (0.09 ± 0.072 ng of ^{125}I-ManBSA per μg of protein). (*B*) Receptor–ligand binding is enhanced by IgG2a (15 μg/ml, 72 hr) whether normalized to cell protein (hatched bars, left ordinate) or cell number (black bars, right ordinate) (n = 3).

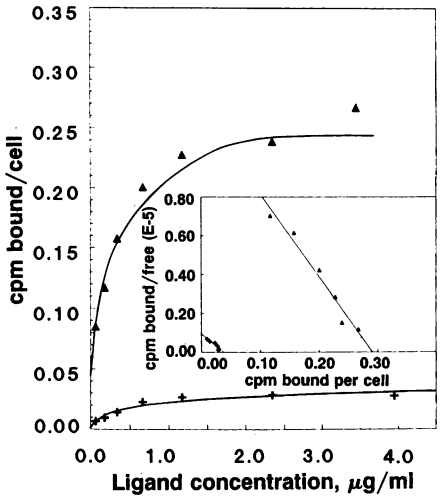

FIG. 2. Binding isotherm and Scatchard analysis of BMMPs exposed to ^{125}I-ManBSA. Marrow cells were plated with or without IgG2a at 15 μg/ml, and 72 hr later specific binding of ^{125}I-ManBSA was assessed. K_d for both control (+) and IgG2a-treated (▲) cells are similar, indicating that differences in ligand binding reflect receptor capacity rather than affinity.

effect of the immunoglobulin on ^{125}I-ManBSA binding reflects differences in MMR density and not ligand affinity.

The specificity of the IgG2a-induced event was investigated by comparison with ^{125}I-labeled α-thrombin binding, another cell-surface glycoprotein-binding site. There were no differences in the number of α-thrombin binding sites expressed by control (353,000 per cell) and IgG2a-treated BMMPs (384,000 per cell) (data not shown).

Effect of IgG2a on MMR Synthesis. BMMPs were biosynthetically labeled with ^{35}S-labeled methionine/cysteine, and the membrane-associated MMR was immunoprecipitated. Fig. 3 demonstrates both enhanced synthesis of the 154-kDa receptor precursor and its rapid conversion to the mature 172-kDa form of the receptor after treatment of cells with IgG2a.

Effects of IgG2a on the Time Course of MMR Expression. In these experiments, IgG2a (15 μg/ml) was added at plating, and MMR expression was followed with time by ^{125}I-ManBSA binding or immunoprecipitation. As demonstrated in Fig. 4, radioligand binding increases with progressive culture under control conditions, but IgG2a accelerates expression of the receptor by several days. Unexposed cells ultimately (after 7–10 days) reach levels of MMR capacity similar to IgG2a-treated macrophages, an effect that indicates IgG2a does not alter their ultimate phenotype. Moreover, surface expression of the receptor closely parallels biosynthesis of the receptor during the first days of induction (data not shown).

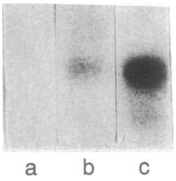

FIG. 3. MMR biosynthesis is stimulated by IgG2a. Immunoprecipitation of biolabeled MMR (3 hr, 37°C, Tran^{35}S-labeled-methionine/cysteine) from cultures incubated 72 hr with IgG2a (lane c) shows a reproducible 5-fold increase, by densitometry, in receptor synthesis compared with control cells (lane b). Lane a represents immunoprecipitation with preimmune rabbit serum. Surface binding from the same set of cells was increased 9.2-fold (one of two identical experiments).

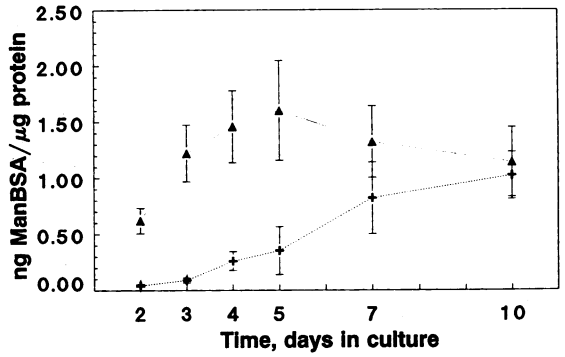

FIG. 4. Time course of MMR expression by IgG2a-treated and control bone-marrow macrophages. Marrow cells were cultured with IgG2a (15 μg/ml at the beginning of culture, ▲) or under control conditions (+), and ^{125}I-ManBSA binding was followed with time. Time course of MMR expression is accelerated, but its maximum level is not enhanced by IgG2a.

Our interest, therefore, focused on regulation of MMR expression in marrow-cell cultures under control conditions, which may involve endogenous monomeric IgG2a and IgG2b. We and others have shown (24, 25) that immunoglobulins of all isotypes are spontaneously secreted by bone-marrow B cells *in vitro* without antigenic or mitogenic stimulation. We therefore placed diffusion chambers containing acrylamide bead-bound rabbit anti-mouse IgG into our marrow cultures at plating, reasoning that antibody would continuously remove IgG secreted by B lymphocytes. Fig. 5 shows that such treatment markedly decreases MMR expression, indicating that endogenous immunoglobulin production may help regulate appearance of the receptor and, perhaps, bone-marrow macrophage differentiation. Rabbit anti-mouse IgG-coated beads saturated with normal mouse serum before the experiment has no effect on MMR expression.

Dependence of IgG2a-Mediated MMR Up-Regulation on Macrophage Differentiation. To explore the impact of *in vitro* BMMP maturation on IgG2a-induced MMR up-regulation, IgG2a (15 μg/ml) was added to marrow-cell cultures during plating and 1, 2, 4, 8, and 24 hr thereafter. MMR surface expression was assessed by ^{125}I-ManBSA binding at 72 (Fig. 6) and 96 hr (data are similar to 72 hr and therefore not shown). BMMPs lost >90% of their responsivity to IgG2a

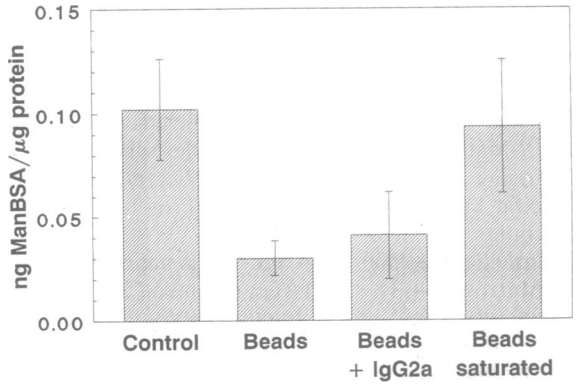

FIG. 5. Endogenous IgG2a regulates "spontaneous" bone-marrow macrophage MMR expression. Marrow cells were cultured with rabbit anti-mouse IgG-coated acrylamide beads, separated from the culture by a permeable membrane. MMR expression in this circumstance is markedly decreased. Preincubation of beads in normal mouse serum, however, obviated their effect on MMR. Exogenous addition of IgG2a (15 μg/ml) in the presence of mouse IgG-binding beads (Beads) did not increase MMR expression, showing the capacity of the system to absorb IgG (n = 4).

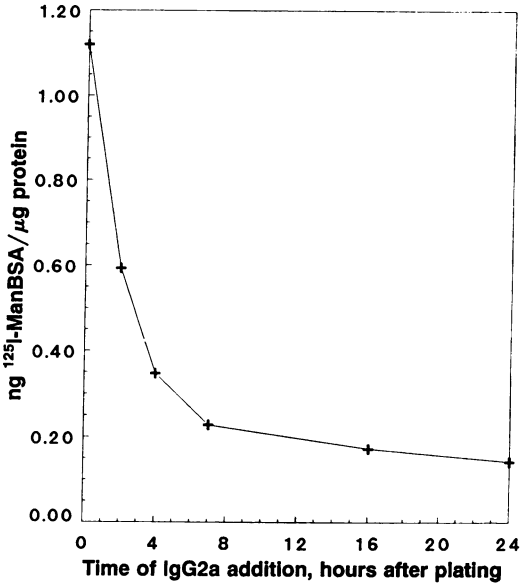

FIG. 6. IgG2a-induced MMR expression is maturation dependent. IgG2a was added to marrow-cell cultures at various time points, and MMR surface expression was assessed at 72 hr by ^{125}I-ManBSA binding. Responsivity to IgG2a with regard to up-regulation of MMR expression is lost with time in culture. One of two identical experiments is shown.

within the first 24 hr of culture, and subsequent addition of immunoglobulin did not affect MMR expression.

In other experiments, IgG2a (15 μg/ml) was added at initiation of marrow-cell culture, and after 24 hr the medium was replaced. Despite the early (24 hr) withdrawal of IgG2a, MMR expression at 72 hr was 83 ± 14% (n = 3) of that of cells continuously exposed to IgG2a. The fact that the response to IgG2a is maximal at initiation of cultures but markedly diminishes with time suggests that a population of early macrophage precursors, which does not self-replenish *in vitro*, may be the targeted cells.

Our attention, therefore, turned to immature macrophage progenitors that have not yet developed the capacity to adhere to plastic. To this end we exploited the F4/80 antigen, a maturation-dependent surface protein specific to the macrophage lineage that is expressed early during differentiation along with the development of plastic adherence (6, 10). IgG2a (15 μg/ml) was added at initiation of marrow-cell culture, and surface F4/80 antigen was measured by a two-step binding assay using rat anti-F4/80 IgG and ^{125}I-labeled sheep anti-rat IgG. Sixteen hours later, IgG2a-treated cells expressed 1.62 ± 0.22 times (n = 4, mean ± SD, P < 0.02) more F4/80 antigen than control. No F4/80 could be detected on marrow cells at initiation of culture. Thus, IgG2a appears to enhance MMR and F4/80 antigen expression by targeting nonplastic adherent, relatively immature bone-marrow macrophage precursors.

Effect of Cell Cycle on IgG2a-Induced MMR Expression. Because MMR expression might be cell cycle-dependent, we exploited our previous observation that BMMP replication can be manipulated by ambient CSF-1 levels. Specifically, 4 hr after exposure to 1000 units of CSF-1 per ml, 40–50% of BMMPs are in the S phase of the cell cycle in comparison with <5% of cells treated with 50 units/ml, a concentration that sustains survival (7). Marrow cells were plated in medium containing CSF-1 at 500, 50, or 1000 units per ml and IgG2a (15 μg/ml) was added at initiation of culture. ^{125}I-ManBSA binding by adherent cells was assessed at 72 hr. No differences in MMR expression occurred regardless of growth factor concentration, indicating that IgG2a-induced MMR expression is independent of proliferation, and, moreover, CSF-1 is not a necessary cofactor for the regulatory effect of IgG2a (Fig. 7).

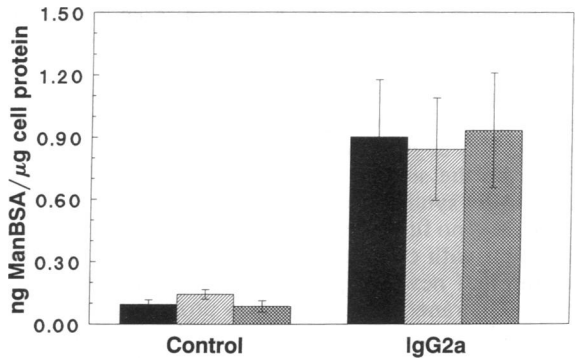

FIG. 7. IgG2a-induced MMR expression is independent of cell cycle and CSF-1 concentration. The replication rate of bone-marrow macrophage precursors was manipulated by ambient CSF-1 levels. Marrow cells were plated in medium containing CSF-1 at 50 units/ml (inducing a resting state) (solid bars), at 500 units/ml (hatched bars), or at 1000 units/ml (inducing a hyperproliferative state) (cross-hatched bars). There are no differences in IgG2a (15 μg/ml, at time of plating)-induced MMR expression [^{125}I-ManBSA binding, 72 hr (n = 3)].

IgG2a-Induced MMR Expression and FC$_\gamma$ Receptors. Monomeric IgG2a-mediated up-regulation of MMR expression is likely to involve signal transduction via FC$_\gamma$ receptors. Fc fragments/F(ab')$_2$ fragments from a variety of IgG2a myeloma proteins were added in an amount equivalent to IgG2a at 15 μg/ml (whole molecule). IgG2a Fc fragments induced MMR expression equivalent to that seen with the whole IgG2a molecule, whereas F(ab')$_2$ fragments have no such effect. These findings are consistent with a Fc$_\gamma$ receptor-mediated mechanism. These results suggest that IgG2a-induced up-regulation of MMR might result from monovalent bindings to Fc receptors rather than via the formation of multivalent "large" ligands that crosslink Fc$_\gamma$ receptors—i.e., by binding of IgG2a to the plastic surface or by spontaneous aggregation of IgG2a. This issue was addressed by two sets of experiments. (*i*) Plastic dishes were precoated with fetal calf serum or IgG2a. We found that fetal calf serum used in this manner does not influence the subsequent response of BMMPs to IgG2a, and precoating with IgG2a only minimally enhances MMR expression (112% ± 23 of non-IgG2a-coated control, n = 3).

(*ii*) IgG was heat aggregated, which, in contrast to IgG2a, when added to marrow cultures down-regulates MMR expression in a dose-dependent manner, and, moreover, primes macrophages for a 6-fold increase of PMA-induced respiratory burst activity (O_2^-) over controls. In contrast, IgG2a-treated cells do not release increased amounts of O_2^-. These findings bolster the hypothesis that IgG2a-induced MMR expression does not involve crosslinking of Fc receptors. Moreover, crosslinking by aggregated IgG-primed macrophages and decreases MMR expression.

DISCUSSION

The process of monocyte maturation is associated with sequential expression of plasma membrane markers, such as the F4/80 antigen (6, 10). Similarly, appearance of the MMR, a protein important to host defense, quantitatively mirrors differentiation of BMMPs into the macrophages (4–9).

This study demonstrates that IgG2a myeloma immunoglobulin promotes bone-marrow macrophage expression of MMR and F4/80. The fact that IgG2a fails to impact on expression of α-thrombin-binding sites indicates that its effect on these differentiation-associated proteins is specific.

Most importantly, as MMR expression is enhanced 7- to 12-fold by IgG2a, this immunoglobulin is the most effective known inducer of the receptor.

MMR expression on the plasma membrane may be influenced by a host of events, such as receptor biosynthesis, recycling, and degradation. We found here that MMR appearance on the surface mirrors its synthesis. Freshly isolated BMMPs synthesize very low or undetectable levels of receptor, and no functional receptor is expressed on the cell surface, but with culture, both processes occur in tandem, indicating that new receptor is rapidly translocated to the plasma membrane. Although IgG2a profoundly impacts on both kinetic events, we found that the immunoglobulin accelerates MMR expression by several days; yet control cells ultimately synthesize and express similar quantities of the protein. Thus, IgG2a enhances BMMP differentiation but does not alter the ultimate cell phenotype.

Our data also indicate that an F4/80-negative, nonplastic adherent BMMP is targeted *in vitro* by IgG2a during a short maturational window at initiation of culture. The induced up-regulation of the MMR is independent of cell cycle and CSF-1 as a cofactor.

The fact that immunoglobulin-induced MMR up-regulation is recapitulated by IgG2a Fc, but not by F(ab')2, fragments, suggests that the event is mediated through an Fc_γ receptor. The high-affinity Fc_γ receptor I, which binds monomeric IgG2a at concentrations used in this study, should not, however, recognize IgG2b (26). A mechanism involving the low-affinity Fc_γ receptor II, which recognizes IgG1, IgG3 (27), aggregated IgG (28), and probably IgG2a (29) also fails to encompass all our findings. Alternatively, responsivity to IgG2a and IgG2b is present only during a short maturational window and, thus, the possibility exists that early BMMPs transiently display a unique Fc_γ receptor with an affinity for these two classes of immunoglobulins (30, 31).

We found that although monomeric IgG2a treatment induces MMR expression, it has no effect on PMA-induced superoxide anion generation. Together with the result that aggregated IgG, in contrast, primes BMMPs to respond to PMA, while at the same time down-regulates the MMR, this finding indicates priming to be unrelated to the IgG2a effect.

These studies document the fact that immunoglobulins can enhance the production of a macrophage glycoprotein receptor. Stimulation of MMR synthesis and subsequent surface expression by cells treated with IgG2a and its Fc fragment points to accelerated monocytic differentiation via a Fc_γ receptor-mediated mechanism. The physiological source of immunoglobulins would, in this case, presumably be marrow-residing B cells, which spontaneously secrete IgA and IgG *in vitro* (32). Evidence that such a system may be physiologically relevant also comes from experiments in which continuous removal of marrow cell-secreted IgG prevents constitutive expression of the MMR. The marrow compartment may also provide a special environment for macrophage differentiation, as the composition of immunoglobulins secreted therein differs considerably from that of plasma as to isotype and subclass distribution (24, 32).

These data suggest that specific immunoglobulins provide a regulatory circuit for monocytic differentiation within the bone marrow. The fact that IgG2a and IgG2b specifically induce BMMP maturation is particularly germane, because both isotypes are primarily expressed during immune responses to a group of bacterial pathogens in which macrophage defense is important (12–14, 25). B cells primed at sites such as intestinal or peripheral lymphoid follicles appear to migrate to the marrow where they may differentiate into immunoglobulin-secreting plasma cells (ref. 33; J. G. Tew, personal communication). These immunoglobulins may not only be important in antigen-specific host defense but also in recruitment of macrophages from marrow progenitors, thereby providing an interesting link between B cells and macrophage maturation.

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