

Brief Definitive Report**Interleukin 4 Potently Enhances Murine Macrophage Mannose Receptor Activity: A Marker of Alternative Immunologic Macrophage Activation**

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*From the Sir William Dunn School of Pathology, Oxford, OX1 3RE, United Kingdom; and the *Department of Pediatrics, Childrens Hospital, Boston, Massachusetts 02115***Summary**

Expression of the macrophage mannose receptor is inhibited by interferon γ (IFN- γ), a T helper type 1 (Th-1)-derived lymphokine. Interleukin 4 (IL-4), a Th-2 lymphocyte product, upregulates major histocompatibility class II antigen expression but inhibits inflammatory cytokine production by macrophages. We have studied the effect of IL-4 on expression of the macrophage mannose receptor (MMR) by elicited peritoneal macrophages. We found that recombinant murine IL-4 enhances MMR surface expression (10-fold) and activity (15-fold), as measured by the respective binding and degradation of ^{125}I -mannose-bovine serum albumin. Polymerase chain reaction analysis of cDNAs from purified primary macrophage populations revealed that MMR, but not lysozyme or tumor necrosis factor α , mRNA levels were markedly increased by IL-4. The above effects were associated with morphologic changes. These data establish IL-4 as a potent and selective enhancer of murine MMR activity in vitro. IL-4 induces inflammatory macrophages to adopt an alternative activation phenotype, distinct from that induced by IFN- γ , characterized by a high capacity for endocytic clearance of mannosylated ligands, enhanced (albeit restricted) MHC class II antigen expression, and reduced proinflammatory cytokine secretion.

The macrophage mannose receptor (MMR) (previously called the mannosyl fucosyl receptor [MFR]) is an important phagocytic receptor mediating the binding and ingestion of micro-organisms with surface mannose residues and soluble mannose-containing glycoproteins. It is expressed on resident and elicited peritoneal macrophages and alveolar macrophages, not expressed on monocytes, and at low levels on Bacillé-Calmette-Guerin (BCG)- or IFN- γ -activated macrophages (1). Therefore, the MMR is a marker of the resident and elicited, but not IFN- γ -activated, macrophage phenotype. MMR activity is increased by steroids and IFN- α and - β , and is inversely correlated with MHC class II antigen expression, which is induced on immunologically stimulated macrophages and used as a marker of activation.

IL-4, predominantly produced by activated Th cells of the type 2 phenotype and non-B, non-T, FcER⁺ cells, has pleiotropic effects on a variety of immune and nonimmune cells. As it induces the expression of MHC class II antigen on B cells and monocytes, and enhances macrophage tumoricidal activity, it has been described as a macrophage-activating factor (2). However, the tumoricidal activity is confined to selected target cell lines, and only HLA-DR and HLA-DP but not HLA-DQ MHC class II molecules are induced by IL-4. In addition, IL-4 induces MHC class II antigen expression on restricted macrophage populations. In contrast, IFN- γ

induces all three class II molecules on most if not all macrophages (3, 4). Furthermore, IL-4 inhibits the expression of pro-inflammatory cytokine genes such as IL-1, TNF, and IL-8, and synergizes with steroids to inhibit macrophage proinflammatory activity (5-9). In addition, IL-4 inhibits superoxide anion release from PMA or zymosan-treated monocytes (10), although this effect depends critically on the particular macrophage source used and on the presence of other cytokines (11, 12). With the exception of MHC class II expression, the influence of IL-4 on macrophage immune function appears to contrast the effect of IFN- γ , the typical product of Th-1 cells and NK cells. Here, the effect of IL-4 on MMR expression and activity is compared with the effect of IFN- γ , a known inhibitor of MMR activity. We provide in vitro evidence that IL-4 greatly enhances MMR activity of murine peritoneal exudate macrophages. The potency and efficacy of IL-4 is unmatched by any other known MMR inducer, such as dexamethasone. The data, taken together with previous studies, indicate that IL-4 induces elicited macrophages to adopt an alternative macrophage phenotype, with very high MMR activity, restricted MHC class II antigen expression, and reduced proinflammatory cytokine secretion. The potential pathophysiologic function of such a macrophage population is discussed.

Materials and Methods

Animals

Adult male BALB/c mice were bred at the Sir William Dunn School of Pathology, University of Oxford.

Media and Reagents

RPMI was obtained from Gibco-Biocult Ltd. (Paisley, Scotland) FCS was obtained from Seralab UK Ltd. (Crawley Down, UK), and routinely heat inactivated for 30 min at 56°C. Media were supplemented with 10% FCS, glutamine (2 mM), penicillin (50 U/ml), and streptomycin (50 µg/ml). Bio-Gel P100 (fine) was obtained from Bio-Rad Laboratories (Richmond, CA). Mannan (from *Saccharomyces cerevisiae*) was obtained from Sigma Chemical Co. (St. Louis, MO).

Antibodies and Cytokines

1B11, an IL-4-blocking rat mAb, was purified by Dr. A. McWilliam in this laboratory from a hybridoma cell line (American Type Culture Collection, Rockville, MD) obtained through Dr. W. E. Paul (National Institutes of Health, Bethesda, MD). 5C6, a mouse complement receptor (CR3)-blocking rat mAb was isolated and purified by Dr. H. Rosen in our laboratory (13). Murine rIFN-γ was a gift from Dr. F. Balkwill (Imperial Cancer Research Fund, London, UK), and murine rIL-4 was a gift from Dr. S. Gillis (Immunex, Seattle, WA).

Cells

Thioglycollate-elicited and biogel bead-elicited peritoneal macrophages were isolated 4–5 d after intraperitoneal injection as previously described (14). Cells were plated at 3×10^5 macrophages/well in 24-well tissue culture plates. The cells were incubated for 1 h at 37°C in a 5% CO₂ incubator and then washed four times with PBS at 4°C to remove nonadherent cells. Thereafter, cells were treated as described in the figure legends. For RNA isolation, Biogel bead-elicited peritoneal macrophages (BgPM) were incubated in 10-cm polystyrene petri dishes as before but left in RPMI with 10% FCS overnight. The cells became nonadherent and were easily washed off the dishes. These cells were centrifuged and spun into a Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) differential density gradient. The macrophage fraction (>99.5% pure by immunocytochemistry) was collected and replated before cytokine treatment.

Macrophage Mannosyl Receptor (MMR) Assays

These assays were performed as described previously (15) with modifications as indicated. Briefly, mannosylated BSA (E-Y Laboratories, San Mateo, CA) was trace labeled with ¹²⁵I-Na by a modified chloramine-T method. Ligand was tested for TCA precipitability before use.

Binding and Internalization of Mannose-specific Ligands. Binding and internalization were assayed at saturating concentrations of ligand using trace-labeled mannose-BSA (saturation, 250 ng/ml ligand/ 5×10^5 MØ) in the presence or absence of mannan (5 mg/ml) or 100-fold excess unlabeled mannose-BSA. Binding was assayed after 1 h at 4°C and internalization was assayed after 20 min at 37°C. Cells were washed in ice-cold PBS with 10 mM sodium azide. Then, 500 µl of 1 N NaOH was added to dissolve the cells and cell-associated radioactivity measured in a Packard gamma spectrometer (Packard Instrument Co. Inc., Downers Grove,

IL). Results were expressed as nanograms of mannose-BSA specifically bound or taken up per 5×10^5 MØ plated.

Degradation of ¹²⁵I-Mannose-BSA by MØ. This was measured by the appearance of TCA-soluble labeled material in the culture medium. Degradation of ¹²⁵I-mannose-BSA is detectable after an ~40-min incubation at 37°C and continues at a linear rate for several days if MØ are maintained in the continuous presence of ligand. Trace amounts of sterile ligand (~10⁶ cpm in 10 µl) were added to monolayers of adherent MØ populations. Cells were incubated for 16 h (unless otherwise indicated), and a 0.4-ml aliquot of medium was removed to microfuge tubes. TCA was added to a final concentration of 10% (wt/vol), the tubes incubated on ice for 30 min, and then spun for 10 min in a centrifuge. Supernatant (0.2 ml) was removed, and 5 µl of potassium iodide (4 M) followed by 10 µl of H₂O₂ were added to each aliquot, incubated for 10 min at room temperature, followed by the addition of 0.8 ml of chloroform. The mixture was vortexed vigorously, spun, and 100 µl of the clear aqueous phase was assayed in a gamma counter. Cell-dependent, saturable degradation of ¹²⁵I-mannose-BSA per unit of time was calculated as a function of MØ number. Cell-free blanks were used routinely.

Reverse Transcription (RT)-PCR Analysis

10⁶ cells, as described in the figure legends, were washed once with PBS (4°C) and lysed with RNazol solution (Cinna/Biotex Laboratories, Friendswood, TX). Total RNA was isolated and reverse transcribed by standard procedures using Moloney Murine Leukaemia Virus reverse transcriptase (British Research Laboratories/Gibco, Paisley, Scotland). Dilutions of cDNA's corresponding to 20-cell equivalents were subjected to PCR (annealing temperature: 60°C [Mg^{2+}] = 2.0 mM) for 30 cycles using the following oligonucleotide primers: MMR (unpublished sequence), 5': AAA CAC AGA CTG ACC CTT CCC; 3': GTT AGT GTA CCG CAC CCT CC; TNF (16), (5': TGG CAG AAG AGG CAC TCC CC; 3': GAG GAG CAC GTA GTC GGG GC); and lysozyme (17), (5': CTA TGG AGT CAG CCT GCC G; 3': CAT GCT CGA ATG CCT TGG GG. PCR products were subjected to agarose gel electrophoresis and visualised by ethidium bromide staining. The specificity of each amplification was verified by restriction enzyme cleavage of the product at an internal site.

Results

The elicited macrophage population used in this study facilitated analysis of the morphological effect of the various cytokine treatments. These cells become rounded and relatively nonadherent to tissue culture plastic after overnight incubation (Fig. 1 A). Striking morphologic changes occurred within 8 h after addition of IL-4. After overnight culture, the cells became firmly adherent and spread out on the tissue culture plastic, as shown in Fig. 1 B. Parallel cultures of the more widely used thioglycollate-elicited macrophage populations were not useful in assessing such changes, since these cells remain tightly adherent to tissue culture plastic under the culture conditions used here. Our findings prompted further analysis of IL-4 modulation of the elicited macrophage phenotype.

As shown in Fig. 2, optimal MMR activity after a 48-h culture in recombinant murine IL-4 was ~15-fold higher than in untreated controls. Half-maximal induction occurred at

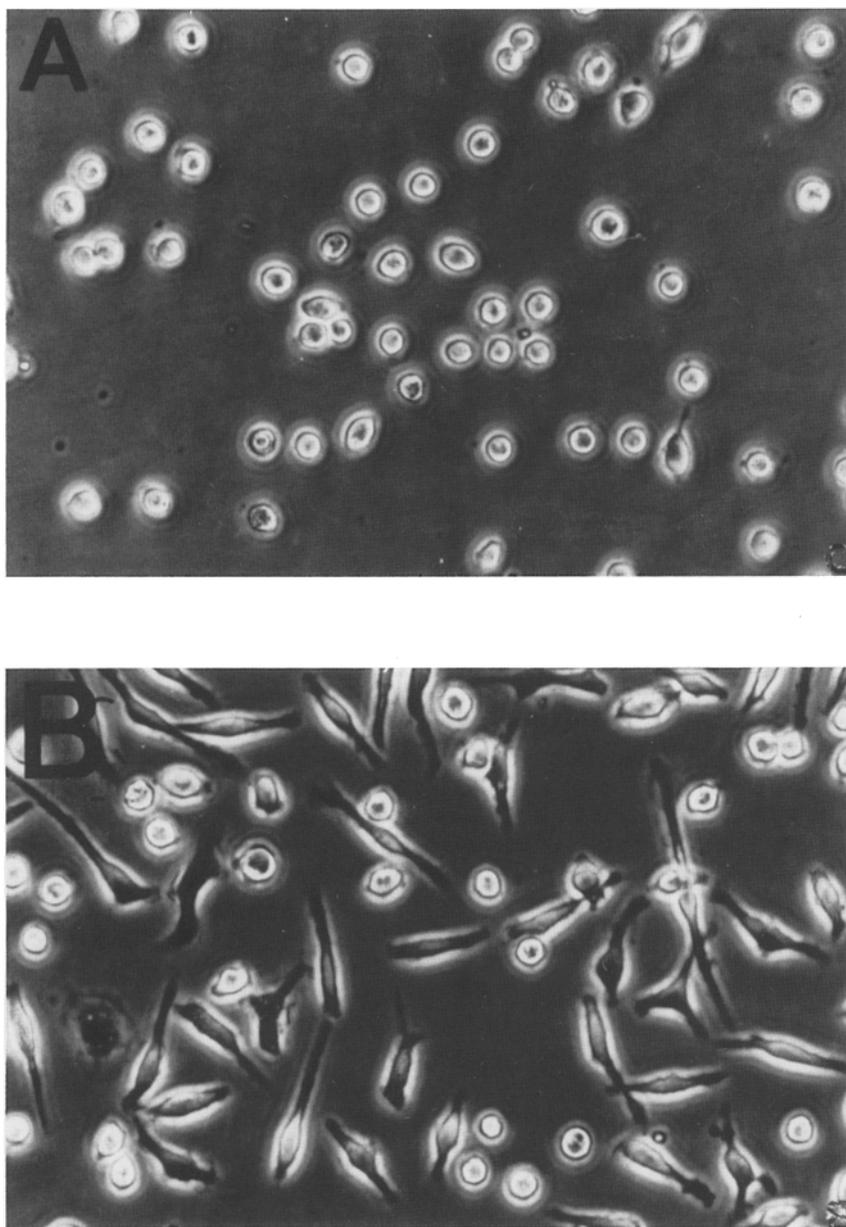


Figure 1. Phase contrast micrographs of BgPM cultured on tissue culture plastic. Cells were harvested and treated as described in Materials and Methods. Control macrophages were rounded and loosely adherent (A). After overnight incubation in the presence of IL-4 (1 ng/ml), the macrophages became tightly adherent and well spread (B).

an IL-4 concentration of <100 pg/ml. IFN- γ decreased and dexamethasone increased MMR activity <2.5-fold. Table 1 shows that maximal degradation activity occurs after 48 h, although increased activity was measurable after 8 h of IL-4 (5 ng/ml) treatment. Addition of an anti-murine IL-4 mAb, 11B11, completely prevented the enhanced MMR activity. The changes in surface MMR expression in response to IL-4 (5 ng/ml) were assessed by high-affinity binding at 4°C in the presence of labeled ligand. Data in Fig. 2 *b* show that saturable binding was increased \sim 12-fold compared with control cultures. The apparent affinity of the receptor-ligand interaction is unaltered by the IL-4 treatment. Note that this analysis is relative to mock-treated controls, since the valency of the mannose-BSA is not known and precludes precise measurement of the number of binding sites. Mannan or excess

cold mannose-BSA effectively competed for binding and degradation of iodinated ligand. Two similar independent binding assays revealed an increase in maximum binding over control cells of 7- and 10-fold, respectively. In addition, internalization of mannose-BSA, as assayed 20 min after the addition of ^{125}I -mannose-BSA at 37°C, was 10-fold enhanced by IL-4 treatment (data not shown).

MMR mRNA is present at only low levels and Northern blot analysis of mRNA induction was not successful. Therefore, we did RT-PCR analysis of MMR mRNA levels. Fig. 3 shows that IL-4, in contrast to IFN- γ or mock-treated control cells, increases MMR mRNA levels as assayed by the RT-PCR using murine MMR-specific oligonucleotides. The unpublished sequence of a mouse MMR cDNA was kindly provided by Dr. Alan Ezekowitz (Harvard Medical School,

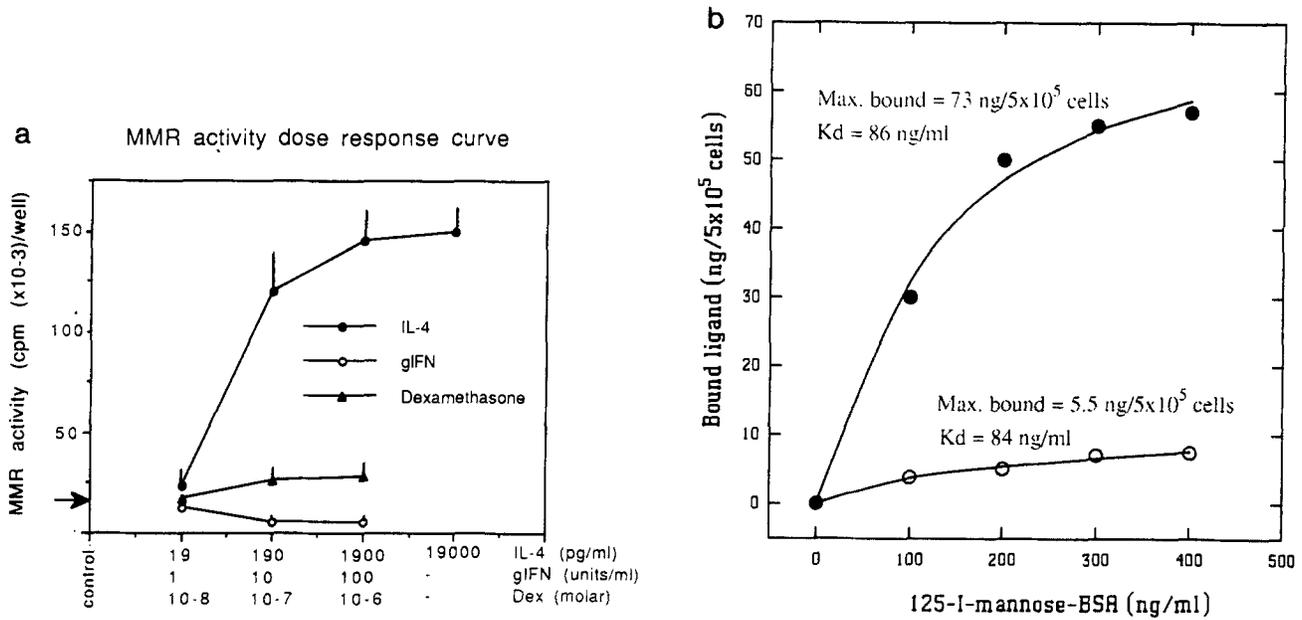


Figure 2. (a) Degradation of ¹²⁵I-mannose-BSA by BgPM in response to increasing doses of murine rIL-4, IFN- γ , and dexamethasone (Dex). Cells were harvested and plated in equal numbers per well as described in Materials and Methods. Cells were incubated in the continuous presence of IL-4, IFN- γ , or Dex for 48 h before the addition of ¹²⁵I-mannose-BSA (0.25 μ g/ml). Specific TCA-soluble counts present in the culture medium after 16 h in the continuous presence of ¹²⁵I-mannose-BSA were used as a measure of ligand degradation as detailed in Materials and Methods. Nonspecific counts, as determined by the addition of 100-fold excess of mannose-BSA, were always <15% of the total. The data shown are from three separate experiments done in duplicate. (b) Binding curve of BgPM incubated with increasing amounts of ¹²⁵I-mannose-BSA. BgPM were incubated with or without IL-4 (5 ng/ml), and 48 h later specific binding of ¹²⁵I-mannose-BSA was measured as described in Materials and Methods. Nonlinear regression analysis of the data was performed using the Marquandt-Levenberg equation (SigmaPlot, version 4; Jandel Scientific, Erkrath, Germany). The K_d for control and IL-4-treated cells is similar, indicating that differences in ¹²⁵I-mannose-BSA binding reveal changes in receptor capacity rather than affinity. The data shown represent one of two similar experiments done in duplicate.

Boston, MA). The same IL-4- or IFN- γ -treated BgPM cDNAs were analyzed for lysozyme and TNF mRNA levels. Fig. 3 (bottom) shows that IFN- γ causes an increase in specific signal for TNF mRNA, as previously reported. Lysozyme mRNA levels were relatively unaltered by the various treatments. Note

that the apparent decrease in lysozyme signal in lane 2 is likely to be due to underloading of the lane since no change in lysozyme signal was seen in four independent experiments, all of which showed a markedly enhanced MMR signal in response to IL-4. Qualitatively similar results were obtained using

Table 1. Time Course of the Effect of IL-4 on MMR Activity

Time in culture	Mock treated	IL-4 (5 ng/ml) treated
h		
8	14 \pm 3	21 \pm 7
20	17 \pm 4	211 \pm 26
48	23 \pm 9	349 \pm 19
72	28 \pm 10	361 \pm 22
16 + 11B11*	53 \pm 8	56 \pm 7

Monolayers of macrophages were cultured in medium, containing 10% FCS, for the time indicated in the presence or absence of IL-4. ¹²⁵I-Mannose-BSA (\sim 0.4 μ g/ml) was added for the last 4 h of the time course and the amount of ligand degraded was measured as described in Materials and Methods. Results reflect the mean \pm SD of triplicate wells and are expressed as nanograms of ligand degraded per 5 \times 10⁵ cells.

* For this time point, the ligand was added for the entire incubation. 11B11 is an IL-4 blocking mAb.

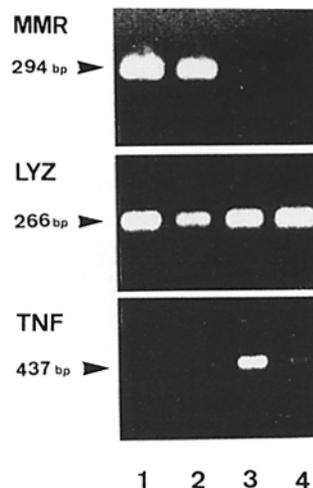


Figure 3. Expression of MMR, lysozyme, and TNF mRNA transcripts by IL-4-treated peritoneal macrophages. Total RNA from control, IFN- γ and IL-4-treated BgPM was reverse transcribed, and cDNA fragments specific for MMR (top), lysozyme (middle), and TNF (bottom) were amplified, as described in Materials and Methods. Lane 1, IL-4 treated (16 h); lane 2, IL-4 treated (4 h); lane 3, IFN- γ treated (16 h); lane 4 = mock treated.

20, 25, and 30 cycles of PCR using serial dilutions of input cDNA corresponding to between 1 and 50 cell equivalents. The PCR data showing IL-4-dependent increase in MMR mRNA levels were confirmed by nuclease protection assays (data not shown).

Discussion

Previously, macrophage activation has been defined in terms of microbicidal and tumoricidal activity. However, cytokines or growth factors may have apparently antagonistic effects depending on the particular micro-organism or cellular target used. Although IL-4 has been regarded as an activator of certain macrophage functions, such as MHC class II expression (2), its effect on microbicidal and cytotoxic activity remains controversial. The effect of IL-4 on modulation of the MMR, long used as negative marker for the IFN- γ -activated macrophage phenotype, is simple to measure at the protein and mRNA level, and does not depend on an additional stimulus or trigger. This distinguishes the use of MMR expression from other more complex assays of macrophage activation.

We have demonstrated that IL-4 potently enhances the expression and activity of the MMR (Fig. 2), an important endocytic receptor known to mediate the binding and ingestion of mannosylated proteins and macromolecules. This effect was similarly demonstrated on thioglycollate-elicited macrophages, another elicited but immunologically nonactivated macrophage population (data not shown). The increased binding and activity are associated with increased MMR mRNA levels (Fig. 3). The RT-PCR analysis used here is qualitative and not quantitative, but further experiments using serial dilutions of input cDNA for PCR indicate that IL-4-treated (48 h) BgPM contain at least eightfold higher levels of MMR mRNA molecules than control populations (not shown). Taken together, the data suggest IL-4 increases MMR surface expression, at least in part, through increased receptor synthesis. Although not experimentally addressed in this paper, it is possible that IL-4 has a further influence on the efficiency of delivery and degradation of ligand within lysosomes, since IL-4 enhanced degradation of ligand 15-fold, as compared with an increase of surface expression and internalization of 7–12-fold each. Further experiments are required to examine the effect of IL-4 on enhanced receptor recycling and lysosomal delivery and degradation of MMR ligands. The effect of IL-4 in upregulating MMR plasma membrane activity is not a general phenomenon since IL-4 reduces transferrin receptor activity and CD14 expression (18).

We conclude that low and possibly physiologic concentrations of IL-4 are able to induce recently recruited inflammatory macrophages to have maximal endocytic clearance capacity for mannosylated ligands. In contrast to the effect of

IFN- γ on TNF expression, IL-4-treated cells remain unprimed with respect to proinflammatory cytokine (e.g., TNF) release. In addition, these data suggest that IL-4 is a candidate regulator of MMR expression in specific tissue micro-environments. Although maximal *in vitro* IL-4 stimulation induces at least 10-fold higher MMR activity than is present in resident peritoneal macrophages, low doses of IL-4, such as those found within tissues, may maintain tissue macrophage MMR expression at high levels, for example on sub-sets of alveolar macrophages (19). Additional circumstantial evidence of an IL-4-like action on alveolar macrophages is their very low proinflammatory secretory activity, but further *in situ* studies are required to test this hypothesis.

Other cytokines tested so far include TGF- β , IFN- α and - β , TNF, IL-2, IL-6, GM-CSF, M-CSF, and IL-10, but these recombinant proteins have only modest or no effect on elicited macrophage MMR activity in comparison to IL-4 (unpublished observations). Recently, monomeric IgG2a was reported to induce MMR expression (20). However, the IgG2a effect was studied in relation to bone marrow-derived macrophage precursor maturation but not elicited monocyte/macrophage populations. Further, while IgG2a greatly enhanced the early expression of MMR on BMM in culture, the maximum level of MMR activity was not greater than mock-treated cells incubated under standard conditions for 7 d. The effects observed in this study were probably not due to IgG2a production by contaminating B cells, as we used highly purified macrophage populations. In addition, IL-4 enhances the release of IgG1 and IgE, but greatly inhibits the release of IgG2a, from activated B cells (21).

The contrasting roles of IL-4 and IFN- γ on macrophage immune activation are reflected in their opposing effects on inflammatory cytokine expression and respiratory burst potential, although both are able to enhance MHC class II antigen expression. Previous studies have documented the priming and inhibitory effects of IFN- γ and IL-4, respectively, on inflammatory cytokine release, and parallel experiments done in this laboratory using BgPM have demonstrated that while IFN- γ greatly enhanced PMA-triggered superoxide release, IL-4 had no effect (not shown). Taken together, these data indicate that helper lymphocyte expression of IL-4 at an inflammatory focus will cause recruited macrophages to acquire an entirely different phagocytic receptor and secretory capability compared with macrophages classically activated by IFN- γ treatment or BCG infection. The MMR, previously used as a marker for immunologically nonactivated macrophages, may instead be a marker of an alternative Th-2 immunologically activated macrophage phenotype. Studies are in progress to further define the role of Th cell-derived cytokines in the modulation of macrophage function.

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