Macrophage Deactivation by Interleukin 10

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Summary

Recombinant mouse interleukin 10 (IL-10) was exceedingly potent at suppressing the ability of mouse peritoneal macrophages (mϕ) to release tumor necrosis factor α (TNF-α). The IC_{50} of IL-10 for the suppression of TNF-α release induced by 0.5 μg/ml lipopolysaccharide was 0.04 $\pm$ 0.03 U/ml, with as little as 1 U/ml suppressing TNF-α production by a factor of 21.4 $\pm$ 2.5. At 10 U/ml, IL-10 markedly suppressed mϕ release of reactive oxygen intermediates (ROI) (IC_{50} 3.7 $\pm$ 1.8 U/ml), but only weakly inhibited mϕ release of reactive nitrogen intermediates (RNI). Since TNF-α is a T cell growth and differentiation factor, whereas ROI and RNI are known to inhibit lymphocyte function, it is possible that mϕ exposed to low concentrations of IL-10 suppress lymphocytes. mϕ deactivated by higher concentrations of IL-10 might be permissive for the growth of microbial pathogens and tumor cells, as TNF-α, ROI, and RNI are major antimicrobial and tumoricidal products of mϕ. IL-10's effects on mϕ overlap with but are distinct from the effects of the two previously described cytokines that suppress the function of mouse mϕ, transforming growth factor β and macrophage deactivation factor. Based on results with neutralizing antibodies, all three mϕ suppressor factors appear to act independently.

IL-10, originally termed cytokine synthesis inhibitory factor (CSIF),[2] was discovered as a product of Th2 cells that suppresses IFN-γ production by Th1 cells (1–3). IL-10 joins TGF-β as one of the few lymphocyte suppressor factors to be purified and cloned (4, 5). The mechanisms by which suppressor factors affect lymphocyte function are not well understood. IL-10 inhibits Th1 cells only in the presence of accessory cells, especially macrophages (mϕ) (6). This raises the possibility that IL-10 might act primarily on mϕ to alter the balance between their lymphocyte-stimulating and lymphocyte-inhibiting secretory products (7), thereby affecting lymphocytes secondarily.

As is the case for lymphocytes, only a few purified cytokines are known to suppress the function of mϕ (8–14). In fact, only two cytokines are known to block or reverse the activation of mouse mϕ: TGF-β (including TGF-β-1, -2, and -3) and mϕ deactivating factor (MDF) (8–10, 15–18; for review see reference 19). The present experiments were designed to test whether IL-10 is a new mϕ deactivation factor, and if so, whether it mediates the mϕ-deactivating effects of TGFβ or MDF.

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[2] *Abbreviations used in this paper:* CSIF, cytokine synthesis inhibitory factor; MDF, macrophage deactivating factor; mϕ, macrophage; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates.

Materials and Methods

Mice. Female CD1 mice (8–12 wk old) were from the Charles River Breeding Laboratories (Wilmington, MA).

Cytokines and Other Reagents. Supernatants from COS7 cells transfected with mIL-10 cDNA (1,000 U/ml, where 1 U caused half-maximal response of the MC/9 mast cell line as described [20]; LPS content at 100 U/ml < 10 pg/ml) and control supernatants from mock transfected cells (LPS content at a 1:10 dilution < 10 pg/ml) were kindly provided by Dr. K. Moore (DNAX, Palo Alto, CA; 1 MC/9 U/ml is equal to 1 CSIF U/ml, personal communication). MDF was purified from the culture supernatants of P815 mouse mastocytoma cells as described (10) or directly extracted from these cells following a similar procedure (Y. Vodovotz, C. Bogdan, and C. Nathan, unpublished results). A unit of MDF is defined as that amount of MDF in a final culture volume of 0.125 ml that causes 50% suppression of PMA-triggered mϕ H_2O_2-releasing capacity after a 48-h incubation (10). rmIFN-γ (protein concentration 1.1 mg/ml; sp act 5.2 × 10^6 U/mg; LPS content <10 pg/ml) and rmTNF-α (protein concentration 0.98 mg/ml; sp act 1.2 × 10^7 U/mg; LPS content <52 pg/ml) were kindly provided by Genentech (South San Francisco, CA). rhTGFβ1 was a gift of Amgen, Inc. (Thousand Oaks, CA). Ascites fluid containing a neutralizing rat IgM mAb against mIL-10 (SXC1 [21]; LPS content <10 pg/ml at a 1:100 dilution) was a gift from Dr. S. Reed (Seattle, WA). A 1:1,000 dilution of this antibody preparation completely neutralized the effect of IL-10 at concentrations ≤100 U/ml and was used in all experiments. Purified rat IgM as an isotype control antibody was obtained from Calbiochem-Behring Corp. (La Jolla, CA). Turkey anti-TGF-β1 IgG and nonimmune turkey IgG were kindly provided by Dr. M. Sporn (National Cancer Institute, Bethesda, MD). LPS, prepared by phenol extraction from *Esche-*

richia coli O111:B4, was from List Biological Laboratories (Campbell, CA).

Mφ Cultures. Periodate- and thioglycolate-elicited mφ were harvested from the peritoneal cavity with αMEM medium (JRH Biosciences, Cambridge, MA) and cultured as published (22).

H_2O_2 Release Assay. 1.5 × 10⁵ Periodate-elicited peritoneal cells were triggered for H_2O_2 release by PMA. H_2O_2 secretion/mg adherent cell protein was determined as described (10).

Reactive Nitrogen Intermediates (RNI) Production and NO_2^- Determination. Monolayers of thioglycolate-elicited mφ were stimulated for RNI production by IFN-γ, IFN-γ/TNF, or IFN-γ/LPS for 48 h. Nitrite concentration in the cell-free mφ supernatants served as a reflection of nitric oxide production and was measured by the colorimetric Griess reaction as described (18, 22).

TNF-α Production and TNF-α Assay. Monolayers of thioglycalate-elicited mφ were obtained by seeding the cells in 24-well plastic tissue culture plates (Costar, Cambridge, MA) at 10⁶/well (final volume of 0.5 ml) for 2–3 h at 37°C in 5% CO_2/95% air. Nonadherent cells were removed by three washes with PBS (37°C) and the monolayers were then stimulated with LPS (± rmIFN-γ) either in the presence or absence of rmIL-10 as indicated in the text. 20 h later, the supernatants were harvested and stored at −20°C. The TNF-α content of the test supernatants was determined in a bioassay with the highly specific and sensitive subclone 13 of the WEHI 164 fibrosarcoma line (23) (kindly provided by Dr. S. Kunkel, Ann Arbor, MI, with permission of Dr. T. Espevik, Trondheim, Norway) as published previously (24). Data from at least 10 serial twofold dilutions per supernatant were subjected to probit analysis using a rmTNF-α standard curve as described (25). Finally, the fold suppression of TNF release in the test supernatants was determined [(ng/ml TNF in medium control)/(ng/ml TNF in test supernatant)]. Use of a polyclonal rabbit anti-TNF-α antiserum (Genzyme, Boston, MA) demonstrated that the cytotoxic activity was completely dependent on TNF-α.

SDS-PAGE and Western Blot. IL-10-containing and mock control COS cell supernatants and partially purified MDF were separated on 20% SDS-PAGE and transferred to nitrocellulose membranes (26, 27).

Protein Synthesis. Monolayers (2 × 10⁶/well) of periodate-elicited mφ were incubated for 48 h in complete RPMI 1640 in the presence or absence of rmIL-10. During the last 6 h of incubation, the medium was exchanged for methionine-free RPMI 1640 with 1% FCS (Gibco, NY), and 50 μCi [³⁵S]L-methionine (> 1,000 Ci/mmol; Amersham Corp., South Clearbrook, IL) was added per 2 × 10⁶ mφ. The cells were then washed and lysed in 1% triton X-100 and processed for determination of radiolabeled protein as described (15). Additional aliquots of the lysates were subjected to SDS-PAGE autoradiography.
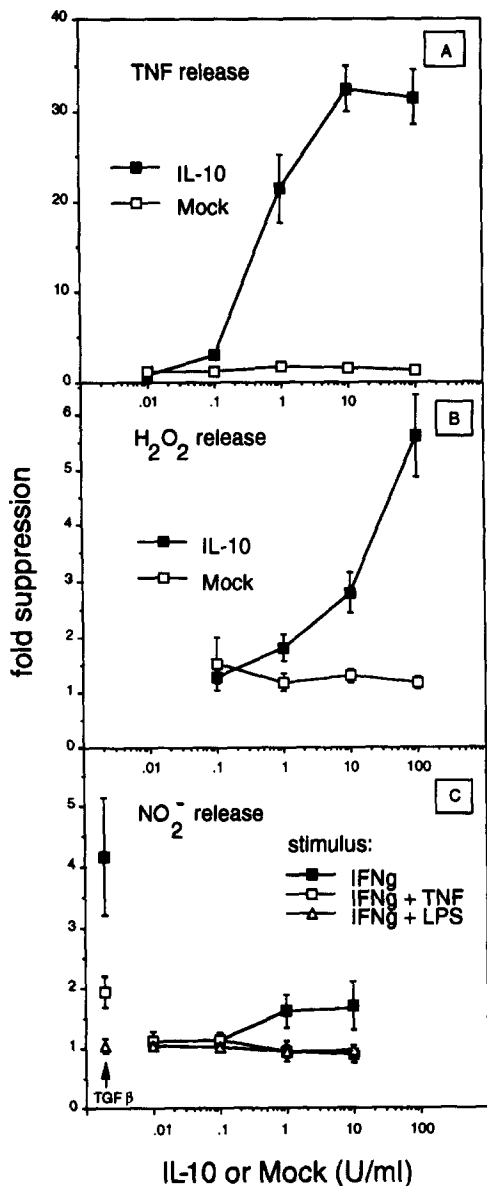
Results

TGF-β and MDF can prevent mφ from releasing all three of their major cytotoxic products: TNF-α (28, and Bogdan, C., Y. Vodovotz, and C. Nathan, manuscript in preparation), reactive oxygen intermediates (ROI) (8, 10, 15–17), and RNI (18, 29). Accordingly, we tested the effects of IL-10 in each of these assays.

Suppression of TNF-α Release. IL-10 was an exceedingly potent inhibitor of mφ TNF-α release (Fig. 1; Fig. 2 A). The inhibitory effect was concentration dependent, reaching its plateau upon exposure of mφ to 10 U/ml IL-10, which cause 32.4 (± 2.8)-fold (97%) suppression (mean ± SEM
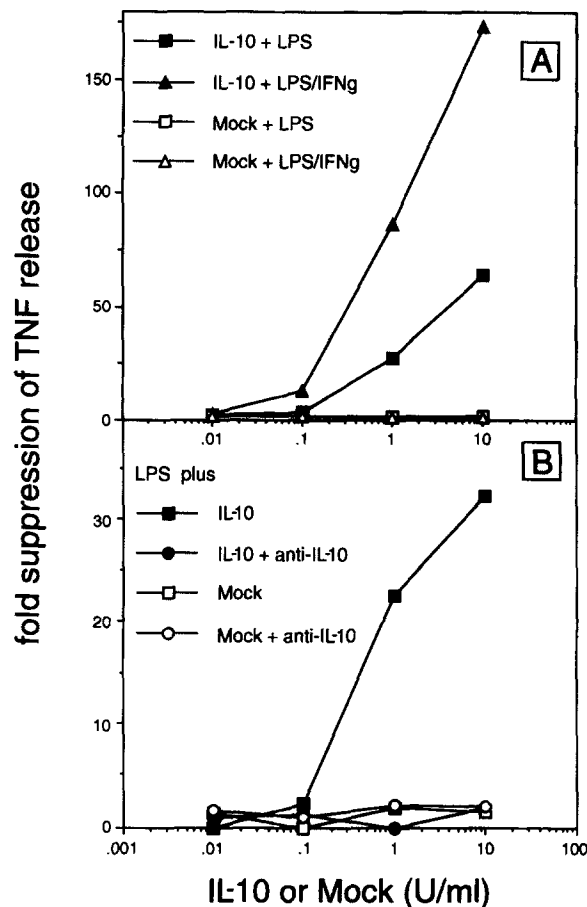
Figure 1. IL-10 suppresses TNF-α release by monolayers of thioglycolate-elicited mφ. (*A*) Mφ were stimulated with LPS (0.25 μg/ml) or LPS (0.25 μg/ml) plus IFN-γ (10 ng/ml) in the absence or presence of IL-10 or an identical amount of COS-cell control supernatant (mock transfectant). (*B*) Mφ were stimulated with LPS (0.5 μg/ml) alone or together with IL-10 or an identical amount of COS-cell control supernatant (mock transfectant) in the absence or presence of anti-IL-10. (*A* and *B*) After 20 h, the TNF content in the cell-free supernatants and the fold suppression of TNF-α release were determined as described in Material and Methods. The TNF release in the medium controls was 4.6 ng/ml (LPS alone) and 41.6 ng/ml (LPS plus IFN-γ) in the experiment shown in *A* and 43.3 ng/ml (LPS alone) in *B*.

of three experiments) of mφ TNF-α release induced by 0.5 μg/ml LPS. 50% suppression required only 0.044 ± 0.031 U/ml (mean ± SD from four experiments). Preincubation of the mφ with IL-10 before stimulation by LPS was not necessary. Anti-IL-10 mAb abolished the suppression afforded by IL-10-containing COS cell supernatant (Fig. 1 B), whereas a rat IgM control antibody failed to do so (not shown). Mock-transfected COS cell supernatant had no effect (Fig. 1 B). IL-10 abolished TNF-α release induced by LPS at concentrations of 0.25 or 0.5 μg/ml (Fig. 1), 1 or 10 μg/ml (not shown), or when TNF-α release was induced by the combination of LPS (0.25 μg/ml) and IFN-γ (10 or 20 ng/ml) (Fig. 1 A). In control experiments, IL-10 did not stimulate the indicator cells in the TNF bioassay; protect them from inhibition by

exogenous TNFα; nor induce mφ to release a factor that could block the action of TNF-α (not shown).

Suppression of H₂O₂ Release. Concentrations of IL-10 50–100-fold higher than those suppressing TNF-α release suppressed the ability of PMA-triggered mφ to release H_2O_2 ($IC_{50} = 3.7 \pm 1.8$ U/ml, mean $\pm$ SD, three experiments) (Fig. 2 B). The range of suppression caused by 10 U/ml IL-10 was 55–79% with a mean ($\pm$ SEM) of 65% ($\pm$ 4.1; four experiments). This action of IL-10-containing COS cell supernatant was abolished by anti-IL-10 mAb and was lacking in mock-transfected COS cell supernatant (Fig. 3).

Relation between IL-10 and MDF or TGF-β. Since suppression of mouse peritoneal mφ H_2O_2 releasing capacity has been the cardinal assay for characterization of MDF (10, 15–17), it was important to test whether MDF consists in, or acts through the induction of, IL-10. As shown in Fig. 3, anti-IL-10 mAb had no effect on the respiratory burst-suppressing action of MDF. Moreover, anti-IL-10 mAb immunoblotted IL-10 in COS cell supernatant, but not a preparation of semi-purified MDF containing a twofold higher bioactivity as determined in the H_2O_2 release assay (Fig. 4).

Likewise, the suppressive action of TGF-β on the mφ respiratory burst (8, 17) or TNF-α release (28) was unaffected by anti-IL-10 mAb (not shown). Finally, abs that neutralize TGF-β but not MDF in the H_2O_2 release assay (10) had no effect on suppression by IL-10 (Fig. 5). Anti-MDF abs are not yet available.

*Effect of IL-10 on RNI Release.* The above results indicated that IL-10 appears to act independently of TGF-β and MDF. Thus, the spectrum of action of IL-10 on mφ may differ from that of TGF-β and MDF. Indeed, unlike TGF-β and MDF (18), IL-10 only variably and weakly inhibited the induction of RNI release by IFN-γ (range of suppression by 10 U/ml in six experiments: 0–60%; mean $\pm$ SEM $= 37 \pm 15\%$) and was unable to suppress RNI release induced by the combination of IFN-γ and TNF-α (Fig. 2 C). Higher concentrations of IL-10 (100 U/ml) did not further increase the suppression of RNI release (three experiments; data not shown). As found for MDF and TGF-β, IL-10 also failed to inhibit RNI release induced by the combination of IFN-γ plus LPS (Fig. 2 C).

Effect of IL-10 on mφ Protein Synthesis. The selectivity of IL-10 effects on mφ was further demonstrated by its inability to alter the incorporation of ³⁵S-methionine into protein, or to affect the overall pattern of protein synthesis as evaluated by one-dimensional SDS-PAGE (not shown).

Discussion

IL-10 appears to be even more potent as a suppressor of mφ TNF-α release than as a suppressor of Th1 cell IFN-γ synthesis (1). A concentration of IL-10 (1 U/ml) that almost completely suppressed mφ TNF-α release (95.4% suppres-

Figure 2. Comparison of the effect of IL-10 on mφ TNF-α production, H₂O₂ secretion, and RNI release. (*A*) Adherent thioglycolate-elicited mφ (10⁶/well) were stimulated with LPS (0.5 μg/ml) for TNF-α release in the presence of IL-10 or a mock-transfectant control supernatant as described in the legend to Fig. 1. (*B*) Periodate-elicited cells (1.5 × 10⁵/well) were incubated in medium alone, IL-10, or a mock-transfectant control supernatant. After 48 h, H₂O₂ release was triggered, the adherent cell protein determined, and the H₂O₂ release/mg adherent cell protein calculated. (*C*) Adherent thioglycolate-elicited mφ (2 × 10⁵) were stimulated with IFN-γ (1 ng/ml), IFN-γ (1 ng/ml) plus TNF-α (20 ng/ml), or IFN-γ (1 ng/ml) plus LPS (100 ng/ml) in the presence of IL-10, supernatant of mock transfectants, TGF-β (10 ng/ml), or medium. After 48 h, the cell-free supernatants were processed for NO₂⁻ determination (nmol release per 2 × 10⁵ mφ) (22). The mean NO₂⁻ release in the control cultures was 6.2 (IFN-γ), 7.8 (IFN-γ + TNF-α), and 12.0 (IFN-γ + LPS) nmol/2 × 10⁵ mφ. The symbols next to the y-axis denote the values for the TGF-β-treated cultures. The remaining symbols are all for cells treated with the indicated concentration of IL-10. The mock-transfectant supernatant-treated cultures revealed <10% suppression and the values are not shown here for the sake of clarity. (*A*, *B*, and *C*) The fold suppression of TNF-α, H₂O₂, and NO₂⁻ release was calculated by comparing the values of the IL-10-, mock-transfectant control supernatant-, or TGF-β-

treated cultures with those of the medium control. Data represent mean values ($\pm$ SEM) of three (TNF-α release), four (H₂O₂ release) or five (NO₂⁻ release) experiments, respectively.
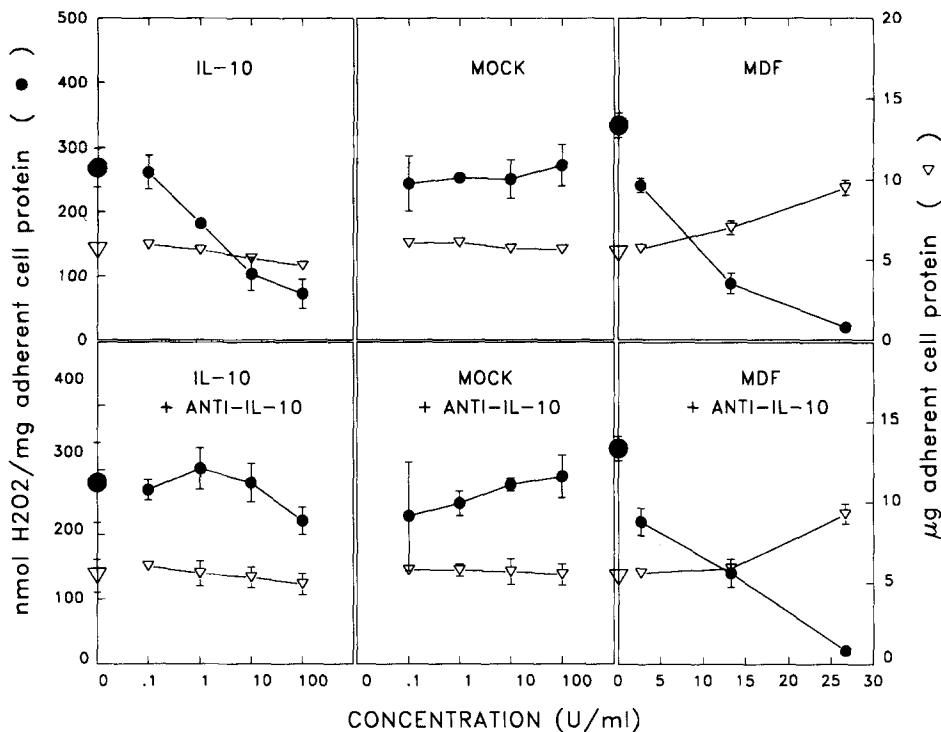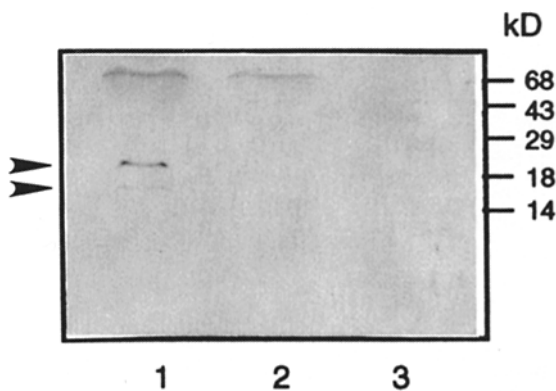
Figure 3. IL-10 suppresses mφ H_2O_2 release, but does not appear to account for the activity of MDF. 1.5×10^5 periodate-elicited cells were incubated either in medium alone or in IL-10, mock COS cell supernatant, or in MDF (C18-RPHPLC-purified) in the presence or absence of anti-IL-10. After 48 h, H_2O_2 release was triggered with PMA and the adherent cell protein (∇) was determined to exclude toxicity. H_2O_2 release is expressed as nmol H_2O_2/mg adherent cell protein (●). Control values are given on the y-axis of each panel. Data represent mean (± SD) from triplicate cultures of a representative experiment. Where error bars are not visible, they fall within the symbols denoting the means.

sion) had little effect on H_2O_2 release (42% suppression) and on the secretion of RNI (36% suppression). TNF-α is a potent growth and differentiation factor for mouse T cells (30–32). In contrast, ROI and RNI suppress lymphocyte function (33–35). Thus, activated mφ exposed to low concentrations of IL-10 are likely to produce ROI and RNI but not TNF-α, and thus may display a suppressor phenotype toward lymphocytes. This may contribute to the CSIF activity of IL-10.
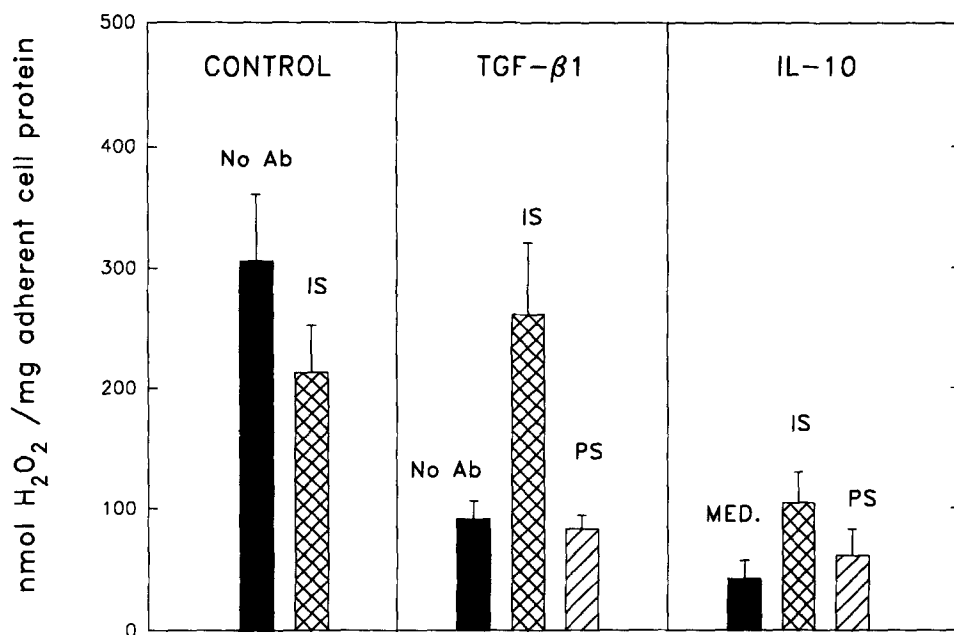

Figure 4. Anti-IL-10 mAb fails to recognize MDF. IL-10 containing COS cell supernatant (50 U, corresponding to ~100 U in the standard H_2O_2 release assay) (lane 1), an equivalent amount of mock transfectant supernatant (lane 2), and MDF (200 U in the H_2O_2 release assay) (lane 3) were separated on 20% SDS-PAGE, electrotransferred to nitrocellulose, immunoblotted with anti-IL-10 mAb (1:100 dilution), and developed with a goat anti-rat IgM antiserum conjugated to alkaline phosphatase. The arrows indicate rmIL-10 in its expected size heterogeneity (2, 21).

On the other hand, at higher concentrations (10 U/ml), IL-10 markedly suppressed not only mφ TNF-α release but also the release of ROI. Finally, although IL-10 only weakly suppressed RNI release induced by IFN-γ, IL-10 might be more effective at suppressing RNI release indirectly in situations where RNI release depends on an autocrine action of TNF-α. Ingestion of pathogens is a strong stimulus for mφ to release TNF-α (24, 36), which in turn helps to induce RNI by interacting synergistically with IFN-γ (22, 37). Since ROI, RNI, and TNF-α are among the major antimicrobial (for review see references 38 and 39) and antitumor products of mφ (for review see reference 40), a second phenotype of the IL-10-treated mφ may be that of a cell permissive for the growth of pathogens and tumor cells. IL-10, therefore, may suppress the antimicrobial and tumoricidal function of mφ in two ways: first, indirectly through inhibition of IFN-γ production by Th1 cells, which then impairs mφ activation; and second, directly through deactivation even in the presence of IFN-γ.

IL-10's actions on mφ function were selective and nontoxic. At the same time that IL-10 abolished TNF-α release and decreased H_2O_2 release, it had only little effect on their release of RNI. IL-10 did not affect the number of mφ remaining adherent to vigorously washed plates (Fig. 3), their overall synthesis of protein, nor their synthesis of major proteins.

IL-10, TGF-β, and MDF appeared to act independently, since abs that neutralized either IL-10 or TGF-β had no effect on the actions of the other two proteins in the same assays. Although MDF remains to be cloned, it appears to be distinct from IL-10 by the following criteria. MDF migrates at ~13 kD on denaturing SDS-PAGE and is purified on the basis of its acid stability (10), while IL-10 migrates at 16–21

Figure 5. H_2O_2 suppression by IL-10 is not mediated through TGF-β1. Periodate-elicited cells (1.5×10^5/well) were cultured in medium alone (control) or in TGF-β1 (10 ng/ml) or in IL-10 (10 U/ml), in the presence of preimmune (PS) or immune (IS) anti-TGF-β1 antiserum or in the absence of antiserum (no Ab). After 48 h, H_2O_2 release was triggered and the adherent cell protein determined. Data represent mean ($\pm$ SD) from triplicate cultures of a representative experiment.

kD on SDS-PAGE and is acid labile (2). MDF (Vodovotz, Y., C. Bogdan, and C. Nathan, manuscript in preparation) but not IL-10 inhibits lymphocyte proliferation (1, 2, 41). Moreover, MDF is much more potent in suppressing RNI release (18). Like TGF-β (8) but unlike MDF (10, 15), IL-10 tends to cause adherent mϕ to round up in culture (not shown). Finally, anti-IL-10 mAb neither neutralizes nor immunoblots MDF.

Several cytokines activate mϕ in ways that are overlapping but distinct, giving rise to diverse phenotypes. The same can now be said of cytokines that deactivate mϕ.

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Note added in proof: After this paper was submitted, we learned of other studies in press or in preparation describing deactivating effects of IL-10 on mouse macrophage or human monocyte MHC class II expression, cytokine release, nitrite production, or killing of parasites (de Waal Malefyt, R., J. Haanen, H. Spits, M.-G. Roncarolo, A. te Velde, C. Fidgor, K. Johnson, R. Kastelein, H. Yssel, and J. E. de Vries. 1991. *J. Exp. Med.* 174:915; de Waal Malefyt, R., J. Abrams, B. Bennett, C. Fidgor, and J. E. de Vries. 1991. *J. Exp. Med.* 174:1209; Fiorentino, D. F., A. Zlotnik, T. R. Mosmann, M. Howard, and A. O'Garra. 1991. *J. Immunol.* In press; Silva, J. S., P. J. Morrissey, K. H. Grabstein, K. M. Mohler, D. Anderson, and S. G. Reed. 1992. *J. Exp. Med.* In press; Gazzinelli, R. T., I. P. Oswald, S. L. James, and A. Sher, personal communication).

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