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 IC₅₀ of IL-10 for the suppression of TNF-α release induced by 0.5 μg/ml lipopolysaccharide was 0.04 ± 0.03 U/ml, with as little as 1 U/ml suppressing TNF-α production by a factor of 21.4 ± 2.5. At 10 U/ml, IL-10 markedly suppressed mφ release of reactive oxygen intermediates (ROI) (IC₅₀ 3.7 ± 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.

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 mlIL-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(10) or directly extracted from these cells following a similar procedure (Y. Vodovozt, C. Bogdan, and C. Nathan, unpublished results).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 mO HzO₂-releasing capacity after a 48-h incubation (10). rmIFN-γ (protein concentration 1.1 mg/ml; sp act 5.2 × 10⁶ U/mg; LPS content <10 pg/ml) and rmTNF-α (protein concentration 0.98 mg/ml; sp act 1.2 × 10⁵ U/mg; LPS content <52 pg/ml) were kindly provided by Genentech (South San Francisco, CA). rhTGF-β1 was a gift of Amgen, Inc. (Thousand Oaks, CA). Ascites fluid containing a neutralizing rat IgM mAb against mlIL-10 (SXCl [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-
reactive oxygen intermediates (ROI) (8, 10, 15-17), and RNI served as a reflection of nitric oxide production and was measured by the colorimetric Griess reaction as described (18, 22).

**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. 2A). 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 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). Pretreatment 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 (mock transfec tant). (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 transfec tant) 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.
Figure 2. Comparison of the effect of IL-10 on mφ TNF-α production, H2O2 secretion, and RNI release. (A) Adherent thioglycolate-elicited mφ (10^6/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 x 10^5/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 x 10^6) 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 x 10^6 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 x 10^6 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-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 H2O2 (IC50 = 3.7 ± 1.8 U/ml, mean ± SD, three experiments) (Fig. 2B). The range of suppression caused by 10 U/ml IL-10 was 55-79% with a mean (± SEM) of 65% (± 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 H2O2 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 H2O2 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 H2O2 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 ± SEM = 37 ± 15%) and was unable to suppress RNI release induced by the combination of IFN-γ and TNF-α (Fig. 2C). 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. 2C).

**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% suppressed treated cultures with those of the medium control. Data represent mean values (± SEM) of three (TNF-α release), four (H2O2 release) or five (NO2⁻ release) experiments, respectively.)
Figure 3. IL-10 suppresses \( H_2O_2 \) release, but does not appear to account for the activity of MDF. 1.5 \( \times 10^6 \) 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 (\( \gamma \)) was determined to exclude toxicity. \( H_2O_2 \) release is expressed as nmol \( H_2O_2/\text{mg adherent cell protein} \) (\( \gamma \)). Control values are given on the y-axis of each panel. Data represent mean (\( \pm \) SD) from triplicate cultures of a representative experiment. Where error bars are not visible, they fall within the symbols denoting the means.

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

IL-10's actions on \( \text{mϕ} \) function were selective and nontoxic. At the same time that IL-10 abolished TNF-\( \alpha \) 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 \( \text{mϕ} \) remaining adherent to vigorously washed plates (Fig. 3), their overall synthesis of protein, nor their synthesis of major proteins. IL-10, TGF-\( \beta \), and MDF appeared to act independently, since abs that neutralized either IL-10 or TGF-\( \beta \) 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 \( \sim 13 \) kD on denaturing SDS-PAGE and is purified on the basis of its acid stability (10), while IL-10 migrates at 16–21

Figure 4. Anti-IL-10 mAb fails to recognize MDF. IL-10 containing COS cell supernatant (50 U, corresponding to \( \sim 100 \) 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).
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.

We are grateful to S. Kunkel (University of Michigan, Ann Arbor, MI), K. Moore (DNAX Research Institute, Palo Alto, CA), M. Palladino (Genentech, South San Francisco, CA), S. Reed (Biomedical Research Institute, Seattle, WA), M. Sporn (National Cancer Institute, Bethesda, MD), and H. Yeganegi (Amgen Biologicals, Thousand Oaks, CA) for gifts of reagents and cells, and to Erika Bach for excellent technical assistance.

This study was supported by National Institutes of Health grant CA-43610. C. Bogdan is supported by grant Bo-996/1-1 from the Deutsche Forschungsgemeinschaft.

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Received for publication 25 June 1991 and in revised form 5 August 1991.

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. Fidgur, J. Johnson, R. Kastelein, H. Yasel, and J. E. de Vries. 1991. J. Exp. Med. 174:915; de Waal Malefyt, R., J. Abrams, B. Bennett, C. Fidgur, 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. Mortissey, 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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