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## IL-10 INHIBITS CYTOKINE PRODUCTION BY ACTIVATED MACROPHAGES<sup>1</sup>

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**IL-10 inhibits the ability of macrophage but not B cell APC to stimulate cytokine synthesis by Th1 T cell clones. In this study we have examined the direct effects of IL-10 on both macrophage cell lines and normal peritoneal macrophages. LPS (or LPS and IFN- $\gamma$ )-induced production of IL-1, IL-6, and TNF- $\alpha$  proteins was significantly inhibited by IL-10 in two macrophage cell lines. Furthermore, IL-10 appears to be a more potent inhibitor of monokine synthesis than IL-4 when added at similar concentrations. LPS or LPS- and IFN- $\gamma$ -induced expression of IL-1 $\alpha$ , IL-6, or TNF- $\alpha$  mRNA was also inhibited by IL-10 as shown by semiquantitative polymerase chain reaction or Northern blot analysis. Inhibition of LPS-induced IL-6 secretion by IL-10 was less marked in FACS-purified peritoneal macrophages than in the macrophage cell lines. However, IL-6 production by peritoneal macrophages was enhanced by addition of anti-IL-10 antibodies, implying the presence in these cultures of endogenous IL-10, which results in an intrinsic reduction of monokine synthesis after LPS activation. Consistent with this proposal, LPS-stimulated peritoneal macrophages were shown to directly produce IL-10 detectable by ELISA. Furthermore, IFN- $\gamma$  was found to enhance IL-6 production by LPS-stimulated peritoneal macrophages, and this could be explained by its suppression of IL-10 production by this same population of cells. In addition to its effects on monokine synthesis, IL-10 also induces a significant change in morphology in IFN- $\gamma$ -stimulated peritoneal macrophages. The potent action of IL-10 on the macrophage, particularly at the level of monokine production, supports an important role for this cytokine not only in the regulation of T cell responses but also in acute inflammatory responses.**

IL-10 was first described as a cytokine produced by Th2 Th cell clones which inhibits macrophage APC-dependent cytokine synthesis by Th1 Th cells (1, 2), and

has subsequently been shown to have a number of effects on other cells (2-6) and to be produced by other cells (2, 7). Th1 cells secrete IL2 and IFN $\gamma$  and preferentially induce macrophage activation and DTH (8, 9), whereas Th2 cells produce IL-4 and IL-5 and provide help for B cell responses (10-12). It has been shown that, once activated, each type of Th cell may be able to regulate the proliferation and/or function of the other (1, 13-15). Such cross-regulation is mediated by various cytokines, and offers a possible explanation for the observation that some antibody and DTH<sup>4</sup> responses can be mutually exclusive (16, 17). We have previously shown that IL-10 acts on the macrophage but not the B cell to inhibit cytokine synthesis by Th1 clones, and that IL-10 exerts a direct effect on the macrophage (18).

Macrophage products, such as IL-1, IL-6, and TNF- $\alpha$  have been implicated in many inflammatory and immunologic responses elicited during infection or tissue injury. TNF- $\alpha$  and IL-1 are endogenous pyrogens (19-24) which in addition cause a number of metabolic changes in a variety of cell types. Moreover, IL-1 and IL-6 are the principal inducers of the synthesis of hepatic acute-phase proteins (24-27). In this study we show that IL-10 inhibits the production of cytokines such as IL-1, TNF- $\alpha$ , and IL-6 by LPS-activated macrophages. Thus, IL-10 may play an important part in inflammatory responses by regulating macrophage function in addition to its role in T cell activation.

### MATERIALS AND METHODS

**Cytokines.** Purified recombinant mouse IL-1 $\alpha$  was a gift from P. Lomedico, Hoffmann-La Roche, Nutley, NJ. Recombinant mouse IL-2 and IFN- $\gamma$  were obtained from Schering Research, Bloomfield, NJ. Recombinant purified mouse IL-6, kindly provided by M. Pearce, DNAX, was expressed in COS7 cells, and immunoaffinity purified. Mouse TNF- $\alpha$  was obtained from Genzyme Corporation (Boston, MA). Recombinant mouse IL-10 (CSIF), obtained by transfecting COS7 cells with the F115 cDNA clone as described previously (2) and control supernatants from mock transfected cells, were used at 2% final concentration unless otherwise indicated. Alternatively, recombinant mouse IL-10, kindly provided by Warren Dang was expressed in *Escherichia coli* and affinity purified by using the SXC2 anti-IL-10 antibody (28).

**Antibodies.** Neutralizing mAb against IFN- $\gamma$  (XMG1.2) (29) and IL-10 (SXC1) (28) have been described previously. J5, an isotype matched control for SXC-1, was kindly provided by Robert Coffman (DNAX). mAb to IL-6 (20F3 and 32C11) (30) and to TNF- $\alpha$  (MP6.XT3.11 and XT22.11) were purified as previously described (30) and were provided by John Abrams, DNAX. Antibodies used for FACS sorting included rat anti-mouse B220 (RA3-6B2) (31) and rat anti-mouse Mac-1 (M1/70) (32). The rat anti-mouse mAb to Fc- $\gamma$ R was 2.4G2 (33).

**Media.** Assay medium (cRPMI) consisted of RPMI 1640 (J. R. Scientific Inc., Woodland, CA) with 10% heat-inactivated FCS (J. R.

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<sup>4</sup> Abbreviations used in this paper: DTH, delayed-type hypersensitivity; KLH, keyhole limpet hemocyanin; PCR, polymerase chain reaction.

Scientific Inc.), 0.05 mM 2-ME (Sigma Chemical Co., St. Louis, MO), and 10 mM HEPES buffer (GIBCO Laboratories, Grand Island, NY). For T cell growth, recombinant mL-2 (330 U/ml) was added to cRPMI.

Ag. KLH obtained from Pacific Bio-Marine Laboratories, Inc. (Venice, CA) or Calbiochem Labs (La Jolla, CA) was used at a final concentration of 100 to 500  $\mu$ g/ml, and OVA from Sigma was used at a final concentration of 1 mg/ml.

**Cell lines.** The Th1 clone, HDK1 (specific for I-A<sup>d</sup>/KLH) (29), and the T cell hybridoma DO11.10 (specific for I-A<sup>d</sup>/ovalbumin) (34), were used for the cytokine synthesis (CSIF) inhibition assay (1). The Th2 clone, D10.G4.1 (D10), AKR/J anti-conalbumin, obtained from C. Janeway (Yale University, New Haven, CT) (35), was used for an IL-1 assay as described in Suda et al. (36). All clones were maintained by periodic stimulation with Ag and irradiated APC, followed by growth in IL-2-containing medium as described elsewhere (29). The cloned 1G.18.LA macrophage cell line (H-2<sup>d</sup>) was derived from thymic stromal cell cultures and maintained in 20% L cell supernatant as previously described (37). The PU5.1 macrophage cell line (H-2<sup>d</sup>) (38) was maintained in cRPMI/10% FCS. For use as APC, the 1G.18.LA and PU5.1 cell lines were previously activated for 20 to 24 h with IFN- $\gamma$  (0.5–2 ng/ml). The WEHI.164.13 cell line, which is responsive to TNF- $\alpha$  and TNF- $\beta$ , (39) was maintained in cRPMI/5% FCS.

**Immunometric assays for cytokines.** Cytokine levels (IL-6, IFN- $\gamma$  and CSIF/IL-10) were measured, in a two-site sandwich ELISA format as previously described (1, 28, 30).

**Bioassays.** The colorimetric [3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide proliferation assay previously described (40) was used for the TNF- $\alpha$  [WEHI 164.13 cells], IL-2 [HT-2 cells] and IL-1 [D10 cells] [ $10^4$ /well for all assays] bioassays. Activity is either expressed as units/ml relative to a known standard or pg or ng/ml. In each case, a unit per ml represents the amount of a particular cytokine which produces 50% of the maximal response of that bioassay.

**Induction and measurement of Th1 cytokine synthesis.** From  $1$  to  $5 \times 10^4$  Th1, HDK.1 cells, or DO-11.10 T cell hybridoma cells were combined with varying numbers of live APC in the presence or absence of Ag in 96-well flat-bottomed microtiter trays in a total volume of 200  $\mu$ l/well. Levels of cytokines were measured in 20- or 48-h supernatants as described previously (1).

**Stimulation of macrophage cell lines.** Macrophage cell lines 1G.18.LA and PU 5.1 were harvested by gentle scraping, washed, and resuspended in cRPMI/5% FCS at  $10^6$  cells per ml in 9.5-cm tissue culture dishes (Becton Dickinson, at 37°C in 5% CO<sub>2</sub>/95% air, for 6 h for RNA expression, or 24 h for cytokine detection in the supernatants. Stimulation with LPS was at 10  $\mu$ g/ml, or in some cases LPS and IFN- $\gamma$  at 100 U/ml, in the presence or absence of IL-10 (200 U/ml) or IL-4 (200 U/ml). Supernatants were collected, centrifuged (800  $\times$  g) and stored at -80°C, and then used to assay levels of IL-1, IL-6, or TNF- $\alpha$ . In addition the macrophage cell lines were stimulated with IFN- $\gamma$ , for 24 h as above, and in some cases further stimulated for 24 h in the presence of the Th1 clone, HDK.1 and its specific Ag KLH. In this case the supernatants were further concentrated by using an Amicon filter with a membrane of 10,000 m.w. cut-off, and depleted of IL-2 and IFN- $\gamma$ , with immunoaffinity columns. These supernatants and the flow-throughs from the concentration step were then tested for their ability to costimulate Ag-specific and APC-dependent Th1 cytokine synthesis. In addition, for use as APC the macrophage cell lines 1G18.LA and PU5.1 were first activated for 20–24 h with IFN- $\gamma$  (0.5–2 ng/ml).

**Purification and stimulation of peritoneal macrophages.** Peritoneal cells were obtained by injection and withdrawal of 7 ml of cold cRPMI/10% FCS, and macrophages were sorted on the basis of Mac-1 and B220 (macrophages are Mac-1<sup>+</sup>, B220<sup>-</sup>) as previously described (18). The cells were stimulated with 10  $\mu$ g/ml LPS in the presence or absence of IL-10, anti-IL-10 mAb, or IFN- $\gamma$ . Supernatants were collected after incubation for 20 h, at  $8 \times 10^6$  cells/ml, in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. The supernatants were assayed for TNF- $\alpha$  and IL-6 using an enzyme-linked immunoassay.

**RNA extraction and analysis of RNA.** Total cellular RNA was extracted from the macrophage cell lines with the guanidinium-isothiocyanate procedure, as described previously (41). The concentration of RNA was measured by absorption at 260 nm. RNA blot analysis was as described in Moore et al. (2) or PCR of reverse-transcribed RNA as previously described (7, 42, 43). A specific amount of each cDNA sample (dilutions of one twentieth of cDNA, as described in Results) was amplified with 2.5 U of Thermalase (*Thermus aquaticus* DNA polymerase) (IBI) and a Cetus/Perkin-Elmer thermocycler under the following conditions: 94°C melting, 30 s; 55°C annealing, 30 s; and 72°C extension, 1 min, by using specific primers for HPRT (house-keeping enzyme), TNF- $\alpha$ , and IL-6 as shown below:

HPRT sense: 5'-GTA ATG ATC AGT CAA CGG GGG AC-3' (422-444)

HPRT antisense: 5'-CCA GCA AGC TTG CAA CCT TAA CCA-3' (598-575)

HPRT probe: 5'-GCT TTC CCT GGT TAA GCA GTA CAG CCC C-3' (543-570)

TNF- $\alpha$  sense: 5'-GCG ACG TGG AAC TGG CAG AAG-3' (4499-4519)

TNF- $\alpha$  antisense: 5'-GGT ACA ACC CAT CGG CTG GCA-3' (5865-5845)

TNF- $\alpha$  probe: 5'-CAG TTC TAT GGC CCA GAC CCT C-3' (5801-5821)

IL-6 sense: 5'-CCA GTT GCC TTC TTG GGA CTG-3' (1520-1540)

IL-6 antisense: 5'-GGT AGC TAT GGT ACT CCA-3' (6093-6075)

IL-6 probe: 5'-GTG ACA ACC ACG GCC TTC CCT ACT-3' (1547-1570).

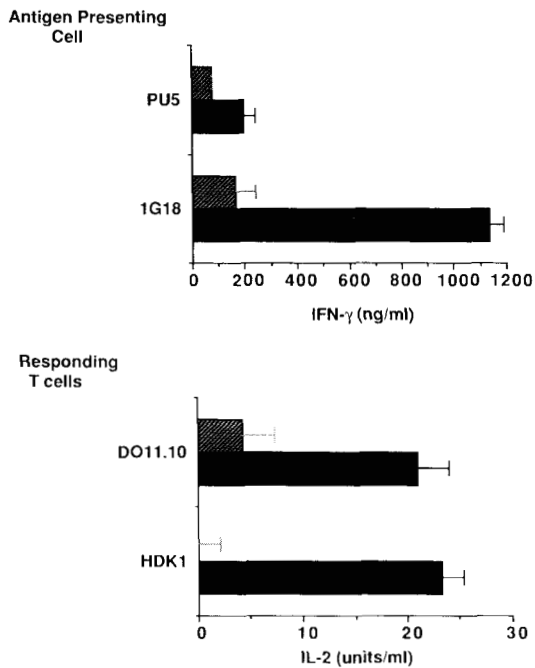
All primers spanned intervening sequences in the gene. Sensitivity and specificity were further increased by probing dot-blots of the amplified products with radiolabeled ( $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ) oligonucleotides internal to the amplified product. Radioactive blots could then be quantitated using the Ambis Image Scanner, and visualized by exposing to x-ray film. In all cases, a standard curve of P388D1 RNA was included to ensure reproducibility of the assay and arbitrary units relative to pg of input RNA in a final dot blot were obtained from it. In addition, an internal standard of the housekeeping enzyme HPRT was used, to ensure that exactly the same amount of RNA was used and that all samples were reverse transcribed and amplified by PCR at the same efficiency.

## RESULTS

**IL-10 inhibits APC function of different macrophage lines.** We have previously shown that IL-10 will inhibit the ability of APC to stimulate IFN- $\gamma$  production by Th1 cells when normal peritoneal or splenic macrophages or the macrophage cell line 1G18.LA were used as APC (18). IL-10 is also effective on the PU5.1 macrophage line (38) which has a different origin from the 1G18.LA (Fig. 1, top) (37), although the stimulation achieved using the PU5.1 cell line as APC for Th1 cells was not as great as that observed with the 1G18.LA macrophage cell line. Figure 1 (bottom) shows that the 1G18.LA cell line can also mediate Ag-specific induction of IL-2 production by both the Th1 clone (29), and the OVA-specific T cell hybridoma DO11.10 (34). Both stimulations were significantly inhibited by IL-10.

**IL-10 induces a morphologic change in IFN- $\gamma$ -stimulated peritoneal macrophages.** FACS-purified peritoneal macrophages (18), were incubated with IFN- $\gamma$  in the presence or absence of IL-10 for various periods of time. Supernatants were then removed, the cells air-dried, fixed and stained with Wright's-Giemsa. As shown in Figure 2, IL-10 induces rounding up of the cells, and de-adherence, which may be of significance with regard to the inhibition of macrophage APC function.

**IL-10 down-regulates production of biologically active or secreted cytokines by activated macrophage cell lines.** Earlier findings suggest that IL-10 has a direct effect on the ability of macrophages to function as APC and activate Th1 cytokine synthesis. For this reason the direct effect of IL-10 on monokine production by these macrophage cell lines was tested. Supernatants collected from macrophage cell lines stimulated with IFN- $\gamma$ , LPS, or IFN- $\gamma$  plus LPS, in the presence or absence of IL-10, were tested for their levels of cytokine(s). Where possible IL-4 was used as a control, because this factor has previously been shown to inhibit cytokine production by activated macrophages (44–46). Stimulation of the macrophage cell lines with IFN- $\gamma$  alone did not induce detectable levels of the monokines, IL-1, IL-6, or TNF- $\alpha$  in the



**Figure 1.** Effect of IL-10 on APC function of macrophage cell lines. (Top) 1G18.LA or PU5.1 macrophage cell lines ( $10^6$  cells/ml) were activated with IFN- $\gamma$  (2 ng/ml). These APC were then washed and incubated ( $10^5$ /well) with HDK.1 Th1 cells ( $5 \times 10^4$ /well) and antigen (TNP-KLH, 10 mg/ml), in the presence (▨) or absence (■) of purified IL-10 (200 U/ml). Supernatants were collected after 48 h and tested for IFN- $\gamma$ . (Bottom) 1G18.LA macrophages were activated, as above, and then incubated ( $10^6$ /well) with either HDK.1 Th1 cells ( $5 \times 10^4$ /well) plus TNP-KLH (10 mg/ml); or DO11.10 T cell hybridoma ( $5 \times 10^4$ /well) plus OVA (2.5 mg/ml), in the presence (▨) or absence (■) of purified IL-10 (bottom) (200 U/ml). Supernatants were collected after 20 h and tested for IL-2. In all panels, the means and SD of triplicate cultures are shown.

supernatants (data not shown). In contrast, LPS, or LPS plus IFN- $\gamma$  induced significant levels of these cytokines (Fig. 3). The D10.G4.1 assay used to detect IL-1, performed in the absence of Con A, does not detect other cytokines expressed by macrophages (36). The 1G.18.LA and PU5.1 macrophage cell lines were both significantly inhibited in their ability to produce IL-1 bioactivity after induction with LPS (Fig. 3). IL-10 also had a very significant inhibitory effect on LPS, or IFN- $\gamma$  plus LPS induced production of TNF- $\alpha$  in the supernatants of both macrophage cell lines, as demonstrated in the WEHI.164.13. bioassay (all the activity in these macrophage supernatants was shown to be attributable to TNF- $\alpha$  using a specific blocking antibody, data not shown). Similarly, IL-10 inhibited the production of IL-6 protein by the macrophage cell lines, stimulated by IFN- $\gamma$  plus LPS and/or LPS induced as measured in an enzyme-linked immunoassay for IL-6. In all experiments tested IL-10 had a much more significant inhibitory effect than IL-4 on LPS or IFN- $\gamma$  plus LPS-induced monokine production (at the protein level).

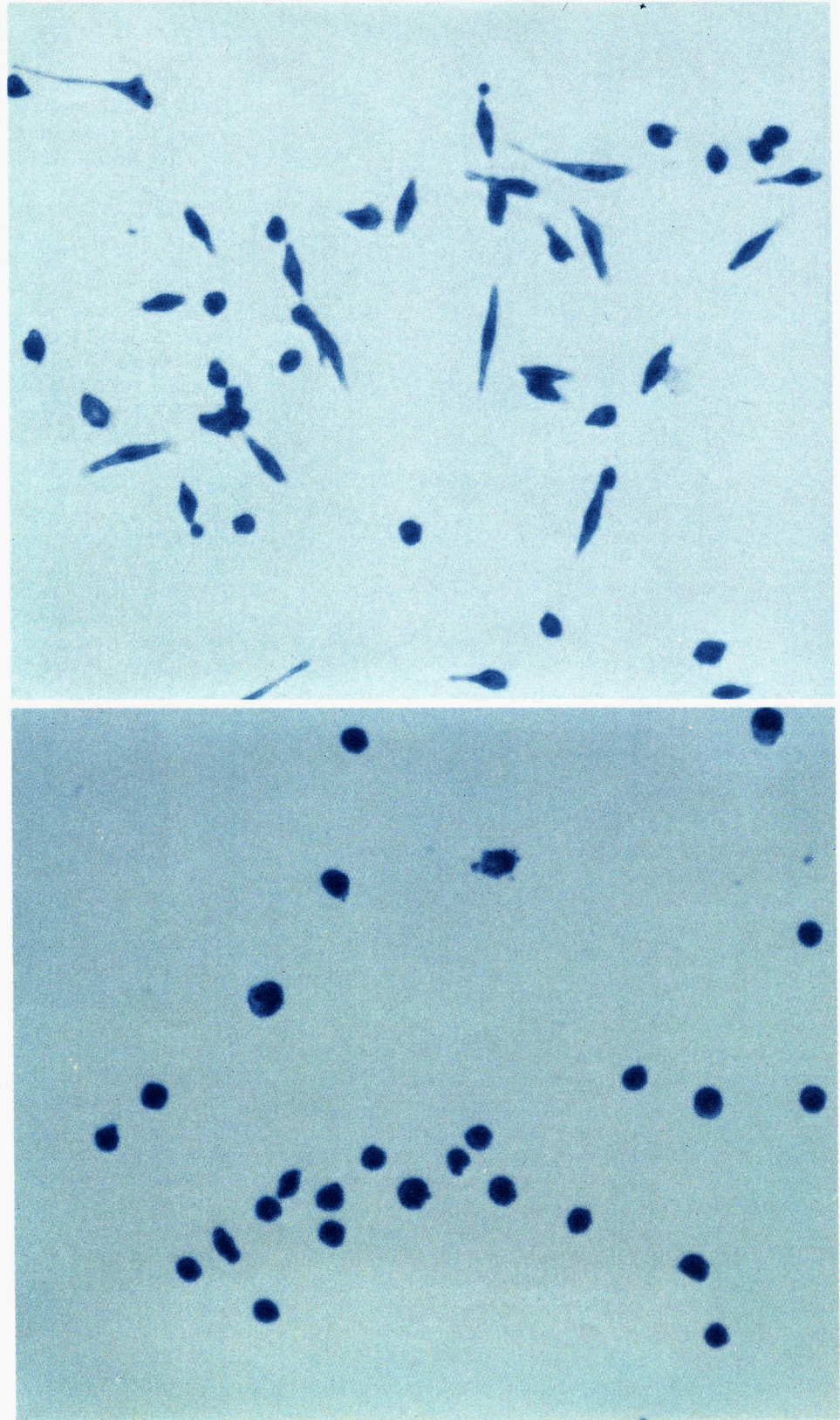
**IL-10 down-regulates cytokine mRNA expression by activated macrophages.** RNA was extracted from the macrophage cell lines, 1G18.LA and PU5.1, 6 h after stimulation with LPS or LPS and IFN- $\gamma$ , in the presence or absence of IL-10 or IL-4, as described in *Materials and Methods*. RNA blot analysis of 10  $\mu$ g total RNA revealed that IL-10 down-regulated expression of TNF- $\alpha$  RNA, induced by LPS or IFN- $\gamma$  plus LPS, in both cell lines (Fig. 4), albeit to a lesser extent in the latter case. This was also demonstrated using a semiquantitative PCR method

for analysing reverse-transcribed RNA, based on a modification by Dallman et al. (47) from both cell lines. By including a standard curve for each cytokine (not shown) as described in Table I, it was possible to obtain arbitrary units relative to the pg of total standard RNA represented in each dot and thus present the data numerically. The data shown in Table I, show that IL-10 and IL-4 inhibit LPS- and IFN- $\gamma$  plus LPS-induced expression of TNF- $\alpha$  in the 1G18.LA macrophage cell line. This can be observed best with values obtained from the linear part of the standard curve (not shown), and Table I legend explains the method used to derive the numerical data. Analysis of RNA expression by the PU5.1 macrophage cell line in response to LPS and IFN- $\gamma$  plus LPS, by using the same method, also showed that expression of IL-6 and TNF- $\alpha$  was also inhibited by IL-10 (Table II).

**IL-10 down-regulates production of IL-6 protein by LPS-activated peritoneal macrophages.** Peritoneal macrophages (sorted on the basis of Mac-1 and B220) (macrophages are Mac-1<sup>+</sup>, B220<sup>-</sup>) which we have shown previously to be inhibited by IL-10 for APC function to Th1 cells (18), were also tested for their production of TNF- $\alpha$  and IL-6 in response to LPS. Unfortunately, TNF- $\alpha$  was not detectable in the supernatants of LPS-stimulated FACS sorted macrophages (cell density,  $7 \times 10^5$ /ml). In contrast, significant levels of IL-6 were detectable in supernatants obtained from peritoneal macrophages from BALB/c or CBA/J mice, stimulated with LPS as above (Fig. 5). IL-6 levels were reduced if cells were stimulated by LPS in the presence of IL-10, but surprisingly, the level of inhibition was less marked (Fig. 5) than that observed with the macrophage cell lines (Fig. 3). However, the level of IL-6 production induced by LPS could be increased by the inclusion of a monoclonal antibody directed against IL-10, in the LPS stimulation (Fig. 5). This suggested that macrophages produced IL-10 in response to LPS and this was confirmed in a specific ELISA for IL-10 (CBA/J or BALB/c peritoneal macrophages stimulated with LPS as above, produced 2 U/ml or 4.5 U/ml of IL-10, respectively).

Figure 5 also shows that purified BALB/c peritoneal macrophages produce higher levels of IL-6, when stimulated with LPS in the presence of IFN- $\gamma$ , as opposed to when stimulated with LPS alone. This may be explained by a further experiment on IL-10 production by peritoneal macrophages from BALB/c mice. In this case, macrophages stimulated with LPS produced 12 U/ml IL-10, and this was reduced to less than 3 U/ml if the cells were stimulated with LPS in the presence of IFN- $\gamma$ .

**Possible mechanisms for IL-10 inhibition of macrophage APC function.** We have previously shown that IL-10 will only inhibit Th1 cytokine synthesis in the presence of live APC (macrophages) (18). A possible mechanism of IL-10 action is suppression of production of a soluble cofactor needed for optimal cytokine release from Th1 cells. Supernatants were obtained from macrophages stimulated with IFN- $\gamma$ , in the presence or absence (not shown) of Th1 cells and specific Ag, as described in *Materials and Methods*. Such supernatants (Fig. 6A), or the flow throughs generated during their concentration (data not shown), were unable to reverse the IL-10-mediated inhibition of macrophage APC function for Th1 cells. These data provide no evidence that IL-10 down-regulates a soluble costimulator required for



*Figure 2.* IL-10 induces a morphologic change in purified peritoneal macrophages. Peritoneal macrophages (Mac-1<sup>+</sup><sub>bright</sub>, B220<sup>-</sup>) were purified on the FACS and then incubated for 72 h, in the presence of IFN- $\gamma$  (2 ng/ml) (*top*), or IFN- $\gamma$  (2 ng/ml) plus IL-10 (200 U/ml) (*bottom*). Supernatants were removed, the cells air-dried, fixed and stained with Wright's-Giemsa, dried, and then photographed.

Th1 cytokine synthesis. Similar experiments provide no evidence that IL-10 induces macrophages to produce a soluble inhibitory factor which acts directly on the T cell (not shown), although such an inhibitor may be labile or membrane bound. To test this a cell mixing experiment was performed as follows. Splenic B cells and macrophages were FACS sorted (on the basis of B220 and Mac-

1) as previously described (18). Graded doses of macrophages, in the presence or absence of B cells, and also in the presence or absence of IL-10, were used as APC for Ag-specific stimulation of HDK.1 cells. Macrophage but not B cell stimulation of Th1 cytokine synthesis was inhibited by IL-10, as previously described (Fig. 6B). Mixing of graded doses of macrophages, with a constant

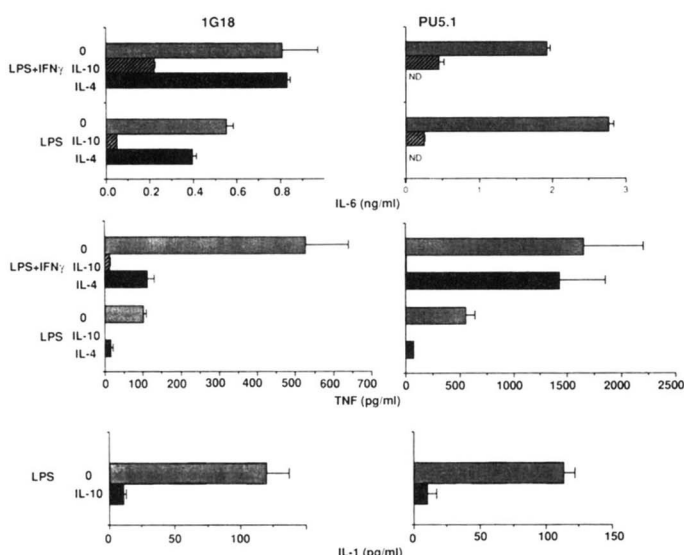


Figure 3. IL-10 inhibits LPS-induced production of IL-1, TNF- $\alpha$ , and IL-6 proteins by macrophage cell lines. The macrophage cell lines 1G18.LA and PU5.1 were incubated ( $10^6$  cells/ml) with LPS ( $10 \mu\text{g/ml}$ ); or LPS ( $10 \mu\text{g/ml}$ ) plus IFN- $\gamma$  ( $2 \text{ ng/ml}$ ); in the presence or absence of IL-10 or IL-4 (in some cases) both at  $200 \text{ U/ml}$ , as indicated. Supernatants were collected and tested for their levels of IL-1, IL-6, or TNF- $\alpha$  as described in *Materials and Methods*.

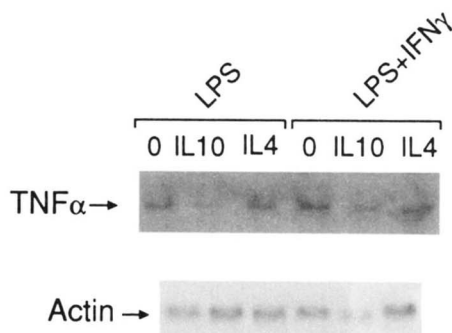


Figure 4. IL-10 inhibits the LPS-induced expression of TNF- $\alpha$  RNA in a macrophage cell line. The 1G18.LA macrophage cell line was stimulated as in Figure 3. Supernatants were removed after 6 h and guanidinium isothiocyanate solution was added for harvest of the cells for RNA preparation. Samples of  $10 \mu\text{g}$  of total RNA of the 1G18.LA cell line stimulated accordingly was loaded on a gel, blotted, and then probed for TNF- $\alpha$  and actin.

number of B cells, gave an additive stimulation of Th1 cytokine synthesis (Fig. 6B). However, addition of IL-10 to this mixture of APC brought the level of Th1 cytokine synthesis only down to the level achieved with B cell APC

alone, an observation that argues against the presence of a short range or labile inhibitor which acts directly on the Th1 cell, or the B cell APC.

DISCUSSION

We have previously shown that IL-10 inhibits the ability of macrophages to stimulate Th1-T cell clones to produce IFN- $\gamma$  (18). In the present study we present data which demonstrate that IL-10 has an inhibitory effect on LPS-induced cytokine production by macrophage cell lines (Fig. 3) and peritoneal macrophages (Fig. 5). These data suggest that IL-10 may not only have an important role in the regulation of T cell responses, but in addition on inflammatory responses elicited during infection or injury.

The effect of IL-10 on the macrophage cell lines is more pronounced than that elicited by similar amounts of IL-4, which has previously been shown to inhibit cytokine production by both murine and human macrophages and monocytes (44–46). Our results show a significant inhibition of LPS-induced TNF- $\alpha$  protein production by IL-4, but a less marked inhibition of IL-6 synthesis, in these cell lines. However, inhibition of TNF- $\alpha$  by IL-4 is not as great as that previously reported for human monocytes (44, 45). This difference may be explained either by the difference in species, the use of differentiated macrophage cell lines rather than monocytes, or that we are used  $10 \mu\text{g/ml}$  of LPS as opposed to the  $100 \text{ ng/ml}$  dose used in one of the human monocyte systems (44). In contrast to IL-4, IL-10 significantly inhibited IL-6, TNF- $\alpha$ , and IL-1 protein production at this high concentration of LPS. Stimulation of the macrophage cell lines with LPS and IFN- $\gamma$  induced a much higher level of TNF- $\alpha$  and IL-6 and in some cases this was refractory to IL-4 action, suggesting that IL-4 and IFN- $\gamma$  may have opposite and counteracting effects on certain aspects of macrophage activation. Again this is in contrast to the studies on human monocytes, although those studies were performed with lower amounts of LPS (44) than used in our system. Once more the IL-10-mediated inhibition of IFN- $\gamma$  plus LPS-induced production of IL-6 and TNF- $\alpha$  protein was more significant than that seen with IL-4. Inhibition of LPS- or IFN- $\gamma$  plus LPS-induced expression of RNA encoding IL-6 and TNF- $\alpha$  was also observed by using a semiquantitative PCR amplification method for reverse-transcribed RNA (47). In some cases, IL-4 inhibited expression to a similar extent as IL-10 (Fig. 4, Table I,

TABLE I  
Inhibition of LPS-induced TNF- $\alpha$  expression in 1G18.LA macrophage cell line by IL-10 and IL-4<sup>a</sup>

Stimulus	cDNA Dilution Factor	0		IL-4			IL-10		
		cpm	*pg RNA	cpm	*cpm	*pg RNA	cpm	*cpm	*pg RNA
LPS + IFN- $\gamma$	1:9	1260	300	1987	2682	Plateau	1232	1034	200
	1:27	984	160	601	769	110	713	589	90
	1:81	722	110	358	332	57	411	312	50
LPS	1:9	1114	210	1620	1587	330	1387	1068	200
	1:27	896	120	517	403	60	460	271	45
	1:81	472	72	480	403	60	310	220	33

<sup>a</sup> The 1G18.LA macrophage cell line was stimulated as in Figure 3. Supernatants were removed after 6 h and guanidinium isothiocyanate solution was added for harvest of the cells for RNA preparation. One microgram of total RNA from the 1G18.LA macrophage cell line was reverse transcribed in a total of  $20 \mu\text{l}$ . Dilutions were made of the cDNA (shown in the figure) and  $1 \mu\text{l}$  of each dilution was amplified by PCR with specific primers for HPRT (internal standard) or TNF- $\alpha$ . Ten microliters of the amplified cDNA product was dot-blotted and further probed with each respective radioactively labeled probe (internal to the primers). The signal shown was measured with the use of an Ambis Image Scanner. For each dilution, the raw cpm for TNF- $\alpha$  were standardized (\*cpm) according to a correction factor (\*) obtained from the HPRT cpm relative to the "0" sample (i.e., no IL-10 or IL-4). Arbitrary units, relative to a titration of the positive control, and corrected for their HPRT content (\*pg RNA/dot) could then be assigned by using a standard curve (not shown).

TABLE II  
*IL-10 Inhibits LPS-induced expression of RNA encoding IL-6 and TNF- $\alpha$  in the PU5.1 macrophage cell line<sup>a</sup>*

Cytokine RNA Tested	Stimulus							
	LPS + IFN- $\gamma$				LPS			
	+0		+IL-10		+0		+IL-10	
	cpm	*pg RNA	cpm	*pg RNA	cpm	*pg RNA	cpm	*pg RNA
TNF- $\alpha$	1031	370	587	80	1864	730	469	23
IL-6	589	1000	207	700	108	90	27	44

<sup>a</sup> Samples of PU5.1 were treated essentially as described in Table I and analyzed for HPRT, TNF- $\alpha$ , and IL-6 levels. The raw cpm shown above were first corrected according to the levels of HPRT per sample as described in Table I. By using the standard curves of known amounts of positive controls for TNF- $\alpha$  or IL-6, an arbitrary unit \*pg RNA per dot blot, was assigned to it as described in Table I. Values for both TNF- $\alpha$  were obtained from a 1 in 27, and IL-6 from a 1 in 9 dilution of 1  $\mu$ l of cDNA (1  $\mu$ g of RNA was reverse transcribed in a total volume of 20  $\mu$ l).

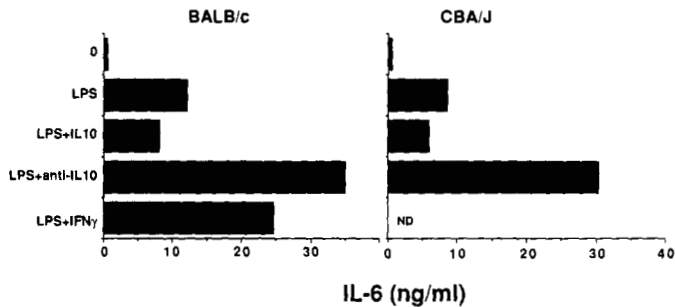


Figure 5. IL-10 inhibits LPS-induced synthesis of IL-6 protein by peritoneal macrophages. Peritoneal macrophages (Mac-1<sup>+</sup>, bright, B220<sup>-</sup>) were purified on the FACS and then incubated alone, or with LPS (10  $\mu$ g/ml) in the presence or absence of IL-10 (200 U/ml), anti-IL-10 (10  $\mu$ g/ml), or IFN- $\gamma$  (2 ng/ml), at  $7 \times 10^5$  cells/ml. Supernatants were harvested and tested for their levels of IL-6, relative to a known standard using a specific immunoassay (ELISA).

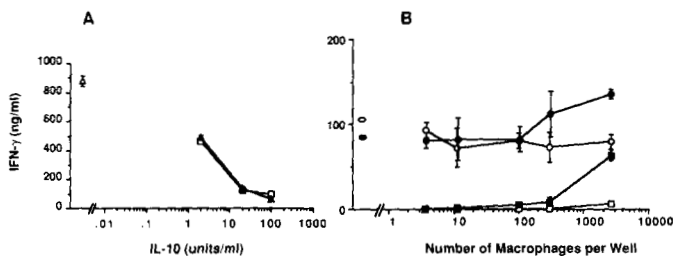


Figure 6. IL-10 does not down-regulate a soluble costimulator or induce a soluble inhibitor from the macrophage. A, Supernatants were obtained from the 1G18.LA macrophage cell line ( $10^6$ /ml) after stimulation with IFN- $\gamma$  for 24 h, and then further stimulation with Th1 cells (HDK-1,  $2 \times 10^5$ /ml) and antigen (KLH, 500  $\mu$ g/ml), for 24 h. The supernatants were concentrated and depleted of IFN- $\gamma$  and IL-2, as described in *Materials and Methods* and then used in a CSIF assay as follows. (These supernatants alone did not contain detectable levels of IFN- $\gamma$  themselves.) IFN- $\gamma$ -activated 1G18.LA macrophage APC ( $10^5$ /well) were incubated with HDK-1 cells ( $5 \times 10^4$ /well) and antigen (TNP-KLH, 10  $\mu$ g/ml) alone ( $\Delta$ ) or in the presence of varying doses of IL-10 plus ( $\square$ ) or minus ( $\blacktriangle$ ) the above supernatants (final 25% concentration). Supernatants from the CSIF assay were collected after 48 h and assayed for IFN- $\gamma$  levels. The means and SD of triplicate cultures are shown. B, Graded doses of purified splenic macrophages, in the absence ( $\blacksquare$ ), or presence of IL-10 ( $\square$ ) (200 U/ml); or a mixture of graded doses of purified macrophages together with B cells ( $2.5 \times 10^5$ /well) in the absence ( $\bullet$ ) or the presence of IL-10 ( $\circ$ ) (200 U/ml); or B cells alone in the absence (left upper oval) or presence of IL-10 (left lower oval) (200 U/ml) were used as APC for the HDK-1 Th1 clone ( $10^4$ /well) with TNP-KLH (10  $\mu$ g/ml). After 48 h supernatants were collected and assayed for IFN- $\gamma$ . The means and SD of triplicate cultures are shown.

and data not shown), suggesting that IL-10 may also have an effect on secretion or stability of the translated proteins. However IL-10 achieves its effects, it would appear that its final inhibitory effect on cytokine production by macrophages is more potent than that seen with IL-4. The effects of IL-10 on monokine production suggest that this powerful inhibitor may have potential as an anti-inflammatory agent perhaps in a wide variety of clinical

manifestations.

Inasmuch as we had also shown that peritoneal macrophages were inhibited by IL-10 from stimulating Th1 cells (18), we attempted to see whether IL-10 inhibited cytokine secretion by this purified cell population. FACS-purified peritoneal macrophages obtained from BALB/c or CBA/J mice, stimulated with LPS produced significant levels of IL-6 protein which was only slightly reduced in the presence of IL-10 (Fig. 5). Since we had preliminary PCR data suggesting that purified macrophages produced IL-10 (not shown) we also included an antibody directed against IL-10 in our LPS stimulations. Addition of this antibody to LPS stimulations in both strains of mouse, elevated the production of IL-6 protein to a much higher level (30–35 ng/ml per  $7 \times 10^5$  cells/ml, in 20 h). This suggests that cytokine production by macrophages is under tight autocrine control, or that more than one population of macrophages is contained in the Mac-1<sup>+</sup>, bright peritoneal macrophages, one of which has control over the other by its production of IL-10. Data obtained in parallel in the human monocyte system by De Waal Malefyt et al. (48), also shows that IL-10 inhibits LPS-induced cytokine production, including the production of IL-10 itself, by human monocytes purified by elutriation (48). In accordance with previously reported contrasting effects of Th2 Th cell derived cytokines such as IL-4 and IL-10 with Th1 Th cell cytokines such as IFN- $\gamma$  (13, 14, 18) we provide further evidence that these cytokines may have counteracting effects on each other. IFN- $\gamma$  increased the level of IL-6 produced in response to LPS, to almost as high a level as that achieved by anti-IL-10, by ostensibly inhibiting the production of IL-10 by the same macrophages (Results and Fig. 5). These data suggest that production of cytokines such as IL-6 and TNF- $\alpha$  is regulated by IL-10, which in turn is under the control of IFN- $\gamma$  produced by activated T cells and NK cells (49). This action of IFN- $\gamma$  may explain our previous data showing that incubation of peritoneal macrophages with IFN- $\gamma$  for 24 h improved their capacity to stimulate Th1 cells (18).

The question regarding how IL-10 inhibits the macrophage from stimulating Th1 cells from synthesizing IL-2 and/or IFN- $\gamma$  still remains. In view of the significant decrease in cytokine synthesis observed when macrophages are stimulated in the presence of IL-10 it is possible that IL-10 down-regulates a costimulatory activity needed for optimal Th1 cytokine secretion in response to macrophages and Ag. By using supernatants obtained from the macrophage cell line 1G18.LA stimulated as described previously, it was not possible to overcome the inhibitory effect of IL-10 on macrophage and Ag-dependent stimulation of Th1 cells (Fig. 6A). This suggests that

IL-10 does not mediate its effects on Th1 cytokine synthesis by down-regulation of a soluble costimulator. It is still possible that IL-10 interferes with the action rather than the production of such a factor, or that such a costimulator is highly labile or absorbed, and thus not present in our supernatant preparations. Alternatively, IL-10 may inhibit the expression of a membrane-bound costimulator, which would also explain previous reports suggesting that stimulation of Th1 cells requires an APC/accessory cell which cannot be replaced by a soluble costimulator (50–52). An alternative explanation for the mechanism of IL-10 inhibition of Th1 cytokine synthesis could be that IL-10 induces production of an inhibitory factor(s) by the macrophage, which then acts on the T cell to inhibit cytokine secretion. Figure 6B shows that mixing of macrophage and B cell APC in an Ag-specific system for stimulation of Th1 cells leads to an additive stimulation of the T cell, as compared to stimulation with each individual APC. The presence of IL-10 inhibits cytokine synthesis by the Th1 cell to the level that the B cell APC achieves on its own. This suggests that the macrophage does not induce the production of an inhibitor which acts directly on the T cell, or the B cell APC. Although unlikely, it is still possible that such an inhibitor may either be specific for macrophage-T cell interactions, or that the B cell APC somehow overcomes or evades the effect of such an activity. Again, data obtained in the human system by De Waal Malefyt et al. (53) suggest that IL-10 does not achieve its inhibition of Th cell proliferation via a soluble inhibitory or costimulatory factor produced by the monocyte. However, their effects can be explained by the down-regulation of MHC-Class II antigens by IL-10 on human monocytes (53), which we as yet have not observed in the mouse system (18).

We have previously suggested that (18) in addition to regulation of effector function, IL-10 may also play a role in the initiation of an immune response toward antibody production or DTH, by activation of different subsets of CD4 Th cells producing different patterns of cytokines (18). That both B cells and macrophages produce IL-10, and also function as APC, while being sensitive to different cytokine modulators (IFN- $\gamma$  inhibits many B cell functions (54, 55); IL-10 inhibits macrophage but not B cell APC function (18)) lends support to this theory. In addition our present study shows that IL-10 has a significant inhibitory effect on cytokine synthesis by macrophages, as well as causing a marked morphological change in peritoneal macrophages. Taken together, our observations suggest an important role for IL-10, not only in the regulation of T cell responses but also as an important modulator of acute inflammatory responses elicited by infection or injury.

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