

# Recruitment of STAT3 for Production of IL-10 by Colon Carcinoma Cells Induced by Macrophage-Derived IL-6<sup>1</sup>

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The immunosuppressive cytokine IL-10 is associated with poor prognosis in colon cancer. Although macrophages are involved in antitumor defenses, production of IL-10 by tumor cells may permit malignant cells escape to cell-mediated immune defenses. To investigate interactions between macrophages and tumor cells in humans, we cultured macrophages isolated from patients and tested the effect of these macrophages on the production of IL-10 by several tumor cell lines. Macrophages were isolated from pleural effusions of patients with malignancy and from noncancer control patients. We demonstrated that culture supernatants of macrophages from both sources strongly stimulated IL-10 production by the three different human colon adenocarcinoma cell lines, Colo 205, Colo 320, and HT29. Recombinant IL-6, but not IL-10, TNF- $\alpha$ , and IFN- $\alpha$ , stimulated the secretion of IL-10 by colon tumor cells. mAbs against IL-6 and IL-6R prevented the effect of macrophage culture supernatants and of rIL-6, respectively, on the production of IL-10 by the three cell lines. Cocultures of macrophages and colon cancer cells showed that these tumor cells first stimulated macrophages to produce IL-6, which was then followed by IL-6-induced IL-10 production by colon cancer cells. Finally, we showed that IL-10 gene regulation was mediated by STAT3, which was phosphorylated after the binding of IL-6 to IL-6R. This is the first demonstration that IL-6, secreted by macrophages, can induce a STAT3-mediated IL-10 production by colon tumor cells. *The Journal of Immunology*, 2004, 172: 4630–4636.

Cytokines are key regulatory molecules of the immune system, and play an important role in antitumor defense (1). Despite the high infiltration into tumor sites by leukocytes, malignant cells frequently evade immune surveillance. It has been demonstrated that some malignant cells can respond to macrophage products by secreting cytokines themselves, such as IL-10 (2), TGF- $\beta$ , and IL-6 (3, 4), resulting in tumor escape from immune system defenses. Some reports have focused on mechanisms of macrophage activation (5–7), but only few studies have evaluated interactions between products secreted by macrophages and tumor cells (8).

IL-10 is produced by various immunocompetent cells, as well as by human cell lines derived from carcinoma of breast, pancreas, kidney, and colon, as well as neuroblastoma (9, 10). IL-10 exhibits various immunosuppressive effects *in vivo*, and seems to play a crucial role in colon cancer. Circulating levels of IL-10 were found to be high in colon cancer patients (11), and IL-10 was shown to be a useful marker for predicting both surgical removal of the tumor and tumor recurrence (12).

The role of IL-6 in neoplastic disease seems to be more complex. This cytokine is produced by a variety of normal and malignant cells. Human IL-6 has been reported to enhance tumor cell growth in myelomas (13) and can promote tumor cell motility *in vitro* (14). High levels of IL-6 were present in ascites of patients

with ovarian and renal cancer (15) and in serum of colon cancer patients (11). Complex relationships between IL-10 and IL-6 were often shown, but the interaction between these two cytokines has not been fully explained. IL-6 binds to a hexameric receptor, composed of an IL-6-specific receptor ( $\alpha$ -chain, IL-6R $\alpha$ ) and a signal transducer, gp130, which is common to other IL-6 family members (16). The IL-6 signal transduction is mediated by the Janus kinase/STAT pathway. Interaction of IL-6 with its receptor induces phosphorylation of STAT3 protein, which binds directly to target genes (17). Constitutive activation of STAT3 is associated with ovarian cancer in human (18) and with severe colitis in mice (19).

Earlier studies have reported an inverse association between the number of macrophages in the pleural effusions of cancer patients and the extent of malignant disease (20–22). Our objective was to study soluble factors produced by human macrophages and determine whether they were capable of stimulating the secretion of immunosuppressive cytokines by tumor cells. We sampled pleural effusions from patients with and without cancer, and used human tumor cell lines as targets. Macrophages isolated from pleural fluids were cultured, and supernatants were used as conditioned medium (CM)<sup>3</sup> for cultures of human cell lines derived from carcinomas of colon (Colo 205, Colo 320, and HT29), breast (MCF7), lung (A549), prostate (PxC3), and cervix (HEC1). We investigated whether IL-6 produced by these macrophages contributes to IL-10 production by colon tumor cell lines. Finally, the level of STAT3 activation was determined in Colo 205 tumor cells.

## Materials and Methods

### Collection of macrophages

Pleural fluids containing tumor cells and leukocytes were collected by drainage thoracocentesis from nine patients displaying effusions related to various cancers (three with pleura mesothelioma, four with lung adenocarcinoma, one with breast adenocarcinoma, and one with colon adenocarcinoma) and from five patients with congestive heart failure as tumor-free

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<sup>3</sup> Abbreviation used in this paper: CM, conditioned medium.

controls, as previously described (20). The thoracocentesis procedure was approved by the Institutional Review Board at the University Hospital Center (Saint-Etienne, France), and signed informed consent was obtained from all the cancer and cardiac patients.

### Preparation of CM

Pleural fluids were centrifuged at  $400 \times g$  for 10 min at  $4^{\circ}\text{C}$ , and cell pellets were resuspended in RPMI 1640 medium containing 20 mM HEPES buffer (Life Technologies, Paisley, U.K.), 5% human AB serum (Western States Plasma-Seracare, Oceanside, CA), 2 mM L-glutamine (Eurobio, les Ulis, France), and antibiotics (penicillin G at 100 U/ml, streptomycin at 100  $\mu\text{g}/\text{ml}$ , and amphotericin B at 0.25  $\mu\text{g}/\text{ml}$ ) (Life Technologies). Macrophage-like cells were allowed to adhere to 75-cm<sup>2</sup> tissue culture flasks (Falcon, Oxnard, CA) at  $37^{\circ}\text{C}$  in an incubator in a humidified atmosphere of 5%  $\text{CO}_2$  for 2 h. Nonadherent cells were removed by two washings with Dulbecco's PBS 0.02% EDTA (Eurobio) at room temperature. The remaining adherent cells were 95% macrophages by light microscopy and May-Grünwald-Giemsa staining (23).

We then plated macrophages at  $5 \times 10^5$  cells/ml culture medium. Adherent cells were cultured for 48 h in the same medium at  $37^{\circ}\text{C}$  in an incubator with a humidified atmosphere of 5%  $\text{CO}_2$ . Supernatants were centrifuged at  $400 \times g$  for 10 min at  $4^{\circ}\text{C}$  and stored as aliquots at  $-80^{\circ}\text{C}$  until use. After thawing, supernatants were filtered through 0.22- $\mu\text{m}$  (pore size) filters and were added as CM to the culture medium of various tumor cell lines.

### Tumor cell culture conditions

Human colon (Colo 205, Colo 320, and HT29), breast (MCF7), lung (A549), prostate (Pc3), and cervical (HEC1) cell lines were purchased from European Collection Cell Culture (Salisbury, U.K.). Tumor cells were cultured under the same conditions used for macrophage cultures, except that RPMI 1640 medium was supplemented with 10% heat-inactivated FCS (Life Technologies). Cells were plated at different numbers in 24-well plates (Falcon) and stained with trypan blue, and viable cells were counted by use of a hemacytometer. Exponential growth was observed at a plating density of 50,000 cells/well. Cells were cultured for an additional 4 days with 50% CM. No contamination with mycoplasma was detected by culture in Hayflick medium (24).

### Macrophage and tumor cell coculture conditions

From our IL-10 production results obtained comparing the three human colon cell lines, we selected the Colo 205 cell line as well-adapted target. Macrophages from patients were seeded at a density of  $5 \times 10^5$  cells/ml, and 0.25, 0.5, 1, or  $2 \times 10^6$  Colo 205 cells/ml were then added to the cultures. Using this approach, we selected four different ratios of macrophages-Colo 205 cells: 1:0.5, 1:1, 1:2, and 1:4. Macrophages and Colo 205 cells were cultured alone as controls. These cocultures were conducted for 2 days in the same manner as described above, and the supernatants were tested for the IL-6 and IL-10 levels.

### Cytokine quantification by ELISA

Levels of IL-6, IL-10, TGF- $\beta$ , and TNF- $\alpha$  were measured in each CM and cell culture supernatant by ELISA, using commercial kits (R&D Systems, Abingdon, U.K.), according to the manufacturer's instructions. Positive controls were supplied in the kit. The IFN- $\alpha$  level was quantified using a high sensitivity commercial kit (BioSource International, Camarillo, CA). The OD was measured at 492 nm on a Titertek Multiskan reader (Flow Laboratories, Irvine, U.K.). Detection ranges of ELISA kits were as follows: 3–300 pg/ml for IL-6, 7–500 pg/ml for IL-10, 31–2000 pg/ml for TGF- $\beta$ , 15–1000 pg/ml for TNF- $\alpha$ , and 12–500 pg/ml for IFN- $\alpha$ . The cytokine content of each of the 14 CM was tested three times. Levels of cytokines were expressed in pg/ml. Cytokines were not detected in the medium used to culture these cells.

### Effects of recombinant cytokines and blocking Abs

Human recombinant IL-6, IL-10, TNF- $\alpha$ , and IFN- $\alpha$  were purchased from PeprōTech (Rocky Hill, NJ). Recombinant IL-6, IL-10, TNF- $\alpha$ , and IFN- $\alpha$  were used at a range from 50 to 40 ng/ml. Human IL-6-blocking and anti-IL-6R mAbs were purchased from R&D Systems. To prevent the effect of IL-6, 1  $\mu\text{g}$  of anti-IL-6 mAb was added to CM or to rIL-6 (10 ng/ml) at  $4^{\circ}\text{C}$  1 h before addition to Colo 205 cells in cultures. To perform the IL-6R-blocking assay, Colo 205 cells were cultured for 4 days in the presence of CM and 10 ng/ml rIL-6 and with or without 10  $\mu\text{g}/\text{ml}$  anti-IL-6R-blocking mAb, as well as with isotype control Ab.

### Measurement of IL-6 and IL-10 mRNA production by RT-PCR

Total RNA was extracted from  $10^6$  Colo 205 cells or  $5 \times 10^5$  macrophages, according to the instructions for use of TRIzol reagent (Life Technologies). Reverse transcription was performed with Super Script Preamplification System for first-strand cDNA synthesis (Life Technologies) using a PerkinElmer (Wellesley, MA) thermocycler. A quantitative IL-10 mRNA expression by competitive RT-PCR was performed to detect low concentration of transcripts with 1  $\mu\text{g}$  of cDNA using a DNA thermal cycler (PerkinElmer) for 33 cycles. A cycle profile consisted of 1 min at  $95^{\circ}\text{C}$  for denaturation, 1 min at  $55^{\circ}\text{C}$  for annealing, and 1 min at  $72^{\circ}\text{C}$  for primer extension. Oligonucleotide primers for human IL-10 were purchased from BioSource International. An internal standard (internal cDNA standard IL-10; BioSource International) was used in competition with IL-10 to ensure the efficiency of the reaction and to evaluate the amount of IL-10 transcript in each sample. A similar intensity between the IL-10 cDNA band and competitor band indicates that the number of copies was the same. Using this method, we determined different levels of IL-10 mRNA production for each culture condition.

A semiquantitative measurement of IL-6 mRNA production was performed using RT-PCR. Oligonucleotide primers for human IL-6 (260 bp) and  $\beta_2$ -microglobulin (180 bp) were produced by M. Dy (25). The housekeeping gene  $\beta_2$ -microglobulin was used as reference for the quantification of the IL-6 transcripts. The cycle profile (30 cycles) consisted in 1-min time period at  $95^{\circ}\text{C}$  for denaturation, 1 min at  $55^{\circ}\text{C}$  for annealing, and 1 min at  $72^{\circ}\text{C}$  for primer extension. All PCR-amplified products were run on 1.5% agarose gel containing 0.5  $\mu\text{g}/\text{ml}$  ethidium bromide and were visualized under UV light.

### Tyrosine phosphorylation analysis

After a 24-h starvation, cells were stimulated for 10 min in presence of 10 ng/ml indicated cytokine. After stimulation, cells were lysed in 10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, proteinase inhibitors (1  $\mu\text{g}/\text{ml}$  pepstatin, 2  $\mu\text{g}/\text{ml}$  leupeptin, 5  $\mu\text{g}/\text{ml}$  aprotinin, 1 mM PMSF), and 1% Nonidet P-40 (26). After pelleting insoluble material, the supernatants were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane (Millipore, Bedford, MA). Membranes were subsequently incubated overnight with an anti-phospho-STAT3 Tyr<sup>705</sup> Ab (New England Biolabs, Beverly, MA) before being incubated with a second Ab coupled to peroxidase for 60 min. Reaction was visualized on x-ray film using the ECL reagent (Amersham, Les Ulis, France), according to the manufacturer's instructions. Membranes were stripped in 0.1 M HCl glycine, pH 2.5, for 1 h, neutralized in 1 M Tris-HCl, pH 7.6, before reblotting (anti-STAT3; Santa Cruz Biotechnology, Santa Cruz, CA).

### Transient transfection and reporter gene activity

The lipid reagent Fugene 6 (Roche Diagnostics, Meylan, France) was used for all transfections. The cDNA-encoding STAT3 $\beta$  was in Pcdna3 expression vector. For each experiment, a control plate was transfected with a  $\beta$ -galactosidase-encoding expression vector. Transient transfection of Colo 205 cells was conducted in 24-well culture plates using Fugene 6 transfection reagent. Cells were transfected with 300 ng of sis-inducible element-luciferase reporter gene alone or together with a vector encoding STAT3 $\beta$ , as described previously (27). Forty-eight hours after transfection, cells were incubated with medium alone or rIL-6. Transfected cells were washed twice with ice-cold PBS, and 100  $\mu\text{l}$  of lysis buffer was added to the cells (0.1 M  $\text{KH}_2\text{PO}_4$ , pH 7.8, 0.1% Triton X-100). Extracts were then used directly to measure the luciferase activity by integrating total light emission over 10 s using a Packard Topcount luminometer (Packard Instrument, Meriden, CT). Luciferase activity was normalized based on protein concentration.

### Statistical analysis

Data are expressed as the mean of three to six experiments, each in triplicate samples for individual treatments or dosage. All comparisons of data were made using Student's *t* test and were considered to be statistically significant at  $p < 0.05$ . Values are represented as the mean  $\pm$  SE.

## Results

### Cytokine content in CM from macrophage cultures

Macrophages were isolated from pleural effusions from cancer patients and control patients with congestive heart failure (tumor-free controls). Macrophages were cultured for 2 days, and the content of cytokines was determined using ELISA. High levels of IL-6

were detected in culture supernatants from macrophages isolated from cancer ( $18,160 \pm 3,014$  pg/ml) and cardiac patients ( $17,972 \pm 2,500$  pg/ml) after 2 days of culture, without any significant differences ( $p < 0.8$ ). Supernatants of macrophages from both groups of patients consistently contained relatively low levels of IL-10 ( $50 \pm 20$  pg/ml), TNF- $\alpha$  ( $180 \pm 30$  pg/ml), and IFN- $\alpha$  ( $25 \pm 6$  pg/ml). TGF- $\beta$  was not detected in any supernatants. These macrophage supernatants were used as CM to stimulate various cell lines in culture. In experiments that follow, both tumor and nontumor CM were systematically investigated.

#### IL-10 production by tumor cells

Cell lines derived from cancers of colon (Colo 205, Colo 320, HT29), cervical (HEC1A), breast (MCF7), lung (A549), and prostate (PxC3) cell lines were cultured in presence of 50% CM from cancer and cardiac patients, and the IL-10, TGF- $\beta$ , and IL-6 levels in supernatants were measured using ELISA. We found that only the cell lines of colon origin produced IL-10 (Fig. 1A). The production of IL-10 was  $120 \pm 15$  pg/ml for Colo 205 cells,  $80 \pm 5$  pg/ml for Colo 320 cells, and  $43 \pm 3$  pg/ml for HT29 after 4 days of culture in presence of CM. As control, we cultured macrophages alone for 4 days and we detected  $2 \pm 1$  pg/ml IL-10 in the culture supernatants (CM). No production of TGF- $\beta$  and IL-6 was detected in supernatants using ELISA (data not shown).

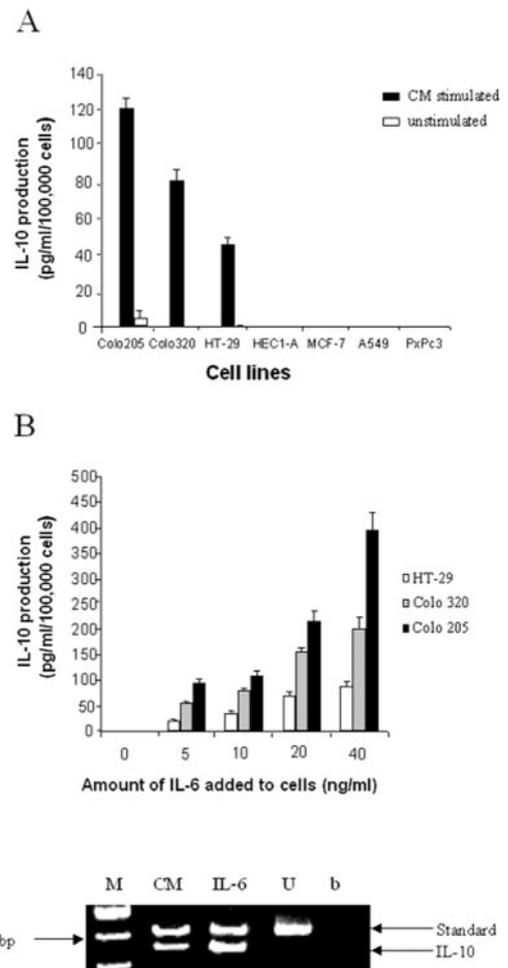
To determine the cytokines produced by macrophages that were responsible for the induction of IL-10 production by colon cell lines, we tested the effects of human recombinant cytokines IL-6, IL-10, TNF- $\alpha$ , and IFN- $\alpha$ , as these cytokines were contained in macrophage CM. We used a range of concentrations from 50 to 40 ng/ml for each cytokine tested, and found that IL-10, TNF- $\alpha$ , or IFN- $\alpha$  did not induce IL-10 production by colon tumor cells. In contrast, addition of increasing amounts of human rIL-6 to cell culture medium resulted in IL-10 production by tumor cells (Fig. 1B). Four-day cultures of the colon tumor cells in presence of 10 ng/ml rIL-6 (which corresponds to the amount of IL-6 when 50% CM was added to the cells) produced similar levels of IL-10 to that obtained when the tumor cells were cultured with 50% CM. In presence of 10 ng/ml rIL-6, the production of IL-10 was  $110 \pm 12$  pg/ml for Colo 205 cells,  $78 \pm 4$  pg/ml for Colo 320 cells, and  $40 \pm 2$  pg/ml for HT29.

IL-10 mRNA was studied using a competitive RT-PCR (Fig. 1C). The IL-10 mRNA band was clearly expressed in Colo 205 cells cultured with 50% CM. Similarly, cells cultured with 10 ng/ml rIL-6 produced IL-10 transcripts in the same proportion as seen above ( $\sim 5000$  copies). Similar results were obtained with Colo 320 and HT29 cells.

#### IL-6- and IL-6R-blocking assays

To confirm that IL-6 present in CM was responsible for inducing the IL-10 production by Colo 205 cells, we performed IL-6-blocking experiments by mixing 1  $\mu$ g/ml anti-human IL-6 mAb with CM (50%) 1 h before adding it to Colo 205 cells, Colo 320 cells, and HT29 cells culture. Addition of IL-6-blocking mAb inhibited IL-10 production in Colo 205 cells by  $85 \pm 10\%$ , in Colo 320 cells by  $79 \pm 5\%$ , and in HT29 cells by  $76 \pm 7\%$  (Fig. 2A). We performed the same experiment using 10 ng/ml rIL-6 instead of 50% CM, and we obtained similar results (data not shown).

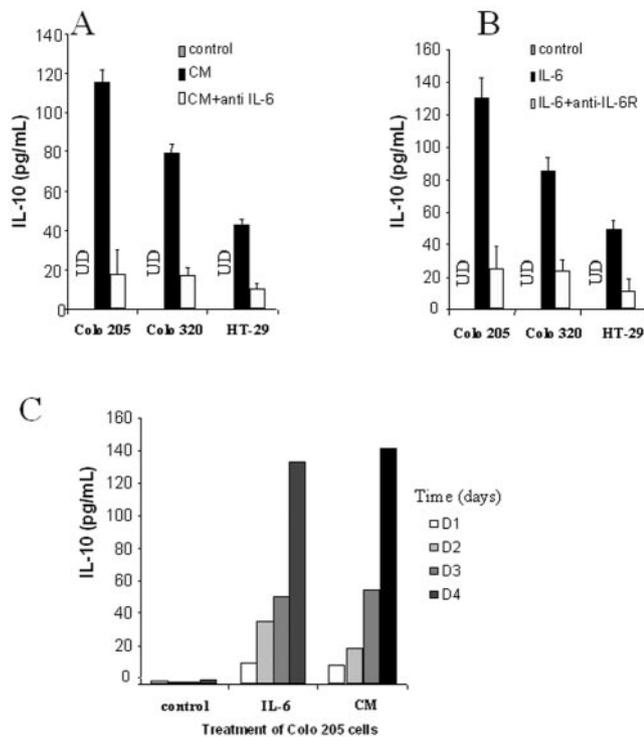
An IL-6R-blocking assay was performed. Colo 205 cells, Colo 320 cells, and HT29 cells were cultured for 4 days in the presence of 10 ng/ml rIL-6 alone or with 10  $\mu$ g/ml IL-6R-blocking mAb. IL-10 production was inhibited by  $80 \pm 10\%$  for Colo 205 cells, by  $72 \pm 8\%$  for Colo 320 cells, by  $78 \pm 8\%$  for HT29 cells, demonstrating that the binding of IL-6 to its receptor was necessary to achieve the biological effect of IL-6 in our model (Fig. 2B).



**FIGURE 1.** A, Effect of macrophage CM obtained by culturing macrophages isolated from pleural effusions of patients with malignant and nonmalignant pleural effusions on IL-10 production by tumor cell lines. Colo 205, Colo 320, HT29, HEC1A, MCF7, A549, and PxC3 cells were cultured during 4 days with or without 50% CM added to culture medium. The level of IL-10 in supernatants was measured using ELISA. Results are expressed in picograms per milliliter per 100,000 cells. B, Effect of rIL-6 on IL-10 production by Colo 205 cells, Colo 320 cells, and HT29 cells. Colo 205 cells, Colo 320 cells, and HT29 cells were cultured during 4 days with increasing IL-6 concentrations (from 0 to 40 ng/ml). Supernatants were analyzed for IL-10 levels by ELISA. Data in A and B are expressed as mean  $\pm$  SE of six independent experiments. C, Detection of IL-10 mRNA in Colo 205 cells cultured with CM (from patients with malignant and nonmalignant pleural effusions) or rIL-6. Colo 205 cells were cultured in the presence of 50% CM or 10 ng/ml human rIL-6 for 4 days. Total mRNA was extracted from  $10^6$  cells, submitted to a RT-PCR with a specific primer for IL-10, and with 50,000 copies of an internal control. Arrows indicate the IL-10 cDNA (lower band, 453 bp) and the internal control (upper band, 503 bp). Similar results were obtained with Colo 320 and HT29 cells, but are not shown here. M = m.w. standard; CM = Colo 205 cells cultured with CM; IL-6 = Colo 205 cells cultured with 10 ng/ml rIL-6; U = untreated cells; b = no cDNA. Results are representative of three independent experiments.

Isotype control mAbs used at the same concentration had no effect. We performed the same experiment using 50% CM instead of rIL-6 and obtained similar results (data not shown).

Finally, we studied the kinetics of IL-10 production by Colo 205 cells cultured with 50% CM or with 10 ng/ml rIL-6. As shown in Fig. 2C, we observed production of IL-10 as soon as 24 h, and an increase for both treatments. The production of IL-10 by tumor



**FIGURE 2.** Effect of IL-6- and IL-6R-blocking mAbs on IL-10 production by Colo 205 cells, Colo 320 cells, and HT29 cells. *A*, Colo 205 cells, Colo 320 cells, and HT29 cells were cultured for 4 days with 50% CM and 1  $\mu$ g/ml IL-6-blocking mAb or isotypic mAb added to CM 1 h before culture. Control = tumor cells (Colo 205 cells, Colo 320 cells, and HT29) cultured with anti-IL-6 mAb, but without CM; CM + isotypic mAb = Colo 205 cells, Colo 320 cells, and HT29 cells cultured with CM with isotypic mAb; CM + anti-IL-6 = Colo 205 cells, Colo 320 cells, and HT29 cells cultured with CM after addition of anti-IL-6 mAb. *B*, Colo 205 cells, Colo 320 cells, and HT29 cells were cultured for 4 days with 10 ng/ml rIL-6 and with 10  $\mu$ g/ml blocking IL-6R mAb or isotypic mAb. Supernatants were analyzed for IL-10 content by ELISA. Control = Colo 205 cells, Colo 320 cells, and HT29 cells cultured with anti-IL-6R mAb, but without IL-6; IL-6 + isotypic mAb = Colo 205 cells, Colo 320 cells, and HT29 cells cultured with rIL-6 with isotypic mAb; anti-IL-6R = Colo 205 cells, Colo 320 cells, and HT29 cells cultured with recombinant IL-6 and IL-6R mAb. UD = undetectable. Data are expressed as mean  $\pm$  SE of three independent experiments for each three CM tested and for each experimental culture condition (for both *A* and *B*). *C*, Kinetic of IL-10 production by Colo 205 cells cultured for 4 days with 50% CM or with 10 ng/ml rIL-6. IL-10 concentration in Colo 205 cell supernatants was measured every 24 h by ELISA. Data are representative of three independent experiments.

cells increased with the time when cultured in presence of CM or rIL-6.

#### Effects of macrophages and Colo 205 cells coculture on IL-6 and IL-10 production

To test whether macrophages and tumor cells could interact directly, cocultures of macrophages from patients' pleural effusions and Colo 205 cells were performed. The cells were cultured for 2 days at macrophage-Colo 205 cell ratios, ranging from 1:0.5 to 1:4, and the production of IL-6 and IL-10 was measured. As shown in Fig. 3*A*, the production of IL-6 was higher in cultures with tumor cells, but was not depending on the number of Colo 205 cells added. The production of IL-10 in coculture was studied in the same way (Fig. 3*B*). Macrophages or Colo 205 cells alone (as shown before) did not produce high amount of IL-10, but we did detect high levels of IL-10 in coculture. Furthermore, we observed

that the amount of IL-10 produced was proportional to the number of Colo 205 cells added. An IL-6-blocking assay was performed to verify that IL-10 production in coculture was due to IL-6. As shown in Fig. 3*C*, anti-IL-6, but not isotype control Abs used at 10  $\mu$ g/ml, were able to inhibit  $75 \pm 6\%$  IL-10 production after 2 days. We confirmed that IL-6 was produced by macrophages and not by Colo 205 cells (after 4 days of culture with CM) by studying IL-6 mRNA by RT-PCR (Fig. 3*D*).

To study the kinetics of IL-6 and IL-10 production by macrophages and Colo 205 cells, respectively, we measured the levels after 1, 2, and 4 h of culture. IL-6 production ( $300 \pm 50$  pg/ml) was observed after 2 h. In contrast, IL-10 could be detected in supernatants ( $20 \pm 5$  pg/ml) only after 8 h of culture. After coculture with macrophages (ratio 1:4), the IL-10 production was  $85 \pm 4$  pg/ml by Colo 320 cells and  $74 \pm 5$  pg/ml by HT29 cells.

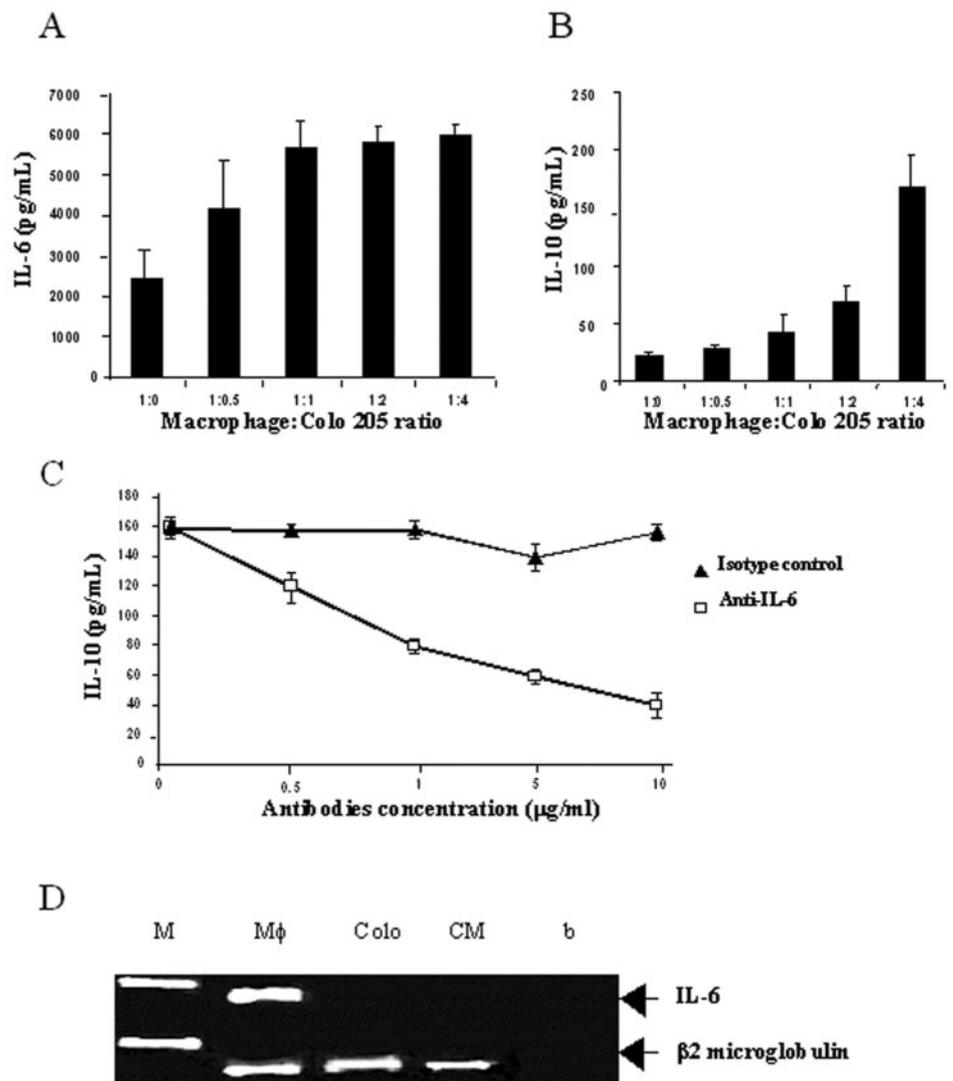
#### Effect of CM and rIL-6 on STAT3 activation in Colo 205 cell line

Because Colo 205 cells produced highest level of IL-10 among the three tumor cell lines studied, we selected Colo 205 cell line for investigating the role of STAT3. Signal transducer gp130 activation by the IL-6 family resulted in the tyrosine phosphorylation of the transducing receptor subunits and downstream regulatory proteins. We tested the possibility that CM containing IL-6 and rIL-6 induces the activation of STAT3, a major transcriptional factor involved in the IL-6 response. Experiments were performed using Colo 205 cells. As shown in Fig. 4, IL-6 and CM elicit the phosphorylation of STAT3. This activation was completely abolished by an anti-IL-6 (Fig. 4*A*) or an anti-IL-6R Ab (Fig. 4*B*), confirming that STAT3 activation was induced by IL-6 present in the CM and that this activation was mediated by IL-6R.

#### Effect of STAT3 $\beta$ in production of IL-10 in Colo 205 cell line

To assess the role of STAT3 in the production of IL-10 by Colo 205 cells, we transiently transfected Colo 205 cells with an expression vector encoding the splice form of STAT3 (named STAT3 $\beta$ ). This splice product is the naturally occurring isoform of STAT3 and encodes an 80-kDa protein that inhibits the *trans* activation of STAT3 (28). The potential for STAT3 $\beta$  involvement in the inhibition of IL-10 production was determined by analyzing the expression of STAT3 $\beta$  proteins in Colo 205-transfected cells. The cells were stimulated for 10 min with 20 ng/ml IL-6. Forty-eight hours after transfection with STAT3 $\beta$  or mock, total protein extracts were probed with a polyclonal Ab that recognizes both STAT3 and the truncated variant STAT3 $\beta$  form (Fig. 5*A*). To analyze the effect of STAT3 $\beta$  on IL-10 production, transfected cells were then stimulated with 20 ng/ml IL-6 for 4 days. As shown in Fig. 5*B*, IL-10 production by Colo 205 cells transfected with STAT3 $\beta$  was largely inhibited, demonstrating that the transcriptional factor STAT3 was necessary to induce the production of IL-10 in these cells. To confirm the primordial effect of STAT3 in the processes of IL-10 secretion, we transfected Colo 205 cells with the expression vector for STAT3 $\beta$  together with a luciferase reporter construct, containing three STAT3 consensus binding sites located upstream of a thymidine kinase minimal promoter (27). The cells were stimulated for an additional 15 h (48 h post-transfection) with a saturating amount of IL-6. Untransfected cells and transfection of the mock vector show an IL-6-dependent 4- to 5-fold increase in luciferase activity. By contrast, transfection of STAT3 $\beta$  gave almost no increase in luciferase activity after IL-6 stimulation (Fig. 5*C*). These data confirm the importance of STAT3 in the initiation of IL-10 secretion by Colo 205 cells.

**FIGURE 3.** IL-6 (A) and IL-10 (B) production in cocultures of macrophages and Colo 205 cells. Macrophages from patients with malignant or nonmalignant pleural effusion were seeded at a density of  $5 \times 10^5$  cells/ml. Different numbers of Colo 205 cells were added to achieve macrophage-tumor cell ratios of  $2.5 \times 10^5$  (ratio 1:0.5),  $5 \times 10^5$  (ratio 1:1),  $10^6$  (ratio 1:2), or  $2 \times 10^6$  (ratio 1:4), respectively. The mixed cells were cultured for 2 days. We also cultured macrophages alone as control (ratio 1:0). IL-6 (A) and IL-10 (B) production were quantified by ELISA. Each bar represents the mean  $\pm$  SE of IL-10 or IL-6 production in picograms per milliliter of six independent experiments. C, Effect of anti-IL-6-blocking Ab on IL-10 production in coculture of macrophages and Colo 205 cells. Macrophages from patients were cocultured with Colo 205 cells (ratio 1:4) for 2 days in presence of IL-6-blocking mAb or of isotypic mAb at 0.5, 1, 5, or 10  $\mu\text{g/ml}$ . IL-10 production was measured by ELISA. Each bar represents the mean  $\pm$  SE of IL-10 production in picograms per milliliter of four independent experiments. D, Detection of IL-6 mRNA production by macrophages and Colo 205 cells by RT-PCR. Macrophages were cultured for 2 days in culture medium. Colo 205 cells were cultured 4 days with or without CM. After mRNA extraction, we performed a semiquantitative IL-6 RT-PCR. M = m.w. standard; M $\phi$  = macrophages; Colo = Colo 205 cells cultured without CM; CM = Colo 205 cells cultured with CM; b = no cDNA. Results are representative of three independent experiments for each of the three CM tested.

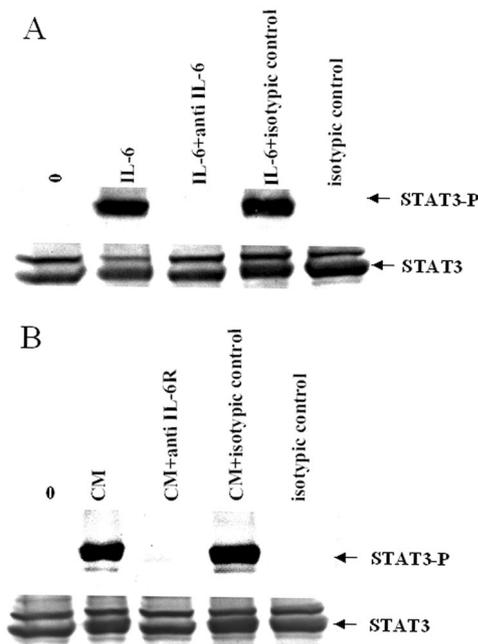


## Discussion

To study interactions between macrophages and tumor cells, we developed a human cell culture model that provides an approximation of physiological conditions. Thus, culture supernatants of macrophages from pleural effusions harvested from cancer and cardiac patients were used as CM to stimulate human tumor cell lines. These macrophages consistently produced high amount of IL-6 and low amount of IL-10, TNF- $\alpha$ , and IFN- $\alpha$ . We found that IL-10, but not TGF- $\beta$  and IL-6, was produced by colon tumor cells (Colo 205, Colo 320, and HT29), but not by cervical (HEC1A), breast (MCF7), lung (A549), and prostate (PxC3) tumor cells after exposure to macrophage supernatants. This finding suggests that colon tumor cells are able to respond to the antitumor effects of macrophages by producing an immunosuppressive cytokine. Thus, we demonstrated that IL-6 secreted by macrophages was responsible for IL-10 production by colon adenocarcinoma cells (Colo 205, Colo 320, HT29). This finding contrasts with the observation using IL-10, TNF- $\alpha$ , and IFN- $\alpha$ , because none of these three cytokines induced the same effect. rIL-6 induced production of high levels of IL-10 in colon tumor cell culture supernatants and IL-10 transcript expression in these cells. Ab-blocking experiments confirmed these results, demonstrating that IL-6 was the only cytokine in CM that induced IL-10 production by Colo 205 cells. This effect of IL-6 was suppressed by IL-6R-blocking Ab, indicating that binding of IL-6 to IL-6R initiated the effect.

IL-6R mRNA is twice as abundant in colon carcinoma as in normal colon (29), and is known to induce a signaling pathway that usually involves STAT3 recruitment (30–32). STAT3 activation appears to play a central role in ovarian (18) and prostate (33) cancer and also in intestinal inflammation (19, 34, 35). One study reported that STAT3 controls expression of the human IL-10 gene in human 8226.1 B cell line and monocytic cell line Mono Mac 6 after LPS stimulation (36). We show in this study that IL-6 induces IL-10 production through the STAT3 signaling pathway. IL-6- and IL-6R-blocking Abs inhibit STAT3 phosphorylation in Colo 205 cells, demonstrating that IL-6 is responsible for STAT3 activation by binding to its receptor. Furthermore, after stimulation by rIL-6, cells transiently transfected in an expression vector encoding STAT3 $\beta$  (a dominant-negative splice form of STAT3) lose the capacity to produce IL-10. These results confirm the importance of STAT3 in production of IL-10 by Colo 205 cells.

We also demonstrated that macrophages and tumor cells could be reciprocally activated in a coculture system. The kinetics studies suggest that colon cancer cells initially promoted IL-6 production by macrophages, which activated tumor cells to produce IL-10. Abs against IL-6 inhibited IL-10 production in the coculture system, confirming the major role of IL-6 in IL-10 secretion. Importantly, Colo 205 cells stimulated IL-6 production by macrophages from both malignant and benign pleural effusions. Taken together, we suggest the following mechanism of interaction between

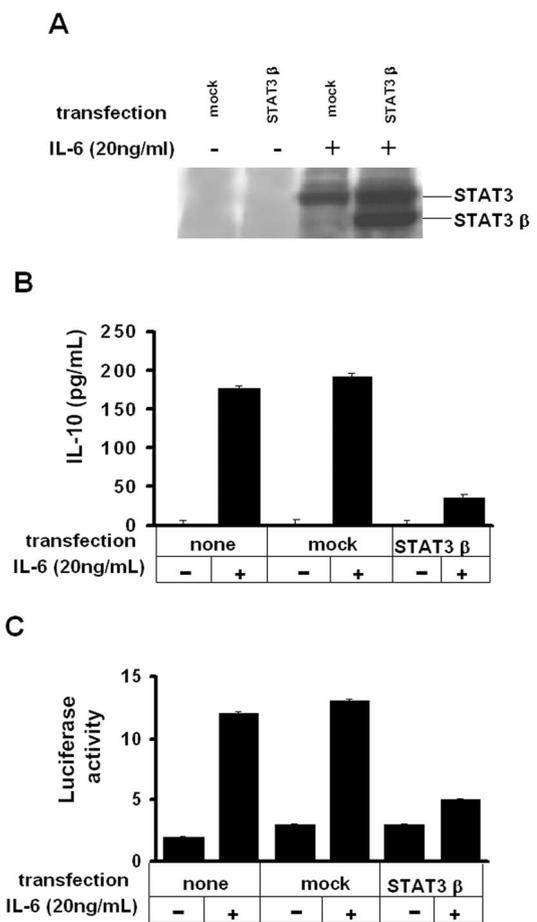


**FIGURE 4.** IL-6 and macrophage CM induce STAT3. Colo 205 cells were preincubated with 10  $\mu\text{g/ml}$  anti-IL-6 mAb or with the same concentration of isotypic IgG1 Ab control for 1 h before being activated for 10 min with 50 ng/ml IL-6. CM was incubated with 1  $\mu\text{g/ml}$  IL-6-blocking mAb or an IgG1 control for 1 h before activation. Following stimulation, cells were lysed in 1% Nonidet P-40, and proteins were subjected to Western blot analysis with Abs specific for a phosphorylated form of STAT3 (STAT3-P). The blots were then stripped and reprobed with Abs to STAT3. Results are representative of three independent experiments.

macrophages and colon tumor cell lines. Macrophages are activated by tumor cells to secrete IL-6. Through the binding to its receptor, IL-6 activates STAT3 signaling pathway, which stimulates tumor cells to produce IL-10, which contributes to the evasion of antitumor activity by local tumor-associated macrophages and T cell immune response. We did not detect any IL-6 mRNA production in colon tumor cells stimulated by macrophage supernatants, although we have not excluded the possibility that some colon tumor cells produce IL-6. However, even if the colon tumor cells in patients can make IL-6, this provides even more importance to our study, because the more IL-6 we add to the colon cancer cells, the more they produce IL-10 and the more they could escape the immune system.

Based on our findings, we develop the following model of interactive regulation between the cells of the immune system and tumor cells. The local cytokine environment in the intestine could explain how colon tumor cells, but not the other neoplastic cells, are able to produce IL-10 in response to IL-6 secretion by macrophages. In the colon mucosa, microbial flora continuously stimulate the production of the proinflammatory cytokine IL-6 by the local immune system (37). IL-6, which is present at high concentrations in colon tissue, could contribute to the selection of tumor cells that express IL-6R (29) and STAT3, and consequently are capable of producing IL-10, which could decrease local immunity, facilitating tumor cell proliferation. Moreover, previous studies reported that: 1) an excess of IL-6 production by immune cells could promote mucosal inflammation, which is considered to be a bad prognostic factor (34), and 2) IL-6 stimulates clonogenic growth of primary and metastatic human colon carcinoma cells (38).

In conclusion, our data are the first to demonstrate that colon tumor cell lines (Colo 205, Colo 320, and HT29) can activate



**FIGURE 5.** STAT3 $\beta$  is an inhibitor of IL-10 secretion by Colo 205 cells. *A*, Whole cell extracts from Colo 205, Colo 205 transfected with STAT3 $\beta$ , or mock followed by stimulation with 20 ng/ml IL-6 for 10 min were monitored for the presence of STAT3 and STAT3 $\beta$  by Western blotting with a specific polyclonal Ab against a phosphorylated form of STAT3. The protein of  $\sim 90$  kDa represents STAT3, while the smaller protein is STAT3 $\beta$ . Results are representative of three independent experiments. *B*, Colo 205 cells were cultured for 4 days with rIL-6 after transfection with the expression vector, as indicated. Supernatants were analyzed for IL-10 content by ELISA. *C*, Colo 205 cells were transfected with a reporter plasmid gene together with an expression vector for STAT3 $\beta$ . After 48 h, the cells were treated with 20 ng/ml IL-6 for an additional 15 h. The cellular extracts were prepared and used to directly measure luciferase activity. Each bar represents the mean  $\pm$  SE of three independent experiments (data in *B* and *C*).

macrophages from either cancer or cancer-free patients to produce IL-6 in a coculture system. This IL-6 can subsequently induce STAT3 activation in colon carcinoma cells, resulting in IL-10 production. Thus, activation of the IL-6/STAT3 pathway may play a role in the pathogenesis and progression of colon cancer.

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