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*J Immunol* 2004; 172:4630-4636; doi: 10.4049/jimmunol.172.7.4630
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Recruitment of STAT3 for Production of IL-10 by Colon Carcinoma Cells Induced by Macrophage-Derived IL-6

Jean-Philippe Herbeuval,* Eric Lelievre,† Claude Lambert,* Michel Dy,‡ and Christian Genin*

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-α, and IFN-α, 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-β, 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 hexametric receptor, composed of an IL-6-specific receptor (α-chain, IL-6Rα) 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)3 for cultures of human cell lines derived from carcinomas of colon (Colo 205, Colo 320, and HT29), breast (MCF7), lung (A549), prostate (PxPc3), 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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Received for publication September 15, 2003. Accepted for publication January 28, 2004.

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This work was supported by a grant from the Ligue Nationale Contre le Cancer.

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Abbreviation used in this paper: CM, conditioned medium.

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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 g for 10 min at 4°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 (Eurogentec, Les Ulis, France), and antibiotics (penicillin G at 100 U/ml, streptomycin at 100 μg/ml, and amphotericin B at 0.25 μg/ml) (Life Technologies). Macrophage-like cells were allowed to adhere to 75-cm² tissue culture flasks (Falcon, Oxnard, CA) at 37°C in an incubator in a humidified atmosphere of 5% CO₂ for 2 h. Nonadherent cells were removed by two washes 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 (21).

We then plated macrophages at 5 × 10⁴ cells/ml culture medium. Adherent cells were cultured for 48 h in the same medium at 37°C in an incubator with a humidified atmosphere of 5% CO₂. Supernatants were centrifuged at 400 g for 10 min at 4°C and stored as aliquots at -80°C until use. After thawing, supernatants were filtered through 0.22-μm (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 (PxPc3), 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 × 10⁵ cells/ml, and 0.25, 0.5, 1, or 2 × 10⁴ 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-β, and TNF-α were measured in each CM and cell culture supernatant by ELISA, using commercial kits (R&D Systems, Minneapolis, MN). Positive cell culture supernatant by ELISA, using commercial kits (R&D Systems, Minneapolis, MN). The OD was measured at 492 nm on a Titertek Multiskan reader (Flow Laboratories, Irvine, U.K.).

Statistics

The data were expressed as the mean ± the standard error of the mean. Differences were analyzed by Student’s t test and were considered to be statistically significant at p < 0.05. Values are represented as the mean ± 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 and IL-10 mRNA production by RT-PCR

Total RNA was extracted from 10⁶ Colo 205 cells or 5 × 10⁶ macrophages, according to the instructions for use of TRIzol reagent (Life Technologies). Reverse transcription was performed with Super Script Preimplication 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 μg of cDNA using a DNA thermal cycler (PerkinElmer) for 33 cycles. A cycle profile consisted of 1 min at 95°C for denaturation, 1 min at 55°C for annealing, and 1 min at 72°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 β2-microglobulin (180 bp) were produced by M. Dy (25). The housekeeping gene β2-microglobulin was used as reference for the quantification of the IL-6 transcripts. The cycle profile (30 cycles) consisted in a 1-min time period at 95°C for denaturation, 1 min at 55°C for annealing, and 1 min at 72°C for primer extension. All PCR-amplified products were run on 1.5% agarose gel containing 0.5 μg/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, protease inhibitors (1 μg/ml pepstatin, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM PMSF), and 1% Nonidet P-40 (26). After pelleting insoluble material, the supernatant was subjected to SDS-PAGE and transferred onto a nitrocellulose membrane (Millipore, Bedford, MA). Membranes were subsequently incubated overnight with an anti-phospho-STAT3 Tyr705 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 STAT3β was in Pcmna3 expression vector. For each experiment, a control plate was transfected with a ß-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 six-inducible element-luciferase reporter gene alone or together with a vector encoding STAT3β, as described previously (27). Forty-eight hours after transfection, cells were incubated with medium alone or ßL-6. Transfected cells were washed twice with ice-cold PBS, and 100 μl of lysis buffer was added to the cells (0.1 M KH₂PO₄, 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.

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Control

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Macrophage and tumor cell coculture conditions

Cytokine quantification by ELISA

Effects of recombinant cytokines and blocking Abs

Human recombinant IL-6, IL-10, TNF-α, and IFN-α were purchased from PeproTech (Rocky Hill, NJ), R&D Systems (Minneapolis, MN), and BioSource International (Camarillo, CA). TNF-α and IFN-α 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 μg of anti-IL-6 mAb was added to CM or to ßL-6 (10 ng/ml) at 4°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 IL-6 and with or without 10 μg/ml anti-IL-6R-blocking mAb, as well as with isotype control Ab.
were detected in culture supernatants from macrophages isolated from cancer (18.160 ± 3.014 pg/ml) and cardiac patients (17.972 ± 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 ± 20 pg/ml), TNF-α (180 ± 30 pg/ml), and IFN-α (25 ± 6 pg/ml). TGF-β 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 (PxPc3) cell lines were cultured in presence of 50% CM from cancer and cardiac patients, and the IL-10, TGF-β, 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 ± 15 pg/ml for Colo 205 cells, 80 ± 5 pg/ml for Colo 320 cells, and 43 ± 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 ± 1 pg/ml IL-10 in the culture supernatants (CM). No production of TGF-β 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-α, and IFN-α, 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-α, or IFN-α 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 ± 12 pg/ml for Colo 205 cells, 78 ± 4 pg/ml for Colo 320 cells, and 40 ± 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 of 10 ng/ml rIL-6 produced IL-10 transcripts in the same proportion as seen above (~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 μg/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 ± 10%, in Colo 320 cells by 79 ± 5%, and in HT29 cells by 76 ± 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 μg/ml IL-6R-blocking mAb. IL-10 production was inhibited by 80 ± 10% for Colo 205 cells, by 72 ± 8% for Colo 320 cells, by 78 ± 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).

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...
Experiments were performed using Colo 205 cells. As shown in Fig. 4, IL-6 and CM elicited the phosphorylation of STAT3. This activation was completely abolished by an anti-IL-6 (Fig. 4A) or an anti-IL-6R Ab (Fig. 4B), confirming that STAT3 activation was induced by IL-6 present in the CM and that this activation was mediated by IL-6R.

**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. 4A) or an anti-IL-6R Ab (Fig. 4B), confirming that STAT3 activation was induced by IL-6 present in the CM and that this activation was mediated by IL-6R.

**Effect of STAT3β 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 STAT3β). 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 STAT3β involvement in the inhibition of IL-10 production was determined by analyzing the expression of STAT3β 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 STAT3β or mock, total protein extracts were probed with a polyclonal Ab that recognizes both STAT3 and the truncated variant STAT3β form (Fig. 5A). To analyze the effect of STAT3β on IL-10 production, transfected cells were then stimulated with 20 ng/ml IL-6 for 4 days. As shown in Fig. 5B, IL-10 production by Colo 205 cells transfected with STAT3β 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 STAT3β 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 posttransfection) 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 STAT3β gave almost no increase in luciferase activity after IL-6 stimulation (Fig. 5C). 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 x 10^5 cells/ml. Different numbers of Colo 205 cells were added to achieve macrophage-tumor cell ratios of 2.5 x 10^5 (ratio 1:0.5), 5 x 10^5 (ratio 1:1), 10^6 (ratio 1:2), or 2 x 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 ± 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 μg/ml. IL-10 production was measured by ELISA. Each bar represents the mean ± 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; Mφ = 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.
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 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.

Acknowledgments
We thank C. Ruesch (University Hospital Center, Saint-Chamond, France), P. Sagnol (University Hospital Center, Firminy, France), M. Perol (University Hospital Center, Lyon, France), J. M. Vergnon, P. Fournel, and M. Cottier (University Hospital Center, Saint-Etienne, France) for providing us with pleural effusions. We thank O. Coqueret (Institut National de la Santé et de la Recherche Médicale E-9928, Centre Hospitalier Universitaire Angers, France) for the kind gift of plasmid-encoding STAT3β.

We thank Dr. G. Shearer (National Cancer Institute, National Institutes of Health, Bethesda, MD), M. Crépin (University of Paris XIII, Paris, France), Dr. O. Garraud, and D. Pearson (University of Saint-Etienne) for providing us with pleural effusions.
helpful discussion and review of the manuscript. We also thank H. Hamzeh, D. Laurent, and F. Duplat for technical help, and C. Moulin (University of Saint-Etienne) for secretarial assistance.

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