Interleukin 17, a T-cell-derived Cytokine, Promotes Tumorigenicity of Human Cervical Tumors in Nude Mice


Introduction
IL-17, previously termed CTL-associated antigen 8 (CTLA-8), is a cytokine secreted mainly by activated human memory CD4 T cells that induces IL-6, IL-8, and nitric oxide. Because IL-6 and IL-8 have been implicated in the pathogenesis of cervical cancer, we investigated the role of IL-17 in human cervical tumor cell lines in vitro and in vivo. We showed that in vitro, IL-17 enhances IL-6 and IL-8 secretion by cervical carcinoma cell lines at both protein and mRNA levels. No direct effect of IL-17 on in vitro proliferation of cervical tumor cell lines could be demonstrated. However, two cervical cell lines transfected with a cDNA encoding IL-17 exhibited a significant increase in tumor size as compared to the parent tumor when transplanted in nude mice. This enhanced tumor growth elicited by IL-17 was associated with increased expression of IL-6 and macrophage recruitment on the tumor site. A potential role of IL-17 in modulation of the human cervical tumor phenotype was also supported by its expression on the cervical tumor in patients with CD4 infiltration. IL-17 therefore behaves like a T-cell-specific cytokine with paradoxical tumor-promoting activity. This may partially explain previous reports concerning the deleterious effect of CD4 T cells in cancer.

ABSTRACT
Interleukin (IL-17) is a proinflammatory cytokine secreted mainly by activated human memory CD4 T cells that induces IL-6, IL-8, and nitric oxide. Because IL-6 and IL-8 have been implicated in the pathogenesis of cervical cancer, we investigated the action of IL-17 on human cervical tumor cell lines in vitro and in vivo. We showed that in vitro, IL-17 increases IL-6 and IL-8 secretion by cervical carcinoma cell lines at both protein and mRNA levels. No direct effect of IL-17 on in vitro proliferation of cervical tumor cell lines could be demonstrated. However, two cervical cell lines transfected with a cDNA encoding IL-17 exhibited a significant increase in tumor size as compared to the parent tumor when transplanted in nude mice. This enhanced tumor growth elicited by IL-17 was associated with increased expression of IL-6 and macrophage recruitment on the tumor site. A potential role of IL-17 in modulation of the human cervical tumor phenotype was also supported by its expression on the cervical tumor in patients with CD4 infiltration. IL-17 therefore behaves like a T-cell-specific cytokine with paradoxical tumor-promoting activity. This may partially explain previous reports concerning the deleterious effect of CD4 T cells in cancer.

INTRODUCTION
IL-17, previously termed CTL-associated antigen 8 (CTLA-8), is a cytokine secreted mainly by activated human memory CD4 T cells (1–3). Its amino acid sequence shares remarkable homology with that of the thirteenth open reading frame of herpesvirus saimiri, a γ herpes virus causing T-cell lymphoma in monkeys and rabbits (4). In vitro, this virus could immortalize human CD4 and CD8 T cells via an unknown mechanism that confers an IL-2-based autocrine growth after activation by CD2 upon these cells (5, 6). The role of IL-17 in the proliferation of T cells is a matter of discussion. In mice, IL-17 plus suboptimal concentrations of phytohemagglutinin resulted in a modest enhancement of T-cell growth (7). No similar studies have been reported in mice, IL-17 plus suboptimal concentrations of phytohemagglutinin resulted in a modest enhancement of T-cell growth (8). The role of IL-17 in the proliferation of T cells is a matter of discussion. In mice, IL-17 plus suboptimal concentrations of phytohemagglutinin resulted in a modest enhancement of T-cell growth (7). No similar studies have been reported in mice, IL-17 plus suboptimal concentrations of phytohemagglutinin resulted in a modest enhancement of T-cell growth (8).

MATERIALS AND METHODS
Tumor Cell Lines. The HeLa cervical tumor cell line was obtained from the American Type Culture Collection (Manassas, VA), and the IC1 cervical carcinoma cell line was provided by Dr. J. Couturier (Institut Curie, Paris, France; Ref. 25). The human melanoma cell lines WM793, 1341D, MZ2, and HT144 have been described previously (26). These cell lines were grown in RPMI 1640 (Biowhittaker, Walkersville, MD) supplemented with 10% FCS, 100 units/ml penicillin-streptomycin, 5% sodium pyruvate, and 0.01% mercaptoethanol (all from Sigma Chemical Co., St. Louis, MO).

Transfection of HeLa and IC1 Cells. A cDNA encoding hIL-17 was inserted into an expression vector under the control of the SRα promoter in pBR322 into which a neomycin resistance gene was introduced (NT-Neo; Ref. 27). NT-Neo containing or lacking the 640-bp IL-17 cDNA was linearized with ScaI restriction enzyme and stably transfected by electroporation into HeLa and IC1 cells. Electroporation was performed with a Bio-Rad Gene Pulser at a voltage of 260 V with a capacitance of 960 μF. At 72 h after electroporation, transfected cells were selected by culture in RPMI 1640 (Life Technologies, Inc.) supplemented with 1 mg/ml G418 (Geneticin; Life Technologies, Inc., Paisley, United Kingdom). G418-resistant clones were expanded in selection medium and tested for IL-17 expression.

Recombinant Proteins and Antibodies. rhIL-17 was purified from the supernatant of IL-17-transfected NSO cells, as described previously (9).

Interestingly, in vitro, IL-6 behaves as a growth factor for many tumor cell lines derived from myeloma, lymphoma, Kaposi’s sarcoma, melanoma, and ovarian and renal bladder cell carcinoma (13). In mice, IL-6-transfected tumors often exhibited increased tumorigenicity (14–16). In myeloma patients, anti-IL-6 mAb administration transiently inhibited myeloma cell proliferation (17). IL-8, a member of the CXC family of chemotactic cytokines, also stimulates the proliferation of tumor cells because IL-8 is an autocrine growth factor for human melanoma (18). Its expression by tumor cells is directly correlated with their metastatic potential in nude mice (19). Therefore, if the activity of IL-17 is mediated via these proinflammatory cytokines, a potential role for IL-17 in tumor cell proliferation may be hypothesized.

Cervical cancer is associated with HPV infection, but additional factors must contribute to its pathogenesis because only a minority of HPV infections result in persistent lesions or progress to malignancy (20). Various arguments suggest that IL-6 may be involved in the pathogenesis and development of cervical cancers. IL-6 stimulates the growth of both normal cervical cells and HPV-immortalized and cervical carcinoma-derived cell lines (21, 22). In vitro, cervical carcinoma cells also secrete higher levels of IL-6 and IL-8 than HPV-infected and normal cervical epithelial cells (23). Finally, an increased expression of IL-6 mRNA was demonstrated in biopsies derived from invasive cervical carcinoma compared to biopsies derived from cervical intraepithelial neoplasia or normal cervix (24). The role of IL-17 in the up-regulation of IL-6 and IL-8 expression therefore prompted us to investigate the action of this cytokine on the in vitro and in vivo proliferation of human cervical tumors.

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1 The abbreviations used are: IL, interleukin; rhIL, recombinant human IL; HPV, human papillomavirus; hIL, human IL; mAb, monoclonal antibody; RT-PCR, reverse transcription-PCR; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TBS, Tris-buffered saline; MCP-1, monocytic chemotactactant protein 1.

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**Assays.** hIL-6 and hIL-8 were assayed using ELISA kits purchased from Immunotech (Marseille, France) and Medgenix (Brussels, Belgium), respectively.

An ELISA was used to measure hIL-17 concentrations in culture supernatants. Briefly, 96 break-away, flat-bottomed-well (Nunc) microtiter plates were coated with 50 μl of anti-hIL-17 mAb 25 (10 μg/ml) diluted in carbonate buffer [0.1 M Na₂CO₃/NaHCO₃ (pH 9.6)] overnight at 4°C. The plate was then saturated with 200 μl of PBS-1% BSA at room temperature. After washes with PBS-0.05% Tween 20 (Merck, Schuchardt, Germany), 50 μl of rhIL-17 or samples diluted in PBS-1% BSA were added and incubated for 3 h at 37°C. After washes, the plates were incubated with 50 μl of peroxidase-labeled anti-IL-17 mAb 16 (5 μg/ml) diluted in PBS-1% BSA for 2 h at 37°C. The reaction was revealed by the addition of the peroxidase substrate (o-phenylenediaminedihydrochloride), and the optical density was read at a wavelength of 492 nm. The lowest concentration of hIL-17 detected was 0.05 ng/ml.

**RT-PCR Amplification.** RT-PCR was performed as described previously (28). The following oligonucleotide primers were used: (a) human β-actin sense, TCTGCAGGGAAGCCTGGATGTC; (b) human β-actin antisense, TTCTGAGGGACCTGAAAGCAGC; (c) hIL-6 sense, AC-GAATTCACAAATACATTGGAATC; (d) hIL-6 antisense, CATCCTAGATCCTTGCCCTTTTCGTC; (e) hIL-8 sense, TTCTGACGCTGGTGTAGGAGG; (f) hIL-8 antisense, GAAGAGGCTGAGATTTCACT; (g) hIL-17 sense, ACTCTCTGGGAAACAGTCTTGG; (h) IL-17 antisense, GGGCA-CATGGGGGAACTTG; (i) human CD4 sense, GGAATCTCCCTTGAAGGCAGCTTCG; (j) human CD4 antisense, GAACCCCACTGTTCCTCCCCCT; (k) human CD8 sense, CTTCTCTGGCCGCGGACGGAAG; (l) human CD8 antisense, GCCGGCCTCCTCCCTCCGGC; (m) murine IL-6 sense, TGGAGTCA-CAGAAGGAGTGGCTAAG; (n) murine IL-6 antisense, TCTGACCACAGT-GAGAAGTCCAC; (o) murine hypoxanthine phosphoribosyltransferase antisense, GTTGGATAACGGCAGACCTTT; and (p) murine hypoxanthine phosphoribosyltransferase antisense, GATTCAACTTGCGCTCATCTGAG;

**In Vitro Growth Assay.** Cells were plated into flat-bottomed 96-well plates at a density of 10⁶ cells/well. The cells were cultured for 3 days, and their proliferation was determined by a MTT assay (29). Briefly, 20 μl of MTT (Sigma) at a concentration of 5 mg/ml were added to each well and incubated for 4 h in the dark. The formazan grain was then dissolved in DMSO, and the absorbance at 570 nm was read using an ELISA plate reader. A standard curve between the absorbance of the MTT test and the number of cells was determined for each cell line. The conversion of MTT to formazan was directly correlated with the number of viable cells.

**Tumor Growth in Nude Mice.** Male 8-week-old athymic nu/nu mice (Ifa Credo, L’Arbresle, France) were used for the experiments described here. Human cervical tumors were injected into mice by subdermal inoculation of 10⁶ cells. A total of 8–10 mice/group were used per experiment. Tumor volume (in mm³) was estimated from the length (a) and width (b) of the tumor by using the following formula: volume = ab²/2. Biopsies were snap-frozen in liquid nitrogen and stored at −70°C for RNA extraction.

**Immunocytochemistry.** Cryostat sections (5 μm) of tumor xenografts were fixed in acetone at 4°C for 5 min. After washes in TBS, they were incubated with biotinylated rat mAb against Mac1 (M1/70 hybridoma) or with isotype-matched control biotinylated rat mAb (PharMingen, San Diego, CA). After washes in TBS, slides were incubated with alkaline phosphatase-conjugated streptavidin (DAKO, Trappes, France), and enzymatic activity was revealed with Fast Red reagent (DAKO) associated with 1 mM of levamisole, a known inhibitor of endogenous alkaline phosphatase. Sections were counterstained with Mayer’s Hematoxylin.

**Statistical Analysis.** The in vivo comparison of tumor size between the various groups of mice was analyzed by the Mann-Whitney test.

**RESULTS**

Analysis of IL-6 and IL-8 Production by Tumor Cell Lines Stimulated with rhIL-17. rhIL-17 significantly increased IL-6 secretion by the two human cervical cell lines, HeLa and IC1 (Fig. 1). This action was not restricted to tumor cell lines derived from cervical carcinoma because two of four melanoma cell lines were also sensitive to rIL-17. To control this result, we showed that the addition of anti-IL-17 mAb inhibited up-regulation of IL-6 production by IL-17 (Fig. 1), whereas an isotype control-matched antibody had no effect (data not shown). Tumor cells did not constitutively secrete significant levels of IL-7, and anti-IL-17 antibody alone had no effect on IL-6 secretion (data not shown). Only cell lines that already produced basal levels of IL-6 appeared to respond to IL-17 (Fig. 1). An increased production of IL-8 was also demonstrated after treatment of the HeLa (Fig. 2A), IC1, WM793, and HT144 cell lines with rIL-17. In contrast, cell lines resistant to rIL-17-induced IL-6 production (MZ2 and 1341D) did not secrete IL-8 after IL-17 treatment (data not shown). The activity of rIL-17 was observed at a dose range between 0.2 and 2 ng/ml (Fig. 2A). We did not observe any effect of IL-17 on granulocyte colony-stimulating factor production (data not shown), which has been shown to be regulated by IL-17 in other cell types (2, 30).

To more thoroughly analyze the mechanisms of action of IL-17, IL-6, and IL-8, mRNA expression was assessed by RT-PCR in rIL-17-treated and untreated cervical carcinoma cells. A clear up-regu-
tion of IL-6 and IL-8 mRNA was observed 6 h after in vitro stimulation of HeLa cells with rIL-17. IL-17 therefore seemed to regulate IL-6 and IL-8 either at the transcriptional level or by increasing the mRNA half-life.

Characterization of IL-17-transfected Human Cervical Carcinoma Cell Lines. To further analyze the role of IL-17 on human cervical carcinoma cell lines, we transfected two tumor cell lines (HeLa and IC1) with a cDNA encoding hIL-17. Stable transfectants were obtained that secreted significant amounts of IL-17 in their supernatant (from 4–10 ng/ml for 5 × 10^5 cells over 24 h; Fig. 3A). No expression of either IL-17 mRNA or protein could be detected in any of the tumor cell lines analyzed before transfection (Fig. 3A; data not shown). Interestingly, IL-17-transfected cell lines produced more IL-6 than the parent cervical carcinoma cell lines or cells transfected with the vector alone (Fig. 3A), which confirms the results obtained with rIL-17. No clear increase in IL-8 secretion was observed in these IL-17 transfectants, which could be explained by the weaker effect of IL-17 on the regulation of IL-8 than IL-6 in these cells (Fig. 2B).

We then investigated the growth rate of the various clones. Fig. 3B shows a typical experiment, in which IL-17-expressing and parent cells (HeLa and IC1) showed identical in vitro growth. Similarly, exogenous recombinant IL-17 did not influence the proliferation of human cervical cell lines (data not shown).

Tumor Growth of Human Cervical Carcinoma Cells and Their IL-17 Transfectants in Nude Mice. In contrast to the absence of any significant effect of IL-17 on the in vitro growth of cervical carcinoma cell lines, the two cervical carcinoma cell lines (HeLa and IC1) transfected with hIL-17 cDNA and then transplanted in nude mice grew faster than mock-transfected cells (Fig. 4, A and B). For example, at day 31 after transplantation, a mean tumor volume of 26 mm^3...
was measured in transplanted parental HeLa cells, whereas the tumor volume of the IL-17-transfected HeLa cell line reached 404 mm$^3$ ($P = 0.0003$). IL-17 did not affect the tumor incidence rate (Fig. 4C), and its effect seemed more pronounced after an initial growth period in nude mice. Therefore, IL-17 did not play a role in the initial stage of tumor development but rather enhanced its progression.

Analysis of the Expression and Activity of the hIL-17 Transgene in Nude Mice. To determine the in vivo persistence of the IL-17 transgene in tumors transfected with IL-17 cDNA and transplanted in nude mice, biopsies were performed at day 56 after transplantation. hIL-17 cDNA was detected in four of four biopsies derived from mice transplanted with HeLa-IL-17 cells but not in mice previously injected with the control transfectant (HeLa-Neo; Fig. 5A), indicating that the transgene was not lost during tumor development.

Because various studies have reported that hIL-17 is biologically active in murine cells (31), we analyzed the in situ expression of genes normally regulated by IL-17, such as IL-6. Increased expression of
murine IL-6 mRNA was demonstrated in biopsies excised from HeLa-IL-17 cells. (Fig. 5B). Murine IL-6 primers used for this study did not cross-react with hIL-6 (data not shown). The same analysis could not be performed for hIL-6 mRNA because the primers used could also amplify murine IL-6 mRNA. The murine counterpart of hIL-8 has not yet been isolated; therefore, its expression was not investigated.

Immunohistochemical analysis of HeLa-IL-17 tumors with anti-Mac1 antibodies revealed marked infiltration by murine macrophages, whereas mock-transfected HeLa tumors did not seem to recruit cells from the monocyte-macrophage lineage (Fig. 6). This selective peri- and intratumoral macrophage recruitment was also detected in IC1-IL-17 tumors using other antibodies (anti-Mac3) recognizing antigenic determinants on macrophages (data not shown). The intratumoral infiltration of other immune cells such as B cells and neutrophils did not seem to differ in tumor xenografts derived from mock-transfected or IL-17-transfected HeLa cells (data not shown).

### hIL-17 mRNA Expression in Biopsies Derived from Invasive Cervical Carcinomas

The possible involvement of IL-17 in the progression of human cervical tumors prompted us to determine whether it is expressed in tumor biopsies from cervical carcinoma patients. We showed a significant expression of IL-17 in four of six samples tested (Fig. 7). It is noteworthy that no IL-17 mRNA expression could be observed in tumors infiltrated only by CD8 T cells (Fig. 7). Equivalent β-actin mRNA in the various samples assessed by RT-PCR excluded a bias in the interpretation of these results due to a variation of the total mRNA levels (data not shown). In this short series, the presence of CD4-positive T cells seems to be associated with IL-17 mRNA expression, in line with previous studies indicating the activated memory CD4 origin of this cytokine (2).

### DISCUSSION

This study demonstrates that IL-17 increases the growth rate of human cervical tumors transplanted in nude mice. A direct effect of IL-17 on the in vivo proliferation of cervical cancer cells seems unlikely because wild-type and mock- or IL-17-transfected tumors exhibited the same in vitro proliferation kinetics (Fig. 3B). Therefore, an indirect mechanism involving a host-dependent growth-promoting effect may be hypothesized because the enhanced tumor growth elicited by IL-17 was associated in vivo with increased expression of IL-6 and macrophage recruitment at the tumor site (Figs. 5 and 6). In previous studies, macrophages were considered to be the main source of IL-6 produced in cervical carcinoma (24). Induction of IL-6 by IL-17 may play a role in the activity of IL-17 because it has been shown previously that IL-6 stimulates the growth of cervical carcinoma-derived cell lines in medium deprived of growth factors (21, 22). However, in line with our experiments, various groups have also reported that IL-6 did not exhibit any in vitro effect on tumor cell proliferation, despite a significant change in the in vivo tumorigenicity of IL-6-transfected tumor cells (32, 33). Recently, in a model of...
human melanoma transplanted in nude mice, Lu et al. (34) demonstrated that IL-6 could indeed function as an in vivo paracrine growth-stimulating factor for advanced-stage human melanoma cells.

A tumor-promoting activity of macrophages acting on the formation of tumor stroma has also been described. For example, tumor growth was markedly impaired in the op/op mouse, which possesses a profound macrophage deficiency compared with normal littermates. Treatment of tumor-bearing op/op mice with recombinant colony-stimulating factor 1 corrected this impairment (35). Moreover, in nude mice, the reduced tumorigenicity of IL-10-transfected Chinese hamster ovary cells compared to parent cells was found to be associated with inhibition of macrophage recruitment at the tumor site (36). The mechanisms that may explain the selective tumor homing of macrophages in IL-17-producing cervical carcinoma remain to be elucidated. It has been shown that IL-17 induces MCP-1, a known selective macrophage chemoattractant, in epithelial cells (37). However, we found no difference in the expression of JE and MCP-5 mRNA, the murine counterpart of MCP-1, in biopsies derived from mock- or IL-17-transfected cervical carcinoma cell lines transplanted in nude mice. In addition, IL-17 did not induce human MCP-1 in human cervical cell lines (data not shown).

Although it has been shown that IL-17 stimulated granulopoiesis and elicited a rise in peripheral neutrophil counts after s.c. administration (2, 38), we could not demonstrate any significant neutrophil infiltrate in established cervical tumor xenografts.

Of course, only neutralization of macrophages and/or IL-6 activity in vivo would constitute direct evidence of the role of these factors as mediators of IL-17 activity on tumor enhancement. Unfortunately, in three independent experiments, the unexpected inhibitory effects of control immunoglobulins administered to tumor-bearing nude mice prevented us from drawing any definitive conclusions about the role of anti-IL-6 or anti-Mac1 antibodies in this model.

Because IL-17 is secreted mainly by CD4-positive T cells in man [Ref. 2; its source in mice has not been thoroughly investigated (3, 31)], it may seem paradoxical that immune cells secrete tumor-promoting factors. However, other studies have also reported that T lymphocytes could secrete basic fibroblast growth factor or vascular endothelial growth factor, which stimulate tumor angiogenesis (39, 40), or heparin binding epidermal growth factor, which is considered to be a tumor survival factor (40, 41).

In mice, although CD4 and CD8 T cells were often both required for induction of immune control on tumor growth (42–44), various models have clearly implicated the CD4 T-cell population as cells that may enhance tumor development (45). A spontaneously arising B-cell lymphoma in SJL/J mice has been shown to depend on host CD4 T cells for its proliferation and growth (46). Treatment with anti-CD4 mAb before or after inoculation of a lethal dose of lymphoma cells inhibited tumor growth and allowed mice to survive (47, 48). Various immunotherapy protocols in mice, including systemic administration of IL-2 or IL-12, were greatly improved after elimination of CD4 T cells (49–51). Similarly, depletion of CD4 T cells increased the efficiency of T-cell-mediated immunity after peptide vaccination in a model of established micrometastases from lung carcinoma (52).

In man, several tumors seem to be sensitive to the enhancing effects of CD4 T cells. Nakamura et al. (53) characterized Kaposi’s sarcoma cell lines that were dependent on growth factors released by CD4 T cells. Umetu et al. (54) also identified a group of follicular B-cell lymphomas highly infiltrated with CD4 T cells that play an essential role in sustaining rather than inhibiting tumor cell growth in vivo. These examples may explain some reports in which a high number of tumor-infiltrating lymphocytes in certain cancers is considered to be a poor prognostic marker (55).

Finally the expression of IL-17 in biopsies derived from cervical cancer patients (Fig. 7), together with the fact that the amount of IL-17 secreted by activated human T cells (2) was in the same range as that produced by the stably transfected cervical carcinoma cell lines, suggests that our findings may have some clinical relevance. Future studies will be designed to assess the clinical prognostic value of this factor.

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