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Tumor-Derived IL-35 Promotes Tumor Growth by Enhancing Myeloid Cell Accumulation and Angiogenesis

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IL-35 is a member of the IL-12 family of cytokines that is comprised of an IL-12 p35 subunit and an IL-12 p40-related protein subunit, EBV-induced gene 3 (EBI3). IL-35 functions through IL-35R and has a potent immune-suppressive activity. Although IL-35 was demonstrated to be produced by regulatory T cells, gene-expression analysis revealed that it is likely to have a wider distribution, including expression in cancer cells. In this study, we demonstrated that IL-35 is produced in human cancer tissues, such as large B cell lymphoma, nasopharyngeal carcinoma, and melanoma. To determine the roles of tumor-derived IL-35 in tumorigenesis and tumor immunity, we generated IL-35–producing plasmacytoma J558 and B16 melanoma cells and observed that the expression of IL-35 in cancer cells does not affect their growth and survival in vitro, but it stimulates tumorigenesis in both immune-competent and Rag1/2-deficient mice. Tumor-derived IL-35 increases CD11b+Gr1+ myeloid cell accumulation in the tumor microenvironment and, thereby, promotes tumor angiogenesis. In immune-competent mice, spontaneous CTL responses to tumors are diminished. IL-35 does not directly inhibit tumor Ag–specific CD8+ T cell activation, differentiation, and effector functions. However, IL-35–treated cancer cells had increased expression of gp130 and reduced sensitivity to CTL destruction. Thus, our study indicates novel functions for IL-35 in promoting tumor growth via the enhancement of myeloid cell accumulation, tumor angiogenesis, and suppression of tumor immunity. The Journal of Immunology, 2013, 190: 000–000.

Interleukin-35 is a dimeric protein composed of an IL-12α-chain and an IL-27β-chain, which are encoded by the IL12A and EBI3 genes, respectively (1–3). A recent study revealed that IL-35 signals through a unique heterodimer of receptor chains IL-12Rb2 and gp130 or the homodimers of each chain in target cells (4). Although the expression pattern of IL-35 may differ in humans (5, 6), IL-35 was shown to be secreted by Foxp3+CD4+CD25+ regulatory T cells (Tregs) in mice (3) or a regulatory T cell population induced by IL-35 (7). IL-35 was also shown to expand Foxp3+ Tregs (2). IL-35–deficient (either deficient for EBI3 or P35) Tregs have significantly reduced regulatory activity in vitro and fail to control homeostatic proliferation or cure inflammatory bowel disease in vivo (3). IL-35 suppresses T cell proliferation (2), Th17 (2), Th2 responses (2), experimental arthritis (2), and airway inflammation (8). Ectopic expression of IL-35 in pancreatic β cells prevents the development of diabetes mellitus in NOD mice (9). Thus, IL-35 is a novel regulatory cytokine that has potent inhibitory effects on T cell responses.

Although the expression and function of IL-35 have only been demonstrated in Tregs, gene-expression analysis revealed that IL-35 may have much broader tissue distribution (10). Reports indicate upregulation of EBI3 and IL-12 p35 expressions in placental trophoblasts (11), and EBI3 associates with p35 in the extract of the trophoblastic components of human full-term normal placenta (1). EBI3 is also expressed in Hodgkin lymphoma cells (12), acute myeloid leukemia cells (13), and lung cancer cells (14). IL-12p35 (12), but not IL-27p28 (15), was detectable in EBI3+ tumor cells; therefore, it is likely that some cancer cells can produce IL-35 but not IL-27. In the tumor microenvironment, Foxp3+ Tregs and other Tregs are common (16, 17); they can provide another source of IL-35. In addition, tumor-infiltrating dendritic cells were found to express EBI3 (12, 15), which could be an additional source of IL-35. Together, IL-35 could be an important factor in the tumor microenvironment that impacts tumor-specific T cell responses and tumor progression.

Treg-derived IL-35 was shown to inhibit antitumor T cell responses (7). Small interfering RNA silencing of EBI3 in lung cancer cells inhibits cancer cell proliferation, whereas stable expression of EBI3 in lung cancer cells confers growth-promoting activity in vitro (14). Moreover, high EBI3 gene expression in human lung cancer cells was shown to be associated with poor prognosis (14). However, it is unclear whether the observed effect was due to the production of the IL-35 heterodimer. Overall, little is known about the roles of tumor-derived IL-35 in tumorigenesis and the antitumor CTL response. Based on the known roles of IL-35, we hypothesized that IL-35 production in the tumor microenvironment could contribute to tumor progression. To test this hypothesis, we generated IL-35–producing cancer cells and found that expression of IL-35 significantly increased tumorigenesis. IL-35 in the tumor microenvironment significantly increased the numbers of CD11b+Gr1+ myeloid cells in tumors and subsequently promoted tumor an-

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Abbreviations used in this article: IHC, immunohistochemistry; MDSC, myeloid-derived suppressor cell; TIDC, tumor-infiltrating dendritic cell; Treg, regulatory T cell.

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The image contains a page of a document discussing IL-35 in tumor growth and immunity. The text is relevant to research involving the expression of IL-35 in cancer tissues and its role in immune responses. Here is a structured representation of the text:

**Materials and Methods**

**Mice**

BALB/c, C57BL/6, and Rag1−/−C57BL/6 mice were purchased from The Jackson Laboratory. Rag2−/−BALB/c mice were purchased from Taconic Farms (Germantown, NY). Transgenic mice expressing a TCR specific for the tumor Ag P1A (P1CTL), whose TCR recognizes the H-2Ld P1A35-43 complex, were described (18). All animal experiments were performed after approval by The Ohio State University Institutional Animal Care and Use Committee.

**Cancer cell lines and tumor establishment in mice**

Mouse plasmacytoma J558 cells (H-2Kd+) were described (19). Mouse plasmacytoma J559 cells or B16F10 melanoma cells were cotransfected with an expression vector pORF9-mIL-35 or pcDNA3-neo (or the control expression vector pORF9-InvivoGen) and pcDNA3-neo. Thereafter, stable cell lines resistant to G418 were generated. RT-PCR was used to screen IL-35+ cell lines, using the following primers: EB13: 5'-ACGTCTCAATTGCAGCTTCGAGCCT-3' (forward), 5'-TGGTCCTTGTTT-3' (reverse) and gp130: 5'-AGTTGTCATTGCACTTGTTT-3' (reverse) and Kit: 5'-AGTTGTCATTGCACTTGTTT-3' (reverse), 5'-CTGAAAGCGT-GAAACGGATGCACA-3' (reverse). RT-PCR was also used to determine the expression of IL-35R subunits (IL-12Rβ2 and gp130) in IL-35–treated or IL-35+ or IL-35− tumor cells. The following primers were used: IL-12Rβ2: 5'-GGATGGGTTGCCGCAAGGAC-3' (forward), 5'-CTTGTTTGCGTAAAGGAGGATTCT-3' (reverse) and gp130: 5'-TGTCGCTTCATTGCCACTTACAGGCT-3' (forward), 5'-ACGTCCTTCATTGCCACTTACAGGCT-3' (reverse). HPRT gene was amplified for PCR loading control, and the primers used were 5'-GATGATCTTGGTTACAGACGTC-3' (forward), 5'-CCACACAGACGTCCTTACACCT-3' (reverse). The generated J558-IL-35, J558-Ctrl or B16-IL-35, B16-Ctrl cells were maintained in RPMI 1640 medium (Life Technologies) supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin, and 5% FBS. To establish tumors in mice, 5 × 105 J558-IL-35, J558-Ctrl or 0.1 × 106 B16-IL-35, B16-Ctrl cells were inoculated s.c. into the flank. The length (a) and width (b) of each tumor were calculated according to the formula V = ab2/2, as described (18–21). 5 × 105 J558-IL-35 or J558-Ctrl cells were injected i.p. in Rag2−/− mice or bone marrow of normal mice. Macrophage cell lines generated from spleens of J558 tumor–bearing Rag2−/− mice or bone marrow of normal mice. Macrophage cell lines generated from spleens of J558 tumor–bearing Rag2−/− mice or bone marrow of normal mice were cultured in complete RPMI 1640 medium. A total of 2 × 105 cells in 200 μl medium was added to the upper chamber of a Transwell system (3.0–8.0 μm pore size polyester; Becton Dickinson Biosciences) and inserted into a 24-well plate. The lower chambers of each Transwell were filled with 800 μl culture medium, with or without IL-35. The cells were incubated for 24 h at 37°C, medium was discarded, the transmembranes were washed, and the numbers of cells that had migrated to the bottom of the transmembranes were counted under the microscope.

**Statistical analysis**

Data are expressed as mean ± SD. The two-tailed Student t test was used for statistical analysis; p < 0.05 was considered significant.

**Results**

**IL-35 is produced in human cancer tissues**

Accumulating evidence suggests that human cancer tissues may produce IL-35. IHC studies revealed that EB13 protein can be detected in Hodgkin lymphoma cells (12), nasopharyngeal carcinoma cells (12), and lung cancer cells (14). IL-2p25 (12), but not IL-27p28 (15), was detectable in EBI3+ tumor cells. Therefore, it is possible that some cancer cells can produce IL-35. Moreover, tumor-infiltrating dendritic cells (TIDCs) also express EB13 protein (12, 15); thus, TIDCs could be an additional source of IL-35. Furthermore, Foxp3+ Tregs are frequently found in human cancer tissues (16, 17) and could be an additional source of IL-35. Accordingly, it is possible that IL-35 can be detected in human cancer tissues. To test this hypothesis, we performed IL-35 IHC analysis on human large B cell lymphoma (Fig. 1A, 1B) and nasopharyngeal carcinoma (Fig. 1C, 1D), two cancer types that are...
associated with EBV infection. In addition, we also examined IL-35 expression in non-EBV–related cancer tissues, such as melanoma. As shown in Fig. 1B, some large B cell lymphoma cells showed positive staining of IL-35. In other cancer types, IL-35+ staining was detected mainly in stromal cells rather than cancer cells (Fig. 1D, 1F, 1H). In addition, IL-35+ cells exhibited various shapes (Fig. 1D, 1F, 1H). Thus, IL-35 can be readily detected in various human cancer tissues and is likely of multiple cellular sources.

**IL-35 production in the tumor microenvironment stimulates tumor growth**

To determine the roles of IL-35 in the tumor microenvironment, we generated IL-35+ or IL-35− mouse plasmacytoma J558 cells and B16 melanoma cells by transfecting J558 and B16 melanoma cells with an IL-35 expression vector or a control expression vector. RT-PCR analysis revealed that J558–IL-35 and B16–IL-35 cells expressed both EBI3 and IL35A mRNA (Fig. 2A, 2E). Moreover, RT-PCR assay using EBI3 forward primer and P35 reverse primer detected the rRNA encoding both EBI3 and IL-35A subunits (Fig. 2A, 2E). Thus, the IL-35 molecule was stably expressed in J558–IL-35 and B16–IL-35 cells. Immunocytochemistry staining of cells and ELISA assay of supernatants from cell cultures revealed that IL-35 protein was produced by J558 (Fig. 2B) and B16 (Fig. 2F) cells. Expression of IL-35 in cancer cells did not alter the expression of MHC class I (Fig. 2C, 2G) and tumor Ag P1A (Fig. 2A) in J558 cells. MTT assay revealed that IL-35 expression did not affect J558 and B16 cell growth in vitro (Fig. 2D, 2H).

To determine whether the expression of IL-35 in tumor cells affects tumor growth in vivo, $5 \times 10^6$ IL-35+ or IL-35− J558 cells were injected s.c. into each BALB/c mouse. As shown in Fig. 3A, IL-35− J558 cells induced tumors in normal BALB/c mice 10–14 d after tumor cell injection. J558–IL-35 tumors grew progressively in BALB/c mice; within 2 wk the tumors grew to a size that required euthanasia. Our results indicated that ex vivo J558–IL-35 tumors were significantly bigger than ex vivo J558-Ctrl

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**FIGURE 1.** Expression of IL-35 in human cancer tissues. H&E staining and IHC were performed on paraffin-embedded serial tissue sections of human large B cell lymphoma (A, B), nasopharyngeal carcinoma (C, D), skin melanoma (E, F), and lymph node metastatic melanoma (G, H). The anti-human IL-35 mAb 15K8D10 was used to stain human cancer tissues. (A), (C), (E), and (G) show H&E staining; (B), (D), (F), and (H) show IL-35–specific staining. Scale bars, 100 μm.

**FIGURE 2.** Generation of IL-35–producing J558 and B16.F10 cells. Mouse plasmacytoma J558 cells or B16F10 melanoma cells were cotransfected with an expression vector pORF9-IL-35 and a selection vector pCDNA-neo or the control expression vector pORF9 and pCDNA-neo. Stable cell lines that were resistant to G418 were generated. RT-PCR was used to detect the expression of transcripts for recombinant IL-35, IL-12A, EBI3, and tumor Ag P1A in J558 cells (A) and IL-35, IL-12A, and EBI3 transcripts in B16F10 cells (E). Immunofluorescence staining and ELISA revealed that IL-35 protein was produced by the generated J558 (B) and B16.F10 (F) cells. Flow cytometry was used for the analysis of MHC class I expression on the generated J558 cells (C) and B16 cells (G). MTT proliferation assay was used to measure growth and proliferation of J558 cells (D) and B16 cells (H). Bars indicate SD of triplicates. Original magnifications ×400 for (B) and (F).
creased tumor angiogenesis was also found in B16–IL-35 tumors. Tumor angiogenesis, as determined by CD31 expression, was significantly increased in J558–IL-35 mice. Tumor angiogenesis, as determined by CD31 expression and CD34 expression, was also significantly increased in J558–IL-35 tumors grown in BALB/c mice (Fig. 3E, 3F). MDSCs (CD11b+Gr1+) were found to be highly recruited into J558–IL-35 tumors, while the numbers of CD11b single-positive cells were lower in J558–IL-35 tumors compared with control tumors (Fig. 3A). However, the numbers of Gr1 single-positive cells were low and did not differ between the two types of tumors (Fig. 3A). Flow cytometry analysis of disassociated tumor cells verified the fluorescence staining results obtained from tumor sections (Fig. 3B). Furthermore, increased numbers of CD11b/Gr1+ cells were also found in J558–IL-35 tumors grown in BALB/c mice (Fig. 3C) and B16–IL-35 tumors from C57BL/6 mice (Fig. 3D) in comparison with control tumors. Anti-Gr1 mAb treatment largely eliminated the tumor growth difference between the two groups of mice (Fig. 3D). Therefore, IL-35 may play a chemotaxis effect on myeloid cells.

**IL-35 enhances myeloid cell accumulation and tumor angiogenesis**

To determine the mechanisms by which IL-35 expression promotes tumor growth, we first examined tumor angiogenesis in established J558–IL-35 and J558-Ctrl tumors collected from Rag2−/− BALB/c mice. Tumor angiogenesis, as determined by CD31 expression and expression of VEGF, was significantly increased in J558–IL-35 tumors in comparison with J558-Ctrl tumors (Fig. 4A, 4B). Increased tumor angiogenesis was also found in B16–IL-35 tumors (Fig. 4C, 4D).

**IL-35 does not enhance migration of myeloid cells in vitro**

IL-35+ tumors also contained increased numbers of myeloid cells in comparison with J558-Ctrl tumors. Fluorescence staining of sections from J558–IL-35 tumors grown in Rag2−/− mice revealed accumulation of more myeloid-derived suppressor cells (MDSCs; CD11b+Gr1+) (Fig. 5A). In contrast, the numbers of CD11b single-positive cells were lower in J558–IL-35 tumors compared with control tumors (Fig. 5A). However, the numbers of Gr1 single-positive cells were low and did not differ between the two types of tumors (Fig. 5A). Flow cytometry analysis of disassociated tumor cells verified the fluorescence staining results obtained from tumor sections (Fig. 5B). Furthermore, increased numbers of CD11b+/Gr1+ cells were also found in J558–IL-35 tumors grown in BALB/c mice (Fig. 5C) and B16–IL-35 tumors from C57BL/6 mice (Fig. 5D) in comparison with their controls. To determine whether increased MDSC accumulation was responsible for enhanced tumor growth, we injected three doses of anti-Gr1 mAb (250 μg/dose) into J558–IL-35 or J558-Ctrl cell–inoculated Rag2−/− BALB/c mice. We previously showed that this regimen could completely deplete Gr1+ cells (22). We found that anti-Gr1 mAb treatment largely eliminated the tumor growth difference between the two groups of mice (Fig. 5E). Thus, MDSC accumulation is largely responsible for the enhanced growth of J558–IL-35 tumors in Rag2−/− BALB/c mice.
different lineages of myeloid cells, including macrophage cell lines Raw264.7 (H-2b) and P338D1 (H-2d), MDSCs purified from the spleen of J558-tumor bearing mice, and bone marrow–derived CD11b+Gr1+ cells, were used in the transmigration assay. As shown in Fig. 6, similar numbers of Raw264.7 cells (Fig. 6A), P338D1 cells (Fig. 6B), spleen MDSCs (Fig. 6C), and bone marrow

**FIGURE 4.** IL-35 production in the tumor microenvironment enhances angiogenesis. J558 tumors (A, B) from Rag2<sup>−/−</sup> mice and B16 tumors from C57BL6 mice (C, D) were analyzed for the expression of CD31 and VEGF by immunofluorescent staining and microscopy. Scale bars, 200 μm. Mean vessel wall area and numbers of VEGF<sup>+</sup> cells for each tumor were analyzed and quantified using ImageJ software. Three random fields from each slide/tumor were analyzed, and each symbol represents data from one microscope field. **p = 0.001, ***p < 0.0001, Student t test.

**FIGURE 5.** Increased numbers of CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid cells in the tumor microenvironment of IL-35<sup>+</sup> tumors. (A) J558–IL-35 and J558-Ctrl tumors from Rag2<sup>−/−</sup> mice were analyzed for the infiltration of myeloid cells by immunofluorescence staining and microscopy. Scale bars, 200 μm. Frozen tissue sections were colabeled for CD11b (Alexa Fluor 488) and Gr1 (Texas Red), and images were analyzed and quantified using ImageJ software. Three random fields from each slide/tumor were analyzed, and each symbol represents data from one microscope field. Arrows indicate CD11b and Gr1 double positive MDSCs. **p < 0.001, Student t test. (B–D) Flow cytometry was also used for the analysis of myeloid cells in IL-35<sup>+</sup> and IL-35<sup>−/−</sup> tumors. Single-cell suspensions were prepared from tumors grown in Rag2<sup>−/−</sup> (B), BALB/c (C), and C57BL6 (D) mice and stained for CD11b and Gr1, followed by flow cytometry analysis. Each symbol represents data from a single tumor. *p < 0.05, **p < 0.01, ***p < 0.001, Student t test. (E) A total of 5 × 10<sup>6</sup> J558–IL-35 or J558-Ctrl cells was injected s.c. into each BALB/c mouse, followed by treatment with 250 μg/mouse of anti-Gr1 mAb (RB6-8C5; Bio X Cell) i.p. on days 0, 5, and 10. Mice were observed for tumor growth over time. Five mice/group were used for this experiment, and the data shown represent two experiments with similar results.
IL-35 does not directly inhibit tumor Ag–specific CTL proliferation and effector functions

To determine whether IL-35 production directly inhibits tumor Ag-specific CD8+ T cell proliferation and effector function, we cultured P1CTL transgenic T cells that recognize tumor Ag P1A in the presence of IL-35 produced by J558 cells. There were no differences in the proliferation and growth of P1CTL cells either in the presence or absence of IL-35, as determined by [3H]thymidine-incorporation assay (Fig. 8A) and MTT assay (Fig. 8B). IL-35–stimulated P1CTL cells also expressed similar levels of IFN-γ and granzyme B (Fig. 8C) and exhibited similar cytotoxicity to P1A+ P815 target cells (Fig. 8D).

IL-35 induces resistance of cancer cells to destruction by tumor Ag–specific CTL

Despite expressing similar levels of P1A Ag and MHC class I (Fig. 2), J558–IL-35 cells were more resistant to P1CTL destruction...
compared with J558-Ctrl cells (Fig. 9A). Similarly, IL-35–stimulated P815 cells were also found to be more resistant to P1CTL lysis (Fig. 9B) compared with control P815 cells. Consistent with this observation, established J558–IL-35 tumors in BALB/c mice contained less apoptotic tumor cells compared with J558-Ctrl tumors (Fig. 9C). Thus, IL-35 renders tumor target cells more resistant to CTL destruction. To determine whether cancer cell resistance to CTL destruction was due to IL-35R signaling, we determined the expression of IL-35R subunits (IL-12Rβ2 and gp130) in IL-35+ and IL-35− tumor cells by RT-PCR. As shown in Fig. 9D, although expression of IL-12Rβ2 and gp130 was barely detectable in B16 cells, increased gp130 expression was detected in J558–IL-35 cells compared with J558-Ctrl cells. To determine whether induction of gp130 was IL-35 specific, J558 and P815 cells were cultured or not with IL-35 for 24 h, and we determined gp130 expression by RT-PCR. As shown in

FIGURE 8. IL-35 does not directly affect differentiation of tumor Ag–specific CTL. Splenocytes from P1CTL-transgenic mice were activated with P1A peptide (0.2 μg/ml) in the presence or absence of IL-35. [3H]tritium-incorporation assay (A) and MTT assay (B) were used to determine cell proliferation and survival. (C) Intracellular staining and flow cytometry were used to determine IFN-γ and granzyme B expression in activated P1CTL cells. (D) [51Cr] release assay was used to determine cytotoxicity of activated P1CTL cells to P815 target cells. Data shown represent at least three experiments with similar results.

FIGURE 9. IL-35 induces tumor cell resistance to CTL destruction. P1CTL cells were activated with P1A peptide (0.2 μg/ml) for 5 d. [51Cr] release assay was used to determine cytotoxicity of activated P1CTL cells to J558–IL-35/J558-Ctrl cells (A) and P815 cells treated or not with IL-35 (B). Data shown represent three experiments with similar results. (C) Frozen tissue sections from J558–IL-35 or J558-Ctrl tumors grown in BALB/c mice were labeled for TUNEL; images were photographed under a fluorescent microscope and quantified using ImageJ software (left panels). Scale bars, 200 μm. Three random fields from each slide/tumor were analyzed, and each symbol represents data from one microscope field (right panel). RT-PCR was used to detect IL-35R subunits in B16–IL-35/B16-Ctrl and J558–IL-35/J558-Ctrl cells (D) or in J558 and P815 cells treated or not with IL-35 (E). Data shown in (D) and (E) represent three experiments with similar results. *p < 0.05, Student t test.
IL-35 IN TUMOR GROWTH AND IMMUNITY

Discussion
Our study validates the hypothesis that IL-35 is produced in human cancer tissues, and tumor-derived IL-35 plays important roles in tumor progression and tumor immune surveillance.

First, we demonstrated that IL-35 is produced in human cancer tissues. Among the three types of human cancer tissues examined, IL-35 is mainly found in tumor stromal cells. Thus, it will be interesting to determine the types of cells in cancer stromas that produce IL-35. Previous studies revealed that TIDCs (12, 15) express EBI3; therefore, TIDCs could be a source of IL-35. Additionally, tumor-infiltrating FOXP3+ Tregs could be another source of IL-35. EBI3 is known to be expressed in EBV-associated Hodgkin lymphoma, diffuse large B lymphoma, nasopharyngeal carcinoma (12, 25), and EBV-malignant tumors (14). In this study, we observed that some diffuse large B lymphoma cells are also positive for IL-35. Therefore, IL-35 is abundantly produced in many human cancer tissues and multiple cell types, including some cancer cells.

We next observed that tumor-derived IL-35 considerably increased tumorigenesis in both immune-competent and immune-deficient mice. The protumor effect is very rapid, which necessitates sacrifice of mice injected with IL-35+ cancer cells within 2–3 wk after tumor cell injection. This protumor effect of IL-35 was confirmed in two tumor models: plasmacytoma J558 and B16.F10 melanoma. Previous studies reported that IL-35–producing Tr1 cells can inhibit tumor growth via suppression of antitumor immune responses (7). Because the protumor effect of IL-35 was also observed in Rag1/2-deficient mice in this study, the rapid tumor growth of IL-35–producing tumors is not solely due to suppression of adaptive immunity. The IL-35 effect in tumors also differs from another EBI3-containing cytokine, IL-27, which has potent tumor-inhibiting effects (26–31). Expression of EBI3 in human lung cancer cells was reported to promote lung cancer cell growth in vitro (14). However, in this study we showed that IL-35 expression did not affect tumor growth and proliferation in vitro. It is not known whether the discrepancy of the results is due to a lack of IL-35 heterodimer formation in lung cancer cells. To our knowledge, this is the first study to show that tumor-derived IL-35 has a protumor effect in vivo.

We demonstrated that IL-35 production in the tumor microenvironment increased CD11b+Gr1+ myeloid cell accumulation and tumor angiogenesis (reflected by increased density of CD31+ blood vessels and VEGF production in the tumor bed). Because IL-35 production by cancer cells does not affect their growth and proliferation in vitro, it is likely that IL-35–producing cancer cells are capable of inducing host cells to promote tumor growth. In this regard, increased numbers of CD11b+Gr1+ MDSCs were present in IL-35+ tumors. MDSCs are a known cell type that can promote tumor angiogenesis via production of VEGF and other proangiogenesis factors (32–34). Thus, it is likely that increased angiogenesis and tumor growth in IL-35+ tumors are due to increased accumulation of CD11b+Gr1+ myeloid cells. Indeed, depletion of Gr1+ myeloid cells abrogated tumor growth enhancement by IL-35. Our in vitro migration assay revealed that tumor-produced IL-35 does not have a direct role in MDSC chemotaxis. Quantitative RT-PCR analysis also revealed that VEGF expression was not altered in J558–IL-35 and B16–IL-35 cells in comparison with their respective control cells (data not shown). Thus, it remains to be determined what signal pathways are activated in tumor cells by IL-35, which are responsible for initially accumulating CD11b+Gr1+ myeloid cells into tumors and, thereby, inducing tumor angiogenesis.

Finally, in this study we demonstrated that tumor-derived IL-35 induces a suppressive tumor microenvironment, which, in turn, inhibits tumor immunity. MDSCs induce immune suppression and inhibit CTL responses (23, 24). Consistent with this observation, we found that spontaneous CTL responses in IL-35+ tumors were inhibited in immune-competent mice. However, IL-35 does not directly inhibit CTL proliferation, differentiation, and effector functions in in vitro assays, and this is in contrast to other IL-12 family cytokines, such as IL-12 and IL-27, which can significantly affect CTL differentiation (35–37). Thus, it is likely that the inhibition of CTL responses in IL-35+ tumors is due to the accumulation of MDSCs therein, which subsequently inhibits CTL responses.

Although IL-35 does not have direct effects on CTL activation and effector functions, we showed that IL-35–treated target cells were less susceptible to CTL-mediated destruction. Consistent with this effect, we found that less apoptotic tumor cells were observed in IL-35+ tumors than in IL-35− tumors grown in immune-competent mice. This effect is unlikely mediated by downregulation of MHC class I or Ag expression, because IL-35–expressing tumor cells have similar levels of MHC class I and Ag. Our RT-PCR results suggest that IL-35 upregulates gp130 expression in tumor cells. Interestingly, gp130 signaling was shown to mediate cancer cell resistance to chemotherapy (38). Because IL-35 is known to signal through the gp130 dimer (4), it is highly suggestive that IL-35 signaling via gp130 induces cancer cell resistance to CTL destruction. Thus, IL-35–induced cancer cell resistance to CTL destruction could be one mechanism by which cancer cells escape CTL destruction in tumors.

Taken together, our investigation determining the role of tumor-derived IL-35 on tumor growth and immunity revealed novel functions for IL-35 in promoting tumor growth and inhibition of antitumor CTL responses. Because IL-35 can be produced by both cancer cells and tumor-infiltrating stromal cells, further investigations elucidating the cross-talk among cancer cells, tumor-infiltrating stromal cells, and MDSCs via IL-35 may help us to better understand tumor progression and immune evasion. Accordingly, targeting IL-35–mediated cross-talk may be a novel immunotherapeutic approach for the treatment of cancer patients.

Disclosures
The authors have no financial conflicts of interest.

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