Viral Interleukin 10 (IL-10), the Human Herpes Virus 4 Cellular IL-10 Homologue, Induces Local Anergy to Allogeneic and Syngeneic Tumors
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Summary
After the cloning of murine cytokine synthesis inhibitory factor, it was recognized that a homologous open reading frame was encoded within the Epstein-Barr virus (human herpes virus 4). This viral protein has now been termed viral interleukin 10 (vIL-10) to reflect its protein sequence homology to “cellular” IL-10 (cIL-10, either murine or human IL-10). It is now widely accepted that vIL-10 shares many functions with cIL-10, principally, the ability to enhance survival of newly infected B cells and to diminish the production of IFN-γ and IL-2 during ongoing immune reactions. The immunomodulatory effect of locally secreted vIL-10 and murine IL-10 (mIL-10) was examined in tumor models using CL8-1 (a BL6 melanoma cell line transfected with the H-2Kb class I gene) in syngeneic animals. Although parental BL6 tumor cells grow in immunocompetent syngeneic hosts, CL8-1 are rejected. To achieve local secretion of vIL-10, we generated vIL-10 retroviral vectors. While nontransduced CL8-1 cells (1 x 10⁴) failed to grow when injected intradermally in C57BL/6 mice, CL8-1 cells (1 x 10⁴) transduced with vIL-10 formed palpable tumors and eventually killed 80% of injected animals. Suppression of tumor rejection was also noted when CL8-1 tumors with or without vIL-10 transfection were admixed with syngeneic vIL-10–transfected fibroblasts and inoculated. Since the in vitro proliferation of the tumor was not altered after transduction with the vIL-10 gene and injection of vIL-10–transduced CL8-1 does not affect the rejection of nontransduced CL8-1 inoculated at a distant site, local vIL-10 secretion appears to suppress the process of immune rejection of the target cells in a dose-dependent manner. Similar results were observed for the H-2k MCA105 sarcoma tumor model in allogeneic BALB/c mice (H-2d). Although all animals that received nontransfected MCA105 rapidly rejected these tumors, MCA105 sarcomas transacted with vIL-10 remained palpable for up to 37 d. The local immunosuppressive effect of gene-delivered vIL-10 could be neutralized by anti–human IL-10 monoclonal antibody or could be reversed by the systemic administration of IL-2 or IL-12. In marked contrast, mIL-10 transfection of CL8-1 significantly suppressed tumor growth and frequently led to the rejection of tumor. Similar results were obtained for the murine tumor cell lines MC38 and MCA102. These results contrast the immunologic effects of vIL-10 and cIL-10 and argue for divergence in the function of the virally captured gene, which is presumably the result of altered interaction with one of the two derived IL-10 receptors. The present study, along with our recent study showing prolonged survival of cardiac allografts transduced with retroviral vIL-10, suggests that local vIL-10, but not mIL-10, secretion can suppress immune reactivity in both the syngeneic and allogeneic settings.

The cytokine IL-10 was initially described as cytokine synthesis inhibitory factor (CSIF),¹ a product of mouse Th2 cells that inhibited cytokine synthesis of Th1 cells (1). The CSIF activity of IL-10 is mediated principally via its inhibitory effects on APC and accessory cell function by macrophages and dendritic cells (2, 3). Murine “cellular” IL-10 (mIL-10) and human cellular IL-10 (hIL-10) cDNA sequences exhibited a strong homology to a previously uncharacterized

¹ Abbreviations used in this paper: CHO, Chinese hamster ovary; cIL-10, cellular IL-10; CSIF, cytokine synthesis inhibitory factor; hIL-10, human IL-10; MCA, methylcholanthrene; mIL-10, murine IL-10; vIL-10, viral IL-10.
open reading frame in the EBV (human herpes virus 4), viral IL-10 (vIL-10), formerly termed BmH1, C fragment rightward reading frame 1 (4, 5). The hIL-10 and vIL-10 mature protein sequences are 84% identical, with most of their divergence found in the NH2-terminal 20 amino acids (4). The vIL-10 gene was initially characterized as a "late" viral gene, expressed during the lytic phase of virus replication (6), but, more recently, vIL-10 has been suggested to be an immediate early gene product (7). The protein product of vIL-10 shares some functional properties with both hIL-10 and mIL-10 (5). vIL-10 shares many functions of cellular IL-10 (cIL-10) and mediates predominantly immunosuppressive effects through the inhibition of cytokine synthesis (IL-2 and IFN-γ) by human PBMC (1, 8, 9). vIL-10 strongly reduces antigen-specific T cell proliferation by diminishing the antigen-presenting capacity of monocytes via down-regulation of class II MHC expression (10). However, vIL-10 does not (a) stimulate the proliferation of murine thymocytes (11), (b) stimulate proliferation of murine mast cell lines (5, 12), or (c) induce increased expression of class II MHC molecules on murine B cells (13). Thus, vIL-10 appears to function as a predominantly immunosuppressive agent. These in vitro results suggest that vIL-10 could be an ideal cytokine for blunting the immune response after transplantation or potentially in the setting of autoimmune disease.

To test this hypothesis, we used a tumor system that directly reflects the in vivo immunologic environment. Some tumors in experimental animals escape T cell-mediated destruction by down-regulation of MHC class I molecule expression (14). The importance of these molecules in tumor rejection is strongly supported by experiments in which transfection of class I MHC gene(s) leads to restoration of tumor cell immunogenicity and prevention of tumor growth in immunocompetent host (15, 16). Although the B16 melanoma expresses low levels of H-2 class I molecules and is poorly immunogenic (17, 18), transfection of B16 melanoma cells with H-2Kb results in increased immunogenicity and rejection (19). We used retrovirus-mediated gene transfer to introduce the vIL-10 and mIL-10 genes into these cells, and we studied the consequences of local cytokine production on the development of tumors in vivo. The results distinguish the effects of vIL-10 and cIL-10 in vivo and support in vitro evidence suggesting that vIL-10 is able to confer only a subset of the activities of the cIL-10. The present results, along with our recent study showing prolonged survival of cardiac allografts after retrovirus-mediated vIL-10 transduction (Qin, L., unpublished observation), indicate that local vIL-10 but not mIL-10 production can suppress immune responses in both syngeneic and allogeneic settings.

Materials and Methods

Mice. Female C57BL/6 mice and BALB/c mice, 8-12 wk old, were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were routinely screened and found to be pathogen free.

Murine Cell Lines. The CL8-1 clone was generously provided by Dr. E. Gorelik (University of Pittsburgh, Pittsburgh, PA) and used in most experiments. CL8-1 (19) was obtained after cotransfection of the BL6-8 (H-2Kb−, H-2Db−) B16 melanoma clone with a plasmid encoding the class I gene H-2Kb and one encoding the bacterial neomycin phosphotransferase gene conferring sensitivity to the neomycin analogue, Geneticin (G418; GIBCO BRL, Gaithersburg, MD). MCA102 and MCA105 are methylcholanthrene (MCA)-induced murine fibrosarcoma cell lines. MC38 is a murine colon adenocarcinoma cell line. All were generously provided by Dr. S. A. Rosenberg (National Cancer Institute, National Institutes of Health, Bethesda, MD) and maintained by serial transplantation in mice (20). Tumor cell lines were maintained as adherent cultures derived from harvesting fresh tumor specimens ∼10 mm in diameter. TIB81 is an H-2● embryonic fibroblast cell line purchased from American Type Culture Collection (Rockville, MD). CL8-1, MCA102, MCA105, and MC38 cells were maintained in RPMI 1640 (GIBCO BRL) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. TIB81 cells were maintained in DMEM (GIBCO BRL) supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, and 100 mg/ml streptomycin.

Reagents. rhIL-2 was obtained from Dr. A. Louie (Chiron, Emeryville, CA) and had a specific activity of 18 × 106 IU/mg (21, 22). Recombinant murine IL-12 (rmIL-12) was kindly provided by Dr. B. Hubbard (Genetics Institute, Cambridge, MA). The specific activity was 5-7 × 106 U/mg as determined in a PHA blast proliferation assay as previously described (23). For in vivo administration, IL-12 was diluted in 0.1% mouse albumin carrier protein. mAb 19F1, which blocks both hIL-10 and vIL-10, was provided by Schering-Plough Research Institute (Kenilworth, NJ).

Construction of Vectors Carrying vIL-10 and mIL-10. The cDNA-encoding vIL-10 and mIL-10 were subcloned into a retroviral–proviral construct termed MFG (4, 24, 25). The cDNAs were inserted into MFG so that the translational initiation codon ATG of vIL-10 and mIL-10 protein was fused to the ATG of the retroviral env gene. We have also subcloned vIL-10 and mIL-10 cDNA into a modified MFG (termed DFG), which simultaneously expresses a neomycin transference gene as a selectable marker (26) (Fig. 1). The packaging cell line termed ΨCRIP (27) was transfected with these proviral plasmids using the calcium phosphate method to generate stable producer cell lines.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Structure of retroviral constructs containing cDNAs of vIL-10 and mIL-10. **(A)** MFG-mIL-10, and **(B)** MFG-vIL-10. vIL-10 and mIL-10 cDNAs were subcloned into the Nco I cloning site, which allows fusion of the protein-coding region of the cloned gene into the ATG of the env-coding region. Both vIL-10 and mIL-10 are expressed with a spliced RNA message. **(C)** DFG-mIL-10-Neo, and **(D)** DFG-vIL-10-Neo. vIL-10 and mIL-10 cDNAs were subcloned into DFG-Neo. Neomycin transference can be translated because of an internal ribosome entry site (IRES), located 5' to the Neo cDNA insert. LTR, long terminal repeat; Ψ, Ψ-packaging sequences; SD, splice donor; SA, splice acceptor.

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Infection and Measurement of vIL10 and mIL-10 Production. Tumor or fibroblast cell lines were infected in culture using viral supernatant from the respective producer cell lines. Cells were incubated with viral supernatant containing polybrene (8 μg/ml) at 37°C for 3 h. After incubation, viral supernatant was replaced with fresh medium. When the cells were infected with DFG, cells were split 48 h after the infection and selected in media containing 0.5-0.75 μg/ml G418. Supernatant of infected cells was harvested after infection and tested for vIL-10 and mIL-10 protein using an ELISA as directed (Pharmingen, San Diego, CA). The amount of vIL-10 was determined with an ELISA, which detects both v- and hIL-10 but does not cross-react with mIL-10. Levels of mIL-10 were measured by ELISA using an mAb specific to mIL-10.

In Vivo Cell Proliferation Assay. Morphology and in vitro cell proliferation patterns were compared between vIL10- and mIL10-transduced and nontransduced tumors (CL8-1, MCA105, MC38). 1 × 10⁴ nontransduced vIL10-transduced, and mIL10-transduced cells were seeded into 10-cm dishes. At certain time points (1, 2, 3, 4, and 5 d after the cell split), the morphology of cells was examined, and the number of total cells in each dish was evaluated after obtaining single cell suspensions by trypsinization.

Murine Studies. The mice used in the experiments were ear tagged and randomized before inoculation with tumor. Tumor cells were inoculated intradermally in the shaved area of the left flank, and tumor establishment was determined by palpation. Tumor size was determined by measuring perpendicular tumor diameters with a vernier caliper. In some experiments, tumor cells were injected intradermally admixed with fibroblasts. All these measurements were done in a coded, blinded fashion. Systemic rIL-2, rML-12, and anti-hIL-10 mAb were administered by intraperitoneal injection. Treatment groups consisted of five mice per group.

Immunohistochemistry. Tumor specimens were harvested and snap frozen 10 d after nontransfected CL8-1, CL8-1-vIL-10, or CL8-1-mIL-10 cells were inoculated into syngeneic mice. Cryostat-cut sections were fixed in cold acetone, hydrated in PBS, and incubated in protein-blocking solution (Lipshaw Immunon, Pittsburgh, PA) for 8 min. They were then incubated overnight at 4°C with rat mAbs directed against mouse CD4, CD8, and macrophages (MOMA-2; Serotec Ltd., Oxon, UK). Isotype-matched rat IgG was used as a negative control. A biotinylated mouse primary antibody against I-A<sup>+</sup> was also used. In this case, an irrelevant biotinylated mouse mAb (anti-BRDU; Caltag Laboratories, San Francisco, CA) was used as a negative control. Positive tissue controls consisted of frozen sections of spleens from B10 and BALB/c mice. After buffer washes, endogenous peroxidase activity was quenched with 0.6% H<sub>2</sub>O<sub>2</sub> in methanol. Species-absorbed biotinylated mouse anti-rat F(ab')<sub>2</sub> (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was applied to sections incubated with unlabeled primary antibodies, followed by streptavidin peroxidase (Boehringer Mannheim, Indianapolis, IN) and 3'-amino-9-ethylcarbazole (Biomedica Corp., Foster City, CA). In the case of anti-I-A<sup>+</sup> and its negative control, the biotinylated secondary antibody was eliminated. Sections were counterstained with hematoxylin and mounted in Crystal Mount (Biomeda Corp.).

Statistical Analysis. The percentage of mice with tumor and tumor size in each group were calculated and compared with other groups using a standard Student's t-test. The differences were considered statistically significant when the P value was <0.05.

Results

Substantial Amounts of vIL10 and mIL10 Proteins Are Secreted after Infection of Cells with Retroviral Vectors. The levels of v- and mIL-10 secreted from target cells infected with retrovirus are shown in Table 1. These levels from infected cells were higher than those from COS-7 cells transfected with an expression plasmid (pJRU3-vIL-10), which has a modified SV40 promoter (28) to drive vIL-10 gene expression (COS-7, 3.2 ng/10⁶ cells per 48 h). Repeated infections have often increased expression of IL-10 protein. No significant difference in cell proliferation (Fig. 2) or H-2K<sup>b</sup> expression (mAb 28-13-3S, anti-H-2K<sup>b</sup>, IgM; American Type Culture Collection) was observed when compared with nontransduced CL8-1 and CL8-1-vIL-10 cells.

In Vivo Growth of Nontransfected Tumor Cells in the Syngeneic or Allogeneic Setting Is Tumor Dose Dependent, and Rejection Is Mediated Immunologically. Class I-transfected CL8-1 cells have been reported to grow less well when compared with parental BL6 melanoma cells in an immunocompetent syngeneic host (19, 29). Titrated doses of CL8-1 cells and BL6 cells were inoculated intradermally into syngeneic mice to confirm this difference (C57BL/6, groups of five mice; data not shown). Parental BL6 cells grew as intradermal tumors in all mice, even when as few as 1 × 10⁴ cells were inoculated. At the same cell dose, CL8-1 was poorly tumorigenic, inducing tumors in no more than 0–20% of mice. Only when >1 × 10³ CL8-1 cells were inoculated intradermally into syngeneic mice did 60–80% of the animals develop a palpable tumor. Expression of the class I gene (H-2K<sup>b</sup>) in tumor cells is associated with a reduced tumorigenic potential in syngeneic recipients. In an allogeneic setting (H-2<sup>b</sup>, BALB/c; data not shown), all tumors were rejected within 2–3 wk after inoculation.

To examine the mechanism of rejection of the CL8-1 cells in syngeneic animals, varying doses of CL8-1 cells were inoculated intradermally into irradiated (a sublethal dose of 500 R to whole body) or nonirradiated C57BL/6 mice (Fig. 3, A and D). While 1 × 10⁴ CL8-1 cells did not grow in nonirradiated immunocompetent mice, they did grow in all the irradiated mice. The size of the tumor at day 25 in irradiated mice (1 × 10⁴, 19.40 ± 4.67 mm²; 5 × 10⁴, 36.40 ± 9.50 mm²; 1 × 10⁵, 56.20 ± 8.77 mm²) was significantly larger than that observed in CL8-nonirradiated mice (1 × 10⁴, 0 ± 0 mm²; 5 × 10⁴, 2.40 ± 5.37 mm²; 1 × 10⁵, 9.60 ± 9.21 mm²; P <0.0001). Thus, failure of tumor growth appears to be mediated by radiation-sensitive, most likely immune, cells.

vIL10 Gene Transduction Can Suppress Immune Rejection of Tumor Cells In Vivo. A series of experiments was performed to determine if local vIL-10 secretion could suppress immunemediated rejection of CL8-1 tumor cells inoculated intradermally into syngeneic animals. 10⁴ CL8-1 cells, transduced with MFG-vIL10 (CL8-1-vIL-10, 72.56 ng/10⁶ cells per 48 h), were injected intradermally into syngeneic C57BL/6 animals. Although nontransduced CL8-1 cells failed to grow in any of these animals, CL8-1-vIL-10 displayed palpable tumors in 80% of injected animals. All of these animals developed progressively growing tumors (Fig. 3, B and E). The size of the CL8-1-vIL-10 tumors at day 35 (89.6 ± 67.7 mm²) was significantly larger than that of nontransfected CL8-1 tumors (0 ± 0 mm², P = 0.018). All of the animals inoc-
# Table 1. Expression of vIL-10 and mIL-10 Proteins After Transfection

| Cells      | Cell type     | Vector       | Selection* | Mean ± SD (experiment No.) |
|------------|---------------|--------------|------------|---------------------------|
| 3T3        | Fibroblast    | MFG-vIL-10   | -          | 30.62 ± 7.45 (5)          |
|            |               | DFG-vIL-10-Neo | +b         | 106.83 ± 37.52 (5)        |
|            |               | MFG-mIL-10   | -          | 37.09 ± 12.6 (2)          |
|            |               | DFG-mIL-10-Neo | +b         | 55.50 (1)                 |
| TIB81      | Fibroblast    | MFG-vIL-10   | -          | 16.72 (1)                 |
|            |               | DFG-vIL-10-Neo | +b         | 32.59 ± 7.45 (11)         |
|            |               | DFG-mIL-10-Neo | +b         | 36.70 ± 8.63 (2)          |
| CL8-1      | Melanoma      | MFG-vIL-10(×1) | -          | 21.49 ± 8.21 (21)         |
|            |               | (×2)         | -          | 43.56 ± 12.50 (13)        |
|            |               | (×4)         | -          | 61.73 ± 12.24 (7)         |
|            |               | DFG-vIL-10-Neo | -          | 78.47 ± 30.03 (7)         |
|            |               | MFG-mIL-10   | -          | 42.06 ± 9.65 (4)          |
| Re-CL8-1   | Melanoma      | MFG-vIL-10(1w) | -          | 62.8 (1)                  |
|            |               | (2w)         | -          | 54.9 (1)                  |
|            |               | (4w)         | -          | 25.4 (1)                  |
| MCA105     | Sarcoma       | MFG-vIL-10   | -          | 26.80 ± 6.05 (4)          |
|            |               | DFG-vIL-10-Neo | +b         | 67.55 ± 18.76 (12)        |
| MCA102     | Sarcoma       | DFG-mIL-10-Neo | +b         | 38.12 ± 20.62 (3)         |
| MC38       | Colon cancer  | DFG-vIL-10-Neo | +b         | 57.71 ± 13.12 (7)         |
|            |               | DFG-mIL-10-Neo | +b         | 45.31 ± 17.37 (3)         |
| COS-7      | Epithelial cell | pJJRU3-vIL-10 | -t         | 3.20 (1)                  |

* +, selected with G418; -, not selected; b, bulk, G418-resistant cells without an attempt of cell cloning; t, transient expression of vIL-10.

† Levels of the vIL-10 and mIL-10 secretions were determined in the supernatant of cell cultures.

§ Repeat infection.

Re-CL8-1-vIL-10: CL8-1-vIL-10 cells were inoculated intradermally, harvested from resultant tumors (1.0-cm diameter) 4 wk after the inoculation, and introduced to cell culture again. Secretion of vIL-10 was tested on the supernatant of the cell culture at 1, 2, and 4 wk in culture.

Cells were transduced with calcium phosphate transfection.

Table 1 shows the expression of vIL-10 and mIL-10 proteins after transfection in various cell types. The secretion levels were measured in the supernatant of cell cultures. The data include the mean ± standard deviation (SD) for each experiment, with the number of experiments in parentheses.

### Figure 2

To determine if transduced fibroblasts could also be used to deliver vIL-10 at the site of tumor cells, CL8-1 or CL8-1-vIL-10 was admixed with TIB81 fibroblasts, which were transduced with both vIL-10 and Neo (TIB81-vIL-10) or Neo only (TIB81-Neo; Fig. 3, C and F). 1 × 10⁵ TIB81-vIL-10 (27.0 ng/10⁶ cells per 48 h) or TIB81-Neo cells were admixed with 1 × 10⁵ CL8-1-vIL-10 (25.2 ng/10⁶ cells per 48 h) or nontransduced CL8-1 cells, and were injected into the left flank of C57BL/6 mice. All mice rejected nontransduced CL8-1 tumor cells admixed with TIB81-Neo within 14 d, while 40% of CL8-1-vIL-10 admixed with TIB81-Neo formed palpable growing tumors. Rejection of nontransduced CL8-1 cells was also inhibited by admixed TIB81-vIL-10 cells at a fibroblast/tumor ratio of 10:1. The size of the nontransfected CL8-1 admixed with TIB81-Neo tumors at day 35 (0 ± 0 mm²) was smaller when compared with those of animals receiving CL8-1-vIL-10 admixed with TIB81-vIL-10 tumors (75.0 ± 51.7 mm², P = 0.012), CL8-1-vIL-10 admixed with TIB81-Neo tumors (19.2 ± 26.9 mm², P = 0.149), or nontransfected CL8-1 admixed with TIB81-Neo (9.4 ± 8.2 mm², P = 0.21).
Figure 4. Paracrine vIL-10 secretion suppresses allorejection of MCA105 sarcoma (H-2b) in BALB/c (H-2d) mice. BALB/c mice (five per group) were injected intradermally into the left flank with 1 x 10^4 MCA105-vIL-10 or wild-type MCA105 cells. Tumor growth was assessed twice or thrice weekly. Shown are percentages of animals with tumor (A-C) and mean tumor area (D-F). (A) VIL-10 was abrogated by total body irradiation. Irradiated C57BL/6 mice (five per group) or nonirradiated C57BL/6 control mice (five per group) were injected intradermally with varying doses of C8-1 cells on the left flank. (C) 1 x 10^4 C8-1 cells were injected into irradiated mice; (△) 5 x 10^4 C8-1 cells were injected in irradiated mice; (□) 1 x 10^4 C8-1 cells were injected in irradiated mice; (●) 1 x 10^4 cells were injected in controls; (▲) 5 x 10^4 cells were injected in controls; (•) 1 x 10^4 cells were injected in controls. (B and E) Effect of vIL-10 on CL8-1 tumor growth. C57BL/6 mice (five per group) were injected intradermally in the left flank with 1 x 10^4 or 5 x 10^4 nontransfected C8-1 cells (controls) or C8-1-vIL-10 cells. (○) 5 x 10^4 C8-1-vIL-10; (△) 1 x 10^4 C8-1-vIL-10; (●) 1 x 10^4 C8-1; (C and F) Tumor progression can be promoted using coadministration of the TIB81-vIL-10 fibroblast with tumor. C57BL/6 mice (five per group) were injected intradermally in the left flank with 1 x 10^4 CL8-1-vIL-10 or nontransfected CL8-1 cells admixed with 1 x 10^3 TIB81-vIL-10 or TIB81-Neo fibroblasts. (○) CL8-1-vIL-10 + TIB81-vIL-10; (△) CL8-1-vIL-10 + TIB81-Neo; (●) CL8-1 + TIB81-Neo; (X) CL8-1 + TIB81-vIL-10.
In group 1, 60% of the animals developed CL8-1-vlL-10 tumors in the left flank within 7 d (day 44, group 1: 78.0 ± 72.5 mm² vs group 2: 0 ± 0 mm², P = 0.043) and died of progressive tumor, but none of the animals developed a palpable tumor in the right flank. These results suggest that local secretion of vlL-10 protein suppressed tumor rejection at that site but did not affect the immune-mediated rejection of nontransduced tumors at a distant site. They also suggest that tumor-reactive T cells primed at a distal site do not impact on the progression of vlL-10 tumors.

The Immunosuppressive Effects of vlL-10 Can Be Reversed by the Administration of Anti-vlL-10 Antibody or by Addition of the Cytokines IL2 or IL12. Anti-hlL-10 antibody (0.5 mg per mouse) was administered as intraperitoneal injections (1.0 ml) 24 h before tumor inoculation and on day 6 after inoculation. C57BL/6 mice were inoculated with 5 x 10⁴ CL8-1-vlL-10 (81.1 ng/10⁶ cells per 48 h) or nontransduced CL8-1 cells on day 0 (Fig. 5, B and E). While 80% of animals inoculated with CL8-1-vlL-10 developed progressively growing tumors, CL8-1-vlL-10 cells failed to grow in animals receiving anti-hlL-10 antibody. The size of the CL8-1-vlL-10 tumors at day 28 (18.0 ± 10.4 mm²) was significantly larger than that of CL8-1-vlL-10 with anti-vlL-10 antibody (0 ± 0 mm², P = 0.005). Significant neutralization of the vlL-10 effect was demonstrated with administration of anti-vlL-10 antibody. The growth of nontransduced CL8-1 tumor was not altered upon administration of anti-vlL-10 antibody. To examine whether other cytokines could reverse the vlL-10 effects, rhIL-2 (600,000 IU daily from day 7 to day 13) or rmIL-12 (0.1 µg daily from day 7 to day 13) was administered systemically (intraperitoneally) into C57BL/6 mice that received intradermal injection of 5 x 10⁴ CL8-1-vlL-10 (67.3 ng/10⁶ cells per 48 h) or nontransduced CL8-1 cells on day 0 (Fig. 5, C and F). All mice that received CL8-1-vlL-10 cells developed palpable tumor within 10 d, while nontransduced CL8-1 tumors progressed in only 40–60% of mice. In this experiment, the growth of CL8-1-vlL-10 tumors as well as of nontransduced CL8-1 tumors was significantly inhibited after systemic IL-2 or IL-12 administration. In the rhL-2–administered animals, the percentage of animals with palpable tumor (20%) and the tumor area (0.8 ± 1.8 mm², P < 0.0001) at day 35 were similar to those in the group administered rmIL-12 (0%, 0 ± 0 mm², P < 0.0001), when compared with that of control or Hanks' solution–administered groups (38.2 ± 7.4 mm² or 37.6 ± 3.2 mm²). Thus, systemic IL-12 or IL-2 administration significantly reversed vlL-10–mediated suppression of tumor rejection.

Profound Differences Exist between vlL-10 and mlL-10 in Im- munomodulatory Functions. To analyze the differences between vlL-10 and mlL-10 in immunomodulatory functions, varying doses of nontransduced CL8-1, CL8-1-vlL-10 (56.5 ng/10⁶ cells per 48 h) and CL8-1-mlL-10 (51.0 ng/10⁶ cells per 48 h) cells were injected into C57BL/6 mice (Fig. 6, A and B). When 1 x 10⁴ cells were injected into the animals, 60% of the animals with CL8-1-vlL-10 displayed palpable growing tumors, while tumor was identified in none of the animals receiving nontransduced CL8-1 or CL8-1-mlL-10. For the size of the tumors at day 23, values were 7.2 ± 6.6 mm² in CL8-1-vlL-10 tumors and 0 ± 0 mm² in both CL8-1-mlL-10 and nontransduced CL8-1 tumors, a statistically significant difference (P = 0.04). When 10⁵ cells were injected into animals, 100% or 60% of the animals receiving nontransduced CL8-1-vlL-10 cells or nontransduced CL8-1 cells developed palpable progressively growing tumors, respectively. All animals receiving

Figure 5. Local secretion of vlL-10 does not affect rejection of nontransfected tumor at a distant site, and immune-suppressive effects can be overcome by administration of antibody to IL10 or by administration of systemic IL-2 or IL-12. (A and D) C57BL/6 mice (five per group) were injected intradermally in the left flank with 1 x 10⁴ nontransfected CL8-1 cells. Mice were concurrently injected intradermally in the right flank with 1 x 10⁴ CL8-1-vlL-10 or 1 x 10⁴ nontransfected CL8-1. (O) group 1, right flank with CL8-1-vlL-10; (I) group 1, left flank with CL8-1; (C) group 2, right flank with CL8-1; (m) group 2, left flank with CL8-1. The effects of vlL-10 transfection can be reversed by IL-2 or IL-12 administration. (B and E) C57BL/6 mice were inoculated intradermally in the left flank with 5 x 10⁴ CL8-1-vlL-10 or nontransfected CL8-1 cells on day 0. On days 7–13, Hanks' solution (1.0 ml), 600,000 IU of systemic rhIL-2, or 0.1 mg of systemic rmIL-12 was administered intraperitoneally once daily. (O) CL8-1-vlL-10; (X) CL8-1-mlL-10 + Hanks' solution; (D) CL8-1-vlL-10 + anti-vlL-10 mAb; (E) CL8-1-mlL-10 + anti-mlL-10 mAb.

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Figure 6. vlL10 and mIL10 differ profoundly in their tumor immunomodulatory functions. C57BL/6 mice were inoculated intradermally in left flank with CL8-1-vlL10, CL8-1-mIL10, or nontransfected CL8-1 cells (A, 1 x 10^4; B, 1 x 10^5). (O) CL8-1-mIL10; (A) CL8-1-vlL10; (□) CL8-1.

the same dose of CL8-1-mIL10 cells initially developed small palpable tumors, but they were rejected within 20 d. The size of the CL8-1-mIL10 tumors at day 23 (0 ± 0 mm²) was significantly smaller than those of CL8-1-vlL10 (18.8 ± 5.9 mm², P < 0.0001) or nontransduced CL8-1 tumors (8.8 ± 8.2 mm², P = 0.043). These results indicated that mIL10, unlike vlL10, transduction of CL8-1 significantly promoted immune rejection of tumor.

Immunohistological Evaluation of CL8-1, CL8-1-vlL10, and CL8-1-mIL10 Tumors. After staining of nontransduced CL8-1, CL8-1-vlL10, and CL8-1-mIL10 tumors with mAbs to T cell subsets, a specific cellular CD4 + and CD8 + T cell infiltrate was identified. Nontransduced CL8-1 tumors were infiltrated with a large number of CD4 + and a moderate number of CD8 + lymphocytes (Fig. 7, A–C). Conversely, only a few CD4 + and CD8 + lymphocytes were observed within CL8-1-vlL10 tumors (Fig. 7, G–I). CL8-1-mIL10 tumors displayed a modest number of both CD4 + and CD8 + lymphocytes (Fig. 7, D–F). Interestingly, CD4 + and CD8 + lymphocytic infiltration of CL8-1-vlL10 tumor differs from the characteristic dense infiltration of CD4 + and CD8 + lymphocytes surrounding regressing nontransduced CL8-1 or CL8-1-mIL10. Additionally, CD8 + lymphocytes were observed primarily in the peritumoral area of CL8-1-mIL10 tumors and within the nontransduced CL8-1 tumors.

Discussion

We have constructed retroviral vectors that allow for high level secretion of vlL10 and mIL10 by transduced cells. Using this vector system, we demonstrated that local vlL10 secretion can inhibit or suppress immune rejection of immunogenic tumor cells in both syngeneic and allogeneic settings. The local secretion of vlL10 does not affect the rejection of a nontransduced tumor at a distant site. These results suggest that the local secretion of vlL10 protein does not have systemic effects but, rather, only local immunosuppressive effects. We have also shown that vlL10–transduced nontransformed fibroblasts, when admixed with tumor cells, yield similar immunosuppressive effects in vivo. Furthermore, when vlL10–secreting fibroblasts are combined with vlL10–secreting tumor cells and inoculated in vivo, the extent of the immunosuppression is greater than that observed with vlL10–secreting tumor cells alone. Thus, the immunosuppressive effects associated with vlL10 are dose dependent, but independent of the source of the vlL10.

These vlL10 effects were abrogated when anti–vlL10 antibody or immunostimulatory cytokines, such as IL-2 and IL-12, were administered systemically to the tumor-bearing animals in vivo. This result suggests that vlL10 protein secreted by the tumor cells is responsible for this observation and that the mechanism of the vlL10 immunosuppression might in-
volve suppression of cytokine synthesis. IL-12 induces T and NK cells to produce IFN-γ at high levels (30) and enhances primarily a Th1 cellular immune response (31). IL-2 is a growth factor that stimulates the proliferation of cytotoxic T cells, Th, and NK cells, and generates LAK activity (32–35). Thus, immunosuppressive effects of vIL-10 might be mediated by inhibition of IL-12-associated functions, including IFN-γ induction, NK or Th1 function and/or differentiation, and IL-2 synthesis by cytotoxic T cells. This interpretation is compatible with the previous findings observed with cIL-10 in vitro.

(1, 8, 9, 36–38).

The effects of vIL-10 we have observed in vivo support the idea that vIL-10 has retained the CSIF or macrophage/dendritic cell–deactivating activities of cIL-10. It has been suggested that the gene transfer of vIL-10 using our retroviral vectors prolonged cardiac allograft survival in a heterotopic, nonvascularized allograft model, and it has been demonstrated that, when allografts were transduced with vIL-10, decreased numbers of CD2+-, CD3+-, CD4+-, and CD8+–expressing lymphocytes were observed (Qin, L., unpublished observation). Our immunohistochemical results also showed that infiltration by CD4+ and CD8+ lymphocytes in animals bearing CL8-1-vIL-10 tumors was markedly reduced compared with that in nontransduced CL8-1 or CL8-1-mIL-10 tumors. These observations are consistent with the notion that h- and vIL-10 block the Ag-specific proliferation of both total T cells and CD4+ TTh1- or Th2-like T cell clones (10). However, the in vivo effect of mIL-10 expression by the same tumor cells was, surprisingly, to stimulate rejection of the tumor. cIL-10 has additional activities, such as costimulation of T cell/thymocyte growth and differentiation (11, 39, 40), chemotactic effects on CD8 cells (41), and induction of adhesion molecule expression by endothelial cells (42), which vIL-10 may lack (11, 39).

Recent studies by others (42, 43) also suggest that cIL-10 may have complex effects in vivo. The expression of cIL-10 under control of the insulin promoter in transgenic mice is associated with pronounced pancreatic inflammation, dominated by the presence of CD4+ and CD8+ T cells and B lymphocytes. The exocrine tissue is completely destroyed, but the islets of Langerhans are intact and appear to function normally (42). In nonobese diabetic mice, production of IL-10 by islet cells markedly accelerated immune-mediated destruction of β cells (43). In contrast, systemic administration of cIL-10 blocked the onset of diabetes in nonobese diabetic mice (44). Thus, our results, along with those of Wogensen and co-workers, suggest that stimulatory effects of cIL-10 on T cells and other cells may obscure the consequences of its "suppressive" effects on the APC and accessory cell functions of macrophages and dendritic cells when cIL-10 is expressed locally at high levels. Our studies with systemic administration of hIL-10 (Berman, R., T. Suzuki, H. Tahara, S. K. Narula, P. D. Robbins, and M. T. Lotze, manuscript submitted for publication) suggest that it has significant immunostimulatory functions, promoting the rejection of established day 7 murine tumors when high doses (higher than 20 μg per mouse) are administered daily for 7 d.

The basis for the apparent differences in cIL-10 and vIL-10 activities is only partially understood. cDNA clones encoding mouse and hIL-10R ligand-binding chains have been isolated and characterized (45, 46). Both rIL-10R bind their respective cIL-10 ligand with high affinity yet exhibit at least a 100-fold lower affinity for vIL-10 than for cIL-10 (46a). Nevertheless, vIL-10 stimulates proliferation of mIL-10R–transduced Ba/F3 cells with a specific activity only 3–10-fold lower than cIL-10, as is observed on normal cells that respond to both cIL-10 and vIL-10 (46a). These and other observations (Moore, K. W., unpublished observations) support the notion that IL-10R expression on vIL-10–responsive cells is composed of the ligand-binding (α) chain that binds, and one or more additional components that are required for interaction with vIL-10. Further studies of IL-10R structure should clarify the nature of the vIL-10 "defect."

The immunosuppressive effects of locally expressed vIL-10 suggest the possible application of vIL-10 in allogenic organ transplantation. Although organ transplantation is now recognized as an established life-saving measure for various diseases (47), most patients who receive organ or cellular transplantation require systemic immunosuppression for the rest of their lives using cyclosporin A or FK506 (48). During the prolonged period of general immunosuppression, patients are susceptible to opportunistic infections and malignancies. In theory, if local immunosuppression similar to that observed in this study could be achieved at the site of the transplanted organ by gene transfer, immune rejection of the graft could be blocked without disturbing systemic immune function.

In contrast to vIL-10, our results with cIL-10 suggest that it could be developed as an antitumor reagent when used in a gene therapy strategy. The possibility of using cIL-10 as an antitumor agent in gene therapy has been reported by Richter et al. (49) using mIL-10–transduced Chinese hamster ovary (CHO) cells. CHO-IL-10 cells grew normally in vitro but did not grow as tumors in immunodeficient animals (either nude or SCID mice). This effect was IL-10 dose–dependent, and CHO-IL-10 cells also suppressed the growth of tumors if coinjected with nontransduced CHO cells. Immunohistologic examination suggested that mIL-10 inhibited the infiltration of macrophages that was observed for tumors containing nontransduced CHO cells. Although direct interpretations of these results are limited because of the nature of the tumor selected and the use of immunodecient animals, they suggest that, under certain circumstances, cIL-10 can stimulate an immune response (Velu, T., and G. Forni, personal communication). Consistent with these results, we have shown that establishment of CL8-1 melanoma cells (Fig. 6), MC38 colon cancer cells, and MCA102 fibrosarcoma cells can be abrogated in syngeneic, immunocompetent mice (C57BL/6) (data not shown). We have also shown that the abrogation of tumor establishment leads to the promotion of protective immunity to a subsequent challenge with nontransduced tumor cells. Therapeutic effects are now being tested to examine the potential clinical application of this strategy. The discordant in vivo effects observed when comparing vIL-10 and mIL-10 should be noted for further characterization of these cytokines with multiple functions.
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