Tumor-Derived Interleukin-4 Reduces Tumor Clearance and Deviates the Cytokine and Granzyme Profile of Tumor-Induced CD8+ T Cells

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Abstract

An interleukin (IL)-4-containing tumor environment is reported to be beneficial for immune clearance of tumor cells in vivo; however, the effect of IL-4 on the effector CD8+ T cells contributing to tumor clearance is not well defined. We have used the immunogenic HLA-CW3-expressing P815 (P.CW3) mastocytoma and investigated whether IL-4 expression by the tumor affects tumor clearance and, if so, whether it alters the tumor-induced Vβ310+ CD8+ T-cell response. P.CW3 were stably transfected with IL-4 or the empty control vector, and independent cell lines were injected i.p. into syngeneic DBA/2 mice. After apparent clearance of primary tumors over 12 to 15 days, secondary tumors arose that lacked surface expression and H-2-restricted antigen presentation of CW3 in part due to the loss of the HLA-CW3 expression cassette. Surprisingly, mice that received IL-4-producing tumor cells showed delayed primary tumor clearance and were significantly more prone to develop secondary tumors compared with mice receiving control tumor cells. Tumor clearance was dependent on CD8+ T cells. The IL-4-secreting P.CW3 tumor cells led to markedly higher mRNA expression of IL-4 and granzyme A and B but no differences in IFN-γ and IL-2 production, cell proliferation, or ex vivo CTL activity in primary Vβ310+ CD8+ T cells when compared with the control tumor cells. We concluded that tumor-derived IL-4 selectively changed the quality of the tumor-induced CD8+ T-cell response and resulted in unexpected negative effects on tumor clearance. These data bring into question the delivery of IL-4 to the tumor environment for improving immunotherapy. (Cancer Res 2006; 66(1): 571-80)

Introduction

Successful tumor immunity relies on innate and adaptive immune responses, with cytokines known to influence tumor clearance in both positive and negative ways. For example, interleukin (IL)-4, the signature cytokine of a type 2 polarized immune response, is known to promote a wide range of immune responses and directs the effector differentiation and function of CD4+ and CD8+ T cells (1-3). These effector cells can eliminate tumors in antigen-specific or nonspecific ways. IL-4 is found in the microenvironment of some tumors, mostly expressed by tumor-infiltrating leukocytes (4-6). Infiltrating CD8+ T cells can produce high levels of IL-4 and low levels of IFN-γ, the key cytokine of a type 1 polarized T-cell response (7, 8).

Several studies have assessed the effects of an IL-4-containing tumor microenvironment on tumor clearance by recombinantly expressing IL-4 in tumor cell lines. In these models, the presence of IL-4 enabled the clearance of various tumor cells in mice that otherwise did not clear the parental tumor; although the precise mechanisms of IL-4 involvement were not defined, innate immune cells [i.e., eosinophils and neutrophils (9-11) and CD8+ T cells (12-14)] were thought to mediate or to be required for the antitumor activity. When IL-4 was produced by nontumor cells (e.g., by type 2 polarized CD4+ T helper cells), the IL-4-containing T-cell milieu also protected against immunogenic murine melanoma via the recruitment and activity of eosinophils (15). Adoptive transfer of tumor-specific CD8+ T cells expressing IL-4 cured lung metastases, with the T-cell-derived IL-4 being instrumental in tumor rejection via the cooperation of bystander leukocytes (16). Conversely, IL-4 ablation diminished the recruitment of tumor-infiltrating CD8+ T cells (17). Together, these reports indicate that IL-4 expressed by tumors or T cells has beneficial effects on tumor clearance and that CD8+ T cells are important effector cells in reducing the tumor load.

However, the effectiveness of IL-4-producing CD8+ T cells in tumor clearance is questioned by results from adoptive transfer studies with tumor-specific CTL. When T-cell receptor transgenic CD8+ T cells were polarized toward a type 1 (Tc1) or type 2 (Tc2) cytokine profile in vitro, the IL-4-producing Tc2 cells were less efficient than the non-IL-4-producing Tc1 cells in tumor rejection in vivo; this lower efficiency was thought to be due to the reduced levels of IFN-γ secretion by Tc2 cells and pointed to different mechanisms whereby Tc1 and Tc2 cells eliminate tumor cells expressing defined tumor antigens (18-20). IL-4 is also reported to increase the expression of antiapoptotic genes in tumors (6, 21), which may boost tumor cell resistance to immune-mediated cytotoxicity. In summary, the effects of IL-4 depend on the tumor model used and the type of effector cell mediating tumor clearance.

Using an immunogenic tumor that induces a potent CD8+ T-cell response believed to contribute to tumor clearance, we investigated whether IL-4 expression by the tumor affects tumor clearance and/or the tumor-specific CD8+ T-cell response. The MHC class Ii class IIα P815 mastocytoma is poorly immunogenic in syngeneic DBA/2 mice and leads to their death. In contrast, P815 cells stably transfected with the HLA-CW3 gene (P.CW3) and injected i.p. into DBA/2 mice induce marked expansion of CD8+ T cells in the peritoneal cavity leading to clearance of the tumor over 12 to 15 days. These T cells express Vβ10 T-cell receptor and recognize the decapeptide HLA-CW3770-779 with H-2Kd (22, 23). We report here that tumor-derived IL-4 caused unexpected detrimental effects on tumor clearance and selective changes in effector gene expression in tumor-induced CD8+ T cells.

Materials and Methods

Animal studies. All animal studies were approved by the Queensland Institute of Medical Research Animal Ethics Committee. Specific...
pathogen-free female DBA/2 mice from the Animal Resources Centre (Perth, Western Australia) were used at 7 to 9 weeks. Tumor cells (2 × 10⁵) were injected ip (day 0), and peritoneal exudate cells (PEC), spleen, and brachial, axillary, and inguinal lymph nodes were subsequently harvested; PECs were obtained by washout with balanced salt solution (2 × 5 mL). Rat anti-mouse CD8α (53-6.7) and anti-CD8β (55-0.8) antibody were protein G purified from cell supernatant. For CD8 cell depletion studies, mice were injected with anti-CD8α antibody, CD8β antibody, rat IgG control (Sigma-Aldrich, Sydney, New South Wales, Australia; 0.5 mg in 0.5 mL PBS), or PBS ip at days −2 and −1 and iv at day 4 (0.2 mg in 0.2 mL PBS).

**Cell lines.** P.CW3 cells are derived from the P815 mastocytoma engineered to express the HLA-CW3 gene and were cultured in hypoxanthine-aminopterin-thymidine medium (HAT medium; Sigma-Aldrich) supplemented with 5% heat-inactivated FCS (CSI Ltd., Parkville, Victoria, Australia; ref. 23). Parental P815 cells were maintained in growth medium (modified DMEM supplemented with 50 μmol/L 2-mercaptoethanol, 216 mg/L L-glutamine, and 5% FCS). Control or IL-4-expressing P.CW3 cells were grown in HAT medium supplemented with hygromycin B (300 μg/mL; Roche Diagnostics, Mannheim, Germany). Generation of HLA-CW3-specific CD8⁺ Vj10⁺ CTL clones and enhanced green fluorescent protein (EGFP)-expressing P815 cells was described elsewhere (24).

**Generation of IL-4-expressing or control P.CW3 cell lines.** The murine IL-4 cDNA was cloned into the Sall-Kpn1 restriction sites of the expression vector EBO-pLPP. This vector replicates as an episome and contains a SV40 expression cassette permitting stable gene expression in growing P.CW3 cells (5 × 10⁵) by electroporation using a Gene Pulser (Bio-Rad Laboratories, Hercules, CA) and anti-receptor (CD3, CD8, CD11a) antibody and IL-2 as described, except using reduced anti-CD3 antibody (1 μg/mL; ref. 27). HLA-CW3 and CD11b⁺ (to exclude contamination with macrophages) tumor cells were sorted with >98% purity. For analysis without sorting, a FACSCalibur was used with CellQuest version 3.1f software (Becton Dickinson).

**Immunoblot analysis.** Detection of vector EBO-pLPP encoded EBNA1 protein was done using human serum as described recently (27, 28). Anti-mouse Bcl-2 (3F11) and Bcl-x (polyclonal; BD PharMingen) and anti-β-actin (AC-40, Sigma-Aldrich) antibodies were used to analyze antiapoptotic protein expression in total SDS or NP40 lysates of 3 × 10⁶ tumor or C57BL/6 lymph node cells.

**IL-4 assays.** Tumor cells were washed and cultured at 10 ml per 0.5 or 1 mL and cultivated for 48 or 96 hours, respectively, in HAT medium (plus hygromycin B where appropriate) in 24-well plates. Duplicate or triplicate serial dilutions of supernatant (50 μL) from in vitro cultivated cells or from peritoneal lavage were assayed for IL-4 by ELISA using the anti-IL-4 antibody BVD4 and biotinylated BVD6 (29). IL-4 activity was standardized by reference to titrations of baculovirus-derived murine recombinant IL-4; 1 unit/mL activity was defined as the amount stimulating half-maximal proliferation of the IL-4-dependent cell line CT.45 (30) and 1 unit/mL baculovirus-derived IL-4 was equivalent to 0.83 μg/mL recombinant IL-4 (R&D Systems, Minneapolis, MN). The cumulative IL-4 levels of different cell lines, initially cultured at 10⁶ cells per 0.5 or 1 mL, ranged from 0.1 to 0.7 unit/mL over a 48-hour period or 0.2 to 1.32 unit/mL.

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**Table 1. Real-time PCR primer and probe sequences**

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over a 96-hour period; none of the control vector-containing cells secreted detectable IL-4 protein.

**CTL assays.** For $^{51}$Cr release assays, cells ($4 \times 10^2$-$5 \times 10^3$) of the mastocytoma line P815 and its derivatives or cell suspensions from PEC or tumor nodules were labeled with Na$^{51}$CrO$_4$ (Amersham Pharmacia, Sydney, New South Wales, Australia) in the absence or presence of peptide CW3$_{170-179}$ (10 µg/ml; synthesized by Mimotopes, Clayton, Victoria, Australia). For the highly sensitive fluorolysis assay, P815 cells ($1 \times 10^4$) stably transfected with the EGFP gene were labeled with or without peptide CW3$_{170-179}$. Target cells were incubated with effector CD8 T cells for 4 to 5 hours ($^{51}$Cr release) or 2 days (fluorolysis) at 37°C; both assays were described in detail previously (24).

**RNA preparation and real-time PCR analysis.** FACS-purified T cells were analyzed ex vivo or after 5-hour restimulation with anti-CD3ε antibody and IL-2 as described (27). RNA was extracted by NP40 hypotonic or Trizol lysis of 5 × 10$^3$ cells and cDNA was prepared as described in detail (27, 31, 32). cDNA was quantified using real-time PCR analysis, labeled primers (Table 1), known copy numbers of cloned DNA, and a Corbett Rotor-Gene 3000 (Corbett Research, Mortlake, New South Wales, Australia) under the following conditions: 95°C for 2 minutes, 95°C for 5 seconds, and 60°C for 30 seconds for 40 cycles. Commercial primer kits were used for the amplification and detection of IL-2, IL-5, and IL-10 (Applied Biosystems, Foster City, CA).

**Statistical analyses.** The Prism 4.0 software package (GraphPad Software, San Diego, CA) was used for statistical analyses.

**Results**

**In vitro characterization of IL-4-expressing and control P.CW3 tumor cells.** The tumor cell line P.CW3 was transfected with an IL-4 expression plasmid or, for controls, with the empty vector; several lines stably expressing either IL-4 (P.CW3-IL4) or the control vector (P.CW3-EOB) were selected after culture at limiting dilution. Secreting IL-4 was quantified by ELISA and a cell proliferation assay using the IL-4-sensitive cell line CT.4S. The *in vitro* growth rates of various cell lines expressing high, low, or undetectable IL-4 were similar when tested over 1 to 5 days by either thymidine incorporation or cell counting with trypsin blue exclusion. FACs analysis showed similar surface expression of the HLA-CW3 molecule among three tested lines of P.CW3-IL4 secreting high levels of IL-4 (i.e., 4H1, 4H2, and 4H3), P.CW3-EOB control transfrectants (i.e., E1, E2, and E3), and the parental P.CW3 cell line. The specific lysis of the 4H1, 4H2, 4H3, E1, E2, E3, and the parental P.CW3 cell line was similar in $^{51}$Cr release assays using a panel of HLA-CW3$_{170-179}$-specific CTL clones (data not shown). These results indicated that (a) IL-4 did not alter the *in vitro* growth rate of the tumor cells and (b) expression and MHC class I–restricted presentation of the HLA-CW3 antigen in the selected tumor cells was not lost or compromised by coexpression of IL-4.

**Effects of IL-4 on development of secondary tumors.** P.CW3-IL4 or control P.CW3-EOB tumor cells were injected i.p. into syngeneic mice. After apparent clearance of the primary tumor load in the peritoneal cavity (as assayed by FACs for HLA-CW3-expressing cells), some mice developed secondary tumors that presented as ascites and solid tumor nodules; the tumor nodules were present mainly in the peritoneal lumen but also attached to extraluminal organs like liver, heart, and lung. The time course of secondary tumor presentation was assessed in three long-term experiments using different tumor cell lines expressing high levels of IL-4 (4H1 and 4H2) or control vector (E1 and E2). Starting from day 30 postinjection, the frequency of mice bearing secondary (S-) tumors was statistically higher for 4H2 than S-E2 tumors (Fig. 1A); this trend was supported by another experiment (data not shown). Similar, statistically significant differences were obtained with 4H1 and E1 cell lines (Fig. 1B). The secondary E2- and E1-derived tumors always occurred as solid large tumor nodules with minimal ascites, whereas the secondary tumors originating from 4H1 and 4H2 cells always presented as smaller and more dispersed nodules with abundant ascites.

We assessed the antigenicity of primary and secondary tumor cells. When PEC from mice injected with 4H2 cells were $^{51}$Cr labeled at day 11, there was specific killing of the target cells by a CW3-specific CTL clone equivalent to the lysis of peptide HLA-CW3$_{170-179}$-coated P815 control cells (Fig. 2A). S-4H2 or S-E2 tumor nodules and ascites presenting at day 55 or 65 post-injection were isolated, radiolabeled, and analyzed for cognate CTL recognition by $^{51}$Cr release assay. The secondary tumor cells were not killed by a CW3-specific CTL clone, but coating with HLA-CW3$_{170-179}$ peptide rendered them susceptible to lysis (Fig. 2A). Parallel FACs analysis showed that the lack of tumor antigen presentation correlated with the absence of surface HLA-CW3 expression for all four secondary tumor types of E2 or 4H2 origin presenting as ascites or nodules (Fig. 2B). In contrast, all the tumors expressed surface MHC H-2d (data not shown). These results were confirmed in analyses of 19 independent secondary tumors derived from E2- or 4H2-injected mice, presenting between days 30 and 338. Together, these results indicated that the lack of CTL recognition was due to loss of the tumor antigen rather than to a defect in MHC class I expression in the secondary tumors. In contrast, the S-E2 or S-H2 tumor cells still contained the empty vector or the IL-4 expression vector, respectively, as indicated by their growth in hygromycin B (the antibiotic selecting for the expression vector) and immunoblotting of tumor
were typically linear and intercepted the input number versus the percentage of nonresponding cultures limiting dilution in different media. Semilogarithmic plots of cell S-4H2 cells presenting as ascites or nodules were cultivated at tumors (data not shown).

14 of 14 S-4H2 tumors secreted IL-4 compared with 0 of 7 S-E2 secondary tumors from individual mice were analyzed by ELISA, (encoded on the vector backbone). Moreover, when independent cell lysates with an antiserum specific for the EBNA-1 protein (data not shown).

To elucidate the basis of loss of HLA-CW3 expression, S-E2 or S-4H2 cells presenting as ascites or nodules were cultivated at limiting dilution in different media. Semilogarithmic plots of cell input number versus the percentage of nonresponding cultures were typically linear and intercepted the Y axis close to the origin, indicating that the response depended on a single limiting cell type (data not shown). Most tumor cells could grow in control or media, indicating no inherent restriction to growth under these conditions. The majority (193) of the 198 clonal or independent P.CW3 lines expressing high levels of IL-4 (4H1) or the clones.

Figure 2. Lack of antigenicity of secondary tumors. A, pools of day 11 PEC from two mice injected with 4H2 tumor cells [4H2 (d11) ascites; primary tumor], PEC from individual mice presenting secondary tumors and injected previously with 4H2 (S-4H2) or E2 (S-E2), and control P815 cells were51Cr labeled in the presence or absence of HLA-CW3 170-179 peptide; lysis of these target cells was tested with a HLA-CW3-specific CTL clone. The time of presentation (days 55 and 65) and phenotype (ascites and nodule) of the secondary tumors are indicated. Columns, lytic units at 30% lysis from three separate experiments; broken line, threshold of detection. B, HLA-CW3 expression by the secondary tumors tested in (A). Solid lines and filled histograms, nodular and ascites forms, respectively, of secondary tumors of 4H2 (left) or E2 (middle) origin. Right, ex vivo staining of primary 4H2 cells by anti-HLA-CW3 (filled histogram) or isotype control (broken line) antibody. The day of tumor presentation is indicated. C, in vitro cloning efficiency of secondary tumors from individual mice injected with E2 (circles) or 4H2 (triangles) was determined by limiting dilution. Open symbols or filled symbols, nodular or ascites tumor presentation. Cells were cloned in medium with or without hygromycin B (hygB) to select for the empty or IL-4 expression vector and/or HAT to select for the HLA-CW3 expression cassette. Data are pooled from two independent experiments using a total of three (S-E2) and six (S-4H2) mice with secondary tumors detected from days 32 to 163. Crosses, cloning efficiencies of the parental E2 and 4H2 tumor cell lines.

Effects of IL-4 on primary tumor clearance. Because the negative effects of IL-4 on the development of secondary tumors were unexpected, we analyzed the time course of primary tumor clearance. At day 12, there was a marked difference in the persisting tumor load of mice injected with 4H2 or E2 tumor cells with most of the control E2 cells being cleared (Fig. 3A). The summary in Fig. 3B shows that at days 9 and 12 postinjection a median 455- or 42-fold, respectively, higher tumor load was found in 4H2-injected mice compared with E2-injected controls; these differences were highly significant (P < 0.004, two-tailed Mann-Whitney test), although the tumor cell numbers were highly variable among different mice in 11 independent experiments. Two experiments at day 12 with independent P.CW3 lines expressing high levels of IL-4 (4H1) or the
control vector (E1) supported these results. The mean tumor cell numbers were $16.4 \pm 13.1 \times 10^6$ (4H1) versus $4.7 \pm 7.2 \times 10^6$ (E1) in experiment 1 and $100.8 \pm 71.2 \times 10^6$ (4H1) versus $0.011 \pm 0.002 \times 10^6$ (E1) in experiment 2 ($n = 3-4$ mice per group); these differences were statistically significant in experiment 2 ($P = 0.03$, two-tailed unpaired $t$ test). Despite these differences, various lines of both control and IL-4-expressing tumor cells were mostly cleared by day 15. This time course of apparent primary tumor clearance is consistent with previously published data for the parental P.CW3 tumor cells (22, 33).

Immunoblot analysis indicated no marked differences in levels of the antiapoptotic proteins Bcl-2 or Bcl-XL among E2 and 4H2 cells cultured in vitro or purified ex vivo at day 9; however, these levels were elevated compared with normal lymph node cells (Fig. 3C). From this and several other experiments, we concluded that the IL-4-induced delay in primary tumor clearance was not due to major differences in the expression of antiapoptotic genes.

**Primary tumor clearance is dependent on CD8$^+$ T cells.** Analysis of PEC at days 9 and 12 postinjection of various control or IL-4-expressing tumor lines detected similar numbers of macrophages, natural killer cells (by FACS analysis of CD11b and anti-CD49b antibody expression), and myeloid cells (using Giemsa histology), suggesting no specific effect of IL-4 on the recruitment of these cell types (data not shown).

To test the contribution of CD8$^+$ T cells to clearance of E2 or 4H2 tumor cells, mice were given depleting anti-CD8 antibody or control rat IgG or PBS. When compared with the controls after 12 days, anti-CD8a antibody treatment of mice receiving E2 or 4H2 tumor cells reduced the frequency of CD8$^+$ T cells 10- to 13-fold in spleen and lymph node; in PEC, the reduction in CD8$^+$ T cells was 128-fold by percentage and 27-fold in absolute cell numbers (data not shown). Moreover, the mean $\sqrt{10^6}$ CD8$^+$ CD4$^+$ T-cell numbers in PEC were reduced 17- or 95-fold by the anti-CD8a antibody treatment in mice injected with E2 or 4H2 tumor cells, respectively (Fig. 4A). CD8$^+$ T-cell depletion was

![Figure 3. Clearance kinetics and phenotype of primary tumor cells.](image-url)

A, mice were injected i.p. with P.CW3 tumor cells expressing IL-4 (cell line 4H2; left) or control vector (cell line E2; right); at day 12 postinjection, PECs were analyzed by FACS for forward scatter, PI exclusion, and binding of antibody to HLA-CW3 and CD11b or isotype control antibody. Filled histograms, live CD11b$^+$ HLA-CW3$^+$ 4H2 or E2 tumor cells; the percentages of tumor cells are indicated. Broken lines, isotype control antibody staining. B, the absolute numbers of HLA-CW3$^+$ tumor cells present in PEC 9 to 25 days after injection of 4H2 (left) or E2 (right) tumor cells are given for each mouse. Line, geometric mean of 11 independent experiments indicated by different symbols. The numbers of experiments were seven at day 9 ($n = 16-17$ mice), eight at day 12 ($n = 18-19$ mice), two at day 15 ($n = 4$ mice), and one at days 19 and 22 ($n = 2$ mice). In some experiments at days 9 and 12, CD11b$^+$ cells were excluded. Broken line, threshold of detection. C, ex vivo HLA-CW3$^+$ CD11b$^+$ E2 or 4H2 tumor cells were purified by FACS at day 9 postinjection and their total SDS lysates were analyzed by immunoblot for expression of the antiapoptotic proteins Bcl-2 and Bcl-XL, and the housekeeping protein \(\beta\)-actin (right). In a separate experiment, the NP40 lysates of the parental in vitro cultured tumor cells or lymph node cells of a naive mouse (cont) were tested (left).
associated with a 83- or 44-fold increase in mean numbers of E2 or 4H2 tumor cells, respectively (Fig. 4B). As treatment with anti-CD8α antibody can lead to depletion of both CD8αβ+ T cells and CD8αα+ cells, such as dendritic cells, we also depleted CD8αβ+ T cells with an anti-CD8β antibody as reported previously (34). Again, Vβ10+ CD8+ CD4− T-cell numbers were markedly reduced (4H2, 58-fold; E2, 132-fold) by the anti-CD8β antibody compared with the rat IgG control; conversely, the E2 and 4H2 tumor loads increased 668- and 383-fold, respectively (Fig. 4C and D). Giemsa histology revealed the presence of very few myeloid cells in the PEC of anti-CD8 antibody-treated mice injected with E2 or 4H2 tumor cells possibly due to dilution with the overwhelming tumor cells (data not shown). We concluded that tumor-specific CD8+ T cells were essential for clearing the tumor in this experimental system.

Effects of IL-4 on tumor-induced Vβ10+ CD8+ T cells. The quantity and quality of the tumor-induced CD8 T-cell response was assessed. FACS analysis at day 12 showed that many of the live CD8+ CD4− T cells expressed Vβ10, with the E2 tumor inducing a markedly higher percentage of Vβ10+ CD8+ T cells than the 4H2 tumor (Fig. 5A, bottom). During the time course analysis, both tumor-induced Vβ10+ CD8+ T-cell responses increased up to days 9 to 12 and subsequently declined to background levels (Fig. 5B; note that these data were obtained in the same 11 independent experiments as in Fig. 3B). At days 9 and 12, there were median 6.9- and 4.0-fold higher frequencies of E2-induced compared with 4H2-induced Vβ10+ CD8+ T cells (P < 0.0004, two-tailed Mann-Whitney test). These data indicated a significantly reduced and delayed Vβ10+ CD8+ T-cell response against 4H2 versus E2 tumor cells. However, other experiments with independent P.CW3 lines expressing high levels of IL-4 (4H1 and 4H3) or the control vector (E1 and E3) did not consistently show these differences (data not shown).

We analyzed phenotype and functional quality of the T cells over time. Both tumor types induced peritoneal Vβ10+ CD8+ T cells with similar levels of activation marker expression (CD62Llow and/or CD44high) detected by FACS, and the frequency of activated cells in the Vβ10+ CD8+ fraction rose over a 12-day period to 100% (data not shown). Real-time reverse transcription-PCR analysis showed that 4H2 cells induced selective up-regulation of effector gene expression in peritoneal exudate Vβ10+ CD8+ CD4− T cells with 60-fold higher mean levels of granzyme A (P = 0.002, two-tailed unpaired t test) and 13-fold higher levels of granzyme B mRNA at day 12 (P = 0.05; Fig. 6A). In contrast, expression levels of perforin, IFN-γ, IL-10, and the housekeeping gene β2-microglobulin were not markedly different (<4-fold). Granzyme C levels were low and varied among different samples and days. At least 10-fold higher expression of IL-4 was seen in 4H2-derived CTL at days 12 and 15 (P < 0.05). IL-5 mRNA was not detected (data not shown). This marked up-regulation of IL-4 and granzyme A and B gene expression in response to 4H2 tumor cells was confirmed in another experiment (data not shown). There were low but similar levels of IL-2 mRNA in both populations of Vβ10+ CD8+ T cells restimulated with anti-CD3 antibody and IL-2 (Fig. 6B); unstimulated cells did not show detectable IL-2 levels. Similar results were obtained in another experiment. In the absence of added IL-2, endogenous IL-2 levels were low, sometimes below the threshold of detection (data not shown). No marked differences in the division profiles of E2- or 4H2-induced Vβ10+ CD8+ T cells were visible when the cells were CFSE labeled and restimulated with anti-receptor antibody and IL-2 for 4 days (Fig. 6C); the cells did not proliferate in the absence of added IL-2. Analysis at earlier time points and in another experiment confirmed these results.

The cytolytic capacity of peritoneal exudate Vβ10+ CD8+ CD4− T cells was tested in the fluorolysis assay, which detects very small numbers of CTL by measuring the lysis of EGFP-expressing target cells (24). At day 12 postinjection, both tumors induced CTL that were highly cytolytic against peptide HLA-CW3*70.179-coated, but not control, P815 target cells (Fig. 6D). Three other fluorolysis experiments at days 12 to 19 postinjection showed that 4H2-induced CTL had similar or slightly stronger (-3-fold) cytolytic activity than E2-induced CTL. Likewise, Vβ10+ CD8+ T cells induced by the independent tumor lines E1 and 4H1 were highly cytolytic when tested in the fluorolysis assay (data not shown) or in the less sensitive 51Cr release assay (Fig. 6D).

Discussion

We report here that tumor-derived IL-4 had unexpected detrimental effects on tumor clearance and altered the cytokine and granzyme profile of tumor-specific CD8+ T cells. The mechanism of tumor clearance in this model was not known previously, although the correlation between tumor clearance and rising Vβ10+ CD8+ T-cell numbers suggested the involvement of CD8+ T cells (22). Our in vivo depletion studies with anti-CD8 antibody showed that CD8+ T cells are indeed essential for clearing the primary tumor cells. Preliminary studies indicated that up to ~16,000- or ~6,000-fold higher mean numbers of E2 or 4H2 tumor cells, respectively, persisted at day 12 in the PEC of mice

Figure 4. Inverse relationship between Vβ10+ CD8+ T cells and tumor cells after CD8+ T-cell depletion. In separate experiments, mice were injected with control (rat IgG, PBS) or anti-CD8 antibody specific for the α (top) or β (bottom) chain before (days −1 and −2) and during (day 4) challenge with tumor cells expressing IL-4 (4H2, open columns) or empty vector (E2; filled columns). At day 12, the PECs were analyzed for CD8+ CD4− Vβ10+ T cells (4A and 4C) and HLA-CW3* tumor cells (B and D). Columns, mean (n = 2-4 mice); bars, SD.
receiving anti-IFN-γ antibody compared with mice receiving the control rat IgG antibody; thus, in vivo IFN-γ depletion can increase the load of both control and IL-4-producing tumors. E2 and 4H2 tumor cells isolated from tumor-bearing mice expressed similarly high levels of IFN-γRα, suggesting that IFN-γ could play a direct role in tumor elimination. Together, these results indicate that both IFN-γ and CD8 T cells are instrumental for clearance of this tumor in vivo.

In the absence of a functional CD8 T-cell response (as in our anti-CD8 antibody depletion studies), a higher tumor load persisted, but there was no consistent decrease in the numbers of tumor cells and no increase in granulocytes present in the PEC of mice injected with the IL-4-producing tumor compared with the control tumor cells. Moreover, the IL-4 expressed by the tumor cells led to a delay in primary tumor clearance that was mostly (but not always) apparent at 9 to 12 days after tumor injection. These findings were unexpected, as several reports indicated that an IL-4-positive tumor environment has beneficial effects on tumor clearance (10–14) and that enhanced tumor clearance by IL-4 was mediated by eosinophils and granulocytes (9, 15). It is important to point out some critical differences between these and our studies. Firstly, the tumor cells in the other studies are normally not rejected when IL-4 is absent. Secondly, tumor clearance in this study is dependent on CD8 T cells. Thirdly, our mastocytoma cells predominantly grow as ascites in contrast to the models using solid tumors in which IL-4 could interfere with stromal elements (e.g., angiogenesis) that are required for growth of solid tumors but not for ascites (35).

Tumor cells are known for their genetic instability that can reduce their antigenicity and lead to the emergence of tumor variants that escape immunosurveillance; this is the basis of the current concept of the three E (elimination, equilibrium, and escape) phases in tumor immunobiology (36). There were unexpectedly increased frequencies of secondary tumors in mice receiving IL-4-expressing primary tumor cells compared with mice receiving control tumors. The secondary tumor cells had lost both CW3 surface expression and presentation of the CW3 170-179 epitope, events that were due to the loss of the CW3 expression cassettes in at least some of the cancer cells. The secondary tumor cells were derived from the primary tumor cells as indicated by the

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Figure 5. Time course of V_{H}10+ CD8+ T-cell induction. A, mice were injected i.p. with P.CW3 tumor cells expressing IL-4 (cell line 4H2; left) or control vector (cell line E2; right), and PECs were analyzed at day 12 postinjection by FACS using forward and side scatter, PI exclusion, and binding of antibody specific for CD8α, CD4, and V_{H}10 or isotype control antibody. Bottom, percentage of V_{H}10+ T cells among the total CD8+CD4+ population induced by the 4H2 or E2 tumor cells. B, all samples correspond to Fig. 3B. The absolute numbers of CD4+CD8+V_{H}10+ T cells present in PEC 9 to 22 days after injection of 4H2 (left) or E2 (right) tumor cells are shown for each mouse. Line, geometric mean of 11 independent experiments indicated by different symbols. The numbers of experiments were seven at day 9 (n = 16-17 mice), eight at day 12 (n = 18-19 mice), two at day 15 (n = 4 mice), and one at days 19 and 22 (n = 2 mice).

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3 Unpublished observations.
presence of the control or IL-4 expression vector and expressed vector backbone genes and/or IL-4. It is noteworthy that mice, which were injected i.p. with the parental P.CW3 cells or a derivative clone (G2) and subsequently cleared the tumor cells, occasionally developed secondary tumors with frequencies around 17% (in three of five different experiments) or 0% (two of five experiments); these secondary tumor cells presented as large, solid nodules and, when tested, were not lysed by CW3-specific CTL in a 51Cr release assay, similar to the secondary tumors derived from the E1 or E2 control tumor cells. Together, these data suggested that the potential for secondary tumor formation and loss of antigenicity was an intrinsic quality of P.CW3 cells and its derivatives and not due to the presence of the expression vector in transfected P.CW3 cells.

We were able to observe the temporal formation of secondary tumors in two mice that were initially injected with IL-4-expressing tumor cells (4H2) and presented the secondary tumors at early time points (days 35 and 36); the PEC showed some (<17%) residual surface HLA-CW3 expression among the mixed population of primary and secondary tumor cells. Thus, one can suggest...
that the tumor escape mutants evolved over time within the population of antigenic primary tumor cells by bypassing immunosurveillance by CD8 T cells. We hypothesize that IL-4 expression by the tumor accelerated the evolution of this tumor escape process. For example, the delayed primary clearance of IL-4-producing tumor cells could affect the dynamics of the equilibrium phase (36) and increase the ratio between the residual minimal tumor load and the tumor-induced immune response. In addition, IL-4 could increase the in vivo growth rate of the tumor cells. Together, these effects could raise the frequency of residual tumor cells and thus increase the chances of acquiring mutations that led to loss of antigenicity. It is not trivial to test this prediction experimentally in vivo as, by definition, it is difficult to measure tumor growth when there is minimal residual disease after apparent clearance of the primary tumor cells. We attempted to address these questions in vitro and found no evidence that IL-4 alters the growth rate of the tumor cells or expression and H-2-restricted presentation of the CW3 antigens in the different cell lines used. Furthermore, in vitro cultured or ex vivo tumor cells expressed high and similar levels of antiapoptotic proteins, making it unlikely that IL-4-producing tumor cells are more resistant to cell death; this result is different to other tumor models in which IL-4 can promote cell survival and expression of antiapoptotic genes (6, 21).

The finding that CD8 T cells and IFN-γ activity were essential for tumor clearance raises the possibility that the increase in granzyme A and B and IL-4 expression in the IL-4 tumor-induced CD8 T-cell response contributes to the increase in tumor escape. To our knowledge, this is the first demonstration that an IL-4-positive tumor environment can increase granzyme A expression levels in the cognate CD8 T-cell response in vivo. This effect of IL-4 on effector gene expression is consistent with our previous in vitro findings that IL-4 mRNA and protein expression was induced and granzyme A mRNA levels were up-regulated in type 2 polarized effector CD8+ T cells (27, 37). The in vitro type 2 polarized CD8+ T cells expressed IL-10 and IL-5, showing a conventional type 2 cytokine expression profile. In contrast, the Vh10+ CD8+ T cells, which were induced by the IL-4-producing or control tumors in vivo, showed no detectable IL-5 mRNA expression and no differences in the levels of IL-10 mRNA. These findings suggested that IL-4 production by the tumor polarized the normally type 1 biased CD8+ T cells toward a limited type 2 polarized profile.

The increased levels of IL-4 and granzyme A in the Vh10+ CD8+ T-cell response might influence the effector response during the equilibrium phase. IL-4 is known to modify antigen presentation in dendritic cells that leads to inhibition in the acquisition of cytolytic and IFN-γ function in the responding CD8+ T cells (38). Previous studies showed that IL-4 can inhibit both IL-2 production by T cells and IL-2-induced lymphocyte proliferation (39, 40). In our study, IL-2 and IFN-γ expression, proliferation, and CTL activity were similar in Vh10+ CD8+ T cells induced by tumor cells that did or did not express IL-4. It is nevertheless possible that the tumor-expressed IL-4 compromised additional effector functions in CD8 T cells or other cells contributing to tumor clearance.

In summary, our results bring into question the delivery of IL-4 to the tumor environment for improving antitumor therapies whose effectiveness is dependent on CD8 T cells. These findings could have significance for current approaches to immunotherapy against human cancer aiming to induce an effective CD8 T-cell response (41–43).

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