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Inhibition of Cytolytic T Lymphocyte Proliferation by Autologous CD4$^+$/CD25$^+$ Regulatory T Cells in a Colorectal Carcinoma Patient Is Mediated by Transforming Growth Factor-β[1]

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ABSTRACT

Cancer patients often develop CTLs that lyse autologous tumor cells in culture. However, tumors can progress *in vivo* despite the presence of CTLs. Various mechanisms have been reported to down-modulate CTL functions. In this study, the role of CD4$^+$/CD25$^+$ regulatory T cells in CTL induction and proliferation of established CTLs was investigated in a patient with CRC. CD4$^+$ cytotoxic and regulatory T-cell lines were derived from the peripheral blood mononuclear cells of the same patient in mixed-lymphocyte tumor culture. The cytotoxic T-cell line and a clonal derivative specifically lysed the autologous tumor cells but not the B lymphocytes. Only HLA-A1-matched allogeneic CRC cells were lysed by the CTL clone. The clone produced IFN-γ and TNF-α. The regulatory CD4$^+$/CD25$^+$ T-cell line was tumor cell-dependent in its growth but did not lyse autologous tumor cells. This T-cell line suppressed pokeweed mitogen responses of allogeneic lymphocytes, proliferative activity of the established, autologous CTLs, and induction of CTLs in autologous, freshly isolated peripheral blood mononuclear cells. The immunosuppressive effect of the CD4$^+$/CD25$^+$ regulatory T cells was mediated by transforming growth factor-β and did not require cell-to-cell contact. Thus, although CRC patients can develop specific CTLs against their tumors, the development of regulatory T cells may allow the escape of tumor cells from immune surveillance by the CTLs *in vivo*.

INTRODUCTION

Infiltration of T lymphocytes into the tumor mass has been correlated with reduced recurrence and increased survival from the disease (1), suggesting that T cells contribute to tumor rejection. However, tumors often grow despite lymphocytic infiltration.

In CRC,[4] there is an apparent imbalance between lymphocytes that can kill tumor cells and those that cannot. CRC patients' lymphocytes stimulated *in vitro* with autologous tumor cells have not been reported to be cytolytic (2–6), although isolation of CTLs from CRC patients has been reported occasionally (7, 8). Nevertheless, the immunological significance of the isolated CTLs for disease outcome remains unclear because these cells either grew slowly in culture, showed unstable lytic activity against autologous tumor cells, or, most likely, were not specific for the tumor cells because allogeneic Ag-presenting cells were used to induce them. Thus, CRC patients can harbor T cells that are noncytolytic or, at most, poorly cytolytic, but the mechanisms underlying the failure of immune surveillance are unclear. At least four possibilities that lead to dysfunctional lymphocytes unable to kill tumor cells can be considered: (*a*) defects in T-cell signaling because of decreased or aberrant expression of the TCR ζ chain (9), which would block target cell lysis by the T cells; (*b*) decreased expression of HLA by CRC cells (10, 11); (*c*) CRC cell expression of molecules that suppress CTL induction and/or function, *e.g.*, TGF-β (12) and Fas ligand (13); and (*d*) appearance of regulatory T cells that suppress CTL induction and/or effector function.

There is limited information on the role of regulatory T cells and the mechanism of their suppressive functions in spontaneous T-cell immunity to human tumors. In melanoma, regulatory CD4$^+$ T cells can inhibit induction of both Ag-specific and nonspecific proliferative and cytolytic T cells in fresh autologous lymphocytes (14–16). However, none of these studies has delineated the mechanism of the suppressive effects exerted by the regulatory T cells. Although CD4$^+$/CD25$^+$ T cells were isolated from the blood and tumor tissues of patients with lung and ovary carcinomas (17) and these T cells produced the immunosuppressive cytokine TGF-β (18), it is not known whether they inhibited the proliferative and/or cytolytic functions of the CD8$^+$ T cells also found in these patients. Recently, CD4$^+$/CD25$^+$ regulatory T cells that inhibited Ag-nonspecific autologous T-cell proliferation in lung carcinoma patients have been described (19). The mechanism of T-cell proliferation inhibition has not been delineated in that study. Furthermore, it is unclear whether the regulatory T cells were induced by tumor Ag.

To investigate the potential defects in the cell-mediated immune responses of CRC patients, we analyzed the role of regulatory T lymphocytes in a patient.

MATERIALS AND METHODS

Abs. Murine anti-CRC mAbs used in this study have been described (8, 20). Murine anti-HLA mAb W6/32 (IgG2a; anti-HLA-A, HLA-B, and HLA-C) and GAPA3 (IgG2a; anti-HLA-A3) were obtained from American Type Culture Collection (Manassas, VA), and mAbs 289HA-1 (IgM; anti-HLA-A1) and 196HA-1 (IgM; anti-HLA-B57) were from One Lambda (Canoga Park, CA). Purified rabbit polyclonal immunoglobulin against TGF-β and purified mouse anti-IL-10 mAb were purchased from R&D Systems (Minneapolis, MN).

Cell Lines. CRC cell lines WC007, WC013, and WC016 (Dukes' stage B) and WC008 and WC010 (Dukes' stage C) were established as described (8). All cell lines were tumorigenic in nude or severe combined immunodeficient mice (8). CRC cell lines SW48, SW480, SW837, LoVo, and HT29, erythroleukemia cell line K562, and Burkitt lymphoma cell line Daudi were obtained from American Type Culture Collection. Leukemia killer T cells TALL-104 (21) were provided by D. Santoli (The Wistar Institute).

EBV-transformed B-cell lines of patient 007 were established as described (8). All lymphoid cell lines were maintained in RPMI 1640 (JRH Biosciences, Lenexa, KS) supplemented with 10% FBS.

HLA Typing. HLA typing of fresh PBMCs and EBV-B or CRC cell lines was performed using tissue typing trays and genomic typing (8).

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[4] The abbreviations used are: CRC, colorectal carcinoma; TGF, transforming growth factor; Ag, antigen; Ab, antibody; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; MLTC, mixed-lymphocyte tumor culture; IL, interleukin; PWM, pokeweed mitogen; PHA, phytohemagglutinin.

Table 1 *Phenotypic markers and cytokine production by cytotoxic and regulatory T lymphocytes*

	Surface markers[a]							Cytokines[b]					
T cell	CD3	CD4	CD8	CD25	TCR α/β	CTLA4	HLA-DR	IL-2	IL-4	IL-10	IFN-γ	TNF-α	TGF-β
CTL007	93	96	0	30	94	NT	90	3	14.5	5.5	25.6	7.6	0
CTL007 clone F8	96	95	NT[c]	93	49.4	4.5	98.4	NT	0	0.9[d]	12	4	NT
Regulatory T007	91	98	<3	60	97	2.0	90	0	7.5	5.0	4.6	2.9	6.4

[a] Fluorescence-activated cell sorter analyses; % of positive cells.
[b] RIA or ELISA; values (units/ml) are shown for optimal time of incubation (24–72 h for the various cytokines).
[c] NT, not tested.
[d] Not significant.

Establishment of T-Cell Lines and Clones. T-cell lines were established in two MLTCs as described (8, 22). One culture (the CTL line designated hereafter as CTL007) received IL-2 from day 7 and thereafter, and the other culture (the regulatory T-cell line, designated hereafter as T007) received IL-2 from day 15 and thereafter. The CTL007 CTL line was cloned by limiting dilution as described (8, 22). The clone was designated as CTL007 clone F8.

Proliferation Assay. Proliferation based on [^{3}H]thymidine incorporation was assessed as described (8, 22).

Coculture Assays. PWM responses of the PBMCs from a healthy donor (RL) in the presence of the regulatory T-cell line T007 of patient 007 were determined in a lymphocyte coculture assay (20). PWM responses of the PBMCs were also tested in the presence of 6-day culture supernatant derived from T007 cells after stimulation with either autologous WC007 CRC cells or EBV-B cells or both. The supernatant was preincubated with 1–10 μg/ml of rabbit immunoglobulin to TGF-β, normal rabbit immunoglobulin, mouse anti-IL-10 mAb, or normal mouse immunoglobulin for 3 h at 37°C before addition to the cells. [^{3}H]Thymidine incorporation was determined after 4–5 days of culture.

Suppression of the CTL007 line or clone F8 proliferation by T007 cells (both lines, 17 weeks in culture) was determined in the second and third coculture assay, respectively. CTL007 cells (5×10^3 cells/well) were stimulated with irradiated WC007 tumor cells (2×10^3 cells/well), and irradiated EBV-B007 cells (5×10^3 cells/well) were stimulated in the presence of irradiated T007 cells (5×10^3 cells/well, *i.e.*, T007:CTL007 ratio, 1.0). In control cultures, T007 cells were substituted with equal amounts of irradiated EBV-B007 cells.

Suppression of CTL007 clone F8 proliferation by T007 cells (21 weeks in culture) was determined in the fourth coculture assay in 24-well transwell plates (0.2 μm; Corning, Corning, NY). In this assay, the effect of soluble factors secreted by T007 cells on CTL proliferation was determined. Stimulated T007 cells (5×10^4) were plated in the top chamber, and stimulated CTL007 clone F8 cells (1×10^5) were plated in the bottom chamber. As controls, T007 cells were replaced by an equal number of autologous PHA blasts or Sepharose beads (Pharmacia, Peapack, NJ). Some wells were incubated with 10 μg/ml (10-fold excess) of rabbit immunoglobulin to TGF-β, mouse anti-IL-10 mAb, normal rabbit immunoglobulin, or normal mouse immunoglobulin. Cultures were harvested on day 3, and [^{3}H]thymidine incorporation was determined.

Suppression of CTL induction in the PBMCs of patient 007 by T007 cells was determined in the fifth coculture assay. Irradiated T007 cells (1×10^5 cells/well) were added to an MLTC containing autologous PBMCs (1×10^5 cells/well) and an equal number of irradiated WC007 tumor cells. Cultures were restimulated (day 7) with irradiated tumor cells in the presence of IL-2, and cells were tested for cytolytic activity (day 15) against autologous WC007 target cells in the ^{51}Cr-release assay described below. As controls, allogeneic PHA blasts were used.

Cytotoxicity Assay. CTL activity was tested in standard 4–18-h ^{51}Cr-release assays in the absence and presence of anti-HLA and the indicated anti-T-cell mAbs (8, 22).

Cytokine Determinations. IFN-γ, IL-4, and TNF-α were measured in cell-free supernatants (48 h) by RIA (8, 22). TGF-β was measured by ELISA using the Quantikine kit (R&D Systems).

Flow Cytometry Analyses. Flow cytometry analyses of T-cell markers was performed as described (8, 22).

Statistical Analyses. Differences between experimental and control values were evaluated by Student's *t* test.

RESULTS

CD4$^+$ CTL007 Line. The CD4$^+$ CTL007 line showed stable growth rates for >4 months. Proliferation of CTL007 was dependent on the presence of autologous WC007 CRC cells and autologous EBV-B cells. CTL007 expressed CD3, CD4, CD25, HLA-DR, and TCR α/β and secreted IL-2, IL-4, IL-10, IFN-γ, and TNF-α (Table 1).

Standard 4–6-h ^{51}Cr-release assays revealed lysis of autologous WC007 tumor cells by the CTL007 cells at E:T cell ratios as low as 10 (Fig. 1). Lysis of WC007 tumor cells was significant throughout the 23-week culture period of the CTL line (Fig. 2*A*). Lytic activity was significantly enhanced (by 15%; $P < 0.05$) by treatment of the tumor cells with IFN-γ (20 units/ml), most likely reflecting up-regulation of HLA class I and/or class II on the tumor cells by the cytokine, because lysis of tumor cells is both HLA class I and II dependent (see below). The CTL line also lysed allogeneic CRC HT-29 cells matched for HLA-A1 and WC016 cells matched for HLA-A3, whereas other HLA-matched and HLA-unmatched CRC cells were not lysed (Fig. 2*A*); presumably because these cells lack the relevant tumor Ag recognized by the CTL line, HLA expression is either too low for lysis to occur (*e.g.*, WC008 and WC013 cells express HLA-A1 only after IFN-γ treatment), or these cell lines express HLA-A1 and HLA-A3 subtypes not shared with those of WC007 cells (23). However, all of the target cells that were not lysed by the CTL line were lysed by TALL-104 leukemic killer cells (results not shown).

Ag-specific lysis of WC007 CRC cells by the CTL line was significantly ($P < 0.05$) inhibited by saturating (as determined by flow cytometry analysis) concentrations of mAb W6/32 to HLA class

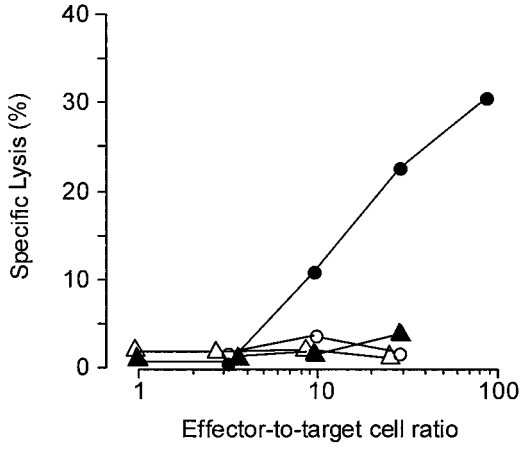

Fig. 1. Cytolytic activity of the CTL007 line against autologous WC007 CRC cells. The CTL007 line (19 weeks in culture) was tested for cytolytic activity (6-h ^{51}Cr-release assay) against autologous WC007 CRC cells (●), autologous EBV-B007 cells (○), K562 cells (▲), and Daudi cells (△) at various E:T cell ratios. ^{51}Cr-release in wells containing WC007 target cells plus CTL007 cells was significantly ($P < 0.05$) higher than in control wells (WC007 cells in the absence of CTL007 cells; EBV-B007, K562 or Daudi target cells in the presence of CTL007 cells) at E:T cell ratios of >10.

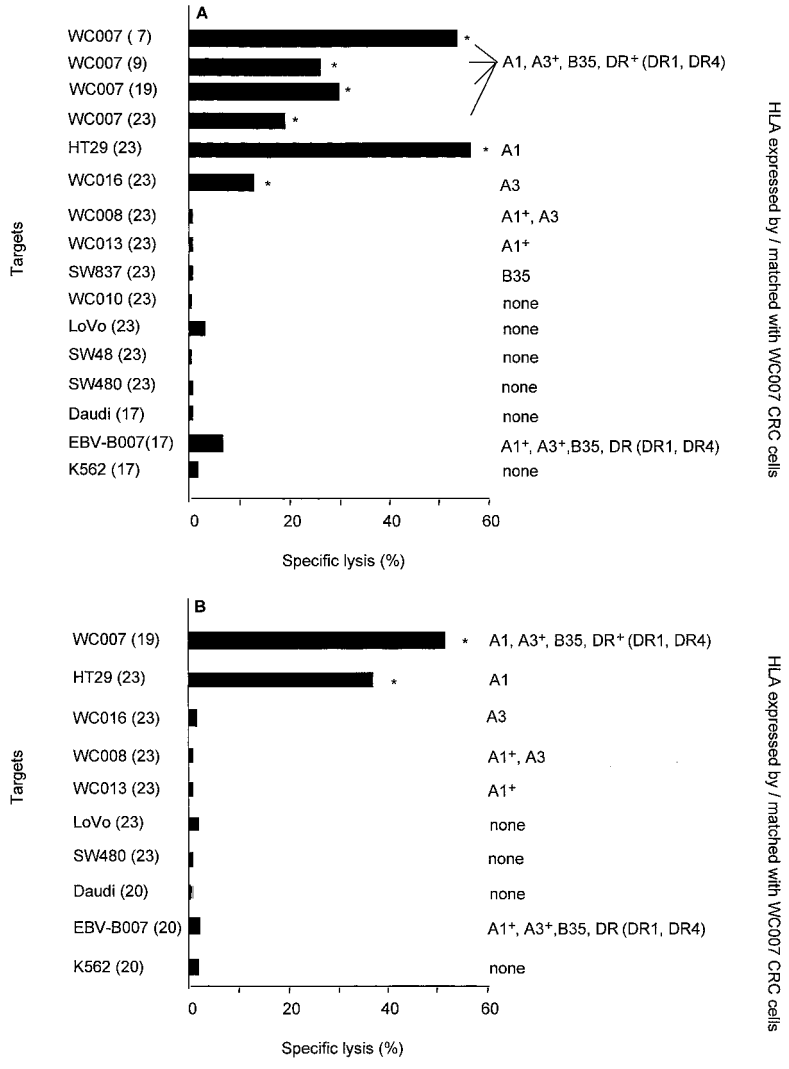

Fig. 2. *A*, cytolytic activity of the CTL007 line against autologous and allogeneic target cells. The CTL007 line (7–23 weeks in culture, see numbers in parentheses) was tested for cytolytic activity in ^{51}Cr-release assay (4-h assay for CTL007 cells at 7, 9, 17, and 19 weeks in culture; 18-h assay for CTL007 cells at 23 weeks in culture) against autologous and allogeneic CRC cells, autologous EBV-B cells, and allogeneic K562 and Daudi cells at an E:T ratio of 20. *B*, cytolytic activity of CTL007 clone F8 against autologous and allogeneic target cells. CTL007 clone F8 (19–23 weeks in culture, see numbers in parentheses) was tested for cytolytic activity in 18-h ^{51}Cr-release assay against target cells as described in *A*. HLA expressed by autologous WC007 CRC and EBV-B007 cells and HLA of allogeneic tumor cells matched with those of autologous WC007 cells are indicated. +, HLA types expressed by cells only after IFN-γ treatment; *, ^{51}Cr-release in test wells (target plus CTLs) significantly ($P < 0.05$) higher than in control wells (target in the absence of CTLs).

I, HLA-A3, HLA-DR, CD3, and CD4 (Table 2). Results of assays using IgM mAb 289HA-1 to HLA-A1 to block CTL line lysis of CRC targets were inconclusive because of high nonspecific inhibition of lysis obtained with two irrelevant IgM control mAbs (mAbs 196HA-1 to HLA-B57 and 1116NS33a to CRC; not shown). We have isolated one additional CD4$^+$ CTL line (CTL007-C11) in MLTC007 (results not shown).

Table 2 *Inhibition of CTL lysis by mAb*

Ab			% lysis inhibition[a]	
Designation	Reactivity	Concentration (μg/ml)[b]	CD4$^+$ CTL line	CTL F8 clone
W6/32	HLA class I	0.5	51.0*	87.6*
GAP A3	HLA-A3	1.0	42.6*	14.3
D1.B.6	HLA-DR	1.0	94.8*	19.2
OKT3	CD3	1.0	95.0*	NT[c]
B66.6	CD4	1.0	28.0*	NT
Mouse immunoglobulin (control)	Unknown	1.0	1.5[d]	10.2[d]

[a] Specific cytotoxicity against WC007 cells (18-h ^{51}Cr-release assay) in the absence of antibody at suboptimal E:T cell ratio of 10:1 was 19.3% for the CD4$^+$ CTL line and 29.2% for the CTL F8 clone (corrected for spontaneous ^{51}Cr-release by the target cells). The percentage of inhibition of target cell lysis by mAb was calculated relative to lysis in the absence of mAb. Similar results were obtained in another independent experiment. *, significantly ($P < 0.05$) different from values obtained with control normal mouse Ig.

[b] All mAbs were used at saturating concentrations. Excess anti-HLA mAb was washed off the WC007 tumor cells after 1-h incubation at room temperature. Anti-CD3 and anti-CD4 mAbs were present in the mixture of tumor cells and CTLs.

[c] NT, not tested.

[d] Inhibition of lysis by control normal mouse Ig was not significant when compared with media controls.

CD4$^+$ CTL007 Clone F8. The CD4$^+$ CTL line most likely was not clonal in origin because it was both HLA class I and class II dependent (Table 1). CTL007 clone F8 produced IFN-γ and TNF-α (Table 1) and showed stable lytic activity against autologous tumor cells for at least 12 weeks in culture (Fig. 2*B*). CTL clone F8 lysed allogeneic HT29 cells matched with the autologous WC007 cells at HLA-A1 but not any of the other HLA-matched and nonmatched CRC cells or lymphoblastoid cells (Fig. 2*B*).

Lysis of WC007 CRC cells by CTL007 clone F8 was significantly ($P < 0.05$) inhibited by saturating concentrations of mAb to HLA class I but not HLA-DR or HLA-A3 (Table 2). Thus, the CD4$^+$ CTL clone is HLA class I dependent (most likely HLA-A1, see Fig. 2*B*; for HLA-A1 blocking, see above).

CD4$^+$ Regulatory T007 T-Cell Line. The T007 cells grew exponentially for ~3 months, slowing thereafter. The proliferative activity of T007 cells was highest ($P < 0.01$) when T cells were cultured in the presence of both autologous EBV-B and tumor cells at a T cell:stimulator cell ratio of 4. T007 cells expressed the markers CD3, CD4, CD25, HLA-DR, and TCR α/β and secreted IL-4, IL-10, IFN-γ, TNF-α, and TGF-β but not IL-2 (Table 1).

The functional activity of the regulatory T-cell line T007 was tested in five different coculture assays. In the first assay, the effect of T007 cells on PWM responses of allogeneic PBMC RL derived from a

healthy donor was investigated. Irradiated T007 cells significantly ($P < 0.001$) and specifically inhibited PWM responses of PBMC RLs at all T007 to PBMC ratios tested (Fig. 3). Culture supernatant of T007 cells also significantly ($P < 0.001$) inhibited the PWM response of PBMC RLs, and this response was partially, but significantly ($P < 0.05$), restored by preincubating the supernatant with anti-TGF-β antibody (Fig. 3). The inhibitory factor was produced by T007 cells in the presence of both autologous tumor cells and autologous EBV-B cells but not in the presence of either cell type alone (not shown).

In the second coculture assay, the effect of T007 cells on the proliferative activity of autologous CTL007 was investigated. The non-CTL line significantly and specifically inhibited the proliferative activity of the autologous CTL007 cells (25% inhibition at a CTL007:T007 cell ratio of 1; $P < 0.01$ as compared with CTL007 proliferation in the presence of irradiated autologous EBV-B cells as controls). As regulatory T cells have been shown to inhibit both proliferation and cytokine release of Ag-specific mouse T cells (24), we investigated possible effects of T007 on IFN-γ release by CTL007. However, IFN-γ production by CTL007 was not inhibited by T007 (results not shown).

In the third coculture assay, the effect of T007 cells on the proliferative activity of autologous CTL007 clone F8 was investigated. T007 cells significantly ($P < 0.01$) inhibited the proliferative activity of clone F8 cells (Fig. 4*A*). The same experiment repeated in transwell plates with T007 cells cultured in the top chamber and clone F8 in the bottom chamber revealed significant ($P < 0.01$) inhibition of the clone F8 proliferative activity by T007 (Fig. 4*B*), indicating that soluble factors, and not cell-to-cell contact, mediate the suppressive effects of T007. T007 cells mediated suppressive effects on CTL007 proliferation only when freshly stimulated with tumor cells, not when left unstimulated for 12 days, suggesting that induction of T007 regulatory activity was tumor Ag dependent. The inhibitory function of T007 was significantly ($P < 0.01$) decreased by rabbit anti-TGF-β immunoglobulin but not by mouse anti-IL-10 mAb (both at 10 μg/ml; Fig. 4*B*). These results indicate that TGF-β is responsible, at least in part, for the inhibitory activity of T007.

In the fifth coculture assay, the effect of T007 cells on the induction of CTLs in fresh autologous PBMCs was investigated. T007 cells significantly ($P < 0.001$) inhibited induction of CTLs in fresh autologous PBMCs in a 15-day MLTC (Fig. 5). In contrast, irradiated allogeneic RD T-cell blasts did not significantly ($P > 0.05$) inhibit CTL induction in the PBMCs.

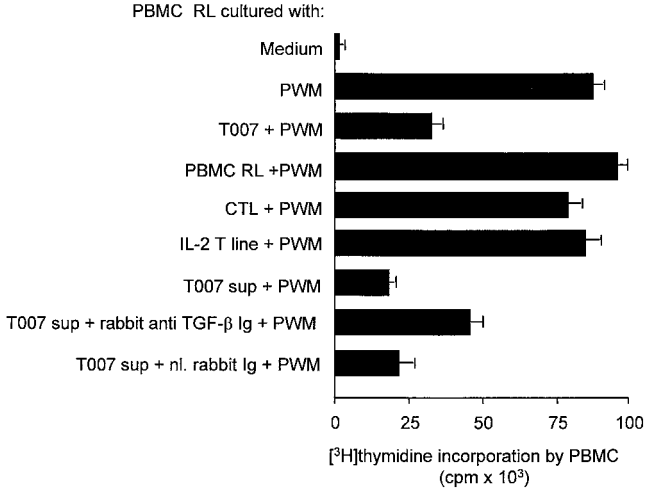

Fig. 3. Suppressor activity of T007 cells against PWM responses of allogeneic PBMCs. PWM proliferative responses of PBMC RLs (1×10^4 cells/well) derived from a healthy donor were determined in the absence (*second column*) and presence (third column) of irradiated T007 cells (week 12 in culture) at an optimal T007:PBMC ratio of 1. As controls, PBMC RLs were cultured with irradiated PBMC RLs, irradiated CTL007, or an IL-2-induced, short-term T-cell line derived from CRC patient 007 (*fourth*, *fifth*, and *sixth columns*, respectively). Data are given as means; *bars*, SE. T007 cells significantly ($P < 0.05$) suppressed PWM responses of PBMC RLs as compared with the responses of PBMC RLs in the presence of either medium or irradiated control cells (PBMC RLs, irradiated CTL007, and an IL-2 induced T-cell line derived from CRC patient 007). Culture supernatant (*sup*) of T007 (*seventh column*) also significantly ($P < 0.001$) suppressed PWM responses of PBMC RLs [$P < 0.001$ *versus* PWM only (*second column*)]. Preincubation of T007-derived culture supernatant with rabbit anti-TGF-β antibody (*eighth column*), but not normal rabbit immunoglobulin (*ninth column*), partially restored PWM response of PBMC RLs [$P < 0.05$, for comparison of cpm obtained with rabbit anti-TGF-β antibody (*eighth column*) *versus* normal rabbit immunoglobulin (*ninth column*), or *versus* no antibody (*seventh column*).

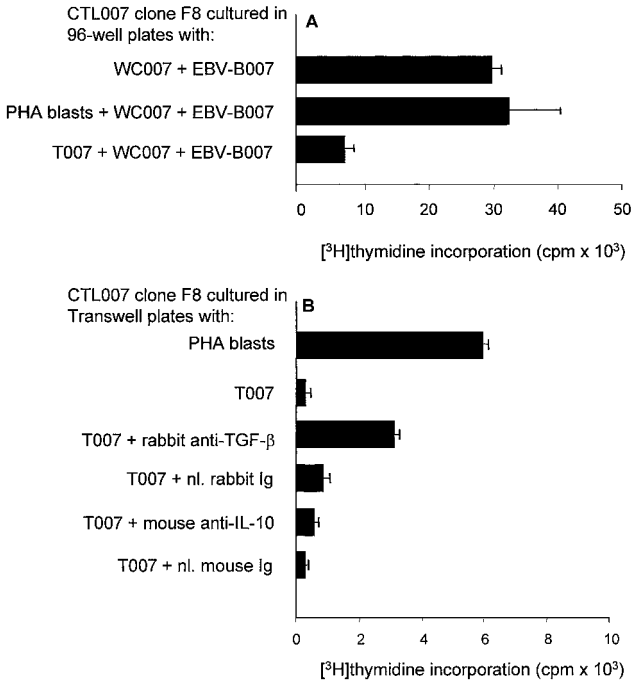

Fig. 4. Inhibition of CTL007 clone F8 proliferation by T007 cells. *A*, the proliferative response of clone F8 (10^4 cells/well) was determined in the absence (*first column*) and presence (*second column*) of irradiated autologous PHA blasts (controls) and the presence (*third column*) of irradiated T007 cells (2×10^4/well). T007 cells significantly ($P < 0.01$) suppressed the proliferative responses of CTL007 clone F8 to tumor cell stimulation as compared with controls. *B*, proliferative activity of clone F8 (5×10^4/well) was determined in transwell plates in the presence of nonirradiated autologous PHA blasts (1×10^5/well; *first column*) or T007 (1×10^5/well; *second column*). Rabbit anti-TGF-β Ig (*third column*), normal rabbit immunoglobulin (*fourth column*), mouse anti-IL-10 mAb (*fifth column*), or normal mouse immunoglobulin (*sixth column*) was added to the cultures of clone F8 and T007. Data are given as means; *bars*, SD. T007 significantly ($P < 0.001$) inhibited the proliferative response of clone F8 (*second column*) when compared with PHA blasts (*first column*). Rabbit anti-TGF-β antibody (*third column*) significantly ($P < 0.01$) inhibited the suppression when compared with normal rabbit immunoglobulin control (*fourth column*), whereas anti-IL-10 mAb had no effect ($P > 0.05$; *last two columns*).

DISCUSSION

The lymphocyte population of a patient with CRC contained at least two types of lymphocytes with tumor specificity, *i.e.*, CTLs and regulatory T cells. The CTLs lysed autologous and HLA-matched allogeneic tumor cells but not autologous B-lymphocytes or HLA-nonmatched allogeneic tumor cells. The CTLs showed stable cytolytic activity over a period of at least 23 weeks. These data suggest that CRC patients have the potential to develop a strong cell-mediated immune response. We attribute our success in establishing CTLs from the peripheral blood to the selection of a patient with primary, not metastatic, disease (8). In other studies, isolation of CTLs was unsuccessful using lymphocytes from metastatic patients (2–6).

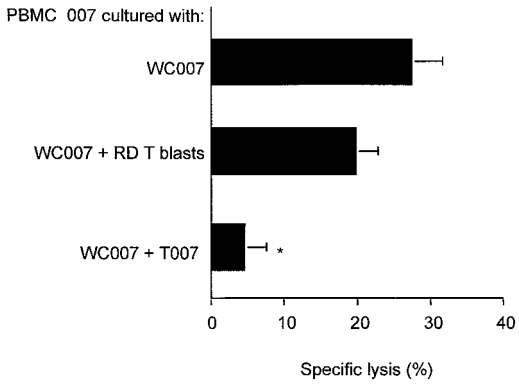

Fig. 5. Inhibition of CTL induction in fresh autologous PBMCs by T007 cells. Cytotoxic responses of lymphocytes derived from a 15-day MLTC of autologous PBMCs and WC007 cells in the absence (*first column*) and presence (*third column*) of autologous regulatory T-cell line or RD T blast control cells (*second column*) were determined in an 18-h ^{51}Cr-release assay using autologous WC007 target cells. Data are given as means; *bars*, SD. Cytolytic activity of PBMC-derived lymphocytes against tumor targets was significantly (*, $P < 0.001$ *versus* controls) inhibited by T007 cells.

The Ag recognized by the CTL clone induced by primary CRC cells is expressed by allogeneic CRC cells regardless of whether they are derived from primary or metastatic lesions. This suggests that CRC cells from patients with metastatic disease harbor the Ag, and that these patients can be considered candidates for future vaccination studies using cloned Ag or Ag-derived peptides.

The CTL clone described here is $CD4^+$ and HLA class I (most likely HLA-A1) restricted. Approximately 15% of the human population is HLA-A1 positive. Target cell recognition by $CD4^+$ CTLs is usually HLA class II dependent (reviewed in Ref. 25), but $CD4^+$ HLA class I-dependent CTLs have also been described, including our CTL clones against CRC and melanoma cells (8, 22, 26–29). In one of these studies (22), HLA class I (B57) restriction of a CTL clone against melanoma cells was demonstrated by CTL lysis of allogeneic tumor cells only after transfection of the cells with cDNA encoding HLA-B57.

We have isolated a $CD4^+/CD25^+$ regulatory T-cell line, a phenotype that has been associated with murine and human regulatory T cells in tumor and autoimmune systems (30, 31). This $CD4^+/CD25^+$ T-cell line suppressed PWM responses of allogeneic lymphocytes and proliferative activity of the established, autologous CTL line and clone. Furthermore, the regulatory T cells inhibited induction of CTLs in fresh autologous lymphocytes. The suppression of CTL proliferation by the regulatory T cells was maintained in the presence of IL-2, arguing against the hypothesis (30–34) that IL-2 abrogates suppressor function of regulatory T cells.

The suppressive effect of our T-cell line was mediated by a soluble factor(s) and did not require cell-to-cell contact, in contrast with a previous report of the requirement for cell-to-cell contact in the immunosuppressive activity of regulatory $CD4^+/CD25^+$ T cells (35). In our study, suppressive activity of the regulatory T cells was mediated by TGF-β but not IL-10, although both cytokines are well known for their immunosuppressive effects (18, 36). In lung carcinoma patients, TGF-β was not involved in the immunosuppressive effects of $CD4^+/CD24^+$ regulatory T cells (19). TGF-β inhibits T-cell growth and CTL induction, differentiation, and cytokine production, as well as Ag-presenting cell function (18). The role of TGF-β in mediating immune response suppression to tumors by regulatory T cells has not been described previously; however, TGF-β was shown to mediate immunosuppressive effects of regulatory T cells in murine autoimmunity systems (37, 38). Recently, TGF-β was shown to induce $CD4^+/CD25^+$ regulatory T cells that prevented $CD8^+$ T cells from proliferating in response to alloantigens and from becoming cytotoxic effector cells in humans (35, 39). Thus, TGF-β can be produced by regulatory T cells (this study; Refs. 37, 38, 40, 41) and can induce these cells (35, 39). Many tumor cells types including WC007 CRCs secrete TGF-β (not shown). In our study, tumor-derived TGF-β does not appear to be involved in regulatory T-cell induction because irradiated WC007 CRC cells did not produce this cytokine (not shown).

The demonstration of regulatory T cells with suppressor function and TGF-β production raises the question of why CTLs are induced in the PBMCs of a CRC patient. Possibly, the regulatory T cells were absent in the separate cultures that gave rise to CTLs, because of the different culture conditions used (IL-2 added at days 7 and 15 to the CTLs and regulatory T-cell cultures, respectively).

The stimulus for induction of both CTLs and regulatory T cells might be a tumor-associated self antigen(s) (42, 43). Regulatory T cells may suppress autologous CTL functions through TGF-β *in vivo*, resulting in escape of the tumor from immune surveillance by CTLs. This may explain why tumors often grow despite the presence of CTLs in the same individual. A T-cell-specific block of TGF-β signaling appears to have therapeutic potential by shifting the balance of the immune responses to favor antitumor immunity (44).

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