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Th1 and Th2 Cell Clones to a Poorly Immunogenic Tumor Antigen Initiate CD8$^+$ T Cell-Dependent Tumor Eradication In Vivo[1]

Francesca Fallarino, Ursula Grohmann, Roberta Bianchi, Carmine Vacca, Maria C. Fioretti, and Paolo Puccetti[2]

Although CD8$^+$ T cells play a central role as immune effectors, CD4$^+$ T cells act to control the activation and persistence of the CD8$^+$ T cell response in autoimmune disease, antiviral immunity, and experimental systems with immunogenic model tumor Ag. However, little information is available on the effects of CD4$^+$ T cells on the function of endogenous CD8$^+$ T lymphocytes recognizing authentic tumor rejection Ag with limited immunogenicity. We report here that the prophylactic or postchallenge administration of T helper Th1-type and Th2-type CD4$^+$ clones specific for an unmutated rejection Ag (murine P815AB, resembling tumor-specific shared Ag in humans) leads to the induction of P815AB-specific reactivity in vivo and concomitant tumor destruction, with quantitative rather than qualitative differences characterizing the antitumor activity of Th1 vs Th2 cells. Because the transferred CD4$^+$ cells lacked direct antitumor activity in vitro and required the de novo generation of P815AB-specific CD8$^+$ T cells in vivo, these findings suggest that CD4$^+$ lymphocytes can enhance the ability of host APC to initiate an endogenous CD8$^+$ T cell response to authentic, poorly immunogenic tumor rejection Ag.   *The Journal of Immunology*, 2000, 165: 5495–5501.

G enetic instability of tumor cells often generates new Ag that may not elicit immune recognition and tumor destruction because of low intrinsic immunogenicity and tumor-associated immunosuppression. Since most nonhematopoietic tumors express MHC class I molecules, which serve as the restricting element for CD8$^+$ T cell recognition, but do not express MHC class II molecules, which are required for CD4$^+$ T cell recognition, it is generally believed that the predominant tumoricidal effector mechanism is killing by CD8$^+$ T cells. Yet, CD4$^+$ T cells may serve to control the activation and persistence of the CD8$^+$ T cell response in antitumor immunity (1, 2), according to a pattern that has been most clearly elucidated in autoimmune disease and antiviral immunity (3–6). To explore the impact of CD4$^+$ T cells on Ag-specific CD8$^+$ T cells in experimental tumor settings, different strategies have been devised to monitor the Th response to tumors made to express evolutionarily distant Ag, including transfer of Th cells from OVA-specific TCR-transgenic mice (7) and Th cells specific for β-galactosidase from *Escherichia coli* (8). These studies have demonstrated that CD4$^+$ T cells control CD8$^+$ T cell reactivity to such Ag (7, 8), emphasizing distinct roles for Th1 and Th2 cells in tumor eradication (7).

Cell-mediated immunity involving CD8$^+$ lymphocytes is effective in mediating rejection of murine mastocytoma cells bearing P815AB, a tumor-associated and self Ag showing similarity to tumor-specific shared Ag in humans (9–11). Although this Ag may act as an efficient target for class I-restricted responses in immunized mice, neither P815AB expressed on tumor cells nor a

related synthetic nonapeptide will activate unprimed CD8$^+$ cells for in vivo reactivity, presumably as a result of poor ability of P815AB to recruit CD4$^+$ T cells to the afferent response (12, 13). However, effective priming to P815AB is achieved by immunizing mice with dendritic cells exposed in vitro to recombinant IL-12 and P815AB-related peptides (14–17). Under such conditions, an immune response is initiated in vivo that involves both class II-restricted (helper) and class I-restricted epitopes of P815AB, and can be detected in vivo to nonameric P815AB (12, 13), the minimal core peptide for CTL recognition (10).

In the present study, we have used this immunizing strategy to induce CD4$^+$ T cell clones recognizing the helper epitopes of P815AB. Two representative clones, one releasing high levels of IFN-γ (Th1 type) and the other high levels of IL-4 (Th2 type), were assayed for antitumor activity when transferred into recipient hosts challenged with P815AB-expressing tumor cells. The prophylactic or postchallenge administration of either clone was found to result in a tumor eradication that was dependent on the de novo generation of P815AB-specific CD8$^+$ T cells in vivo.

Materials and Methods

Mice and cell lines

DBA/2J (H-2^d) mice were obtained from Charles River Laboratories (Calco, Milan, Italy). Male mice were used at the age of 2–4 mo. Mastocytoma P815 was cultured in DMEM supplemented with 10% FCS and incubated at 37°C in an 8% CO$_2$ atmosphere. Various clones of P815 were used in this study: P1, a tumorigenic clone of P815; P1.204, a P815AB-negative variant obtained from an in vivo escaping tumor population and carrying a deletion of gene *P1A*; and P1.HTR, the highly transfectable variant of P1 (18, 19).

Peptides, cytokines, and Abs

Peptides were synthesized as previously described (12, 17), purified by means of reversed-phase HPLC, and characterized by amino acid analysis. The single letter code sequence of the peptides used in this study is as follows: P815AB.9, LPYLGWLVF; P815AB.12, EILPYLGWLVFA; and

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OVA-(323-339), ISQAVHAAHAEINEAGR. Murine rIL-12 was a generous gift from B. Hubbard (Genetics Institute, Cambridge, MA). IL-12 was 98.8% pure, as assessed by SDS-PAGE, and endotoxin contamination was <0.9 endotoxin units/mg on *Limulus* amebocyte assay. The specific activity of the purified rIL-12 preparation, measured as the ability to stimulate proliferation in human PHA-activated blasts, was 3.1×10^6 U/mg. Human rIL-2 was obtained from Chiron (Emeryville, CA). Murine rIFN-γ and rIL-4 were purchased from PharMingen (San Diego, CA). PE-labeled antimouse CD8α and IFN-γ, and FITC-labeled antimouse CD4 and IL-4 were obtained from PharMingen. Isotype-matched irrelevant mAbs were used as controls in the analysis of CD4/CD8 Ag expression. Antimouse IL-4 mAbs 11B11 and biotinylated BVD6-24G2 and antimouse IFN-γ mAbs R4-6A2 and biotinylated XMG1.2 were purchased from PharMingen. The anti-I-A^d (MK-D6, mouse IgG2a) mAb was purified from hybridoma culture supernatant (17). Source and characteristics of the monoclonal rat-mouse hybrid cell line producing 53.6.72 (IgG2a anti-CD8) mAb were as described previously (14), and Abs were purified by means of affinity chromatography.

Generation of helper T cell clones

Five $\times 10^6$ highly purified splenic dendritic cells (16), >96% CD11c$^+$, were incubated overnight with 100 ng/ml rIL-12 (14–16) and pulsed with 5 μM P815AB.12 peptide at 37°C for 2 h. Cells were then irradiated (30 Gy) and washed, and each mouse received an intrafootpad injection of 5 $\times$ 10^5 peptide-pulsed dendritic cells. After 7 days, popliteal lymph nodes were harvested and T cells were plated at 1×10^6 cells/well in 24-well plates in complete media (20) containing 50% of supernatant from secondary mixed leukocyte culture as a source of T cell growth factors. Irradiated syngeneic splenocytes pulsed with 5 μM P815AB.12 peptide were added at 6×10^6 cells/well every week. After 2 wk, contaminating CD8$^+$ T cells were removed using a positive selection column and CD8α MicroBeads (Miltenyi Biotec, Bergish Gladbach, Germany). The recovered CD4$^+$ T cells, >99% pure, were cloned by limiting dilution. The resulting clones were maintained by weekly restimulation of 1×10^5 cells with 5 μM P815AB.12 peptide and 6×10^6 irradiated spleen cells, in complete medium containing 40 U/ml human rIL-2.

Cytokine activity and intracellular cytokine expression

CD4$^+$ T cells (10^5) were cultured with peptide-pulsed syngeneic irradiated spleen cells (10^6) in 96-well plates. After 48 h, supernatants were harvested and cytokine contents were evaluated by sandwich ELISA. The IFN-γ activity was determined using R4-6A2 and biotinylated XMG1.2, whereas IL-4 measurements involved the use of mAb 11B11 and biotinylated BVD6-24G2. The sensitivity limit was ~20 pg/ml for both cytokines assayed by ELISA. To evaluate the cytoplasmic IFN-γ and IL-4 contents of T cell clones, cells stimulated with immobilized anti-CD3 mAb for 5 h in the presence of brefeldin A (1 μg/ml, during the final 1.5 h) were stained by the paraformaldehyde-saponin procedure, as described previously (21). After extensive washing in immunofluorescence buffer (PBS with 3% FCS and 0.02% NaN$_3$), cells were fixed in 2% paraformaldehyde for 10 min at room temperature, washed again, and incubated with PE-labeled anti-IFN-γ and FITC-labeled anti-IL-4 Abs in immunofluorescence buffer containing 0.3% saponin for 30 min on ice. Isotype-matched irrelevant mAbs were used as controls in the analysis of intracellular IFN-γ/IL-4 expression.

Delayed-type hypersensitivity assay

An assay system for measuring the delayed-type hypersensitivity (DTH)[3] response to tumor Ag was employed (22) in which 10^6 irradiated P1 cells and 10^5 CD4$^+$ T cells were inoculated into the left footpads of naive mice, whereas the right footpads received 10^6 irradiated P1-204 control cells and 10^5 CD4$^+$ T cells. The DTH reaction was recorded at different times after challenge by measuring the footpad thickness using a digital micrometer (Mitutoyo, Tokyo, Japan). The results were expressed as the increase in footpad thickness (left hind) over that of the control (right hind) counterpart. Data are the means ± SD of six to eight mice per group.

In vivo tumor experiments

Cultured tumor cells were washed three times with PBS and resuspended in PBS for implantation into mice. Living cells (10^6) were injected in 100 μl via a 27-gauge needle on the left flank. Tumor size was assessed twice per week using calipers, the longest and the shortest diameters were measured, and a mean was calculated. Data from groups of eight mice were analyzed at each time point, and a mean and a SE were determined. Mea-

surements were continued for 4 wk. F2 or F76 T cells (5 $\times 10^6$/injection) were i.v. transferred into mice either 3 days before tumor challenge (prophylactic administration) or on days 7, a time when the tumor mass became palpable (4–6 mm), 14, and 21 after tumor challenge (postchallenge administration). In selected experiments, mice also received IL-12-treated, P815AB.9-pulsed dendritic cells (5 $\times 10^5$) on day 6 after tumor challenge. For CD8$^+$ T cell depletion, mice received multiple i.p. injections of purified anti-CD8 mAb: once before tumor challenge (1 mg on day −1) and twice a week for 3 wk after tumor challenge (0.25 mg/injection). Control groups for mAb treatment received equal amounts of affinity-purified rat IgG (Zymed, San Francisco, CA).

CTL generation

Cytolytic assays were performed as described elsewhere (23). Briefly, splenocytes (5 $\times 10^6$) were harvested 20 days after in vivo tumor challenge and stimulated with irradiated (10,000 Gy) P1 cells (2.5 $\times 10^5$) in a volume of 2 ml. After 5 days, responder cells were recovered and tested for lytic activity against 2×10^3 ^{51}Cr-labeled tumor target cells according to standard conditions. Results were expressed as the mean of quadruplicate samples at different E:T ratios.

Results

Generation of P815AB-specific Th1 and Th2 cell clones

To study the effect of Ag-specific CD4$^+$ T cells on the function of endogenous CD8$^+$ T cells recognizing the same tumor Ag, we generated CD4$^+$ T cell populations from mice immunized with dendritic cells treated in vitro with IL-12 and pulsed with a P815AB-related dodecameric peptide (P815AB.12). Lymph node cells were restimulated in vitro with the eliciting peptide and then enriched for CD4$^+$ T cells. The resulting T cell line was cloned by limiting dilution, yielding several types of specific clones, including those that secreted predominantly IFN-γ and those that secreted predominantly IL-4 (Fig. 1 *A*). Of >30 clones characterized in detail, F76, which appeared to be CD4$^+$ and expressed intracellular IFN-γ by FACS analysis, and F2, also CD4$^+$ but expressing IL-4 (Fig. 1, *B* and *C*), grew continuously for over 12 mo, allowing for further study. Functional characterization confirmed that F76 was Th1-type and mediated Th1-associated effector responses in vivo, in contrast to Th2-type F2. When admixed with P815AB-expressing tumor cells before intrafootpad injection, F76 elicited a classical DTH response that peaked at about 12 h postchallenge. No such response was observed in mice injected with F2 cells (Fig. 1*D*). In the latter animals, a negligible nonspecific DTH was detected that did not increase in the presence of specific Ag.

Disparate but significant productions of IFN-γ and IL-4 were also exhibited by F76 and F2 clones when cocultured in vitro with class II-negative, P815AB-expressing P1.HTR cells in the presence of splenocytes as APC (Fig. 1*E*), thus confirming that P815AB is efficiently processed and presented by professional APC (24). In addition, Fig. 1*E* shows that cytokine production by the two clones was specific, being negligible in response to an OVA peptide; furthermore, the helper epitopes recognized by the CD4$^+$ cells induced by vaccination with P815AB.12 were also present in P815AB.9, the minimal core peptide for CTL recognition. We have previously shown that P815AB.9 does possess class II-restricted epitopes that appear nevertheless to be suboptimal for priming in vivo in the absence of immunoadjuvants (15, 17). Finally, cytokine secretion in response to specific Ag in vitro was restricted by I-A^d class II molecules. In experiments not reported here, we also found that neither clone was cytotoxic in vitro to radiolabeled P815AB-expressing (P1) tumor cells.

Antitumor effects of P815AB-specific CD4$^+$ T cell clones administered before challenge with P815AB-expressing tumor cells

To evaluate whether the prophylactic injection of F76 and F2 CD4$^+$ T cell clones could mediate tumor regression, prospective

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[3] Abbreviation used in this paper: DTH, delayed-type hypersensitivity.

FIGURE 1. Generation and characterization of Th cell clones from mice immunized to a tumor rejection Ag, P815AB. An Ag-specific T cell line was obtained from the popliteal lymph nodes of mice injected intrafootpad with IL-12-treated dendritic cells pulsed with P815AB.12 peptide under the conditions described in *Materials and Methods*. *A*, Clones obtained by limiting dilution of the CD4$^+$ bulk line were assayed for release of IFN-γ and IL-4 when cocultured in the presence of splenocytes pulsed with the eliciting peptide. The expressions of CD4/CD8 Ag (*B*) and cytoplasmic IFN-γ/IL-4 (*C*) were determined by flow cytometric analysis, and the percentage of unstimulated cells expressing either cytokine was <2%. *D*, Functional activity of F76 and F2 clones was examined in a DTH assay by intrafootpad injection of lymphocytes admixed with P815AB-bearing tumor cells. At different times postchallenge, the DTH reaction was determined as footpad thickness increase of experimental footpads over that of control counterparts injected with a mixture of F76 or F2 cells and Ag-loss variant cells. *, $p < 0.01$, experimental vs control footpads (Student's *t* test). *E*, Cytokine production by F76 and F2 clone cells was also assayed in response to APC cultured with P815AB-bearing (P1.HTR) cells, OVA peptide, or a P815AB-nonameric peptide representing the minimal core peptide for class I-restricted responses. The cytokine response to P815AB.12 was also tested in the presence of anti-I-A^d (MK-D6) mAb. IFN-γ production by F2 clone cells in response to APC + P815AB.12 was 110 pg/ml whereas IL-4 production by F76 was 62 pg/ml.
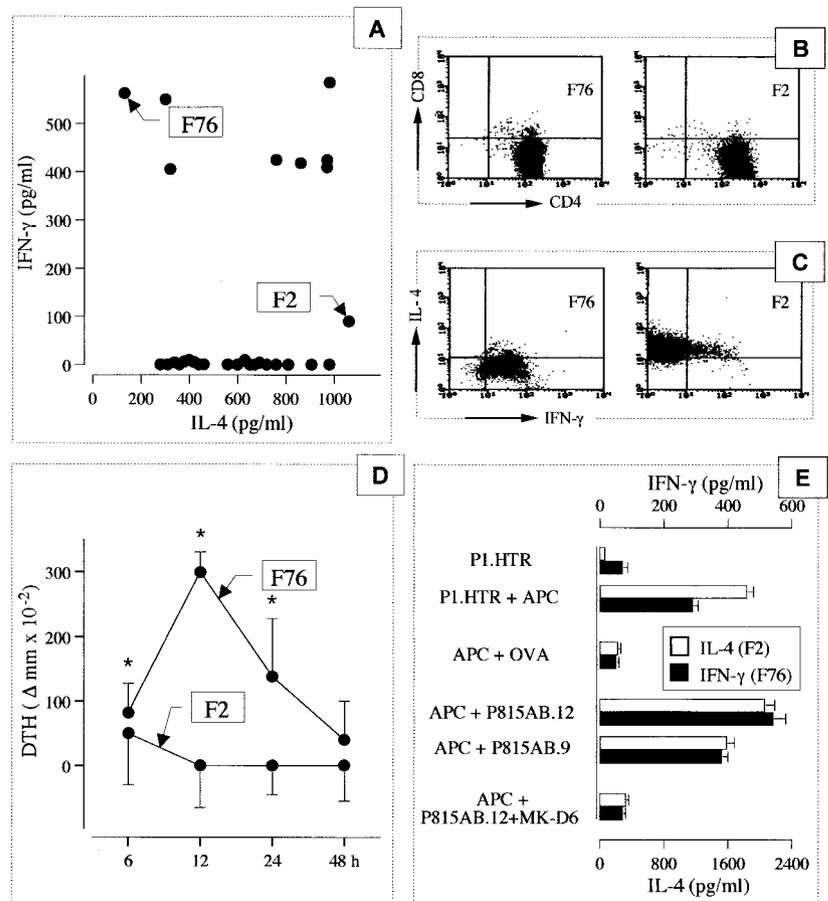
recipients of a s.c. challenge with P815AB-expressing (P1.HTR) cells or an Ag-loss variant (P1.204) were administered an i.v. injection of either clone. Control mice received PBS. Tumor growth was monitored in terms of mean tumor diameter assessed at different times postchallenge. All recipients of F76 cells rejected P1.HTR but not P1.204 tumor cells. Complete rejection of P1.HTR but not P1.204 cells was observed in 60–80% of mice receiving F2 clone cells (representative experiment shown in Fig. 2, out of five performed with analogous results). Histochemical analysis showed that no significant lymphocyte infiltration was observed over time in control P1.HTR tumor tissue preparations in the absence of cell transfer; in contrast, at 10–14 days after tumor challenge, marked and qualitatively comparable lymphocyte infiltra-

tions were present in tumor tissue of mice that had been treated with F76 or F2 cells (data not shown).

Induction and requirement of CD8$^+$ T cell reactivity in adoptive tumor immunotherapy initiated by CD4$^+$ T cells

In our immunization model system, dendritic cells likely present P815AB.12 in the context of class II molecules. However, the interaction of host APC with Ag-specific CD4$^+$ T cells could make APC capable of stimulating naive, endogenous CD8$^+$ cytotoxic T cells, which might then function as specific effectors capable of lysing tumor. Such a mechanism has previously been shown to be at work in a system with a model tumor Ag (8). To directly assess
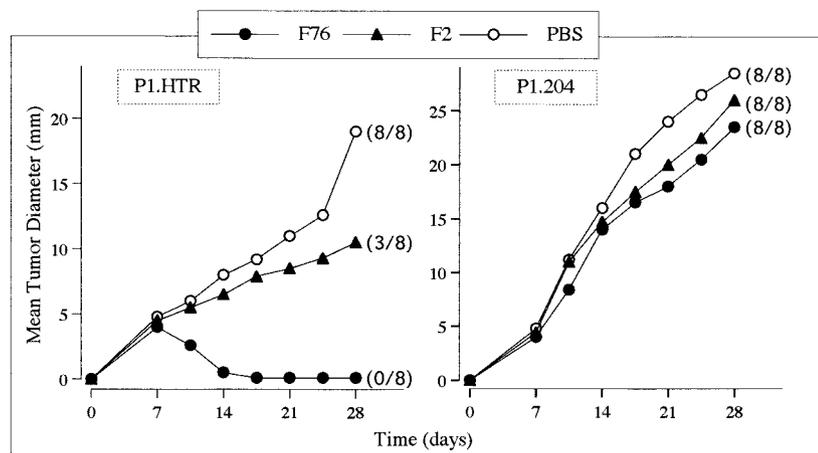

FIGURE 2. Adoptive immunotherapy of P815AB-expressing tumor cells by prophylactic injection of Ag-specific CD4$^+$ T cell clones. Mice were injected i.v. with 5×10^6 F76 or F2 clone cells or vehicle alone (PBS) 3 days before s.c. challenge with P1.HTR cells or a P815AB-negative (P1.204) variant (day 0). Mean tumor diameter (average of two perpendicular measurements) was assessed on the indicated days. SEs were usually <10% of the mean, except for P1.HTR-bearing mice treated with F2 cells in which the value gradually increased up to ~50% of the mean by days 21–28. Numbers in parentheses indicate dead mice over total animals injected. The tumor-free mice were followed for >90 days.

the induction of P815AB-specific host CD8$^+$ T cells, fresh spleno-cytes from tumor-bearing mice that had been treated with the F76 or F2 clones were harvested 20 days after tumor implantation and were cocultured with P1 cells. The recovered cells were assayed for cytotoxic activity to P815AB-positive (P1) or -negative (P1.204) cells. Fig. 3 shows that potent CD8$^+$ T cell responses were induced to P1 cells by the immunotherapy regimen that would result in a 100% cure rate, namely, the adoptive transfer of F76 cells. Although less remarkable, a cytotoxic response was also induced by transfer of the F2 clone, a procedure that would result in cure of 60–80% mice, as illustrated above. No cytotoxic activity was displayed to P815AB-negative variant cells as target cells in the cytotoxicity assay. Control cultures not reported in Fig. 3 showed that no cytotoxic activity was generated by coculturing tumor cells with splenocytes from tumor-bearing mice unexposed to F76 or F2 cells.

When P1.HTR tumor-challenged mice, recipients of F76 or F2 clone cells, were assayed at 3 wk postchallenge for class I-re-stricted skin test reactivity in vivo to the P815AB.9 peptide (24), all recipients of F76 cells uniformly displayed high levels of spe-cific reactivity; in contrast, among recipients of F2 cells, ~30% tumor-challenged mice gave negligible responses (data not shown). Interestingly, these mice had the largest tumor diameters.

To directly assess the possible requirement of CD8$^+$ T cells in the protective effect induced by transfer of F76 or F2 cells, we resorted to serologic ablation of CD8$^+$ cells by means of specific Ab. Fig. 4 shows that the depletion of CD8$^+$ lymphocytes con-current with adoptive transfer followed by tumor challenge com-pletely ablated the protective effect of T cell clone transfer.

Regression of established P815AB-expressing tumor by transfer of CD4$^+$ T cell clones

To evaluate whether the CD4$^+$ T cell clones could mediate tumor eradication when injected after challenge, the P1.HTR tumor was injected s.c. and allowed to establish for 7 days, a time when it became palpable. The tumor-bearing mice were then treated with an i.v. injection of 5×10^6 F76 or F2 cells. Fig. 5A shows that cure

FIGURE 4. Requirement for CD8$^+$ cells in the efficacy of Th cell trans-fer. Concurrent with F76 or F2 cell injection and P1.HTR tumor challenge, mice received several injections of anti-CD8 mAb as specified in *Materials and Methods*. Control mice received vehicle alone at the time of cell trans-fer and were treated with rat IgG in the place of anti-CD8 mAb. Mean tumor diameter was assessed on the indicated days. Numbers in parenthe-ses indicate dead mice over total animals injected. One representative ex-periment is shown of three performed.

of mice was only achieved by transfer of F76 cells, whereas, under these conditions of treatment, immunotherapy with F2 cells re-sulted in no significant beneficial effect in terms of tumor growth and survival rate of the tumor-challenged mice. This suggested that the F2 cells by themselves might not be enough to induce a suf-ficient and/or appropriate immune response when administered postchallenge. We thus examined the effect of multiple injections of F76 or F2 cells, either alone or in combination with transfer of dendritic cells preexposed to IL-12 and P815AB.9 (Fig. 5B). When mice were treated on days 7, 14, and 21 with F76 or F2 cells, the recipients of the former cells exhibited accelerated rejection of the implanted tumor; among recipients of three injections of F2 cells, <40% survived challenge. However, on combining lymphocyte transfer with dendritic cells administered on day 6, the vast ma-jority of F2 cell recipients resisted challenge, according to a growth and rejection pattern of the P1.HTR tumor similar to that observed in the recipients of a single injection of F76 cells. Inter-estingly, no effect was displayed by peptide-pulsed dendritic cells administered 6 days after challenge, as opposed to the complete protection observed when transfer precedes tumor challenge (13). Most strikingly, all mice cured by immunotherapy with the F76 clone or a combination of F2 plus dendritic cells were later (i.e., at 4–6 wk) found to resist challenge with large inocula of P1.HTR cells. Under the latter conditions of testing, a peculiar growth and rejection pattern of the implanted tumor was observed in that no initial tumor development was observed as in the primary challenge.
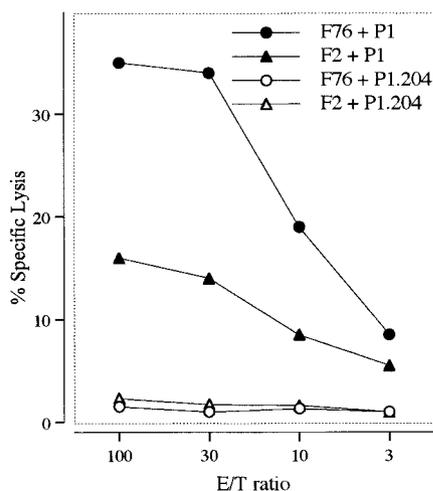

FIGURE 3. Detection of CTL activity in mice cured by immunother-apy. P1.HTR tumor-challenged mice, transferred with either F76 or F2 cells, served as donors of spleen cells to be restimulated in vitro before assaying CTL activity to radiolabeled, antigenically relevant P1 cells. Per-cent specific lysis was measured in a standard ^{51}Cr release assay at different E:T ratios. Specificity controls included the use of antigenically irrelevant, Ag-loss variant cells (P1.204) as target cells. Additional controls (data not reported in the figure) included the use of radiolabeled YAC-1 target cells to search for possible effects mediated by NK cells, and none was found.
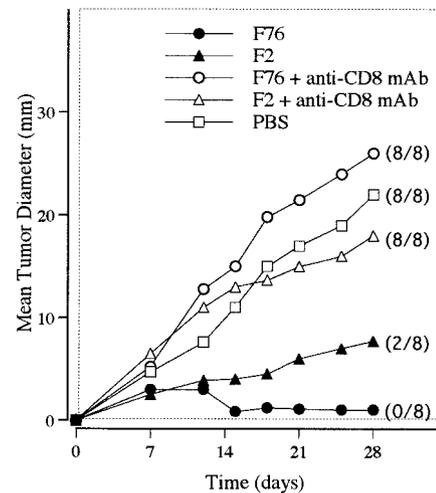
Discussion

Transfer of CD4$^+$ T cell clones, specific for a molecularly defined and poorly immunogenic tumor Ag with class I- and class II-re-stricted epitopes, was shown in this study to induce de novo gen-eration of CD8$^+$ T cells specific for that same Ag. Moreover, activation of host CD8$^+$ T cells and tumor eradication appeared to be initiated by both Th1-type and Th2-type cell clones. This report adds to previous data on the cooperation between adoptively trans-ferred Th cells and host CD8$^+$ T cells in tumor eradication in vivo
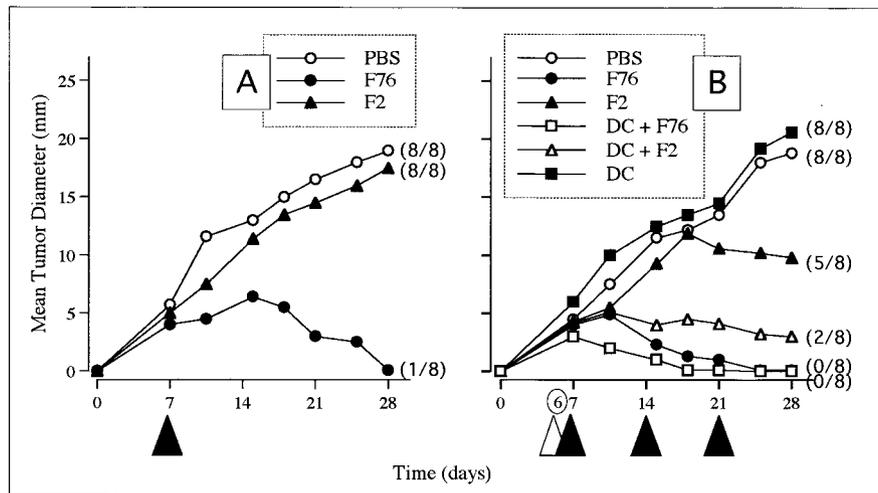
FIGURE 5. Eradication of established tumor by Ag-specific Th cells. *A*, T cell therapy was conducted using mice challenged s.c. with 10^6 P1.HTR cells at a time when the tumor mass became palpable. *B*, Effects of repeated injections of F76 or F2 cells either alone or in combination with transfer of dendritic cells. On days 7, 14, and 21 after tumor challenge, mice received injections of either type of cell clone. Groups of mice were treated on day 6 with dendritic cells (DC) exposed in vitro to IL-12 and P815AB.9. Controls included mice treated with PBS in the place of either cell clone and mice injected with dendritic cells in the absence of further treatment. The antitumor activity was determined by recording changes over time of the means of two perpendicular diameters of the tumor mass. Numbers in parentheses indicate dead mice over total animals challenged with the tumor.

using Th1/Th2 cell lines (7) or a Th1 clone (8) specific for highly immunogenic model tumor Ag, or a Th2 clone recognizing undefined rejection Ag on a methylcholanthrene-induced sarcoma (25).

Tumor-specific shared Ag in humans, such as members of the MAGE, BAGE, and GAGE families found in melanoma and other cell types, derive from nonmutated self proteins and have an expression pattern similar to P815AB, a poorly immunogenic tumor-associated Ag of murine mastocytoma cells (26). We have generated Th cell clones specific for P815AB by immunizing mice with syngeneic dendritic cells activated with IL-12 in vitro and pulsed with a synthetic P815AB-related peptide (13). We have previously shown that under these conditions of priming, an immune response is initiated in the host which is directed to both class II-restricted and class I-restricted epitopes of P815AB (15). One of the resulting CD4$^+$ cell clones, F76, produced high levels of IFN-γ but not IL-4 in vitro and mediated a classical DTH response in vivo when admixed with antigenically relevant tumor cells (Fig. 1). It could thus be defined functionally as a Th1-type clone. In contrast, F2, which secreted high levels of IL-4 but not of IFN-γ in vitro in response to specific Ag, failed to mediate DTH in vivo (Fig. 1) and could thus be defined as a Th2-type clone (27). Both clones secreted the respective cytokine pattern when stimulated in vitro with class II-negative, P815AB-bearing cells in the presence of APC, presumably recognizing helper epitopes also present in P815AB.9 (Fig. 1*E*), the minimal core peptide for CTL recognition (10, 28).

When administered i.v. to prospective recipients of a s.c. challenge with P815AB-positive cells, both Th1 and Th2 cells prevented tumor growth and lethality in the totality (F76) or the majority (F2) of the tumor-challenged mice (Fig. 2). In a recent report using Th1 and Th2 cell lines from OVA-specific TCR-transgenic mice in combination with OVA gene-transfected tumor cells, Th1 and Th2 cells were found to mediate tumor regression. However, the CD4$^+$ T cells in that system appeared to act through distinct mechanisms, namely, induction of cellular immunity for Th1 cells and tumor necrosis for Th2 cells. Another important difference between Th1 and Th2 cell therapy in that model is that Th1 therapy was able to induce a strong immunological memory which was suitable for CTL generation and was associated with resistance

against rechallenge with parental tumor cells (7). In our model system with P815AB, histochemical analysis of tumor tissues from the recipients of Th1 or Th2 cells did not suggest the involvement of distinct mechanisms in tumor eradication by either type of T cell clone (data not shown). In addition, the rejection of P815AB-expressing tumor cells initiated by postchallenge transfer of either clone was associated with strong immunological memory, as evidenced by the growth and rejection pattern of the P815AB-expressing tumor used for rechallenge. It has been suggested that the distinct antitumor activities mediated by OVA-specific Th1 and Th2 cells can be traced to different cell adhesion interactions involved in the migration of these cells into tumor tissues across endothelia. Yet, unknown mechanisms appear to be involved in Th2-induced tumor necrosis, although recruitment of eosinophils and macrophages could be a likely possibility (29). Shen and Fujimoto (25) also described regression of a methylcholanthrene-induced sarcoma mediated by a Th2-type clone. No evidence was obtained in that model for either a direct cytotoxic activity of the CD4$^+$ T cells or recruitment of host eosinophils to the antitumor effector response. It was rather hypothesized that the transferred CD4$^+$ T cells would migrate into tumor sites to be restimulated by APC in tumor tissue. This would lead locally to the secretion of cytokines necessary for CTL priming.

Although the mechanisms underlying the therapeutic activity of Th1 and Th2 cells in our model system are unclear, the antitumor effect initiated by transfer of either clone was associated with the detection of CTL activity in vitro (Fig. 3) and required the presence of CD8$^+$ T cells in vivo (Fig. 4). An absolute requirement for host CD8$^+$ T cells has been found not only in the model system with the Th2 cell clone mentioned above (25) but also in the immunotherapy with model tumor Ag. In particular, the therapeutic efficacy of Th2 cells in conjunction with tumor cells expressing OVA Ag has been suggested to be mediated by the recruitment of Ag-nonspecific CD8$^+$ killer T cells as the final effectors of the response (7). In the case of the Th1 clone recognizing Ag on a β-galactosidase-transduced tumor (8), it has been suggested that the antitumor effect of CD4$^+$ T cells occurs through an indirect mechanism involving the recruitment of host Ag-specific CD8$^+$ T cells. It has been proposed that the interaction of host APC with

Ag-specific CD4$^+$ T cells can make the former cells capable of stimulating naive, endogenous CD8$^+$ cytotoxic T cells, which may then function as specific effectors capable of destroying tumor. It is likely that CD8$^+$ T cells are important effector cells in our model of Th1 and Th2 cell therapy, because Ag-specific CTL activity could be detected in vitro, and depletion of CD8$^+$ T cells abrogated the therapeutic effect of cell transfer. Interestingly, the higher efficacy of F76 cell transfer was associated with higher levels of cytotoxic activity generated in vitro. In experiments not reported here, we also found that CD8$^+$ T cell responses were not induced in nontumor-bearing mice receiving F76 or F2 cells when their splenocytes were stimulated in vitro with P815AB-expressing tumor cells.

CD4$^+$ T cells are known to interact with class II-expressing host cells, especially dendritic cells, affecting their ability to modulate different components of the immune system. Presentation of P815AB by dendritic cells and host production of IL-12 are required for the initiation of a class I-restricted response to P815AB that is dependent on the presence of an intact CD4$^+$ cell compartment (12, 14). In our adoptive transfer model with CD4$^+$ cells, it is possible that the transferred cells specifically interact with dendritic cells at the tumor site, resulting in CD40-CD40 ligand interactions and production of IL-12. The cytokine may further activate the dendritic cell in an autocrine fashion (16, 30) and contribute to the activation of proximate CD8$^+$ T cells recognizing class I-restricted epitopes of P815AB on the dendritic cell. This would be consistent with our finding that preactivation in vitro of P815AB-pulsed myeloid dendritic cells with IL-12 results in the development of strong class I-restricted responses when the dendritic cells are transferred into recipient hosts (31).

The adoptive transfer of CD4$^+$ T cells was also found to treat established tumor in our model system (Fig. 5). However, whereas the Th1 clone was fully capable of eradicating tumor, the Th2 clone required repeated injections and optimal protection was only achieved by combining Th2 cell transfer with the injection of peptide-pulsed dendritic cells. The combined effects of T cell transfer and dendritic cell transfer under conditions in which neither treatment alone would be effective further substantiate the hypothesis of an interaction taking place in vivo between the transferred T cells and dendritic cells, resulting in effective host production of endogenous IL-12. The hypothesis of a primary role of dendritic cell "conditioning" (8, 32–34), as mediated by a CD40 engagement that results in up-regulation of CD80/CD86, class I/class II, and IL-12 expression, would be consistent with the finding of therapeutic efficacy of both Th1 and Th2 cells, largely independent of their cytokine secretion profile. This would imply that quantitative rather than qualitative differences may characterize the antitumor immunity mediated by Th1 and Th2 cell clones. From the data in Fig. 2, it would appear that there is a quantitative inferiority of the Th2 clone relative to Th1 cells in promoting tumor rejection. Preliminary experiments based on transfer of graded numbers of either clone cells seem to suggest that the difference is indeed quantitative.

Independent of the cytokine secretion profiles, the adoptive transfer of CD4$^+$ cells has also been reported to eradicate established tumors in a variety of experimental models (35–37). In most of these systems, the therapeutic activity of CD4$^+$ T cells was traced to activation of NK or macrophage effector cells or to direct lysis of class II-expressing tumor cells. In addition, specific Th cell requirement for optimal CTL induction was also found when the tumor cells were class II negative (38). Although our Th cell clones lacked direct cytotoxic activity in vitro to antigenically relevant target cells, it is possible that one or more of these mechanisms may contribute to the therapeutic efficacy of Th cell clones.

However, our present data suggest that activation of endogenous CD8$^+$ effector cells is a major therapeutic effect initiated by CD4$^+$ T cell transfer. Although CD8$^+$ T cells have been used successfully to transfer antitumor resistance in adoptive immunotherapy models (39, 40), it has been recently found that transgenic mice that express the TCR from a CTL clone recognizing P815AB are no more resistant to a P815AB-bearing plasmacytoma than nontransgenic littermates (41). Our data suggest that the activation of specific CD8$^+$ T cells by CD4$^+$ T cells may be most efficient when cells recognize the same Ag presented by dendritic cells in the context of both class I and class II molecules. The effect would require an action on dendritic cells, perhaps largely mediated by CD40-CD40 ligand interactions, leading to enhanced ability of the dendritic cell to stimulate responder CD8$^+$ T cells. Finally, the production of IL-12 by dendritic cells at the tumor site may be a critical step with the cytokine acting at both the dendritic cell (16) and T cell (42) levels. Lack or suppression (43) of inflammatory signals in a growing tumor could explain the failure of the host to locally recruit helper and cytotoxic cells to the antitumor response. However, provision of specific CD4$^+$ T cells by adoptive transfer or by activation in vivo after vaccination might lead to the de novo induction of CD8$^+$ T cell reactivity.

Acknowledgments

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