Expanded Tumor-reactive CD4+ T-Cell Responses to Human Cancers Induced by Secondary Anti-CD3/Anti-CD28 Activation

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ABSTRACT
Generation of tumor-reactive T cells in large numbers ex vivo is a requisite step in the adoptive immunotherapy of patients. We examined the immune responses of T cells derived from tumor vaccine-primed lymph nodes activated with anti-CD3 alone and with an anti-CD3/anti-CD28 combination. Nylon wool-purified CD3+ cells were isolated from vaccine-primed lymph nodes obtained from melanoma, renal cell, and head and neck cancer patients. In the absence of antigen-presenting cells, activation with anti-CD3/anti-CD28 greatly enhanced subsequent T-cell expansion in interleukin 2 (>100-fold), compared to anti-CD3 alone. CD4+ T cells were preferentially stimulated. In four of eight patients, we found evidence of CD4+ cellular responses to autologous tumors by cytokine release assays. Positively selected CD4+ cells activated with anti-CD3/anti-CD28 released greater amounts of cytokine (IFN-γ and granulocyte macrophage colony-stimulating factor) in response to autologous tumors compared to cells activated by anti-CD3 alone. The CD4+ reactivity was MHC class II restricted and appeared to be associated with the expression of class II molecules on the vaccinating tumor cells. The CD4+ T-cell responses to class II-restricted tumor-associated antigens in patients with renal cell cancers represent unique findings.

INTRODUCTION
The adoptive transfer of immune lymphoid cells has been reported by our laboratory and many other investigators to be efficacious in mediating the regression of established tumor burdens in experimental models (1–5). From these studies, it is readily apparent that the antitumor responses achieved with adoptive immunotherapy are directly related to the quantity and immunological reactivity of the lymphoid cells infused. Hence, methods that are capable of expanding lymphoid cells while still maintaining or augmenting antitumor reactivity are critical to the development of clinical T-cell therapy. We have previously described an in vitro activation procedure that involved the sequential culture of tumor-primed lymphoid cells with anti-CD3 mAb3 (anti-CD3) followed by IL-2 that resulted in the expansion of immune T cells that are capable of mediating tumor regression in vivo (3). Lymphoid cells generated in this manner resulted in the preferential expansion of CD8+ T cells with reactivity against autologous tumor cells that was MHC class I restricted (6).

During T-cell activation, ligation of CD28 is incorporated to provide a second signal to lymphoid cells in concert with a stimulus via the TCR-CD3 complex (7). This latter interaction via the TCR-CD3 complex is optimally initiated by antigen but can also be triggered by anti-CD3. Ligation of CD28 is through the interaction between CD28 and its natural ligands, namely, CD80 (B7–1) and CD86 (B7–2), which are expressed on APCs (8, 9). In the absence of APCs, such as when purified T-cell populations are used, complete activation of T cells depends on an interaction between the costimulatory molecule CD28 and its mimic ligands (i.e., anti-CD28 mAbs) after an activation signal is delivered to the TCR-CD3 complex (10, 11).

We have shown, in both animal models and clinical studies, that immune lymphoid cells can be retrieved from either tumor-draining LNs or VPLNs for use in adoptive immunotherapy (3, 4, 6). We are actively engaged in evaluating the antitumor reactivity of VPLN cells adoptively transferred into patients with various metastatic malignancies. This report describes our initial observations on an alternative approach to activate purified human T cells derived from VPLN using the combination of anti-CD3 plus anti-CD28 mAb (anti-CD28), followed by expansion in IL-2.

We observed that the activation of T cell-enriched human VPLN cells by an anti-CD3/anti-CD28 combination resulted in significantly enhanced T-cell proliferation as well as IFN-γ and GM-CSF secretion compared with anti-CD3 alone. More importantly, the use of anti-CD3 plus anti-CD28 as activating reagents, in the absence of APCs, allowed us to detect tumor reactivity manifested by CD4+ T cells that was MHC class II restricted. The T-cell activation procedure reported herein may

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3 The abbreviations used are: mAb, monoclonal antibody; IL, interleukin; TCR, T-cell receptor; APC, antigen-presenting cell; LN, lymph node; VPLN, vaccine-primed LN; GM-CSF, granulocyte macrophage colony-stimulating factor; RCC, renal cell cancer; SCC, squamous cell cancer; CM, complete medium; FACS, fluorescence-activated cell sorting.

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facilitate the identification of CD4+ T cell reactivity to MHC class II restricted tumor-associated antigens that have been less well characterized.

MATERIALS AND METHODS

Clinical Protocols. Patient samples were obtained during the course of separate clinical trials being performed at the University of Michigan Cancer Center. These studies had been previously reviewed and approved by the University of Michigan Institutional Review Board. One of the clinical trials represents a Phase II evaluation of the treatment of stage IV RCC patients with adoptively transferred VPLN cells plus IL-2. Patients were vaccinated intradermally with irradiated autologous tumor cells admixed with Tice Bacillus Calmette-Guérin (Organon Inc., West Orange, NJ) in the anterior thigh followed 1 week later by retrieval of VPLN in the inguinal region. The VPLN cells used for transfer were activated and expanded by an anti-CD3/IL-2 culture method previously described (6). Another trial represents an exploratory Phase I study of this same adoptive immunotherapy approach in patients with recurrent head and neck SCC. The other clinical trial involves the treatment of stage IV melanoma patients with LN cells primed by autologous tumor cells transduced to secrete GM-CSF (12). Patients are vaccinated intradermally with irradiated autologous tumor cells transduced by a retroviral vector encoding for human GM-CSF (MFG-S-GM-CSF; provided by Somatix Therapy, Inc., Alameda, CA). One week later, VPLNs were harvested, and the whole population of LN cells was activated and expanded by the anti-CD3/IL-2 culture method noted above (6). The VPLN cells were subsequently transferred i.v., in conjunction with IL-2 (12). All these clinical trials have as their common feature the activation of VPLN cells with anti-CD3. The data presented in this report focused on studies evaluating the in vitro reactivity of purified VPLN T cells activated by anti-CD3 alone, compared with the combination of anti-CD3 plus anti-CD28 mAbs.

Tumor Cell Cultures and MHC Class II Modulation. Patients in these trials were required to have autologous tumor cells for tumor vaccination and in vitro immunological assays. Tumor cells were prepared as described previously (6). Aliquots of cells were then cryopreserved at −180°C in liquid nitrogen for subsequent immunization and immunological assays. For in vitro immunological assays, cultured tumor cell lines were established and used, or if they were not available, cryopreserved tumor cells were thawed and used. Histologically matched allogeneic tumor cell lines were used for specificity controls. Tumor cell lines were maintained as adherent monolayers in CM, which consisted of RPMI 1640 supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 1× nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin sulfate, 0.5 μg/ml fungizone, and 0.05 mM 2-mercaptoethanol (all from BioWhittaker, Walkersville, MD). Cells were passaged in CM and harvested by trypsinization (6). To induce expression of MHC class II molecules on class II-negative tumor cells, the tumor cells were cultured in the presence of recombinant human IFN-γ (Genentech, Inc., San Francisco, CA) at a concentration of 1000 units/ml for 3 days.

VPLN Preparation. For vaccination, cryopreserved tumor cells were thawed and washed in HBSS (BioWhittaker) twice. Viable tumor cells were irradiated to a dose of 25 Gy, counted by trypan blue exclusion, and resuspended so that a volume of 0.2–0.4 ml contained 1 × 10^7–2 × 10^7 viable tumor cells. Patients were vaccinated intradermally at two sites ~10 cm below the inguinal crease. As indicated above, RCC and SCC patients were inoculated with irradiated tumor cells admixed with Bacillus Calmette-Guérin (6), and melanoma patients were inoculated with irradiated GM-CSF-secreting tumor cells (12). Seven to 10 days later, draining LN pairs were surgically removed for culture. A single-cell suspension of LN cells was obtained by mechanical dissociation. Briefly, LNs were minced into 2-mm³ pieces in cold HBSS with a scalpel. The fragments were then pressed through a stainless steel mesh with a glass syringe plunger. The resultant cell suspension was filtered through nylon mesh and washed in HBSS. The cells were counted and suspended in X-Vivo-15 medium (BioWhittaker) at 2 × 10^6 cells/ml. Aliquots of cells were then cryopreserved in liquid nitrogen for future use.

T-Cell Purification and Fractionation. VPLN T cells were enriched by passing the single-cell suspension prepared above through 1-g quantities of sterile brushed nylon wool (Cellular Products, Inc., Buffalo, NY) column, using a 45-min absorption period at 37°C, followed by elution of the nonadherent T cells with warm RPMI 1640 without additives. VPLN CD4+ or CD8+ T cells were positively selected from T cell-enriched cell suspensions (>95% CD3−, as indicated by FACS analysis) by treatment with superparamagnetic microbeads conjugated with monoclonal antihuman CD4 or antihuman CD8 antibodies, respectively, followed by separation using the MACS Separator. The microbeads coupled with antibodies and separator were both purchased from Miltenyi Biotec Inc., Sunnyvale, CA) and applied using the manufacturer’s directions.

T-Cell Activation and Expansion. Twenty-four-well cell culture plates (Costar, Cambridge, MA) were coated with antihuman CD3 mAb (OKT3; Ortho Pharmaceutical Corp., Raritan, NJ) at a concentration of 2 μg/ml in PBS or coated with the combination of antihuman CD3 plus antihuman CD28 mAb (PharMingen, San Diego, CA) at a ratio of 1:1. The concentration of each antibody in the combination was 2 μg/ml in PBS. Each well of a 24-well plate was coated with 600 μl of anti-CD3 or the anti-CD3/anti-CD28 antibody combination at 4°C overnight or at room temperature for 5–6 h. The coated plates were then washed twice with PBS. Purified VPLN T cells were placed on the precoated and washed plates at 1 × 10^5–2 × 10^6 cells/ml in 2 ml of X-Vivo-15 medium and cultured for 4 days in 5% humidified CO₂. After exposure to the immobilized antibodies, the cells were harvested and counted. The cells were then expanded in IL-2 (Chiron, Emeryville, CA) containing X-Vivo-15 medium starting at a concentration of 3 × 10⁵ cells/ml in six-well culture plates (Costar) for 7 days. Each well of IL-2 was 50 units/ml. During the 7-day cell expansion period, additional IL-2-containing medium was added and cells were split as they grew to keep the cell density below 3 × 10⁶ cells/ml.

Phenotypic Analysis. All of the FITC-labeled mouse antihuman antibodies used for immunofluorescence assays were
purchased from PharMingen (San Diego, CA) and included: CD3 (clone UCHT1, IgG1); CD4 (clone RPA-T4, IgG1); CD8 (clone RPA-T8, IgG1); CD25 (IL-2 receptor, clone M-A251, IgG1); HLA-A, -B, and -C (clone G46–2.6, IgG1); and HLA-DR, -DP, and -DQ (clone TU 39, IgG2a). Control staining was performed using FITC-conjugated mouse monoclonal immunoglobulin isotype control, also purchased from PharMingen. Each sample that was analyzed contained 0.3 × 10^6–1 × 10^6 cells. Cells were stained after centrifugation by being mixed with 3–10 μl of appropriate antibody and incubated at 4°C for 30 min. The sample was then washed with 4 ml of PBS containing 5% FBS, centrifuged and fixed in 1.0 ml of 1% paraformaldehyde. Analysis of cells was performed within 3 days after fixation in a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). A scatter window was set to eliminate the dead cells. The cytometer was interfaced to a Hewlett-Packard 310 computer, and data analysis was performed using Lysis software (Becton Dickinson).

Assessment of Cytokine Release. Cytokines (GM-CSF, IFN-γ, and IL-10) released by activated CD3^+, CD4^+, or CD8^+ VPLN T cells were measured in response to tumor stimulation. T cells activated with the anti-CD3/anti-CD28 antibody combination were compared with those activated with anti-CD3 alone. After T-cell activation and expansion, 2 × 10^6 activated VPLN T cells were cocultured with 0.5 × 10^6 of irradiated autologous tumor cells or histologically matched allogeneic tumor cells. The assay was carried out in 2 ml of CM containing 4 units/ml IL-2 in 24-well culture plates. Tumor cells were irradiated with 6,000 cGy for RCC and SCC or 20,000 cGy for melanoma by a ^137^Cs source (500 cGy/min; Gamma Cell Irradiator, Atomic Energy of Canada). After 48 h, culture supernatants were collected and centrifuged at 1500 rpm for 5 min to separate any cells. Culture supernatants were then measured for cytokine release with commercially available ELISA reagents (PharMingen) using the manufacturer’s directions. For GM-CSF, a standard curve starting at 20,000 pg/ml with serial 2-fold dilutions was performed. For IFN-γ and IL-10, standard curves starting at 10,000 pg/ml were established in similar fashion. Experimental values were calculated after cytokine release by T cells or tumor cells alone (if any) was subtracted.

Antibody Blocking Studies. To inhibit VPLN T cell recognition of tumor-specific antigen on target tumor cells, mAbs were added at the same time that tumor cells were mixed with the activated T cells for the cytokine release assessment. Antibodies directed against HLA determinants included anti-class I (HLA-A, -B, and -C) mAb and anti-class II (HLA-DR, -DP, and -DQ) mAb (PharMingen). Antibodies directed against HLA counterpart ligands on T cells included anti-CD4 and anti-CD8 mAbs (PharMingen). The cocultures were maintained for 48 h at 37°C in the continued presence of the mAbs.

RESULTS

Costimulatory Function of Anti-CD28 mAb on T-Cell Proliferation. We investigated the ability of an anti-CD28 mAb to function as a second signal in T-cell proliferation together with a high-affinity anti-CD3 mAb, in the absence of APCs. Culture plates precoated with anti-CD3 (2.0 μg/ml) alone or anti-CD3 (2.0 μg/ml) plus anti-CD28 at 0.5, 2.0, or 8.0 μg/ml, respectively, were tested for T-cell activation using purified T cells (>95% CD3^+) derived from VPLNs. Activation with the anti-CD3/anti-CD28 combination greatly enhanced T-cell expansion in IL-2 following antibody activation with anti-CD3 alone (Fig. 1). Specifically, T-cell activation with anti-CD3 at 2 μg/ml plus anti-CD28 at 2 μg/ml led to a cell expansion of >100-fold starting from 2.5 × 10^6 cells, whereas T cells activated by anti-CD3 alone expanded to a very limited extent. T-cell expansion in the presence of anti-CD28 at either higher (8 μg/ml) or lower (0.5 μg/ml) concentrations was less than that at 2 μg/ml (Fig. 1). Hence, the optimal ratio of anti-CD3/anti-CD28 was determined to be 1, with the concentration of each being 2 μg/ml.

We further studied the proliferation of T cells activated by the optimal anti-CD3/anti-CD28 combination compared to anti-CD3 alone for different periods of time (Fig. 2). In these studies, purified T cells were activated with antibodies for 2 or 4 days before they were transferred to IL-2-containing medium and cultured for 9 or 7 days, respectively, to achieve a total culture period of 11 days. In this experiment, we noticed that T cells activated with anti-CD3 alone for a shorter period of time (2 days) favored cell growth versus a longer period of time (4 days). By contrast, cell activation with the anti-CD3/anti-CD28 combination for 4 days lead to a cell expansion of ~120-fold, whereas only one-third of the proliferation could be achieved cells were activated with the same antibody combination for only 2 days. Additionally, cell death was frequently observed when cells were activated in antibody longer than 5 days. Therefore, the optimal activation time with an anti-CD3/anti-CD28 combination at 2 μg/ml each was determined to be 4 days. This became our standard T-cell activation protocol for all of the experiments reported herein unless otherwise indicated.

![Fig. 1](clincancerres.aacrjournals.org) Proliferation of purified CD3^+ T cells in IL-2 following antibody activation with anti-CD3 alone (2 μg/ml) or anti-CD3 (2 μg/ml) plus anti-CD28 at 0.5, 2, or 8 μg/ml. Equal numbers of cells were added to each well of the precoated plate and incubated at 37°C for 4 days. The antibody-activated T cells were then transferred to fresh medium containing 50 units/ml IL-2 and cultured for 7 days. Cells were then harvested and counted by trypan blue exclusion.
Phenotype Analysis of T-Cell Activation. To characterize the costimulatory function of anti-CD28 on T cell proliferation, we examined the expression of several T-cell markers, including CD25 (IL-2 receptor) before and after T-cell activation with anti-CD3 alone or anti-CD3 plus various concentrations of anti-CD28. The expression of CD25 on the CD3^{+} T cells of VPLN prior to antibody activation was low (10%; Fig. 3A). T-cell activation with anti-CD3 alone increased the CD25 expression to 22% (Fig. 3B). In contrast, T-cell activation with a combination of anti-CD3 plus anti-CD28, regardless of the concentrations of anti-CD28 used within the range tested, upregulated the CD25 expression to nearly 100% (Fig. 3, C–E). At the end of T-cell expansion following antibody activation, T-cell markers were examined to determine the CD4^{+} and CD8^{+} T-cell subpopulations contributing to the whole expanded CD3^{+} population. FACS analysis indicated that anti-CD28 preferentially stimulated the growth of CD4^{+} T cells (Fig. 4) because the percentage of CD4^{+} T cells in the final CD3^{+} T cell population increased gradually from 45 to almost 80% with the increase of anti-CD28 used for cell activation, whereas the percentage of CD8^{+} T cells, by contrast, declined from 55 to nearly 20%.

Immunological Reactivity of Anti-CD3/Anti-CD28-activated VPLN T Cells. To examine the immune responses of anti-CD3 activated VPLN T cells in the presence or absence of anti-CD28, we assessed the cytokines released by nonfractionated CD3^{+} cells or fractionated CD4^{+} and CD8^{+} VPLN T cells after cell activation and expansion. After the activation procedure, the cells were washed and restimulated with irradiated autologous tumor cells for cytokine release measurement. T-cell activation by the anti-CD3/anti-CD28 combination resulted in significantly enhanced IFN-γ and GM-CSF (Fig. 5) secretion by CD3^{+} or CD4^{+} VPLN T cells in response to autologous tumor cells in 2 of 3 patients examined (P < 0.05, Student’s t test). In all three patients, CD4^{+} VPLN T cells activated by anti-CD3/anti-CD28 mediated greater release of IFN-γ and GM-CSF to autologous tumor cells than did anti-CD3 activation alone (P < 0.05). Each experiment was repeated at least three times. In these experiments, we failed to grow the positively selected CD8^{+} population of VPLN T cells of the RCC patient to a total cell number needed for the cytokine assay. Although we could grow positively selected CD8^{+} T cells from the melanoma and SCC patients, their cytokine (both IFN-γ and GM-CSF) release response to autologous tumor remained very low or absent under either antibody activation conditions (Fig. 5).

MHC Class II Restriction of Cytokine Release. We further evaluated the specificity of the VPLN response and its
MHC restriction. Cytokine secretion in response to histologically matched allogeneic melanoma or RCC tumor targets was assessed in parallel to autologous tumor targets and found to be negligible (Figs. 6 and 7), which demonstrated that the observed IFN-γ and GM-CSF release in response to autologous tumor cells was immunologically specific. The autologous tumor cells of a melanoma patient (patient 1, Table 1) and the allogeneic melanoma tumor line under study (data not shown) expressed both MHC class I and class II molecules. The observed cytokine release by CD4+ VPLN cells to tumor antigen stimulation suggested an MHC restricted T-cell response. To assess the nature of this response, we assessed the cytokine released by positively selected CD4+ T cells (>95% by FACS) from this melanoma patient in response to autologous tumor with or without the presence of blocking mAbs to MHC class I or class II epitopes. As shown in Fig. 6, cytokine release by positively selected and activated CD4+ VPLN T cells to autologous tumor stimulation was blocked significantly by anti-class II mAb (64, 53, and 77% reduction of GM-CSF, IFN-γ, and IL-10 secretion, respectively), whereas no apparent blocking effect was observed with anti-class I mAb. In addition, anti-CD4 mAb blocked the release of cytokines, whereas anti-CD8 mAb did not (Fig. 6). These results indicated that the immunological reactivity manifested by CD4+ VPLN T cells was MHC class II restricted.

The tumor cells of a RCC patient under study expressed only MHC class I (>95%), not class II, molecules (patient 3, Table 1). Initially, VPLN T cells from the RCC patient failed to release significant amounts of GM-CSF or IFN-γ in response to stimulation with autologous tumor cells, even if the T cells were activated with the anti-CD3/anti-CD28 combination (Fig. 7). To assess whether these cells were responsive to a class II-restricted antigen on autologous tumor, we induced the expression of MHC class II molecules on the RCC tumor cells before using them as stimulators in the cytokine release assay. Induction of class II by IFN-γ was effective with 90% of the RCC tumor cells expressing class II after incubation (Table 1). IFN-γ treated autologous RCC tumors successfully stimulated GM-CSF and IFN-γ release of anti-CD3/-anti-CD28-activated CD4+ VPLN T cells at a much higher level than the cytokine release of the same CD4+ cells in response to the untreated class II-negative autologous tumor cells (Fig. 7). Cytokine secretion of CD3+ T cells was also elevated in response to IFN-γ treated autologous tumors due to the presence of CD4+ cells (~75% of the CD3+ T cells were CD4+, as indicated by FACS; data not shown). Again, the specificity of this enhanced cytokine release was clearly suggested by the failure of the T cells to respond to the IFN-γ-treated MHC class II-positive allogeneic RCC tumor cells. These results demonstrate the requirement of class II expression by tumor cells for CD4+ immune responses. This may have clinical relevance, as will be discussed later.

Summary of Tumor-reactive CD4+ T-Cell Responses. We have evaluated the CD4+ cellular responses in VPLNs obtained from eight patients to date. As summarized in Table 1, these patients included individuals diagnosed with melanoma, RCC, or head and neck SCCs. For purposes of this study, we evaluated whether there was evidence of tumor-reactive CD4+ responses within the VPLN using our method of anti-CD3/anti-CD28 activation. A total of 23 independent cultures were set up among these eight patients (range, 2–5 cultures per patient). Among the eight patients, four manifested CD4+ T-cell tumor-reactive responses, as indicated by IFN-γ release. Of these four patients, three (patients 1, 6, and 7) had MHC class II molecules expressed on their tumor cells. The one patient (3) who had evidence of CD4+ T cell tumor reactivity and no detectable MHC class II expression by autologous tumor did have inducible class II molecules after culture in IFN-γ. However, the ability to induce MHC class II expression by IFN-γ in class II negative tumors did not ensure the detection of CD4+ T cell tumor reactivity, as noted in two patients (patients 2 and 5).

DISCUSSION

We previously described a protocol to activate and expand tumor-primed T cells by using an anti-CD3/IL-2 culture method for the adoptive immunotherapy of both murine and human tumors (3, 4, 6). In those studies, whole populations of VPLN cells were activated and expanded without prior nylon wool treatment to enrich CD3+ T cells. Cell activation by anti-CD3 alone followed by expansion in IL-2 resulted in efficient cell proliferation without the use of anti-CD28. Because LNs typically consist of 40–60% T cells with the remaining cells being B cells, NK cells, macrophages, dendritic cells, and other cell types, the costimulatory signals needed in addition to anti-CD3 activation may have been provided by the APCs present in the whole VPLN cell preparation. In contrast, the nylon wool enriched T cells in this study (>95% CD3+ as indicated by FACS) proliferated poorly in IL-2-containing medium after activation with anti-CD3 alone due to the lack of costimulatory signals provided by CD28 ligation. One of the unique observations made in this study is that secondary activation of purified VPLN T cells by an anti-CD3/anti-CD28 combination led to expanded tumor-reactive CD4+ cell responses, which were MHC class II restricted.

Our interest in examining the activation of tumor-primed T cells with anti-CD28 was based upon extensive observations
documenting the costimulatory effects of signaling provided by CD28 ligation in a variety of nontumor systems (8, 9, 11, 13–18). Complete activation of T cells depends on an interaction between CD28 and related costimulatory molecules after a specific antigen is delivered to the TCR. In the absence of adequate costimulation, T cells reveal a form of “partial activation,” in which they fail to proliferate, produce lymphokines, or promote B cell blastogenesis (10, 11). Purified murine or human CD4\(^+\) T cells stimulated by anti-CD3 mAb have been shown to die through programmed cell death, whereas costimulation with anti-CD28 protected target cells from apoptosis (13, 14). A recent study demonstrated that CD28 costimulation can support long-term proliferation of CD4\(^+\) cells in the absence of exogenous feeder cells (15). Studies in man and mice have indicated that anti-CD28 antibodies augmented CD3-induced T cell proliferation (18) and that the CD28/B7 pathway costimulates the response of T cells to conventional mitogens as well as superantigens (9). Using T cells derived from VPLN of cancer patients, we have shown that stimulation with an anti-CD3/anti-CD28 antibody combination resulted in significantly enhanced cytokine secretion to tumor antigen(s) compared to stimulation with anti-CD3 alone.

On the basis of our prior animal studies, we have demonstrated that the amount of certain cytokines (i.e., IFN-γ and GM-CSF) released by adoptively transferred effector T cells in response to tumor was directly correlated with their therapeutic efficacy in vivo (19). Hence, we focused our attention on the functional activity of activated VPLN cells by examining cytokine release to autologous tumor targets. In this study, we found that VPLN T cells activated with the combination of anti-CD3/anti-CD28 released significantly higher levels of cytokines in response to tumor antigen compared with the cells activated by anti-CD3 alone. This would suggest that tumor-reactive T cells were preferentially activated by the anti-CD3/anti-CD28 combination compared with anti-CD3 alone. Both type 1 (i.e., IFN-γ) and type 2 (i.e., IL-10, data not shown) cytokine release profiles were found to be up-regulated by activation with the combination of mAb in this report. We have not detected IL-4 release by ELISA assays in the supernatants generated in this study. In a previous report, we showed that type 1 release of cytokines (i.e., IFN-γ and GM-CSF) was associated with in vivo tumor recognition and destruction, whereas type 2 cytokine release (i.e., IL-10) appeared to suppress antitumor reactivity (20). Taken together, our data would indicate that we should be able to preferentially enrich the number of tumor-reactive T cells using the combination of anti-CD3/anti-CD28. However,
the heterogeneous function of these activated T cells with respect to type 1 and type 2 cytokine release needs to be further explored. One approach would be to positively select for T cell subpopulations with type 1-associated tumor reactivity and to expand these lymphoid populations for subsequent adoptive transfer. We have previously demonstrated that tumor-reactivity based upon Vβ restriction can discriminate between type 1 versus type 2 effector cells with disparate in vivo antitumor function in a mouse model (20). We plan to examine whether or not there exists analogous functional differences (i.e., type 1 versus type 2 cytokine secretion) between Vβ subpopulations within human tumor-primed VPLNs in the future.

In this study, we found that the enhanced proliferation of purified CD3+ T cells in the presence of anti-CD28 was associated with an increase in the percentage of CD4+ cells. CD28 ligation was found to provide a costimulatory signal that had a much stronger effect on the proliferation and cytokine secretion of CD4+ cells compared to CD8+ cells. It has been reported that purified CD4+ cells proliferated vigorously in response to anti-CD3/anti-CD28 and that their proliferation was CD8+ cell independent (21). However, the proliferation of purified CD8+ cells under the same activation conditions was critically dependent upon the availability of the supernatants generated by CD4+ cells, indicating that the development of CD8+ cells require CD4+ T-cell help (21–23). A similar phenomenon was observed in our experiments because we found it very difficult to grow purified CD8+ cells alone when activated with anti-CD3/anti-CD28. In some of our experiments in which we were able to expand the CD8+ cells to a limited extent, they demonstrated a very low or absent cytokine release in response to tumor stimulation. These results initially appear to be different from our prior report in which we identified MHC class I restricted antitumor reactivity of VPLNs after activation (6). In those studies, anti-CD3 activation resulted in preferential stimulation of CD8+ T cells. One significant difference in the activation protocol of our previous report involved the use of the whole population of VPLN cells without prior nylon wool treatment to

**Fig. 6** Cytokine secretion of anti-CD3-/anti-CD28-activated VPLN T cells in response to autologous melanoma tumor cells versus histologically matched allogeneic melanoma tumor cells and inhibition of cytokine secretion of anti-CD3/anti-CD28 activated VPLN CD4+ T cells. Blocking antibodies directed against MHC class I, class II, CD8, or CD4 were applied as described in “Materials and Methods.”

**Fig. 7** Cytokine secretion of anti-CD3-/anti-CD28-activated VPLN T cells in response to untreated or IFN-γ preincubated autologous or allogeneic RCC tumor cells. RCC tumor cells were treated by preincubation in IFN-γ at 1000 units/ml for 3 days to up-regulate MHC class II expression before being used as stimulators to T cells.
purify T cells. The activation protocol of the current report used purified T cells that were devoid of APCs. Recent reports have demonstrated that dendritic cells in concert with CD4+ cells are necessary in the induction of antigen-reactive CD8+ T cells (24–27). This explains the relative lack of CD8+ T cells seen with our current culture conditions. In preliminary studies, we have examined the activation of VPLN cells with anti-CD3/anti-CD28 mAbs, without prior nylon wool purification and have found enhanced CD8+ stimulation compared with anti-CD3 alone (data not shown). Hence, the cell components present during the culture period dictate what kind of effector cell is generated.

Our results indicated that the immunological reactivity manifested by CD4+ VPLN cells against tumor-associated antigens was MHC class II restricted. Limited progress has been made in the past several years in the endeavor to identify MHC class II restricted tumor-associated antigens. Potential HLA class II restricted tumor-associated antigens have been described or suggested in melanoma (27–29), sarcoma (30), leukemia (31), and SCC (32). Unlike the well-documented MHC class I-restricted tumor-associated antigens characterized during the past few years, no cDNA or corresponding amino acid sequence has been defined yet from the described potential MHC class II-restricted tumor-associated antigens (36). Limited progress has been made in the past several years in the endeavor to identify MHC class II-restricted tumor-associated antigens. This explains the relative lack of CD8+ T cells seen with our current culture conditions. In preliminary studies, we have examined the activation of VPLN cells with anti-CD3/anti-CD28 mAbs, without prior nylon wool purification and have found enhanced CD8+ stimulation compared with anti-CD3 alone (data not shown). Hence, the cell components present during the culture period dictate what kind of effector cell is generated.

Our results indicated that the immunological reactivity manifested by CD4+ VPLN cells against tumor-associated antigens was MHC class II restricted. Limited progress has been made in the past several years in the endeavor to identify MHC class II-restricted tumor-associated antigens. Potential HLA class II restricted tumor-associated antigens have been described or suggested in melanoma (27–29), sarcoma (30), leukemia (31), and SCC (32). Unlike the well-documented MHC class I-restricted tumor-associated antigens characterized during the past few years, no cDNA or corresponding amino acid sequence has been defined yet from the described potential MHC class II-restricted tumor-associated antigens (28, 33). Although two separate studies have identified tumor-associated antigens expressed on RCCs, they were both MHC class I-restricted antigens (34, 35). The CD4+ T cell responses we described here suggest that MHC class II-restricted tumor-associated antigens are also expressed by RCC, and this represents a unique finding that has not been described previously. The techniques we have described to isolate and expand tumor-reactive CD4+ T cells from VPLNs offers potential opportunities to identify and clone novel class I-restricted tumor-associated antigens.

Our data suggested that the expression of MHC class II on the tumor cells used for priming LNs was associated with the induction of CD4+ T cell tumor reactivity (Table 1). This has clinical relevance because many tumors lack MHC class II expression. In one report, the clinical responses associated with IL-2-containing regimens observed in melanoma patients appeared to be associated with the induction of MHC class II molecules on tumor cells (36). The induction of class II molecules can be achieved by the transfection of tumor cells with IFN genes or by exposure of tumor cells to IFNs. In the therapy of established tumors, this can be accomplished by the exogenous administration of IFN. In animal studies, the exogenous administration of IFN-α in conjunction with IL-2 was shown to have synergistic antitumor effects (37). The potential contribution of CD4+ T cell responses in mediating tumor regression has not been examined closely in these models. We hypothesize that CD4+ T cell tumor reactivity may have clinical utility in cancer immunotherapy.

In summary, we have examined the antitumor reactivity of VPLN T cells after combined anti-CD3/anti-CD28 mAb activation in the absence of APCs. Our culture procedure resulted in the preferential proliferation of CD4+ T cells with tumor reactivity in a subset of patients. Our approach of in vivo priming of LNs followed by isolation and expansion of VPLN T cells may provide cellular reagents useful in the identification of CD4+ T cell reactive, MHC class II restricted tumor-associated antigens. This also raises the prospects of evaluating CD4+ T cells as cellular agents in adoptive immunotherapy. In preclinical animal models, we are evaluating this alternative approach to activating tumor-primed LN cells and comparing their in vivo antitumor reactivity with that obtained using previously described methods with anti-CD3 alone. These studies will help us characterize the relative merits of CD4+ and CD8+ T cells in the adoptive immunotherapy of established tumor.

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