Prolonged Culture of Vaccine-Primed Lymphocytes Results in Decreased Antitumor Killing and Change in Cytokine Secretion

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ABSTRACT

Adoptive transfer of effector T cells has been used successfully to eliminate metastases in animal models. Because antitumor activity depends on the number of effector cells transferred, some human trials have used in vitro-repetitive activation and expansion techniques to increase cell number. We hypothesized that the prolonged culture period might contribute to the lack of human trial success by decreasing the potency of the effector T cells. Lymph nodes draining a progressively growing murine melanoma tumor transduced to secrete granulocyte/macrophage colony-stimulating factor were harvested and activated in vitro with anti-CD3 monoclonal antibody followed by expansion in IL-2 for a total of 5 days in culture. Some lymphocytes were reactivated and further expanded for a total of 9 days in culture. In vivo activity of the effector T cells was measured by the reduction in lung metastases and is shown to be dose dependent. The prolonged culture period resulted in nearly 3-fold more T cells but at least 8-fold less antitumor activity. This was accompanied by decreased secretion of the proinflammatory cytokine, IFN-γ, and increased secretion of the anti-inflammatory cytokine, IL-10. Thus, although increased cell number is important to maximize the effectiveness of adoptive immunotherapy, some culture conditions may actually be counterproductive in that decreases in cell potency can outweigh the benefits of increased cell numbers. The T-cell cytokine secretion pattern predicts decreased effector cell function and may explain the decreased antitumor effect.

INTRODUCTION

Numerous adoptive immunotherapy models have shown that the transfer of effector T lymphocytes into tumor-bearing hosts can mediate the regression of both primary and metastatic tumors (1–5). Lymph nodes draining a progressively growing tumor have been shown to be a particularly effective source of such antitumor T cells (5–12). To become fully functional, harvested lymphocytes need to be stimulated in vitro with an anti-CD3 monoclonal antibody (mAb) and then further matured and expanded with low-dose interleukin-2 (IL-2). These T cells then contain a subpopulation of tumor-reactive effector cells specific for the sensitizing tumor that was originally drained by the harvested lymph nodes. This antitumor activity is dose (or cell number) dependent and will establish long-term immunity in successfully treated animals.

Since these initial observations, however, human clinical trials that use this approach have resulted in less encouraging therapeutic responses (13–17). The limited efficacy of this method of adoptive immunotherapy may be because of (a) a low total number of effector cells relative to tumor burden, (b) decreased sensitivity of human tumors to these effector cells, and/or (c) decreased potentiation or suppression of the human antitumor lymphocytes as opposed to the murine antitumor lymphocytes, which could be related to poor localization, limited survival, and/or decreased functional activity. To address the first concern, some human trials have altered in vitro activation and expansion conditions to increase effector cell number (13, 18–21). However, the effects of these alterations on antitumor activity and cell potency are unknown.

The goals of this study were to examine the antitumor efficacy of tumor vaccine primed lymphocytes (VPLs) that have undergone either a single (5-day ex vivo culture) or double (9-day ex vivo culture) anti-CD3/IL-2 activation protocol. The single activation protocol has been well established in murine studies whereas the double activation culture method has been used in human clinical trials to increase effector cell number (13, 18–21). We showed that the double-activated VPLs lose in vivo therapeutic function in excess of any gains achieved by expansion in cell number. A possible mechanism is suggested as the double activation method produces T cells that secrete more IL-10 and less IFN-γ in response to tumor antigen stimulation than do single-activated VPLs. A high ratio of IFN-γ:IL-10 production has been correlated with improved antitumor activity in this and other model systems (22–26).

MATERIALS AND METHODS

Mice. Female C57BL/6 (B6) mice between 4 and 6 weeks of age were purchased from Charles River Laboratories (Frederick, MD) and maintained in the animal colony at the Cincinnati Veterans Affairs Medical Center Research Facility with free access to food and water. They were used for experiments at the age of 6 to 8 weeks. All protocols were approved by the Cincinnati Veterans Affairs Animal Care and Use Committee, and all mice were treated in accordance with the institutional guidelines for animal care.

Tumor Cells. D5 is a B16-derived murine melanoma cell line chosen for its poor immunogenicity, high metastatic potential and aggressive growth characteristics. It was used for antigenic stimulation during in vitro cytokine release assays and for initiation of pulmonary metastases in vivo. D5-G6 (G6) is a D5 cell line stably transfected to secrete granulocyte/macrophage colony-stimulating factor (27). G6 was used as the tumor vaccine for induction of vaccine primed pre-effector cells. Tumor cell lines were kindly provided by Dr. Alfred E. Chang (University of Michigan, Ann Arbor, MI) and Dr. Suyu Shu (Cleveland Clinic, Cleveland, Ohio). Tumor cell lines were maintained in culture in complete medium (CM, RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 μmol/L sodium pyruvate, 50 μg/mL gentamicin, 1 × 10−5 mol/L 2-mercaptoethanol (Sigma, St. Louis, MO), 100 unit/mL penicillin/100 μg/mL streptomycin/0.25 μg/mL Fungizone mixture, and 0.1 mmol/L nonessential amino acids). Cell lines are adherent and harvested with EDTA/trypsin followed by washing in CM and HBSS before use (all from BioWhitaker, Walkersville, MD, except as noted).

Monoclonal Antibodies and Flow Cytometry, mAbs specific for the CD3ε chain of the murine TCR/CD3 complex (2C11), CD4 (FITC-GK1.5), CD8 (FITC-2.43), CD25 (FITC-7D4), L-selectin (FITC-MEL-14), rat κ-chain (MAR-18.5), and Escherichia coli β-glucosidase (GL117) were produced as ascites in pristane-primed mice and purified and labeled with FITC as described previously (28). Cell surface phenotype analyses were conducted by direct immunofluorescence staining of 1 × 106 cells with 10 μg of FITC-conjugated mAbs. A FACScan or FACS caliber flow microfluorometer (Becton Dickinson, Sunnyvale, CA) was used for flow cytometric analysis. Result-
ant fluorescent profiles are presented as the log of fluorescence intensity versus cell number, gated to remove dead cells as determined by propidium iodide staining.

**Anti-CD3/IL-2 Activation of Tumor Vaccine-Draining Lymph Node Cells.** For clarity, Fig. 1 depicts the schema for the preparation and use of VPLs. B6 mice were inoculated with $1 \times 10^6$ G6, the granulocyte/macrophage colony-stimulating factor producing murine melanoma cell line. The tumor was injected intradermally in 0.1 mL of HBSS in both flanks or flanks and shoulder regions. Eight to 9 days later, the draining vaccine-primed inguinal or inguinal and axillary lymph nodes were harvested, and a single-cell suspension was prepared by mechanical dissociation and filtering through 53 μm nylon mesh fabric (Small Parts Inc., Miami Lakes, FL). $4 \times 10^6$ VPLs/well in 2 mL of CM were activated in 24-well culture plates (Corning Inc., Corning, NY) precoated with 2 μg/mL anti-CD3ε mAb. After 2 days of incubation at 37°C/5% CO₂, activated cells were washed in HBSS and suspended in fresh CM with 24 IU/mL recombinant human IL-2 (a kind gift from Chiron Corporation, Emeryville, CA). Cells were diluted to 1.5 $\times 10^6$ cells/mL of CM in polystyrene culture flasks (Corning Inc.) for 3-day expansion at 37°C/5% CO₂ to yield functional effector cells, termed activated and expanded vaccine-primed lymphocytes. In some experiments, normal splenocytes that were similarly activated and expanded were used as controls. Splenocytes were harvested from tumor-free animals and prepared by the same single suspension method as the VPLs but were also treated with ammonium chloride-potassium bicarbonate lysing buffer to remove red cells. The double activation protocol included a second, 1-day activation with plate-bound anti-CD3 mAb after the 5-day initial activation. The cells were then harvested, washed, and cultured for 3 additional days in 24 IU/mL IL-2 to increase overall cell number.

**In vitro Purification of Vaccine Primed Lymphocyte Subpopulations by Magnetic Beads.** In some experiments, VPLs were fractionated into two subpopulations based on their expression of L-selectin (CD62L). L-Selectin–negative cells were purified by negative selection with L-selectin mAb-conjugated magnetic beads (Miltenyi Biotec, Auburn, CA). VPLs were suspended in fresh CM at $1 \times 10^6$ cells/mL and incubated with 15 μL of L-selectin mAb-conjugated magnetic beads/mL at 4°C for 25 minutes. After washing, magnetic bead-labeled cells were passed over a magnetic depletion column that had been placed within a VarioMACS magnetic separator or autoMACS magnetic separator (Miltenyi Biotec) according to the manufacturer’s instructions. Nonadherent cells were washed from the column to yield the L-selectin–negative cell fraction. Adherent cells were then eluted by removing the depletion column from the magnetic field to yield the L-selectin–positive cell fraction.

**Adoptive Immunotherapy.** Naïve B6 mice were inoculated with $2.5 \times 10^5$ D5 tumor cells intravenously in 0.5 mL of HBSS to establish pulmonary metastases. To allow for establishment of micrometastases, mice were inoculated with the tumor 3 days before receiving the activated and expanded VPLs, also by tail vein injection. Mice also received $3 \times 10^6$ IU recombinant human IL-2 intraperitoneally in 0.5 mL of HBSS beginning the day of adoptive cellular transfer and twice daily thereafter for a total of 6 doses. Approximately 17 to 18 days post-tumor inoculation, mice were randomized, killed, and their lungs insufflated with formalin solution and harvested for enumeration of black pulmonary metastatic nodules. Metastatic foci too numerous to count were assigned an arbitrary value of >250 because these lungs were so saturated with tumor that individual metastases could not be resolved. Each group consisted of five mice, and no animal was excluded from statistical analysis.

**In vitro Cytokine Release Assay.** After activation and expansion, $1 \times 10^5$ or $10^6$ VPLs were restimulated with $2 \times 10^5$ D5 tumor cells or on anti-CD3ε mAb-coated plates respectively in CM with 24 IU/mL IL-2 at 37°C/5% CO₂. After 2 days, culture supernatants were aspirated from cultures with D5 tumor, or after 1 day from cultures with anti-CD3 and stored at $-70°C$ for cytokine measurements in triplicate by ELISA. Cytokine concentrations were determined by the use of standard curve regression analysis.

**Statistical Analysis.** The significance of the differences in pulmonary metastases between groups of the in vivo adoptive immunotherapy assay was analyzed by the nonparametric Wilcoxon’s rank-sum test. Differences in cytokine secretion levels between cell populations were analyzed by the Student’s $t$ test. A two-sided $\alpha$ value of $P < 0.05$ was considered significant. Regression curves were generated by Sigmaplot (SPSS Inc., Chicago, IL).

**RESULTS**

**Antitumor Activity Is Cell Dose Dependent.** The antitumor activity is cell dose dependent. Approximately $10^8$ unfractionated effector cells resulted in eradication of all pulmonary metastases (Fig. 2). Ten-fold fewer cells had minimal effect. The L-selectin–negative fraction has been shown to contain the active subpopulation of effector T-cells (29). L-Selectin mediates leukocyte rolling along vascular endothelium at sites of inflammation and attachment to endothelium within peripheral lymphoid tissue. Lack of L-selectin expression is a marker for recently activated T cells such as those in the draining lymph node exposed recently to a tumor vaccine (30–33). Approximately half as many L-selectin–negative 5-day activated and expanded VPLs are needed to achieve 50% reduction in the number of pulmonary metastases, compared with unfractionated effectors. Similarly, the dose-response curve is rather sharp with loss of activity when 10-fold fewer cells are administered.

Figure 2 (inset) depicts the lungs of representative mice that had received either $10^8$ unfractionated 5-day activated and expanded VPLs (bottom half of figure) or $10^5$ unfractionated and similarly activated and expanded normal splenocytes (top half of figure). The mean number (SEM) of enumerated pulmonary metastases in this representative experiment were $9 (6)$ versus $162 (26)$, $P < 0.01$ for VPLs versus splenocyte controls, respectively. The results of IL-2 treatment alone without cell adoptive transfer was not significantly different from splenocyte transfer (data not shown).

**Double Activation and Prolonged Culture Yields Greater Cell Numbers for Adoptive Transfer.** Because antitumor activity is cell number dependent, investigators have sought to maximize cell numbers for clinical trials. One method to further expand VPLs is to restimulate the VPLs after initial activation and expansion with immobilized anti-CD3 and subsequently culture the effectors with...
L-Selectin–Negative Vaccine Primed Lymphocytes after Double Activation Exhibit Significantly Decreased In vivo Antitumor Efficacy. Double activation decreased per cell potency by a factor of 8 (Table 1). Although $1 \times 10^7$ single-activated L-selectin–negative cells reduced the number of pulmonary metastases to approximately half (mean of 146 pulmonary nodules, experiment 1), it took eight times as many double-activated cells to achieve a similar tumor reduction. Single and double-activated effectors (group A and B cells, respectively) originated from the same set of lymph nodes and were transferred into different cohorts of mice with 3-day lung metastases because the cultured periods were either 5 days or 9 days in length. As an additional control, single-activated VPL cells (group C) were cultured from a different set of lymph nodes initiated 4 days after group A and B lymph nodes and transferred into mice with 3-day lung metastases established at the same time as the animals receiving double-activated effectors (group B). There was no significant difference between the single-activated and expanded VPLs from groups A and C. Similar results were seen in experiment 2 with again at least 8-fold decrease in per cell potency.

**Double Activation of L-Selectin–Negative Vaccine Primed Lymphocytes Switches T-Cell Cytokine Secretion Profile.** We hypothesized that the prolonged culture period may have altered the cytokine phenotype of the effector cells in a way that might explain the decreased antitumor activity seen above. Therefore, single-activated VPLs were compared with double-activated VPLs for the amount and type of cytokines secreted in response to tumor and anti-CD3 stimulation in vitro.

Double activation resulted in VPLs that secreted less IFN-γ and more IL-10 than single cycle VPLs when exposed to D5 tumor (Fig. 4A). IFN-γ decreased 2.9 ± 1.4-fold whereas IL-10 increased 4.2 ± 0.7-fold in an average of four independent experiments. This inversed ratio of IFN-γ:IL-10 production was also seen in response to nonspecific anti-CD3 stimulation by immobilized antibody (Fig. 4B). Minimal background levels of IFN-γ and IL-10 were produced by VPLs alone and tumor alone and were subtracted from VPLs plus tumor levels. Others have shown the specificity of cytokine production of VPLs toward the tumor to which the lymphocytes were

### Table 1 Reduction in pulmonary metastases by adoptive transfer of vaccine primed lymphocytes

<table>
<thead>
<tr>
<th>Activation protocol</th>
<th>No. of cells transferred</th>
<th>Mean no. of pulmonary metastases (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
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<tr>
<td>A, single activation</td>
<td>0</td>
<td>$&gt;250$</td>
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<tr>
<td></td>
<td>$1 \times 10^7$</td>
<td>146 (11)*</td>
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<tr>
<td></td>
<td>$4 \times 10^7$</td>
<td>0 (0.2)*,†</td>
</tr>
<tr>
<td>B, double activation</td>
<td>0</td>
<td>$&gt;250$</td>
</tr>
<tr>
<td></td>
<td>$1 \times 10^7$</td>
<td>$&gt;250$†,‡</td>
</tr>
<tr>
<td></td>
<td>$4 \times 10^7$</td>
<td>155 (20)*,‡</td>
</tr>
<tr>
<td>C, single activation</td>
<td>(delayed vaccine control)</td>
<td>$1 \times 10^7$</td>
</tr>
<tr>
<td></td>
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<td>$4 \times 10^7$</td>
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* Significantly different from groups that did not receive cells.
† Significantly different from single activation $1 \times 10^7$ groups.
‡ Significantly different from single activation $4 \times 10^7$ groups.

NOTE. Indicated number of single and double L-selectin–negative activated effector cells adoptively transferred into mice bearing 3-day–established pulmonary metastases. Group A and B cells originated from the same set of lymph nodes and were transferred into different cohorts of mice with 3-day lung metastases because the cultured periods were either 5 or 9 days in length, respectively. As an additional control, single activation VPL cells (group C) were cultured from a different set of lymph nodes initiated 4 days after group A and B lymph nodes and transferred into mice with 3-day lung metastases established at the same time as the animals receiving double-activated effectors (group B). Number of metastases depicted as mean ± SE.

**Fig. 2.** Dose-response curve relating the number of VPLs adoptively transferred intravenously to their effectiveness in eliminating 3-day–established pulmonary metastases of the parental tumor, D5. Number of metastases depicted as mean ± SE, representative of multiple experiments. Closed circles are unfractionated effector cells; open circles are L-selectin–negative subpopulation. Curves are best-fit 4 parameter logistic regression of representative lungs of representative of multiple experiments.

**Fig. 3.** Representative experiment depicting cell numbers at the different stages of activation and expansion during the generation of VPLs.

more IL-2 for an additional 3 days. For the purposes of this report, this is termed double activation. Double activation resulted in nearly 3-fold more cells than single activation and expansion. There was some cell loss during the activation steps that was more pronounced during the second activation period, but cell expansion during the second IL-2 culture period was significantly increased (Fig. 3). If effector cell potency were unchanged, one would expect to see significantly improved tumor reduction in the double activation group as compared with single activation as more cells could be transferred relative to the same starting number of lymph nodes harvested.
sensitized (23). IL-4 was not detectable in the supernatant of any cell group tested with tumor stimulation. IL-5 was detectable at low amounts (100–200 pg/mL) but was seen with or without D5 tumor stimulation at the same levels and was therefore not specific to tumor stimulation (data not shown).

Double Activation of L-Selectin–Negative Vaccine Primed Lymphocytes Results in a Loss of CD4+ Cells and Increase in CD25+ Expression. Single activation resulted in a population of effector T cells that are all CD3+ (308), and the L-selectin–positive cells represent approximately 75% of the total unfractionated VPLs.
After L-selectin–negative separation, the VPLs remain CD3 activity and limited cytokine secretion in response to tumor (34). After L-selectin–negative separation, the VPLs remain CD3−, and most of these cells are CD8+, but approximately 5% CD4+ cells are observed (Fig. 5). After double activation, although the L-selectin–negative phenotype was maintained, the CD4+ cell population was lost. Others have shown CD4+ cells provide T-cell help but can be eliminated as long as IL-2 is given along with the adoptive transfer as was done in these experiments (35–37). Double activation also increases the number of IL-2 low-affinity receptor (CD25+) expressing cells from 52 to 66%. This likely represents an increase in cell surface expression because of prolonged culture with low-dose IL-2 as elimination of CD25+ cells after adoptive transfer also reduces antitumor activity (Fig. 6). This is opposite to what one would expect if the CD25+ cells represented a suppressive regulatory T-cell population.

DISCUSSION

From lymphokine-activated killer cells to tumor-infiltrating lymphocytes to VPLs, cellular therapy has been exploited in animal systems and with various degrees of success in human clinical trials (2, 17, 38). Its attractiveness lies in the potential for high specificity, low incidence of adverse effects, and perhaps most importantly the hope that the self-sustaining nature of such therapies may lead to a complete and durable response.

Previous studies have shown the existence of a population of immune cells within tumor-draining lymph nodes that differentiate into tumor antigen-specific effector cells with an associated down-regulation of cell surface L-selectin expression (29, 39). After in vitro activation by sequential treatment with anti-CD3 mAb and IL-2, these cells are able to mediate regression of established tumors when systemically administered to tumor-bearing animals. These findings are congruent with the in vivo function of L-selectin as a homing receptor capable of recirculating immune cells into peripheral lymphoid tissues by interaction with activated endothelium. Thus, correlation between down-regulation of L-selectin and increased antitumor efficacy in adoptive immunotherapy models may, at least in part, be a function of activated immune cells becoming capable of leaving lymphoid structures and localizing in tumor deposits (40). Indeed, recent evidence in a similar adoptive immunotherapy model indicates that down-regulation of L-selectin on tumor-reactive T cells is essential for tumor infiltration (41). However, subsequent tumor regression is dependent on immunologically specific interactions between transferred VPLs and the host tumor.

One mechanism that may account for tumor destruction is direct lytic action by the transferred cells against the tumor cells. However, classical in vitro cytotoxicity has not been shown against tumor cell targets by the activated VPLs and the exact mechanism of tumor destruction has not been well-defined (42–45). This has limited in vitro determinations of VPLs effectiveness. Previous studies have shown a correlation between cytokine profiles secreted by tumor-reactive T cells and in vivo therapeutic response (13, 22–24, 36, 46–58). Elevated IFN-γ and decreased IL-10 production correlates with improved in vivo function. Our study is consistent with those findings.

Additional evidence that tumor elimination is mediated by type 1 inflammatory cytokotmics includes studies showing (a) IL-12, TNF-α, IFN-γ, IL-12R, and IL-2R mRNA transcripts associated with tumor-infiltrating lymphocytes within regressing tumors (46, 49, 59); (b) increased circulating levels of IFN-γ and IL-4 in patients with progressive metastatic disease (60–63); and (c) that in vitro IL-4 or IL-10 treatment of type-1 antitumor cells reduced their IFN-γ, TNF-α, and IL-12 production, increased IL-4, IL-5, and IL-10 production and reduced antitumor activity whereas in vitro exposure to IL-1, TNF, or IL-12 increased antitumor activity (24, 64–70). An increase in the IL-10 production seen with prolonged culture would also be congruent with an increase in regulatory cells (71).

A major impediment to human clinical adoptive transfer is the need for large numbers of effector cells. The elimination of pulmonary metastases requires approximately 10^8 VPLs for a 20-g mouse in our model. This D5 line was purposely chosen for its lack of immunogenicity and rapid growth characteristics. Other tumor model systems are more sensitive to immune destruction and require fewer adoptively transferred cells. On a per kilogram basis for an average weight human however, this would translate to 3,500 times as many cells or approximately 3.5 x 10^11, a formidable number of effector cells to generate. To assist in achieving such large quantities of effector cells for human trials, investigators have used more prolonged in vitro culture techniques to expand VPLs to large numbers (13, 18–21). However, lymphodepletion of the host before cell transfer has been shown recently to also greatly reduce the number of cells needed for success (72, 73). Other protocol modifications may also be needed to limit the number of effector cells required for successful human application.

Although there are obvious limitations to extrapolating murine studies to human trials, we have shown that repeat activation and expansion of VPLs causes a significant loss in antitumor function that we have correlated with decreased IFN-γ and increased IL-10 secretion. It remains to be determined whether a similar change occurs with human double activation adoptive immunotherapy protocols.

Other methods to expand VPLs cell number without loss of activity may be possible. However, we were unable to maintain a type-1 phenotype and increased potency of the double-activated T-cells with addition of IL-12 or anti-IL-10/anti IL-10 receptor/anti-IL-4 mAbs. Higher doses of IL-2 or longer expansion time in IL-2 culture without a second exposure to anti-CD3 activation also increased cell number but decreased cell potency (data not shown). IL-7 and IL-15 have been used in other systems to promote naive and memory CD8+ cell phenotype (74–80). These cytokines alone or in combination with IL-2 could be useful to prevent the functional antitumor loss of these VPLs in culture and should be the subject to additional study.

We have shown a loss in the CD4 lymphocytes in the prolonged

Fig. 6. Number of pulmonary metastases enumerated from mice treated with single-activated VPLs with and without administration of anti-CD25 monoclonal antibody. +, group receiving VPLs without anti-CD25 significantly different from others.

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Our unpublished observations.
culture protocol. Whether the CD4 subpopulation of cells is important for maintenance of tumor cell reactivity during expansion in vitro is not fully delineated. We have depleted single-activated effectors of CD4+ cells, and they are equally effective as the whole population in our system (data not shown). Others have also shown CD4 cells not to be required for in vivo function (36, 37); however, their presence may be more important in the described longer double activation culture system. Alternatively, the CD4+ subpopulation decrease seen may have no impact on antitumor function but rather be a coincidental result of the longer culture period. Others have indeed shown that longer-term culture of VPLs increases the predominance of CD8+ cells in the resulting T-cell populations (81).

Investigators have shown CD25-positive cells to be associated with T-cell regulatory function in patients immunized against melanoma antigens (82). Although others have shown prolonged vaccination leads to regulatory L-selectin+–positive cells but without association with CD25 (IL-2 low-affinity receptor) expression (83) or have shown generation of regulatory T cells by ex vivo culture with IL-2 and transforming growth factor-β (84). The increase we have seen in CD25-positive subpopulations may explain the decreased function of the double-activated cells as well. In fact, depletion of the CD25+ cells before tumor vaccination leads to increased potency of the subsequently harvested and activated VPLs (85, 86). However, in our laboratory, depletion of the CD25+ cells after adoptive transfer does not improve antitumor efficacy as is shown in Fig. 6. The increased CD25-positive population may simply represent the selective pressure of prolonged culture with low-dose IL-2. The transcription factor, Foxp3, which is associated with regulatory T cells, may help to better delineate regulatory T-cell populations in the future (87, 88).

Future human trials may need to measure proinflammatory/anti-inflammatory cytokine secretion in response to in vitro stimulation to optimize antitumor cell potency as well as cell expansion. If the mechanisms of tumor cell killing by these cells were better understood, then in vitro optimization of effector cell generation and expansion might be better achieved. Abbreviated cell culture protocols may be optimal and would also decrease media and reagent expense as well as decrease the risk of bacterial and fungal contamination.

In summary, we have shown that secondary activation of tumor vaccine-primed lymphocytes via a second round of sequential anti-CD3/IL-2 treatment results in increased expansion of T cells with significantly reduced in vivo antitumor efficacy. Furthermore, these cells show a shift away from a proinflammatory to an anti-inflammatory cytokine phenotype. Thus, these findings may provide some guidance for future studies to improve the generation of potent, tumor-reactive T cells for human use.

**REFERENCES**


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