Comparative Analysis of Regulatory and Effector T Cells in Progressively Growing versus Rejecting Tumors of Similar Origins

Jack D. Bui,1 Ravindra Uppaluri,2 Chyi-Song Hsieh,3 and Robert D. Schreiber1

1Center for Immunology, Department of Pathology and Immunology, 2Department of Otolaryngology, and 3Division of Rheumatology, Washington University School of Medicine, St. Louis, Missouri

Abstract

Although regulatory T cells (Tregs) have been detected in clinically apparent and experimentally induced tumors, the significance of their presence is obscured because past studies examined late-stage tumors that had formed in immunocompetent hosts and thus had evolved mechanisms to escape immunologic recognition and/or elimination. Herein, we report the first comparative analysis of the antitumor response to 3’-methylcholanthrene–induced tumors, which either grow progressively (progressor tumors) or are rejected by the immune system (regressor tumors). Surprisingly, we found that both progressor and regressor tumors harbored proliferating (i.e., activated) Foxp3+CD25+Tregs. However, progressor tumors contained a higher percentage of Tregs in the lymphocyte subset versus regressor tumors. The Tregs in progressor tumors were derived from peripheral CD25+ natural Tregs, accumulated early after tumor challenge and were actively proliferating, suggesting that progressor tumors recruited and/or activated endogenous Tregs as a mechanism of escaping immune destruction. To explore whether Tregs directly contributed to the progressive growth phenotype of progressor tumors, we monitored tumor outgrowth in naive wild-type recipients pretreated with either a control monoclonal antibody (mAb) or a depleting CD25-specific mAb. In mice predepleted of CD25+ cells, the tumors that subsequently developed displayed an increased accumulation of proliferating CD8+ T cells and were rejected. These results show that, although Tregs are activated in both regressor and progressor tumors, the ratio of regulatory to effector T cells is critical in determining whether the host successfully rejects the tumor or eventually succumbs to tumor outgrowth. (Cancer Res 2006; 66(14): 7301-9)

Introduction

Extensive studies in mice have shown that the immune system plays an important role not only in detecting and eliminating carcinogen-induced and some spontaneous cancers but also in promoting tumor growth (1–5). We have named this process cancer immunoediting (3, 6–8). Using rodent models, our group and others have identified many of the molecular and cellular mechanisms of escaping immune control (7, 8, 24). Even more important, these studies used tumor cells derived from immunocompetent hosts that presumably had already developed mechanisms of escaping immune control (7, 8, 24). Nevertheless, the emergence of a clinically apparent tumor is evidence that endogenous tumor surveillance mechanisms have failed (24). The cancer immunoediting hypothesis suggests that, because of its interaction with the immune system, a tumor may either be altered in a way so that it escapes immune recognition or may escape immune destruction by actively suppressing the antitumor immune response (3, 7). Over the past 10 years, a subset of T cells, which exert immunosuppressive function, has been characterized (25–27) and was shown not only to prevent autoimmunity but also to inhibit immune responses against developing tumors. These cells are known as regulatory T cells (Tregs) and are a lineage of CD4+ T cells that express the surface molecule CD25 and the transcription factor Foxp3. Tregs suppress T-cell proliferation and cytokine secretion via contact-dependent and cytokine-mediated mechanisms.

In mice, the participation of Tregs in promoting tumor escape from immune control was inferred from the observation that pretreatment of mice with a single dose of the CD25-specific, depleting PC61 monoclonal antibody (mAb) before tumor transplantation converted the growth phenotype of some but not all tumors from progressor to regressor (28–31). Furthermore, the combination of PC61 pretreatment and subsequent vaccination against a tumor led to enhanced protection of mice to tumor challenge (32, 33). However, these experiments used tumor cells derived from immunocompetent hosts that presumably had already developed mechanisms of escaping immune control (7, 8, 24). Even more importantly, these studies did not explore whether all tumors, even those which eventually regress, might evoke a natural Treg...
response due to expression of endogenous self-antigens (25). In humans, a causal role for Tregs in tumor escape has yet to be clearly established (reviewed in refs. 24, 25, 27). Thus, the issue of whether Tregs function as a physiologically relevant mechanism of tumor escape remains unresolved.

We previously reported the development of a unique mouse model, in which similar tumors arising in immunocompetent versus immunodeficient mice display reproducibly different growth behaviors when transplanted into naive, syngeneic wild-type (WT) recipients (3). 3’-Methylcholanthrene (MCA) sarcomas from immunodeficient 129/Sv strain RAG2−/− mice, generated in the absence of adaptive immunity (i.e., unedited sarcomas), display high immunogenicity, and many are rejected when transplanted into naive, syngeneic WT hosts. For this reason, these tumors are called regressor tumors. In contrast, MCA sarcomas derived from 129/Sv strain WT mice (progressor tumors) are able to grow when transferred into naive recipients presumably because they arose by evading cancer immunosurveillance. In the current report, we present the first in-depth comparison of the antitumor response to these regressor and progressor tumor cell lines, placing special attention on the presence and/or action of Tregs in the developing tumor. Our results document that one factor regulating the regressor versus progressor phenotypes of MCA sarcomas is their differential capacity to recruit and/or activate regulatory versus effector T cells.

Materials and Methods

All experiments involving animals were approved by the Washington University Animal Studies Committee (St. Louis, MO).

Tumor cell lines. Regressor and progressor MCA sarcoma cell lines were derived from 129/Sv RAG2−/− or WT mice by injecting MCA (Sigma, St. Louis, MO) as described (3). The growth characteristics have been described elsewhere (3, 8). All the sarcomas grow with similar kinetics in vitro and following s.c. injection of 1 × 105 cells into RAG2−/− mice. When injected into WT mice (1 × 105 s.c.), regressor tumors grow to 5 to 10 mm (average diameter) and then are rejected 8 to 10 days after tumor transplant. Progressor tumors all reach 20 mm by 20 to 30 days after tumor transplant. Progressor tumors all reach 20 mm by 20 to 30 days after tumor transplant. After which time the mice are sacrificed. Cell lines were maintained in RPMI 1640 (Cambrex, East Rutherford, NJ) supplemented with 10% FCS (HyClone, Logan, UT) as described previously. The BALB/c-derived fibrosarcoma cell line CMS-5 was obtained from Dr. Hiroshi Shiku (MIE University School of Medicine, MIE, Japan; ref. 34).

Mice, 129/Sv strain or (129/Sv × C57BL/6) F1 mice were purchased from Taconic Farms (Germantown, NY). BALB/c (Thyl.2) and BALB/c-scid (Thyl.2) mice were from the National Cancer Institute (Bethesda, MD). BALB/c (Thyl.1) mice were a gift from Paul Allen (Washington University, St. Louis, MO).

Antibodies and fluorescence-activated cell sorting analysis. CD45-FITC, CD4-APC, CD8-APC, CD25-PE (PC61 clone), CD25-FITC (7D4 clone), B220-PE, CD44-FITC, CD62L-PE, MAC1-APC, F4/80-PE, CD69-FITC, purified anti-CD16/32, and the bromodeoxyuridine (BrdUrd) staining kit were from BD PharMingen (San Diego, CA). The Foxp3 staining kit was from eBioscience (San Diego, CA). Staining was conducted for 15 to 20 minutes at 4°C in fluorescence-activated cell sorting (FACS) tubes containing 1 to 2 million total cells, 0.5 to 1 µl antibody, 1 µl Fc block (anti-CD16/32), and 100 µl staining buffer [PBS with 1% FCS and 0.05% NaN3 (Sigma)]. Propidium iodide (PI) was from Sigma and was added at 1 µg/ml immediately before FACS analysis. For quantitative analysis of tumor-infiltrating lymphocytes (TILs) and lymph node populations, a CD45/PI- gate was used. For Foxp3 and BrdUrd analysis of lymph node and TILs, small lymphocytes were analyzed base on a forward and side scatter gate rather that CD45 and PI because the cells had to be permeabilized. Gated events (10,000) were collected on a FACS Calibur and analyzed using Flojo software. The mAb PC61 was generated from purified supernatant of the PC61 hybridoma obtained from the American Type Culture Collection (Manassas, VA). Control rat IgG was obtained from Sigma.

PC61 depletion. Four days before tumor transplant, mice were injected i.p. with a single dose of 250 µg of purified PC61 or control Rat IgG. PC61 treatment led to a 70% depletion of CD25+ cells as detected by flow cytometry using the 7D4 CD25-specific mAb that recognizes an epitope on CD25 separate from that recognized by PC61 (data not shown). After 7 days, an increase in 7D4-staining cells could be detected in the thymus of nontumor-bearing mice, suggesting that the depletion was transient, although full recovery of CD25+ cells was not evident even at day 14 (data not shown).

Tumor transplantation. Tumor cell lines were washed thrice with PBS and resuspended at 5 × 10^6/mL. Recipient 129/Sv strain or (129/Sv × C57BL/6) F1 strain mice were either pretreated with a single i.p. injection of 250 µg PC61 or control rat IgG at day −4 or used untreated and then were injected with 1 × 10^6 tumor cells in 0.2 mL s.c. along the flank on day 0. The mice were monitored for tumor growth by measurement of mean tumor diameter, defined as the average of the two maximum dimensions of the tumor mass. Some mice also received a single injection of 100 µg BrdUrd (Sigma) i.p. and subsequently were maintained on water containing 0.8 mg/mL BrdUrd and 1% sucrose, which was changed daily.

Tumor, draining lymph node, and nondraining lymph node harvest. On various days after transplant, tumor was excised from mice, minced, and treated with 1 mg/mL type IV collagenase (Sigma) in HBSS for 2 hours at room temperature. The ipsilateral inguinal (tumor draining) or contralateral (nondraining) lymph nodes were also harvested, and single-cell suspensions were made by crushing the lymph nodes between two glass slides. All cell suspensions were vigorously resuspended, washed in FACS staining buffer, and filtered before staining.

Foxp3 real-time PCR. The tumor cell suspension was incubated in a Petri dish at 37°C for 2 hours to remove tumor cells and macrophages. The lymph node cell suspensions were incubated with anti-B220 Dynabeads (DynaBiotec, Brown Deer, WI) to deplete B cells using a magnet (DynaBiotec). Partially purified cell suspensions were stained with CD4-APC and CD25-PE and sorted on a FACS Vantage. RNA was prepared from sorted cells using the RNA-Beet protocol (Tel-Test, Friendswood, TX). cDNA was made using the Applied Biosystems (Branchburg, NJ) protocol. Real-time PCRs were done using the following primers: Foxp3-1, GGGCCTTTCAGGACACAGA; Foxp3-2, GCTGATCATGGCCTGGGTGTG; and Foxp3-probe, 6FAMACCTCATGCATCGCTTCACTGTTGATTAMRA (35, 36). CD3 primers were ordered through Assays-on-Demand (Applied Biosystems) and used according to the manufacturer’s instructions.

Adaptive transfer. Lymph nodes and spleens from BALB/c mice were homogenized and pooled. For CD25-depleted cells, Thy1.1 mice were used. Cells were stained with CD25-PE, incubated with anti-PE magnetic-activated cell sorting (MACS) beads (Miltenyi Biotech, Auburn, CA), and loaded onto a MACS MS column (Miltenyi Biotech). The flow through was collected and analyzed by FACS. The population was >99% CD25 negative. For CD25-enriched cells, Thy1.2 mice were used. Cells were magnetically depleted using B220 and CD8 Dynabeads, stained with CD25-PE, incubated with anti-PE MACS beads, and loaded onto a MACS MS column. The bound cells were eluted and consisted of >85% CD4+CD25+ cells. The purified cells were mixed and injected i.v. into BALB/c-scid mice. The ratio of the transferred CD4+CD25+ Thy1.1 to CD4+CD25+Thy1.2 cells was 10:1.

Results

Progressor tumors contain fewer CD45+ cells but a higher percentage of CD4+CD25+ cells than regressor tumors. Using a modification of a FACS-based assay described previously (37–40), TILs could be discerned from tumor cells and dead cells based on the high expression of CD45 and exclusion of PI (Fig. 1A, bottom, left). Further analysis of cells within the TIL gate revealed the presence of lymphocyte populations that expressed T and B lineage markers with similar staining intensity to that of lymph node lymphocytes, although the distribution of cells within the TIL population differed significantly from lymph node cells (Fig. 1A, middle and right).
Using the gates defined in Fig. 1A, we found that the percentage of CD45+ TILs was higher in regressor than progressor tumors (Fig. 1B, left). When the CD45+ cells were stained for other immune cell markers, the enhanced content of hematopoietic cells in regressor tumors correlated with a decrease in the percentage of CD4+ cells that coexpressed CD25 (Fig. 1B, right). On the other hand, the percentage of total CD4+ and CD8+ cells within the CD45+ population did not correlate with the growth phenotype of the tumor (Fig. 1C, left and right). These results suggest that the ratio of CD4+CD25+ cells to other lymphocyte subsets within the tumor is a reliable indicator of successful versus unsuccessful antitumor rejection responses.

The CD4+CD25+ cells that infiltrate progressor and regressor tumors express Foxp3. Because CD25 can be expressed on either activated helper cells or Tregs, we next determined whether the CD25+ cells that infiltrated the tumors expressed the transcription factor Foxp3, a marker that is pathognomonic of the Treg lineage (26). We therefore stained TILs or draining lymph node cells for intracellular Foxp3. Anti-Foxp3 stained a subset of CD4+ cells, which did not stain with control IgG (Fig. 2A). The subset of Foxp3+ cells was also identified by anti-CD25 and was at a higher percentage in progressor WT-P1 than in regressor RAG2-R1 CD4+ TILs (Fig. 2B), confirming the findings in Fig. 1B (right). When CD4+CD25+ cells from either progressor or regressor TILs were

**Figure 1.** Increased CD45+ and decreased CD25+ cell percentages in regressor versus progressor tumors. A, one million WT-P1 progressor sarcoma cells were transplanted into syngeneic 129/Sv mice and harvested on day 8. FACS analysis of the draining lymph node and tumor mass. B and C, four regressor and three progressor tumors were each transplanted into groups of 3 to 10 syngeneic mice and harvested on day 8. B, left, percentage of CD45+ cells within the tumor mass, calculated by using the following formula: % 100 × [TIL gate / (TIL gate + tumor gate)]; right, percentage of CD4+CD25+ cells within the CD4 total population. C, percentage CD4+ or CD8+ cells within the CD45+ population. Symbol, one mouse.
sorted, the expression of Foxp3 (as assessed by real-time PCR) was similar to that of endogenous Tregs isolated from naive animals (Fig. 2C). Thus, the CD25+ cells that infiltrate progressor and regressor tumors express amounts of Foxp3 similar to endogenous Tregs. Our results are consistent with the findings in patients that increased percentages of Foxp3-expressing cells in a tumor mass are indicators of poor prognosis (21, 22).

**Predepletion of CD25+ cells by PC61 leads to rejection of some progressor tumors and a concomitant increase in CD45+ and CD8+ cells within the TILs.** To determine whether the CD25+ cells that accumulated within progressor tumors were causally related to tumor progression, we used the CD25-specific PC61 mAb to deplete these cells before tumor challenge. CD25+ cells were substantially reduced in TIL, draining lymph node, and non-draining lymph node cell populations in mice that were pretreated with PC61 on day –4, challenged with progressor tumor cells on day 0, and analyzed on days +6, +8, +10, and +14 (Fig. 3A). The magnitude of the reduction was consistently greater in the nondraining lymph node than the draining lymph node and tumor tissue. The depletion lasted at least 2 weeks after PC61 administration (10 days after tumor transplant), as evidenced by the low percentage of CD25-staining cells in the nondraining lymph node at day 10 compared with cells from control rat Ig–treated mice (Fig. 3A, right).

When two of the progressor cell lines examined in Fig. 1 (WT-P1 and WT-P2) were transplanted into mice pretreated with PC61, they were rejected (Fig. 3B). Both cell lines grew progressively for 10 days in WT mice pretreated with PC61 but then regressed during the 2nd week. In contrast, these tumor cells grew progressively in WT mice pretreated with control rat Ig.

---

**Figure 2. CD4+CD25+ cells within the tumor express Foxp3.** A, WT-P1 progressor tumor cells were transplanted into syngeneic mice, and at day 8 after transplant draining lymph node and tumor were harvested and stained with control IgG or anti-Foxp3. Cells within a small lymphocyte gate based on forward and side scatter. B, regressor or progressor tumor cells (1 × 10^6) from RAG-R1 or WT-P1 tumors were transplanted into syngeneic mice, and tumor was harvested at day 8 after transplant. CD4+ TIL gate based on forward and side scatter. Numbers, percentage of CD25+Foxp3+ cells of total CD4+ TILs. C, CD4+ cells were sorted into CD25+ and CD25− populations from tumor or naive lymph node (LN), and Foxp3 transcript levels were quantitated by real-time PCR.
We next examined the effect of depleting CD25+ cells on the kinetics of accumulation of different immune cells in the tumor. As shown in Fig. 3C (left), PC61 pretreatment led to a maintenance and even slight increase of CD45+ cells in the tumor over the 14-day observation period. In contrast, CD45+ TILs decreased in control Ig–treated mice. By day 14, a 1.6-fold difference in TILs was observed ($P = 0.0079$). Concomitantly, PC61 pretreatment caused a 2.7-fold increase in CD8+ cell percentages in the tumor compared with control Ig pretreatment ($P = 0.028$; Fig. 3C, middle) but did not affect the percentage of CD4+ cells within the TIL population (Fig. 3C, right). The selective CD8+ T-cell increase within the tumor coincided with tumor regression, suggesting that Tregs limited the participation of CD8+ T cells in controlling tumor growth.

Because other groups have shown a function for Tregs in the lymph node, we also examined draining lymph node and nondraining lymph node cells of tumor-bearing mice pretreated with PC61 or control rat Ig. We did not find differences in the percentages of CD25+ cells within the CD4+ subset in the draining lymph node versus nondraining lymph node of tumor-bearing mice during the time course of our experiment (Fig. 3A, middle). Furthermore, no change in the composition of the major T-cell subsets was observed in PC61 versus rat Ig pretreatment in the draining and nondraining lymph nodes over the 2-week tumor challenge (data not shown).

PC61-treated mice have larger draining lymph nodes after tumor challenge compared with control Ig–treated mice. Although there was no change in the composition of the draining lymph nodes of PC61-treated, tumor-bearing mice, there was a 2.7-fold increase in cellularity in lymph nodes from PC61-treated and rat Ig–treated mice (Fig. 4A). To test whether the increased cellularity was due to enhanced proliferation, we measured the incorporation of BrdUrd into CD8+ and CD4+ draining lymph node versus nondraining lymph node cells from tumor-bearing mice pretreated with PC61 or rat Ig. Although PC61 treatment enhanced the incorporation of BrdUrd into CD8+ and CD4+ draining lymph node cells from tumor-bearing mice pretreated with PC61 or rat Ig, the enhanced incorporation was not statistically significant ($P = 0.163$ and 0.052, respectively) and did not correspond to the 2.7-fold increase in tumor-draining lymph node cellularity. Thus, the major effect of PC61 was to increase lymph node cellularity without affecting changes in any particular cellular subset. This finding suggests that Tregs limit the recruitment and/or migration of polyclonal lymphocytes to areas of immune activation.

PC61 pretreatment leads to increased CD8+ cell proliferation within the tumor. Because one effect of PC61 was to enhance the percentage of CD8+ TILs (Fig. 3C), we examined whether this result was explained by enhanced proliferation of CD8+ cells within the tumor. We therefore measured the incorporation of BrdUrd...
into CD8+ and CD4+ TILs in mice pretreated with PC61 or rat Ig. Compared with the draining lymph node, there was significant BrdUrd incorporation in CD8+ and CD4+ TILs in both rat Ig-pretreated and PC61-pretreated mice (Fig. 4B-C, right). Importantly, PC61 pretreatment significantly ($P = 0.027$) increased the percentage of BrdUrd+ CD8+ cells within the tumor versus rat Ig treatment (Fig. 4B). BrdUrd incorporation could be seen in the TILs even after 1 day of BrdUrd administration, suggesting that the mechanism was due to increased in situ proliferation rather than enhanced migration of proliferated cells from the draining lymph node (data not shown). The CD8+ TILs were uniformly CD44hi and CD62Llo, thus documenting that they were activated (data not shown). Taken together, the data suggest that one mechanism by which PC61 causes tumor regression is to deplete CD25+ Tregs, which accumulate in progressively growing tumors and thereby permit enhanced proliferation of CD8+ effector cells within the tumor mass.

**CD25+CD4+ cells are actively proliferating within tumors.** In many tumor systems, it is unclear whether tumor-infiltrating Tregs are activated and/or proliferating. Recently, it was shown that colon and melanoma tumors contain immature dendritic cells that can induce Treg proliferation through a transforming growth factor-$\beta$ (TGF-$\beta$)-dependent mechanism (41). To determine whether CD25+CD4+ TILs were actively proliferating in progressor and regressor MCA sarcomas, we monitored CD4+ TILs and draining lymph node cells from tumor-bearing mice for BrdUrd incorporation and CD25+ positivity. As shown in Fig. 5A, proliferating cells from a representative mouse bearing a progressor tumor could be distinguished from nonproliferating cells within both CD25+ and CD25− subsets in TIL and draining lymph node cell preparations. When these analyses were conducted using four mice per group, 63% of CD4+CD25+ TILs incorporated BrdUrd compared with 14% of CD4+CD25− draining lymph node cells, a 4.5-fold increase (Fig. 5B, left). Furthermore, there was a greater percentage of BrdUrd+ cells within the CD25+ subset versus the CD25− subset of CD4+ TILs in both progressor and regressor tumors (Fig. 5B), confirming previous studies that Tregs may represent a subset of CD4+ cells, which are partially activated and have higher turnover. We were unable to co-stain BrdUrd and Foxp3 because the fixation conditions were different for the different antibodies. However, because virtually all CD25+ TILs were Foxp3+ (Fig. 2B), we conclude that Tregs are activated to proliferate within both progressively growing and rejecting MCA sarcomas.

**CD25+CD4+ cells within tumors derive from natural Tregs.** To determine whether the tumor microenvironment caused CD25− cells to differentiate into CD25+Foxp3+ cells, we used an adoptive transfer approach that used congenic strains of BALB/c mice pretreated with PC61 or control rat IgG at day −4 and transplanted with WT-P1 progressor tumor cells on day 0. BrdUrd was given on days 8 to 10. On day 10, draining lymph node (DLN) was harvested and analyzed for BrdUrd incorporation by FACS (B-C). Symbol, different mouse.
differing only in the Thy1 marker and the BALB/c-derived progressor MCA sarcoma CMS-5. This tumor behaved in a manner similar to progressor 129/Sv tumors in that, when transplanted into WT BALB/c mice, it grew progressively and accumulated a high percentage of CD25+ TILs (data not shown). Moreover, when transplanted into PC61-pretreated mice, CMS-5 was rejected and the mAb treatment induced increases in draining lymph node cellularity and CD8+ TIL proliferation versus control Ig treatment (data not shown).

We sorted CD25+CD4+ cells from the lymph nodes and spleens of naive BALB/c mice congenic for the Thy1.2 marker and mixed these cells with Thy1.1+ BALB/c splenocytes that had been depleted of CD25+ cells. We then reconstituted BALB/c strain severe combined immunodeficient (SCID) mice with the mixture of Thy1.1+CD25+CD4+ and Thy1.2+CD25- cells and challenged the reconstituted host with CMS-5. As observed in WT mice, reconstituted SCID mice responded to tumor challenge by recruiting CD25+CD4+ cells into the developing tumor mass (Fig. 6). These cells represented 30% to 50% of CD4+ cells within the TIL population (data not shown). Notably, 80% of CD25+CD4+ TILs expressed Thy1.2, showing that they were indeed derived from native peripheral CD25+ cells rather than from CD25- cells that were induced to express CD25. Thus, the tumor microenvironment preferentially recruits and activates natural peripheral Tregs rather than inducing Treg development from CD25- cells.

**Discussion**

The cancer immunosurveillance/immunoediting model predicts that clinically apparent, growing tumors must have evaded immune detection and/or elimination, and many of the mechanisms that underlie the escape phase of immunoediting have long been recognized (3, 7, 8, 24). Recent studies have implicated Tregs as an additional mechanism contributing to tumor escape (24, 42, 43), but the relative importance of this mechanism has remained unclear. Specifically, because earlier studies used tumors from immunocompetent hosts, it is unknown whether Tregs infiltrate all tumors, even highly antigenic regressor tumors, or only a subset of tumors that has been selected to evade the immune response. We document herein that both regressor and progressor tumors contain Tregs and thus show that even highly immunogenic tumors can attract and/or activate these cells. However, we go on to show that the relative percentage of Tregs among the lymphocytes in progressor tumors is on average twice that in regressor tumors. Thus, our results provide the basis for the novel conclusion that, although Tregs are activated in both regressor and progressor tumors, a successful antitumor response can be achieved as long as effector T cell activation outweighs Treg activation. These findings confirm and extend the observations from other studies, which have shown that Treg depletion can produce an effective antitumor response (31, 41). We conclude that the ratio of regulatory to effector T cells and not simply the presence or absence of Tregs is a critical determining factor in the in vivo growth behavior of a tumor.
For progressively growing tumors, this ratio is tipped to favoring Tregs. In contrast, for regressor tumors, the balance is tipped toward the effector T cells and, thus, the tumor is rejected. This hypothesis is supported by the findings that manipulations, which decrease the regulatory to effector T-cell ratio, such as depletion with PC61 or increasing effector T-cell precursors through immunization (44), can lead to a successful antitumor response and rejection of progressor tumors. In addition, manipulations that increase the regulatory to effector T-cell ratio, such as immunization of naive mice with known Treg epitopes, establish an in vivo environment that is less capable of eliminating developing tumors (45, 46). Moreover, our conclusions using an experimentally tractable mouse tumor transplantation approach are consistent with those stemming from analysis of Treg to CD8+ T-cell ratios found in naturally occurring human primary tumors (22). Specifically, cancer patients, whose tumors contain high levels of Tregs compared with CD8+ cells, have a poor clinical prognosis. Conversely, patients with tumors containing high numbers of CD8+ T cells relative to Tregs have a favorable prognosis.

Several experimental findings support the hypothesis that the increase in Tregs in progressor tumors is causally related to tumor progression. First, Tregs accumulated early within the tumor. Second, depletion of the Tregs before tumor challenge led to tumor rejection, a result that correlated with increased CD8+ proliferation and accumulation in the tumor. Third, the Tregs within progressor tumors were actively proliferating, suggesting that they were activated and could exert their immunosuppressive function at a time point early enough to influence subsequent immune events.

We also present the novel finding that the CD4+CD25+ tumor-infiltrating cells are derived from the peripheral CD4+CD25+ Treg pool. Previous studies have shown that Tregs can be induced from CD25- cells under conditions that lead to tolerance to foreign antigens (38, 47). Although many groups have shown Treg infiltration into tumors, it has, until now, not been clear whether tumor-infiltrating Tregs are converted from CD4+CD25- precursors or derived from the preexisting CD4+CD25+ cellular pool. Our results clearly establish that the vast majority of tumor-infiltrating Tregs originates from peripheral CD4+CD25+ cells in the MCA sarcoma model. The implication of these findings is that the specificity of tumor-infiltrating Tregs must reflect that of the peripheral CD4+CD25+ cellular pool, which is thought to be directed toward self-antigens (48). This conclusion suggests that the specificity of the Treg populations that participate in suppressing tumor immunity would be distinct from that of CD4+CD25+ cells, which display a distinct spectrum of T-cell receptor sequences (48) and which may be the cellular sources of CD4+ helper T cells needed to promote the antitumor response. Thus, our data provide support for the tenet that presentation of endogenous self-antigens by tumors recruits and activates peripheral native Tregs, which function to protect the tumor from "autoimmunity" and thus facilitate escape from immune rejection (25, 30).

We speculate that the number of regulatory and effector T cells within a developed tumor reflects not only the inherent host response against antigens recognized by these two cell populations but also the immunologic environment in which the tumor originally formed. We propose that progressor tumors, because they arose in an editing environment, express antigens with either a higher quantity and/or quality of Treg epitopes compared with effector T-cell epitopes. In contrast, regressor tumors, which arose in a nonediting environment, may have stronger and/or greater numbers of effector versus regulatory T-cell epitopes. This antigen imbalance model will need to be tested further by identifying the relative distribution of regulatory and effector T-cell epitopes within regressor versus progressor tumors. Several Treg antigens have already been identified in fibrosarcomas from immunocompetent BALB/c mice (45). These include the self-proteins Dnal-like 2, Galectin-8, and Ligase 1, and their common characteristic is their expression of CD4+ epitopes but not CD8+ epitopes. However, although highly relevant in the context of BALB/c tumor models, these same antigens are not differentially expressed in our 129/Sv strain progressor versus regressor tumors. Thus, the antigen imbalance model must also take into account the contextual immunogenicity of a multi-antigenic tumor.

The differential accumulation of Tregs into progressor versus regressor tumors can also be attributed to differences in recruitment and/or expansion of Tregs within the tumor microenvironment. In particular, the secretion of TGF-β, which often occurs within the tumor microenvironment, has been shown to enhance Treg proliferation (41, 43, 47). In our studies, we found that CD25+ cells in regressor tumors incorporated BrdUrd at an equivalent rate as in progressor tumors, suggesting that the microenvironments (e.g., TGF-β production) of both regressor and progressor tumors may be similar in their capacity to support Treg proliferation. Further studies will delineate the relative contribution of tumor antigen content versus TGF-β production in setting the regulatory to effector T-cell ratio during tumor development.

In conclusion, we have taken advantage of the availability of large cohorts of well-characterized MCA sarcomas from WT and immunodeficient mice to show that one major difference between tumor progression and rejection is the relative proportion of

---

*J.D. Bui and R.D. Schreiber, unpublished observation.*
CD4+CD25+Foxp3+ cells that become associated with the tumor mass. These Tregs are activated to proliferate, derive from peripheral CD4+CD25+ cells, and function to inhibit CD8+ cell proliferation and potentially effector functions in progressor tumors. We propose that both regressor and progressor tumors contain effector and regulatory T-cell antigens. However, the balance of these antigens may have been preestablished by a cancer immunoediting process that acted during tumor development. From an evolutionary perspective, we hypothesize that evoking Treg activity may represent the most direct and generalizable mechanism by which tumors evade immune destruction, as Tregs would exert a “dominant” form of immunotolerance on many different cell types, such as NK and CD8+ cells (25, 27, 49, 50). In contrast, other immunoediting changes that result in the elimination of specific immune recognition structures on the tumor might be considered “recessive,” as other forms of immune attack on the tumor would remain unaltered. Future studies will be able to determine whether this dominant form of tumor escape can be modulated to effect clinical tumor regression.

Acknowledgments

Received 2/13/2006; revised 4/21/2006; accepted 4/27/2006.

Grant support: NIH grants CA43059 and CA107527, Ludwig Institute for Cancer Research grant, and Cancer Research Institute Bhea Rosemary Finnell Clinical Investigation grant (R.D. Schreiber); Cancer Research Institute Postdoctoral Fellowship (J.D. Bul); grant KO8 CA90036 (R. Uppal); and National Center for Research Resources grant C06 RO128466.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Hiroshi Shiku for CMS-5, Paul Allen for the congenic BALB/c mice, Ken Matsui for help with the adoptive transfers, and Lloyd Old, Catherine Koebel, Lyse Norian, Gavin Dunn, and Fei Shih for critical discussion of the article.

References


www.aacrjournals.org

Downloaded from cancers.cancerres.org on April 13, 2017. © 2006 American Association for Cancer Research.
Comparative Analysis of Regulatory and Effector T Cells in Progressively Growing versus Rejecting Tumors of Similar Origins

Jack D. Bui, Ravindra Uppaluri, Chyi-Song Hsieh, et al.