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**Supplementary Material**

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**Errata**

An erratum has been published regarding this article. Please see next page or:
/content/191/10/5319.full.pdf
Immune cells comprise a substantial proportion of the tumor mass in human nonsmall cell lung cancers (NSCLC), but the precise composition and significance of this infiltration are unclear. In this study, we examined immune complexity of human NSCLC as well as NSCLC developing in CC10-TAg transgenic mice, and revealed that CD4+ T lymphocytes represent the dominant population of CD45+ immune cells, and, relative to normal lung tissue, CD4+Foxp3+ regulatory T cells (Tregs) were significantly increased as a proportion of total CD4+ cells. To assess the functional significance of increased Tregs, we evaluated CD8+ T cell–deficient/CC10-TAg mice and revealed that CD8+ T cells significantly controlled tumor growth with antitumor activity that was partially repressed by CD45+ immune cells, and, relative to normal lung tissue, CD4+Foxp3+ regulatory T cells (Tregs) were significantly increased as associated with poor prognosis in NSCLC and other carcinomas. However, whereas treatment with anti-CD25–depleting mAb as monotherapy preferentially depleted Tregs and improved CD8+ T cell–mediated control of tumor progression during early tumor development, similar monotherapy was ineffective at later stages. Because mice bearing early NSCLC treated with anti-CD25 mAb exhibited increased tumor cell death associated with infiltration by CD8+ T cells expressing elevated levels of granzyme A, granzyme B, perforin, and IFN-γ, we therefore evaluated carboplatin combination therapy resulting in a significantly extended survival beyond that observed with chemotherapy alone, indicating that Treg depletion in combination with cytotoxic therapy may be beneficial as a treatment strategy for advanced NSCLC.

Lung cancer is the most common cause of cancer-related mortality worldwide, with ~85% of all nonsmall cell lung cancer (NSCLC) histological subtype, and associated with prior tobacco use (1). Despite advances in treatment modalities, survival rates for advanced lung cancer remain poor; thus, innovative therapeutic approaches are urgently needed.

Retrospective analysis of most human tumors (2), including lung (3–7), has revealed a significant correlation between immune infiltration by CD8+ cytotoxic T cells and improved outcome. In contrast, infiltration of tumors by regulatory T cells (Tregs) expressing the lineage-specific transcription factor FOXP3 is instead associated with poor prognosis in NSCLC and other carcinomas. As the tumor immune microenvironment and the immunosuppressive cell types that function in tissues are distinct, we first evaluated leukocyte complexity of human NSCLC and found that CD4+ T cells were significantly increased relative to adjacent normal lung tissue, and that CD4+Foxp3+Tregs constituted a significant proportion of these tumor-infiltrating cells. To determine the functional significance of these adaptive leukocytes, and the cellular and molecular mediators of pro- versus antitumor immunity, we used a transgenic mouse model of multistage lung carcinogenesis, namely CC10-TAg mice, in which SV40 T Ag–driven carcinogenesis mirrors that of aggressive human lung cancers (14). We revealed that, whereas CD8+ T lymphocytes are critical in restraining lung tumor growth, their recruitment into tumors and bioeffector functions are inhibited by CD4+ Foxp3+Treg depletion of which significantly prolongs survival of tumor-bearing mice in combination with chemotherapy (CTX).

Materials and Methods

**Human tissue samples**

Patients with NSCLC who had not received neoadjuvant therapy were recruited into the study under approval of local Institutional Review Boards.
Informed written consent was obtained from the patients. Tumor tissue, adjacent normal tissue, and blood were collected from patients following surgical resection at University of California, and histopathological diagnosis was obtained at the same center.

**Animal studies**

Generation of CC10-TAg mice and characterization of their neoplastic/histopathological stages have been previously reported (15). CC10-TAg mice deficient in B cells (JH−/−), CD4+ T cells (CD4−/−), CD8+ T cells (CD8−/−), and both CD4+ and CD8+ T cells (CD4+CD8−/−) were generated by backcrossing JH−/−, CD4−/−, CD8−/−, and CD4+CD8−/− mice, respectively, into the FvB/n strain to at least N5 (16, 17), followed by intercrossing with CC10-TAg mice. All animal studies and procedures conformed to National Institutes of Health guidelines and were approved by University of California Institutional Animal Care and Use Committee. For in vivo depletion studies, mice were injected i.p. with 400 μg anti-CD25 mAb (clone PC61) and 500 μg anti-CD8 mAb (clone YTS169-4) every 5 d for the respective time periods, as indicated. For survival studies, control from 8 wk of age until end stage (defined by 15% weight loss). Carboplatin ( Hospira) was injected i.p. at 50 mg/kg every 5 d for three doses starting at 13 wk of age.

**Histology and tumor size**

Mice were sacrificed at indicated time points, and all tissues were collected following intracardiac PBS perfusion. Tissues were fixed in 10% neutral-buffered formalin or frozen in OCT. Tumor burden of each mouse was quantified in five H&E-stained serial sections (100 μm apart) of lungs using Image J software.

**Immunohistochemistry**

The 5-μm sections of formalin-fixed paraffin-embedded tissues were deparaffinized in xylene and rehydrated by immersion in reducing concentrations of alcohol, followed by PBS. Ag retrieval for CD45, CD8, Foxp3, cleaved caspase-3, and BrdU staining was performed by boiling in citrate buffer (BioGenex), followed by incubation with protease K (Dako) for CD31. Endogenous peroxidase activity was quenched by incubation in hydrogen peroxide (Sigma-Aldrich) and methanol at 1:50. Following blocking of nonspecific binding by application of blocking buffer (PBS containing 5% goat serum, 2.5% BSA, and 0.1% Tween 20), tissue sections were incubated overnight with primary Abs, for example, anti-CD45, CD8+ and B cells (JH−/−), CD4+ T cells (CC10-TAg/CD4−/−), CD8+ T cells (CC10-TAg/CD8−/−), and mice lacking both CD4+ and CD8+ T cells (CC10-TAg/CD4−/−CD8−/−) were evaluated for tumor (eBioscience). All samples were analyzed on a LSRII flow cytometer (BD Biosciences).

**Quantitative PCR assays**

mRNA was obtained by processing tissue samples as per recommendations using RNeasy Micro/Mini Kit (Qiagen) and quantified with NanoDrop ND-1000 (Thermo Fisher Scientific). cDNA was prepared from mRNA by reverse transcription using Superscript III. Preamplification of cDNA for genes of interest was performed using TaqMan PreAmp Master Mix Kit (Applied Biosystems). PCR amplification to 40 cycles was performed using TaqMan gene expression assays (Applied Biosystems) for respective genes and TaqMan gene expression master mix (Applied Biosystems) in 20 μl reactions at recommended cycle temperature conditions on an ABI 7900HT quantitative PCR machine (ABI Biosystems). Differences in gene expression were determined by calculating relative expression as fold change over TBP used as the housekeeping gene.

**Statistical analyses**

Statistical analyses were performed using Prism 4.0 (GraphPad Software). Differences between groups for all parameters were determined using Mann-Whitney U test (unpaired, nonparametric, two tailed), except for survival studies in which log rank test was used. *p < 0.05, **p < 0.01, ***p < 0.001 are shown for all figures.

**Results**

**Human NSCLC are infiltrated by CD4+ T and B lymphocytes**

Using immunohistochemical and flow cytometric approaches, we evaluated the immune microenvironment within tumors of patients with CTX-naïve NSCLC (Supplemental Table I), and found increased presence of CD45+ leukocytes within tumors as compared with adjacent normal tissue (Fig. 1A, 1B). Both adaptive lineage (T and B lymphocytes) and innate lineage cells (macrophages, dendritic cells [DCs], and granulocytes) were observed in normal adjacent lung and tumor tissue. However, as compared with adjacent normal lung tissue, the relative composition of leukocytes within tumors was skewed toward higher proportions of CD4+ T and B cells (Fig. 1C, 1D). In all of the tumors examined, both CD4+ and CD8+ T cells displayed activated phenotypes, with most samples displaying higher percentage of CD69+ cells in tumors as compared with normal adjacent tissue (Fig. 1E).

**Immune complexity of CC10-TAg NSCLC mirrors human NSCLC**

CC10-TAg mice express the SV40 large T Ag under control of the Clara cell promoter, and as a consequence develop multifocal pulmonary adenocarcinoma (15) with a gene signature correlated with that of aggressive subtypes of human lung cancers, and thus represent a relevant preclinical model to study NSCLC development (14). In CCT10-TAg mice, hyperplastic and dysplastic lung tissue is prominent as early as 4 wk of age, and develops into adenomas by 8 wk, with invasive NSCLC in 100% of mice on the FVB/n strain background between 12 and 16 wk of age (15). Similar to human NSCLC, CC10-TAg tumors are characterized by marked CD45+ leukocytic infiltration (Fig. 2A, 2B) with an increased percentage of CD4+ T lymphocytes (Fig. 2C, 2D).

**Endogenous CD8+ cytotoxic T cell responses restrain lung tumor growth in CC10-TAg mice**

Because our data indicated that human NSCLC were predominantly infiltrated by activated T lymphocytes, we investigated the functional significance of CD4+ T, CD8+ T, and B cells in CC10-TAg mice by generating mice harboring homozygous null mutations in genes controlling lineage development. CC10-TAg mice deficient for B220+CD19+ mature B cells (CC10-TAg/JH−/−), CD4+ T cells (CC10-TAg/CD4−/−), CD8+ T cells (CC10-TAg/CD8−/−), and mice lacking both CD4+ and CD8+ T cells (CC10-TAg/CD4−/−CD8−/−) were evaluated for tumor
burden at 12 wk of age. CC10-TAg mice lacking CD8+ T cells, but not CD4+ T cells or B cells, exhibited increased tumor burden (Fig. 3A, 3B), accelerated progression to end stage, and reduced survival (Fig. 3C), indicating that endogenous CD8+ T cell responses played a critical role in limiting tumor growth and progression. To demonstrate that the phenotype of CC10-TAg/CD82/2 mice was not a side effect of genetic manipulation, we depleted CD8+ T cells from CC10-TAg mice from 8 to 12 wk of age using anti-CD8–depleting Abs that efficiently depleted CD8+ T cells in both spleen and lungs (Supplemental Fig. 1A). Ab-mediated depletion phenocopied the CC10-TAg/CD82/2 mice (Fig. 3D), thereby demonstrating that CD8+ T cells were...

**FIGURE 1.** Immune complexity of human NSCLC. (A) H&E staining of human NSCLC and adjacent normal tissue (top panel) with representative images showing staining for CD45 (bottom panel). (B) Numbers of CD45+ leukocytes per square millimeter of tissue sections as assessed by immunohistochemistry. n = 8 samples per group. (C) Flow cytometric analysis of immune cell infiltrates within human NSCLC represented as percentage of total CD45+ leukocytes. n = 6 samples per group. (D) CD19+CD20+HLA-DR+ B cell and CD3+CD4+ T cell infiltrate within human NSCLC, as assessed by flow cytometry, shown as a percentage of total CD45+ cells. (E) Percentage of CD4+ and CD8+ T cells staining positive for CD69, as assessed by flow cytometry, with representative histograms of CD69 expression shown to the right. **p < 0.01, ***p < 0.001.

**FIGURE 2.** Immune complexity of NSCLC in CC10-TAg mice. (A) H&E staining of lungs from negative littermates (−LM) and CC10-TAg mice showing adenomas and adenocarcinoma (top panel), with representative staining for CD45 (bottom panel). (B) Numbers of CD45+ leukocytes per square millimeter of tissue, as assessed by immunohistochemistry. n = 5 mice per group. (C) Flow cytometric analysis of immune cell infiltrates in CC10-TAg lungs assessed at various stages of neoplastic development, namely hyperplasia/dysplasia (4 wk), adenomas (8 wk), and adenocarcinomas (16 wk), represented as percentages of total CD45+ leukocytes. (D) CD4+ T cell lung infiltrate, as assessed by flow cytometry, shown as a percentage of total CD45+ cells. n = 5–8 mice per group. Significant differences are shown relative to negative littermates. **p < 0.01, ***p < 0.001.
functionally important in restraining tumor growth in the CC10-TAg model.

**Human and CC10-TAg lung tumors are infiltrated by CD4⁺Foxp3⁺Tregs**

CD8⁺ cytotoxic T cells infiltrate lung tumors, where they functionally regulate tumor growth; nevertheless, CC10-TAg tumors continue to progress with mice eventually succumbing to respiratory insufficiency. Given that CD4⁺ T cells abundantly infiltrate tumors relative to nontumor-bearing lungs, we hypothesized that Foxp3⁺ Tregs might be enriched within tumors where they functioned to suppress productive CD8⁺ T cell responses. To investigate this, we first ascertained whether Tregs were present in tumors by intracellular staining for Foxp3 by flow cytometry and immunohistochemistry. We observed that indeed within human NSCLC tumors (Fig. 4A), there was enrichment of CD4⁺Foxp3⁺ Tregs relative to adjacent normal lung tissue. These findings were mirrored in CC10-TAg lung tumors at multiple stages of tumor development (Fig. 4B), where upregulation of CD11c surface expression in tumor-infiltrating Tregs, as compared with normal lungs, was also observed, thus indicating their activated phenotype (Fig. 4C).

**Treg depletion diminishes tumor burden in CC10-TAg mice**

To examine the functional significance of Treg infiltration of lung tumors, we examined the effects of partial Treg depletion. Although complete and specific elimination of Tregs can be achieved by use of scurfy mice (20) harboring a loss-of-function mutation in the Foxp3 gene, or by administration of diphtheria toxin to mice following activation. Hence, we first determined the profile of cells expressing CD25 in lung tumors and observed that, within CC10-TAg tumors, the majority of CD25 expressing T cells coexpressed Foxp3 (Supplemental Fig. 1B). Administration of a single dose of the anti-CD25 mAb resulted in progressive diminution of Tregs in peripheral blood, attaining a maximum reduction of 70% as compared with control mice 5 d postinjection, with some evidence of recovery by day 11 (Supplemental Fig. 1C).

Treatment of 4-wk-old CC10-TAg mice every 5 d with anti-CD25 mAb until mice were 8 wk old (Fig. 5D) significantly reduced presence of Tregs within spleen and tumor-bearing lungs (Fig. 5E) and led to a significant, albeit minor, reduction in tumor burden (Fig. 5F). This reduction in tumor burden was not due to reduced presence of proliferating malignant lung epithelia (Fig. 5G), or changes in vascular architecture (Fig. 4H), but instead by a marked increased presence of cleaved caspase-3–positive cells (Fig. 4I) that correlated with increased presence of CD8⁺ T cells infiltrating lung parenchyma and tumors (Fig. 4J–L). Together these data indicated that Tregs were most likely involved in restricting antitumor activity of tumor-infiltrating CD8⁺ T cells.

**Enhanced recruitment of CD8⁺ T cells restricts NSCLC development**

Analysis of infiltrating CD8⁺ T cells in Treg-depleted CC10-TAg mice revealed no difference in in vivo proliferation as measured by BrdU incorporation (Fig. 5A) or activation as determined by CD69 expression (Fig. 5B). Instead, gene expression analysis of FACs-sorted CD8⁺ T cells isolated from CC10-TAg/CD8⁻⁻ mice, where, as expected, anti-CD25 mAb administration from 4 to 8 wk of age failed to alter tumor burden at end stage (Fig. 5G)

As other studies have reported that Treg suppression of effector T cells may be mediated by cross-talk with APCs (22–25), we also examined whether CD11c⁺MHCIIC⁺ alveolar macrophage (Supplemental Fig. 2) or CD11chighMHCIIChigh DC (Supplemental Fig. 3) polarization might be altered following partial Treg depletion. Although a significant reduction of CCL17 and CCL22, chemokines known to promote recruitment of Tregs into tumors, was observed in tumor-isolated CD11c⁺MHCIIC⁺ alveolar macrophages, baseline expression of these genes was 100-fold lower.
compared with DCs, which did not display altered gene expression. Hence, we reasoned this was unlikely to account for changes in CD8+ T cell activity. Based on the modest changes in macrophage and DC transcriptomes, we therefore speculated that Tregs were the major leukocyte population repressing CD8+ T cell presence and effector function.

**FIGURE 4.** Functional significance of Tregs in NSCLC. (A) Frequency of FOXP3+ Tregs within the CD4+ T cell compartment in human NSCLC assessed by flow cytometry, with representative FOXP3 immunohistochemistry shown on left. n = 6 per group. (B) Frequency of Foxp3+ Tregs within the CD4+ T cell compartment in CC10-TAg tumors at various ages, as assessed by flow cytometry, with representative Foxp3 immunohistochemistry shown on left. n = 5–8 mice per group. (C) Percentage of CD3+CD4+Foxp3+ cells expressing CD103+ in CC10-TAg tumors. n = 5–8 mice per group. (D) Treg depletion was assessed in CC10-TAg mice in a prevention trial by i.p. injections of anti-CD25 mAb every 5 d from 4 wk until 8 wk of age. (E) Frequency of Foxp3+ Tregs represented as percentage of CD3+CD4+ T cells in spleen (left) and lung tumors (right) following treatment with anti-CD25 mAb. (F) Tumor burden represented as percentage of lung area following anti-CD25 treatment in CC10-TAg mice. (G) Number of BrdU+ tumor cells per square millimeter of lung tumors. (H) Angiogenic vasculature represented as percentage of positive pixels of CD31 staining by automated quantification of representative stained sections. (I) Number of cleaved caspase-3+ tumor cells per square millimeter of lung tumors. (J) Immune cell complexity of lung tumors following Treg depletion represented as percentage of CD45+ leukocytes assessed by flow cytometry. (K) CD8+ T cell infiltrate of lung tumors, as assessed by flow cytometry, shown as a percentage of total CD45+ cells. (L) Absolute numbers of CD8+ cells per square millimeter of lung tumor with representative immunohistochemistry shown to the right. (E–L) n = 12–13 mice per group with data obtained over three independent cohorts of animals. *p < 0.05, **p < 0.01, ***p < 0.001.

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Treg depletion attenuated tumor burden in CC10-TAg mice in a prevention trial, we sought to evaluate whether Treg depletion in combination with cytotoxic CTX might extend survival of CC10-TAg mice in a more clinically relevant setting when mice with late-stage NSCLC were treated. Because platinum compounds are first-line standard-of-care chemotherapeutic agents for human NSCLC, we first conducted dose-response experiments with cisplatin in CC10-TAg mice to determine the maximum tolerated dosage that would not produce total leucopenia for use in survival studies. We observed that 50% of CC10-TAg mice did not tolerate administration of both cisplatin and mAb despite the reported safety profile of combinatorial cisplatin and mAbs in clinical trials (27, 28). We therefore conducted a similar dose-response study with carboplatin, and determined the maximum tolerated dose in combination with anti-CD25 mAb to be 50 mg/Kg, with peripheral blood erythrocytes, leukocytes (lymphocytes and granulocytes), and platelets showing reduced, but not abnormal levels in mice (data not shown).

Thus, CC10-TAg mice were randomized and recruited into four arms to evaluate survival. Mice received control IgG or anti-CD25 mAb as monotherapy from 8 wk of age until end stage (15% weight loss), or received mAbs in combination with carboplatin CTX administered in three doses, 5 d apart, commencing at 13 wk when CC10-TAg mice histopathologically exhibit features of invasive adenocarcinomas. Whereas administration of anti-CD25 mAb as a monotherapy yielded no survival benefit as compared with control

**FIGURE 5.** CD8⁺ T cells in NSCLC following Treg depletion. (A) Frequency of BrdU⁺ proliferating CD8⁺ T cells represented as percentages of total tumor-infiltrating CD8⁺ T cells. (B) Frequency of CD69-expressing activated CD8⁺ T cells represented as percentages of total tumor-infiltrating CD8⁺ T cells. (A and B) n = 7 per group; one of two representative experiments is shown. (C-F) Relative expression of Ifng (C), Gzma (D), Gzmb (E), and Prf1 (F) mRNA in flow-sorted CD8⁺ T cells represented as fold change over Tbp, as assessed by quantitative PCR. n = 7 per group, with data obtained over two independent cohorts of animals. (G) Tumor burden represented as percentage of lung area, following treatment with anti-CD25 mAb from 4 wk until 8 wk of age in CC10-TAg mice deficient in CD8⁺ T cells. n = 10 per group, with data obtained over three independent cohorts of animals. *p < 0.05, **p < 0.01.

**FIGURE 6.** Treg depletion in combination with chemotherapy extends survival. Percentage of survival of CC10-TAg mice treated with control IgG or anti-CD25 mAb as monotherapy from 8 wk of age until end stage (15% weight loss), or received mAbs in combination with carboplatin CTX administered in three doses, 5 d apart, commencing at 13 wk when CC10-TAg mice histopathologically exhibit features of invasive adenocarcinomas. Whereas administration of anti-CD25 mAb as a monotherapy yielded no survival benefit as compared with control.
IgG-treated mice, mice that received combination anti-CD25 mAb plus carboplatin exhibited a significant (p < 0.05) extension of survival relative to carboplatin alone (Fig. 6).

Discussion

In this study, we evaluated leukocyte complexity of human NSCLC from CTX-naïve patients and in a mouse model of de novo NSCLC development. Results from these studies indicate that, whereas lymphocytes and myeloid cells infiltrate both NSCLC and normal lung, the immune complexity of human NSCLC is dominated by T cells and, in particular, CD4+ T and B cells as compared with adjacent normal lung tissue. Interestingly, in over half of the patient tissues examined, both CD4+ and CD8+ tumor-infiltrating T cells exhibited an activated phenotype based upon expression of CD69, as compared with those in adjacent normal tissue, indicating that these lymphocytes may be functionally significant. CD4+ T cells can protect against methylcholanthrene-induced sarcomas (29) and human papillomavirus type 16–induced cervical carcinogenesis (30), whereas other tissues instead promote carcinogen-induced (31) or human papillomavirus type 16–induced squamous cancer (32). In a similar manner, B cells have been found to dampen antitumor immune responses in some murine tumors (33, 34), although augmenting them to enable tumor rejection in others (35, 36). It has therefore become increasingly clear that tumor-infiltrating immune cells exert different bioactivities depending on context, namely tumor etiology and tumor microenvironment.

In CC10-TAg mice harboring NSCLC, neither CD4+ T cell nor B cell deficiency significantly altered tumor growth or progression; in contrast, CD8+ T cell deficiency led to an acceleration of tumor growth and reduction in survival, thus indicating their critical role in thwarting tumor development in lung. Nevertheless, all CC10-TAg mice succumbed to their disease, indicating tumor immune escape. In keeping with previous reports (37, 38), we found enhanced T cell infiltration in both human and murine NSCLC relative to normal adjacent or nonneutrogenic lung, respectively. If CD4+Foxp3+Tregs infiltrating NSCLC were functionally significant in promoting tumor immune escape in NSCLC, the expectation would instead be tumor regression in CD4+ T cell–deficient CC10-Tag mice, a result that was not observed. However, the conflict in our observation may be accounted for by the simultaneous absence of conventional CD4+ T cells in CD4-deficient TAg mice, which may be essential for providing help to CD8+ T cells (39, 40).

The functional significance of Tregs in several malignancies has been elucidated using mouse models (41–45); however, their precise role in lung cancer is unclear. Furthermore, the in vivo mechanism, the target cell types, and molecular mediators used by Tregs to exert their suppressive function in the tumor microenvironment are incompletely understood. In this study, we report that, in CC10-TAg mice, depletion of Tregs using the anti-CD25 mAb (PC61) at an early stage of tumor development significantly reduced tumor burden in a manner dependent upon infiltration of functionally active CD8+ T cells. Tumor-infiltrating CD8+ T cells in Treg-depleted mice did not display enhanced activation or in vivo proliferation, indicating that increased CD8+ T cell infiltration observed following Treg depletion was most likely a result of increased recruitment rather than local proliferation. That said, CD8+ T cells infiltrating tumors of Treg-depleted mice were characterized by upregulation of effector cytotoxic genes, including granzyme A, granzyme B, and perforin, indicating enhanced functional capacity following release from Treg-mediated suppression. Taken together, these findings indicate that CD8+ T cells recruited to NSCLC following Treg depletion were functionally empowered to better kill malignant cells (as indicated by increased presence of cleaved caspase-3 cells), leading to increased tumor cell death. Our data implicating Treg suppression of CD8+ T cells are supported by several studies (41, 42), although other cell types, such as conventional CD4+ T cells and NK, have also been reported to be involved (46, 47). Unexpectedly, a recent study by Teng and colleagues revealed a requirement for Th2 cytokines, IL-4 and IL-13, in addition to the Th1 cytokine IFN-γ in achieving tumor control following Treg depletion using respective cytokine-deficient mice (48).

Tregs regulate APC function as a means of regulating immune responses. Tregs establish direct interactions with DCs in lymph nodes, leading to impaired ability to engage and activate T effector cells (49). Treg modulation of macrophages also results in reduced activation, blunted proinflammatory cytokine secretion, upregulation of CD206 and CD163, and reduced macrophage cytotoxicity (50, 51). Both DCs and macrophages can be stimulated by Tregs to produce immnosuppressive molecules such as IDO, IL-10, and TGF-β (22, 25, 52). We assessed whether Tregs exerted their suppressive effect on APCs in the lung tumor microenvironment and found no significant changes in either DC or macrophage gene expression profiles when comparing cells isolated from Treg-depleted versus control tumor tissue, indicating that Treg most likely directly suppress CD8+ T cells in the lung.

Prophylactic Treg depletion in many experimental murine cancer models results in tumor protection when Treg depletion precedes tumor cell implantation (41, 43, 53, 54). In contrast, Treg depletion as monotherapy in large established tumors exhibits minimal impact (26). Recent studies have also revealed that conventional CTX causes tumor regression not just by direct tumor cell killing, but also by eliciting an antitumor cytotoxic immune response (55). Thus, we hypothesized that depletion of Tregs in combination with CTX would exert synergistic effects in restraining established tumors. Indeed, we revealed that CC10-TAG mice display enhanced survival when treated with a combination of anti-CD25 mAb and carboplatin, as compared with either treatment alone. This study thus highlights that, even in established tumors, manipulation of Tregs may be beneficial in combination with standard-of-care conventional CTX. Survival benefits have also been reported for early treatment of implanted mesothelioma tumors using anti-CD25 mAb and pemetrexed (56). Interestingly, two other reports revealed that complete and selective Treg depletion using DEREG mice controls growth of established implanted tumors in isolation (57) or in combination with vaccination (58). As Tregs from transgenic murine tumors have been described to derive from the thymus (59), it will be interesting to determine whether this is also true for implantable tumor models, and whether these cells are functionally equivalent.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References


Corrections


The fourth author’s name was published incorrectly. The correct name is Adam Yagui-Beltrán.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1390059
Supplementary Figure Legends

**Supplemental Figure 1:** (A) Frequency of CD8<sup>+</sup> T cells in spleen and tumor bearing lungs following treatment of CC10-TAg mice with αCD8 mAb from 8 weeks to 12 weeks of age, represented as percentage of CD3<sup>+</sup> T cells and assessed by flow cytometry. n=5 mice per group, one of three representative experiments is shown. (B) Frequency of CD25-expressing cells represented by different T cell subsets in tumor-bearing lungs of CC10-TAg mice, shown as percentage of total CD3<sup>+</sup>CD25<sup>+</sup> T cells. n=5 mice per group. (C) Frequency of Foxp3<sup>+</sup> Tregs in the peripheral blood of CC10-TAg mice following a single injection of αCD25 mAb, represented as a percentage of CD4<sup>+</sup> T cells. n=5 per time-point post injection. *p<0.05; **p<0.01, ***p<0.001.

**Supplemental Figure 2:** Relative expression of chemokine (A), receptor/ligand (B), cytokines (C) and mRNAs related to metabolism and angiogenesis (D) in flow-sorted CD11c<sup>+</sup>MHCII<sup>+</sup> alveolar macrophages from tumor-bearing lungs following treatment with of αCD25 mAb, represented as fold change over Tbp. n=7 per group, with data obtained over 2 independent cohorts of animals. *p<0.05; **p<0.01, ***p<0.001.

**Supplemental Figure 3:** Relative expression of chemokines (A), receptor/ligand (B), cytokine (C) and mRNAs related to metabolism and angiogenesis (D) in flow-sorted CD11c<sup>hi</sup>MHCII<sup>hi</sup> DCs from tumor-bearing lungs following treatment with of αCD25 mAb, represented as fold change over Tbp. n=7 per group, with data obtained over 2 independent cohorts of animals.
Table S1. Clinical characteristics of patients with NSCLC.
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A. Cytokine gene expression

B. Receptor/Ligand expression

C. Cytokine expression

D. Metabolic/angiogenic gene expression