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Antigen Specificity Determines the Pro- or Antitumoral Nature of CD8$^+$ T Cells

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Although CD8$^+$ T cells are usually considered antitumoral, several recent studies report that the cells can also promote tumor progression. Using the melanoma cell line B16 as a murine model of pulmonary metastasis, we examined whether the pro- versus antitumoral effects of CD8$^+$ T cells relate to their Ag specificity. Results of the study indicate that although CD8$^+$ T cells specific for tumor Ags promote tumor rejection, CD8$^+$ T cells specific for unrelated Ags promote tumor progression. We found the effect to be partly attributable to CD8$^+$ T cells dampening effective antitumor NK cell responses. Notably, activation of CD8$^+$ T cell responses by an unrelated stimulus, in this case infection with influenza virus, increased the number of pulmonary tumor nodules. These data provide a rationale for previously unexplained data identifying contrasting roles for CD8$^+$ T cells in tumor progression. ***The Journal of Immunology*****, 2010, 184: 607–614.**

It is widely recognized that CD8$^+$ T cells are important for antitumoral responses. The expansion of tumor-specific CD8$^+$ responses can result in effective antitumoral immunity, and indeed this has been the basis for a number of vaccine trials. In contrast to the adaptive immune response, an inflammatory innate response may be ineffective against tumors, and the release of inflammatory mediators from macrophages and neutrophils has been found to augment cancer growth in a number of systems (reviewed in Refs. 1, 2). Thus, an innate immune response is often associated with tumor enhancement, whereas a strong adaptive response is usually associated with an antitumoral effect. Interestingly, recent reports indicate that CD8$^+$ T cells may also be capable of protumoral activity in some systems (3); however, it is not known how these observations can be reconciled with previous data.

Although tumor Ag-specific immune responses are usually antitumoral, there has been little work examining how the adaptive immune response to unrelated Ags might affect tumor growth. This is likely to be of significance in patients who are exposed to Ags and infectious agents throughout tumor growth. There are suggestions that activation of CD8 responses by virus infection can have long-term effects on cell migration to the lung and consequent pulmonary immune responses (4), indicating that a similar situation might occur for tumors.

The current study investigates the hypothesis that CD8$^+$ T cell responses to nontumor Ags can affect growth of melanoma using the B16 model. We found that CD8$^+$ T cells had the capacity to be powerfully protumoral as well as antitumoral and that this occurred in the context of pulmonary but not subcutaneous tumor growth. Protumoral activity did not require Ag stimulation, but was enhanced by it and could occur if stimulated by viral infection. This study has implications for how we view the immune environment in the context of cancer and in the treatment of patients at risk for metastatic cancer.

Materials and Methods

Mice

Specific pathogen-free wild-type (WT) C57BL/6 (B6) mice, Rag.1$^{-/-}$ (5), IL-10$^{-/-}$ (6), IFN-$\gamma^{-/-}$ (7), and F5 TCR transgenic mice (8) were bred and maintained by the Biomedical Services Unit, Cardiff University, Cardiff, U.K. All mice were on a B6 background. Mice were kept in filter top cages or scantainers throughout experiments and provided with water and standard mouse chow ad libitum. All experiments were conducted in accordance with the U.K. Animal (Scientific Procedures) Act 1986.

Cells and cell culture

B16F10 (B16) is a melanoma cell line derived from B6 mice (ATCC number CRL-6475). Cells were maintained in RPMI 1640 with 10% FBS, supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate. The B16 influenza nucleoprotein (np) cell line was generated by transducing B16F10 cells with a retrovirus encoding the enhanced GFP with the A/NT/60/68 (NP68) epitope of influenza A strain E61-13-H17 (H17) (9) as a C-terminal fusion (10).

Tumor model

To model metastatic melanoma growth, mice were injected i.v. with 1–5 × 10^5 B16 cells. Lung nodules were counted after 14 d. To assess the effect of viral infection on lung nodule numbers, mice were infected intranasally with 20 hemagglutination units influenza-H17 at the stated times before or after B16 injection. To model subcutaneous growth, 10^5 B16 were injected s.c. and tumors assessed by palpation and measurement twice weekly.

Cell subset depletions

For depletion of CD8$^+$ cells, mice were treated with 100 μg each of YTS169 and YTS156 mAbs the day prior to injection with B16. NK cells were depleted with 250 μg PK136 (anti-NK1.1) and granulocytic cells depleted with 250 μg RB6-8C5 (anti-Gr1). All depleting Abs were kindly purified from hybridoma supernatants by Siôn Morgan Jones (Cardiff University).

T cell reconstitution

Mice were irradiated with 650 cGy from a [^{51}Cr] source 24 h prior to repopulation with between 1 and 3 × 10^6 CD8$^+$ T cells purified from lymph node suspensions by MACS (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Twenty-four hours after transfer, an emulsified mix

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Abbreviations used in this paper: IFA, incomplete Freund's adjuvant; np, influenza nucleoprotein; WT, wild-type.

of 100 μl incomplete Freund's adjuvant (IFA) and 900 μl PBS with or without 100 μg NP68 peptide was injected s.c. After a further 3 d, 10^5 B16 or B16np were injected i.v., and lung nodules were counted as previously. For reconstitution of Rag.$1^{-/-}$ mice, splenocytes were isolated from B6 or IL-$10^{-/-}$ mice and T cells isolated by MACS. Mice were then injected with 2×10^6 T cells from the relevant genotype. One week after transfer, mice were immunized with IFA/peptide and PBS as above, and after a further 3 d, 10^5 B16 were injected i.v. Lung nodules were counted after 14 d.

Flow cytometric and cytokine analysis

PBS-perfused lungs were homogenized in ice-cold PBS plus 1% FCS (FACS buffer). For cell surface stains, cell suspensions from lung homogenate or bronchoalveolar lavage were washed twice before incubation with appropriate Abs for 20 min on ice. Cells were washed a further two times then resuspended in FACS buffer plus 1% formaldehyde. For intracellular cytokine and CD107a staining, cells were incubated with 20 ng/ml PMA and 1 μg/ml ionomycin for 4 h after harvest with or without CD107a-FITC. One hour after beginning the incubation, Golgi-stop (BD Biosciences, San Jose, CA) was added to the medium. Cells were resuspended in fixation and permeabilization buffer (eBioscience, San Diego, CA) for 20 min on ice, then incubated with appropriate Abs for 20 min in permeabilization buffer. Samples were washed then FACS analyzed as for cell surface staining. Samples were run on a BD FACScaliber and analyzed using CellQuest (BD Biosciences) and FlowJo (TreeStar, Ashland, OR). All Abs were sourced from Pharmingen (BD Biosciences) except CD11b-PE (Caltag Laboratories, Burlingame, CA) and FoxP3-PE (eBioscience).

Statistical analysis

Appropriate tests as shown on individual figures were performed using GraphPad Prism version 3.02 for Windows (GraphPad, San Diego, CA). Probability values <0.05 were considered statistically significant.

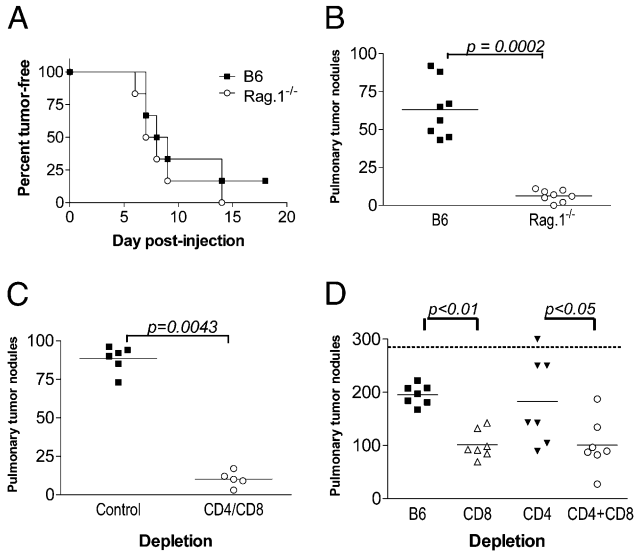

FIGURE 1. T cell-deficient mice are resistant to pulmonary but not subcutaneous B16 growth. *A*, Groups of six mice were injected with 10^5 B16 cells s.c. and observed twice a week for 20 d for appearance of a palpable tumor. *B–D*, Groups of greater than five mice were injected with 10^5 B16 i.v. 14 d before assessing lungs for the number of metastases. *C* and *D*, B6 mice were depleted of CD4 and/or CD8 cells 24 h prior to injection. Lungs in which metastases were uncountable were assigned a value of 300. Data analyzed using Mann-Whitney *U* test (*B* and *C*) or one-way ANOVA (*D*). Data for each set of conditions are representative of at least two separate experiments.

Results

CD8+ T cells can be protumoral for pulmonary melanoma

We initially compared the impact of T cells on s.c. versus pulmonary melanoma growth. T cell deficient Rag.$1^{-/-}$ mice or control immunocompetent B6 (WT) mice were injected with 10^5 B16 cells s.c. or i.v. and monitored for tumor growth. The melanoma grew equivalently in WT and Rag.$1^{-/-}$ mice when injected subcutaneously, but a marked difference in the growth of lung metastases was observed in the two sets of mice (Fig. 1*A*, 1*B*). Surprisingly, T cell–deficient mice typically carried only 10–20% of the tumor burden found in identically treated WT mice. The same effect could be seen in B6 mice concurrently depleted of CD4+ and CD8+ T cells (Fig. 1*C*). Thus, T cells were protumoral in pulmonary but not s.c. growth of B16 melanoma. To determine which T cell subset contributed to tumor growth, mice were depleted either of the CD4+ or CD8+ subsets 24 h prior to i.v. injection with B16 cells. As previously, depleting both subsets led to decreased tumor growth (Fig. 1*D*; $p < 0.01$). Depleting CD8+ cells alone led to a reduction in tumor numbers indistinguishable from depleting both subsets, whereas depleting CD4+ cells had no significant effect. These data indicate that the CD8+ T cells can promote pulmonary growth of B16 melanoma.

The process of metastatic tumor development can be separated into two broad phases: initial tumor take and subsequent tumor growth. Pro- or antitumoral agents can be effective at either or both of these phases. We next asked when in tumor development CD8+ T cells might be important. CD8+ cells were depleted either before i.v. injection of melanoma cells, 24 h postinjection, or well after tumor establishment (6 d). Fig. 2*A* shows that the CD8+ T cell numbers did not recover within the 15 d window of time used. Mice in which CD8+ T cells had been depleted prior to cell injection showed the smallest number of metastases. The number of tumor nodules was still significantly below those seen in undepleted mice if CD8+ cells were depleted 24 h after cell injection ($p = 0.044$). However, if treatment did not begin until 6 d after B16 injection, the number of metastases was very similar to that in control mice. Interestingly, it was found that the early activation marker CD69 was upregulated on >80% of pulmonary CD8+ T cells within 24 h of injection of the B16 cells, then downregulated to background levels within a further 48 h (Fig. 2*C*). CD8 depletion after this time did not have an observable effect on the number of tumor nodules. No changes were seen in the marker of recent degranulation CD107a or the general activation marker CD25 (data not shown). Likewise, no change in CD4 T cell activation was noted (CD25, CD69; data not shown). Thus, the protumoral activity of CD8+ T cells occurs within the very early stages of tumor development, affecting tumor take and possibly to a lesser extent tumor growth.

Protumoral activity of CD8+ T cells is decreased by depleting NK cells

The early protumoral effect of CD8+ T cells appeared at odds with the 5–7 d required even in strongly antigenic situations for the T cell response to become fully activated and significant numbers of T cells to be recruited to the lung (11, 12). It was possible that either the administration of anti-CD8 Abs was directly affecting cells of the innate immune system or that resident pulmonary CD8+ cells were responsible. To investigate possible alterations in other immune cell subsets, mice were treated with anti-CD8 or control Ab as previously and cell populations isolated from the lungs over the crucial first 3 d postinjection and 8 d postinjection. Fig. 3*A* and 3*B* show that numbers of macrophages, eosinophils, and CD4+ T cells were similar between CD8-ablated and control mice over the time course. A small but reproducible decrease was seen in the number of neutrophils attracted into the lungs 24 h after B16 injection. The largest differences in cell numbers between experimental and control mice were within the NK cell population, which was significantly increased in CD8-depleted mice from 48 h postinjection. A comparison of the number of CD69+ NK cells also showed that almost 85% of pulmonary NK cells from CD8-depleted mice were

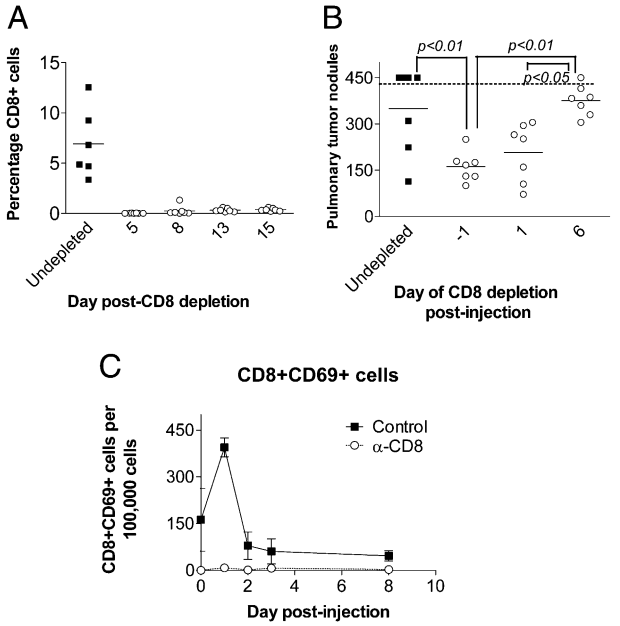

FIGURE 2. CD8 T cells are protumoral in the early stages of tumor development. *A* and *B*, Groups of six to seven B6 mice were depleted of CD8 cells with a single injection of Ab at various times pre- or post-injection with 2×10^5 B16 i.v. The percentage of $CD8^+$ T cells in the spleen was determined at various times postdepletion by FACS analysis (*A*). Lung tumor burden was assessed 14 d later (*B*). Lungs in which metastases were uncountable were assigned a value of 400. Data analyzed using one-way ANOVA. *C*, Pulmonary $CD69^+CD8^+$ T cells were enumerated by FACS as described in *Materials and Methods*. Groups of three per time point; data are representative of two experiments.

activated 24 h after B16 injection compared with ~55% in control mice (Fig. 3*C*). This indicates that pulmonary NK cell activity was increased in CD8-depleted mice.

Given that NK cells are efficiently antitumoral in many tumor models including B16 melanoma (13–16), it could be hypothesized that the removal of $CD8^+$ T cells had uncovered an NK cell response sufficient to reduce the number of tumors by 80%. Thus, it could be further hypothesized that the effect of removing $CD8^+$ T cells could be counteracted by depleting NK cells. We therefore depleted mice of CD8 alone or CD8 as well as NK1.1. Depletion of NK cells substantially reduced the impact of CD8 depletion on tumor numbers (Fig. 3*D*). In control mice depleted of $CD8^+$ T cells, the number of tumors was reduced by 71%. When mice were concurrently depleted of CD8 and NK1.1, tumor numbers were only reduced by 30% (Fig. 3*E*). Thus, anti-CD8 treatment is less effective in NK1.1-depleted animals. In order to determine whether NK cell-mediated cytotoxic activity was enhanced in CD8-depleted mice, NK cells from these and control, unmanipulated mice were analyzed for CD107a expression. It was found that a higher proportion of NK cells expressed CD107a in mice depleted of $CD8^+$ T cells (Fig. 3*F*). Together, these data support a protumoral role for $CD8^+$ T cells by downregulation of otherwise antitumoral NK cells.

IFN-γ and IL-10 have roles in the protumoral activity of CD8 T cells

Previous studies have suggested that IL-10 and IFN-γ secreted by $CD8^+$ T cells or interacting cells may be instrumental in their protumoral activity (3, 17). We investigated each of the cytokines for potential activity.

There is now a body of work supporting $CD8^+$ T regulatory cells in limiting immune responses to tumors, in particular through the expression of IL-10 (18, 19). We examined production of IL-10 postinjection of B16 and detected a rapid increase in IL-10 production from lung $CD8^+$ cells, which was reduced to background levels within 72 h (Fig. 4*A*). This was not seen in $CD4^+$ T cells, which showed minimal changes in IL-10 production. To determine whether T cell production of IL-10 was important for the observed protumoral activity, T cell–deficient Rag.$1^{-/-}$ mice were reconstituted with $CD8^+$ T cells from either WT or IL-$10^{-/-}$ mice before injection with B16 as before. We found that reconstitution of Rag.$1^{-/-}$ mice with WT $CD8^+$ T cells led to a 3.25-fold increase in tumor cell numbers, compared with a 2-fold increase after reconstitution with IL-$10^{-/-}$ $CD8^+$ T cells (Fig. 4*B*). Thus, IL-$10^{-/-}$ $CD8^+$ T cells were less efficiently protumoral.

We had also found a substantial increase in IFN-γ in $CD8^+$ T cells after B16 challenge (Fig. 4*C*). This was not reflected by a concomitant increase in IFN-γ^+CD4^+ T cells. To investigate the role of IFN-γ, control and IFN-$\gamma^{-/-}$ mice were depleted of CD8 as previously and growth of tumor cells examined in the lungs. Depletion of CD8 cells in IFN-$\gamma^{-/-}$ mice did not reduce tumor growth to the same extent as depletion of $CD8^+$ T cells in the control animals (Fig. 4*D*). Interestingly, the increase in NK cells seen in WT mice after CD8 depletion did not occur in IFN-$\gamma^{-/-}$ mice and numbers of NK cells remained below levels seen in CD8-sufficient control mice (Fig. 4*E*). Thus, the data support an instrumental role for IFN-γ in the antitumoral activity of pulmonary NK cells uncovered by depletion of $CD8^+$ T cells.

Protumoral activity is mediated by non–tumor-specific $CD8^+$ T cells

The data thus far indicated that $CD8^+$ T cells are indirectly protumoral through downregulation of potentially antitumoral NK cells. The early stage at which the effect occurs implies that resident $CD8^+$ T cells may be responding nonspecifically to the inflammation stimulated by entry of the tumor cells into the pulmonary circulation rather than through conventional TCR-mediated priming and activation. This implies that the activation is not Ag-specific. To test the role of tumor Ag recognition in the response of T cells to the tumor cell burden, F5 TCR transgenic mice were used. The $CD8^+$ T cells of F5 mice are specific for NP68, a D^b-restricted peptide derived from the np of influenza virus H17 (8). The mice were challenged with either the parent B16 line or B16 engineered to express NP68 (B16np). F5 mice were efficient at clearing B16 cells expressing NP68, with or without further immunization with peptide and adjuvant (Fig. 5*A*). However, the F5 mice proved susceptible to B16 melanoma development. Further, immunization with NP68 peptide and adjuvant led to increased tumor numbers ($p < 0.02$). This demonstrated that F5 mice are capable of a strong antitumoral response if the $CD8^+$ T cells recognize tumor Ag, but have increased tumor development if the T cells recognize an irrelevant Ag.

To ensure that the effect seen was specifically due to $CD8^+$ T cells, the $CD8^+$ T cells were transferred from F5 mice to irradiated recipients, where they were activated with IFA and NP68 peptide. Control mice were immunized with IFA alone or irradiated and immunized with IFA and peptide without reconstitution of the $CD8^+$ T cell population. Three days later, all mice were challenged with control B16 cells or B16np. As can be seen in Fig. 5*B*, B16 and NP68-expressing B16 grew to similar levels in mice immunized with adjuvant alone or in irradiated mice treated with adjuvant and peptide. Irradiation significantly reduced the number of tumor nodules that developed with both cell lines. This could be reversed in B16-challenged mice by transfer of F5 $CD8^+$ T cells and immunization with adjuvant and peptide. Indeed, the number of nodules increased to significantly greater levels than seen in B6 mice treated with adjuvant alone ($p < 0.05$). In contrast,

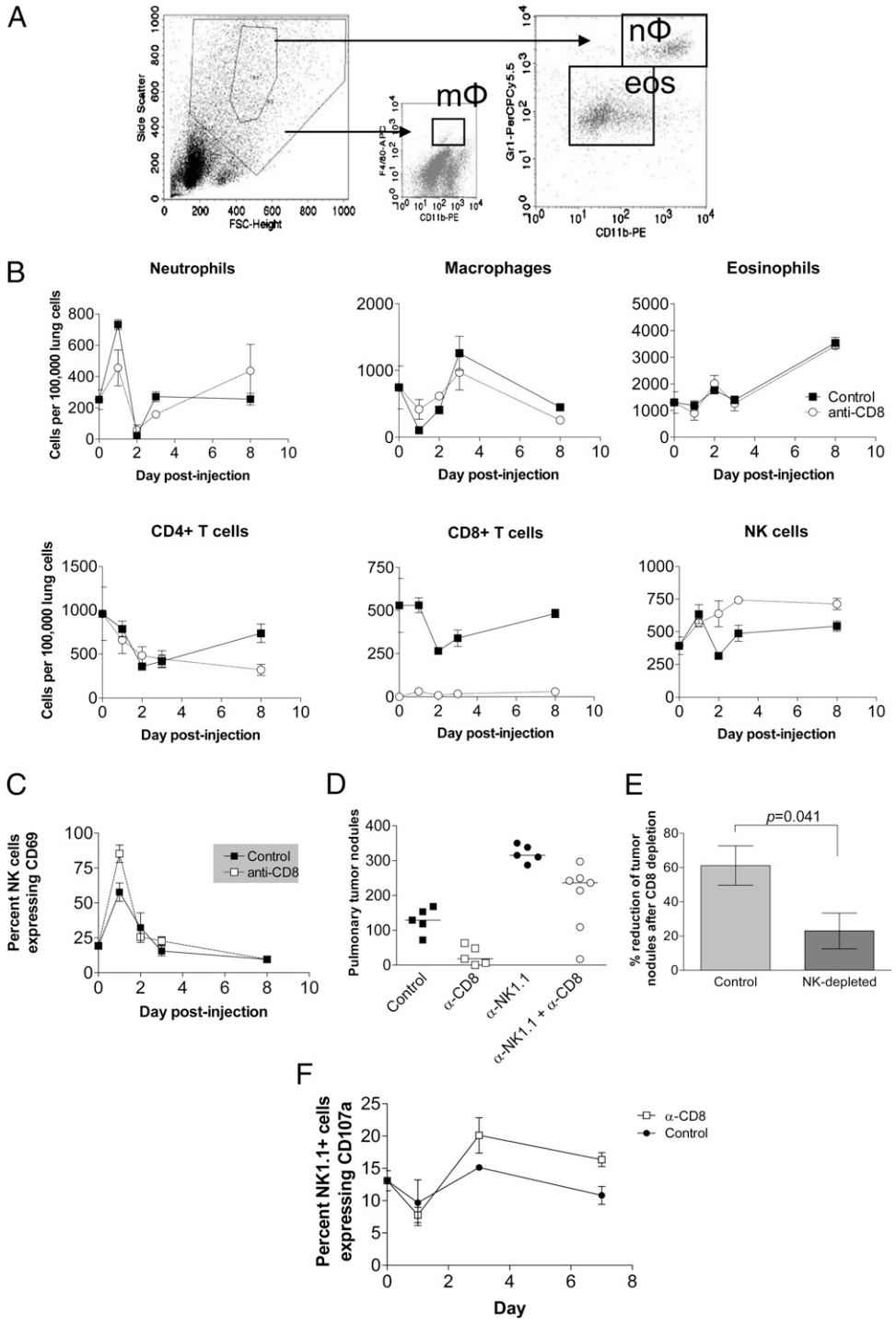

FIGURE 3. Effect of CD8 depletion on other pulmonary immune cell populations. *A*, Classification of eosinophils (eos), macrophages (mΦ), and neutrophils (nΦ) in a typical lung by FACS analysis. *B*, B6 mice were depleted of $CD8^+$ cells (open circles) or treated with a control Ab (closed squares) as previously. Pulmonary cell populations from three mice per group were isolated as per *Materials and Methods* at the given time points and enumerated by FACS. *C*, Bronchoalveolar fluid of three mice per group from CD8-depleted (open circles) or control mice (closed squares) was stained with CD69, NK1.1, CD4, and CD8. $CD69^+NK1.1^+CD8^-CD4^-$ cells were then measured as a percentage of the overall number of $NK1.1^+CD4^-CD8^-$ cells present. *D*, Groups of five or more B6 mice were treated with the indicated Abs 24 h prior to i.v. injection with B16 and lung metastases were counted 14 d later. Data are representative of two separate experiments. *E*, Reduction of tumor nodules in control or NK-depleted mice. The percent reduction of tumor nodules after CD8-depletion was significantly less in NK-depleted mice than control mice (Mann-Whitney *U* test). *F*, Depletion of $CD8^+$ cells results in increased cytolytic activity of NK cells. B6 mice were depleted of $CD8^+$ cells as previously, or treated with a control Ab 24 h before i.v. injection with B16. At various time points, pulmonary cells were collected and stained for NK1.1 and CD107a as per *Materials and Methods*. $CD107a^+NK1.1^+$ cell numbers were significantly higher in CD8-depleted mice at d3 and d8 (Mann-Whitney *U* test).

reconstitution with F5 $CD8^+$ T cells enabled rejection of B16np tumor cells. These data show that $CD8^+$ T cells that are not responding to tumor Ags have protumoral activity that can be amplified by exposure to their cognate Ag. Further, $CD8^+$ T cells in otherwise identical situations can be either pro- or antitumoral depending on whether the cells are specific for a tumor Ag and

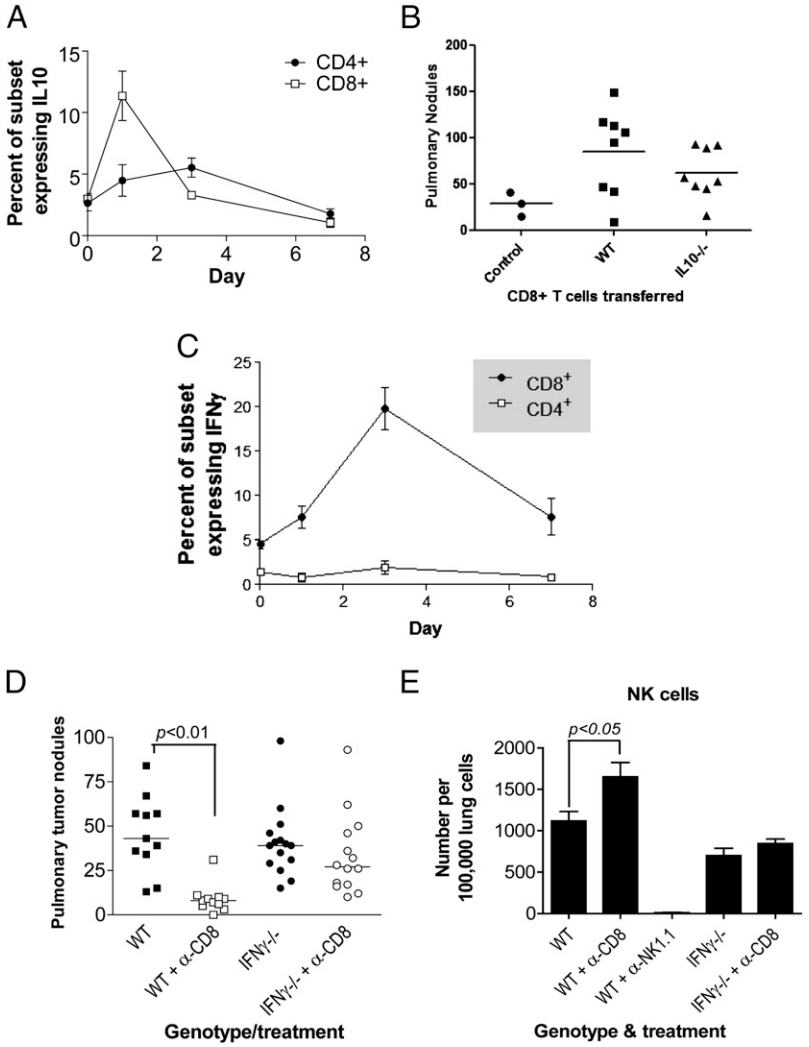

FIGURE 4. IFN-γ and IL-10 are important for differing aspects of the observed protumoral effect of CD8+ T cells (*A*). IL-10 expression in $CD4^+$ and $CD8^+$ T cells was determined by intracellular cytokine staining of lung lymphocytes. B6 mice were treated with anti-CD8 or a control Ab as previously. Cell populations from three mice per group were isolated from the lungs at the given time points and Ab stained as per *Materials and Methods* before enumeration by FACS. *B*, Groups of greater than seven Rag.$1^{-/-}$ mice were either left unpopulated or repopulated with $CD8^+$ T cells derived from B6 or IL-$10^{-/-}$ mice as per *Materials and Methods*. After repopulation, the T cells were rested for 1 wk before i.v. injection with 10^5 B16. Lungs were assessed for the number of metastases after 14 d. *C*, IFN-γ expression in $CD4^+$ and $CD8^+$ T cells was determined by intracellular cytokine staining of lung lymphocytes. B6 mice were treated with anti-CD8 or a control Ab as previously. Cell populations from three mice per group were isolated from the lungs at the given time points and Ab stained as per *Materials and Methods* before enumeration by FACS. *D*, Groups of five or more mice were treated with anti-CD8 24 h prior to i.v. injection with a tumorigenic dose of B16 (10^5 B16 for B6 [WT] and 2×10^4 B16 for IFN-$\gamma^{-/-}$ mice). Lung metastases were counted 14 d later. Data pooled from two separate experiments with equivalent results. The number of nodules was significantly different after CD8 depletion in B6 ($p < 0.01$) but not IFN-$\gamma^{-/-}$ mice by one-way ANOVA. *E*, B6 (WT) and IFN-$\gamma^{-/-}$ mice were depleted of CD8 or treated with a control mAb as previously, and NK cells in the lung were enumerated by FACS. Depleting CD8 resulted in significantly more NK cells in the lungs of B6 mice ($p < 0.05$) but not in IFN-$\gamma^{-/-}$ mice. Data compared using one-way ANOVA.

argue against the $CD8^+$ T cells belonging to separate suppressor or regulatory subsets. Thus, although activation of tumor-specific pulmonary $CD8^+$ T cells is antitumoral, activation of unrelated $CD8^+$ T cells enhances the development of melanoma nodules in the lung.

Activation by flu infection can enhance tumor growth

The data thus far describe an antitumoral role for $CD8^+$ T cells that recognize tumor Ags and a protumoral capacity for those directed toward nontumoral Ags. This implies that activation of a T cell response by a pathogen may promote tumor progression. To determine whether $CD8^+$ T cell activity might have a role in metastatic tumor growth in a physiologically relevant situation, we next examined whether T cell activation by a pulmonary viral infection could affect tumor growth in the lung. Influenza virus creates an effective T cell response that peaks from days 7 to 10 of infection (20). To determine whether the response to a virus could affect tumor growth, mice were infected with influenza 8 d prior to injection with B16 or B16np. For one group, mice were also depleted of $CD8^+$ cells 24 h before injection. Fig. 6*A* shows that at 8 d postinfection, the number of tumor metastases was enhanced for B16 compared with uninfected mice. This corresponded to increased pulmonary $CD8^+$ T cell numbers (Fig. 6*B*). Removing the CD8 response after influenza infection by Ab treatment resulted in an 85% reduction in tumor numbers. The T cells themselves were clearly functional, as B16 bearing the flu np Ag were cleared, and numbers of $CD8^+$ T cells were similar in both groups receiving B16 cells expressing and not expressing the np (Fig. 6*A*, 6*B*). In addition, the number of $CD69^+$ NK cells was selectively increased in the group in which $CD8^+$ cells had been depleted (Fig.6*C*), consistent with previous experiments. Hence the $CD8^+$ response to the virus was capable of clearing Ag-bearing tumor cells, but enhanced the growth of tumor cells not expressing the appropriate Ag. This corresponded with an enhancement in NK cell activation.

Discussion

There is emerging evidence that $CD8^+$ T cells have a Jekyll-and-Hyde–like aspect to their responses to cancer. There have been many studies correlating infiltration of $CD8^+$ T cells with a good prognosis (21–24), and tumor-specific $CD8^+$ T cell responses have been shown to be capable of robust antitumoral activity. However, Filaci and colleagues (19) recently characterized $CD8^+CD28^-$ regulatory T cells in a number of different human cancers and found a clear correlation between increased infiltration and the later stages of cancer. Protumoral $CD8^+$ T cells have also recently been described in a chemically induced tumor model (17) in which perforin-negative $CD8^+$ T cells were found to infiltrate s.c. carcinomas. These cells expressed increased inflammatory cytokines and depletion of $CD8^+$ cells resulted in a reduction in tumorigenicity. However, the in situ development of the tumors did not allow the Ag specificity of the $CD8^+$ T cells to be examined. The current study provides clear evidence that $CD8^+$ T cells can

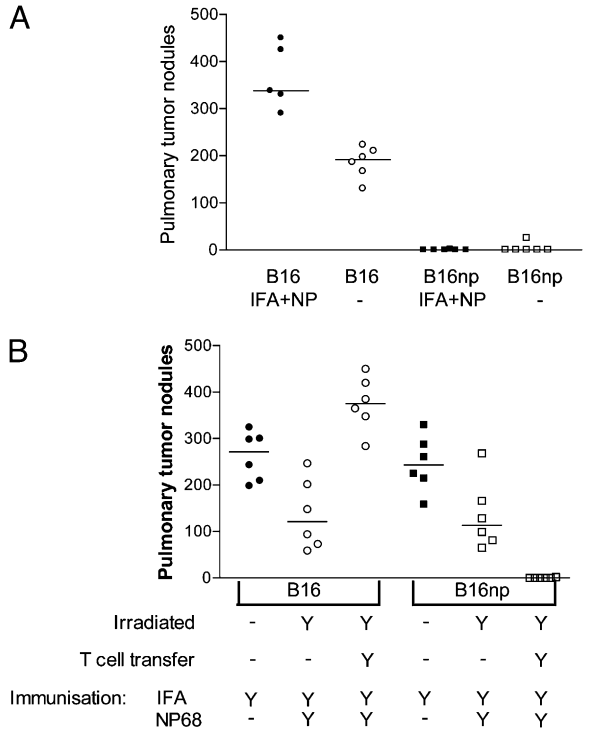

FIGURE 5. Tumor-specific activation of CD8+ T cells results in tumor elimination, whereas immunization by unrelated Ags results in enhanced tumor growth. *A*, Groups of five F5 mice were injected s.c. with 100 μg NP68 peptide in 100 μl IFA or left unimmunized. Three days later, mice were injected i.v. with 10^5 wt B16 (B16) or B16 carrying the NP68 epitope (B16np). Metastases were evaluated at 14 d post B16 injection. More metastases were seen in IFA + NP68 immunized mice than control mice ($p < 0.001$) and WT B16 in both immunized and unimmunized mice resulted in more metastases than B16np-injected mice ($p < 0.001$). Data compared using one-way ANOVA. *B*, Groups of six B6 mice were sublethally irradiated and either left unpopulated or repopulated with F5 CD8 T cells. Control groups, indicated by filled symbols, were not irradiated. Mice were immunized with 100 μl IFA alone or 100 μl IFA with 100 μg NP68 and 10^5 B16 or B16np injected i.v. 3 d later. After 14 d, lungs were assessed for the number of metastases. Data compared using one-way ANOVA.

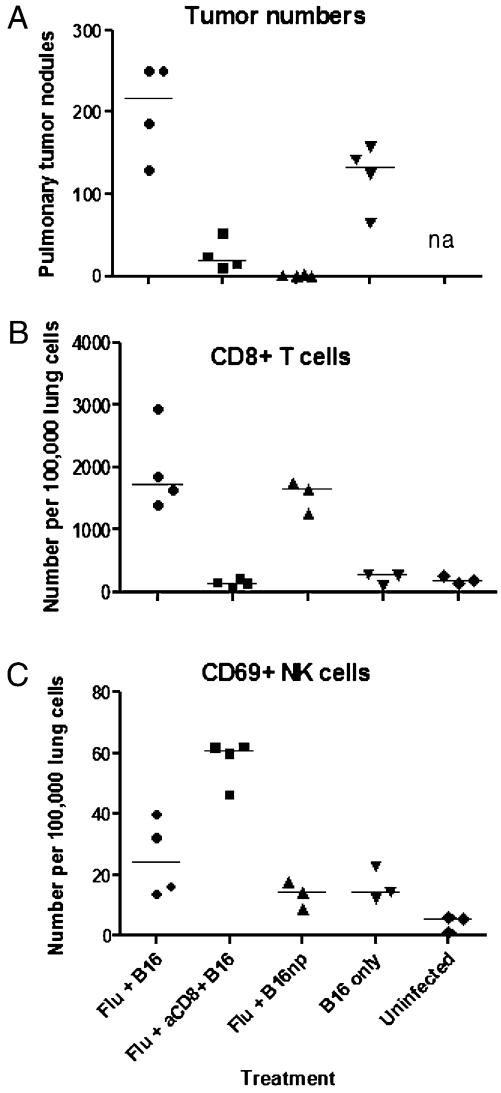

FIGURE 6. Tumor growth is enhanced by a pre-existing inflammatory response. B6 mice were infected intranasally with 20 hemagglutination units of influenza. At 7 d, one group was depleted of CD8 as previously. At 8 d postinfection, mice were injected with 2.5×10^5 B16 or B16np i.v. Mice were assessed for tumor growth 14 d later (*A*) or assessed for pulmonary $CD8^+$ T cell numbers (*B*) and $CD69^+$ NK cell numbers (*C*) by FACS 24 h after tumor cell injection. Data were compared using one-way ANOVA with Tukey's post hoc test. Tumor numbers were greater in mice infected with influenza than all other groups ($p < 0.05$). $CD69^+$ NK cell numbers were greater in the absence of $CD8^+$ T cells than in any other group ($p < 0.01$).

be protumoral and helps explain the contradictory data showing that $CD8^+$ T cells are capable of both pro- and antitumoral effects.

In these experiments, we have shown that mice treated with CD8-specific depleting mAbs exhibited a decrease in the number of lung nodules following i.v. inoculation with B16 cells. Cells other than T cells (e.g., $CD8^+$ dendritic cells) could be targeted by the depleting CD8-specific mAb. However, we observe that mice lacking T cells, but not $CD8^+$ dendritic cells, are protected against tumor growth (Fig. 1) and that reconstitution of Rag.$1^{-/-}$ mice with purified $CD8^+$ T cells exhibit enhanced tumor growth (Fig. 4). Collectively, these data support a central role for $CD8^+$ T cells in the observed protumoral effects.

The current study does not support the hypothesis that the protumoral $CD8^+$ T cells are a separate T suppressor cell or $CD8^+$ regulatory T cell lineage. The most compelling evidence is that generated in the NP68-specific $CD8^+$ T cell transfer experiments. These clearly showed that in otherwise identical conditions, activated F5 $CD8^+$ T cells enhanced the growth of B16 lung metastases that could not be recognized, but were capable of clearing B16 lung metastases bearing the cognate Ag. These data indicate that same $CD8^+$ T cell population has the ability to promote or control tumor cells, at least in the lung. In the absence of specific tumor cell recognition, the dominant effect of the T cell population is to promote tumor progression, but when the tumor is specifically recognized by $CD8^+$ T cells, tumor cells are killed by the cytotoxic action of these T cells, thus the net effect of the interaction is to limit rather than facilitate tumor progression.

The ability of $CD8^+$ T cells to facilitate tumor progression can be attributed, at least in part, to their ability to suppress the activity of NK cells. This is supported by our findings that NK cell numbers are increased in the lungs of CD8-depleted mice, and the impact of $CD8^+$ depletion on tumor development is reduced in mice lacking NK cells. Indeed, it is clear from several other studies that NK cells can play an important antitumoral role (13–16). Data from virus infections may parallel our findings. Recent studies of mouse hepatitis virus and murine cytomegalovirus have shown that depletion of T cells (25), specifically of $CD8^+$ T cells (26), results in a stronger antiviral NK response. Further, Sun and colleagues (27) have described IL-10 and IFN-γ as being central

to the $CD8^+$ T cell–mediated suppression of innate immune responses. Interestingly, the latter has only examined this question through whole mouse blockade of the cytokine receptor; our data now pins this specifically to T cell–derived IL-10. Our data suggests that a similar suppression of innate responses occurs when $CD8^+$ cells are depleted during tumor development. A possible model is that NK cells and T cells exist in a dynamic balance within non-inflamed tissues. Inflammatory signals would allow the NK cell response to emerge, and, if effective, the inflammatory stimulus would be removed, thus the adaptive T cell response would not be induced. If the NK cell response is not effective, the upregulation of T cells would then tip the balance toward a dampening of the NK response. However, if the adaptive response is directed toward an inappropriate Ag, it may impede the NK response while itself being ineffective in clearing the pathogen or tumor. Further, in the case of tumors, the ongoing though ineffective immune response could facilitate chronic inflammation, which may further promote tumor progression (reviewed in Ref. 1). In support of this model, data has been previously published supporting downregulation of NK cell activity against tumor cells, in vitro and in vivo, by T cells isolated from B16 tumors (28). The same study reported that the supernatant from the T cells was also capable of preventing NK cytotoxicity and suggested that the suppression was cytokine mediated. These data are consistent with our findings that IFN-γ and IL-10 are central to pulmonary protumoral responses.

The trigger for the early increase in IL-10 and IFN-γ production from $CD8^+$ T cells is not yet clear. The increase in CD69 expression on $CD8^+$ T cells in the lung within 24 h of injection of B16 (Fig. 2*C*) shows that the tumor cells induce T cell activation, perhaps indirectly through activation of the innate immune system. Thus, $CD8^+$ T cells may be triggered to produce IL-10 in response to such nonspecific inflammatory stimuli. We further hypothesize that the immune response to the tumor cells differs between tissues. We found that depletion of $CD8^+$ cells promoted tumor rejection in the lung, but had no impact on s.c. tumor growth. Differences in the antitumoral activity of treatments in the pulmonary and s.c. systems have also been noted by other investigators (29). Although the basis of this finding must be explored further, it appears that immune cells exert distinct effects in different microenvironments.

A similar situation may occur in humans. Although melanoma metastases per se have not, to our knowledge, been examined in patients with respect to previous infection, several epidemiological papers have examined whether previous infections correspond with lung cancer. Chronic bronchitis, pneumonia, and emphysema are all consistently associated with an increased risk of lung cancer; interestingly, data are less consistent with respect to asthma and tuberculosis (30–33). It is of note that damage is Th1- and/or CD8-dependent in chronic bronchitis, pneumonia, and emphysema (34, 35), whereas the responses are more ambiguous in tuberculosis (36) and Th2-skewed in asthma (37). Data presented in the current study provide an intriguing potential link between epidemiological findings and the immunology of cancer.

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Disclosures

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