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Cutting Edge: CD4$^+$ T Cell Control of CD8$^+$ T Cell Reactivity to a Model Tumor Antigen

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Neoantigens resulting from the inherent genomic instability of tumor cells generally do not trigger immune recognition. Similarly, transfection of tumors with model Ags often fails to elicit CD8$^+$ T cell responses or alter a tumor's growth rate or lethality. We report here that the adoptive transfer of activated Th1-type CD4$^+$ T cells specific for a model tumor Ag results in the de novo generation of CD8$^+$ T cells with specificity to that Ag and concomitant tumor destruction. The anti-tumor effects of the CD4$^+$ T cells required the presence of both MHC class I and class II on host cells, as evidenced by experiments in knockout mice, suggesting that CD4$^+$ T cells enhanced the ability of host APC to activate endogenous CD8$^+$ T cells. These results indicate that the apparent inability of tumor cells expressing highly immunogenic epitopes to activate tumor-specific CD8$^+$ T cells can be altered by activated CD4$^+$ T cells. *The Journal of Immunology*, 2000, 164: 562–565.

Most tumor cells are characterized by genetic instability, which results in deletions and additions to the tumor genome (1) and leads to the generation of new tumor Ags. These neoantigens often fail to elicit immune recognition and tumor destruction. In mouse models, tumor cells have been transfected with genes encoding a variety of highly immunogenic Ags with no change in the tumor's growth rate or lethality. These Ags include OVA (2), glycoprotein from lymphocytic choriomeningitis virus (3), nucleoprotein (4), and hemagglutinin (5) from influenza A virus and β-galactosidase (β-gal)[2] from *Escherichia coli* (6).

CD8$^+$ T cells have long been thought to play a central role as immune effectors in the tumor setting. However, while *LacZ*-transfected tumor cell lines can be recognized by β-gal-specific CD8$^+$ T cells, they do not elicit β-gal-specific CD8$^+$ T cells. Why is this the case? β-Gal is large (1023 aa) and contains many potential immunogenic epitopes. Ag-specific cytolytic responses are efficiently elicited after immunization of mice with a variety of different recombinant viruses encoding *LacZ* (7).

CD4$^+$ T cells may serve to control the activation and persistence of the CD8$^+$ T cell response, because they play a major role in the induction of autoimmune disease and in the orchestration of antiviral immunity (8–11). CD8$^+$ T cells specific for viral Ags have been found to be inhibited under conditions in which CD4$^+$ T cell help was lacking (12). To explore the impact of CD4$^+$ T cells on Ag-specific CD8$^+$ T cells in the tumor setting, we generated a β-gal-specific, IL-2-secreting, Th1-type CD4$^+$ T cell clone, B12, and used a *LacZ*-transduced tumor, WP4.β-gal, as a target. Using this experimental system, we address the question: What are the effects of Ag-specific CD4$^+$ T cells on the function of endogenous, Ag-specific CD8$^+$ T cells?

Materials and Methods

Generation of CD4$^+$ T cell clones

C57BL/6 mice (National Cancer Institute, Frederick, MD) were immunized with a vaccinia virus expressing β-gal (13). Three weeks later, spleens were harvested and CD4$^+$ cells were isolated by passage over a negative selection column (R&D Systems, Minneapolis, MN). CD4$^+$ T cells were plated at 8×10^5 cells/well in 24-well plates in complete media (6). Irradiated syngeneic splenocytes pulsed with 100 μg/ml of β-gal protein (Sigma, St. Louis, MO) were added at 2×10^6 cells/well every 2 wk. Recombinant IL-2 (Chiron, Emeryville, CA) was added on day 3 of culture at 5 CU/ml. Reactivity after limiting dilution cloning was assessed by incubation of 2×10^5 CD4$^+$ T cells with 2×10^5 splenocytes pulsed with 100 μg/ml β-gal protein. Supernatants were evaluated for the presence of IL-4 and IL-2 by ELISA (Endogen, Woburn, MA). Control CD4$^+$ T cell clones were similarly generated from mice immunized with OVA protein (Sigma) in IFA. FACS analysis was performed on the T cell clone using anti-CD4 (L3T4) and anti-CD8 (Ly-2) Abs (PharMingen, San Diego, CA). Stained cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA).

For Ab blocking experiments, 10^5 effectors were incubated with 1×10^5 splenocytes in the presence of 20 μg/ml of Abs specific for I-A^b (M5/114.15.2), CD4 (L3T4), or MHC class I (28-8-6) (PharMingen). After 30 min, 100 μg/ml of OVA or β-gal protein was added. Supernatants were assayed for the presence of IL-2 by ELISA (Endogen).

Tumors

The weakly immunogenic, methylcholanthrene-induced MCA-205 fibrosarcoma and a clone of this line, WP4.WT, have been described previously (14). WP4.WT was retrovirally transduced with an LSXN-based retrovirus encoding the *LacZ* gene driven by a long terminal repeat to produce the WP4.β-gal line, which was cloned by limiting dilution and selected for high expression of β-gal as assessed by 5-bromo-4-chloro-3 indoyl β-D-galactoside (X-Gal) staining.

In vivo experiments

A total of 5×10^5 tumor cells were injected i.v., and CD4$^+$ T cells were transferred i.v. 3 days later. Mice were sacrificed on day 14–16 after tumor

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[2] Abbreviations used in this paper: β-gal, β-galactosidase; KO, knockout; β_2m, β_2-microglobulin; DC, dendritic cell.

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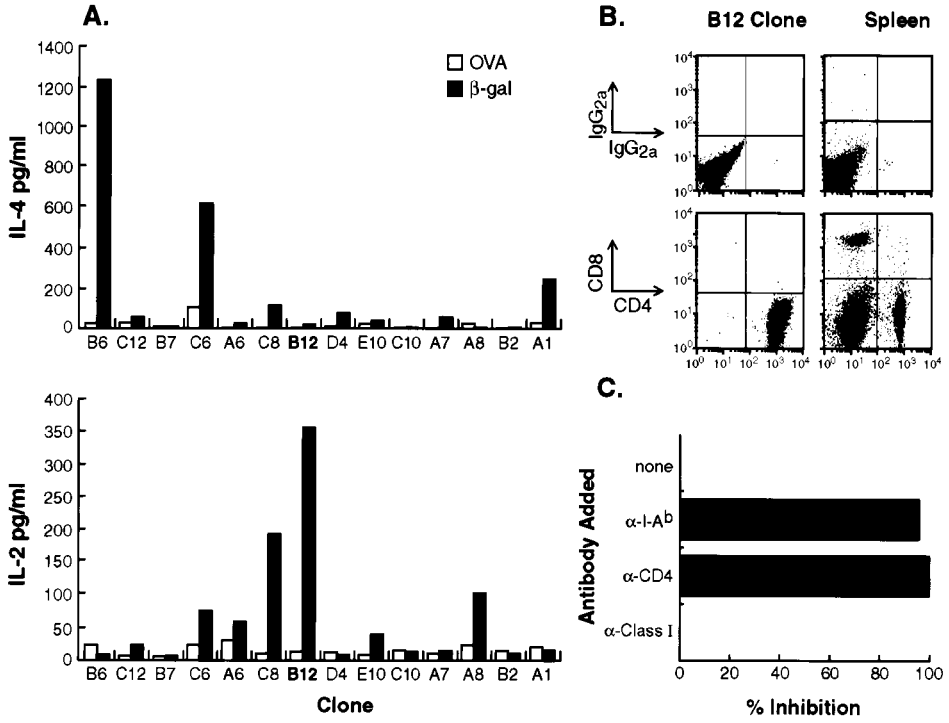

FIGURE 1. Generation of a I-A^b-restricted, β-gal-specific, CD4$^+$, Th1-type clone. *A*, Clones obtained by limiting dilution of a β-gal-specific, CD4$^+$ bulk line were assayed for release of IL-4 (*top*) or IL-2 (*bottom*) when cocultured in the presence of splenocytes pulsed with OVA or β-gal protein. *B*, The B12 clone was confirmed to be CD4$^+$ by FACS analysis. *C*, β-gal-specific IL-2 secretion by the B12 clone was CD4 dependent and I-A^b restricted as evidenced by blocking experiments.

injection, lungs were removed and stained with india ink, and lung nodules were counted in a blinded fashion. In Ab-depletion experiments, mice were given the mAb 2.43 as described (2). Depletion was confirmed by FACS analysis. Class II knockout (KO) and β_2-microglobulin (β_2m) KO mice (C57BL/6TacfBR-[KO]Aβ^bN5 and C57BL/6GphTacfBR[KO]β_2mN5) and their respective controls are on a B6 background and were obtained from Taconic (Germantown, NY).

To generate CD8$^+$ cultures, mice were inoculated with tumor cells then treated with 3×10^5 CD4$^+$ T cells 3 days later. Fifteen days later, splenocytes were harvested then incubated in upright T25 flasks (two spleens pooled per three flasks) in 20 ml of culture medium with 1 μM peptide for 6 days. The β-gal$_{96-103}$ peptide used was DAPIYTNV (15). Irrelevant peptides varied between experiments and included NP$_{366-374}$ (ASNENMETM), hgp100$_{25-33}$ (KVPRNQDWL), or mgp100$_{25-33}$ (EGSRNQDWL). IFN-γ release was measured in supernatants after overnight incubation of 2×10^5 cells with 1 μM peptide added directly to the culture well.

Results and Discussion

Generation of an experimental tumor system using β-gal as a model Ag

To study the effects of Ag-specific CD4$^+$ T cells on the function of endogenous, Ag-specific CD8$^+$ T cells, we generated a β-gal specific, CD4$^+$ T cell population from mice immunized with a vaccinia virus containing the *LacZ* gene. After enrichment for CD4$^+$ T cells, splenocytes were restimulated in vitro with β-gal protein. The β-gal-specific T cell line was cloned by limiting dilution, yielding two types of specific clones: those that secreted predominantly Th1-type cytokines, and those that secreted predominantly Th2-type cytokines (Fig. 1*A*).

One Th1-type CD4$^+$ (Fig. 1*B*) clone, B12, produced IL-2 and IFN-γ, but not IL-4 or IL-10, and grew continuously for over 9 mo, allowing for further study. Ab-blocking experiments showed that the β-gal reactivity was class II restricted and CD4 dependent (Fig. 1*C*). As a β-gal-expressing tumor target, we employed a *LacZ*-transfected WP4.β-gal subclone, which was shown to be able to process and present Ags on its surface because it could be recognized by K^b-restricted, β-gal-specific CD8$^+$ T cells (not shown).

CD4$^+$ T cell clone mediates regression of β-gal-expressing tumor in vivo: requirement for host MHC class I and class II molecules

To evaluate if the B12 β-gal-specific, CD4$^+$ T cell clone could mediate tumor destruction, the WP4.β-gal tumor was injected i.v. and allowed to establish for 3 days. Groups treated with the B12 clone had significantly fewer pulmonary nodules than untreated controls at doses as low as 1×10^5 cells in titering experiments (data not shown). Treatment of WP4.β-gal with 3×10^5 B12 clone significantly reduced the average number of lung nodules in repeated experiments (for example from 85 to 17 in Fig. 2*A* or 174 to 6 in Fig. 2*D*). The presence of host CD8$^+$ cells was required for this effect (Fig. 2*B*). To determine the specificity of the observed tumor treatment, the parental WP4.WT was included in the same experiment. No therapy was observed in mice bearing the parental tumor, indicating that tumor expression of β-gal was required (Fig. 2*C*). Adoptive transfer of a control Th1-type CD4$^+$ T cell clone specific for OVA had no effect on the β-gal expressing tumor (not shown).

No specific release of IL-2 was observed upon coculture of the CD4$^+$ B12 clone with tumor. As is characteristic of mouse sarcomas, the WP4.WT and WP4.β-gal tumor cell lines stained positive for MHC class I but negative for MHC class II (not shown), even after treatment with IFN-γ. In contrast, the expression of I-A^b was up-regulated on the B16 melanoma control cell line after incubation with IFN-γ, as previously shown (16).

CD4$^+$ T cells are known to interact with MHC class II-expressing host cells, especially dendritic cells (DC), altering their ability to activate other components of the immune system. To assess whether the presence of host MHC class I and MHC class II was required for tumor eradication after adoptive transfer of the CD4$^+$ B12 clone, we performed experiments using the WP4.β-gal tumor in KO mice with homozygous deficiencies in β_2m (Fig. 2*E*) or MHC class II (Fig. 2*F*). The B12 clone was therapeutically ineffective against the WP4.β-gal tumor in both strains, indicating

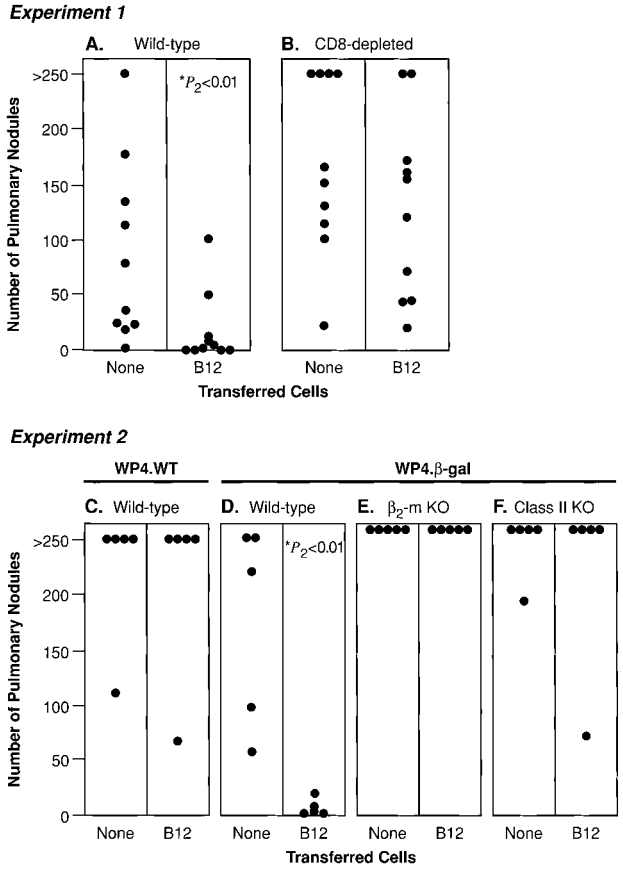

FIGURE 2. Adoptive transfer of the B12 clone results in eradication of pulmonary nodules established for 3 days. Mice were given 3×10^5 B12 clone 3 days after i.v. injection of tumor. Pulmonary nodules were enumerated in a coded, blinded fashion 14–16 days later in repeated experiments. Representative experiments are shown. The ability of the B12 clone to mediate tumor regression was assessed in wild-type mice (*A*) and in mice that were depleted of $CD8^+$ cells (*B*). Ag specificity was determined by treatment of mice bearing either the WP4.WT (*C*) or the WP4.β-gal tumor (*D–F*). Identically treated β_2m KO mice and MHC class II KO mice are shown in *E* and *F*, respectively (*, $p_2 = 0.007$ in *A* and $p_2 = 0.008$ in *D*, no treatment vs treatment with B12, as assessed by the Kruskal-Wallis test).

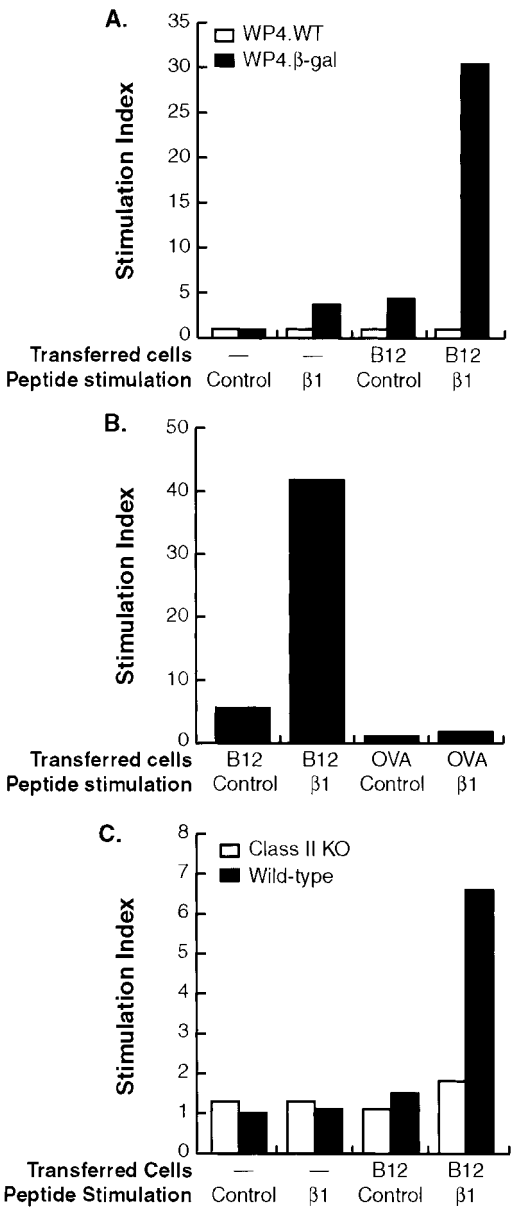

FIGURE 3. De novo generation of β-gal-reactive $CD8^+$ T cells occurs only in mice treated by adoptive transfer of B12 clone. *A*, Spleens from mice bearing WP4.WT or WP4.β-gal tumor and treated with 3×10^5 B12 clone or with nothing were removed 12 days after treatment and restimulated for 6 days with the MHC class I-restricted β-gal$_{96-103}$ peptide or control peptide. Splenocytes were then cultured for 24 h with β-gal$_{96-103}$ or control peptide. Stimulation index was calculated by dividing the amount of Ag-specific IFN-γ released by nonspecific release. *B*, Mice receiving WP4.β-gal tumor were treated with B12 clone or an OVA-reactive clone. Twelve days later, spleens were removed, restimulated, and assayed as above. *C*, Class II KO or wild-type mice bearing WP4.β-gal were treated with B12 clone or with nothing. Spleens were removed, restimulated, and assayed as above. The results shown are the averages from three independently performed experiments in *A* and *C* and two independently performed experiments in *B*.

a requirement for both MHC class I and MHC class II expression by host cells.

Antitumor effect of $CD4^+$ T cells is through an indirect mechanism involving the recruitment of host Ag-specific $CD8^+$ T cells

APC, such as DC, can cross-present shed β-gal tumor Ag in the context of both MHC class I and class II molecules. The interaction of a host APC with Ag-specific $CD4^+$ T cells can make it capable of stimulating naive, endogenous $CD8^+$ cytotoxic T cells, which may then function as specific effector cells capable of lysing tumor. $CD8^+$ T cells were important effector cells in our model because depletion of $CD8^+$ T cells in wild-type mice abrogated the therapeutic effects of the $CD4^+$ T cell clone (Fig. 2, *A* and *B*).

To directly assess the induction of Ag-specific host $CD8^+$ T cells, fresh splenocytes from tumor-bearing mice that had been treated with the B12 clone were cocultured with the K^b-restricted, immunodominant β1 epitope from β-gal (15). In three experiments, potent β-gal-specific $CD8^+$ T cell responses were elicited (Fig. 3*A*). $CD8^+$ T cells were generated only from mice receiving the B12 helper clone that were then exposed to the β-gal peptide, but not a control peptide, ex vivo. $CD8^+$ T cell responses were not elicited in nontumor bearing mice whose peptide-stimulated splenocytes exhibited a stimulation index of 0.9, nor were any Ag-specific $CD8^+$ T cells seen in mice bearing the parental tumor.

The transfer of a similarly generated, IL-2-secreting, OVA-specific $CD4^+$ Th clone into WP4.β-gal bearing mice did not induce

β-gal-specific $CD8^+$ T cells (Fig. 3*B*). These findings are consistent with experiments described above in which we failed to observe a reduction of WP4.β-gal nodules in mice treated with OVA-specific $CD4^+$ T cells. Finally, no specific $CD8^+$ T cells were induced after transfer of the B12 clone to MHC class II KO mice (Fig. 3*C*). This finding correlated with the failure of the B12 clone to eradicate tumor in MHC class II KO mice (see Fig. 2).

Implications for immune activation of anti-tumor $CD8^+$ T cells

The data presented here point to an active cooperation between $CD4^+$ and $CD8^+$ T cells in the eradication of tumor cells. The adoptive transfer of $CD4^+$ T cells has been reported to treat established tumor (17–19); however, $CD4^+$ T cells in these systems were hypothesized to act through NK or macrophage effector cells or by direct lysis of a MHC class II-positive tumor. Ossendorp et al. have found that generation of specific $CD4^+$ T cells through immunization with a helper epitope resulted in increased anti-tumor activity that is mediated by $CD8^+$ effector cells, even when the tumor cells used are MHC class II negative (20). The present manuscript is the first in which the transfer of $CD4^+$ T cells specific for a model tumor Ag have been found to elicit the de novo generation of $CD8^+$ T cells specific for that same Ag.

$CD8^+$ T cells have been widely reported to transfer tumor immunity and to treat established tumors upon adoptive transfer (21, 22). They are thought to work by directly destroying tumor cells in situ. In contrast, adoptive transfer of the $CD4^+$ T cell clone described here activates endogenous $CD8^+$ effector cells. The activation of specific $CD8^+$ T cells by $CD4^+$ T cells may be most efficient when cells recognize the same Ag presented by a DC, in the context of both MHC class I and class II molecules. Recognition of DC by $CD4^+$ T cells can lead to IL-2-production resulting in proliferative effects on proximate $CD8^+$ T cells. Perhaps more importantly, $CD4^+$ T cells activate DC, enhancing their ability to stimulate naive $CD8^+$ T cells (23, 24). DC "conditioning" is characterized, in part, by the up-regulation of CD80/CD86 and MHC class I and II levels and the production of IL-12 (25), a process mediated in large part by the interaction of CD40, expressed on the APC, and CD40 ligand, expressed on activated helper cells (26).

Thus, we propose that DC do not fully mature in the microenvironment of the tumor, where help is limiting and inflammatory signals are lacking. The striking ability of $CD4^+$ T cells to treat established tumors in this model system suggests that these DC are then unable to activate naive $CD8^+$ T cells, important mediators of tumor destruction. Provision of Ag-specific $CD4^+$ T cells by adoptive transfer or by activation in vivo after vaccination may result in the de novo induction of $CD8^+$ T cell function in patients with cancer.

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