Melan-A–specific Cytotoxic T Cells Are Associated with Tumor Regression and Autoimmunity Following Treatment with Anti-CTLA-4

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Abstract **Purpose:** Ipilimumab is a monoclonal antibody that blocks the immune-inhibitory interaction between CTL antigen 4 (CTLA-4) and its ligands on T cells. Clinical trials in cancer patients with ipilimumab have shown promising antitumor activity, particularly in patients with advanced melanoma. Often, tumor regressions in these patients are correlated with immune-related side effects such as dermatitis, enterocolitis, and hypophysitis. Although these reactions are believed to be immune-mediated, the antigenic targets for the cellular or humoral immune response are not known.

Experimental Design: We enrolled patients with advanced melanoma in a phase II study with ipilimumab. One of these patients experienced a complete remission of his tumor. The specificity and functional properties of CD8-positive T cells in his peripheral blood, in regressing tumor tissue, and at the site of an immune-mediated skin rash were investigated.

Results: Regressing tumor tissue was infiltrated with CD8-positive T cells, a high proportion of which were specific for Melan-A. The skin rash was similarly infiltrated with Melan-A–specific CD8-positive T cells, and a dramatic (>30-fold) increase in Melan-A–specific CD8-positive T cells was apparent in peripheral blood. These cells had an effector phenotype and lysed Melan-A–expressing tumor cells.

Conclusions: Our results show that Melan-A may be a major target for both the autoimmune and antitumor reactions in patients treated with anti-CTLA-4, and describe for the first time the antigen specificity of CD8-positive T cells that mediate tumor rejection in a patient undergoing treatment with an anti-CTLA-4 antibody. These findings may allow a better integration of ipilimumab into other forms of immunotherapy.

CTL antigen 4 (CTLA-4), a member of the CD28 family, is one of the key molecules involved in regulating the activity of T lymphocytes. It is mobilized from an intracellular pool to the cell surface of T lymphocytes following activation. It then competes with the costimulatory molecule CD28 for binding to their shared ligands, CD80 and CD86, on antigen-presenting cells (1). As a result of this interaction, the CD28-mediated costimulatory signal is shut off, preventing further T cell activation and leading to decreased T cell survival, proliferation, and effector functions such as cytokine production or cytotoxicity. Supporting its crucial role in regulating T lymphocyte activity, CTLA-4 knockout mice develop a lethal lymphoproliferative disorder a few weeks after birth (2, 3).

A number of animal studies have shown that blocking the activity of anti-CTLA-4 with neutralizing antibodies can greatly enhance immune responses, including those against tumor-associated antigens (4–7). These findings have since paved the way for the use of anti-CTLA-4 antibodies in cancer immunotherapy trials in an attempt to augment antitumor immune responses in cancer patients, particularly those with advanced melanoma (8–11). Treatment with ipilimumab, a monoclonal IgG1 anti-CTLA-4 neutralizing antibody, has shown objective clinical response rates of around 15% in heavily pretreated patients with advanced melanoma. The patterns of response in these patients may differ from those seen following cytotoxic chemotherapy. For instance, late responses may be seen and these may occur after an initial period of tumor progression. Additionally, long-lasting disease stabilization may occur under treatment (12). Patients treated with ipilimumab also frequently develop immune-related manifestations such as dermatitis

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Translational Relevance

Successful treatments for patients with advanced melanoma are not presently available. There is increasing evidence, however, that treatment with blocking anti – CTL antigen 4 (CTLA-4) antibodies can achieve long-lasting tumor remissions in 10% to 15% of these patients. Although the effect of this treatment is thought to be immune-mediated, a clear understanding of its mechanism in humans is still missing. A better definition of the immune responses that take place in patients responding to treatment with anti-CTLA-4 blockade might allow an integration of this new class of drugs into preexisting immunotherapy approaches and improve the efficacy of these treatments in patients with advanced melanoma.

and enterocolitis, and interestingly, the frequency and severity of these side effects correlate closely with the effectiveness of tumor regression (9, 13, 14).

Until now, the immunologic mechanism responsible for anti-CTLA-4 – induced tumor regression in melanoma patients has not been well defined, and although CD8-positive CTL are likely to play a major role, the tumor antigen(s) targeted by this response are unknown. Furthermore, it is unclear whether these same T cells are also responsible for the observed immune-related side effects, or whether anti-CTLA-4 treatment results in the development of separate T cell populations with antitumor and antihost specificity. We therefore carried out a detailed examination of the antitumor immune response in a patient who experienced a complete remission and immune-related skin complications following treatment with the CTLA-4 blocking antibody ipilimumab.

Materials and Methods

Clinical protocol. This study was conducted as part of a randomized, double-blind, phase II fixed-dose study of ipilimumab monotherapy (Bristol Myers Squibb protocol number CA184022) comparing three different doses of ipilimumab (0.3, 3, or 10 mg/kg). At present it is not known which dose of ipilimumab a patient was receiving. The study was approved by the Austin Health Human Research Ethics Committee, and the patient provided written informed consent before any study-specific procedures.

Immunohistochemical analysis of tumor and skin biopsies. Preparation of paraffin-embedded tissue sections and staining were done as previously described (15). Anti-CD8 antibody (clone C8/144B) was purchased from Dako Cytomation.

Characterization of antigen specificity of T lymphocytes in tumor and skin biopsies. Single-cell suspensions were prepared from biopsy tissue

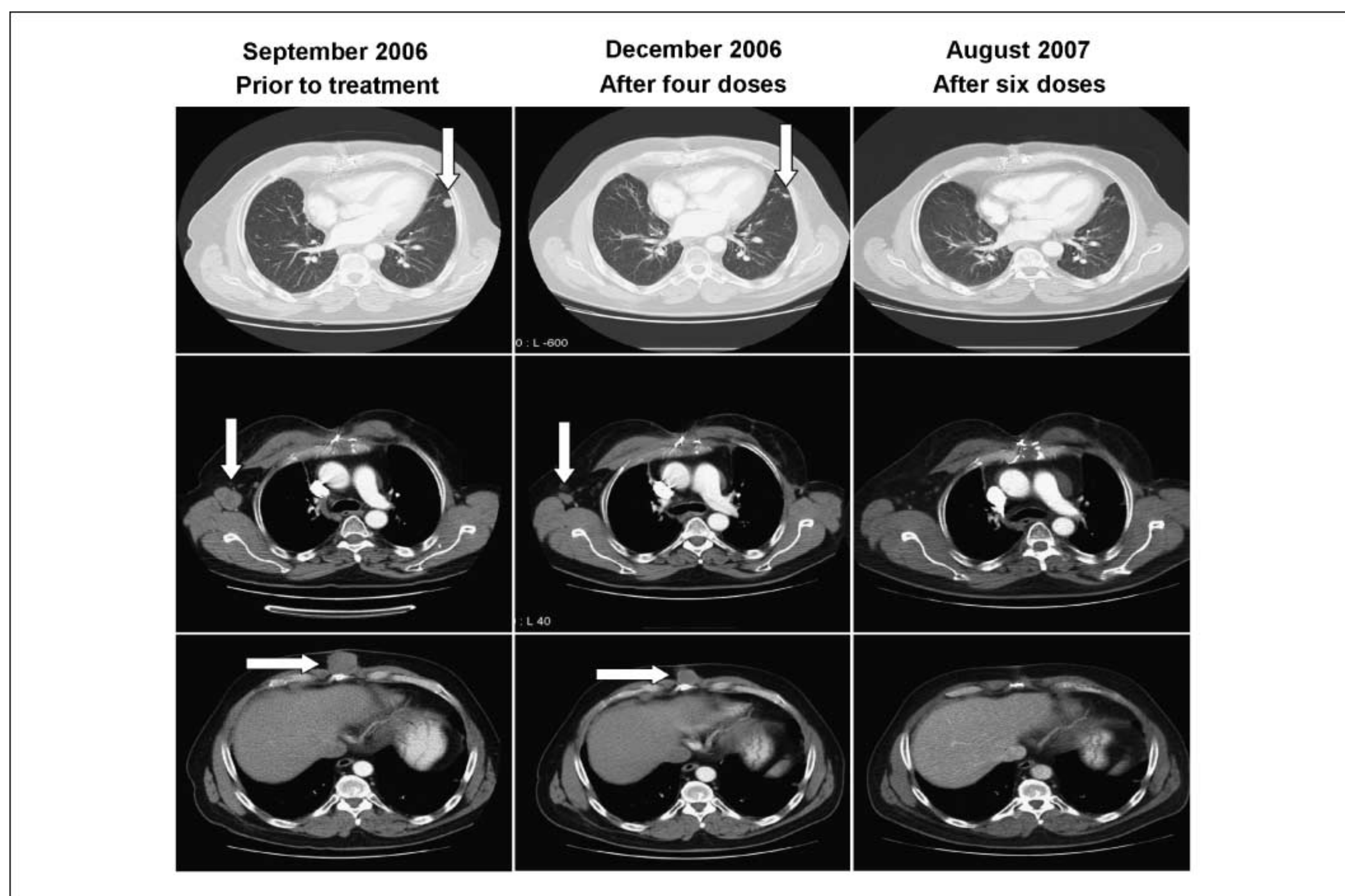

Fig. 1. Treatment with CTLA-4 antibody ipilimumab leads to a complete tumor remission in a patient with metastatic melanoma. Computer axial tomography scans illustrating disease status pretreatment, after four cycles and after six cycles of treatment with anti-CTLA-4 antibody ipilimumab. Arrows, representative sites of disease (lung metastasis, axillary lymph node metastasis, and chest wall metastasis).

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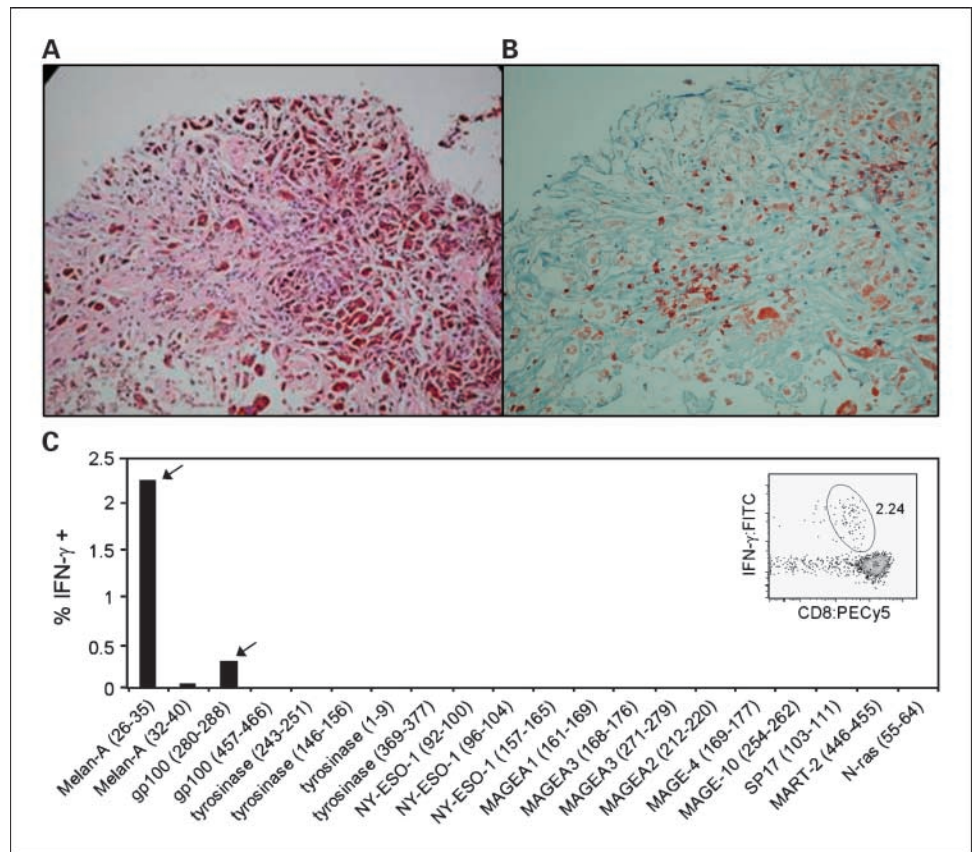

Fig. 2. Anti-CTLA-4 treatment leads to tumor regression associated with infiltration of Melan-A – specific CD8-positive T cells. *A,* H&E staining of a formalin-fixed paraffin section prepared from the regressing chest wall metastasis. *B,* immunohistochemical staining of the same tissue with an anti-CD8 antibody. All photomicrographs are at 200× magnification. *C,* the specificity of the tumor-infiltrating CD8-positive T cells was determined by testing a T-cell line generated from the same tumor biopsy sample against a variety of tumor antigenic epitopes in an intracellular cytokine staining assay. Inset, staining pattern observed for the Melan-A_{26-35} epitope; arrows, responses confirmed by tetramer staining.

by mechanical disruption followed by digestion using 1 mg/mL Collagenase D (Roche) and 100 U/mL DNase (Sigma) at 37°C for 1 h. Cells were expanded in complete RPMI with 10% FCS (RF-10) in the presence of 1 μg/mL phytohemagglutinin (PHA), 100 U/mL interleukin-2 (IL-2), and irradiated feeder cells for ~2 wk, as described (16). To assess reactivity to defined tumor antigen epitopes, cells were stimulated for 5 h with the indicated synthetic peptide at 100 μmol/L (Multiple Peptide Systems, EZBiolab, and Mimotopes) in the presence of 10 μg/mL Brefeldin A (Sigma) and then subjected to intracellular cytokine staining for IFN-γ as previously described (17).

Analysis of antigen-specific T cell frequency in peripheral blood. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient from blood and stained with human leukocyte antigen (HLA)-A2 multimers ("tetramers") formulated using the following peptides: Melan-A_{26-35} (ELAGIGILTV), NY-ESO-$1_{157-165}$ (SLLMWITQC), EBV $BMFL1_{280-288}$ (GLCTLVAML), and flu $matrix_{58-66}$ (GILGFVFTL) for 30 min at room temperature. Cells were then stained with anti-CD8:FITC (BD) and analyzed by flow cytometry. Tetramers were synthesized at the Ludwig Institute for Cancer Research. For detailed phenotypical characterization, PBMC were stained with Melan-A tetramer followed by anti-CD28:FITC, anti-CD45RA:PECy5, anti-CD27:APC (all from BD), and anti-CD8:APC/Alexa750 (Caltag).

Cytotoxicity assays. PBMC collected 3 wk after beginning treatment were expanded with Melan-A_{26-35} peptide (1×10^{-6} mol/L) for 13 d. These cells were used as effectors in calcein-release cytotoxicity assays, essentially as described (18). Briefly, tumor cells were treated for 40 h with IFN-γ (Peprotech), labeled with 15 μmol/L Calcein-AM (Invitrogen) at 37°C for 30 min, washed, and 5×10^3 cells were mixed with T cells at the indicated ratios. After 4 h at 37°C, calcein release was quantified in the supernatant by measuring fluorescence in a Wallac plate reader (Perkin Elmer). Alternatively, to assess mobilization of CD107b to the cell surface, T cells were incubated with an equal number of tumor cells for 4 h in the presence of anti-CD107b:FITC (BD) and 2 μmol/L monensin (Sigma). T cells were then stained with Melan-A_{26-35} tetramer and anti-CD8 as described above. Where indicated, tumor cells were pulsed with 1×10^{-4} mol/L Melan-A_{26-35} peptide at 37°C for 20 min prior to the assays.

Results

Anti-CTLA-4 treatment leads to tumor regression, associated with infiltration with Melan-A – specific CD8-positive T cells. On the basis of promising preclinical and early clinical data (4, 8, 11), we enrolled several advanced melanoma patients in a phase II study of ipilimumab (anti-CTLA-4) monotherapy. One of the patients enrolled was a 46-year-old man who had been heavily pretreated with surgery, radiotherapy, and temozolomide chemotherapy, and had also been vaccinated with an experimental NY-ESO-1/ISCOMATRIX vaccine (19). Despite these treatments, he failed to develop an anti-NY-ESO-1 T-cell response and his disease was progressing. He was therefore enrolled in the ipilimumab study. He initially received an induction treatment course, consisting of four antibody doses administered at three-weekly intervals, followed by maintenance treatment with one dose every three months. A first tumor restaging by computed tomography scan at the end of the induction treatment at week 12 showed partial remission. Subsequent computed tomography scans at weeks 24 and 36 showed further tumor regression with complete disappearance of all metastases nearly one year after beginning treatment at week 48 (Fig. 1). This clinical course is consistent with other patients who have responded to anti-CTLA-4 treatment (9, 11).

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One of the regressing tumor lesions located on the chest wall was biopsied 10 weeks after the fourth dose of treatment. Histologically, lymphocytic infiltration of the tumor was visible, with evidence of extensive necrosis of the tumor tissue and numerous melanin-laden macrophages (Fig. 2A). Immunohistochemistry revealed that most lymphocytes were CD8-positive T cells (Fig. 2B), with smaller numbers of CD4-positive T cells also present (not shown). To examine the antigen specificity of CTL within the tumor specimen, the same biopsy tissue was used to generate a short-term T-cell line by culturing disaggregated tumor tissue with PHA, IL-2, and feeder cells for 2 to 4 weeks. These T cells were then stimulated with a panel of peptides corresponding to relevant (HLA-matched) epitopes of various melanoma antigens, and responsiveness was determined by intracellular IFN-γ staining. The patient's class I expression is HLA-A*0101, 0201; HLA-B*0801, 4001; HLA-Cw*0304, 0701; and the HLA restriction of the epitopes is detailed at http://www.cancerimmunity.org/peptidedatabase/differentiation.htm. As shown in Fig. 2C, the T-cell line generated from regressing tumor showed a remarkably restricted specificity profile, with a high frequency of T cells responsive to Melan-A and a much smaller response to gp100. Both of these antigens are differentiation antigens, expressed specifically on cells of the melanocyte lineage (and tumors derived from them; refs. 20 – 23). The expression of these antigens could not be determined on this biopsy tissue due to the lack of viable tumor cells. However, previous immunohistochemistry studies confirmed that the patient's tumor expressed high levels of Melan-A and gp100 (not shown). No significant response was detected to a third differentiation antigen (tyrosinase) nor toward cancer/testis antigens (NY-ESO-1, Sp17, and MAGE family members) or mutated antigens (n-ras and MART2). Responses to the Melan-A_{26-35} and $gp100_{280-288}$ determinants were confirmed by staining with specific peptide-MHC multimers ("tetramers"; not shown).

An inflammatory skin rash induced by anti-CTLA-4 treatment is also infiltrated with Melan-A–specific CD8-positive T cells. One week after the first dose of anti-CTLA-4, the patient developed a patchy macular rash on his trunk which resolved without treatment within several weeks. Of note, this rash was especially pronounced around nevi, suggesting that the inflammatory response was directed toward melanocytes. A skin biopsy showed a dense inflammatory dermal infiltrate including many CD8-positive lymphocytes, which were associated with Melan-A – positive nevoid structures (Fig. 3A and B). To examine the antigen specificity of these CD8-positive T cells, the biopsy tissue was used to generate a T-cell line by PHA expansion, as described above. These T cells were then stimulated with the same panel of peptides as above and responsiveness was determined by intracellular IFN-γ staining. Similar to the tumor specimen, the dominant response detected in the skin was toward Melan-A, with smaller responses identified to additional differentiation antigens gp100 and tyrosinase (Fig. 3C). The response to the Melan-A_{26-35} and $gp100_{280-288}$ determinants was again confirmed by tetramer staining (not shown). Also, analysis of a second T-cell line generated from a skin biopsy collected approximately two weeks later showed a virtually identical pattern of reactivity to that illustrated in Fig. 3C (not shown). The predominance of T cells specific for differentiation antigens, especially Melan-A,

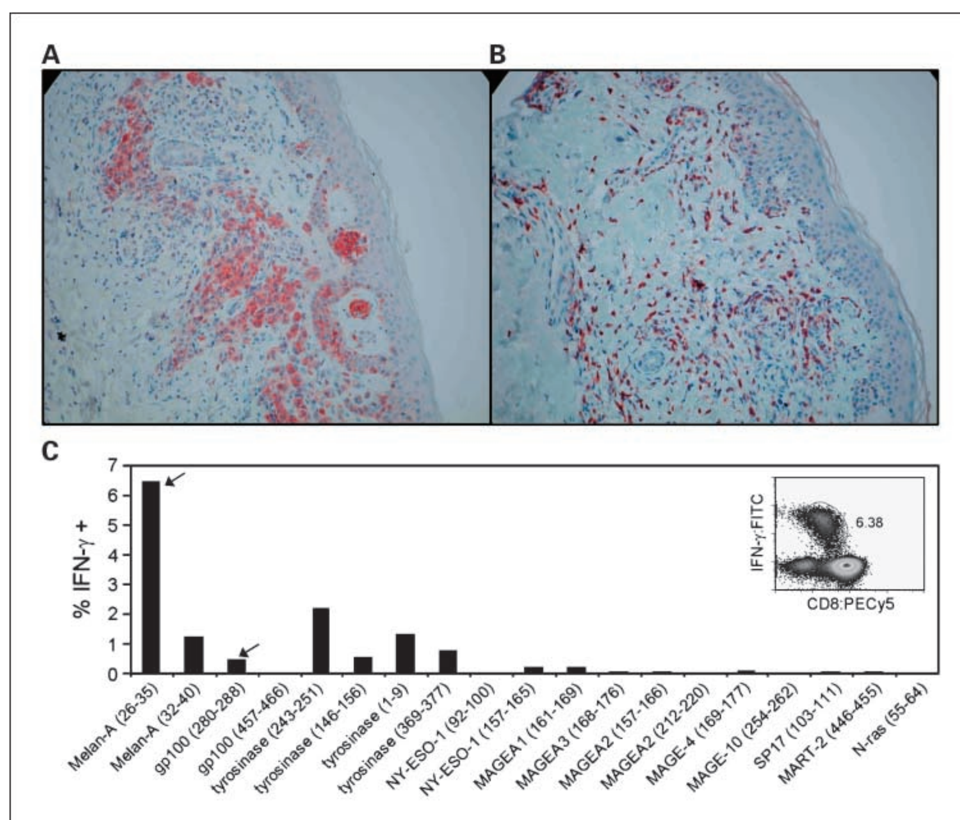

Fig. 3. Melan-A – specific CD8-positive T cells infiltrate an inflammatory skin reaction induced by anti-CTLA-4 treatment. *A* and *B,* an area of skin affected by the rash was biopsied and sections were stained with antibody to Melan-A (to visualize nevoid structures; *A*) or CD8 (to visualize CD8-positive T cells; *B*). *C,* the specificity of skin-infiltrating CD8-positive T cells was determined by testing a T-cell line generated from the same tissue biopsy against a variety of tumor antigenic epitopes in an intracellular cytokine staining assay. Inset, staining pattern observed for the Melan-A_{26-35} epitope; arrows, responses confirmed by tetramer staining.

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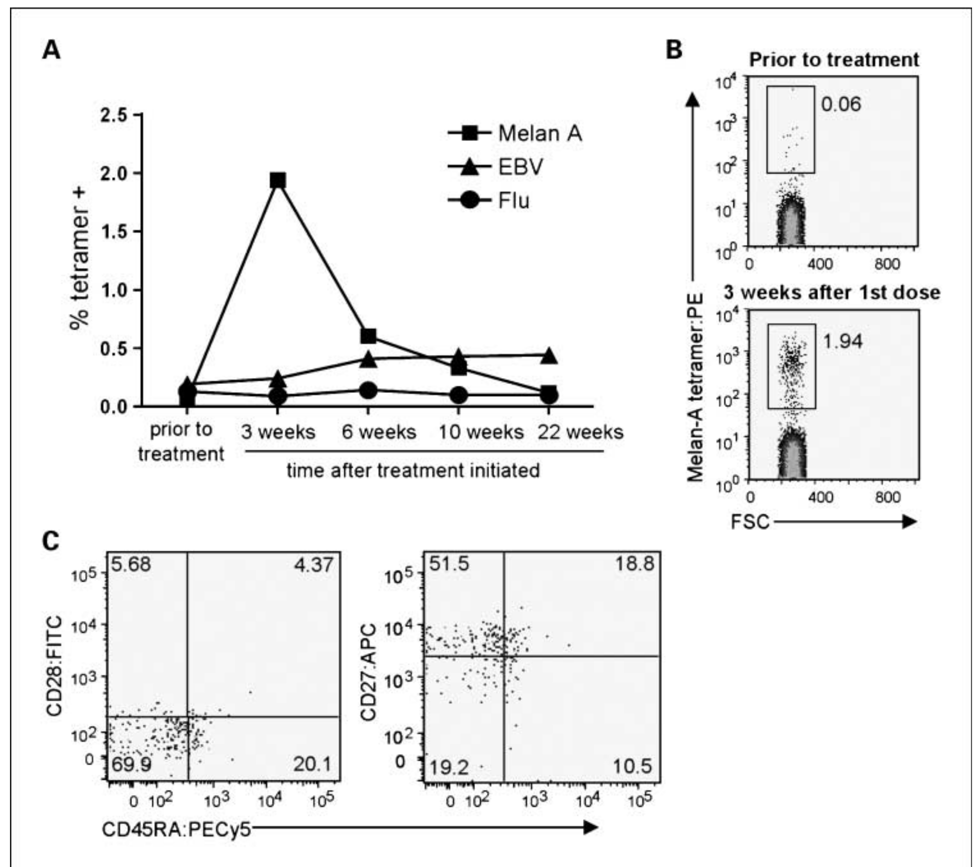

Fig. 4. Dramatic increase in Melan-A – specific CD8-positive T cells in the peripheral blood in response to anti-CTLA-4 antibody treatment. *A,* proportion of peripheral blood CD8-positive T cells specific for Melan-A, EBV, and flu virus during the treatment period. PBMC were isolated from blood drawn prior to the start of treatment and immediately before each dose of anti-CTLA-4 antibody and stained with HLA-A2 tetramers formulated using the following peptides: Melan-A_{26-35}, $EBV_{280-288}$, and flu $matrix_{58-66}$. *B,* flow cytometry density plots illustrating the increase in CD8-positive T cells staining with the Melan-A tetramer after the first dose of anti-CTLA-4. *C,* phenotype of the expanded Melan-A – specific CD8-positive T cells in the peripheral blood 3 wk after the start of treatment. CD8-positive Melan-A tetramer – positive cells were gated and analyzed for staining for CD45RA, CD28, and CD27.

in both the skin and the tumor is in keeping with the shared expression of such antigens on both melanoma cells and normal melanocytes, and suggests that, at least in this patient, the same T cells may have mediated both clinical reactions.

The frequency of Melan-A – specific CD8-positive T cells in peripheral blood increased dramatically after the first dose of anti-CTLA-4. As Melan-A – responsive T cells dominated within the regressing tumor as well as the skin rash, the frequency of these cells was also examined in the peripheral blood using the Melan-A_{26-35} tetramer (directly *ex vivo*). After the first antibody dose, a >30-fold expansion in tetramer-positive CD8-positive T cells was detected (Fig. 4A and B). The frequency subsequently declined but still remained above baseline for up to 22 weeks. In contrast, the frequency of T cells stained with an influenza virus tetramer (HLA-A2/$matrix_{58-66}$) was stable during treatment, whereas staining with an EBV-specific tetramer (HLA-A2/$BMFL1_{280-288}$) showed a slight increase over the time course (Fig. 4A). It is unclear why Melan-A – specific responses were preferentially amplified. One possibility is that this is related to the levels of antigen that were available to induce an active T cell response that could be enhanced by CTLA-4 blockade.

To address the phenotype of the Melan-A – specific T cell population, PBMC were costained with the Melan-A_{26-35} tetramer and antibodies against memory/activation markers. This analysis revealed that the majority of tetramer-positive cells had a CD45RA-negative/low CD28-negative CD27-positive phenotype (Fig. 4C). These characteristics were described previously for cytotoxic effector T cells in virally infected individuals (24, 25), suggesting that circulating Melan-A – specific T cells were functional CTL capable of mediating tumor cell lysis.

Expanded Melan-A – specific T cells kill tumor targets. To investigate the CTL activity of Melan-A-specific T cells, PBMC collected at the peak of the expansion in the blood were cultured with Melan-A_{26-35} peptide for 13 days to generate a short-term T-cell line containing 74% Melan-A_{26-35} tetramer – positive cells. As illustrated in Fig. 5A, these cells showed potent cytotoxic activity against a HLA-A2 – positive Melan-A – positive melanoma cell line (SK-Mel-14). In contrast, no cytotoxic activity was observed against a second HLA-A2 – positive melanoma cell line (LM-Mel-26) that lacked Melan-A expression, unless these cells were loaded with the Melan-A_{26-35} peptide. To further confirm the cytotoxic activity of Melan-A – specific cells, we also analyzed mobilization of the cytotoxic granule marker CD107b to the cell surface, which occurs following degranulation (26). This analysis was done in conjunction with tetramer staining, such that the cytotoxic response of antigen-specific cells could be directly assessed. Figure 5B shows that tetramer-positive cells expressed CD107b on the cell surface only after exposure to the Melan-A – positive SK-Mel-14 cell line, and not the LM-Mel-26 cell line (which lacks Melan-A expression), unless the latter is loaded with peptide. Importantly, the small population of CD8-positive T cells within the culture that did not stain with the Melan-A tetramer predominantly failed to mobilize CD107b in response to SK-Mel-14 cells (Fig. 5C). This confirms that the killing activity shown in Fig. 4A is due to recognition of Melan-A and not other tumor antigens or allo-antigens that this mixed T-cell line could potentially recognize.

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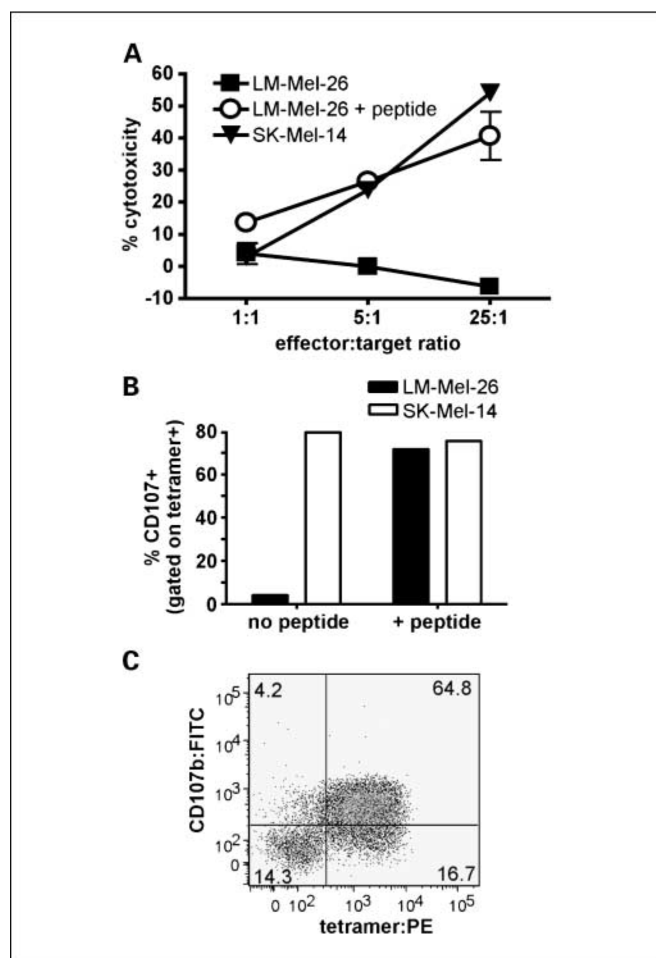

Fig. 5. The expanded Melan-A – specific T cells in the blood kill Melan-A – positive tumor targets. *A,* a Melan-A – specific T cell line was generated from PBMC collected 3 wk after beginning treatment and tested in a Calcein-release cytotoxicity assay, using the HLA-A2 – positive melanoma cell lines SK-Mel-14 (Melan-A – positive) and LM-Mel-26 (Melan-A – negative) as targets. Where indicated, tumor cells were pulsed with Melan-A_{26-35} peptide. *B* and *C,* the same T cell and melanoma cell lines were cocultured in the presence of anti-CD107b and 4 h later, mobilization of CD107b to the T cell surface was assessed by flow cytometry. A summary of results (gated on CD8-positive tetramer-positive cells) is shown in (*B*), and the staining pattern observed following T cell coculture with SK-Mel-14 cells is shown in (*C*), gated on CD8-positive T cells.

Discussion

Neutralizing antibodies against CTLA-4 represents one of the most promising advances in the nonsurgical treatment of advanced melanoma, a disease poorly responsive to standard systemic therapy approaches. Although objective clinical responses have been reported, until now the specificity of T cells mediating the observed tumor regressions has not been defined (27). Furthermore, although previous studies with anti-CTLA-4 antibodies have shown that tumor regression is frequently associated with immune-related manifestations, it was unclear whether this is due to T cells specific for antigens shared by tumor and normal cells, or concomitant activation of multiple populations with separate antihost and antitumor activity (9, 13, 14). Our study shows that Melan-A, an antigen shared by melanoma cells and normal melanocytes, was associated with both tumor regression and the immune-related skin reaction in this patient. A much smaller response was also detected with two other differentiation antigens (gp100 and tyrosinase) in the skin, although only T cells specific for gp100, and not tyrosinase, were also identified in the tumor. Due to the small number of cells obtainable from biopsy tissue, these studies were conducted on T cells expanded by PHA and IL-2. However, given that PHA is a nonspecific mitogen that should expand T cells of all specificities equally, these short-term expanded lines are likely to represent well the T cells originally present in the tissue.

It is known that tumor-infiltrating lymphocytes in patients with melanoma may commonly contain Melan-A – specific cells (28, 29), but their role in controlling tumor progression is unknown. In our patient, however, there was marked Melan-A – specific T cell reactivity in tumor and skin tissue, with CD8-positive cells localizing to nevi. Most importantly, this was accompanied by a very substantial and simultaneous increase in Melan-A – specific CD8-positive T cells in the peripheral blood. The parallel appearance of these cells in tissues and the temporal relationship with those in blood provide strong circumstantial evidence that these were mediators of the antitumor immune response seen in this patient.

Melan-A is unusual among human tumor antigens in that the naïve T-cell repertoire contains a remarkably high proportion of Melan-A – specific T cells (1 in 2,500 CD8-positive T cells; ref. 28). Our observation that the antimelanoma immune response is preferentially directed toward Melan-A may relate to this unusually high precursor frequency. This suggests that increasing the frequency of T cells specific for additional melanoma antigens (by vaccination) may significantly broaden the response, thereby increasing the effectiveness of anti-CTLA-4 treatment.

Anti-CTLA-4 treatment resulted in a dramatic increase in the frequency of Melan-A – specific CD8-positive T cells in the blood immediately after the first infusion. This was not, however, paralleled by notable increases in EBV- or flu-specific cells. These results suggest that the dramatic increase in Melan-A – specific T cells was not simply due to a nonspecific expansion of memory T cells, which should be reflected equally in all three populations, but instead seems to be related to antigen availability. Thus, large amounts of Melan-A antigen would be expected to be available to T cells due to the presence of the Melan-A – expressing tumor mass. In contrast, influenza antigens will not be present in the absence of active infection, resulting in a lack of flu-specific T cell expansion following anti-CTLA-4 administration. EBV, however, persists as a chronic infection, with periodic cycles of viral reactivation that will result in low-level antigen release (29), potentially explaining the slight increase in EBV-specific T cells during treatment.

The expanded population of Melan-A – specific T cells had an effector memory phenotype, and could efficiently lyse Melan-A – expressing tumor cells. Together, our observations suggest that blocking the function of CTLA-4 with ipilimumab releases the regulatory mechanism that is holding the anti-Melan-A CTL response in check. As a result, these cells undergo expansion and differentiation to cytotoxic effector cells, which subsequently migrate to the skin, inducing a transient inflammatory response, and to the tumor, where they mediate tumor regression. These findings are consistent with observations in a B16 mouse melanoma model combining CTLA-4 blockade with granulocyte monocyte colony-stimulating factor – transduced tumor cell vaccination, in which tumor rejection and

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skin autoimmunity were both dependent on CD8-positive T cells specific for the melanocyte differentiation antigen TRP-2 (30). However, our findings contrast with those of Phan et al. who investigated ipilimumab in combination with a different melanosomal antigen, gp100, as a peptide vaccine and found no evidence for *ex vivo* anti-gp100 immune responses in their patients. Immune responses against Melan-A were not assessed (31). Our findings provide a mechanistic explanation for the previously observed correlation between tumor regression and immune-related manifestations in the skin (8, 11), and suggest that treatment efficacy could be improved (with no increase in side effects) by simultaneous vaccination against tumor antigens that are expressed on melanoma cells but not skin, such as the cancer/testis antigen family (32).

Disclosure of Potential Conflicts of Interest

A. Hoos is an employee of Bristol-Myers Squibb. The authors have no other conflicting financial interests.

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