Combined IL-21–primed polyclonal CTL plus CTLA4 blockade controls refractory metastatic melanoma in a patient

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Adoptive transfer of peripheral blood–derived, melanoma-reactive CD8$^+$ cytotoxic T lymphocytes (CTLs) alone is generally insufficient to eliminate bulky tumors. Similarly, monotherapy with anti-CTLA4 infrequently yields sustained remissions in patients with metastatic melanoma. We postulated that a bolus of enhanced IL-21–primed polyclonal antigen-specific CTL combined with CTLA4 blockade might boost antitumor efficacy. In this first-in-human case study, the combination successfully led to a durable complete remission (CR) in a patient whose disease was refractory to both monoclonal CTL and anti-CTLA4. Long-term persistence and sustained anti-tumor activity of transferred CTL, as well as responses to nontargeted antigens, confirmed mutually beneficial effects of the combined treatment. In this first-in-human study, Chapuis et al. demonstrate that the combination of adoptive cellular therapy with CTLA4 blockade induces long-term remission in a melanoma patient resistant to both modalities administered serially and individually.

Patients with metastatic melanoma rarely display tumor-specific immune cells sufficient for thwarting disease progression (Restifo et al., 2012). Adoptive transfer of autologous peripheral blood (PB)–derived, antigen-specific T cells can increase the frequency of melanoma-specific T cells with a very tolerable safety profile (Yee et al., 2002; Wallen et al., 2009; Chapuis et al., 2012). When used as monotherapy, this approach has been effective in delaying disease progression. But sustained, complete tumor regression is rare, in part due to the short in vivo survival of transferred cells, as well as inhibitory signals limiting full T cell activation (Yee et al., 2002; Wallen et al., 2009; Chapuis et al., 2012).

Antibodies that block human CTL–associated antigen 4 (CTLA4) function in part by preventing inhibitory signals after antigen-specific engagement of the TCR, releasing the brake on the population of extant tumor-specific CTL (Sharma et al., 2011). Used alone, CTLA4 blockade produces disease control in 22% of patients with metastatic melanoma (Hodi et al., 2010; Schadendorf et al., 2015). However, durable complete remissions (CRs), reflecting the development of long-term immune-mediated tumor control, have been observed in a minority of patients (range 0–7%; Hodi et al., 2010; Prieto et al., 2012; Wolchok et al., 2013). Most nonresponders do not benefit from additional courses of anti-CTLA4 (Wolchok et al., 2013).

As the quality and magnitude of T cell responses prompted by anti-CTLA4 alone is usually insufficient to eradicate the tumor, we hypothesized that an ex vivo source of melanoma-reactive CTL might not only provide sufficient substrate for anti-CTLA4 to enhance tumor lysis, but also trigger the development of de novo responses to nontargeted antigens (epitope spreading; Ribas et al., 2003).

To generate tumor-specific CTL with enhanced in vivo persistence, we primed antigen-specific cells in vitro in the

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Abbreviations used: AE, adverse event; CR, complete remission; CTL, CD8$^+$ cytotoxic T lymphocyte; CTLA4, CTL-associated antigen 4; CY, cyclophosphamide; gp, glycoprotein; HLA, human leukocyte antigen; HTTCS, high-throughput TCRβ sequencing; MAGE, melanoma-associated antigen; MART1, melanoma tumor antigen 1; NY-ESO1, New York esophageal protein 1; PD1, programmed cell death protein 1; pMHC, peptide-MHC; RECIST, response evaluation criteria in solid tumors.

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presence of IL-21 (Li et al., 2005) to enrich a population of central memory type CTL (Cui et al., 2011; Chapuis et al., 2013) and selected polyclonal CTL from autologous melanoma-specific T cell lines using a clinical grade sorter (Pollack et al., 2014), thereby limiting the ex vivo expansion required to achieve target CTL numbers.

We then assessed whether combining CTLA4 blockade plus infusion of enhanced tumor-specific CTL could indeed improve antitumor efficacy in a patient who presented with metastatic melanoma, resistant to both monoclonal CTL and anti-CTLA4 administered serially.

RESULTS AND DISCUSSION

Clinical evaluation

This 53-yr-old male presented with stage III melanoma on his lower right thigh. He underwent wide local excision and inguinal node dissection (Clark level 4, Breslow 1.5 mm, no ulceration, 3/8 lymph nodes involved), followed by 12 mo of adjuvant IFN-α treatment. 4 yr later, he developed metastatic disease with supraclavicular, subcarinal, and right hilar masses uniformly positive for melanoma tumor antigen 1 (MART1). In anticipation of adoptive transfer studies, his PBMCs were collected. He then received four cycles of high-dose IL-2, but experienced disease progression.

He received two infusions of monoclonal MART1-specific T cells (10^{10} cells/m^2) 30 d apart, each followed by low-dose s.c. IL-2 (250,000 U/m^2 every 12 h). The second infusion was preceded by selective $CD25^{hi}$ T regulatory cell depletion (denileukin diftitox); the melanoma progressed. The patient received anti-CTLA4 monoclonal antibody (ipilimumab 3 mg/kg × 4 doses) that partially slowed tumor growth initially, but 4 mo later, he developed new metastases.

Finally, he received IL-21–primed polyclonal MART-1–specific T cells at a dose of $10^{10}/m^2$, immediately followed by a single course of ipilimumab (same dosing as above; Pollack et al., 2014). The T cell infusion was preceded by low-dose cyclophosphamide (CY) conditioning (300 mg/m^2 × 1) and followed by a 2-wk course of low-dose s.c. IL-2. Although the entire regimen could be administered in the ambulatory setting, he was hospitalized for monitoring of potential cell infusion–associated adverse events (AEs).

No serious AEs were observed, apart from expected transient (<24 h) culture-negative fevers (≥38.3°C) associated with CTL-induced cytokine release syndrome, and lymphopenia lasting 10 d (Chapuis et al., 2013). Before CTL infusions (Fig. 1 A), the patient presented with PET^+ subcarinal and right hilar masses (Fig. 1, B and C). Serial imaging demonstrated progressive disease after the first course of CTL infusions, followed by ipilimumab monotherapy. The patient had bulky disease in the paratracheal, supraclavicular, and subcarinal regions at the time the combined therapy with IL-21–primed, polyclonal MART-1–specific T cells plus ipilimumab was initiated. 12 wk (day 384) after the start of the combined treatment, progressive tumor reduction was associated with the development of vitiligo (Fig. 1 D), manifest as depigmentation of the eyebrows and eyelashes. The patient achieved a CR by response evaluation criteria in solid tumors (RECIST) and immune-related response criteria (Wolchok et al., 2009) at year 3, and remains disease-free 5 yr later, with no additional therapy or long-term immune side effects other than persistent vitiligo.

Persistence, clonality, phenotype, and function of monoclonal and polyclonal CTL in vivo

The first course of monoclonal MART1-specific CTL yielded a peak CTL frequency of 2.1% of total $CD8^+$ T cells 1 d after the second infusion; CTL rapidly disappeared. Combined therapy resulted in a 4% peak 1 wk after infusion and frequencies of 1.2 and 4% 2 and 4 yr later, respectively (Fig. 2 A). Tracking of the monoclonal CTL (clone 120; Fig. 2 B, inset pie chart), using high-throughput TCRβ sequencing (HTTCS), confirmed that the peak response was a result of the infused clone, which was not detected in pre-infusion PBMC samples or in PBMC samples taken at any time point after day 29 (Fig. 2 B, graph). Polyclonal CTL included 13 clonotypes with frequencies >0.1% (Fig. 2 C, inset pie chart). HTTCS tracking revealed that one clonotype (TCR-1) represented the majority of detected antigen-specific CTL after infusions (Fig. 2 C, graph). To assess phenotypic and functional differences that could account for the preferential survival of long-lived IL-21-primed CTL, compared with monoclonal CTL, we assessed surface expression of markers associated with long-lived memory T cells (CD28, CD62L, C-C Chemokine Receptor 7 [CCR7]), activation/exhaustion (programmed cell death protein 1 [PD1]), and function (IFN-γ, TNF, and IL-2 production in response to cognate antigen). The polyclonal CTL product expressed CD28 (55.1%) and low PD1 (12%), and produced IFN-γ, TNF, and IL-2 (Fig. 2 D, top graph), whereas monoclonal CTL expressed none of the memory markers, a higher fraction of PD1 (44%), and produced only IFN-γ in response to cognate antigen (Fig. 2 D, bottom graph). After adoptive transfer, the polyclonal CTL additionally expressed CD62L in vivo (Fig. 2 E). After 12 wk (day 385), they expressed all three memory markers (CD28, CD62L, and CCR7), maintained low PD1 expression (12%) and produced IFN-γ, TNF, and IL-2 in response to cognate antigen (Fig. 2 F), all features consistent with central memory T cells (Restifo et al., 2012).

Response to nontargeted antigens (antigen spreading)

The reactivity of $CD8^+$ and $CD4^+$ T cells to overlapping peptides spanning known melanoma-associated proteins MART1, New York esophageal protein 1 (NY-ESO1), glycoprotein (gp) 100, tyrosinase, and melanoma-associated antigen (MAGE) A3, independent of human leukocyte antigen (HLA) restriction, was tested at indicated time points throughout the patient's treatment course (Fig. 3). PBMCs taken before and up to 80 d after the first infusion of monoclonal CTL showed low/no reactivity to the overlapping peptides (<10 IFN-γ spots/10^5 PBMC). After the initial ipilimumab treatment,

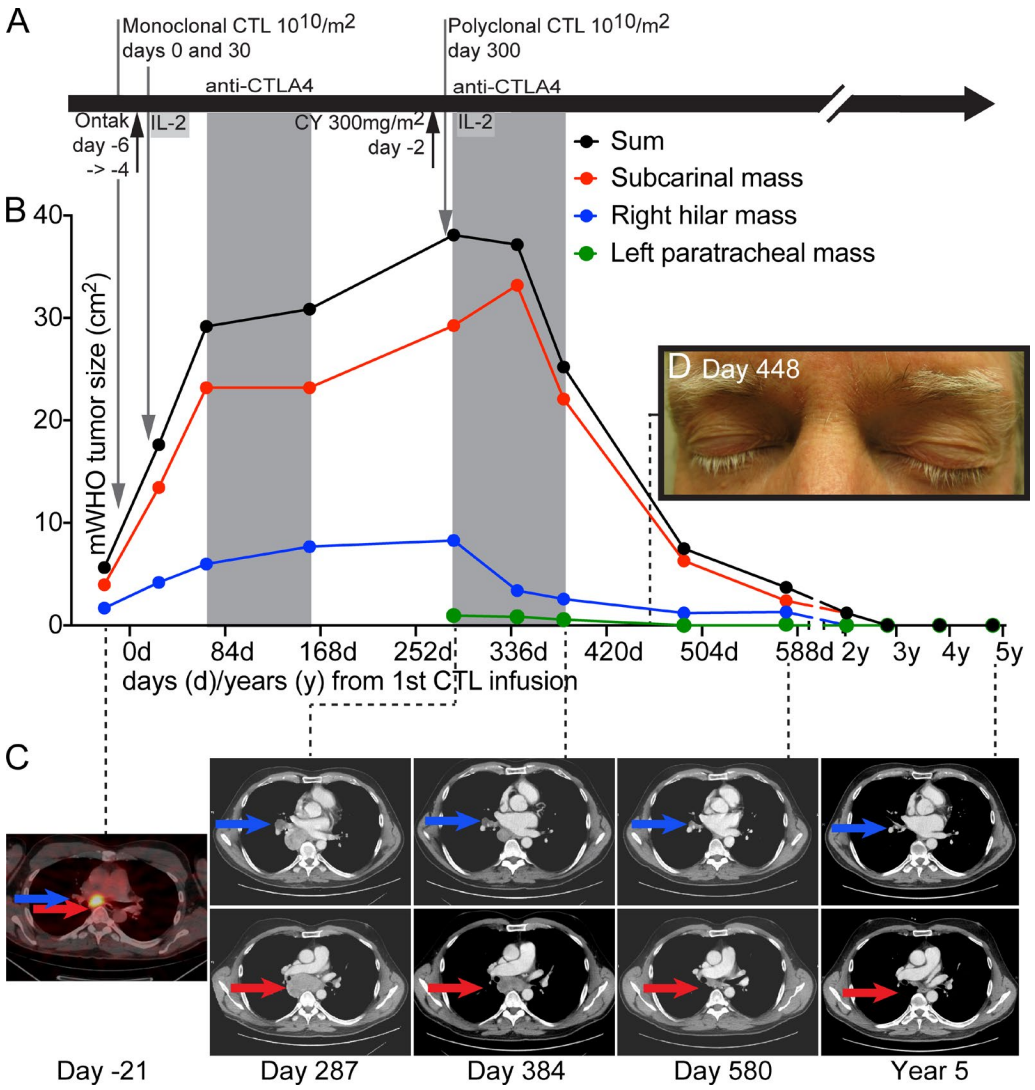

Figure 1. **Tumor regressions after melanoma-reactive polyclonal CTL combined with anti-CTLA4.** (A) Timeline of successive therapies. (B) Kinetics of response for three target lesions (y axis) spanning 5 yr (x axis). (C) Serial PET (leftmost image) and CT images at indicated time points. Arrows indicate the location of the right hilar (blue) and subcarinal (red) masses. (D) Photograph of the patient's depigmented eyelashes and eyebrows ∼5 mo after the start of the combined treatment.

antigen-spreading was still not detected (day 295). However, 6, 12, and 27 wk (days 349, 390, and 495) after the start of combined therapy the patient developed a marked response to multiple peptides within each melanoma-associated protein. Frequencies matched or exceeded MART1-specific responses in some cases, suggesting antigen spreading, coincident with a >80% reduction in tumor burden (Fig. 1).

Thus, we have shown that IL-21–primed, polyclonal MART1-specific CTLs plus ipilimumab achieved complete, durable tumor eradication with minimal side effects in a patient whose melanoma was refractory to monoclonal MART1-specific CTL and subsequent single-agent ipilimumab. Although the immune component responsible for tumor eradication cannot be precisely determined here, our observations elucidate three critical factors for immune-mediated tumor regression.

First, polyclonal IL-21–primed CTLs achieved higher peak frequencies and longer persistence in vivo, compared with identical doses of monoclonal CTLs. Reduced ex vivo manipulation (≤6 wk vs. ≥12 wk) plus IL-21 addition during priming (Pollack et al., 2014) generated CTLs that had undergone fewer divisions and had characteristics associated with enhanced survival. Specifically, expression of CD28 and a retained capacity to secrete IL-2 after exposure to cognate antigen (Topp et al., 2003) likely facilitated the robust persistence of transferred tumor-specific cells.

Second, ipilimumab exposure likely enhanced the antitumor activity of the transferred cells. By enabling unob-

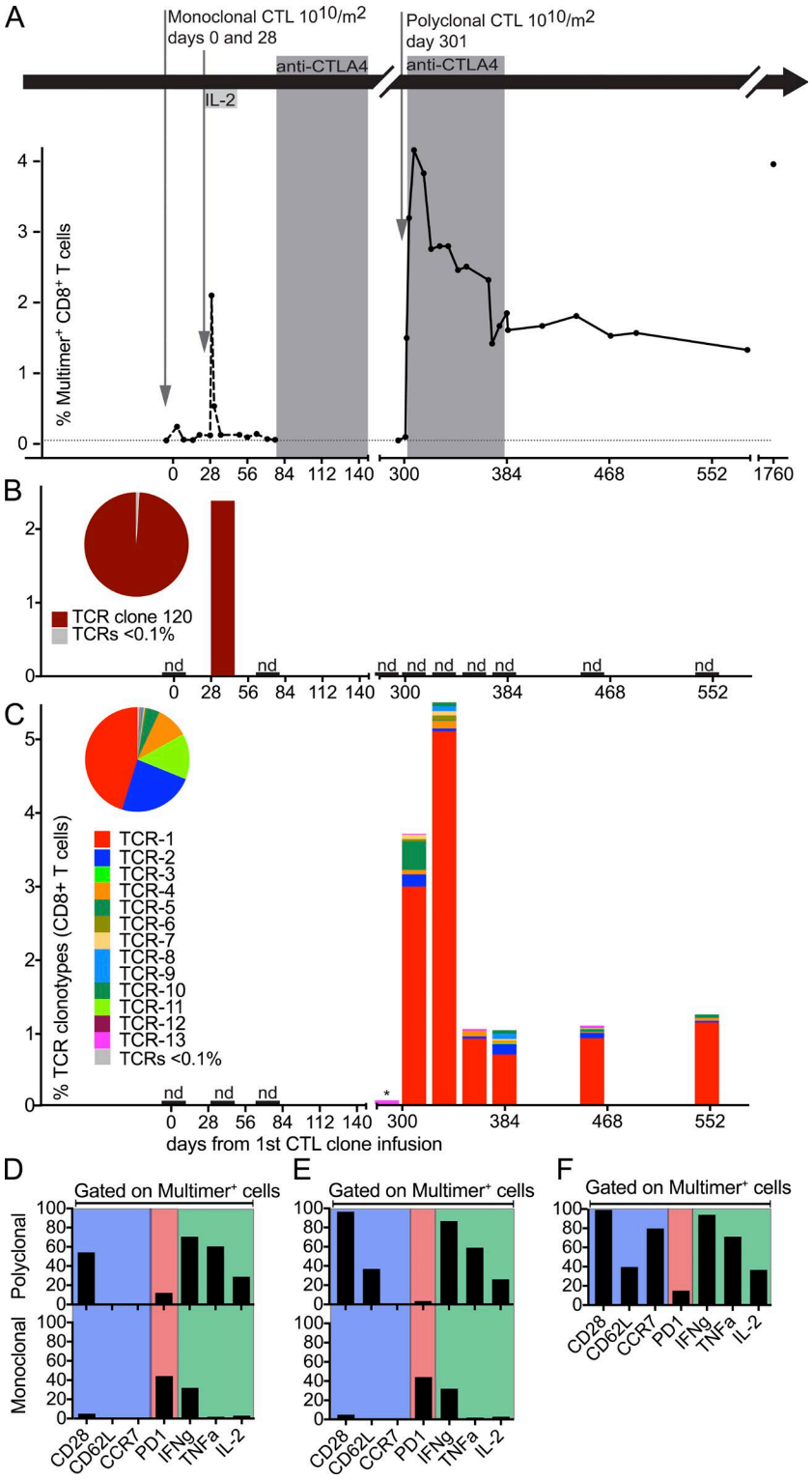

Figure 2. **Kinetics, clonality, phenotype, and function of monoclonal and polyclonal CTL in vivo.** (A) Percent multimer$^+$CD8$^+$ T cells (left y axis) in PBMCs (solid circles) collected before and at defined time points after monoclonal (dashed line) and polyclonal (solid line) CTL infusions (indicated). Gray shaded areas indicate anti-CTLA4 treatment. (B and C) Inset pie charts represent individual clonotypes composing the monoclonal (B) and polyclonal (C) infused CTL. Graphs track the corresponding unique (B) and sum of clonotypes (C) as a percentage of total CD8$^+$ T cells (y axis). Time points in which the corresponding clones were assessed but not detected (nd) are indicated. *, only clone TCR-13 was detected immediately before the polyclonal infusion with a frequency of 0.054%. (D) Percent expression of CD28, CD62L, CCR7 (long-lived memory markers, blue shade), PD1 (activation/exhaustion marker, red shade), IFN-γ, TNF, and IL-2 (functional markers, green shade) on polyclonal (top) and monoclonal (bottom) infused CTL. (E and F) The same analysis performed on multimer$^+$ cells 1 d (E) and 86 d (F) in vivo after infusion.

structed engagement of B7 with CD28 (instead of CTLA4), the CD28$^+$ CTL subset may have preferentially survived/expanded through continued production/secretion of autocrine IL-2. Consistent with this hypothesis, CTL examined in vivo months after transfer nearly all expressed CD28$^+$, retained the capacity to secrete IL-2 in response to cognate antigen, and had low PD1 expression (Freeman et al., 2000). In contrast, CD28$^-$, IL-2$^-$, and PD1^{hi} monoclonal cells did not survive

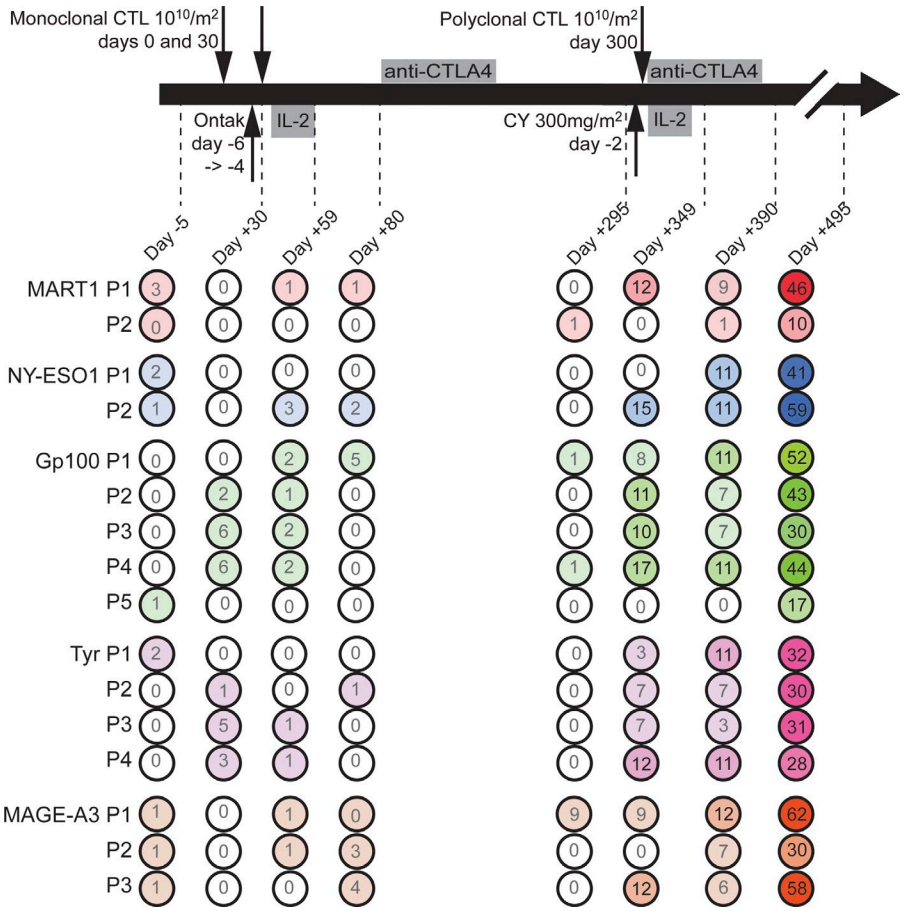

Figure 3. **Reactivity to nontargeted epitopes.** Heat map summarizing responses of $CD8^+$ and $CD4^+$ T cells independent of HLA restriction to pools of 20–30 peptides spanning MART1 (red), NY-ESO1 (blue), gp100 (green), tyrosinase (violet), and MAGE-A3 (orange). The color scale (light to dark) reflects the response magnitude at indicated time points before and after administration of monoclonal and polyclonal CTL during the patients' treatment course (top schema). Inset numbers indicate IFN-γ spots per 10^5 PBMC for each peptide pool.

beyond 1 d after transfer without ipilimumab exposure. With ipilimumab, the transferred cells further acquired the canonical markers of long-lived memory cells CD62L and CCR7, suggesting that the remaining cells were now programmed to persist (Unsoeld et al., 2002; Wölfl et al., 2011).

Finally, the targeted immune response provided by tumor-specific CTLs, plus the proinflammatory context fostered by anti-CTLA4 blockade, were both required for epitope spreading (Ribas et al., 2003). Whereas epitope spreading has been demonstrated in some patients receiving anti-CTLA4 antibody monotherapy (Kvistborg et al., 2014), no evidence of antigen spreading or a clinical response was evident in this patient before receiving the combination therapy. Although delayed responses can occur after ipilimumab alone, this usually occurs by 3 mo (Wolchok et al., 2009). Our patient demonstrated unequivocal disease progression with the appearance of a new lesion 7 mo after ipilimumab monotherapy. As melanoma is a highly mutated tumor, antigen-spreading may have increased the number and strength of T cells targeting multiple antigens beyond the ones assessed here (Schreiber et al., 2011). Multivalent responses may have blocked the outgrowth of antigen-loss tumor variants such that complete tumor eradication could occur.

We conclude that combining CTLA4 blockade with the transfer of well-characterized, robust antitumor CTLs represents an encouraging strategy to enhance the activity of the adoptively transferred CTL and induce de novo antitumor responses. This strategy may hold broad promise for immune checkpoint blockade–resistant melanomas.

MATERIALS AND METHODS

Clinical protocols. All clinical investigations were conducted according to the Declaration of Helsinki principles. This patient was first enrolled in protocol FHCRC #2271 (monoclonal CTL plus denileukin diftitox, three patients treated), then #2225 (polyclonal CTL plus ipilimumab, 10 patients treated). Of the 10 patients treated on protocol #2225, two, including the patient described here, achieved ongoing CRs (beyond 12 wk), two achieved partial responses, three achieved stable disease, and three had progressed at 12 wk. Both protocols were approved by the FHCRC Institutional Review Board and the U.S. Food and Drug Administration and were registered at MD Anderson Clinical Trials as NCT00945269 and NCT00871481.

Treatment plans. Enrolled in protocol #2271, the patient received $10^{10}/m^2$ monoclonal (Yee et al., 2002; Wallen et al.,

2009) A*0201-restricted MART1-specific T cells 28 d apart. The second infusion was preceded by Ontak (18 mcg/kg i.v. 6, 4, and 2 d before infusion), and both infusions were followed by low-dose IL-2 (250,000 U/m^2 s.c. twice daily for 14 d). In protocol #2225, the patient received CY (300 mg/m^2 i.v.) before the infusion of 10^{10} polyclonal, IL-21–primed (Pollack et al., 2014) $MART_{127-35}$ CTL/m^2, immediately followed by low-dose s.c. IL-2 and ipilimumab (3 mg/kg every 3 wk × 4 doses; Hodi et al., 2010). Radiological responses were evaluated according to the mWHO-based immune-related response criteria (Wolchok et al., 2009).

Isolation and expansion of monoclonal MART1-specific CTLs (without IL-21). PBMCs were collected by leukapheresis, and all ensuing ex vivo manipulations were performed in the clinical Good Manufacturing Practices Cell Processing Facility of the FHCRC. Donor PBMCs were stimulated three times for 7–10-d cycles with autologous DCs pulsed with the HLA*0201-restricted $MART\text{-}1_{26-35}$ (EAAGIGILTV) peptide (Anaspec) at a DC to effector ratio of 1:2–10 to obtain sufficient frequencies (>5%) of MART1-reactive $CD8^+$ T cells. On day 2 of each stimulation, the γ_c-chain cytokines IL-2 (12.5 IU/ml), IL-7 (5 ng/ml), and IL-15 (1 ng/ml) were added. Cultures that contained ≥5% specific $CD8^+$ T cells, assessed by multimer analysis, were cloned by limiting dilution, and then stimulated twice using the rapid expansion protocol (Riddell et al., 1992; Ho et al., 2006). CTL products were frozen, thawed, and washed before infusion, for a total production time of 12–13 wk.

Isolation and expansion of polyclonal MART1-specific CTLs (with IL-21). PBMCs were depleted of $CD25^+$ T cells (Miltenyi Biotec) to eliminate regulatory T cells, and stimulated twice for 7 d with autologous DC pulsed with $MART1_{26-35}$. DC stimulations were supplemented with the same γ_c-chain cytokines plus IL-21 (30 ng/ml) on day 1. Cultures that contained ≥5% specific $CD8^+$ T cells were clinical-grade sorted (Influx cell sorter; BD) and stimulated twice using the Rapid Expansion Protocol. The total production time was 6 wk. The purity and phenotype, as well as the Vβ repertoire of each CTL product immediately before infusion, are shown in Fig. S1 and Table S1, respectively. There was no overlap in the Vβ repertoire of both products.

T cell tracking by peptide-MHC (pMHC) multimers. Allophycocyanin-conjugated MART1-specific antigen pMHC multimers (FHCRC Immune Monitoring Core Facility) were used to detect transferred CTL in PBMCs collected after infusions, with a staining sensitivity of 0.05% of total $CD8^+$ T cells, as previously described (Chapuis et al., 2012).

T cell tracking by HTTCS. To guarantee that all tracked clonotypes were tumor-specific, only pMHC multimer-binding cells within the CTL infusion products were selected by flow cytometry before DNA isolation for HTTCS. The HTTCS limit of detection was set at 0.001% of all TCR reads, below which frequencies could not be reliably determined (Robins et al., 2012). The HTTCS frequency of each clonotype is based on all TCR Vβ chain reads, from both $CD4^+$ and $CD8^+$ T cells (Robins et al., 2009). To compare tracking by HTTCS versus multimer staining, expressed as a percentage of $CD8^+$ T cells, HTTCS results are reported as a percentage of $CD8^+$ cells using the formula: HTTCS frequencies × ([% total $CD8^+$ T cells] + [% total $CD4^+$ T cells]/[% total $CD8^+$ T cells in each sample]).

Flow cytometry. Monoclonal and polyclonal CTL products pretransfer and PBMCs after transfer were identified by binding to a specific pMHC multimer, and analyzed by flow cytometry after staining with fluorochrome-conjugated mAbs to CD4, CD16, CD19 (exclusion channel), CD8, CD28, CD62L, CCR7, and PD1 (BD). Assessments of the intracellular cytokine expression of IFN-γ, TNF, and IL-2 in response to cognate antigen were performed, as previously described (Papagno et al., 2007). Cells were analyzed on an LSRII instrument (BD) using FACSDiva software.

ELISpot assays. Amino acid peptides, 15 aa in length offset by 5 aas, were grouped into pools of 20–30 peptides spanning MART1, NY-ESO-1, gp100, tyrosinase, and MAGE A3 (2, 2, 5, 4, and 3 pools, respectively; Sigma-Aldrich). Peptide pools were used to stimulate PBMCs at indicated time points, and T cell reactivity was quantified using a human IFN-γ ELISpot assay, as previously described (Scheibenbogen et al., 2000). This method quantifies $CD8^+$ and $CD4^+$ T cell reactivity to peptide pools independent of HLA restriction.

Online supplemental material. Fig. S1 shows purity and phenotype of infused monoclonal and polyconal products. Table S1 shows Vβ repertoire of infused monoclonal and polyclonal CTL products. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20152021/DC1.

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C. Yee is on the advisory board of Adaptive Biotechnology whose technology was used to sequence the TCRs. A conflict management board was established at Fred Hutchinson Cancer Research Center when these assays were performed. The authors declare no additional competing financial interests.

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