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Tracking the Fate and Origin of Clinically Relevant Adoptively Transferred CD8+ T Cells *In Vivo*

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Abstract

Adoptively transferred tumor-specific cells can mediate tumor regression in cancers refractory to conventional therapy. Autologous polyclonal tumor-specific cytotoxic T cells (CTL) generated from peripheral blood and infused into patients with metastatic melanoma show enhanced persistence, compared to equivalent numbers of more extensively expanded monoclonal CTL, and are associated with complete remissions (CR) in select patients. We applied high-throughput T cell receptor Vβ sequencing (HTTCS) to identify individual clonotypes within CTL products, track them *in vivo* post-infusion and then deduce the pre-adoptive transfer (endogenous) frequencies of cells ultimately responsible for tumor regression. The summed *in vivo* post-transfer frequencies of the top 25 HTTCS-defined clonotypes originally detected in the infused CTL population were comparable to enumeration by binding of antigen peptide-HLA multimers, revealing quantitative HTTCS is a reliable, multimer-independent alternative. Surprisingly, the polyclonal CTL products were composed predominantly of clonotypes that were of very low frequency (VLF) in the

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endogenous samples, often below the limit of HTTCS detection (0.001%). In patients who achieved durable CRs, the composition of transferred CTLs was dominated (57–90%) by cells derived from a single VLF clonotype. Thus, HTTCS now reveals that tumor-specific CTL enabling long-term tumor control originate from endogenous VLF populations that exhibit proliferative/survival advantages. Along with results indicating that naïve cell populations are most likely to contain cells that exist at VLF within the repertoire, our results provide a strong rationale for favoring T cells arising from VLF populations and with early-differentiation phenotypes when selecting subset populations for adoptive transfer.

Introduction

Adoptive transfer of tumor-specific cytotoxic T cells (CTL), a strategy demonstrating the capacity for eliminating cancer in an increasing number of settings, requires the *in vivo* establishment of a robust, persistent population of tumor-specific cells to prevent tumor recurrences (1, 2). Our group has utilized the endogenous autologous T cell repertoire of patients as a readily accessible source of antigen-specific cells. In this approach, bulk peripheral blood mononuclear cells (PBMC) are repetitively stimulated by dendritic cells pulsed with specific HLA-restricted peptides from tumor antigens to increase the frequency of antigen-specific T cells. In our initial efforts, the responding cells were cloned by limiting dilution and CTL infusion products represented one expanded clone/cell (3–8). This strategy facilitated tracking by quantitative polymerase chain reaction (PCR)-based approaches, using primers flanking the clonal complementarity determining region 3 (CDR3) (5, 7). However, such monoclonal T cell products exhibited only a short survival *in vivo* (<14 days) in the majority of patients, likely reflecting the extensive expansion required to attain therapeutic cell doses from a single reactive T cell *in vitro*, and the associated terminal differentiation (3, 9–11).

The availability of high-speed cell sorting based on peptide-MHC (pMHC) binding now enables infusion of CTL that stem from a polyclonal tumor-specific repertoire (12). Additionally, in both murine and human studies, $CD8^+$ T cell culture conditions that include exposure to IL-21 during *in vitro* priming has been shown to facilitate greater expansion of the antigen-responding cells with less apoptosis (13–17). By employing such strategies, therapeutic cell doses can be produced by *ex vivo* expansion in 4–6 weeks rather than 3–4 months, and the tumor-specific cells generated have a higher replicative capacity and less terminally differentiated phenotype *in vivo* (8, 12, 18). As a likely consequence of such technologies, infusion of polyclonal CTL now produces enhanced *in vivo* persistence (>6 months) in a larger fraction of patients, which correlates with long-term tumor regression and improved disease control in a subset of patients (19, 20).

Ample evidence suggests that the nature of the original parent T cell population is critical in determining the *in vivo* behavior of the expanded infused CTL (10, 17, 21–23). To elucidate the factors that might contribute to the persistence of these polyclonal, IL-21 primed CTL that lead to clinical responses, we investigated the endogenous frequencies of the infused clonotypes that contribute to mediating and sustaining tumor regression. In the case of autologous adoptive transfers, expanded/infused polyclonal tumor-specific T cells

necessarily originate from a patients' pre-existing circulating T cell repertoire. Following *ex vivo* stimulation and culture, the phenotype and frequency of the expanded CTL are generally sufficiently altered such that the qualities of the parent cells cannot be determined from the characteristics of the cells at the time of infusion (22). However, the CTL that persist express/acquire phenotypic and functional characteristics associated with long-lived memory T cells, including markers associated with survival (CD28, CD27 and CD127) (24, 25), lymph-node homing (CD62L and CCR7) (26, 27), and exhibit the capacity for poly-functional cytokine production (IFNγ, TNFα and IL-2) (28), suggesting some of these characteristics are critical for persistence and tumor control (20).

We therefore utilized high-throughput T cell receptor Vβ sequencing (HTTCS) to sequence T cell receptor (TCR) Vβ regions in a massively parallel fashion and thereby assess clonotype evolution after infusions, determine the contribution of distinct cells and investigate the original source of the cells which progeny most likely made a major contribution to mediating tumor regression/control. HTTCS can discern individual TCR clonotypes in a polyclonal population with a limit of detection of 1/100,000 (0.001%) (29–31), and we first assessed whether this could enable *in vivo* tracking of the potentially thousands of infused individual tumor-specific clonotypes. After demonstrating the accuracy of this approach, we then applied HTTCS to a set of 10 patients with metastatic melanoma who had detectable frequencies of transferred T cells after infusions. All patients had received polyclonal IL-21-primed T cell products expanded from unselected circulating T-cell populations. We investigated the number of clonotypes that persisted after infusions, their individual frequencies and the characteristics of the original source of the cells whose progeny most likely made a major contribution to mediating tumor regression/control.

Results

Validation of HTTCS for tracking the frequency of transferred monoclonal CTL *in vivo*

Among patients (Pts) who received monoclonal (M) T cells in a previous study (on **Protocol #2140** (7), **see Materials and Methods**), we analyzed the two (of eleven infused) in whom the transferred cells were detectable *in vivo* for >40 days after infusions (Table 1). Pt M2140-1 was infused with cells expanded from a melanoma-specific $CD8^+$ T cell clone: 99.4% of the cell infusion product bound the HLA A*0201-restricted $TCR1_{127\text{-}35}$ (A2/MART1) pMHC multimer (Figure 1A). HTTCS revealed one clonotype comprised 99.7% of all TCR reads; 29 other clonotypes detected with frequencies ≥0.001% (Table S1) comprised the remaining 0.3% and were likely bystander/contaminant clonotypes derived from the irradiated allogeneic PBMCs used to expand the cells (Figure 1B, **upper pie plot**). Pt M2140-2 was infused with $CD8^+$ T cells recognizing the HLA B*4402-restricted $Tyrosinase_{192\text{-}200}$ epitope (B44/Tyr), for which a pMHC multimer could not be synthesized. The cell product included a total of 868 clonotypes of which the most prevalent single clonotype represented 94% of all TCR reads (Figure 1B, **lower pie plot**). For both patients, the most prevalent HTTCS-detected clonotype was confirmed as the infused CTL clone by TCR Vβ quantitative PCR (Table S2). We then compared the post-transfer *in vivo* frequencies of the most prevalent clonotype in each infusion product, as assessed by HTTCS, p-HLA multimer binding and/or TCR Vβ quantitative PCR. For Pt M2140-1,

frequencies obtained by HTTCS were 42.1%, 0.84% and 0.61% of total TCR reads in PBMC collected on days 4, 56 and 508 after transfer. By comparison, the frequencies by multimer staining were 40.0%, 0.21% and 0.74%, and 48.5%, 0.57% and 0.28% by TCR Vβ-specific PCR (Figure 1C, **upper graph**). For Pt. M2140-2, HTTCS frequencies were 21.5%, 6.75% and 1.25% at days 4, 48 and 117, compared to 15.8, 3.0% and 0.71% using TCR Vβ-specific PCR (Figure 1C, **lower graph**). Thus, for frequencies ≥0.001% of total T cells, HTTCS frequencies yielded results concordant to TCR Vβ-specific PCR and pMHC multimer binding, suggesting HTTCS can be used to quantitatively track the frequency of infused monoclonal $CD8^+$ T cells *in vivo*.

HTTCS accurately tracks the *in vivo* frequencies of adoptively transferred polyclonal CTL

We next analyzed PBMC from 10 patients who had each received one infusion of $10^{10}/m^2$ polyclonal (P) CTL specific for A2/MART1 on a previous study (on **Protocol #2225** (20), **see Materials and Methods**) (Table 1). HTTCS detected between 56 and 2036 (mean 555.4) clonotypes in the polyclonal infusion products from Pts P2225-1-10 (Figure 2A). The most prevalent clonotype comprised between 4 and 77% of the total cells in each infusion (mean 33.3%), and the 25 most prevalent clonotypes together comprised between 35.0 and 99.9% (mean 78.4%) of the total cell products. This HTTCS analysis was performed on infusion products selected for binding pMHC multimers with >99% purity. The limit of pMHC detection is typically ≥0.01% of $CD8^+$ T cells, which is at least 10-fold less sensitive than HTTCS. Thus, HTTCS of infusion products that have been selected by pMHC multimer-binding is likely to also include contaminant clonotypes. To avoid tracking these bystander clonotypes after adoptive transfer, we excluded from analysis those clonotypes that had higher frequencies in pre-infusion PBMC compared to their frequencies within the CTL products, as these clonotypes likely reflected non-expanded bystander cells that decreased in frequency through the cell culture process (Table S3). A total of 86 clonotypes (average 0.014% of total CTL products) with frequencies of 0.001% to 8.85% (mean 0.35%) were thus identified and removed from our analysis. Tracking the sum of the frequency of all infused, expanded clonotypes by HTTCS yielded near concurrent results with those obtained by tracking pMHC multimer binding for all pts who received polyclonal products (Figure 2B). Thus, HTTCS appears to accurately track the *in vivo* frequencies of infused polyclonal CTL.

In vivo specific clonotype frequencies increase as a consequence of adoptive transfer

To affirm that the post-infusion increase in clonotype frequencies is a reproducible consequence of the CTL infusions, we assessed the frequency of individual infused clonotypes for two patients who each received two polyclonal infusions, 30 or 37 days apart (**Protocols #2504 and #2558**(32), **see Materials and Methods**) (Table 1). Although the clonotypes constituting the largest fraction of the patients' first and second infusates were shared, clonotype frequencies did vary as a likely consequence of the culture process, which uses an aliquot of the first CTL culture/infusion to expand sufficient cells for a second infusion (Figure 3A). The sum of all shared clonotypes was tracked in PBMC before and after each infusion (Figure 3B), as was the individual contribution of the top 25 and all 10 clonotypes for Pts P2504-1 and P2558-1, respectively (Figure 3C). Before infusions, all clonotypes were detected in PBMC with frequencies of <0.01% (max 0.0097, median

0.001%) and 0.09% (max 0.085%, median 0.0028%) for Pt P2504-1 and P2558-1, respectively. The frequency of the clonotypes peaked between 1 and 7 days after the first infusion (Pt P2504-1 - max. TCR seq. 2: 1.5%, median for first 25 clonotypes: 0.06%; Pt P2558-1 - max. TCR seq. 5: 2.73%, median for all 10 clonotypes: 0.35%), and the frequencies decreased over 14 or 30 days to near or at baseline levels. The same clonotypes then peaked again 1 to 7 days after the second infusion, albeit at lower frequencies (Pt P2504-1- max. TCR seq. 11: 0.64%; median: 0.05%; Pt P2558-1- max. TCR seq. 1: 0.68%; median: 0.15%). Thus, the post-infusion changes in clonotype frequency appear to be a consequence of the infusion of antigen-specific CTL and not co-incidental variations within the endogenous TCR repertoire. Of note for these 2 patients, no correlation could be established between the frequency of the clonotypes in the infused product and the peak frequencies after infusion.

Single, immunodominant persistent clonotypes are associated with CRs after adoptive transfer

Of the ten patients with metastatic melanoma who received A2/MART1-specific polyclonal CTL, two achieved CRs as evaluated by the immune-related response criteria (IrRC)(33), two a partial remission (PR), three stable disease (SD) and three had progressive disease (PD) as best response (Table S4 and (19, 20)). The frequencies of the first 25 most prevalent clonotypes detected in each cell product were individually tracked *in vivo* after adoptive transfer (Figure 4A, **colored lines**), as were the sum of remaining clonotypes (Figure 4A, **grey lines**). In the two patients who achieved a CR after infusion (Pt P2225-1, Pt P2225-7), only one individual clonotype remained detectable at sustained frequencies higher than those of other infused clonotypes (>0.056% at 280 days and 0.093% at 175 days post-infusion for Pts. P2225-1 and 7 respectively) and represented the majority (>99%) of detected antigen-specific cells post-infusion (Figure 4A, **red arrows**). Clonotype half-lives (t½, see **Statistical Analysis**) were determined for each patient and grouped according to best clinical response (Figure 4B). For patients who achieved a CR (Pts P2225-1 and 7), the immunodominant clonotypes (Figure 4B, **red arrows**) had t½ of 173 and 132 days, which was respectively ~7 and 5 times longer than the median t½ of all infused clonotypes for Pts P2225-1-10 (24.8 days). Along with the higher expression of Ki67 by the transferred pMHC multimer-binding cells, - composed by a majority of the dominant clonotype - compared to multimer$^-$ cells (Table S5 and (7, 20)), these findings indicate the immunodominant CTL had the capacity to expand and persist. For the remaining patients, the average t½ of clonotypes after transfer was 53 and 31 days for patients who achieved a PR as best response (Pts P2225-9 and 10), 44, 46 and 21 days for patients who achieved SD (Pts P2225-2, 4 and 6), and 13, 15 and 14 days for patients who progressed (Pts P2225-3, 5 and 8). When all clonotype t½ were grouped according to patients' best response (Figure 4C), the t½ differences were statistically significant between patients who obtained CRs, PRs, SDs and PDs. Thus, prolonged *in vivo* persistence of transferred clonotypes is associated with, and presumably important for, tumor control by transferred CTL.

Pre-existing clonotype frequencies provide insights into the nature of persisting CTL

The number of clonotypes comprising the polyclonal cell products for Pts. P2225-1 to 10 was between 56 and 2036, with a median of 262.5 clonal sequences (Table S2). However,

most clonotypes present in cell products (range 43 to 1275, median 160) were detected by HTTCS with frequencies below 0.001% (the limit of detection) in PBMC obtained before and after infusion, and constituted only a minor fraction of the total infusion product (range 0.82 – 24.7%, median 2.26%) (Figure 5A, **grey areas**). As stated before, a clonotype with a frequency in the CTL product that was less than its corresponding frequency in the pre-infusion PBMC was defined as having not expanded during the culture process (Table S2, bottom row). These clones comprised a minor fraction of the final infusion product, with a median of 0.02% (range 0–0.61%) (Figure 5A, **purple areas**), and were not further monitored. We found that clonotypes detected in any post-infusion PBMC sample, but not detected in pre-infusion samples (range 10–205, median 56 sequences), were the dominant components of the final cell product (range 74.68–98.97%, median 97.67%) (Figure 5A, **blue areas**), implying preferential expansion from individual parental clonotypes with frequencies < 0.001%; i.e. a very low frequency (VLF) population. The immunodominant clonotypes in both patients who achieved CRs (Pts P2225-1 and 7, Figure 4A and 4B, **red arrows**) were derived from respective VLF populations. Clonotypes expanded in the cell infusion product that were detectable in PBMC before infusion were less prevalent (range 0–6, median 2 sequences), comprising <10% (range 0–9.22%, median 0.03%) of the final cell products that were infused (Figure 5A, **orange areas).** The frequencies of all expanded clonotypes were summed at multiple timepoints after infusion (Figure 5B, **solid red circles**) and further subdivided into previously undetected (Figure 5B, **open blue circles)** versus previously detected clonotypes (Figure 5B **orange open circles).** Previously undetected clonotypes represented the majority of cells in both infusion products and the detected clonotypes in post-transfer PBMC, whereas previously detected clonotypes comprised a small fraction of infused products and a minority of post-transfer clonotypes.

To assess the likelihood that the preferentially expanded and infused clonotypes originated from a distinct parental T cell subpopulation, we assessed the propensity for clonotypes present in three sets of normal donor PBMC to exhibit a mostly antigen-experienced or naïve phenotype. Overall, clonotypes with frequencies <0.001% in PBMC were 15 times more likely (72% ± 2% vs 5% ± 3%) to have originated from a $CD3^+CD45RA^+$ (primarily naïve T cells, T_N) VLF population than from a $CD3^+CD45RO^+$ (mostly antigen-experienced cells) population (Figure S1).

Discussion

The use of HTTCS to examine TCR clonotypes of defined antigen specificity pre-infusion, through *in vitro* culture and following adoptive transfer has provided a means to monitor the accumulation and *in vivo* persistence of individual clonotypes within a polyclonal population. This technology provides concordant results with frequency analyses by qPCR of defined TCRs, which has more limited utility but can be used for tracking monoclonal cell products, and by pMHC multimer stains, which can be used to track polyclonal as well as monoclonal products but doesn't distinguishing individual clonotypes (5, 7, 20, 34, 35). HTTCS essentially provides a 'natural barcode sequence' through the specific CDR3 TCR sequences of all clonotypes in an infused CTL cell product, enabling the identification of the cell/clonotype of origin within pre-infusion donor PBMC, measuring the expansion of each individual clonotype in the infusion product, and monitoring their individual *in vivo* fates

after infusions (29–31). These results can be correlated with clinical outcomes, which should facilitate the development of methods to preferentially isolate/generate cells that will have greater therapeutic efficacy.

We were surprised to observe that the vast majority of CTL expanded in the infusion product originated from rare T cells. To increase the probability of generating autologous antigen-specific cells for adoptive transfer, the culture methods used in this study were aimed at expanding as many T cells as possible, without regard for a specific substrate T cell subset (20). Thus, autologous MART1-specific T cells "competed" with each other in the culture system, and certain clonotypes with pre-infusion frequencies <0.001% demonstrated an increased fitness and preferentially expanded. These were also the cells that comprised the majority of antigen-specific cells that were detectable and persisted post-infusion. Thus, the culture system fostered the expansion of fit cells that, despite having the capacity to recognize the tumor antigen, had a lower likelihood of already having been expanded *in vivo* in the tumor-bearing patient. By contrast, tumor-specific clonotypes that were more abundant in pre-infusion PBMC, with frequencies >0.001%, were generally less numerous in the final cell products and persisted less robustly *in vivo* after infusion, suggesting these cells both expanded less well *ex vivo* and were less fit for survival post-infusion. This latter observation is consistent with the poor expansion of tumor-specific cells that have been driven to exhaustion by a persistent antigenic burden (36).

It is known that the qualities of the parent T cell from which tumor-specific CTL originate before *ex vivo* expansion plays a critical role in *in vivo* persistence, survival and proliferation after transfer (1, 2). $CD8^+$ T cells can be separated into distinct subsets based on phenotypic markers that distinguish cells with different molecular programs and biologic functions. These CD8 subsets include T_N and antigen-experienced cells, with the latter further subdivided into at least memory stem (T_{SCM}) cells central (T_{CM}), effector (T_{EM}), and terminal effector (T_{eff}) cells (10, 14, 17, 21, 22, 37, 38). In prevention models, T_{CM} are generally superior to T_{EM}, presumably reflecting the distinct ability of T_{CM} to form long-lasting memory, rapidly proliferate and expand after re-encountering antigen, and produce abundant differentiated T_{EM} (10, 21, 22). By contrast, in therapy models of established tumors and of chronic infection, transferred cells derived from T_N are generally superior to T_{CM}, with an enhanced ability to provide sustained proliferation and *in vivo* expansion leading to enhanced persistence and anti-tumor activity. This likely reflects both greater replicative potential of T_N-derived cells and also that T_{CM} may already be programmed to undergo apoptosis or become functionally exhausted if stimulation persists and the Ag is not rapidly cleared (17, 23). Within the T_N compartment ($CD45RA^+62L^+$) the small subset of T_{SCM}, distinguished by CD95 expression, appears to retain the plasticity of T_N cells and the self-renewal capacities of T_{CM} (38), functioned better than T_{CM} in tumor therapy models and persisted for years after transfer into humans (39–41).

Although available clinical reagents could not distinguish the origin of cells at the time of infusion as expanded CTL derived from T_N, T_{CM} or T_{EM} mostly display a differentiated T_{EM} phenotype after *in vitro* expansion (8, 20, 22), our data set does provide insights into the likely origin(s) of the most effective cells. First, all cells obtained from the patients were stimulated with antigen-pulsed dendritic cells in the presence of IL-21 that facilitates the

expansion of antigen-specific CTL with low initial endogenous frequencies (15). The effects of IL-21 exposure during priming of antigen-specific $CD8^+$ T_N have been best characterized and shown to help imprint a central memory program following antigen-specific stimulation, with limited differentiation of the expanded cells promoting survival, expansion and *in vivo* persistence post-infusion in murine models and human trials (8, 14, 42, 43). However, the effect of IL-21 on previously exposed antigen-experienced memory cells during stimulation is less clear (43, 44). Our analysis of normal donors suggest that clonotypes with PBMC frequencies <0.001% were 15 times more likely to be included in a $CD45RA^+$ subset which includes mostly T_N but also T_{SCM}, compared to $CD54RA^-$ subsets, which include T_{eff}, T_{EM} and T_{CM}. These findings are consistent with murine studies where antigen-specific naïve precursor frequencies were estimated at ≤1 in 200,000 cells (45), and where the majority of naïve CD8 T cells TCRβ sequences were found at very low clonotype frequency (46). Together these data support the notion that antigen-naïve T cells are included in VLF populations. Although speculative, cells derived from VLF populations in our study might have been more likely to originate from a naïve rather than antigen-experienced subset. However, this could not be ascertained due to the logistical impossibility of sampling all pre-infusion cells to obtain their phenotypes (7, 20). It is plausible that some T_{CM} and T_{SCM} cells may also be found among the VLF populations, as these early-differentiation phenotypes may have experienced limited *in vivo* expansion (38). However, regardless of origin, parent cells whose progeny have the potential to ultimately persist, either assisted by IL-21 exposure or due to their intrinsic nature, are clearly rare in the repertoire. By contrast, *in vivo* expanded cells with higher intrinsic frequencies are apparently not suitable substrates for achieving sustained responses by T cell infusion.

Our findings also show that within VLF populations, only a minority of CTL are poised to produce offspring that can mediate sustained clinical responses. In this series of patients with metastatic melanoma, HTTCS tracking of infused clonotypes showed the antigen-specific response in PBMC of patients who reached CRs was largely dominated by a single immunodominant CTL clone, derived from a VLF population, which remained detectable at high frequencies over time. Although no pre- or post-tumor biopsies were obtained from these patients to assess the presence of these clonotypes at the tumor site, the development of vitiligo in Pt P2225-1(19), suggests an ongoing MART1-specific on-target, off-tumor effect. Along with the capacity of the immunodominant CTL for sustained proliferation, reflected by their Ki67 expression and markedly prolonged t½ (>100 days) in PBMC compared to other simultaneously transferred clonotypes, these CTL were likely instrumental for tumor elimination either through a direct anti-MART1 effect or by facilitating the development of secondary responses (19, 20). For these two patients, only 1 in 56 (1.8%) and 1 in 76 (1.3%) infused clonotypes persisted and mediated/contributed to antitumor responses. These results are reminiscent of the proliferation and expansion of a very limited number of T cell clones at the tumor site in patients who presented clinical responses to immunomodulatory agents (47).

The application of HTTCS to this clinical trial has provided unique insights by enabling tracking of individual clonotypes. Whether the properties of the parental cells from which the clonotypes were derived, and/or the affinity of the specific TCRs, conferred superior efficacy in the patients who experienced a CR remains unclear. Since long-term T cell

survival can be enhanced by antigenic exposure, cells that carry high-affinity TCRs that can signal in the context of low-levels of antigen presentation (2, 48, 49), as is the case for most "self" tumor-antigens (50), are likely to have a post-infusion survival advantage compared to cells that carry low-affinity TCRs (51). Of the ten patients we studied, only two of 4,722 MART1-specific infused clonotypes (0.04%) demonstrated an immunodominant anti-tumor effect. Thus, a better understanding of such TCRs, and the intrinsic cellular properties associated with this rare feature, could direct strategies to endow cell therapy products with enhanced anti-tumor efficacy. Such TCRs can be identified by HTTCS and are paramount candidates for isolation use in adoptive T cell therapies in which TCR genes are transduced into optimal T cell subsets for patients with appropriately matched HLA types. This strategy might be used to broadly confer anti-tumor efficacy to a wider range of patients with metastatic disease.

Materials & Methods

Clinical protocols and patient characteristics

All clinical investigations were conducted according to the Declaration of Helsinki principles. All clinical protocols were approved by the Fred Hutchinson Cancer Research Center Institutional Review Board, and the US Food and Drug Administration. All patients provided written informed consent.

Treatment Plans (Table 1): Protocol #2140

Pts M-2140-1 and M-2140-2 with metastatic melanoma received cyclophosphamide (CY) 4000mg/m^2 administered over 2 days before the infusion of 10^{10} *monoclonal* melanoma-specific CTL/m^2 followed by low-dose subcutaneous (s.c.) IL-2 (250,000 U/m^2) twice daily for 14 days (NCT 00438984) (7). Pts M1 and M2 received CTL specific for HLA A*0201-restricted $MART1_{127\text{-}35}$ (AAGIGILTV) and HLA B*4403-restricted $Tyrosinase_{192\text{-}200}$ (SEIWRDIDF), respectively. We specifically analyzed the 2 of 11 patients who received monoclonal products and demonstrated post-infusion *in vivo* persistence for a direct assessment of the equivalence of multimer staining, CDR3 PCR and HTTCS on a unique CTL clone. ***Protocol #2225:*** Pts P2225-1-P10 with metastatic melanoma received CY 300mg/m^2 before the infusion of 10^{10} *polyclonal* A*0201-restricted $MART_{127\text{-}35}$ CTL/m^2, immediately followed by low-dose s.c. IL-2 and ipilimumab (anti- Cytotoxic T-Lymphocyte Associated Protein 4, Yervoy®, Bristol Myers Squibb) (52) 3mg/kg every 3 weeks for a total of 4 doses (NCT 00871481) (20). Radiologic responses were evaluated post-infusion according to the IrRC (Figure S1) (33). Compared to monoclonal products, a higher fraction of polyclonal products expressed the markers CD27, CD28 and CD127 associated with memory. Whereas 100% (10/10) of polyclonal products persisted, only 18% (2/11) of monoclonal products persisted beyond 40 days in vivo (Table 1). ***Protocol #2504***: Single patient protocol for a Pt P2504-1 with metastatic breast cancer whose tumor expressed NY-esophageal antigen 1 (NY-ESO1). The patient received CY 2000mg/m^2 administered 3 days before infusion of 10^{10} *polyclonal* HLA A*0201-restricted $NY\text{-}ESO1_{157\text{-}165}$ (SLLMWITQC) CTL/m^2. Thirty days later, the patient received the same cell dose preceded by CY 300mg/m^2. ***Protocol #2558***: Single patient protocol for Pt P2558-1 with metastatic Merkel cell carcinoma whose tumor expressed the Merkel cell polyoma virus (MCPyV)

(32). The patient received 10^{10} *polyclonal* HLA A*2402-restricted MCPyV LT-$Ag_{92\text{-}101}$ (EWWRSGGFSF) preceded by intralesional IFN-β-1B (3×10^6 IU) to a pancreatic metastasis and followed by low-dose s.c. IL-2. Thirty-one days later, the patient received the same cell dose, this time preceded by a single 8 gray (Gy) fraction of radiation to a remaining metastasis, followed by 14 days of low-dose s.c. IL-2.

Generation and expansion of monoclonal tumor-specific CTL products (without IL-21)

For protocol #2140, which involved monoclonal tumor-specific cells, cell processing was as previously described (7). Briefly, PBMCs were collected by leukapheresis and all ensuing *ex vivo* manipulations were performed in the clinical Good Manufacturing Practices (cGMP) Cell Processing Facility of the FHCRC. (12) Donor PBMC were stimulated three times for 7–10 day cycles with autologous dendritic cells (DC) pulsed with the HLA*0201-restricted MART-$1_{26\text{-}35}$ (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 (12.5IU/ml), IL-7 (5ng/ml) and IL-15 (1ng/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.(4) CTL products were frozen, thawed and washed before infusion, for a total production time of 12–13 weeks.

Generation and expansion of polyclonal tumor-specific CTL products (with IL-21)

For protocols #2225, #2504 and #2586, polyclonal tumor-specific cell products were generated as previously described.(12, 20) Briefly, PBMCs were depleted of $CD25^+$ T cells (Miltenyi Biotec Inc.) to eliminate regulatory T cells, and stimulated for seven days twice with autologous DC pulsed with MART$1_{26\text{-}35}$. 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 (BD Influx cell sorter, BD Biosciences) and stimulated twice using the Rapid Expansion Protocol. The total production time was 6 weeks.

CTL tracking by peptide-MHC multimers

The sensitivity of multimer staining was fixed at 0.1% of total $CD8^+$ T cells for monoclonal products (7), and at 0.05% for polyclonal products(20), as previously described. To compare the multimer stain tracking results expressed as a percentage of $CD8^+$ T cells, to HTTCS expressed as percentage of $CD4^+$ and $CD8^+$ T cells, multimer results are reported as a percent of $CD4^+$ and $CD8^+$ cells using the formula: (%Multimer$^+$ $CD8^+$ T cells) × ([% total $CD8^+$ T cells in each sample]/([% total $CD8^+$ T cells]+[% total $CD4^+$ T cells])).

CTL tracking by quantitative PCR

Primers flanking the CDR3 region of infused melanoma-specific CTL clones were designed, as previously described (5, 7). Total $CD4^+$ and $CD8^+$ T cells were determined by flow cytometry and the following formula was used to determine TCR copies per 100 T cells: 100/([% total $CD8^+$ T cells in each sample]+[% total $CD4^+$ T cells in each sample]*[TCR copies/{β-actin copies/2}*100]).

DNA extraction and immunosequencing

DNA was extracted from CTL products and whole PBMC using Qiagen Maxi DNA isolation kits (QIAGEN Inc.). TCRβ CDR3 regions were amplified and 750 ng of extracted DNA sequenced by Adaptive Biotechnologies Corp (Seattle, WA) using the "deep" resolution ImmunoSEQ assay, as previously described (53). Raw sequence data was filtered using the Adaptive bioinformatic website based on the TCRβ V, D and J gene definitions provided by the International ImMunoGeneTics collaboration (IMGT) (54), using the IMGT database (www.imgt.org). Productive nucleotide sequences were used for all tracking experiments. The data was further filtered to exclude sequences with no identifiable V and J removing PCR errors such as primer dimer and mispriming, as well as sequences with a raw read count <2, removing nucleotide sequencing errors (29).

CTL tracking by HTTCS

Only cells that bound pMHC multimers were selected by flow cytometry before DNA isolation for HTTCS. The limit of detection of HTTCS was set at 0.001% of all TCR reads below which frequency could not be reliably determined (31). Only clonotypes present in the CTL products were tracked in PBMC obtained after infusions. The frequency of each clonotype detected by HTTCS is based on all TCR Vβ reads from $CD4^+$ and $CD8^+$ T cells.

Normal Donor Sorts

PBMC were collected from three healthy adult donors, and processed into three populations for TCR sequencing: i.e. whole PBMC, flow-sorted $CD3^+CD45RA^+$ and $CD3^+CD45RO^+$, representing the naïve and antigen-experienced T cell populations, respectively. TCRs from ~1 million cells from each population were sequenced by HTTCS. Clonotypes were tracked in the original PBMC samples and considered part of a $CD3^+CD45RA^+$ versus an $CD3^+CD45RO^+$ phenotype if it was observed in one population but not the other, or if its abundance was ten-fold greater in one population compared to the other.

Statistical Analysis

Correlation between values obtained by HTTCS, multimer and TCR PCR: The Pearson's R was calculated on the log TCR frequency using frequencies obtained by HTTCS and either % $multimer^+$ T cells or TCR copied per 100 cells and treating each biological sample (i.e., timepoint) as an independent observation. P values were generated by normal approximation. Half-lives of persistent TCR clonotypes were determined using the formula $t_{1/2} = t/(\log_2[N_0/N_t])$. Only clonotypes that had at least 3 consecutive values above the limit of detection (0.001%) and for which R^2 was >0.3 were used in the analysis. Comparison of clonotype $t_{1/2}$ obtained in patients grouped according to their best clinical response: the Wilcoxon rank sum test was used to obtain p values.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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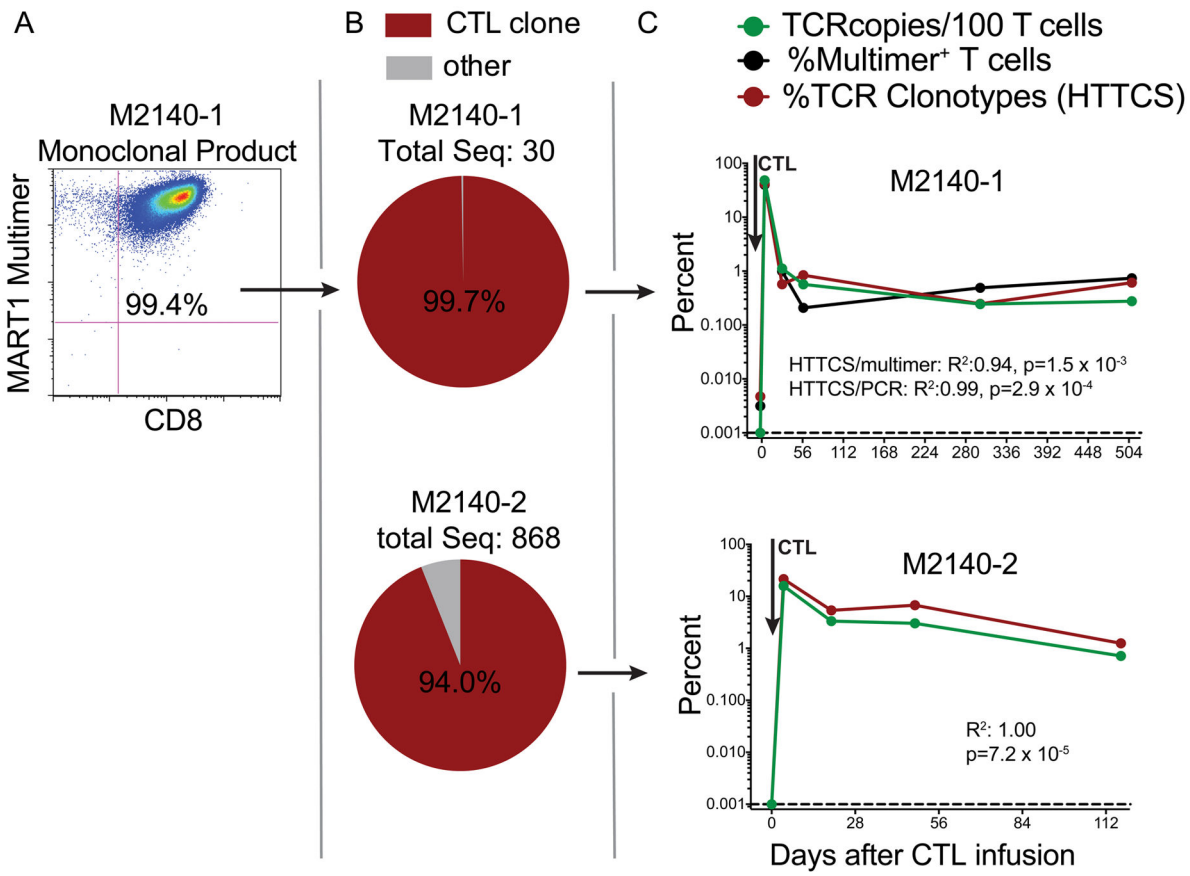

Figure 1. Characteristics of monoclonal CTL products and concurrence of clonotype frequencies determined by TCR Vβ PCR, multimer stains and HTTCS *in vivo*

(A) Scatter plot showing binding of the monoclonal cells to CD8 (x-axis) and HLA A*0201-restricted $MART1_{27-35}$ (y-axis). **(B)** Pie plots showing the percent of individual clonotypes composing the monoclonal CTL products. The frequency of the specific clone is overlayed on the plots. The total number of sequences detected in the products is stated above each plot. **(C)** Pre- and post- infusion TCR-Vβ copies/100 CD8 T cells (solid green circles), % $multimer^+$ T cells (solid black circles) and % clonotypes in PBMC (solid red circles) are shown for 2 patients who received monoclonal CTL products. Vertical arrows indicate CTL infusions. Dashed red lines represents limit of detection of frequencies by HTTCS. Significance of the correlation (R^2 and p values) between HTTCS/multimer and HTTCS/TCR copied per 100 cells are depicted below each graph.

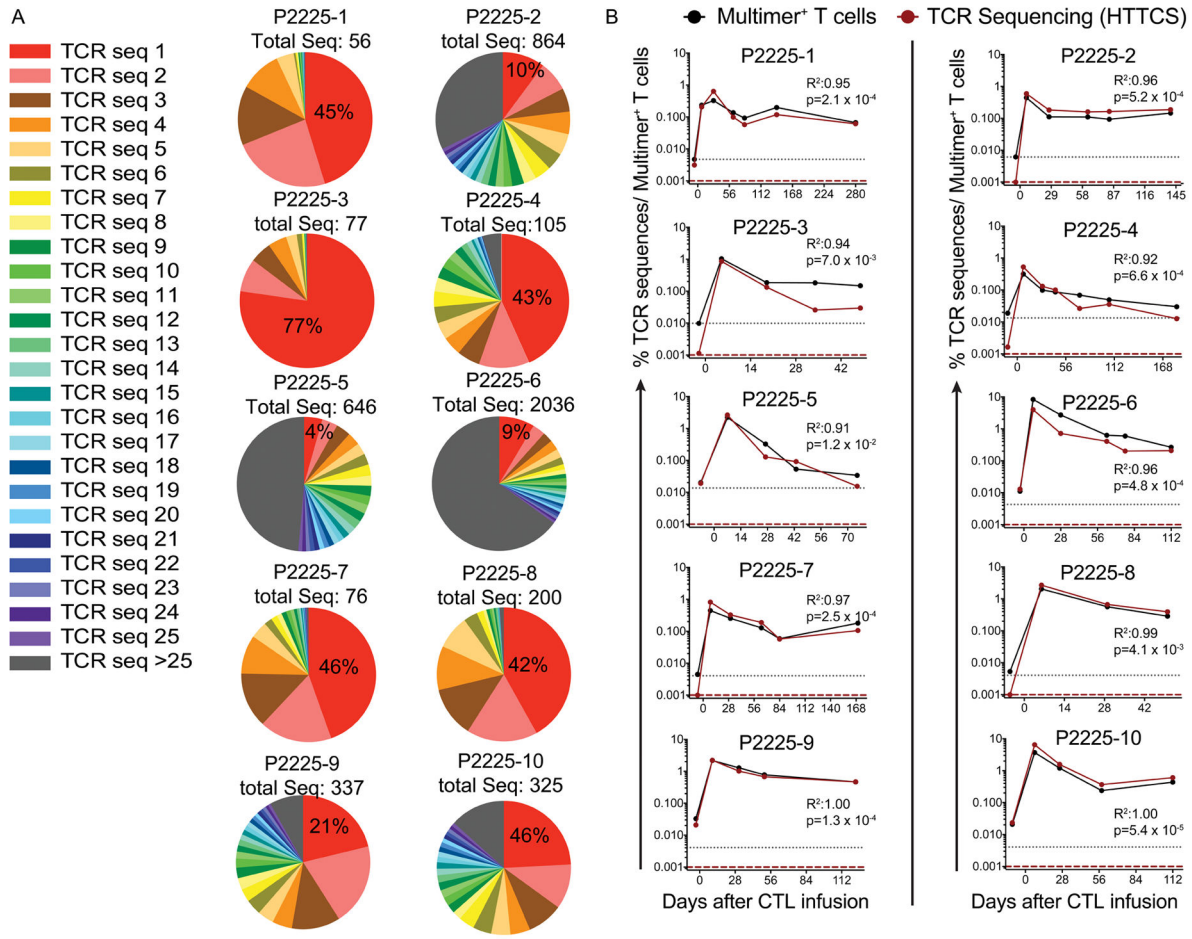

Figure 2. Characteristics of polyclonal products and concurrence of clonotype frequencies determined by HTTCS and multimer staining

(A) Pie plots show the frequency of the first 25 TCR clonotypes in polyclonal products (shaded in color) as well as the remainder of the clones (shaded in grey). The total number of clonotypes in the products is indicated above each plot. The frequency of the most prevalent clonotype (in red) is indicated. **(B)** Percent antigen-specific clonotypes obtained by HTTCS (solid red circles), and % multimer$^+$ T cells (solid black circles) before (left-most timepoint) and after infusions (infused on day 0) are shown for 10 patients who received polyclonal antigen-specific products. Vertical arrows indicate CTL infusions. Dashed red lines represent the limit of detection for HTTCS and dotted grey lines represents limit of detection for multimer staining, which is dependent on the ratio of CD8$^+$ to CD4$^+$ T cells and varies for each patient. Significance of the correlation (R^2 and p values) between HTTCS and multimer are depicted on the right side of each graph.

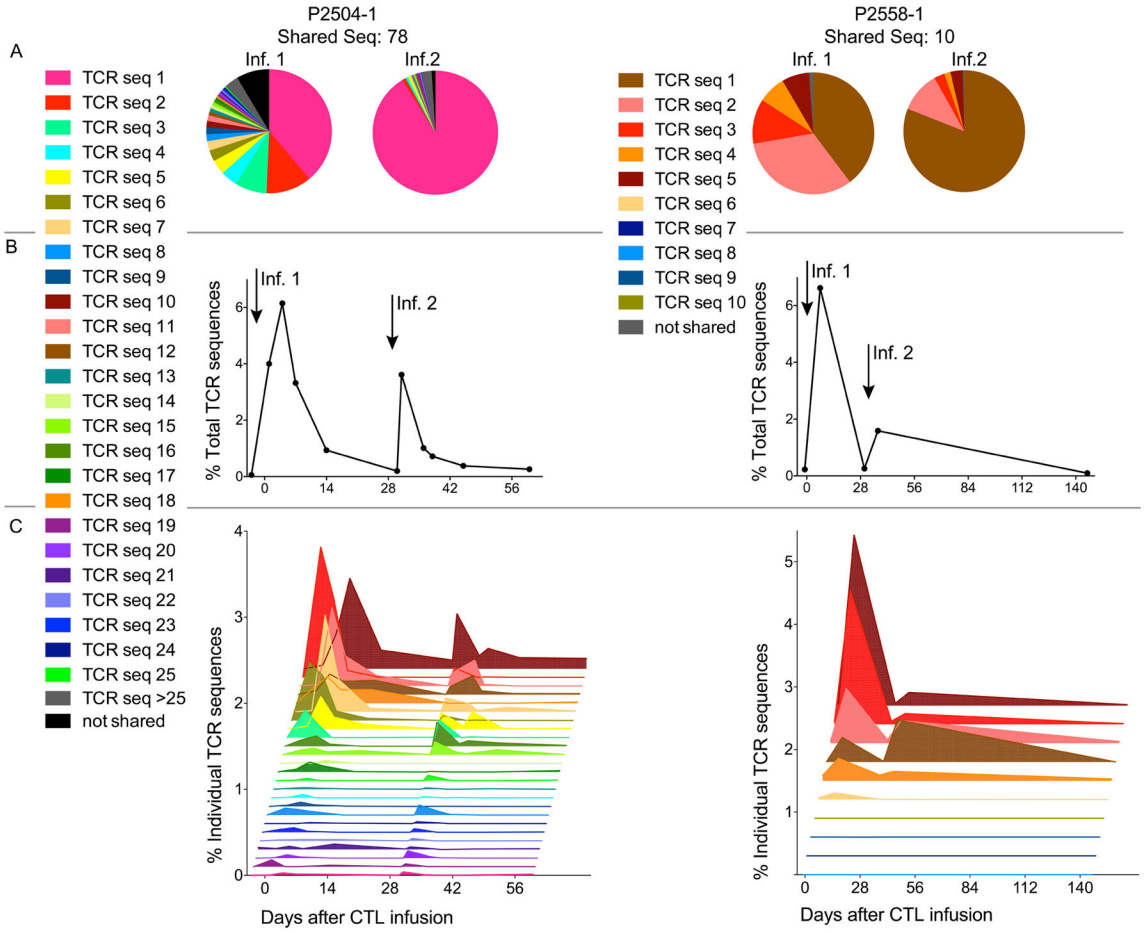

Figure 3. Reproducibility of peak clonotype frequencies in vivo after infusions

(A) Pie plots showing the most prevalent 25 clonotypes shared for infusion 1 and 2 for Pt P2504-1 (left column) and the most prevalent 10 clonotypes for Pt P2558-1 (right column, shaded in color), as well as the remaining, not shared clonotypes present in the infusion products (shaded in grey). TCR sequences that were not shared between the 2 products are shaded in black. The number of shared sequences are indicated above the pie plots. **(B)** The sum of the percents of antigen-specific clonotypes (left y-axis) in PBMC before and after infusions are shown for Pts P2504-1 (left) and P2558-1 (right). **(C)** The individual percent contribution of the most prevalent 25 shared antigen-specific clonotypes (left y axis) are shown for patient P11 (left) and P12 (right).

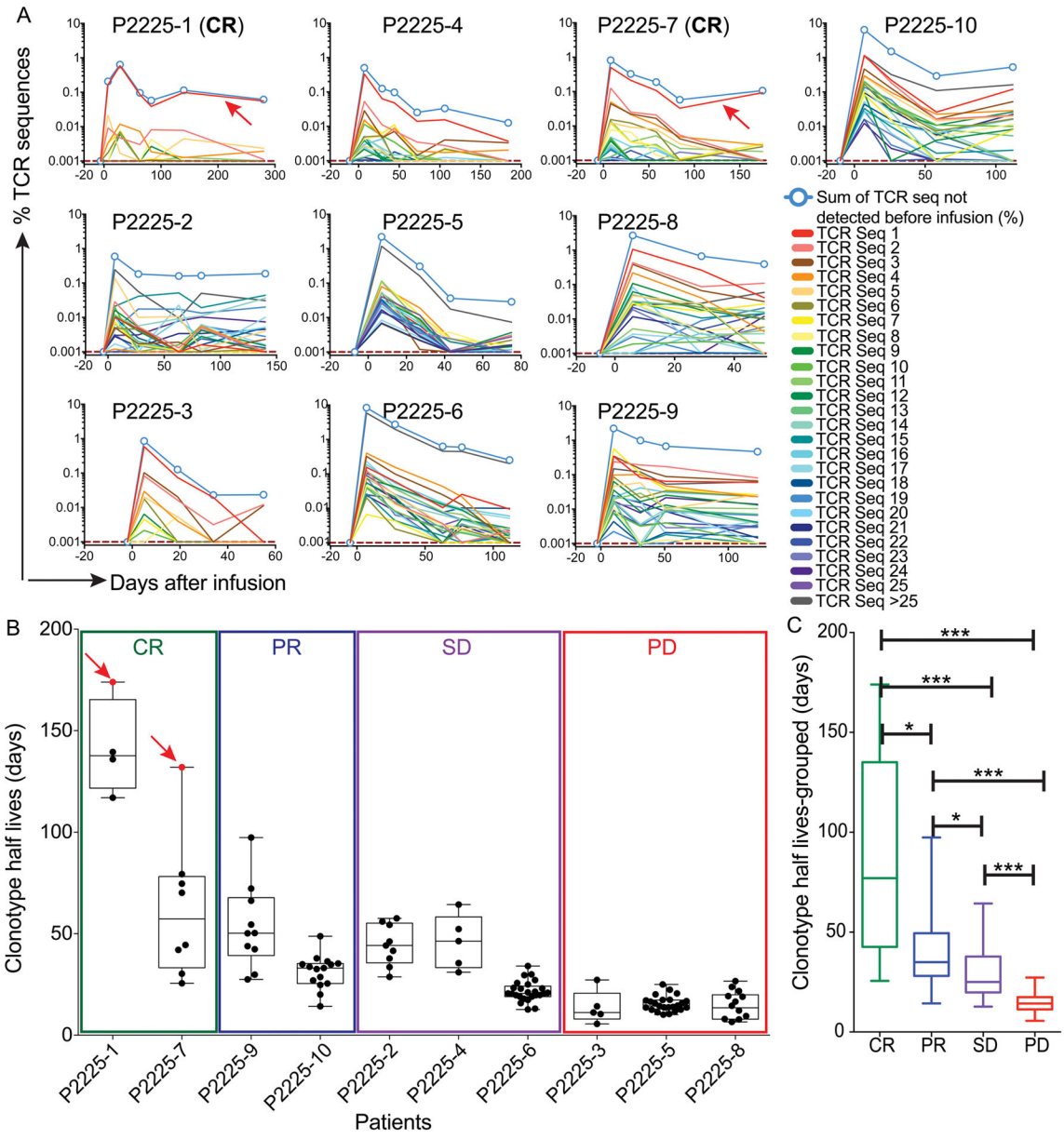

Figure 4. Composition of infused products and persistence/frequency of TCR subsets *in vivo*

(A) Percent clonotypes detected at any time-point after infusions (open blue circles). The individual percent contribution of the most prevalent 25 antigen-specific clonotypes in the infused products (left y axis) are shown (colored lines) as well as the sum of the remaining sequences in the products (grey lines), before (left-most timepoint) and at selected time-points after infusions (day 0). Red arrows indicate the immunodominant clonotypes for Pts P2225-1 and 7 who achieved a CR (indicated) as best response. **(B)** Half-lives of clonotypes for each Pt grouped according to their best response. Green box: CRs, blue box: PR, purple box: SD, red box: PD. Arrows pointing to red circles indicate the immunodominant clonotypes for patients P2225-1 and 7. **(C)** Box and whisker plots of the t½ grouped according to patient best responses. *p≤0.05, ***p≤0.0005.

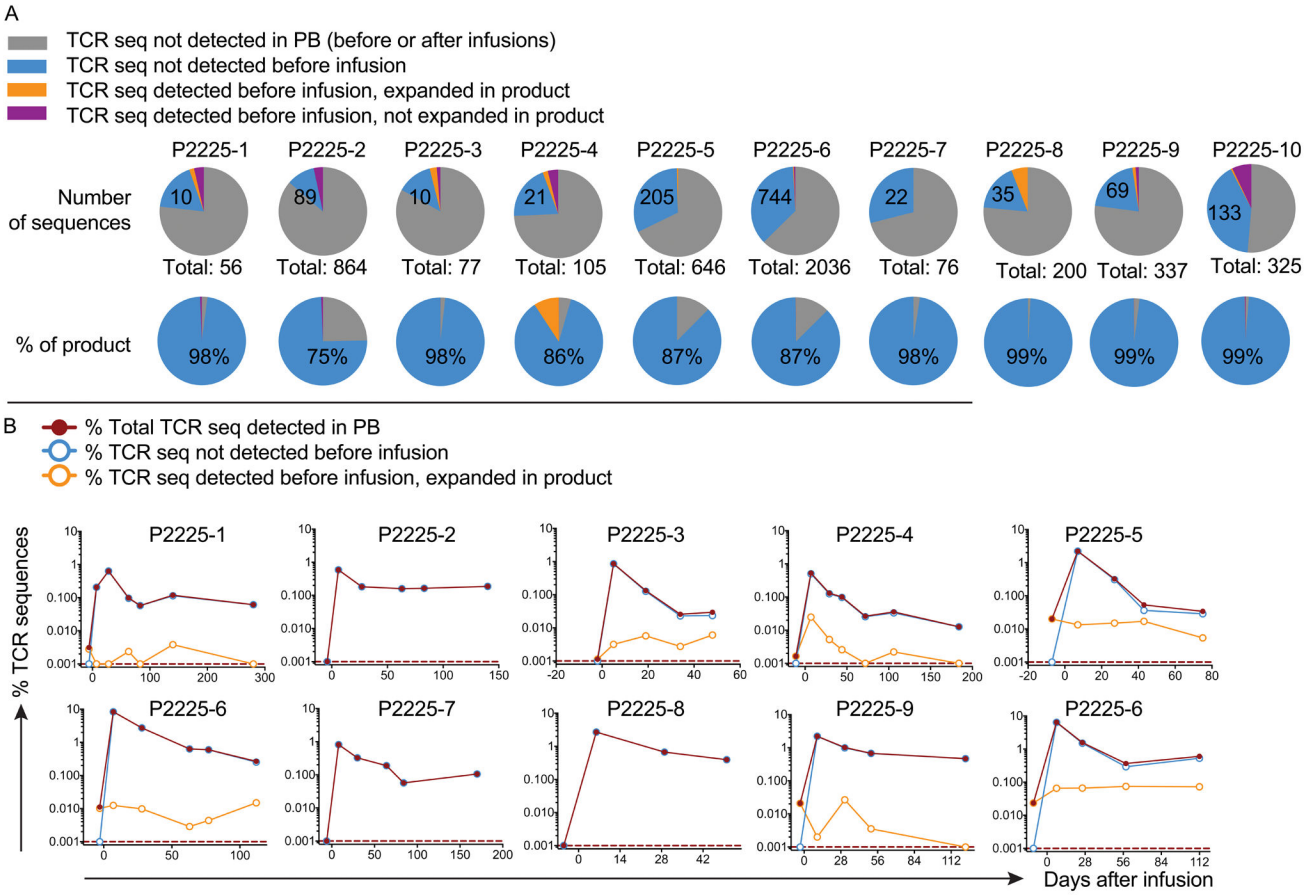

Figure 5. Persistence/frequency of different clonotype subsets *in vivo*

(A) Pie plots showing the number (top row) and percentage (bottom row) of clonotypes composing the infused polyclonal products. Clonotypes in the infusion products which were below the limit of detection either before or at any time-point after infusions (grey area). Clonotypes detected at any time-point after infusions which were not detected in PBMC before infusions (blue area). The number of clonotypes composing the blue area for each patient are shown on the top row and their respective percentage are shown on the bottom row. Sum of clonotypes detected in PBMC before infusions expanded in the infusion product (orange area). Sum of clonotypes detected in PBMC before not expanded in the infusion product (purple area). **(B)** Percent total antigen-specific TCR sequences (solid red circles); percent total TCR sequences detected at any time-point after infusions which were not detected in PB before infusions (open blue circles); percent total TCR sequences detected in PB before infusions expanded in the infusion product (open orange circles), before and at selected timepoints after infusions.

Table 1

Overview of Protocol Characteristics

Protocol #	#2140	#2225	#2504	#2558
Disease Type	Melanoma	Melanoma	Breast cancer	Merkel cell carcinoma
Target cell dose	10^{10} cells/m^2	10^{10} cells/m^2	10^{10} cells/m^2	10^{10} cells/m^2
CTL Specificity	MART1/Tyrosinase/gp100 *	MART1	NY-ESO1	MCPyV
Use of peptide pulsed DCs	Yes	Yes	Yes	Yes
Use of IL-21 in cultures	No	Yes	Yes	Yes
Product clonality	Monoclonal	Polyclonal	Polyclonal	Polyclonal
Product median CD27 expression	52.70%	63.50%	ND	ND
Product median CD28 expression	7.10%	72.40%	ND	6%
Product median CD127 expression	0%	36.20%	ND	0%
Product median CD62L expression	0%	0%	ND	ND
Product median CCR7 expression	0%	0%	ND	ND
Pre-infusion conditionning	CY 4000mg/m^2 over 2 days	CY 300mg/m^2	CY 2000mg/m^2	None
Number of patients infused	11	10	1	1
Persistence ** >40 days?	2 of 11 (18%)	10 of 10 (100%)	1 of 1	1 of 1

* gp100: glycoprotein 100;

** %multimer$^+$ CD8$^+$ cells ≥0.1%;

ND: not done.