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***In vivo* 6-thioguanine-resistant T cells from melanoma patients have public TCR and share TCR beta amino acid sequences with melanoma-reactive T cells**

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Abstract

In vivo hypoxanthine-guanine phosphoribosyltransferase (*HPRT*)-deficient T cells (MT) from melanoma patients are enriched for T cells with *in vivo* clonal amplifications that traffic between blood and tumor tissues. Melanoma is thus a model cancer to test the hypothesis that *in vivo* MT from cancer patients can be used as immunological probes for immunogenic tumor antigens. MT were obtained by 6-thioguanine (TG) selection of lymphocytes from peripheral blood and tumor tissues, and wild-type T cells (WT) were obtained analogously without TG selection. cDNA sequences of the T cell receptor beta chains (TRB) were used as unambiguous biomarkers of *in vivo* clonality and as indicators of T cell specificity. Public TRB were identified in MT from the blood and tumor of different melanoma patients. Such public TRB were not found in normal control MT or WT. As an indicator of T cell specificity for melanoma, the >2600 MT and WT TRB, including the public TRB from melanoma patients, were compared to a literature-derived empirical database of >1270 TRB from melanoma-reactive T cells. Various degrees of similarity, ranging from 100% conservation to 3-amino acid motifs (3-mer), were found between both melanoma patient MT and WT TRBs and the empirical database. The frequency of 3-mer and 4-mer TRB matching to the empirical database was significantly higher in MT compared with WT in the tumor ($p=0.0285$ and $p=0.006$, respectively). In summary, *in vivo* MT from melanoma patients contain public TRB as well as T cells with specificity for characterized melanoma antigens. We conclude that *in vivo* MT merit study as novel probes for uncharacterized immunogenic antigens in melanoma and other malignancies.

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Keywords

T cell receptor; CDR3 sequencing; Tumor immunity; Hypoxanthine-guanine phosphoribosyltransferase

1. Introduction

Metastatic melanoma is a refractory disease and few therapies have an impact on overall survival (Hodi et al., 2010). Similar to other cancers, progressive growth of melanoma occurs in the face of existing tumor-specific immunity to characterized tumor antigens (Rosenberg, 2001). Various methods have been used to identify tumor-specific T cells in the blood, draining lymph nodes, and tumors of melanoma patients (Albertini et al., 2001, Nuovo et al., 2001, Killian et al., 2002, Degauque et al., 2004, Baner et al., 2005). Using these methods, vaccination strategies have been demonstrated to boost the *in vivo* immune response to characterized melanoma-associated antigens (Rosenberg, 2001, Ralph, 2007, Appay et al., 2006). However, these responses have seldom correlated with disease stabilization or tumor regression. Moreover, the recruitment of regulatory T cells to the tumor has been proposed to play a critical role in the suppression of anti-tumor immune responses (Rosenberg, 2001, Curiel et al., 2004, Miller et al., 2006, Viguier et al., 2004) and regulatory T cells are present in increased numbers within melanoma tumors and the blood of melanoma patients (Viguier et al., 2004, Mourmours et al., 2007). An antigen-independent method to identify T cells responding *in vivo* to melanoma could provide new therapeutic opportunities as it would identify T cells responding to both characterized and uncharacterized immunogenic melanoma-associated antigens.

To perform their functions, T cells must become activated through recognition of their cognate antigen presented in context of the appropriate MHC. Following activation, T cells enter the cell cycle, resulting in proliferation, with each daughter cell bearing an identical TCR. The diversity of human $\alpha\beta$ -T cells stems from both the number of T cells in the body ($\sim 10^{12}$) and the diversity of the TRB ($\sim 10^6$, which can each can pair with multiple alpha chains) (Arstila et al., 1999). The TCR sequence is therefore a biomarker of *in vivo* clonality. Indeed, numerous studies have shown restricted TCR repertoires resultant from an immune response (Turner et al., 2006). Moreover, in studies of higher resolution, some TCR restrictions were attributable to clonal expansion of a T cell (Turner et al., 2006). Intriguingly, T cells with public TRB (i.e., T cells with identical TRBV, CDR3, and TRBJ in multiple individuals) have been identified in several diseases, often with known specificity to a shared epitope (Argaet et al., 1994, Callan et al., 1998, Tynan et al., 2005, Moss et al., 1991, Lehner et al., 1995, Trautmann et al., 2005, May et al., 2002, Oksenberg et al., 1993, Prisco et al., 1997, Le Gal et al., 2005, Wieckowski et al., 2009, Jager et al., 2002).

T cell activation requires a very specific interaction, therefore most mature T cells *in vivo* are in the quiescent G_0 stage of the cell cycle at any given time. Thus, T cells proliferating *in vivo* to melanoma, or any antigen, likely represent a minority of the total lymphocyte pool in the individual. As mutations are predicted to occur preferentially in dividing cells and spontaneous gene mutations arising *in vivo* in T cells are rare events, with typical T cell MF in humans between 10^{-6} – 10^{-5} (Albertini, 2001), it follows that *in vivo* mutant T cells should be enriched for disease-specific proliferating T cells. We are investigating a strategy that utilizes *in vivo* mutation in the *HPRT* gene as a means to enrich for such dividing T cells. Our underlying hypothesis is that selection for *in vivo* *HPRT*-deficient T cells (MT) from melanoma patients will enrich for *in vivo* proliferating melanoma-specific T cells. Both MHC Class I and Class II epitopes from melanoma-associated antigens and tumor-associated antigens have been identified and can effectively stimulate CD8+ and CD4+ T

cell responses. As some of these melanoma and other tumor-associated antigens can be shared across patients tumors, we also hypothesize that proliferating MT may be enriched for T cells with public TCRs.

T cells with *in vivo* mutation in *HPRT* have been studied in individuals with ongoing immunological processes. Findings in multiple sclerosis (Allegretta et al., 1990), other demyelinating disorders of the nervous system (Van den Berg et al., 1995), systemic lupus erythematosus (Gmelig-Meyling et al., 1992), rheumatoid arthritis (Cannons et al., 1998), and diabetes mellitus (Falta et al., 1999) suggest that selection for *in vivo* MT T cells serves as a surrogate for selecting for immunological reactivity *in vivo*. Our previous findings in melanoma patients identified an increase in *HPRT* MF in melanoma patients compared to normal controls (Albertini et al., 2001) as well as clonal expansions of *in vivo* MT in the blood and tumor of melanoma patients (Albertini et al., 2008).

Presently, we examined the TCR repertoire of MT and WT from melanoma patients for public TRB features. This model was chosen because, 1) we have previously demonstrated T cell clonal amplifications in MT from melanoma patients; 2) melanoma-specific T cells exist in melanoma patients (and some normal donors); and 3) the literature of melanoma-specific TCRs is extensive. Together, these provide a means by which to demonstrate proof-of-principle that *HPRT* surrogate selection can identify T cells with a potential role in the immune response to melanoma. To assess the “public” nature of T cells from melanoma patients, we compared the TRB features of MT and WT versus each other as well as versus a database of melanoma-reactive T cells compiled from the primary literature and GenBank. Our results suggest that the MT repertoire in melanoma patients contain several public clonotypes. No such public TRB were found in normal control MT or WT. Collectively, these data provide proof-of-principle that *in vivo* MT from melanoma patients contain T cells with *in vivo* specificity for melanoma, extending previous findings from the field of autoimmune disorders (Allegretta et al., 1990, Van den Berg et al., 1995, Gmelig-Meyling et al., 1992, Cannons et al., 1998, Falta et al., 1999).

Moreover, because *HPRT* surrogate selection does not rely on a preconceived “relevant” antigen, it follows that *HPRT* surrogate selection could identify T cells specific to novel tumor-associated antigens. This is important because, as mentioned, immunotherapies focused on currently characterized melanoma-associated antigens are largely ineffective. If clonally amplified T cells responding to novel melanoma-associated antigens are identified in melanoma patients, potential clinical implications include the expansion and subsequent infusion or depletion of T cells with anti-tumor or regulatory functions, respectively.

2. Materials and Methods

2.1. Melanoma patients and normal controls

The melanoma patients in this study were seen at the William S. Middleton Memorial Veterans Hospital or at the University of Wisconsin Hospital and Clinics between December 2004 and July 2006 and had biopsy-confirmed diagnoses of melanoma that was either American Joint Committee on Cancer (AJCC) Stage III or IV (Balch et al., 2009). Melanoma patients provided blood and/or tumor samples. Blood samples were also obtained from normal control blood donors at the William S. Middleton Memorial Veterans Hospital or at the University of Wisconsin Hospital and Clinics. This study is approved by the Health Sciences Institutional Review Board that serves both the William S. Middleton Memorial Veterans Hospital and the University of Wisconsin-Madison. Written informed consent was obtained from all participants in this study.

2.2. Cloning and Mass Culture Conditions

Peripheral blood mononuclear cells (PBMCs) were obtained by standard techniques (Albertini et al., 2008). PBMCs from subjects in this study were cryopreserved in medium containing 90% fetal calf serum (FCS) (HyClone, Logan, UT) and 10% dimethylsulfoxide for subsequent analysis or were incubated immediately for 16–18h in RPMI-1640 supplemented with 20% HL-1 medium (Mediatech, Herndon, VA), and 5% FCS. The T cell cloning assay, in the presence and absence of 6-thioguanine (TG), has been previously described (Albertini et al., 2008, O'Neill et al., 1987). Briefly, PBMC were plated by limiting dilution into 96-well round-bottom plates, with or without TG. Plates were incubated and cultured for 14 days at which time expanding T cells were identified and further expanded.

PBMC and tumor-derived mass cultures were prepared as previously described (Albertini et al., 2008). Surgical specimens from tumor-infiltrated lymph nodes (TILN) or from tumor-infiltrating lymphocytes (TIL) from any metastasis histologically positive for melanoma were obtained (Albertini et al., 2008). Mass cultures were prepared from PBMC or TIL(N), cultured with or without TG as bulk cultures in vertical T25 flasks until the desired number of cells was obtained.

2.3. Preparation and Sequencing of cDNAs Encoding the TRB

Details of the methodology and primers used to sequence the TRB from single-cell derived isolates (SCD-T) are published (Albertini et al., 2008). Briefly, total RNA was extracted from expanded SCD-T, reversed transcribed into cDNA, and then amplified with Taq DNA Polymerase using a TRB constant region primer and a set of 26 different human variable TRB primers corresponding to the different TRBV gene families. Analysis of the cDNA sequence and identification of the TRBV, CDR3 and TRBJ regions of the TRB mRNA was performed using the IMGT/V-QUEST tool provided by the International Immunogenetics Information System (IMGT) (Brochet et al., 2008) or the DNA sequence analysis tools on the National Center for Biotechnology Information website (NLM, NIH). TCR gene rearrangements and CDR3 regions are presented as the converted amino acid sequence, and IMGT nomenclature and numbering are followed. The TRB JUNCTION-IMGT (TRB, hereafter) begins at the conserved cysteine at position 104 and ends at the conserved phenylalanine at position 118, encompassing the TRB CDR3-IMGT (positions 105 to 117) (Brochet et al., 2008).

The methods and primers used for the mass culture analysis are published (Albertini et al., 2008). Briefly, total RNA was extracted from mass culture T cells. 5' RACE cDNA synthesis of the TRB transcripts from total RNA was conducted with a gene specific 3' oligonucleotide complementary to the constant region of the TRB mRNA. TRB transcripts were subsequently amplified by PCR using a TRB constant region primer and the GeneRacer™ 5' Nested primer. TRB cDNAs were cloned into plasmid PCR®4TOPO® (Invitrogen) using topoisomerase, and plasmids containing TRB cDNA were sequenced and analyzed as described above.

2.4. Generation of an empirical anti-melanoma TRB database

A review of the melanoma primary literature was performed and TRBs from T cells demonstrated to have anti-melanoma reactivity were identified and compiled. In addition, the GenBank database was searched for melanoma-reactive T cells. Data include: T cell specificity, HLA restriction, TRBV and TRBJ gene usage, CDR3 amino acid sequence, cell source (PBMC, tumor), and the subject designation (if available from the indicated reference).

2.5. Comparison of current data to empirical anti-melanoma TRB database

TRBs from MT and WT from melanoma patients and normal controls in this study were compared with the empirical database for amino acid sequence similarity. In addition to 100% identical matches, the datasets were probed for less stringent matches including those of four, five, or six amino acid residues and those allowing amino acid mismatches. These comparisons were accomplished with Microsoft Excel 2007 using a sliding search window to alter the query size and wild-card characters.

Germline-encoded amino acids from the carboxyl terminal end of the TRBV and the amino terminal end of the TRBJ of the TRB were determined by alignment to the reference sequence for the particular TRBV or TRBJ used (Brochet et al., 2008). For statistical analysis, the germline amino acids were trimmed from the TRB. This approach was used because the majority of sequences in the empirical database are reported solely as amino acid sequences. Therefore, to standardize the trimming, this method was applied uniformly across all sequences.

2.6. Statistical Analysis

HPRT MF and cloning efficiency (CE) values were computed for SCD-T as previously described (Albertini et al., 2008). Fisher's exact test was extended in order to compare the rates at which MT and WT TRBs match sequences in the empirical database. The matching frequencies of various k-mer subsequences were compared between MT and WT. The extended Fisher procedure accommodated violations of binomial sampling caused by population bottleneck after TG exposure and the possibility that clones have variable *in vitro* growth rates in MT mass culture conditions. Markov chain Monte Carlo computations were used to simulate latent counts and parameter values conditionally upon observed MT frequencies in a hierarchical model of MT TRB counts, providing conditional predictive p-values in the comparison of the MT and WT frequencies (Bayarri and Berger, 1999, Robins et al., 2000, Newton et al., 2006). Briefly, the hierarchical model considered that a Poisson number of each TRB type would survive TG selection (latent counts), with means depending on the MF, the number of input cells in the mass culture, and TRB-specific mean parameters. Then observed counts of the various TRB types, obtained after *in vitro* growth, fluctuated according to an over-dispersed Dirichlet-multinomial distribution. Markov chain Monte Carlo sampled latent counts and governing parameters; each imputed set of latent MT counts gave an ordinary Fisher p-value, and then these were averaged over imputed counts to obtain the conditional predictive p-values.

3. Results

3.1. Characteristics of melanoma patients and normal controls

The age, gender, PBMC CE and MF, stage of disease, and HLA-type of the melanoma patients and normal controls are shown in Table 1. A total of seven biopsies were obtained from five patients: three patients each underwent one tumor resection and two patients each had two resections. Three of seven biopsies were TILN (patients 1 and 13 (2 biopsies)). The remaining four were TIL (patient 2, lung nodule; patient 5, subcarinal lung mass; and patient 9, back nodule and neck nodule). We examined the TRBV, CDR3, and TRBJ from 2624 sequences from the blood and tumor samples from 6 melanoma patients and from 1036 sequences from the blood of 5 normal controls (Table 2).

3.2. T cell clonal expansions in melanoma patients and in normal controls

We determined the extent of *in vivo* T cell clonality in MT and WT from PBMC and TIL(N). The TRB sequence analysis was previously reported for five of these melanoma patients (patients 1, 2, 3, 5, and 9) (Albertini et al., 2008). The term "expanded T cell clone"

refers to a TRB found at least twice (PBMC or TIL(N)) in a given individual. The distribution of TRBs per clonotype for melanoma patient and normal control MT SCD-T are shown in Fig. 1A. PBMC from six melanoma patients and from five normal controls were also cultured as mass populations. For each sample, the MT and WT were cultured for the same duration (range: 13–29 days). The clonotypic profile of melanoma patient and normal control MT mass cultures are shown in Fig. 1B.

TIL(N) from seven tumor samples obtained from five melanoma patients were obtained, processed, and cultured as mass populations. The duration of TIL(N) mass cultures varied among different melanoma patients, but the MT and WT culture durations for each individual were the same (range: 19–40 days). The clonotypic structure of MT TIL(N) mass cultures is shown in Fig. 1C. The TRB of MT and WT from the TILN mass culture of patient 1 is summarized in Table 3 to facilitate interpretation of the distributions of TRBs shown in Figure 1.

The mass culture provides a less labor-intensive method, compared with T cell cloning, to obtain a snapshot of the TCR repertoire. Statistical analysis, adjusting for *in vitro* growth, has shown this methodology to yield significant differences in MT versus WT (Albertini et al., 2008). The finding of identical TRBs using the *HPRT* cloning assay and the *HPRT* mass culture demonstrate that the two assays can identify the same *in vivo* proliferating *HPRT*- T cell clones (Albertini et al., 2008).

3.3. Public TRBs are detected in the majority of the melanoma patients in this study

Analysis of MT and WT TRBs from PBMC and TIL(N) revealed TRB sharing between melanoma patients (Table 4). Three public TRBs were shared amongst 4 (of 6 analyzed) melanoma patients, and were found both in PBMC and at sites of tumor. The public TRBs were derived from *in vivo* expanded T cells, as well as from subdominant T cells. We identified i) a TRBV10-3 TRBJ2-1 sequence shared by two patients (CAISEGRSSYNEQFF); ii) a TRBV27 TRBJ2-1 sequence shared by two patients (CASSLTLPGTSGGASEQFF); and iii) a TRBV7-9 TRBJ2-5 sequence shared by three patients (CASSLGSGGEETQYF). Importantly, the public TRB identified in the WT were only found in the TIL(N). T cells from normal controls did not exhibit TRB sharing between individuals, and there was no TRB sharing between melanoma patients and normal controls.

The present analyses also revealed an association between the level of clonal diversity in the TIL(N) and public or conserved TRB features. In patient 1, the MT TILN was monoclonal (45 of 45 sequences) with a TRBV7-8 TRBJ2-1 sequence of CASSPGQGVYNEQFF. The PGQG motif was also present in melanoma-reactive T cells (see section 3.4). Similarly, in patient 5, the previously mentioned public clonotype, CASSLGSGGEETQYF, comprised 94.5% (69 of 73) of the sequenced MT TIL. This sequence, being found in two other patients, is the most frequent public sequence in the patients studied here (Table 4). In contrast, the vast majority of singlet MT TRB were private (i.e., only found in one individual) (Fig. 1).

3.4. TRB similarity to T cells with known anti-melanoma activity

3.4.1. Database comparison methodology—To address our hypothesis that *in vivo* MT from melanoma patients contain T cells with specificity for melanoma, we compared patient TRBs to those from the empirical database. The analysis was divided into complementary, but distinct subparts. In the first, two TRBs (one patient or normal control and one database) were aligned and examined for a 100% amino acid-to-amino acid match (full-mer match). In subsequent analyses, the stringency of the match was relaxed and matches of at least 4 amino acid residues were permitted. Additionally, sequences trimmed

to remove TRBV and TRBJ amino acids were parsed into k-mers (where 'k' indicates the number of residues) and the presence or absence of unique k-mers in the MT, WT, or empirical database tallied. The normal control analyses were handled analogously.

3.4.2. Empirical melanoma database—The human melanoma literature is extensive, with >61,000 articles deposited in the NCBI database, ~3800 of which pertain to T cell responses (PubMed, 2009). This represents a rich resource to probe questions of melanoma-specific TCR structure. A review of this literature identified TRBs that had demonstrated anti-melanoma reactivity. An empirical database containing 1273 unique TRBs was compiled from 63 primary publications and from GenBank. The database is comprised of TRBs from T cells specific for immunodominant peptides from melanoma-associated antigens such as Melan-A_{26–35}, gp100_{209–217}, gp100_{154–162}, and gp100_{280–288}, and several MAGE proteins (supplementary Table 1). Sequences from these three groups of immunodominant peptides comprise ~74.5%, 5.3%, and 1.5%, respectively, of the database. The remaining TRBs are from T cells specific for other tumor associated antigens (ex. NY-ESO-1, tyrosinase), as well from T cells specific for autologous or allogeneic tumor, or from TIL(N) harvested from regressing melanomas (supplementary Table 1). While the majority of the database involves HLA-A2 restricted T cells, the database also contains examples of T cells restricted by other Class I molecules (HLA-B and –C), as well as by Class II molecules.

3.4.3. Public anti-melanoma TRBs—TRB from two melanoma patients and 1 normal control in this study had full conservation of CDR3 sequences compared to CDR3 sequences of melanoma-reactive T cells from the melanoma empirical database (Table 5). All three of these sequences were derived from T cells from PBMC (2 were WT and one was MT). The WT clones were detected only once in each respective individual. In contrast, the MT clone was detected five times in the blood of patient 5. Two of the three matching database sequences were from Melan-A-specific T cells (note the predominance (>70%) of Melan-A-specific T cells in the empirical database in Supplementary Table 1). Similar to previous findings in Melan-A-specific T cells, such public TRBs were encoded by different TRBV genes in each individual (Wieckowski et al., 2009).

We subsequently examined whether public virus-specific T cells could also be identified in *HPRT*- T cells from melanoma patients. T cell clonal proliferation has been demonstrated in *HPRT*- T cells from patients with chronic viral infection (Albertini et al., 2000). We generated a second empirical database from the published literature containing >360 TRB sequences from virus-specific T cells and identified an exact match between a T cell specific for EBV BMLF1_{280–288} and an *HPRT*- T cell clone from the blood of patient 2 (data not shown). There were no identical matches identified between the virus-derived sequences and patient wild-type T cells.

3.4.4. Similar anti-melanoma TRBs—In addition to identical matches, marked amino acid conservation in the CDR3 β were observed. A recurrent 3-mer CDR3 β motif GLG has been observed in Melan-A-specific T cells (Wieckowski et al., 2009, Serana et al., 2009) and contributes to the binding energy of the TCR to the peptide-MHC complex (Cole et al., 2009). Similarly, we have observed 4-mer motifs in melanoma-reactive T cells. Therefore to increase the stringency of this *in silico* approach we used 4-mer matches as the minimum criterion. For brevity, we present here examples from three patients, two of which themselves share a motif (Table 6). In patient 1, the oligoclonal MT clone (CASSPGQGVYNEQFF) present in the TIL (detailed in section 3.3) and also found in the peripheral blood has marked conservation with two Melan-A-specific T cells as well as with a T cell from a regressing melanoma. All four sequences contain the 4-mer motif PGQG (Table 6) (Serana et al., 2009, Dietrich et al., 2003, Willhauck et al., 2003). Interestingly, a

T cell clonotype detected in the wild-type and *HPRT*- tumor in patient 2 shared the same PGQG motif. Moreover, this 4-mer motif was extended at the carboxyl terminus by one amino acid, yielding a 5-mer motif of PGQGS, which was strikingly present in two of the three melanoma empirical database sequences. Finally, the clone CASSQDSSGANVLTF from patient 5, discussed in section 3.4.3 as fully conserved with a clone from the empirical database (see Table 5), had high similarity to a second database sequence (Table 6) (Manne et al., 2002). These two sequences were derived from the same TIL, and the three TRBs differ at only one amino acid (Manne et al., 2002). Of note, four of five clonotypes that were detected either in both the blood and tumor of an individual or that were public had at least one 4-mer match within the CDR3 to the empirical database.

3.4.5. *HPRT*- T cells with a conserved CDR3 β motif have anti-melanoma functional activity—In preliminary experiments, we assessed the function of the oligoclonal *HPRT*- tumor mass culture from patient 1. This culture was comprised of a single TRB of CASSPGQGVYNEQFF, which contains a recurrent motif found in melanoma-reactive T cells (Table 3, and see section 3.4.4). In contrast, the analogous wild-type mass culture is polyclonal and no CDR3s contain the PGQG motif (Table 3). Following co-culture with irradiated melanoma cell lines, the *HPRT*- T cells exhibited a mixed Th1/Th2 response to both autologous and allogeneic melanoma cell lines (Table 7). This response was elevated over that of the wild-type mass culture.

3.4.6. Comparison of TRB amino acid usage—Finally, the overall amino acid composition of both melanoma patient and normal control TRBs was compared to the empirical database. A high degree of conservation exists in the 3' end of many TRBV and the 5' end of several TRBJs. Therefore, amino acids attributable to germline TRBV and TRBJ were trimmed from the sequences for this analysis to limit inflated matching due to this germline conservation. Analysis revealed significantly greater 3-mer and 4-mer ($p=0.0285$ and $p=0.006$, respectively) matching to the empirical database in the MT TIL(N) from melanoma patients compared to WT TIL(N). No differences were noted between MT and WT from the PBMC of either melanoma patients or normal controls, or between MT from PBMC of melanoma patients and MT from PBMC of normal controls.

4. Discussion

We utilized *HPRT* surrogate selection as an antigen-independent method to study the *in vivo* T cell response to melanoma since this strategy can enrich for T cells with *in vivo* clonal amplifications and can identify T cell that traffic between blood and tumor tissues in patients with progressive metastatic melanoma (Albertini et al., 2008). In addition to identifying *in vivo* clonally expanding T cells, *HPRT* surrogate selection has several potential advantages over other assays used to detect antigen-specific T cell responses. Methods such as intracellular cytokine staining, ELISpot, and real-time qPCR detect antigen-induced cytokine production but require permeabilization or lysis of the cells. In contrast, *HPRT* surrogate selection permits isolation of viable cells for further analysis. Additionally, it does not focus on any one functional parameter and as such is a broader assay. Furthermore, *HPRT* surrogate selection is antigen-independent, obviating the requirement for autologous antigen presenting cells or antigen for initial *in vitro* stimulation, sources of which can be limited in human studies (Zaunders et al., 2009, Wolfl et al., 2007). Adoptive cellular immunotherapy using T cells non-specifically expanded *in vitro* with IL-2 and anti-CD3 antibody, i.e., an antigen-independent method, has shown anti-tumor activity (Rosenberg and Dudley, 2009). Although clinical responses have been achieved, overall responses remain low (Rosenberg and Dudley, 2009). It is interesting to speculate that selectively expanding *in vivo* proliferating T cells, as identified by *HPRT* surrogate selection, would complement this approach. Furthermore, as evidenced by comparison of *HPRT*- to wild-type

TRBs, the addition of a selection strategy (i.e., growth in 6-thioguanine) to a non-specific mitogen- and cytokine-driven stimulatory culture (i.e., LAK supernatant and PHA) permits the identification of T cells with a potential role in the *in vivo* immune response to melanoma.

In this study, we report finding public TRB in *HPRT*- T cells in four melanoma patients. Further, when our dataset was compared to TRB from melanoma-reactive T cells compiled from the literature, we observed public TRB sequences as well as conserved CDR3 β motifs. The phenomenon of TCR bias, whereby T cells specific to a particular antigen within an individual exhibit conserved TCR Variable and/or Joining gene usage, is well documented in the literature (Turner et al., 2006, May et al., 2002, Le Gal et al., 2005, Wieckowski et al., 2009, Dietrich et al., 2003). Preferential use of TRBV or TRBJ by antigen-specific T cells can also extend across individuals. Melan-A-specific T cells were noted to frequently or preferentially use a limited set of TRBV and TRBJ genes (Wieckowski et al., 2009, Serana et al., 2009). Interestingly, the sequences from our patients or normal control presented in Tables 4–6 utilize TRBV, TRBJ, or both from this restricted set. Moreover, public TCRs have a solid basis in the literature of chronic viral infections (Argaet et al., 1994, Callan et al., 1998, Tynan et al., 2005, Moss et al., 1991, Lehner et al., 1995, Trautmann et al., 2005), autoimmune disorders (May et al., 2002, Oksenberg et al., 1993, Prisco et al., 1997), and cancers, including melanoma (Le Gal et al., 2005, Wieckowski et al., 2009, Jager et al., 2002). Our finding of public TRB between melanoma patients in this study, as well as public TRB between these melanoma patients and published sequences from melanoma-reactive T cells, are consistent with this literature. Using our *in silico* matching strategy, we also identified a public TRB between an *HPRT*- T cell from a melanoma patient and an EBV-specific T cell from the literature (data not shown). Together, these data indicate that *HPRT* surrogate selection can identify proliferating T cells that are disease-specific. The immune response to melanoma is likely broad with T cells activated and dividing to an array of epitopes. Thus, a potential application of *HPRT* surrogate selection is the identification of *in vivo* proliferating T cells specific to novel tumor epitopes.

All three of the clonotypes shared between melanoma patients from this study were found in the tumor environments. The localization of these T cell clones to a site of pathology suggests a potential role in the immune response to melanoma. Our data agree with previous studies demonstrating public TRB in the brains, intestinal mucosa, and synovial fluid of patients with multiple sclerosis, celiac disease, and reactive arthritis, respectively (May et al., 2002, Oksenberg et al., 1993, Prisco et al., 1997). These sites are considered the locations of untoward T cell-mediated immune responses in the respective disease. To our knowledge, our data represent the first report in melanoma patients of public TRB from tumor-resident T cells. In our review of the literature, we discovered a previously unrecognized instance of TRB sharing in the tumor of two distinct melanoma patients, patient 2 (Cole et al., 1997) and patient 0831 (Zeuthen et al., 1995). These data may have remained undetected by others (Serana et al., 2009) due to a mistranslation of two cDNA codons in one of the reports (Zeuthen et al., 1995).

The finding of public TRBs from tumor-infiltrating T cells shared between patients suggests T cell responses to common melanoma-associated antigens. It is important to note that an analysis of inter-patient codon redundancy revealed that the TRB shared between patients 3 and 5, CAISEGRSSYNEQFF, was encoded by distinct nucleotide sequences in these individuals, thus excluding possible PCR contamination. Although the remaining public TRB were encoded by identical nucleotide sequences within the CDR3 in the respective patients, there was nucleotide heterogeneity, not attributable to the polymerase, in the non-CDR3 regions of these TRB. Furthermore, the lack of shared TRB sequences between

normal controls as well as between normal controls and melanoma patients suggests that the sharing of TRB is not due to PCR contamination.

Two patients in this study (patients 1 and 2) had TRBs with the conserved motif PGQG[S]. Importantly, this motif was also found in the melanoma empirical database. The finding of such a conserved CDR3 motif indicates that these residues may be critical for T cell recognition of melanoma epitopes. Similarly, T cells with the public GLG motif were isolated from melanoma patients vaccinated with the natural or analog Melan-A peptide and these cells were peptide cross-reactive (Wieckowski et al., 2009). The GLG motif was also detected in TRBs in the present study (data not shown). Interestingly, preliminary functional data (Table 7) and observations made here indicate that T cells from patient 1 bearing the conserved PGQG motif may recognize a peptide from Melan-A in either a DR17- or DQ6-restricted fashion (manuscript in preparation). The melanoma cell lines used in these preliminary experiments both express the Melan-A protein as well as both MHC Class I and II antigens (data not shown). Furthermore, the allogeneic line expresses higher levels of Class II antigens than the autologous line (data not shown), possibly explaining the more robust cytokine induction with this line.

Melan-A-specific T cells are also found at high frequency in the periphery of HLA-A2+ normal controls (Dietrich et al., 2003, Dutoit et al., 2002, Pittet et al., 1999, Zippelius et al., 2002). Normal control 30 from this study is HLA-A2+; therefore, the finding of an identical TRB (CASSLGQPQHF) in this normal control and in the Melan-A-specific T cell M77/86 was not unexpected (Table 5). It is interesting to note that both WT sequences with full conservation to empirical database sequences from Melan-A-specific T cells were germline encoded (Table 5). In each case, the TRBV gene segment is present in its entirety and joins an amino acid contributed by the TRBD gene. TRBJ1-5, used by each of these T cells, is preferentially used by Melan-A-specific T cells (Wieckowski et al., 2009). These two WT sequences may represent melanoma-specific T cells with sufficient precursor frequency, due to the “ease” in producing germline encoded TRBs (Venturi et al., 2006), to be detected in our system. Alternately, such public germline encoded TRB could contribute substantially to the binding energies of the TCR-peptide-MHC complex, thus explaining their prevalence and detection here (Cole et al., 2009).

Collectively, our findings provide proof-of-principle evidence that *HPRT* surrogate selection can identify T cells with a potential role in the *in vivo* immune response to melanoma. The public TRBs expressed by MT from melanoma patients will be studied as candidate probes for novel immunogenic melanoma antigens. While TRB amino acid matching to the empirical database of melanoma-reactive sequences was significantly higher in MT compared with WT in the tumor, this finding did not extend to the PBMC of these patients with progressive metastatic disease. These data suggest localization of proliferating disease-specific T cells to the tumor microenvironment. Additional studies are needed to determine whether *in vivo* MT in melanoma patients can be used to identify an effective immune response against melanoma. We conclude that *HPRT* surrogate selection provides a novel strategy to study the *in vivo* immunobiology of human melanoma, and we predict that this approach can also be utilized for a wide spectrum of other cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard abbreviations used

CE	cloning efficiency
HPRT	hypoxanthine-guanine phosphoribosyltransferase
IMGT	International Immunogenetics Information System
MF	mutant frequency
MT	<i>HPRT</i> -deficient T cells
SCD-T	single-cell derived T cell isolate
TIL(N)	tumor-infiltrating lymphocytes (TIL) or tumor-infiltrated lymph nodes (TILN)
TRB	T cell receptor beta chains
TG	6-thioguanine
WT	wild-type T cells

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Fig. 1.

The clonotypic profile of MT T cells from PBMC of melanoma patients and normal controls and from TIL(N) of melanoma patients. (A) The number of occurrences of TRBs in the MT SCD-T from melanoma patients or normal controls (note, there are no SCD-T for normal control 31); (B), (C) as in (A), but for PBMC or TIL(N) mass cultures, respectively. The black bar represents TRBs found in singlet and in only one individual, i.e., private TRB. Each shaded bar represents a distinct clonally expanded and/or public TRB, and the size of each bar indicates the size of that particular T cell clone.

Table 1

Characteristics of melanoma patients and normal controls.

	Melanoma patients	Normal controls
Age ^a (years)		
mean	57.9	36.2
range	36.4–71.1	33.9–43.9
Sex		
Male	6	2
Female	0	3
CE ^b (range)	0.08–0.15	0.12–0.44
MF ^b (range)	17.6×10^{-6} – 97.3×10^{-6}	13.0×10^{-6} – 18.8×10^{-6}
Stage of disease ^c		na ^d
III	1	
IV	5	
HLA-type, MHC Class I ^e		
Pt 1 ^f	A*0101/*0101; B*0801/*3701; C*0701/*0602	NC 26 NC 29
Pt 2	A2+ ^g	NC 30
Pt 3	A1, 2; B44, 57; Cw5, 6	NC 31
Pt 5	A2, 3; B35, 57; Cw4, 7	NC 32
Pt 9	A1, 24; B8, 61; Cw2, 7	
Pt 13	A2, 24; B7, 35; Cw4, 15	
HLA-type, MHC Class II ^e		
Pt 1 ^f	DRB1*0301/*1302; DRB3*0101/*0301; DQB1*0201/*0604	NC 26 NC 29
Pt 2	nd	NC 30
Pt 3	nd	NC 31
Pt 5	DR1, 7; DQ5, 9	NC 32
Pt 9	DR13, 17; DR52; DQ2, 6	
Pt 13	DR4, 11; DR52, 53; DQ7, 8	

^a Age at the time of blood sample acquisition up to 16 days prior to resection of either TIL(N).^b Determined for PBMC.^c AJCC stage refers to the stage of disease at the time of blood sample acquisition.^d not applicable^e Determined by PCR, with sequence specific primers.^f Determined by PCR with sequence specific oligonucleotides.^g Determined by flow cytometry.^h not determined

Table 2

Summary of TRB sequences analyzed from melanoma patients and normal controls.

	Melanoma patients		Normal controls	
	MT	WT	MT	WT
PBMC SCD-T				
No. of individuals	6		4	
Total no. of sequences	337	151	136	165
Mean no. of sequences per individual	56.1	25.2	34	41.3
Range of no. of sequences per individual	39–69	11–49	20–45	36–54
PBMC mass culture				
No. of individuals	6		5	
Total no. of sequences	543	532	422	413
Mean no. of sequences per individual	90.5	88.7	84.4	82.6
Range of no. of sequences per individual	81–107	78–97	74–94	74–93
TIL(N) mass culture				
No. of individuals	5		na ^a	
No. of cultures ^b	7			
Total no. of sequences	600	461		
Mean no. of sequences per culture	85.7	65.9		
Range of no. of sequences per culture	45–167	35–88		

^a not applicable.^b Two melanoma patients underwent resection of multiple (n=2) distinct tumor samples.

Table 3

MT and WT TRB from the TILN mass culture of Patient 1.

WT				MT			
TRBV	JUNCTION	TRBJ	Freq ^a	TRBV	JUNCTION	TRBJ	Freq
4-3	CASSQDIRGIQETQYF	2-5	22.9	7-8	<u>CASSPGQGYNEQFF</u>	2-1	100
10-3	CASSQDIRGIQETQYF	1-4	14.3				
12-5	CASGLMSSNEQFF	2-1	8.6				
6-5	CASSYLTKNETQYF	2-5	8.6				
6-5	CASRQLMNTAEFF	1-1	5.7				
6-5	CASSYSRQHEAFF	1-1	5.7				
27	CASTDGVGSYNEQFF	2-1	5.7				
6-4	CAGSDSIAGADTQYF	2-3	2.9				
6-5	CASSASTDTQYF	2-3	2.9				
28	CASSFPKPGSYTGELFF	2-2	2.9				
11-2	CASSLDGGDQPQHF	1-5	2.9				
18	CASSPLAETVNEQFF	2-1	2.9				
28	CASSPLGTGPNYEQYF	2-7	2.9				
28	CASSPSQTRYNEQFF	2-1	2.9				
30	CAWSPKNQPQHF	1-5	2.9				
29-1	CSVERGQGSTDTQYF	2-3	2.9				
28	RASSLSPGTIVYGYTF	1-2	2.9				
Number of sequences			35	Number of sequences			45

^aPercentage of each clonotype in the sample.

Underline and bold in the Junction column indicate the clonotype was found in the blood of the same individual as a MT.

Table 4

Public TRB sequences found in distinct melanoma patients from this study.

TRBV	TRB Junction	TRBJ	Patient	Tissue	MT/WT	Frequency ^a
10-3	CAISEGRSSYNEQFF	2-1	3	PBMC ^b	MT	10/95
			5	PBMC ^c	MT	1/55
			5	TIL ^b	WT	12/87
27	CASSLTLPGTSGGASEQFF	2-1	5	TIL ^b	WT	1/87
			9	TIL ^b	MT	1/167
7-9	CASSILGSGGEETQYF	2-5	5	TIL ^b	MT	69/73
			9	TIL ^b	MT	1/167
				PBMC ^b	MT	1/81
			13	TIL ^b	MT	1/75
				TIL ^b	WT	1/88

^aNumber of T cells with the indicated TRB sequence out of the total number sequenced for that sample.

^bmass culture

^cT cell isolate

Table 5
MT and WT TRB sequences identical to TRB sequences from anti-melanoma T cells.

Donor ID ^a	Tissue	MT/WT	TRBV ^b	TRBJ	JUNCTION										Specificity/Source	Ref.					
Pt. 1	PBMC	WT	28	1-5	C	A	S	S	L	D	S	N	Q	P	Q	H	F	TBD ^c	Dietrich et al., 2003		
LAU 337	PBMC	-	5-1	1-5	C	A	S	S	L	D	S	N	Q	P	Q	H	F	Melan-A ^d			
Pt. 5	PBMC	MT	7-2	2-6	C	A	S	S	Q	D	S	S	G	A	N	V	L	T	F	TBD	
20249	TIL	-	4	2-6	C	A	S	S	Q	D	S	S	G	A	N	V	L	T	F	inflamed lesion ^e	Manne et al., 2002
NC 30	PBMC	WT	12-3	1-5	C	A	S	S	L	G	Q	P	Q	H	F					TBD	
M77	TIL	-	27	1-5	C	A	S	S	L	G	Q	P	Q	H	F					Melan-A ^d	Romero et al., 1997

^aMelanoma patients 1 and 5 (Pt. 1; Pt. 5) and normal control 30 (NC 30) are from this study. The subject ID numbers (LAU 337; 20249; M77) refer to the designation given in the indicated reference citations.

^bIMGT nomenclature used for TRBV and TRBJ.

^cTo be determined.

^dReactivity to Melan-A26–35(27L) or Melan-A27–35

^eTIL harvested from a clinically inflamed melanoma metastasis following an intra-tumor vaccination.

Table 6

MT and WT TRB sequences similar to TRB sequences from anti-melanoma T cells.

Donor ID ^a	Tissue	MT/WT	TRBV ^b	TRBJ	JUNCTION												Specificity/Source	Ref.
Pt. 1	PBMC TIL	MT	7-8	2-1	C A S S	P G Q G V Y N E Q F F	TBD ^c											
Pt. 2	TIL	MT, WT	28	2-1	C A S S K . . .	P G Q G S N E Q F F	TBD											
HD 009	PBMC	-	5-6	2-7	C A S S	P G Q G L Y E Q Y F	Melan-A ^d											Dietrich et al., 2003
22	PBMC	-	6	1-6	C A S S	P G Q G S P L H F	Melan-A ^d											Serana et al., 2009
3	TIL	-	25-1	2-2	C A S S A K S S P G Q G S T G E L F F	regressing lesion ^e												Willhauck et al., 2003
Pt. 5	PBMC	MT	7-2	2-6	C A S S Q D S S G A N V L T F	TBD												
20249	TIL	-	4	2-6	C A S S E D S S G A N V L T F	inflamed lesion ^f												Manne et al., 2002

^aMelanoma patients 1, 2 and 5 (Pt. 1; Pt. 2; Pt. 5) are from this study. The subject ID numbers (HD 009; 22; 3; 20249) refer to the designation given in the indicated reference citations.

^bIMGT nomenclature used for TRBV and TRBJ.

^cTo be determined.

^dReactivity to Melan-A26–35(27L) or Melan-A27–35

^eTIL harvested from a regressing melanoma metastasis.

^fTIL harvested from a clinically inflamed melanoma metastasis following an intra-tumor vaccination.

.. indicate gap introduced for alignment

Table 7Tumor cell recognition by TILN mass cultures from Patient 1.^a

Melanoma cell lines			
	Cytokine	Autologous Tumor A2- (A387)	Allogeneic Tumor A2+ (A508)
WT (42) ^b		98	34
<i>HPRT</i> - (49)	IFN- γ	451	532
Tumor cells		4	4
WT (8)		36	17
<i>HPRT</i> - (6)	TNF- α	20	86
Tumor cells		4	5
WT (5)		11	1917
<i>HPRT</i> - (82)	IL-10	2308	>5000
Tumor cells		2	2138
WT (5)		406	1589
<i>HPRT</i> - (3)	IL-6	463	3422
Tumor cells		17	65
WT (6)		43	8
<i>HPRT</i> - (35)	IL-4	1644	3048
Tumor cells		4	4

^aValues indicate cytokine concentration (pg/ml) in supernatant following 24 hr co-culture of T cells with indicated melanoma cell line.^bValues in parentheses indicate cytokine concentration (pg/ml) in cultures with T cells alone.