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Clonal Expansions of 6-Thioguanine Resistant T Lymphocytes in the Blood and Tumor of Melanoma Patients¹

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Abstract

The identification of specific lymphocyte populations that mediate tumor immune responses is required for elucidating the mechanisms underlying these responses and facilitating therapeutic interventions in humans with cancer. To this end, mutant hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) deficient (*HPRT*⁻) T-cells were employed as probes to detect T-cell clonal amplifications and trafficking *in vivo* in patients with advanced melanoma. Mutant T-cells from peripheral blood were obtained as clonal isolates or in mass cultures in the presence of 6-thioguanine (TG) selection, and from tumor-bearing lymph nodes or metastatic melanoma tissues by TG-selected mass cultures. Non-mutant (wild-type) cells were obtained from all sites by analogous means, but without TG selection. cDNA sequences of the T-cell receptor (TCR) beta chains (TCR- β), determined directly (clonal isolates) or following insertion into plasmids (mass cultures), were used as unambiguous biomarkers of *in vivo* clonality of mature T-cell clones. Clonal amplifications, identified as repetitive TCR- β V-region, complementarity determining region 3 (CDR3), and J-region gene sequences, were demonstrated at all sites studied, i.e., peripheral blood, lymph nodes, and metastatic tumors. Amplifications were significantly enriched among the mutant compared with the wild-type T-cell fractions. Importantly, T-cell trafficking was manifest by identical TCR- β cDNA sequences, including the hyper-variable CDR3 motifs, being found in both blood and tissues in individual patients. The findings described herein indicate that the mutant T-cell fractions from melanoma patients are enriched for proliferating T-cells that infiltrate the tumor, making them candidates for investigations of potentially protective immunological responses.

¹Running title: T cell mutant clonal expansions in human melanoma

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Keywords

human melanoma; T cells; T cell receptors; tumor immunity; molecular biology; hypoxanthine-guanine phosphoribosyltransferase

INTRODUCTION

Studies of hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) mutations in human peripheral blood T-cells have often revealed evidence of clonal amplifications within the mutant (MT) but not wild-type (WT) fractions [O'Neill et al., 1994]. Several explanations have been offered for this, e.g., T-cell replication in response to exogenous antigen, restricted genomic instability, or, in some individuals, selection by purine analogue therapy [reviewed in Albertini RJ, 2001]. Indeed, using T-cell mutations as mechanistic probes, each has been shown to produce clonality of T-cell mutants [Albertini, et al., 1998; Allegretta et al., 1990; Gmelig-Meyling et al., 1992; Dawisha et al., 1994; Holyst et al., 1994; Van den Berg et al., 1995; Ansari et al., 1995; Falta et al., 1999; Finette et al., 2000]. Although clonality does not seriously bias quantitative results of mutant frequency determinations when group mean values for normal individuals are considered [O'Neill et al, 1994], it can have a large effect when evaluating individual values. As quantitative mutation studies in humans was the reason for developing the *HPRT* cloning assay in the first place, factors that tend to accentuate clonality must be understood. A corollary to this, however, is that interpreting clonality allows this phenomenon to be used as a mechanistic probe.

The finding of enhanced clonality of T-cell mutants in patients with autoimmune disorders or after organ grafts [above references and Albertini RJ, 2001] supports the dual hypotheses that (i) proliferation underlies function for immunologically reactive T-cells *in vivo* and (ii) that somatic gene mutations arise preferentially in actively dividing as opposed to quiescent cells. According to these hypotheses, mutant T-cell populations should be enriched for proliferating and, therefore, immunologically active cells. This is the rationale for using selection for T-cell mutants as a surrogate for selecting specific T-cell populations that mediate immunological reactions in individuals in whom such reactions are ongoing. The current study is seeking evidence of such populations in patients with advanced melanoma.

Post-thymic (mature) T-cells possess molecular signatures of their clonal origins. Like all cells of the immune system, T-cells recognize the universe of antigens to which they must respond via surface receptors called T-cell receptors (TCRs). An enormous TCR diversity is generated at the genetic level by somatic rearrangement of relatively few germline-encoded variable (V), diversity (D), junctional (J), and constant (C) region genes, a process that occurs during differentiation in the thymus [Tonegawa, 1983; Alt et al., 1992; Lewis, 1994]. For humans, this is usually during fetal life and childhood, up to mid- to late-adolescence. Once a TCR gene rearrangement has occurred, it remains fixed for the remainder of the life of the now-mature T-cell, being present in all clonal descendants. A T-cell's rearranged TCR genes, including the hyper-variable complementarity determining region 3 (CDR3), provide an unambiguous molecular signature that defines its clonal relationships.

Although clonality has often been described for *HPRT* mutants isolated by limiting dilution from blood (i.e., in cloning assays), little is known about its extension to other tissues. In an earlier study, *HPRT* T-cell mutant frequencies (MFs) were determined for peripheral blood lymphocytes from 48 melanoma patients and 38 normal controls [Albertini MR et al., 2001]. MFs in the patients were significantly higher than in controls, a finding not entirely explained by previous chemotherapy. In nine patients, lymphocytes isolated from melanoma containing lymph nodes (LNs) were also studied for mutations, with the finding that lymph

node MFs were slightly but not significantly lower than those of peripheral blood lymphocytes from the same individual. An intriguing finding in one patient was evidence of a MT isolate from the tumor-bearing LN sharing a TCR- β beta chain (TCR- β) gene rearrangement with two mutant isolates from peripheral blood. The rearrangements in this study were characterized by Southern blot analysis, and identity of the rearrangement identified by the sharing of a restriction pattern after multiple enzymatic digests. Nonetheless, this was the first demonstration of clonality of T-cell mutants that encompassed both blood and tissue (LN).

In the present study, we describe a large-scale analysis of the TCR- β gene sequences from WT and *HPRT* MT T-cells from melanoma patients to investigate the hypothesis that *HPRT* mutations can be used as biomarkers to study T-cell proliferation and to provide insights into mechanisms underlying the *in vivo* immune response to human melanoma. We developed a rapid multiplex PCR-based method for sequencing the TCR- β genes from T-cell isolates, and we extended the method to analyses of T-cells from mass cultures. The mass culture system was investigated in lieu of direct cloning to facilitate subsequent functional studies of *HPRT*- T-cells in melanoma patients. The value of the mass culture is the ability to compensate for cell interactions that may be important for: (a) any functions that are being assessed and (b) growing the cells *in vitro*. This second benefit may be missed if only the isolates derived from cloning assays are assessed for functional characteristics. In addition, mass culture selection is faster, cheaper, and less time consuming than working with T-cell isolates. Mass culture selection can be applied to blood, to LNs, and to other tissues of interest. We describe these methods here, and their application in demonstrating that mutant T-cell clonal amplifications have representatives in blood, in LNs, and in tumor tissue in melanoma patients. Implications and potential applications of these observations are discussed.

MATERIALS AND METHODS

Human Subjects

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 May 2006 and had the diagnosis of biopsy-confirmed melanoma that was either American Joint Committee on Cancer (AJCC) Stage III or IV [Balch et al., 2001]. This study is approved by the Health Sciences Institutional Review Board that serves the William S. Middleton Memorial Veterans Hospital and the University of Wisconsin-Madison. Written informed consent was obtained from the participants in this study.

Nomenclature

The TCR- β gene nomenclature is in accordance with the IMGT gene-segment names that were approved by the HUGO Gene Nomenclature Committee in 1999 [Lefranc et al., 2005; Lefranc MP, and Lefranc G, 2001]. For brevity, we have defined the CDR3 amino acid sequence as the non-templated N-region and the D-region amino acid sequence between the last amino acid encoded by the V-region genomic sequence and the first amino acid encoded by the J-region genomic sequence. The term TCR- β gene pattern refers to TCR- β V-region, CDR3, and J-region (V-D-J). All members of a TCR- β defined clone have identical TCR- β gene patterns.

PBMC Cloning Conditions

Peripheral blood mononuclear cells (PBMCs) were obtained from peripheral blood by standard techniques. PBMCs from subjects in this study were cryopreserved in medium containing 90% fetal calf serum (FCS) (HyClone, Logan, UT) and 10% dimethylsulfoxide

(DMSO) for subsequent analysis or were incubated immediately in the cloning assay. To accommodate for cell death that may occur following thawing of cells, all PBMC, whether used fresh or cryopreserved, were cultured at 1×10^6 cells/ml for 16–18 hours in modified RPMI-1640 supplemented with 20% HL-1 medium (Mediatech, Herndon, VA), and 5% FCS, prior to cloning and set-up of mass cultures. We used this approach for all cells, whether fresh or cryopreserved, to maintain consistency of culture conditions for all samples. Following this rest period, the number of viable cells was determined and the cells used in cloning assays. Methods for the T-cell cloning assay for this study have been previously described [O'Neill et al., 1987; Albertini MR et al., 2001]. Briefly, PBMCs were plated by limiting dilution into 96-well microtiter plates to establish cloning plates. PBMCs were plated at 1 to 5 cells/well in the absence of 6-thioguanine (TG) and at 1×10^4 cells/well in the presence of TG (10^{-5} M) in cloning medium containing RPMI-1640 supplemented with 10% lymphokine activated killer cell supernatant, 20% HL-1, 5% FCS and 0.25 μ g/ml PHA (Sigma, St Louis, MO: Patients 1 and 2; Remel, Lenexa, KS: Patients 3, 5 and 9). Accessory feeder cells used were a derivative of WIL-2 lymphoblastoid cells designated as TK6, and these cells were grown in RPMI-1640 containing 10% FCS. Irradiated TK6 cells (9×10^3 cGy) were plated at an initial cell density of 1×10^4 cells/well in both selection and non-selection plates. An inverted phase-contrast microscope was used to score wells for visible colony growth on Day 14 of incubation. The calculations for cloning efficiency (CE) and MF determinations are described in the Statistical Analysis Section and are based on the colony growth on Day 14 of incubation. Single-cell-derived T-cell isolates (SCD-T) were then expanded in cloning medium containing 2.0×10^5 irradiated TK6 cells/ml either with or without TG (10^{-5} M). SCD-T were split and advanced into larger wells every 3–4 days and maintained in culture until a final lymphocyte count of $1\text{--}30 \times 10^6$ cells/isolate was obtained. SCD-T cells were then cryopreserved to allow for subsequent molecular analyses.

Mass Culture Conditions

PBMCs were cultured at a cell density of 1×10^6 cells/ml in T25 flasks in a total volume of 20 ml of modified RPMI-1640 (i.e., 2×10^7 cells/flask) supplemented with 20% HL-1 medium and 5% FCS for 16–18 hours. For MT mass cultures, PBMCs were centrifuged and suspended at approximately 1×10^6 viable cells/ml and cultured in the presence of TG (10^{-5} M) in cloning medium (*HPRT* selection cultures). WT (non-selection) mass cultures were expanded in an analogous fashion, but with a lower starting number of cells (5×10^3 cells/flask) and without TG. Irradiated TK6 were added to the T25 flask at a density of 2.5×10^5 cells/cm² surface area. Cultures were incubated and sub-cultured every 4 days, or as needed, using these methods until the required number of viable cells was obtained.

Tumor-Infiltrated Lymph Node (TILN) and Tumor-Infiltrating Lymphocyte (TIL) Mass Culture Conditions

Surgical specimens from TILN from regional LNs or TIL from any metastasis histologically positive for melanoma were initially rinsed in RPMI-1640 medium and then finely minced into less than 1-mm $\times$ 1-mm pieces with surgical iris scissors. The mechanically disaggregated specimens were enzymatically digested with Pronase (type XIV Protease, Sigma) by performing 4 to 6 sequential 20-min incubations of the mechanically disaggregated specimen in RPMI-1640 containing 20 U/ml Pronase. The cells in suspension were added to an equal volume of RPMI-1640 with 16% FCS after each incubation, while the mechanically disaggregated specimen received an additional incubation with 20 U/ml Pronase. Following the disaggregation procedure, cells were washed with Phosphate Buffered Saline (PBS) and processed as described above for PBMC mass cultures.

Preparation and Sequencing of cDNAs Encoding the TCR- β from SCD-T

Total RNA was extracted from each individual expanded SCD-T using an RNeasy Mini Kit (Qiagen Inc., Valencia, CA). Total RNA was reversed transcribed into cDNA in a reaction primed by oligo-deoxythymidine (oligo-dT) using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). TCR- β transcripts were subsequently amplified for each SCD-T in a single PCR reaction using Taq DNA Polymerase in Storage Buffer A and a TCR- β constant region primer (C β Internal, Table I) at a final concentration of 0.2 pmol/ μ l together with a complete mixture of 26 different human variable TCR- β primers (HUV Beta, Table I) corresponding to the different variable region beta chain (V β) gene families, each at a final concentration of 0.2 pmol/ μ l, according to the manufacturer's instructions (Promega, Madison, WI). The design of these primers and development of these methods were adapted from the doctoral dissertation of one of us (unpublished data; Judice, S.A., 2001, Human and mouse T-cell trafficking, mutation, and kinetics, University of Vermont, Burlington, VT). The PCR cycling consisted of 30 cycles (95°C for 45 sec; 60°C for 1 min; 72°C for 2 min) followed by a final extension (72°C for 5 min) and a cool-down hold (4°C hold). The TCR- β PCR products were gel purified and sequenced at the University of Wisconsin Biotechnology Center. Analysis of the cDNA sequence and identification of the TCR- β variable, CDR3, and joining regions of the TCR- β mRNA was performed using the DNA sequence analysis tools on the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>). By convention, TCR- β CDR3 motifs are presented below as the converted amino acid sequence, i.e., as the phenotype, which is the antigen reactive product of the rearrangement.

Preparation and Characterization of cDNAs Encoding the TCR- β from Mass Culture T-cells

Total RNA from mass culture T-cells was prepared as described in the above section. 5' RACE cDNA synthesis of the TCR- β transcripts from total RNA was conducted using GeneRacerTM RLM-RACE kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions using a gene-specific 3' oligonucleotide complementary to the constant region of the TCR- β mRNA (24TRBC, Table I). TCR- β transcripts were subsequently amplified using BD Advantage 2 PCR Enzyme System (BD Biosciences Clontech, Palo Alto, CA), a TCR- β constant region primer (24TRBC2, Table I), and the GeneRacerTM 5' Nested primer at a final concentration of 0.2 pmol/ μ l, each according to the manufacturer's instructions. The 24TRBC and 24TRBC2 oligo sequences were obtained through a personal communication and are published [Lefranc, 2004; Lefranc et al., 2005; IMGT website (<http://imgt.cines.fr>)]. The PCR cycling consisted of 20 cycles (95°C for 15 sec; 65°C for 30 sec; 68°C for 2 min) followed by a final extension (68°C for 5 min) and a cool-down hold (4°C hold). These PCR conditions were performed to avoid PCR bias inherent in the system [Butler et al., 2005]. TCR- β cDNA was cloned into PCR@4TOPO® (Invitrogen, Carlsbad, CA) using topoisomerase according to manufacturer's instructions. Plasmids containing TCR- β cDNA were sequenced and analyzed as described in the above section.

Statistical Analysis

HPRT clonal assay data were processed by initially calculating a CE for each assay and a PBMC MF for each individual. To determine standard estimates for CE and MF, CE was calculated by use of the Poisson relationship, $P_0 = \exp(-x)$, where P_0 is the fraction of wells without colony growth and x is the average number of clonable cells per well. The value of x divided by the number of cells added to each well (n_0) defines the cloning efficiency. The *HPRT* MF with this calculation is the ratio of the mean CE in the presence (selection) and absence (non-selection) of TG. Averaging yielded CE and MF values in cases with multiple dilutions.

The comparison of TCR- β V-D-J usage between WT T-cells and MT T-cells involves an evaluation of clustering patterns compared with what would be expected in a sample if the *HPRT* selection process were not enriching for amplifying T-cell clones. On that null hypothesis, the population sampled in WT and that sampled in MT are equivalent, and observed differences are attributable to stochastic variation alone. Fisher's exact test for comparing two multinomial samples is therefore appropriate [Agresti, 2002]. This test utilizes data on the frequency of every distinct CDR3 sequence, and allows that even in the absence of enrichment by the selection to MT, there may be variation in the *in vivo* size of T-cell clones. It is sensitive to violations of the null hypothesis that would make TCR- β V-D-J usage patterns distinct in the selected population, such as excessive clustering on one or two particular sequences. We implemented Fisher's test using independent Monte Carlo sampling with $B=50000$ samples per comparison.

We developed an extension to Fisher's exact test in order to accommodate the possibility that *in vitro* growth after selection in the MT mass culture cells could create excessive clustering, even if selection is not enriching for amplifying T-cell clones (a population bottleneck could occur if initial cell frequency is low compared with MF). Recall that Fisher's test may be derived by modeling the number X_i of occurrences in the WT of a TCR- β V-D-J sequence i as a Poisson variable with rate $n\theta_i$, where n is the number of clones sequenced and θ_i is the abundance of i *in vivo*. Similarly Y_i , the analogous count for the MT cells has the same Poisson distribution, on the null hypothesis that selection is not enriching for amplified clones. Fisher's test emerges by conditioning on totals $\sum X_i$, $\sum Y_i$, and $X_i + Y_i$ for all i . Our extended method followed this logic, but we replaced the Poisson model for the sample abundance Y_i in the MT with a Negative Binomial model having the same mean, but having a variance inflated by an amount determined by MF and cell density. The conditional probability mass function for TCR- β V-D-J counts is intractable analytically but is amenable to Monte Carlo simulation, which we used to derive a p-value measuring the significance of the most frequent MT TCR- β V-D-J (details in statistics manuscript in preparation). P-values <0.05 were considered significant.

RESULTS

Cloning Assays: *In vivo* Clonality of SCD-T from Peripheral Blood

Two-hundred ninety-eight MT and 133 WT SCD-T, isolated from cloning assays in five patients in which non-selected cloning efficiencies ranged from 0.08 to 0.15 and MFs ranged from 17.6×10^{-6} to 97.3×10^{-6} (Table II), were analyzed for TCR- β V-region, CDR3, and J-region sequences (Table III). Using the criterion that an identical TCR- β gene pattern in two or more independent SCD-T from a given individual reflects their *in vivo* clonal relationship, MT SCD-T from Patient 1 showed evidence of four expanding *in vivo* clones, with 3, 9, 14, and 2 representatives of each among the 69 MT SCD-T studied. Unexpectedly, evidence of a single *in vivo* expanding clone reflected in 2 WT SCD-T sharing a TCR- β gene pattern without a counterpart among the mutants was found among the 36 WT SCD-T studied (see below and Table VI for MT-WT sharing of TCR- β gene patterns). In this Patient, 41 MT and 34 WT SCD-T showed unique TCR- β gene patterns, i.e., specific TCR- β V-D-J sequences in only a single isolate. The sequence analysis of the TCR- β V-D-J for MT and WT SCD-T from Patients 1,2,3,5 and 9 is shown in Table III.

The Fisher's test was used to examine the differences in clonality distributions between MT and WT T-cell SCD-T for this set of patients. This difference was significant for Patient 1 ($p<0.0001$) and Patient 9 ($p = 0.002$), but not for Patient 2 ($p = 0.068$) or Patient 3 ($p = 0.10$). There was no *in vivo* clonality identified in either the MT or WT SCD-T from Patient 5.

Mass cultures: *In vivo* Clonality of Mass Cultures Derived from Peripheral Blood

Peripheral blood T-cells from each of the five patients were propagated in mass *in vitro* cultures, with or without TG selection, as described above, to obtain populations of MT and WT T-cells, respectively. The duration of cultures differed among the patients to obtain the requisite numbers of cells for analyses, but the selection and non-selection culture durations for each individual were the same. These were 25, 13, 18, 19, and 17 days for patients 1, 2, 3, 5, and 9, respectively. Total RNA from these mass populations were then obtained and analyzed as described.

A total of 107 MT cDNAs and 67 cDNAs from WT cDNAs were analyzed for Patient 1 (Table IV). Seven clonal clusters were detected among the cDNAs from the MT T-cells, with 6, 16, 3, 55, 3, 7 and 14 copies of each defining TCR- β V-D-J sequences (Table IV). There were only three unique TCR- β gene patterns among these cDNAs from the MT T-cells. However, all 67 cDNAs from the WT T-cells derived from this patient showed unique TCR- β gene patterns. The sequence analysis of the TCR- β V-D-J for MT and WT PBMC mass cultures from Patients 1,2,3,5 and 9 is shown in Table IV. The generalized Fisher's test was used to examine the differences in clustering as an indicator of clonality distributions between the cDNAs derived from MT and those derived from WT T-cells. The differences between cDNAs derived from MT and WT T-cells was highly significant for Patient 1 ($p < 0.0001$), Patient 2 ($p = 0.007$), Patient 3 ($p < 0.0001$), Patient 5 ($p = 0.0006$), and Patient 9 ($p < 0.0001$).

In both Patient 1 and Patient 9, identical TCR- β gene patterns were found among the MT SCD-T derived from cloning assays, and in the cDNAs derived from mass cultures of MT T-cells, indicating that mass cultures of peripheral blood T-cells can identify the same *in vivo* proliferating MT T-cell clones (Tables III, IV, and summarized in Table VI).

Mass cultures: *In vivo* Clonality of Mass Cultures Derived from TILN and TIL

T-cells derived from tumor-containing LNs (Patient 1) or metastatic melanoma tissue (Patients 2, 5 and 9) were propagated *in vitro* to obtain sufficient cells for analysis. Two anatomically separated subcutaneous nodules were harvested from Patient 9, from a back nodule (9A) and from a neck nodule (9B). Due to low numbers of cells in some of these samples, T-cells from Patients 1, 2, and the 9A tumor sample required growth *in vitro* for 12, 22, or 10 days, respectively, prior to their culture with or without TG selection for an additional 40, 25, or 21 days, respectively. Adequate numbers of T-cells were obtained directly from Patient 5 and from the Patient 9B tumor sample allowing direct *in vitro* culture with or without TG selection for a total of 29 or 19 days, respectively. Insufficient cells for tissue analyses were obtained from Patient 3.

In Patient 1, 45 cDNAs from the MT T-cells were analyzed for TCR- β V-D-J gene sequences, and all showed the identical gene patterns (Table V). Therefore, this single clonal cluster accounted for all MT T-cells propagated from a tumor-bearing LN in this patient. By contrast, seven clusters of TCR- β gene patterns, represented by 9, 3, 2, 5, 2, 3 and 2 repeats, were observed among the 36 cDNAs obtained from the WT T-cells grown from this lymph node, while 10 showed unique TCR- β gene patterns. The sequence analysis of the TCR- β V-D-J for MT and WT TIL and TILN mass cultures from Patients 1,2,5 and 9 is shown in Table V. Note that sequence analysis of TIL from a back nodule (9A) and from a neck nodule (9B) is provided for Patient 9. As for the studies for the mass cultures obtained from peripheral blood, a generalized Fisher's test was used to examine the differences in cluster distributions between MT and WT and to accommodate the likelihood that *in vitro* growth after selection of mutant cells would create clustering. Again, the difference between cDNAs derived from MT T-cells and those derived from WT T-cells were highly significant

for Patients 1, 2, 5, and the Patient 9A tumor sample ($p < 0.0001$) as well as for the Patient 9B tumor sample ($p = 0.004$).

It is noteworthy that in Patient 1 and in Patient 9, identical TCR- β gene patterns were seen for MT T-cells derived from peripheral blood as SCD-T and/or mass cultures and from mass cultures derived from TILN and/or TIL (Table VI). This indicates not only that proliferating MT T-cell clones can be identified in mass cultures of blood, but that they are also present at tumor sites in melanoma patients.

Shared TCR- β Gene Patterns Between MT and WT T-cell Fractions

In addition to clonality, as manifest by TCR- β gene patterns in the MT and WT fractions from peripheral blood and TIL/TILN from patients with melanoma, the unique TCR- β gene patterns within each fraction were analyzed for MT-WT sharing. A single MT isolate obtained as a SCD-T from peripheral blood of Patient 5 showed an identical gene pattern with that seen in WT T-cells obtained from TIL in that same patient (Table VI). The possibility that this *in vivo* clone grown in mass culture without TG might be an *HPRT* mutant has not been formally excluded. However, this finding reflects clonality, with representatives in both the MT and WT fractions of T-cells.

DISCUSSION

T-cell cloning for *HPRT* mutations is arguably the most frequently used assay for assessing somatic gene mutations arising *in vivo* in humans. Although its major use is in population monitoring for environmental mutagens, there are other applications of the assay. One is in characterizing the target cells in which the mutations arise, i.e., the T-cells. That use is illustrated by the results presented here. The goal of *HPRT* T-cell selection in melanoma patients is to identify a probe for the *in vivo* T cell response to human melanoma that provides mechanistic insights and helps direct subsequent monitoring and/or treatment strategies for patients with melanoma.

Malignant melanoma, a malignancy present in all five patients reported here, is expected to elicit a T-cell immunological response against tumor antigens, with resultant T-cell proliferation. To provide the rationale for studying surrogate selection in the context of melanoma, additional background information about T-cell responses to melanoma will be provided. Definitive evidence that melanoma-associated antigens (MAA) can stimulate cytotoxic and regulatory T-cell responses against human melanoma as well as serve as tumor-rejection antigens for T-cells in model systems has been present for many years [Mukherji et al., 1989; Van der Bruggen et al., 1991]. The characterized MAA can effectively stimulate cellular immune responses *in vitro*, and immunodominant epitopes have been identified and characterized [Kawakami et al., 1995; Kawakami et al., 1994; Kierstead et al., 2001; Topalian et al., 1994; Zarour et al., 2002]. Ongoing investigations are attempting to translate this understanding of MAA into effective *in vivo* therapeutic strategies [Pardoll, 2002]. However, clinical responses remain infrequent. While tumor-rejection antigens on human melanomas would provide an opportunity for effective targeted immune-based therapy, the MAA associated with effective *in vivo* anti-melanoma T-cell activity still need to be identified. It is possible that study of *in vivo* expanding T-cells in melanoma patients will provide insights into the *in vivo* immune response to this disease.

Several methods have been utilized to investigate *in vivo* clonally amplified T-cells including TCR- β analysis by Southern blot [Albertini MR et al., 2001], MaGiK method [Killian et al., 2002], RT *in situ* PCR [Nuovo et al., 2001], padlock probes and microarrays [Baner et al., 2005] and quantitative RT-PCR with spectratyping [Degauque et al., 2004]. These methods can provide a global look at the TCR- β repertoire in melanoma patients and

have identified CDR3 length alterations [Degauque et al., 2004; Speiser et al., 2006] over-expression of TCR- β V-region subfamilies [Degauque et al., 2004; Sensi et al., 1997; Speiser et al., 2006; Willhauck et al., 2003] and clonal expansion of T-cells after melanoma vaccination or cytokine immunotherapy [Degauque et al., 2004; Meidenbauer et al., 2004; Sensi et al., 1997; Speiser et al., 2006; Willhauck et al., 1996]. Additional insights into the detailed antigen specificity of *in vivo* T-cell responses to melanoma could be provided by identifying T cells undergoing ongoing or repetitive *in vivo* cell division and using a multiplex PCR-based method and subsequent analysis of TCR- β V-D-J gene sequences.

Elsewhere, we have suggested that a preferential occurrence of clonality among MT T-cells compared to WT T-cells is a result of an increased likelihood of spontaneous mutations in rapidly proliferating cells as opposed to quiescent G_0 cells — the condition of the vast majority of mature T-cells *in vivo* [O'Neill et al., 1994; Falta et al., 1999; Albertini RJ, 2001]. It may be that the rapidly proliferating cells may have more opportunity to make replication errors or less time to repair DNA damage. In part, preferential clonality in the MT fractions of T-cells may be a reflection of the need for amplification of *HPRT* mutations in order to be recovered in cloning assays. In any case, we have suggested that this phenomenon could be exploited for isolation of T-cells of immunological relevance — a form of surrogate selection. This strategy has been used to recognize such cells in autoimmune disorders and in organ transplantation [Albertini RJ, 2001 and references therein]. The study reported here suggests its application in cancer patients.

It may be asked —as was done in this study — the degree to which T-cell clonality identified by cloning assays represents the broader *in vivo* situation. Does this clonality reflect what is present in peripheral blood? More importantly for cancer patients, does it reflect what might be occurring within the tumors themselves?

In vivo clonality, as defined here, is a characteristic of the cells used to detect *HPRT* mutations, and not necessarily of the *HPRT* mutations themselves. Cellular clonality for the post-thymic, mature T-cells originates when the TCR genes undergo rearrangement, usually during thymic differentiation. *HPRT* mutational clonality begins with the occurrence of that mutation, which may be before, during, or after thymic differentiation. The timing of *HPRT* mutations relative to T-cell maturation has implications as to the anatomical sites of mutation [Albertini RJ, 2001]. Post-thymic mutation implies origination in the peripheral lymphoid tissue, i.e., LNs, spleen, peripheral blood, or other tissues where T-cells circulate; pre-thymic mutation implies origination in bone marrow or in the thymus itself.

In this regard, the finding of an identical TCR- β V-D-J sequence in a MT SCD-T clone and in the WT fraction of TIL derived mass culture cells from Patient 5 (Table VI) is of note. We have only rarely observed MT–WT sharing among clonal SCD-T derived from *HPRT* cloning assays, i.e., as SCD-T clones [O'Neill et al., 1994]. However, similar analyses of MT and WT fractions obtained from mass cultures of PBMCs or tissue-derived lymphocytes (TILN and TIL in the current study) have not previously been undertaken. The studies reported here are being extended to examine potential preferential usage of V-regions as well as potential clonal relationships between patients, with the addition of more patients and analyses of restricted TCR- β gene usage among and within patients. Indeed, we have observed MT–WT sharing of TCR- β gene patterns in mass cultures derived from blood and TILN/TIL (data not shown). The MT–WT sharing data at the mass culture level suggests that the preferential recovery of expanding MT T-cell clones is not simply due to dilution of clonality of WT T-cells because clonality, in this fraction, can be observed if it is extensive enough. However, when it is this extensive, an increased frequency of somatic mutations occur, as reflected in the presence of *HPRT* mutations, with the finding that these expanding *in vivo* clones usually have MT representatives. Importantly, the presence of identical TCR-

β gene patterns in both the MT and WT fractions of expanding T-cell clones indicates that the *HPRT* mutation has occurred in mature T-cells in the periphery, i.e., after the TCR gene rearrangement has occurred.

As shown in Tables IV, V and VI, preferential clusterings of TCR- β gene patterns were clearly reflected in the cDNAs derived from mass cultures of T-cells in peripheral blood and in tumors (in LN or other tissue). There were more clusters, and clusters of larger sizes, in the cDNAs derived from MT cultures than in those derived from WT cultures. These differences were statistically significant for all patients. Although some preferential clustering may have been expected because of size differences in starter population between the selected and non-selected *in vitro* cultures (the MT populations were much smaller based on calculations involving starting numbers of cells and MF) and/or differences in *in vitro* outgrowth of selected cells, allowances in the statistical analyses were made for that consideration.

The most compelling demonstration that MT T-cell clonality as reflected in cloning assays represents the broader *in vivo* situation is the identity of the TCR- β gene rearrangements in MT SCD-T to the rearrangement clusters of TCR- β cDNAs derived from both PBMC and TILN/TIL MT mass cultures in Patients 1 and 9 (Table VI). The finding of identical MT T-cells both in the peripheral blood and at sites of tumor suggests trafficking of expanding MT *in vivo* T-cells clones between the peripheral blood and sites of tumor. These findings validate analysis of MT isolates from blood when the target organ is not accessible, and these analyses can be used to investigate T-cells potentially involved in the *in vivo* T-cell response to human melanoma. Members of these expanding MT T-cell clones are expected to be present at frequencies difficult to detect within the WT fraction of T-cells from the same patient.

In conclusion, *in vivo* clonal amplifications of MT T-cells are present in the peripheral blood and at sites of tumors of melanoma patients. These expanding *in vivo* T-cell clones can be identified by analysis of TCR- β gene usage of TG-selected clonal SCD-T and of TG-selected mass cultures. *In vivo* MT T-cells in melanoma patients provide candidate probes to investigate the *in vivo* T-cell response to human melanoma.

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Abbreviations

AJCC	American Joint Committee on Cancer
C	constant
cDNA	complementary DNA
CDR3	third complementarity determining region
CE	cloning efficiency

D	diversity
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
FCS	fetal calf serum
HL-1	type of tissue culture media
HPRT	hypoxanthine guanine phosphoribosyltransferase
HPRT-	<i>HPRT</i> deficient
IMGT	Immunogenetics
J	junctional
LN s	lymph nodes
MAA	melanoma-associated antigen
MF	mutant frequencies
mRNA	messenger RNA
MT	mutant
NIH	National Institute of Health
NLM	National Library of Medicine
Oligo-dT	oligo-deoxythymidine
PBMC s	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	phytohemagglutinin
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RPMI-1640	type of tissue culture media
SCD-T	single-cell-derived T-cell
TCR	T-cell receptor
TCR-β	T-cell receptor beta
TG	6-thioguanine
TIL	tumor-infiltrating lymphocyte
TILN	tumor-infiltrated lymph node
TK6	lymphoblastoid cell line (derivative of WIL-2)
V	variable
WIL-2	lymphoblastoid cell line
WT	wild type

References

Agresti, A. Categorical Data Analysis. 2. New York: Wiley-Interscience; 2002. 734 pages

Environ Mol Mutagen. Author manuscript; available in PMC 2011 February 11.

- Albertini MR, King DM, Newton MA, Vacek DM. In vivo mutant frequency of thioguanine-resistant T-cells in the peripheral blood and lymph nodes of melanoma patients. *Mutat Res* 2001;476:83–97. [PubMed: 11336986]
- Albertini RJ. HPRT mutations in humans: biomarkers for mechanistic studies. *Mutat Res* 2001;489:1–16. [PubMed: 11673087]
- Albertini RJ, Nicklas JA, Skopek TR, Recio L, O'Neill JP. Genetic instability in human T-lymphocytes. *Mutat Res* 1998;400:381–389. [PubMed: 9685698]
- Allegretta M, Nicklas JA, Sriram S, Albertini RJ. T cells responsive to myelin basic protein in patients with multiple sclerosis. *Science* 1990;247:718–721. [PubMed: 1689076]
- Alt FW, Oltz EM, Young F, Gorman J, Taccioli G, Chen J. VDJ recombination. *Immunol Today* 1992;13:306–314. [PubMed: 1510813]
- Ansari AA, Mayne A, Sundstrom JB, Gravanis MB, Kanter K, Sell KW, Villinger F, Siu CO, Herskowitz A. Frequency of hypoxanthine guanine phosphoribosyltransferase (HPRT-) T cells in the peripheral blood of cardiac transplant recipients. A noninvasive technique for the diagnosis of allograft rejection. *Circulation* 1995;92:862–874. [PubMed: 7641367]
- Balch CM, Buzaid AC, Soong SJ, Atkins MB, Cascinelli N, Coit DG, Fleming ID, Gershenwald JE, Houghton A Jr, Kirkwood JM, McMasters KM, Mihm MF, Morton DL, Reintgen DS, Ross MI, Sober A, Thompson JA, Thompson JF. Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. *J Clin Oncol* 2001;19:3635–3648. [PubMed: 11504745]
- Baner J, Baner J, Marits P, Nilsson M, Winqvist O, Landegren U. Analysis of T-cell receptor V beta gene repertoires after immune stimulation and in malignancy by use of padlock probes and microarrays. *Clin Chem* 2005;51:768–775. [PubMed: 15695323]
- Butler JE, Wertz N, Sun J, Sacco RE. Comparison of the expressed porcine Vbeta and Jbeta repertoire of thymocytes and peripheral T cells. *Immunology* 2005;114:184–193. [PubMed: 15667563]
- Dawisha SM, Gmelig-Meyling F, Steinberg AD. Assessment of clinical parameters associated with increased frequency of mutant T cells in patients with systemic lupus erythematosus. *Arthritis Rheum* 1994;37:270–277. [PubMed: 8129782]
- Degauque N, Schadendorf D, Brouard S, Guillet M, Sebille F, Hohn H, Pallier A, Ruiz C, Dupont A, Chapin S, Hofmann U, Maeurer M, Souillou JP. Blood T-cell Vbeta transcriptome in melanoma patients. *Int J Cancer* 2004;110:721–729. [PubMed: 15146562]
- Falta MT, Magin GK, Allegretta M, Steinman L, Atkinson MA, Brostoff SW, Albertini RJ. Selection of hpert mutant T cells as surrogates for dividing cells reveals a restricted T cell receptor BV repertoire in insulin-dependent diabetes mellitus. *Clin Immunol* 1999;90:340–351. [PubMed: 10075863]
- Finette BA, Homans AC, Albertini RJ. Emergence of genetic instability in children treated for leukemia. *Science* 2000;288:514–517. [PubMed: 10775110]
- Gmelig-Meyling F, Dawisha S, Steinberg AD. Assessment of in vivo frequency of mutated T cells in patients with systemic lupus erythematosus. *J Exp Med* 1992;175:297–300. [PubMed: 1730923]
- Holyst, MM.; Hill, DL.; Sharp, GC.; Hoffman, RW. *Int Arch Allergy Immunol*. Vol. 105. The international ImMunoGeneTics information system (IMGT); 1994. Increased frequency of mutations in the hpert gene of T cells isolated from patients with anti-U1–70kD-autoantibody-positive connective tissue disease; p. 234–237. (<http://imgt.cines.fr>)
- Kawakami Y, Eliyahu S, Jennings C, Sakaguchi K, Kang X, Southwood S, Robbins PF, Sette A, Appella E, Rosenberg SA. Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression. *J Immunol* 1995;154:3961–3968. [PubMed: 7706734]
- Kawakami Y, Eliyahu S, Sakaguchi K, Robbins PF, Rivoltini L, Yannelli JR, Appella E, Rosenberg SA. Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. *J Exp Med* 1994;180:347–352. [PubMed: 7516411]
- Kierstead LS, Ranieri E, Olson W, Brusic V, Sidney J, Sette A, Kasamon YL, Slingluff CL Jr, Kirkwood JM, Storkus WJ. gp100/pmel17 and tyrosinase encode multiple epitopes recognized by Th1-type CD4+T cells. *Br J Cancer* 2001;85:1738–1745. [PubMed: 11742496]

- Killian MS, Matud J, Detels R, Giorgi JV, Jamieson BD. MaGiK method of T-Cell receptor repertoire analysis. *Clin Diagn Lab Immunol* 2002;9:858–863. [PubMed: 12093686]
- Lefranc MP. IMGT-ONTOLOGY and IMGT databases, tools and Web resources for immunogenetics and immunoinformatics. *Mol Immunol* 2004;40:647–660. [PubMed: 14644091]
- Lefranc MP, Giudicelli V, Kaas Q, Duprat E, Jabado-Michaloud J, Scaviner D, Ginestoux C, Clement O, Chaume D, Lefranc G. IMGT, the international ImMunoGeneTics information system®. *Nucl Acids Res* 2005;33:D593–597. [PubMed: 15608269]
- Lefranc, MP.; Lefranc, G. The T cell receptor FactsBook. Academic Press; London, UK: 2001. (398 Pages)
- Lewis SM. The mechanism of V(D)J joining: lessons from molecular, immunological, and comparative analyses. *Adv Immunol* 1994;56:27–150. [PubMed: 8073949]
- Meidenbauer N, Zippelius A, Pittet MJ, Laumer M, Vogl S, Heymann J, Rehli M, Seliger B, Schwarz S, Le Gal FA, Dietrich PY, Andreesen R, Romero P, Mackensen A. High frequency of functionally active Melan-a-specific T cells in a patient with progressive immunoproteasome-deficient melanoma. *Cancer Res* 2004;64:6319–6326. [PubMed: 15342421]
- Mukherji B, Guha A, Chakraborty NG, Sivanandham M, Nashed AL, Sporn JR, Ergin MT. Clonal analysis of cytotoxic and regulatory T cell responses against human melanoma. *J Exp Med* 1989;169:1961–1976. [PubMed: 2471770]
- Nuovo GJ, Nuovo GJ, Morrison C, Porcu P, Caligiuri MA, Suster S. In situ determination of T-cell receptor beta expression patterns. *J Histochem Cytochem* 2001;49:139–145. [PubMed: 11156682]
- O'Neill JP, McGinniss MJ, Berman JK, Sullivan LM, Nicklas JA, Albertini RJ. Refinement of a T-lymphocyte cloning assay to quantify the in vivo thioguanine-resistant mutant frequency in humans. *Mutagenesis* 1987;2(2):87–94. [PubMed: 3331707]
- O'Neill JP, Nicklas JA, Hunter TC, Batson OB, Allegretta M, Falta MT, Branda RF, Albertini RJ. The effect of T-lymphocyte 'clonality' on the calculated hprt mutation frequency occurring in vivo in humans. *Mutat Res* 1994;313:215–225. [PubMed: 7523907]
- Pardoll DM. Spinning molecular immunology into successful immunotherapy. *Nat Rev Immunol* 2002;2:227–238. [PubMed: 12001994]
- Sensi M, Farina C, Maccalli C, Lupetti R, Nicolini G, Anichini A, Parmiani G, Berd D. Clonal expansion of T lymphocytes in human melanoma metastases after treatment with a hapten-modified autologous tumor vaccine. *J Clin Invest* 1997;99:710–717. [PubMed: 9045874]
- Speiser DE, Baumgaertner P, Barbey C, Rubio-Godoy V, Moulin A, Corthesy P, Devedre E, Dietrich PY, Rimoldi D, Lienard D, Cerottini JC, Romero P, Rufer N. A novel approach to characterize clonality and differentiation of human melanoma-specific T cell responses: spontaneous priming and efficient boosting by vaccination. *J Immunol* 2006;177:1338–1348. [PubMed: 16818795]
- Tonegawa S. Somatic generation of antibody diversity. *Nature* 1983;302:575–581. [PubMed: 6300689]
- Topalian SL, Rivoltini L, Mancini M, Markus NR, Robbins PF, Kawakami Y, Rosenberg SA. Human CD4+ T cells specifically recognize a shared melanoma-associated antigen encoded by the tyrosinase gene. *Proc Natl Acad Sci U S A* 1994;91:9461–9465. [PubMed: 7937789]
- Van den Berg LH, Mollee I, Wokke JH, Logtenberg T. Increased frequencies of HPRT mutant T lymphocytes in patients with Guillain-Barre syndrome and chronic inflammatory demyelinating polyneuropathy: further evidence for a role of T cells in the etiopathogenesis of peripheral demyelinating diseases. *J Neuroimmunol* 1995;58:37–42. [PubMed: 7730448]
- Van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 1991;254:1643–1647. [PubMed: 1840703]
- Willhauck M, Mohler T, Scheibenbogen C, Pawlita M, Brossart P, Schmier JW, Keilholz U. T-cell receptor beta variable region diversity in melanoma metastases after interleukin 2-based immunotherapy. *Clin Cancer Res* 1996;2:767–772. [PubMed: 9816229]
- Willhauck M, Scheibenbogen C, Pawlita M, Mohler T, Thiel E, Keilholz U. Restricted T-cell receptor repertoire in melanoma metastases regressing after cytokine therapy. *Cancer Res* 2003;63:3483–3485. [PubMed: 12839930]

Zarour HM, Maillere B, Brusic V, Coval K, Williams E, Pouvelle-Moratille S, Castelli F, Land S, Bennouna J, Logan T, Kirkwood JM. NY-ESO-1 119-143 is a promiscuous major histocompatibility complex class II T-helper epitope recognized by Th1- and Th2-type tumor-reactive CD4+ T cells. *Cancer Res* 2002;62:213–218. [PubMed: 11782380]

TABLE IDNA Oligonucleotides Used for Amplification of Human TCR- β .

	Oligo Name ^a	Oligo Sequence	Annealing Region on TCR- β cDNA
Constant region primers	C Beta Internal	GCGGCTGCTCAGGCAGTA	Constant
	TCR C Beta External	CCAGAAGGTGGCCGAGAC	Constant
	C Beta Sequencing Primer	CGACCTCGGGTGGGAACA	Constant
	24TRBC	CGGGCTGCTCCTTGAGGGGCTGCG	Constant
	24TRBC2	GCGGCTGCTCAGGCAGTATCTGGAGTC	Constant
Variable region primers	HUV Beta 1	CACAACAGTTCCTGACTTGAC	Variable 9–1
	HUV Beta 2	CATCAACCATGCAAGCCTGACC	Variable 20–1
	HUV Beta 3	CTCTAGAGAGAAGAAGGAGCGC	Variable 28–1
	HUV Beta 4	CTGACGATCCAGTGTCAAGTCG	Variable 29–1
	HUV Beta 5.1	GTCGATTCTCAGGGCGCC	Variable 5–1, 5–4
	HUV Beta 5.2	CGCCTTGTTGCTGGGGG	Variable 5–5, 5–6, 5–7, 5–8
	HUV Beta 6	AGGCCTGAGGGATCCGTC	Variable 7–2, 7–3, 7–4, 7–6, 7–7, 7–8, 7–9
	HUV Beta 7	CCTGAATGCCCCAACAGCTC	Variable 4–1, 4–2, 4–3
	HUV Beta 8	GATTCAGGGATGCCCAGG	Variable 12–3, 12–4, 12–5, 17–1
	HUV Beta 9	CTGGAGCTTGGTGACTCTGC	Variable 3–1
	HUV Beta 10	GTCCACGGAGTCAGGGG	Variable 21–1 (Pseudo)
	HUV Beta 11	GTTCTCAAACCATGGGCCATGAC	Variable 25–1
	HUV Beta 12	GGGCATGGGCTGAGGC	Variable 10–1, 10–2, 10–3
	HUV Beta 13	GGCATGGGCTGAGGC	Variable 6–1, 6–2, 6–3, 6–4, 6–5, 6–6, 6–8, 6–9
	HUV Beta 14	CCCAAGTGACCCAGAACCC	Variable 27–1
	HUV Beta 15	GTGTCTCTCGACAGGCACAG	Variable 24–1
	HUV Beta 16	CACAGCGTAATAGAGAAGGGCC	Variable 14–1
	HUV Beta 17	GGTACAGCGTCTCTCGGG	Variable 19–1
	HUV Beta 18	CTGGTCAGGAGGAGGGG	Variable 18–1
	HUV Beta 19	GCGATTCTCATCTCAATGCCCC	Variable 23–1 (Pseudo)
	HUV Beta 20	AGACCCAGGACCGGC	Variable 30–1
	HUV Beta 21	CCTATTCTGGCCATGCTACCC	Variable 11–1, 11–2, 11–3
	HUV Beta 22	CCAGGTCAGGACCCCC	Variable 13–1
	HUV Beta 23	GATCTTGCGCTGTGTCCCC	Variable 2–1
	HUV Beta 24	CAGAAGTCAAGTCAGGCCCC	Variable 15–1
	HUV Beta 25	GCTGCCTGCTCTTCCCC	Variable 16 (Pseudo)

^a These oligo designations were assigned at the time of synthesis (see 'Annealing region on TCR- β cDNA').

TABLE II

Patients in the Study.

Patient	Stage of Disease ^{a,b}	Age ^c	Gender ^d	CE ^e	MF ^f × 10 ⁻⁶
1	III	70.2	M	0.12	97.3
2	IV	44.6	M	0.08 ^g	17.6 ^g
3	IV	36.4	M	0.15 ^h	29.3 ^h
5	IV	55.5	M	0.09	39.4
9	IV	71.1	M	0.08	40.3

^aThe American Joint Committee on Cancer (AJCC) stage refers to the stage of disease at the time of blood sample acquisition.

^bBlood samples were obtained from 5 to 16 days prior to resection of either Tumor infiltrated lymph node (Patient 1) or Tumor infiltrating lymphocytes (Patient 2, lung nodule; Patient 3, subcutaneous metastasis; Patient 5, subcarinal lung mass; and Patient 9, back nodule and neck nodule).

^cAge at the time of blood sample acquisition is given in years.

^dAll 5 patients were male (M).

^eCloning Efficiency (CE) determined for peripheral blood mononuclear cells (PBMC).

^fMutant Frequency (MF) determined for PBMC.

^{g,h}Two separate cloning assays were performed on the same blood sample; CE^g corresponds to MF^g, and CE^h corresponds to MF^h.

Clonality Analysis of Mutant (MT) and Wild Type (WT) Single-Cell-Derived T-cells (SCD-T) from Peripheral Blood Mononuclear Cells (PBMC).

TABLE III

Patient	MT Sequence Analysis Summary				WT Sequence Analysis Summary			
	V Region	CDR3 Motif	J Region	No. of Occurrences ^a	V Region	CDR3 Motif	J Region	No. of Occurrences ^b
1	20-1	AIPPGLAGEHHR	2-3	3	6-5	STPQV	1-5	2
	7-3	FVMG	2-2	9	unique	unique	unique	34
	7-8	PQGQV	2-1	14				
	11-2	SFKG	1-1	2				
	unique ^c	unique	unique	41				
Total				69				36
2	19	APEKEGGDRR	2-5	2	unique	unique	unique	49
	28	EPRQS	1-6	3				
	4-2	VGGAG	1-5	2				
	unique ^c	unique	unique	60				
Total				67				49
3	10-3	RGAVS	2-7	6	unique	unique	unique	17
	11-2	TSGN	2-7	3				
	unique ^c	unique	unique	54				
				63				17
Total								
5	unique ^{c,d}	unique	unique	60	unique	unique	unique	20
				60				20
Total								
9	20-1	AKGGWN	2-7	2	unique	unique	unique	11
	4-1	FRDRE	2-2	2				
	27	GVG	1-6	2				
	9	PQGLSFS	2-2	10				
	20-1	QTGYG	1-2	5				
	unique ^c	unique	unique	18				
Total				39				11

^aTotal MT cDNAs sequenced.

^bTotal WT cDNAs sequenced.

^cIn each instance 'unique' refers to a TCR- β gene pattern detected only once within the total pool of SCD-T for that sample; for example, there were 41 distinct TCR- β gene patterns that were detected just once in the MT SCD-T from Patient 1.

^dA single unique SCD-T had the following TCR- β sequence: V β 10-3, CDR3 GRS, J β 2-1. This MT SCD-T is described in Table VI as 1 cDNA detected with this TCR- β sequence from a total of 60 cDNAs sequenced for that sample.

TABLE IV
Clonality Analysis of Mutant (MT) and Wild Type (WT) Mass Cultures from Peripheral Blood Mononuclear Cells (PBMC).

Patient	MT Sequence Analysis Summary				WT Sequence Analysis Summary			
	V Region	CDR3 Motif	J Region	No. of Occurrences ^a	V Region	CDR3 Motif	J Region	No. of Occurrences ^b
1	4-2	EEGVR	1-4	6	unique	unique	unique	67
	4-1	GGTGEG	2-7	16				
	10-3	GGTSG	2-7	3				
	4-2	LAGGP	2-2	55				
	20-1	MRTAGG	1-6	3				
	7-8 ^c	PGQGV	2-1	7				
	7-3	SGL	2-1	14				
	unique ^d	unique	unique	3				
	Total				Total			
				107				67
2	19	DLVG	2-1	13	28	DA	2-2	2
	28	FVRY	2-2	2	28	FDP	2-2	2
	19	GGRD	1-5	5	28	FQG	2-7	2
	20-1	GGT	2-7	5	6-1	GQGI	2-7	2
	10-3	HGI	2-3	6	2	GREA	1-2	3
	27	IRDRGH	2-7	4	28	INSPLD	2-7	4
	20-1	LAGTSGV	2-7	21	28	PVEAL	1-1	2
	10-1	QRRG	2-3	4	2	RGDRGP	2-3	2
	7-9	SDRGE	2-3	3	28	YYGDSG	1-3	2
	19	TSLGSG	1-1	2	unique	unique	unique	76
	28	VSSRAG	1-1	9				
	unique ^d	unique	unique	7				
	Total				Total			
				81				97
3	6-2	LDRH	2-3	41	20-1	EDPRTDRGH	2-1	2
	10-3	GRS	2-1	9	2	GGQYLA	1-2	2
	29-1	GPMNRSGR	2-3	8	5-1	RDT	2-2	2
	9	ATMGG	2-7	2	20-1	RDT	2-3	2

MT Sequence Analysis Summary					WT Sequence Analysis Summary				
Patient	V Region	CDR3 Motif	J Region	No. of Occurrences ^d	V Region	CDR3 Motif	J Region	No. of Occurrences ^b	
5	4-2	LPRGQD	1-4	11	28	RIQNP	2-7	2	
	20-1	GGAAAF	1-4	5	7-4	STGLQ	1-2	2	
	5-6	RLSGDT	2-7	4	unique	unique	unique	79	
	20-1	SIRRD	1-2	2					
	10-3	GGA	2-3	3					
	7-2	PPGLAPS	2-3	3					
	unique ^d	unique	unique	5					
Total				93				91	
9	7-2	AGQGR	1-1	6	2	SSG	2-1	2	
	15	DFDP	2-2	2	28	YTG	2-5	2	
	18	EMG	1-1	5	unique	unique	unique	88	
	5-5	GGQP	1-3	2					
	27	KIGGSA	1-1	7					
	6-6	KTAQA	2-7	6					
	6-6	PPGQI	1-1	29					
	7-2	QDS	2-6	5					
	11-2	RDRE	1-6	2					
	2	SMGTS	1-1	23					
	unique ^d	unique	unique	5					
	Total				92				92
	9	29-1	GSGT	2-1	73	6-3	EEYLEA	2-7	2
		5-1	GT	1-1	2	29-1	GNULLP	2-5	2
		9 ^c	PQGLSFS	2-2	11	29-1	GNULLRP	2-5	4
		3-1	SNGGGG	2-1	2	7-3	GSGA	2-7	2
11-3		TDGQGA	1-3	2	19	IGQGSP	2-7	2	
unique ^d		unique	unique	4	4-1	KRQG	2-7	2	
					6-6	PGAH	2-7	2	
					7-4	PLAGGPSN	2-1	2	

Patient	MT Sequence Analysis Summary				WT Sequence Analysis Summary			
	V Region	CDR3 Motif	J Region	No. of Occurrences ^a	V Region	CDR3 Motif	J Region	No. of Occurrences ^b
					11-2	SRG	1-3	2
					7-9	TDRAP	1-3	3
					25-1	TQGGM	1-1	2
					2	TSQTGQK	2-7	3
					9	VG	2-3	2
					unique	unique	unique	59
Total				94				89

^aTotal MT cDNAs sequenced.

^bTotal WT cDNAs sequenced.

^cThis TCR-β gene pattern was also detected in the mutant single-cell-derived T-cells from the same patient (Table III).

^dIn each instance 'unique' refers to a TCR-β gene pattern detected only once within the total pool of cDNAs for that sample; for example, there were 3 distinct TCR-β gene patterns that were detected just once in the MT mass culture from Patient 1.

Clonality Analysis of Mutant (MT) and Wild Type (WT) Mass Cultures from Tumor-Infiltrated Lymph Node (TILN) and Tumor-Infiltrating Lymphocytes (TIL).

TABLE V

Patient	MT Sequence Analysis Summary				WT Sequence Analysis Summary			
	V Region	CDR3 Motif	J Region	No. of Occurrences ^a	V Region	CDR3 Motif	J Region	No. of Occurrences ^b
1	7-8	PGQGV	2-1	45	4-3	DIRGI	2-5	9
	unique ^c	unique	unique	0	12-5	MSS	2-1	3
					7-4	RQLM	1-1	2
					10-3	SADFD	1-4	5
					27	TDGVG	2-1	2
					7-4	YLTNK	2-5	3
					7-4	YSRQH	1-1	2
					unique	unique	unique	10
				45				36
	Total							
2	5-1	AEM	2-2	2	10-2	DLPPRRGEA	2-5	5
	28	SAQQTSG	2-7	90	5-6	LR	2-1	3
	unique ^c	unique	unique	8	3-1	PRNSGGA	2-3	3
					7-7	PYREG	2-2	19
					28	RGYQD	1-6	2
					28	SAQQTSG	2-7	29
					29-1	SPGAGK	1-4	2
					unique	unique	unique	9
				100				72
	Total							
3	-	-	-	none	-	-	-	none
5	7-9	GSGGE	2-5	71	14	DLGD	2-2	3
					7-2	GLRV	1-1	2
					10-3	GRS	2-1	12
					4-1	KEIAGN	2-5	2
					6-1	LP	2-1	2
					14	PGLP	2-1	2

MT Sequence Analysis Summary					WT Sequence Analysis Summary			
Patient	V Region	CDR3 Motif	J Region	No. of Occurrences ^a	V Region	CDR3 Motif	J Region	No. of Occurrences ^b
9A	9	ADRDYS	1-2	23	7-9	PGSN	2-3	14
	28	SPQGGFG	1-1	51	7-9	PPT	2-6	2
	unique ^c	unique	unique	2	6-1	QQG	2-3	2
					30	TWTGS	2-1	2
					7-2	VSGA	2-2	2
Total				71	unique	unique	unique	43
				71				
9B	9	ADRDYS	1-2	23	20-1	DIPSGDN	2-7	4
	28	SPQGGFG	1-1	51	6-2	PSGRIAGD	2-1	2
	unique ^c	unique	unique	2	2	QLADD	1-6	2
					unique	unique	unique	54
					unique	unique	unique	54
Total				76				62
				76				
9B	29-1	AS	2-6	7	3-1	DPDRGA	2-3	2
	5-1	ASGSE	2-3	3	4-1	DPSAADTGA	2-3	3
	4-1	FRDRE	2-2	2	27	GD	2-5	2
	5-1	GPGS	2-1	21	29-1	HPSGTSG	2-1	2
	12-3	KG	1-2	5	28	YHGRD	2-7	2
	10-3	KPRGRD	2-2	4	unique	unique	unique	77
	9	PQGLSFS	2-2	81				
	20-1	QTGYP	1-2	10				
	19	TG	1-2	2				
	6-3	TPGTGRA	2-2	28				
Total	unique ^c	unique	unique	10				88
				173				

^aTotal MT cDNAs sequenced.

^bTotal WT cDNAs sequenced.

^cIn each instance 'unique' refers to a TCR-β gene pattern detected only once within the total pool of cDNAs for that sample; for example, there were no distinct TCR-β gene patterns that were detected just once in the MT mass culture from Patient 1.

Analysis^a of *in vivo* Expanded Mutant (MT) and Wild Type (WT) T-cell Clones Present in Peripheral Blood Mononuclear Cells (PBMC) and Tumor-Infiltrated lymph Node (TILN) or Tumor-Infiltrating Lymphocytes (TIL) from Patients 1, 5, and 9^{b,c}.

TABLE VI

Patient	TCR-β Gene Pattern			MT T-cell Cultures			WT T-cell Cultures		
	V Region	CDR3 Motif	J Region	PBMC SCD-T ^f	PBMC MC ^g	TIL/TILN MC	PBMC SCD-T	PBMC MC	TIL/TILN MC
1	7-8	PGQGV	2-1	14/69	7/107	45/45	0/36	0/67	0/36
5 ^d	10-3	GRS	2-1	1/60	0/92	0/71	0/20	0/92	12/88
9 ^e	4-1	FRDRE	2-2	2/39	0/94	2/173	0/11	0/89	0/88
9 ^e	9	PQGLSFS	2-2	10/39	11/94	81/173	0/11	0/89	0/88
9 ^e	20-1	QTGYP	1-2	5/39	0/94	10/173	0/11	0/89	0/88

^aTotal cDNAs positive for indicated clone/total cDNAs sequenced.
^bSamples from Patient 9 included TIL from a back nodule (9A) and a neck nodule (9B).
^cT-cells with shared TCR-β gene patterns were not identified in PBMC and TIL for Patient 2. Sufficient TIL sample was not available for Patient 3.
^dThis TCR-β sequence was detected only once in the MT SCD-T from Patient 5, and so was grouped with the 'uniques' in Table III. However, this TCR-β sequence was detected 12 times in the WT TIL from this patient, and as such is represented in Table V.
^eThis TCR-β sequence was detected both in the PBMC (SCD-T or MC) and in the 9B TIL, but not in the 9A TIL.
^fSingle-cell-derived T-cells (SCD-T)
^gMass culture (MC)