T-cell Receptor Repertoire in Matched MART-1 Peptide-stimulated Peripheral Blood Lymphocytes and Tumor-infiltrating Lymphocytes

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ABSTRACT

Characterization of tumor-associated antigens (TAAs) recognized by CTLs makes the consideration of therapeutic strategies based on peptide stimulation of peripheral blood lymphocytes (PBLs) feasible. Several such approaches are adoptive transfer of peptide-stimulated PBLs, ex vivo peptide stimulation of dendritic cells, and direct vaccination with TAA-derived peptides. A critical component of any of these peptide-based strategies is the requirement that the patient's PBLs are able to react productively against the presented TAA. The purpose of this study, through the study of T-cell receptor (TCR) usage, was to evaluate the T-cell response in matched MART-1, peptide-stimulated PBLs and tumor-infiltrating lymphocytes (TILs). MART-1, peptide reactive PBL and TIL cultures were generated from three patients by in vitro stimulation with an immunodominant peptide of MART-1. All cultures had a human leukocyte antigen A2-restricted, MART-1, peptide specific CTL response. The TCR usage of each was assessed by the DNA sequence analysis of 50 TCR B clones obtained by rapid amplification of cDNA ends and culture. TCR analysis suggests a TCR repertoire that differed from patient to patient (8-16 subfamilies were used) and a predominant usage of a different variable B chain (BV) by each of these MART-reactive T cells. These predominant BV rearrangements were derived from multiple clonotypes because different variable, diversity, and junctional regions were observed. However, a similar pattern of expansion was present for both PBLs and TILs; the relative usage of each prevailing BV was more marked in TILs (36, 50, and 78% of TILs versus 26, 20, and 24% of PBLs, respectively), a broader TCR repertoire was used by PBLs (P > 0.05), and similar TCR subfamily usage was noted when TIL and PBL cultures from the same patient were compared (8 of 11, 7 of 9, and 7 of 8 for patients 1, 2, and 3, respectively). Furthermore, the exact same clonotypes derived from predominant TCR subfamilies in the PBLs and TILs were present in each patient, suggesting peptide-stimulated expansion in both biological compartments. These studies suggest that there will not be a limited and predictable TCR subfamily response to a specific TAA, although reproducible patterns of PBL and TIL expansion are present from patient to patient. Additionally, identical T-cell clonotypes having the same potential for antigen-driven expansion were present in a patient's PBLs and TILs. As such, our data support the conceptualization of approaches using adoptive transfer or vaccination based on TAA-derived peptide stimulation of PBLs.

INTRODUCTION

Tumor-specific CTLs derived from TILs that specifically recognize and respond to both autologous and allogeneic tumor cells in a MHC-restricted manner can be isolated in vitro (1-3). These T cells are able to effectively treat tumor-bearing animals (4) and can mediate tumor regressions in select melanoma patients with metastatic disease (5, 6). Recent progress in our knowledge of antigen processing/presentation and techniques for the isolation of peptides presented in a MHC-restricted format has led to the identification of murine (7, 8) and human TAAs recognized by T lymphocytes (9-16). Antigen-specific T-lymphocyte reactivity is provided by the TCR a and B chain heterodimer. This occurs via binding of the TCR to a short peptide fragment derived from an antigenic protein, presented in the context of a MHC molecule (17, 18). On the basis of CTL-specific recognition of TAAs, several common melanoma-associated antigens have been identified. In some cases, the functional specificity of TCR heterodimer recognition has been characterized (9-16, 19). These antigens are present in the majority of melanoma cell lines and are also expressed in normal melanocytes (tyrosinase, gp100, and MART-1) or testes (Mage-1 and Mage-3). Among these, the MART-1 antigen appears to be one of the most prevalent in terms of T-cell reactivity, as it is specifically recognized on melanoma cells by the large majority of TIL cultures (12). CTLs derived from TILs are capable, therefore, of playing a significant role in an antigen-specific immune response to melanoma and provide evidence that manipulation of the human immune system can result in the regression of cancer (20).

Although the in vivo significance of each of these antigens in the recognition of melanoma by T cells is yet to be defined, it is clear that CTLs specifically recognizing tumor antigens in the context of MHC do exist in vivo. The cloning of the genes that encode TAAs and the description of their class I MHC restricted epitopes, therefore, now provide opportunities for the development of new therapeutic strategies (21-23). Several such approaches actively being pursued are the adoptive transfer of PBLs stimulated in vitro with peptides derived from TAA (24), the direct administration of peptide to host dendritic cells ex vivo prior to adoptive transfer (25), and vaccination with TAA-derived peptides to a patient in a manner capable of enhancing antigen-presenting cell uptake and presentation (26).

A critical component of any of these peptide based strategies is the requirement that patient's peripheral T-cell compartment react productively against the TAAs presented. In this setting, understanding and characterizing the PBL response to TAA peptide presentation becomes important (27, 28). Although IL-2-expanded TILs are capable of mediating an antitumor response in select patients, the response of TILs and/or PBLs to TAA peptide stimulation, the relationship between the PBLs and the TILs as biological compartments, and the potential ability of PBLs to act as an antitumor effector cell population per se is largely unknown. The purpose of this study, through the study of TCR usage, was to evaluate the T-cell response in matched MART-1 peptide-stimulated PBLs and TILs. By analyzing the PBLs and TILs from three different metastatic melanoma patients, we sought to (a) evaluate the TCR repertoire in the patients' matched PBLs and TILs stimulated in vitro with TAA peptide; (b) determine whether the same T-cell clonotypes are expanded from TILs and PBLs in individual patients; (c) assess whether a patient-to-patient TCR usage consistency was present in either the TILs or PBLs; and (d) evaluate whether the tumor site is accessible to the population of lymphocytes present in the peripheral blood or represents a privileged biological compartment.
MATERIALS AND METHODS

Isolation of PBLs and TILs. Three tumor-bearing HLA-A2+ melanoma patients being evaluated for IL-2/TIL-based therapy at the National Cancer Institute Surgery Branch had PBLs and TILs isolated after appropriate informed consent was obtained. Briefly, heparinized whole blood was obtained and diluted 1:4 with HBSS (Life Technologies, Inc., Gaithersburg, MD). PBLs were then separated from the whole blood using a standard Ficoll-Hypaque gradient (Pharmacia, Uppsala, Sweden). TILs were generated by mincing surgically excised tumor and then digesting it with a triple enzyme solution into a single-cell suspension overnight (RPMI 1640 with 0.01% hyaluronidase type V (Sigma Chemical Co., St. Louis, MO), 0.002% DNase type I (Sigma), 0.1% collagenase type IV (Sigma); Ref. 3). The single-cell suspension was passed through a wire grid, and viable lymphocytes were separated on a Ficoll-Hypaque gradient (LSM, Bioinecs, Kensington, MD). Resultant PBLs and TILs were resuspended for subsequent use in Iscove’s medium with 10% pooled human AB serum (Sigma) at a concentration of 5 × 10^6 cells/ml.

Peptide Synthesis. Peptides (kindly provided by Drs. K. Sakaguchi and E. Appella, NIH, Bethesda, MD) were synthesized by a solid-phase method using a multiple peptide synthesizer (model AMS 422, Gilson Co., Inc., Worthington, OH) and purified by high-performance liquid chromatography on a C-4 column (VYDAC, Hesperia, CA) with 0.05% TFA/water-acetonitrile. The MART-1 peptides are located in a hydrophobic putative transmembrane domain in MART-1 (12). MART-1_27-35 (AAGIGILTV) is the immunodominant epitope recognized by MART-1-reactive T lymphocytes, whereas MART-1_122-130 is a nonimmunogenic peptide (12) that was used as a control.

Cell Lines. Melanoma cell lines were obtained from the Surgery Branch, National Cancer Institute, Bethesda, MD, as described (3). 624 mel+ and 624 mel— (established in the Surgery Branch, National Cancer Institute, Bethesda, MD, as described (3)) and T2 cells were maintained in RPMI with 10% FCS. 624 mel was cloned in the cultures with melanoma-associated antigen (MART-1_27-35), and IL-2 as a positive control. MART-1_27-35 iS a nonimmunogenic peptide (12) that was used as a control.

Generation of Cytotoxic T Cells from PBL and TIL Cultures. Cytotoxic T-cell cultures were generated from both PBL and TIL cultures by stimulating the cultures with melanoma-associated antigen, (MART-1_27-35), and IL-2 as described (24). Lymphocytes were cultured in Iscove’s medium (Biofluids, Rockville, MD) containing 10% human AB serum, 1-glutamine, antibiotics (common media), and 1 μg/ml MART-1_27-35 peptide at 1.5 × 10^6 cells/ml in 24-well plates. Two days later, 12 IU/ml IL-2 (Chiron Co., Emeryville, CA) was added to the cultures. Lymphocyte cultures were restimulated weekly as follows: proliferating lymphocyte lines were washed once and resuspended in 24-well plates at a concentration of 2.5 × 10^6 cells/ml. Frozen autologous PBLs were thawed, washed twice in PBS (Life Technologies, Inc.), and resuspended at 5–8 × 10^6 cell/ml in 5 ml of medium with 1 μg/ml MART-1_27-35 peptide for 3 h at 37°C. These stimulator PBLs were then irradiated at 3000 rads, washed once in PBS, and added to the proliferating culture of lymphocytes in the 24-well plates at ratios ranging between 1:3 and 1:10 (proliferating lymphocytes:stimulator PBLs). The following day, 12 IU/ml IL-2 were added to the cultures. This process was performed weekly for 5 weeks (24). Resultant cell lines were CD4+ cell depleted by negative selection using standard AIS Microcellular T25 anti-CD4+ antibody-coated flasks (Applied Immune Sciences Inc., Santa Clara, CA).

^{3}Cr Release Cytotoxicity Assay. Tumor and peptide specificities of the MART-1_27-35 peptide-stimulated PBLs and TILs were assayed by standard ^{3}Cr release cytotoxicity assay as described (3). Briefly, 5 × 10^6 target cells (either 624+, 624−, T2 alone, or T2 plus peptide) were harvested and labeled. For all studies, T2 target cells were cocultured in RPMI with 10% FCS for 1 h at 37°C either with (T2 plus peptide) or without (T2 alone) 1 μg/ml of peptide. The effector cells (either peptide-stimulated TILs, peptide-stimulated PBLs, or positive controls TIL 1235 and TIL 1200) were washed, resuspended, and diluted appropriately for the desired E:T target cell ratios. Controls included medium alone, 51Cr-labeled target cells alone (spontaneous minimum value), and 51Cr-labeled target cells with 100 μl of 2% SDS (maximum value). The plate was incubated at 37°C for 4 h in a 5% CO₂ incubator, and chromium release was assayed using a standard Skatron harvesting system (Skatron Instruments, Sterling, VA). The results were averaged and expressed as a percentage of specific lysis by comparison to the minimum and maximum control values as follows:

\[
\text{% specific lysis} = \frac{\text{cpm experimental} - \text{cpm spontaneous}}{\text{cpm maximum} - \text{cpm spontaneous}} \times 100
\]

RCA and 5' RACE. Whole RNA was isolated from the six PBL and TIL cell lines (using 10^6 cells from each condition) with guanidium isothiocyanate buffer and cesium chloride as described (29). The resultant RNA pellet was isolated, washed twice with 70% ethanol, and dried at room temperature for 15 min. Each RNA sample was then resuspended in 25 μl of diethyl pyrocarbonate water and stored at −70°C. 5' RACE of the RNA samples was performed to isolate the TCR β genes as described (Life Technologies, Inc.; Ref. 19). Briefly, cDNA was synthesized using 1 μg of total RNA, 25 ng of a 3' TCR β constant region-specific primer (5'-CCCTCCCATCCACCCACC-3'), and 1 μl of SuperScript II reverse transcriptase as described, with RNase H subsequently used to degrade the RNA. The resultant cDNA was then purified over a GlassMAX column and subjected to the 5' end end using dCTP nucleotides and 1 μl of terminal deoxynucleotidyltransferase. The terminal deoxynucleotidyltransferase was inactivated by incubating the reaction at 70°C for 10 min. PCR amplification of the tail cDNA was performed using a nested 3' TCR β constant region-specific primer (5'-GCTGCTACCTCAGTGGTGTG-3') and anchor primer (5'-CUACUCUAACUGCGCCGCG-3'). After 35 cycles of amplification with Taq polymerase, the anchor PCR reaction was analyzed on a 1% agarose gel.

Cloning, Screening, and Isolation of TCR Genes. Anchor PCR products were ligated into pCRII and then transformed into competent TOP10B alpha (Invitrogen, San Diego, CA). Transformed cells were plated onto LB/ampicillin plates and incubated overnight at 37°C. Using a blue/white screen, colonies were picked and grown in overnight bacteria cultures for screening by PCR using flanking primers that amplify the pCRII poly linker (BV41, 5'-GAAAGCAGTATGACCATGATTA-3'; BV40, 5'-AGGTTGAAACAGACGGCCGATG-3'). Clones with PCR inserts larger than 250 bp were saved, and plasmid DNA isolation was subsequently performed using Promega Wizard Mini-preps (Promega, Madison, WI).

Sequencing. The plasmid DNA was sequenced using cycle sequencing reactions performed on a Perkin-Elmer Corp. GeneAmp PCR System 2400 machine (Foster City, CA). The sequencing primer (BQONSEQ2, 5'-CCCTCCCATCACCACCCACC-3') was a 3' primer specific for a homologous region of the β constant region 5' from the two constant region primers used in the 5' RACE PCR protocol described above. After PCR, the samples were purified to remove unincorporated dye terminators by precipitating the reaction with 2 μl of 3 M NaAc, pH 4.6, and 50 μl of 95% ethanol, washing with 70% ethanol. The pellet was dried in a vacuum centrifuge for 10 min and then frozen until it was ready to be loaded on the gel. The sequencing reactions were analyzed on a standard 4.75% acrylamide gel using a 373 automated sequencer (Perkin-Elmer Corp.). The gel was run for 14 h at 40 W, and data were collected using the ABI Prism DNA sequencing software, version 2.1.1, which provided a visual representation of the fluorescent data (chromatograph) and the most likely sequence of bases for each sample.

RESULTS

Specificity and Reactivity of MART-1_27-35 Peptide-stimulated TILs and PBLs. Prior to initiating a clonal analysis of the TCR BV subfamily usage by the MART-1_27-35, peptide-stimulated PBLs and TILs, we determined the tumor reactivity and peptide specificity of each culture. To this end, the CTL response from all three patient’s peptide-stimulated PBLs and TILs were evaluated using a standard 4-5^3Cr release assay. All six MART-1_27-35, peptide-stimulated PBL and TIL cultures lysed HLA-A2-positive human melanoma tumor cells expressing MART-1 antigen (624+), but they did not lyse HLA-A2-negative human melanoma tumor cells expressing MART-1 and MART-2.
antigen (624–; Fig. 1). Furthermore, the PBL and TIL cultures lysed T2 cells that were incubated with the MART-1(27-35) peptide, but not the irrelevant MART-1(22-30)-negative control peptide or T2 cells alone (Fig. 1). These peptide-stimulated PBL and TIL cultures, therefore, were reactive toward HLA-A2+ human melanoma expressing MART-1 antigen, specifically recognizing MART-1(27-35) peptide when it was presented in the context of HLA-A2 class I MHC.

**TCR BV Usage in Matched MART-1(27-35) Peptide-stimulated PBLs and TILs.** The pattern of TCR BV subfamily usage for each culture condition was assessed to evaluate the intra- and interpatient TCR repertoire in matched MART-1(27-35) peptide-stimulated PBL and TIL cultures. To achieve an appropriate sampling of TCR BV subfamily usage for TIL and PBL culture, random TCR β chains were isolated using standard RACE PCR. The TCR BV subfamily and junctional usage of these cDNA clones was then assessed by DNA sequence analysis. For each condition we examined 50 sequentially isolated clones that were productively rearranged, with an identifiable >10-bp variability at the 5' carboxyl-tailed (RACE cDNA product) end of the BV region for T-cell clones using the same V/D/J region sequence to ensure that we did not amplify sister clones.

When PBL and TIL responses for each individual patient were compared, a relatively diverse TCR repertoire was noted in both biological compartments. For each condition, there were 8–16 subfamilies used; PBLs had a broader repertoire usage than the TILs (mean PBL subfamily usage, 14.0; SD, 2.16; mean TIL subfamily usage, 9.33; SD, 1.25; P < 0.05; Fig. 2). When comparing PBL and TIL cultures from the same patient, there was a similar BV subfamily usage. For example, the PBLs of patient 1 used 8 of the 11 subfamilies compared, a relatively diverse TCR repertoire was noted in both biological compartments. For each condition, there were 8–16 subfamilies used; PBLs had a broader repertoire usage than the TILs (mean PBL subfamily usage, 14.0; SD, 2.16; mean TIL subfamily usage, 9.33; SD, 1.25; P < 0.05; Fig. 2). When comparing PBL and TIL cultures from the same patient, there was a similar BV subfamily usage. For example, the PBLs of patient 1 used 8 of the 11 subfamilies present in that patient's TIL culture (7 of 9 and 7 of 8 for patients 2 and 3, respectively; Fig. 2). Despite these similarities in TCR usage, the predominant subfamilies present in the patients' PBL and TIL cultures were different.

When the TCR repertoire between patients was compared, the patterns of TIL and PBL response were the same except that the relative usage of the prevailing BV was more marked in the TIL lines and the PBLs tended to have a broader repertoire than the TILs (as noted above). The predominant subfamilies in the TIL cultures (BV 6, 4, and 5 for patients 1, 2, and 3 respectively) represented 36, 50, and 78% of the clones analyzed for patients 1, 2, and 3, respectively, as compared to 26, 20, and 24% for each patient's comparative predominant PBL cultures (BV 2, 8, and 6 for patients 1, 2, and 3 respectively; Fig. 2). No predominant subfamily usage patterns from patient to patient could be identified, although BV 2 was used by five of the six MART-1(27-35) peptide-stimulated PBL and TIL cultures as either the dominant BV subfamily (patient 2 PBLs) or the second most prevalent BV subfamily (patient 1, PBLs; patient 2, TILs; patient 3, TILs and PBLs; Fig. 2). Furthermore, it is interesting to note the presence of BV 1, 2, 6, 9, 13, 14, 21, and 22 in all three PBL cultures, with the concomitant absence of BV 11, 23, and 25 from all three conditions.

**TCR V/D/J Region Usage in Predominant TIL and PBL Subfamily Clonotypes.** Comparison of the TCR V/D/J region usage by the predominant TIL and PBL subfamilies for each culture condition was performed to (a) determine whether the subfamily prevalence noted in the TCR BV usage analysis was the result of a clonal or oligoclonal expansion of a T-cell population in response to the specific culture conditions (as opposed to a diverse T-cell expansion represented by unique subfamily and junctional usage in the prevalent subfamily); (b) identify the presence of any unique junctional motifs used by the predominant TCR subfamilies; and (c) evaluate whether TCR V/D/J usage by a predominant subfamily is present in the remaining TIL or PBL conditions.

Evidence of clonal expansion by the presence of multiple copies of the same clone was present in each culture condition and was more marked in the TIL cultures (the most notable of which is the clonal expansion of patient 3 TILs, with 39 of 39 BV 5.1/BJ 1.4/CB 1). A diversity of response was present with usage of multiple subfamily V/D/J regions for both TIL and PBLs (Table 1). Alignment and
Fig. 2. TCR BV usage in matched MART-127-35 peptide-stimulated PBLs and TILs. TCR BV usage in matched MART-127-35, peptide-stimulated PBLs and TILs graphed in order of relative frequency for patient 1 PBLs (A), patient 2 PBLs (B), patient 3 PBLs (C), patient 1 TILs (D), patient 2 TILs (E), and patient 3 TILs (F). Fifty productively rearranged TCR BV chains per culture condition were isolated using standard RACE technology, which we defined as a >95% match with a gene bank BV variable region, which, when translated into amino acid sequence, includes a 3' cysteine (as part of the 3' β variable terminal region CASS motif) leading into an identifiable BV junctional region, which, when translated into amino acid sequence, contains no stop codons and allows a productive amino acid sequence in either a CB1 or CB2 region. The TCR BV subfamily and junctional usage of these cDNA clones was assessed by DNA sequence analysis with an ABI fluorescence sequencer and dye terminator PCR sequence reactions.

Translation of the subfamily junctional regions used by the dominant subfamilies for each of the six culture conditions found no conserved junctional motifs in the amino acid or DNA sequences of the predominant BV subfamily within each sample (Fig. 3). Furthermore, there was no homology in the CDR3 regions from clones of a predominant subfamily with any of the other TCR sequences obtained from the other TIL/PBL conditions.

Frequency of Identical Clonotypes in an Individual Patient's TIL and PBL Compartments. It might be expected that for an individual patient, the same clonotypes would be expanded from both the PBL and TIL compartments in response to a specific peptide stimulation. We examined, therefore, the frequency of identical clonotypes (which we defined as a TCR junctional clone matched bp-for-bp with the same V/D/J/C regions with an identifiable >10-bp variability at the 5' carboxyl-tailed (RACE cDNA product) end of the BV). For each patient, TCR clones using the exact same V/D/J/C regions were identified in both the TIL and the PBL compartments, suggesting that both biological compartments were available to the same population of lymphocytes and that peptide-stimulated expansion occurred in both (Table 2). These clonotypes were members of the predominant subfamily used for either TIL (patient 3), PBL (patient 2), or both (patient 1) conditions (Table 2). In the case of patient 3, this represented 42 of the 50 TIL clones identified.

DISCUSSION

This study represents the first comparison of human TCR usage in matched PBLs and TILs after TAA stimulation. In cultures stimulated five times with the MART-1 peptide, a relatively diverse PBL and TIL TCR repertoire was noted in both biological compartments, with 8–16 subfamilies used per condition. No predominant TCR BV subfamily usage from patient to patient could be identified although BV 2 was present in 5 of 6 MART-127-35 peptide-stimulated PBL and TIL cultures as either the dominant or subdominant BV subfamily (Fig. 2). This is despite long-term culture using peptide-specific stimulation and is consistent with prior TCR repertoire analysis of long-term melanoma-specific TIL cultures stimulated with IL-2 (30). The presence of shared TAA in melanoma (with the possibility of a limited TAA-specific TCR repertoire) has led to several conflicting studies of T-cell variable gene usage in TIL cultures (30–33). This controversy
takes on practical importance as one considers the potential advantage of a limited and predictable T-cell response to a specific TAA given our current need for more effective biological markers for monitoring tumor-specific T-cell responses (21, 27, 28). Analysis of our data would suggest a relatively diverse TCR repertoire for TAA peptide-stimulated PBLs and TILs.

Despite an overall diversity of subfamily repertoire, certain patterns of response were observed. The relative usage of the predominant BV was more marked in the TIL lines (TIL cultures represented 36, 50, and 78% of the clones analyzed for patients 1, 2, and 3, respectively, as compared to 26, 20, and 24% for their comparative PBL cultures (Fig. 2)), and PBLs had a broader repertoire than the TILs (mean PBL subfamily usage, 14.0; SD, 2.16; mean TIL subfamily usage, 9.33; SD, 1.25; P < 0.05; Fig. 2). Additionally, when PBL and TIL cultures from the same patient were compared, there was similar BV subfamily usage (the PBLs of patient 1, for example, used 8 of the 11 subfamilies present in that patient's TIL culture; corresponding values were 7 of 9 and 7 of 8 for patients 2 and 3, respectively; Fig. 2). In each case, the predominant subfamily present was different, illustrating that the oligoclonal expansion was due to peptide stimulation. However, without knowing the frequency of each BV subfamily in each TIL population prior to in vitro stimulation, we cannot definitively conclude that the oligoclonal expansion was due to peptide stimulation. No statements can be made concerning the specificities of any particular subfamily in each T-cell culture. Furthermore, it is difficult to evaluate whether the findings noted in the TIL and PBL compartments in terms of expansion are representative of true differences present in each compartment or are amplifications of minor differences as a result of long-term in vitro stimulation.

A more detailed analysis was performed evaluating the TCR V/D/J region usage within the predominant TIL and PBL subfamily clonotypes for each culture condition. Evidence of clonal expansion by the presence of multiple copies of the same clone was present in each condition, and it was more marked with TILs. The most significant piece of evidence was the clonal expansion of the TILs of patient 3; 39 of 50 of the isolated TCR used the same subfamily clonotype (BV 5.1/BJ 1.4/CB 2). This level of clonal expansion would under other conditions be strongly suggestive of an antigen-driven phenomenon resulting in antigen-reactive cells. However, the overall diversity of PBL and TIL TCR repertoire was still present in these predominant subfamilies, with approximately 50% of all predominant clones identified using multiple subfamily V/D/J regions (Table 1).

Alignment and translation of the subfamily junctional regions used by the dominant subfamilies for each of the six culture conditions demonstrated no identifiable junctional motifs in terms of nucleic acid or amino acid sequence homology, which were common only to the predominant subfamilies (Fig. 3). Prior work using a PCR-based methodology evaluating VDJ junction size has noted a dominant and virtually monoclonal TCR expansion in long-term TIL cultures based on size uniformity of the CDR3 region (33). Comparison of the CDR3 sequences of our dominant subfamilies, however, illustrated no uniform CDR3 length. The observed diversity in the CDR3 regions suggests either the expansion of multiple specific clonotypes that use CDR3 of different lengths or the presence of subfamily clonotypes of irrelevant specificities within the dominant subfamilies.

Similarities in the frequency and number of tumor-reactive T cells between the PBL and TIL biological compartments is poorly defined. It is not clear how easily tumor-reactive CTLs can migrate from the peripheral blood to the tumor site in vivo. This limits our ability to predict how well the TIL-mediated antitumor response can be translated into PBL-based therapies. It is encouraging that T-cell clones using the exact same V/D/J/C regions have been identified in both the TIL and PBL compartment from each patient (Table 2). The presence of T-cell clones derived from predominant TCR subfamilies in the PBLs and TILs implies that the biological compartments were available to the same population of lymphocytes and that peptide-stimulated expansion occurred in both. Furthermore, these findings indicate the presence of exact T-cell clonotypes in both the PBLs and TILs, which should be able to expand with the same specificity in response to antigen stimulation. This provides support to the premise that the PBL

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### Table 1 TCR V/D/J/C region usage in predominant TIL and PBL subfamily clonotypes

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<th>Predominant TCR V/D/J/C usage</th>
<th>No. of clones</th>
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<td><strong>Patient 1 (TIL)</strong></td>
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<td>BV6S1/BDSI1/BJS7/CB2</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>BV4S3/BDSI1/BJS3/CB2</td>
<td>1</td>
</tr>
<tr>
<td>BV6S5/BDSI1/BJS4/CB2</td>
<td>2</td>
</tr>
<tr>
<td>BV6S7/BDSI1/BJS3/CB2</td>
<td>1</td>
</tr>
<tr>
<td>BV6S14/BDSI1/BJS1/CB2</td>
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</tr>
<tr>
<td>BV6S14/BDSI1/BJS2/CB2</td>
<td>5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>12</td>
</tr>
</tbody>
</table>

*No. of TCR clones having the exact same V/D/J/C usage.

*Clones that use the same V/D/J/C regions but a different junctional rearrangement (thus not representing clonal expansion of the same TCR)*.
Fig. 3. Alignment of junctional sequences from predominant subfamily clonotypes of patients 1, 2, and 3. The 3' ends of the variable genes and the 5' ends of the carboxyl regions.

compartment may be able to react productively against TAA peptides. This hypothesis is the basis for several approaches actively being pursued, which include the adoptive transfer of PBLs stimulated in vitro with peptides derived from TAA (24), the direct presentation (26). In the face of recent reports outlining a possible that PBLs may in fact be a preferable reagent to TILs for effective CTL expansion (37).

In contrast to strong immunogenic antigens such as influenza peptide (38), our results support previous findings in the setting of TAA stimulation. These results suggested that it would be unlikely that a particular V gene subfamily or subfamilies would be predominant from patient to patient because of the ability of the immune system to provide more than one TCR capable of recognizing a specific tumor antigenic epitope (39). It is also interesting to note recent work evaluating T-cell response to MART-1 antigen, which
would propose a role for epitope mimicry from nonrelated antigens leading to a diversity of TCR and junctions capable of responding to the MART-1 epitope (40, 41). Furthermore, our findings are consistent with the current understanding of T-cell responses toward a given MHC-peptide complex is recognized by a very limited number of clonotypes (42). Several studies have noted that a given class II MHC-peptide complex is recognized by a very limited number of clonotypes (42), whereas a class I MHC-peptide complex demonstrates a less restricted V gene usage (44–46).

In conclusion, our studies demonstrate a relatively diverse TCR response to TAA peptide-driven TIL and PBL expansion from patient to patient, suggesting that a limited and predictable T-cell subfamily response to a specific TAA is not present as a marker for tumor-specific T-cell reactivity. A similar pattern of expansion, however, was present for both PBLs and TILs; the relative usage of each prevailing BV was more marked in TILs, and a broader repertoire was used by PBLs. Furthermore, the exact same clonotypes derived from predominant TCR subfamilies in the PBLs and TILs were present, suggesting that the biological compartments were available to the same population of lymphocytes and that peptide-stimulated expansion occurred in both. Therefore, it appears that both PBLs and TILs contain T-cell clonotypes that would have the potential for antigen-driven expansion. Given (a) the ability to stimulate TAA-specific PBLs ex vivo; (b) reproducible patterns of expansion from patient to patient; and (c) the suggestion that the same T-cell clonotypes can be expanded whether present in PBLs or TILs, our data support the conceptualization of approaches using adoptive transfer or vaccination based on TAA-derived peptide stimulation of PBLs.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical support and advice from the Center for Molecular and Structural Biology at the Hollings Cancer Center, Medical University of South Carolina. A portion of this work was performed to complete the Masters of Biological Sciences requirements from Hood College (Frederick, MD) for Michael C. Wilson.

REFERENCES


T-cell Receptor Repertoire in Matched MART-1 Peptide-stimulated Peripheral Blood Lymphocytes and Tumor-infiltrating Lymphocytes

David J. Cole, Michael C. Wilson, Licia Rivoltini, et al.