INTRODUCTION
Adoptive immunotherapy has been shown to be an effective therapy in mice and human patients with melanoma.1–3 We have previously shown that when tumor-sensitized T cells from tumor-draining lymph nodes were activated with bryostatin 1 (B) and ionomycin (I) subsequent culture in the combination of interleukin (IL)-7 and IL-15 produced markedly increased expansion of cell numbers compared with IL-2, in both a melanoma and a breast cancer model.4–6 Antitumor efficacy of adoptive immunotherapy with cells expanded in IL-7/15 after activation with B/I was shown previously that when tumor-sensitized T cells from tumor-draining lymph nodes were activated with bryostatin 1 (B) and ionomycin (I) subsequent culture in the combination of interleukin (IL)-7 and IL-15 for 1, 3 or 6 days. T cells were harvested and analyzed using microarray, real-time quantitative polymerase chain reaction (RT-QPCR) or sorted into T-cell subsets and analyzed. We found significant differences in gene expression for T cells cultured in IL-2 versus IL-7/15, starting on day 3. This was not a function of subset differentiation; when T cells were divided into subsets, the central memory (T_{CM}), effector memory (T_{EM}) and effector (T_{E}) T cells cultured in the IL-2 more closely resembled each other than the identical phenotypic subset exposed to IL-7/15. Thus, the differences in gene expression induced by culture in IL-2 versus IL-7/15 do not merely reflect differences in the frequency of T_{CM} versus T_{EM} versus T_{E} cells, but rather reflect that the gene expression levels of those T-cell subsets when exposed to different cytokines are fundamentally different.

We have previously demonstrated that expansion of activated tumor-sensitized T cells in interleukin (IL)-7/15 results in greater expansion and antitumor activity than expansion in IL-2. We sought to determine whether T cells exposed to IL-2 versus IL-7/15 exhibited distinct gene expression patterns. Lymphocytes were harvested from Pmel-1 mice immunized with B16-GMCSF melanoma cells, activated in vitro, and cultured in IL-2 or IL-7/15 for 1, 3 or 6 days. T cells were harvested and analyzed using microarray, real-time quantitative polymerase chain reaction (RT-QPCR) or sorted into T-cell subsets and analyzed. We found significant differences in gene expression for T cells cultured in IL-2 versus IL-7/15, starting on day 3. This was not a function of subset differentiation; when T cells were divided into subsets, the central memory (T_{CM}), effector memory (T_{EM}) and effector (T_{E}) T cells cultured in the IL-2 more closely resembled each other than the identical phenotypic subset exposed to IL-7/15. Thus, the differences in gene expression induced by culture in IL-2 versus IL-7/15 do not merely reflect differences in the frequency of T_{CM} versus T_{EM} versus T_{E} cells, but rather reflect that the gene expression levels of those T-cell subsets when exposed to different cytokines are fundamentally different.

MATERIALS AND METHODS

Mice

T-cell receptor transgenic Pmel-1 mice, with T-cell receptor specific for the peptide KVPRNQDWL derived from gp100 bound to class I of H-2b, were produced by breeding on-site from breeding pairs obtained from Jackson Laboratories (Bar Harbor, ME, USA). All the guidelines of the Virginia Commonwealth University Institutional Animal Care and Use Committee, which conform to the American Association for Accreditation of Laboratory Animal Care and the US Department of Agriculture recommendations for the care and humane experimental use of animals, were followed.

Draining lymph node sensitization and activation

Pmel-1 mice were inoculated in the hind footpads with 1 × 10^6 B16-GMCSF cells. Ten days after footpad vaccination, popliteal tumor-draining lymph nodes were harvested under sterile conditions. Draining lymph nodes were harvested and dispersed into single-cell suspensions in complete RPMI media at 1 × 10^6 cells per ml. The cells were then activated by incubation with 5 nM Bryostatin 1 (provided by Sigma Aldrich) and 1 μM Ionomycin (Calbiochem, San Diego, CA, USA; B/I) and 80 U ml^-1 of rIL-2 (Peprotech, Rocky Hill, NJ, USA) at 37 °C for 18 h. Cells were then washed three times with warm complete RPMI and resuspended in 1X phosphate-buffered saline. Cells were filtered before injection through a 70-μm cell strainer. All cells were incubated in 250 ml T-flasks (PGC, Gaithersburg, MD, USA) at 37 °C in humidified air with 5% CO_{2}.

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allowed to proliferate in culture for an additional 6 days and were split every 2–3 days in order to maintain 1×10^6 cells per ml concentration. Additional cytokine at the above doses was also added when the cells were split. Our previous work with this activation strategy has demonstrated that almost all remaining cells in culture after exposure to B/L and subsequently to cytokines are T cells.

Flow cytometry and sorting for T-cell subsets
Cells isolated from draining lymph nodes and expanded as described above were stained with a panel of antibodies and analyzed or sorted based on surface marker expression on a BD FACSC Aria II High-Speed Cell Sorter (BD Biosciences, Franklin Lakes, NJ, USA) at days 0 (after B/L), 1, 3 and 6 of expansion. Fluorescent-labeled antibodies directed against the following markers were obtained from Biolegend (San Diego, CA, USA): CD4 (GK1.5), CD8 (53–6.7), CD44 (IM7) and CD62L (MEL-14). Appropriate isotype and single color controls were used in all cases. T-cell subsets analyzed were Te, CD44+CD62L−, TEM, CD44+CD62L low and TCM, CD44+CD62L high.

Microarray and real-time quantitative polymerase chain reaction (RT-QPCR) preparation
Cultured lymphocytes were harvested on days 0, 1, 3 and 6 after activation, and without any fractionation, 1 million cells were suspended in 300 μl of trizol solution and then frozen for later analysis (‘unsorted’ cells). Microarray analysis was also performed on T cells that were sorted using FACs (fluorescence-activated cell sorting) directly into 300 μl of trizol for each cell phenotype and then frozen for later analysis.

RNA extraction
Total RNA was extracted and the quality evaluated using a sample-processing method previously established in our laboratory.7 Total RNA was extracted from 1×10^6 cells (unsorted or sorted cells) using the MagMAX-96 for the Microarrays Total RNA Isolation Kit (InvitrogenTM Life Technologies) in an automated manner using the magnetic particle processors MagMAX Express. RNA purity was judged using spectrophotometry at 260, 270 and 280 nm. RNA integrity as well as cDNA and cRNA synthesis products were assessed by running 1 μl of every sample in RNA 6000 Nano LabChips on the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA was extracted and the quality evaluated using a sample-processing method previously established in our laboratory. Total RNA was extracted from 1×10^6 cells (unsorted or sorted cells) using the MagMAX-96 for the Microarrays Total RNA Isolation Kit (InvitrogenTM Life Technologies) in an automated manner using the magnetic particle processors MagMAX Express. RNA purity was judged using spectrophotometry at 260, 270 and 280 nm. RNA integrity as well as cDNA and cRNA synthesis products were assessed by running 1 μl of every sample in RNA 6000 Nano LabChips on the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). After a 37°C incubation for 16 h, the labeled cRNA was purified using the cRNA cleanup reagents from the GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA, USA). As per the Affymetrix protocol, 10 μg of fragmented cRNA were hybridized on the GeneChip Mouse Genome 430A 2.0 array (Affymetrix) for 16 h at 60°C in a 4°C hybridization oven. The GeneChip Mouse Genome 430A 2.0 array 230 provides a comprehensive coverage of the transcribed murine genome by including over 22,600 probe sets that analyze the expression level of over 14,000 well-characterized mouse transcripts. The arrays were washed and stained with streptavidin phycoerythrin (SAPE; Molecular Probes, Eugene, OR, USA) in the Affymetrix fluidics workstation. Every chip was scanned at a high resolution, on the Affymetrix GeneChip Scanner 3000 7G according to the GeneChip Expression Analysis Technical Manual procedures (Affymetrix). After scanning, the raw intensities for every probe were stored in electronic files (in.DAT and CEL formats) with the GeneChip Operating Software v1.4 (GCOS; Affymetrix). Overall quality of each array was assessed by monitoring the 3′/5′ ratios for the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase, and the percentage of ‘Present’ genes (%P). Arrays exhibiting glyceraldehyde 3-phosphate dehydrogenase 3′/5′ < 3.0 and %P>40% were considered good-quality arrays.

RT-QPCR
RT-QPCR was used to validate gene expression levels of selected genes using TaqMan chemistry. Probes and primer sets specific for detection of mouse RNA transcripts were purchased from Applied Biosystems (Foster City, CA, USA). These included gene-specific probes for the following mouse genes: Igk-V28, assay ID no. Mm01742005_g1; Ccr9, assay ID no. Mm02620030_s1; Foxp3, assay ID no. Mm0047162_m1; Lta, assay ID no. Mm00440228_gH; Cd66, assay ID no. Mm01313342_m1; Jun, assay ID no. Mm0495062_s1; and Nov, assay ID no. Mm00458655_m1. Gene-specific probes labeled with FAM (6-carboxyfluorescein) in the 5′ end, and with a dark quencher in the 3′ end, were used for all the target genes of interest. For each sample, glyceraldehyde 3-phosphate dehydrogenase was used as the endogenous control gene (assay ID no. Mm99999915_g1) using a mouse-specific probe labeled with FAM (6-carboxyfluorescein) in the 5′ end, and with a dark quencher in the 3′ end. The experiments were performed in the ABI Prism 7500 Sequence Detection System (Applied Biosystems) using the TaqMan Reverse Transcription and Universal PCR Master Mix Reagents. All the samples were tested in triplicate. The cycle conditions were 48°C for 30 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. The 2−ΔΔCt method was used to calculate fold changes in the expression levels of the genes of interest.

Statistical analysis
Microarray data analysis, background correction, normalization and estimation of probe set expression summaries were performed using the log-scale robust multiarray analysis method.9 Hierarchical cluster analyses were performed with the BRB-ArrayTools v3.1.0 (Biometric Research Branch, National Cancer Institute), an Excel add-in that collates microarray data with sample annotations. In order to identify differentially expressed genes between the different classes, we performed t-tests for each probe set from biological replicates in each class. Statistical significance for multivariate analysis to assess probe set-specific false discovery rates was performed by estimating the q-values, using the Bioconductor q-value package.10 Pearson's correlation coefficient was calculated to examine the relation between microarray and RT-QPCR results. P-values < 0.05 were considered significant.

Functional analysis of differentially expressed genes
A data set containing Affymetrix probe set IDs as gene identifiers and corresponding fold changes in expression levels and their associated significance (P-value) was uploaded into the Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com) application. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. These genes, called Focus Genes, were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these Focus Genes were then algorithmically generated based on their connectivity, or interactions between one another. Biological networks were ranked by score, where the score corresponds to the likelihood of a set of genes being found in the networks because of random chance; that is, a score of 3 indicates that there is a 1/1000 chance that the Focus Genes are in a network because of random chance. A score of 3 was used as the cutoff for identifying gene networks significantly involved in differences observed in IL-2 versus IL-7/15-treated cells.

RESULTS
Gene expression analysis for IL-2- and IL-7/15-expanded cells
To identify genes that were significantly differentially expressed, microarrays were performed in triplicate on unsorted T cells harvested after activation with B/L and expansion for 6 days in either IL-2 or IL-7/15. Sixty-three probe sets/gene had significantly different levels of expression between the two cytokine regimens (Table 1—most significant 26 shown). In order to delineate the kinetics of the divergence in the gene expression patterns after B/L activation and separation of the cells into the two different cytokine culture conditions, we performed an unsupervised cluster analysis on T cells immediately after 18 h activation with B/L and then subsequently after 1, 3 and 6 days of expansion in either IL-2 or IL-7/15 (Figure 1). This analysis demonstrated that the two populations were similar after 1 day.

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Table 1. Twenty-six of 63 genes that were significantly differentially expressed between T cells cultured for 6 days in IL-2 versus IL-7/15

| Gene symbol | IL-2 mean (log2) | IL-7/15 mean (log2) | Fold Change (geometric) | P-value ($\alpha = 0.005$) | q-value (FDR < 15%) |
|-------------|------------------|---------------------|-------------------------|------------------------|---------------------|
| Nov         | 2.91             | 8.18                | 38.5                    | 2.43E−04               | 1.28E−01            |
| Nov         | 4.12             | 8.81                | 25.8                    | 8.06E−05               | 1.28E−01            |
| Mylc2pl     | 6.53             | 10.23               | 13.1                    | 1.41E−04               | 1.28E−01            |
| Aldoc       | 7.19             | 9.56                | 5.2                     | 2.91E−03               | 1.40E−01            |
| Jun         | 6.35             | 8.45                | 4.3                     | 2.09E−03               | 1.40E−01            |
| Rcn1        | 7.85             | 9.49                | 3.1                     | 6.15E−04               | 1.30E−01            |
| Cdk6        | 4.71             | 6.29                | 3.0                     | 2.10E−03               | 1.40E−01            |
| Lta         | 8.44             | 9.99                | 2.9                     | 7.83E−04               | 1.30E−01            |
| LOC100044677 // Tox | 8.11 | 6.54                | −3.0                    | 3.91E−03               | 1.50E−01            |
| Phlda1      | 8.99             | 7.42                | −3.0                    | 3.21E−03               | 1.42E−01            |
| Cpa3        | 7.38             | 5.79                | −3.0                    | 2.69E−03               | 1.40E−01            |
| Cd81        | 9.08             | 7.49                | −3.0                    | 8.31E−04               | 1.30E−01            |
| Cnn3 /// LOC100047856 | 9.30 | 7.71                | −3.0                    | 1.65E−03               | 1.35E−01            |
| Egr2        | 7.03             | 5.39                | −3.1                    | 3.09E−03               | 1.42E−01            |
| Mgst1       | 5.50             | 3.75                | −3.4                    | 2.50E−03               | 1.40E−01            |
| Il1r1       | 10.22            | 8.34                | −3.7                    | 1.37E−03               | 1.33E−01            |
| Lzrf1       | 9.47             | 7.57                | −3.7                    | 2.30E−03               | 1.40E−01            |
| Cd9         | 7.51             | 5.55                | −3.9                    | 3.19E−03               | 1.42E−01            |
| Cd36        | 7.07             | 5.00                | −4.2                    | 1.82E−03               | 1.39E−01            |
| Foxp3       | 6.93             | 4.83                | −4.3                    | 2.59E−03               | 1.40E−01            |
| Bcl6        | 7.35             | 5.35                | −4.5                    | 3.19E−04               | 1.28E−01            |
| Lpl         | 7.67             | 5.49                | −4.5                    | 1.07E−03               | 1.32E−01            |
| Rgs2        | 8.58             | 6.34                | −4.7                    | 4.08E−03               | 1.50E−01            |
| Rgs2        | 8.35             | 5.84                | −5.7                    | 2.68E−03               | 1.40E−01            |
| Igk-V28     | 9.22             | 6.65                | −6.0                    | 2.06E−03               | 1.40E−01            |
| 2010205A11Rik | 11.12       | 8.46                | −6.4                    | 4.35E−04               | 1.28E−01            |
| Igk-V28     | 11.21            | 8.54                | −6.4                    | 1.94E−05               | 9.55E−02            |
| Mmp13       | 6.54             | 3.74                | −6.9                    | 7.01E−04               | 1.30E−01            |
| Ccr9        | 8.06             | 5.18                | −7.4                    | 4.23E−04               | 1.28E−01            |
| Igk-V28     | 10.71            | 7.74                | −7.8                    | 5.60E−04               | 1.30E−01            |

Abbreviations: FDR, false discovery rate; IL, interleukin. These results are based on the analysis of three biological replicates for each cytokine. Seven of these were chosen for further analysis: Nov, Jun, Lta, Ccr9, Cdk6, Foxp3 and Igk-V28.
of exposure to the different cytokines but gene expression patterns diverged dramatically based on their Euclidean distance on day 3 and remained different on day 6. To confirm the reproducibility of these observations, we performed a supervised cluster analysis on three biological replicates (Figure 2). The cluster analysis, based on 119 probe sets, demonstrated that the divergence after 6 days of exposure to either IL-2 or IL-7/15 was consistent.

**RT-QPCR data**

RT-QPCR was then performed to confirm the microarray results showing significant differences in gene expression between cells grown in IL-2 or IL-7/15. We chose seven genes of interest that were among the most significantly different (higher or lower) in the microarray analysis. The seven genes chosen were Ccr9, Cdk6, Foxp3, Nov, Igk-V28, Jun and Lta. We observed the time course of differential gene expression on day 0 (after B/I pulse and before separation into different cytokine(s)), and after 1 (day 1), 3 (day 3) and 6 (day 6) days of exposure to either IL-2 or IL-7/15. We then calculated the Pearson correlation coefficients (r) to determine whether the gene expression findings from the microarray data correlated with the PCR results and found significant positive correlations for the following genes: Ccr9 (r = 0.975; P = 8.5 × 10⁻⁶), Igk-V28 (r = 0.982; P = 2.7 × 10⁻⁶), Nov (r = 0.845; P = 0.00413), Foxp3 (r = 0.842; P = 0.00433), Lta (r = 0.897; P = 0.00104) and Jun (r = 0.683; P = 0.04244). The correlation for the Cdk6 gene was borderline-positive but not significant (r = 0.598; P = 0.08913). Of those seven genes, Foxp3, Igk-V28 and Ccr9 demonstrated higher expression in T cells cultured in IL-2 for 6 days compared with T cells cultured in IL-7/15. The expression of Nov was higher in cells cultured in IL-7/15 for 6 days compared with those cultured in IL-2.

**Functional analysis of the differentially expressed genes**

The 63 differentially expressed probe sets/genes were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these genes were then algorithmically generated based on their connectivity, or interactions between one another. Five significant biological networks were ranked by score, identifying Cellular Growth and Proliferation, Hematological System Development and Function, and Inflammatory Response. P < 10⁻⁴¹ is shown. The meaning of the network node shapes is also indicated.

**Figure 3.** Functional networks. Interconnection of significant functional networks, where gene nodes are shown in different shades of red, green or white depending on being upregulated, downregulated or no change, respectively, in T cells exposed to either interleukin (IL)-2 or IL-7/15 for 6 days. For each node, the fold change and significance are indicated. The most significant functional network corresponding to Cellular Growth and Proliferation, Hematological System Development and Function, and Inflammatory Response. P < 10⁻⁴¹ is shown. The meaning of the network node shapes is also indicated.
cytokine they were exposed to. In other words, T<sub>CM</sub>, T<sub>EM</sub> or T<sub>E</sub> cells expression patterns resembling each other, regardless of which' 'stimulated with the same cytokines, rather than the subsets

IL-7/15 for 6 days closely resembled the unsorted T cells subsets that were produced after expansion in either IL-2 or IL-7/15 (Figure 5). In fact, the genes expressed in T<sub>CM</sub>, T<sub>EM</sub> and T<sub>E</sub> cell expression differences between different cytokine conditions that differential phenotype distribution did not account for gene sorter. Interestingly, we found, after unsupervised cluster analysis, the similar gene expression between T<sub>CM</sub>, T<sub>EM</sub> and T<sub>E</sub> cells grown in IL-7/15. We then used the same RT-QPCR probes as those used for microarrays of unsorted cells. Thus, we found significant differences of T-cell subsets, we sorted T cells exposed to either IL-2 or IL-7/15 on day 3 and day 6 of culture into their CD8<sup>+</sup> T-cell subsets using flow cytometry—T<sub>CM</sub>, T<sub>EM</sub> and T<sub>E</sub>—using a FACS Aria cell sorter. Interestingly, we found, after unsupervised cluster analysis, that differential phenotype distribution did not account for gene expression differences between different cytokine conditions (Figure 5). In fact, the genes expressed in T<sub>CM</sub>, T<sub>EM</sub> and T<sub>E</sub> cell subsets that were produced after expansion in either IL-2 or IL-7/15 for 6 days closely resembled the unsorted T cells stimulated with the same cytokines, rather than the subsets’ gene expression patterns resembling each other, regardless of which cytokine they were exposed to. In other words, T<sub>CM</sub>, T<sub>EM</sub> or T<sub>E</sub> cells produced after exposure to IL-2 had different patterns of gene expression from the corresponding T<sub>CM</sub>, T<sub>EM</sub> or T<sub>E</sub> cell subsets produced after exposure to IL-7/15. Having made this observation, we then performed a supervised cluster analysis with the same samples, which confirmed the similar gene expression between unsorted T cells and T-cell subsets T<sub>CM</sub>, T<sub>EM</sub> and T<sub>E</sub> exposed to the same cytokine regimen for 6 days; however, significant differences in gene expression between T<sub>CM</sub> cells grown in IL-2 versus IL-7/15 and likewise between T<sub>EM</sub> cells and T<sub>E</sub> cells grown in IL-2 versus IL-7/15 (Figure 6). We then used the same RT-QPCR probes as before to confirm the differential expression of genes among T-cell subsets on day 3 and day 6 that were significant on the microarrays of unsorted cells. Thus, we found significant positive correlation for all the genes analyzed: Nov (r = 0.989; P = 4.7 × 10<sup>−5</sup>), Foxp3 (r = 0.998; P = 4.2 × 10<sup>−5</sup>), Igk-V28 (r = 0.924; P = 4.2 × 10<sup>−5</sup>), Ccr9 (r = 0.561; P = 4.0 × 10<sup>−5</sup>). We had hypothesized, once we saw a distinct difference in gene expression between unsorted T cells cultured in IL-2 versus IL-7/15, that this difference might be attributable to a greater functional networks (P = 10<sup>−41</sup>). The most significant network is shown in Figure 3.

Analysis of T-cell subsets
One possible explanation for the differential gene expression patterns would be the differences in T-cell differentiation we have observed when comparing cells cultured in IL-2 versus those cultured in IL-7-15. We have previously shown that IL-2 preferentially supports or expands T<sub>E</sub> populations, whereas IL-7/15 preferentially expands T<sub>CM</sub> populations in culture.<sup>4,5</sup> In a repeat experiment using the same method of activation and expansion described above, this difference remained true (Figure 4). Moreover, it has been shown that different subsets of CD8<sup>+</sup> T cells have different patterns of gene expression.<sup>11</sup> In order to test the hypothesis that the differences in gene expression that we observed in different cytokines merely reflect different distributions of T-cell subsets, we sorted T cells exposed to either IL-2 or IL-7/15 on day 3 and day 6 of culture into their CD8<sup>+</sup> T-cell subsets using flow cytometry—T<sub>CM</sub>, T<sub>EM</sub> and T<sub>E</sub>—using a FACS Aria cell sorter. Interestingly, we found, after unsupervised cluster analysis, that differential phenotype distribution did not account for gene expression differences between different cytokine conditions (Figure 5). In fact, the genes expressed in T<sub>CM</sub>, T<sub>EM</sub> and T<sub>E</sub> cell subsets that were produced after expansion in either IL-2 or IL-7/15 for 6 days closely resembled the unsorted T cells stimulated with the same cytokines, rather than the subsets’ gene expression patterns resembling each other, regardless of which cytokine they were exposed to. In other words, T<sub>CM</sub>, T<sub>EM</sub> or T<sub>E</sub> cells produced after exposure to IL-2 had different patterns of gene expression from the corresponding T<sub>CM</sub>, T<sub>EM</sub> or T<sub>E</sub> cell subsets produced after exposure to IL-7/15. Having made this observation, we then performed a supervised cluster analysis with the same samples, which confirmed the similar gene expression between unsorted T cells and T-cell subsets T<sub>CM</sub>, T<sub>EM</sub> and T<sub>E</sub> exposed to the same cytokine regimen for 6 days; however, significant differences in gene expression between T<sub>CM</sub> cells grown in IL-2 versus IL-7/15 and likewise between T<sub>EM</sub> cells and T<sub>E</sub> cells grown in IL-2 versus IL-7/15 (Figure 6). We then used the same RT-QPCR probes as before to confirm the differential expression of genes among T-cell subsets on day 3 and day 6 that were significant on the microarrays of unsorted cells. Thus, we found significant positive correlation for all the genes analyzed: Nov (r = 0.989; P = 4.7 × 10<sup>−5</sup>), Foxp3 (r = 0.998; P = 4.2 × 10<sup>−5</sup>), Igk-V28 (r = 0.924; P = 4.2 × 10<sup>−5</sup>), Ccr9 (r = 0.561; P = 4.0 × 10<sup>−5</sup>). We had hypothesized, once we saw a distinct difference in gene expression between unsorted T cells cultured in IL-2 versus IL-7/15, that this difference might be attributable to a greater functional networks (P = 10<sup>−41</sup>). The most significant network is shown in Figure 3.

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T cell gene expression depends on cytokine

Figure 6. Supervised cluster analysis of unsorted T cells after activation with B/I and 6 days of culture with IL-2 or IL-7/15 (two replicates: a and b) and T cells sorted into subsets: TCM, TEM, and TE after activation with B/I and 6 days in culture with IL-2 or IL-7/15. B/I, brystatin/interonymcin; IL, interleukin; TE, T effector; TEM, T effector; TCM, T central memory.

The frequency of TE cells in the IL-2 group and a greater frequency of TCM cells seen in the IL-7/15 group, as we have previously published. Surprisingly, however, even when the T cells from these two cytokine exposure groups were sorted into their respective TCM, TEM, and TE subsets, these gene expression differences held true, differentiating T cells in the same subsets but grown in different cytokines from each other more than differentiating the subsets from each other. Thus, TCM cells exposed to IL-2 in culture after activation with B/I are significantly different from those exposed to IL-2/15 in culture after B/I activation, and so on for the other T-cell subsets. The increased proliferation and survival of the cells expanded in IL-7/15 correlates with the findings from our functional pathway analysis, derived from differences in gene expression described here. Thus, among the 63 significantly altered probe sets/gene, we identified genes significantly involved in cellular growth and proliferation, hematological system development and function and inflammatory response. For the seven genes chosen for RT-QPCR confirmation and analysis, four were markedly different between IL-2-expanded cells and IL-7/15-expanded cells both for unsorted as well as sorted T cells. The Nov gene (nephroblastoma overexpressed) was significantly more highly expressed in unsorted and all subsets of T cells exposed to IL-7/15. Nov has been shown to be involved in cell adhesion, migration, proliferation, differentiation, survival and angiogenesis through actions on integrin, NOTCH1 and fibulin 1C receptors. In cancer cells, it inhibits proliferation but promotes metastasis in patients with Ewing’s sarcoma, melanoma and breast cancer. The other three genes, Ccr9, Foxp3 and Igk-V28 were more highly expressed in unsorted and sorted T cells exposed to IL-2 compared with T cells exposed to IL-7/15. Ccr9 has been shown to be important for migration of T cells; Igk-V28 gene’s function is relatively unknown; and Foxp3 has been shown to have a strong correlation with CD4+ Treg cells. The importance of these genes in T-cell subset function is unknown, and further studies are needed to elucidate why these differences exist between T cells cultured in IL-2 or IL-7/15. Most importantly, the ‘standard’ way of evaluating T-cell subsets used for immunotherapy based on phenotypic surface markers alone may not directly explain the relative efficacy of therapy with these cells in vivo. The differences in gene expression patterns we have found may well relate to increased expansion of cells in culture as well as the survival, trafficking or antitumor effectiveness of adoptively transferred T cells in vivo.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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