Profiling of $CD4^+$, $CD8^+$, and $CD4^+CD25^+CD45RO^+FoxP3^+$ T Cells in Patients with Malignant Glioma Reveals Differential Expression of the Immunologic Transcriptome Compared with T Cells from Healthy Volunteers

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Abstract

Purpose: Analyses of T-cell mRNA expression profiles in glioblastoma multiforme has not been previously reported but may help to define and characterize the immunosuppressed phenotype in patients with this type of cancer.

Experimental Design: We did microarray studies that have shown significant and fundamental differences in the expression profiles of $CD4^+$ and $CD8^+$ T cells and immunosuppressive $CD4^+CD25^+CD45RO^+FoxP3^+$ regulatory T cells (T_{reg}) from normal healthy volunteers compared with patients with newly diagnosed glioblastoma multiforme. For these investigations, we isolated total RNA from enriched $CD4^+$ and $CD8^+$ T cell or T_{reg} cell populations from age-matched individuals and did microarray analyses.

Results: ANOVA and principal components analysis show that the various T cell compartments exhibit consistently similar mRNA expression profiles among individuals within either healthy or brain tumor groups but reflect significant differences between these groups. Compared with healthy volunteers, $CD4^+$ and $CD8^+$ T cells from patients with glioblastoma multiforme display coordinate down-regulation of genes involved in T cell receptor ligation, activation, and intracellular signaling. In contrast, T_{regs} from patients with glioblastoma multiforme exhibit increased levels of transcripts involved in inhibiting host immunity.

Conclusion: Our findings support the notion that key differences between expression profiles in T-cell populations from patients with glioblastoma multiforme results from differential expression of the immunologic transcriptome, such that a limited number of genes are principally important in producing the dysregulated T-cell phenotype.

Over the past 30 years, tremendous gains have been achieved and documented with clinically approved therapies for patients with various types of cancer (1–4). Unfortunately, this has largely not been realized in patients with glioblastoma multiforme (5). Despite a negligible incidence of metastasis, aggressive surgery, and the application of novel chemotherapeutics, patients with glioblastoma multiforme, the most common and lethal form of primary brain cancer, typically live <1 year from the time of diagnosis (6). Recurrence of tumor following surgical resection and chemotherapy is very high (>90%), and survival of these patients is usually <12 weeks (7). A better understanding of the mechanisms of pathogenesis in this disease will likely be necessary before the design and application of new therapies will be successful.

It has been well shown that peripheral blood leukocytes from patients with glioblastoma multiforme proliferate poorly in response to T-cell mitogens, anti-CD3, and T-dependent B-cell mitogens (8–14). In addition, severely reduced total $CD4^+$ counts (15) and diminished delayed type hypersensitivity (16, 17) responses are hallmarks of patients with glioblastoma multiforme. Although the exact nature of T-cell lymphopenia in glioblastoma multiforme is not well understood, recent reports have suggested that mechanisms contributing to this deficiency may include dysregulation of thymic output (6), increased regulatory T-cell (T_{reg}) fraction (15), and tumor-induced immunosuppression (8). Severe T-cell lymphopenia, especially in the $CD4^+$ compartment, is commonly observed in patients with glioblastoma multiforme (15). We hypothesized that a

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limited number of genes are principally important in producing and defining this dysregulated T-cell phenotype.

The ability to explore gene expression levels has become both routine and necessary in differentiating cell characteristics and functions (18–20). Our rationale for the studies conducted here was based on the premise that microarray technology provides a tremendous opportunity to explain and define the phenotypes observed for entire immune cell populations in patients with glioblastoma multiforme. To investigate the possibility that the immunosuppressive phenotype in patients with glioblastoma multiforme is defined at the level of transcription in the T cell, we initiated studies to quantify the expression profiles of freshly isolated $CD4^+$ and $CD8^+$ T cells and $CD4^+CD25^+CD45RO^+FoxP3^+$ T_{regs} from patients with newly diagnosed glioblastoma multiforme before resection compared with these same T-cell subsets from normal healthy volunteers. Our investigations gave particular consideration to genes and pathways previously reported to have a central role in immunity and in tumor-induced immunosuppression.

The studies described herein show quantitative and qualitative differences in the expression profiles of $CD4^+$ and $CD8^+$ T cells and T_{regs} from patients with newly diagnosed glioblastoma multiforme when compared with normal healthy volunteers. We provide evidence that genes related to T-cell activation are significantly decreased in $CD4^+$ and $CD8^+$ T cells in patients with glioblastoma multiforme, whereas inhibitory gene expression is increased in the immunosuppressive T_{reg} subset. These findings suggest that the presence of an intracranial glioblastoma multiforme is sufficient to induce potent changes in several different T-cell subsets systemically. In fact, these differences are so profound that we were able to identify and validate specific T-cell expression signatures that could be used as training sets for modeling immunologic dysfunction that defines patients with glioblastoma multiforme.

Materials and Methods

Patient selection. Over the span of 2 years, leukaphereses were obtained from five age-matched healthy volunteers and five patients with newly diagnosed glioblastoma multiforme before surgical resection under a protocol approved by the Duke University Medical Center Institutional Review Board (IRB#1509-05-9R6). Karnofsky performance scale scores for patients with glioblastoma multiforme were clinically determined at the time of leukapheresis. Age and Karnofsky performance scale have previously been determined to be clinically important prognostic factors for glioblastoma multiforme survival. The cohort of glioblastoma multiforme patients analyzed here are well controlled and homogenous for these criteria that can influence gene expression.

Isolation of $CD4^+$ and $CD8^+$ T cells and $CD4^+CD25^+CD45RO^+$ T_{reg} cells from leukapheresis. Leukapheresis samples were diluted 1:1 with 1× Dulbecco's PBS (pH 7.4; Invitrogen, Grand Island, NY), underlayed with Ficoll (Histopaque 1077, Sigma, St. Louis, MO), and centrifuged for 25 min at 1,300 × *g* in a refrigerated (4°C) table-top centrifuge. Interfaces were collected, washed extensively with 1× Dulbecco's PBS, and subjected to a 2-h adherence step to remove monocytes. Nonadherent peripheral blood mononuclear cells were frozen until needed. Upon use, cells were rested overnight at 37°C/5% CO_2 and then resuspended in cold 1× Dulbecco's PBS with 2 mmol/L EDTA and 0.5% bovine serum albumin (Sigma). A $CD4^+$ T-cell Isolation kit II or $CD8^+$ T-cell Isolation kit II (Miltenyi Biotech, Auburn, CA) was used to isolate untouched $CD4^+$ or $CD8^+$ cells, respectively, according to the manufacturer's instructions. Untouched, enriched $CD4^+$ or $CD8^+$ T cells were checked for purity by fluorescence-activated cell sorting (purity >95%) with PerCp-Cy5.5-anti-CD4 (BD Biosciences, San Jose, CA) or FITC-anti-CD8 (BD Biosciences) on a FACSVantage SE flow cytometer (BD Biosciences). For isolation of T_{regs}, $CD4^+$ cells were isolated as described above and were labeled with PE-anti-CD25 (BD Biosciences) and APC-anti-CD45RO (BD Biosciences) and sorted into $CD25^+CD45RO^+$ T_{reg} and $CD25^-$ populations on a FACSDiVa flow cytometer (BD Biosciences). To ensure purity of isolated cell populations, a portion of each sample was reanalyzed, and purity was determined to be >95%. We and others (21) have also done intracellular staining of $CD4^+CD25^+CD45RO^+$ cells and found them to be >78% positive for FoxP3 (15).

FoxP3 quantitative real-time reverse transcription-PCR. Purified $CD4^+CD25^+CD45RO^+$ T_{regs} and $CD4^+CD25^-$ T cells were collected from leukapheresis samples and fluorescence-activated cell sorting as described above and analyzed for FoxP3 expression by real-time reverse transcription-PCR (RT-PCR) to validate their phenotype as immunosuppressive T_{reg} cells, as previously described in the literature (15). cDNA was synthesized from appropriate amounts of each cell type by oligo (dT) with μMACS One-Step cDNA kit (Miltenyi Biotec) according to the manufacturer's instructions. Human FoxP3 mRNA expression levels were measured by real-time quantitative PCR and detected with SYBR Green dye (Bio-Rad, Hercules, CA) on a Bio-Rad iCycler in 25 mL of PCR reaction at 40 cycles at 95°C for 15 sec, 60°C for 1 min, and 72°C for 30 s. Each sample was run in triplicate and normalized with human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All primers spanned intron/exon boundaries to minimize genomic DNA amplification. One hundred base pairs of FoxP3 PCR products were amplified from human FoxP3-3 (5′-GAAACAGCACATTCCCAGAGTTC-3′) and human FoxP3-4 (5′-CCACATCGCTCAGATGAG-3′), and 114 bp of human GAPDH were produced from GAPDH-1 (5′-CCACATCGCTCAGACACCAT-3′) and GAPDH-2 (5′-GGCAACAATATCCACTTTACCAGAGT-3′). The human *FOXP3* gene was relatively quantified by real-time PCR using a Taqman gene expression assay (Applied Biosystems, Foster City, CA; 5′-GCACATTCCCAGAGTTCCTCCACAA-3′) between the 9 and 10 exon boundary of the gene, producing a 107-bp PCR product (reference sequencing no. NM_0140009) normalized to human GAPDH (Applied Biosystems; 5′-GCGCCTGGTCACCAGGGCTGCTTTT-3′), which produced a 122-bp PCR product (reference sequencing no. NP_002046). All $CD4^+CD25^+CD45RO$ T_{reg} samples were confirmed in this manner to be positive for FoxP3, whereas $CD4^+CD25^-$ T cells were determined to have little or no FoxP3 expression.

RNA isolation, amplification and generation of cDNAs, probe preparation, and microarray hybridization. Total RNA from 1×10^5 purified, untouched $CD4^+$ or $CD8^+$ T cells or fluorescence-activated cell–sorted T_{regs} were isolated using an RNeasy RNA extraction kit (Qiagen, Inc., Valencia, CA). Total RNA quality was assayed using an Agilent bioanalyzer (Silicon Genetics, Redwood City, CA), per the Duke Microarray Core Facility. Isolated total RNA passing quality control assessment was then amplified as previously described (22). Briefly, amplified mRNA was assayed on "in-house" human printed DNA microarrays per the Duke Microarray Core Facility, using the Operon Human Genome Oligo Set version 3.0 (Operon, Huntsville, AL) that possess 34,580 optimized 70 mers, representing 24,650 genes. Therefore, and as is customary, these printed arrays contain different sequence but functionally redundant oligomers for many annotated genes. Data analyses were done using Genespring software v. 7.2 (Silicon Genetics). Total RNA (2 μg) from each sample and a reference RNA (Universal Human Reference RNA, Stratagene, La Jolla, CA) was used in probe preparation. Briefly, reverse transcription is driven by an oligo (dT) primer bearing a T7 promoter using ArrayScript (Ambion, Austin, TX). The cDNA then undergoes second strand synthesis and clean-up to become a template for *in vitro* transcription with T7 RNA polymerase. To maximize RNA yield, Ambion's proprietary MEGAscript *in vitro* transcription technology is used to generate amplified RNA. The antisense amplified RNA is then fluorescently labeled with Cy3 (reference) and Cy5 (sample). Sample and reference amplified RNAs were pooled; mixed with 1× hybridization buffer (50% formamide,

5× SSC, and 0.1% SDS), COT-1 DNA, and poly-dA to limit nonspecific binding; and heated to 95°C for 2 min. This mixture was pipetted onto a microarray slide, a coverslip was placed, and hybridized overnight at 42°C. The array was then washed at increasing stringencies, and scanned on a GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA). Detailed protocols are available on the Duke Microarray Core Facility web site. All steps involved in RNA processing, probe preparation, microarray hybridization, and data processing used Minimal Information About a Microarray Experiment guidelines established by the Microarray Gene Expression Data Society.

Confirmation of microarray results by real-time RT-PCR. To validate the data generated in the microarray study, quantitative RT-PCR was done on selected candidate genes. Two genes were chosen according to multiple testing procedures, and their differential levels of expression were assessed across all samples. The PCR primers specific to these genes were designed using ABI Primer Express Software version 2.0 (Applied Biosystems). All of the primers were designed with the melting temperatures 58°C to 60°C and resulting products between 100 and 150 bp. For each healthy donor and glioblastoma multiforme patient, a small aliquot of total RNA extracted from each T-cell subset was saved for quantitative RT-PCR (separate from the total RNA used for linear amplification). cDNA was transcribed from total RNA using Powerscript reverse transcription kit (Invitrogen) in a 20-μL reaction volume. One microliter of cDNA was then used in triplicate reactions using iQ SYBR Green Supermix (Bio-Rad). The PCR conditions were as follows: 3 min at 95°C, 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s in the Bio-Rad iQ Real-time sequence Detection System (Bio-Rad). The human *pleckstrin* homology gene was relatively quantified with Taqman gene expression assay (Applied Biosystems) in five glioblastoma multiforme patients' $CD8^+$ cells versus five control volunteers' $CD8^+$ cells. The human *decorin* precursor gene expression level was checked in five glioblastoma multiforme T_{reg} and five control T_{reg} individual samples by Taqman gene expression assay (Applied Biosystems). All gene expression levels were normalized with human GAPDH in Taqman gene expression assays (Applied Biosystems).

Data processing. Genespring v. 7.2 (Silicon Genetics) was used to perform initial data analyses. Intensity-dependent (Lowess) normalization was done on the entire data set. Based on triplicates of each condition, a threshold of 2-fold change in expression relative to control and a two-way ANOVA with a P cutoff of 0.05. Expression of each gene was reported as the ratio of the value obtained for each condition relative to control conditions after data normalization.

Genotypic expression with binary regression analyses. Data values generated from the microarray analysis of isolated $CD4^+$ and $CD8^+$ T cell or T_{reg} cell populations were used in comprehensive statistical comparison and cross-validation analyses. The methods of analysis of the gene expression profiles follow similar methods used by West et al. in their study of human breast cancer samples (23). Briefly, instead of focusing on a simple fold change to identify gene expression patterns, special attention was paid to the profile of groups of genes ("metagene") whose expression highly correlates with a distinctive cellular state of normal healthy control or glioblastoma multiforme T cells. This included taking the average difference values for each gene on the array over the entire cohort populations and then identifying the genes whose expression represents a distinct genotype of interest. This group was then used for binary regression analysis to define factors that represents underlying structure in the data for each T-cell population. This factor analysis, which defines a structure in the data, is representative of a group of genes that exhibit a consistent pattern of expression in relation to an observable phenotype. The probability that the gene expression values that distinguish T cells in normal health versus glioblastoma multiforme disease was due to chance was verified with a cross-validation ("hold-one-out") analysis (23). Briefly, as described previously by Potti et al. (22), analysis was done using metagene construction and binary prediction analysis using MATLAB Software, version 7.1 (MathWorks, Natick, MA) to analyze gene expression patterns predictive of glioblastoma multiforme disease.

Table 1. Patient characteristics at time of leukapheresis

Patient	Diagnosis	Gender	Race	Age	KPS (%)
Control 1	Normal	F	Caucasian	48	100
Control 2	Normal	M	Caucasian	45	100
Control 3	Normal	M	Caucasian	49	100
Control 4	Normal	F	Caucasian	42	100
Control 5	Normal	F	Caucasian	43	100
GBM 1	GBM	M	Caucasian	46	100
GBM 2	GBM	M	Caucasian	49	90
GBM 3	GBM	M	African American	40	80
GBM 4	GBM	F	Caucasian	50	100
GBM 5	GBM	M	Caucasian	62	90

NOTE: Characteristics of normal healthy control volunteers and patients with newly diagnosed primary glioblastoma multiforme. Leukapheresis samples were collected from age-matched healthy volunteers (n = 5) or patients with newly diagnosed primary glioblastoma multiforme (n = 5) under a protocol approved by the Duke University Medical Center Institutional Review Board. Karnofsky performance scale scores for patients with glioblastoma multiforme were clinically determined prior to leukapheresis and were based on evaluations of a physical exam and their ability to successfully perform routine activities of daily living.
Abbreviations: GBM, glioblastoma multiforme; KPS, Karnofsky performance scale; M, male; F, female.

Results

Hierarchical clustering analyses of T-cell gene expression in patients with glioblastoma multiforme compared with healthy volunteers have shown significant differences between these groups. To gain a better understanding of the underlying cause of T-cell dysfunction and lymphopenia in patients with glioblastoma multiforme, we determined gene expression at the level of mRNA in $CD4^+$ and $CD8^+$ T cells and T_{reg} cells from healthy volunteers and compared this with expression of the same genes in patients with newly diagnosed primary glioblastoma multiforme. We obtained leukapheresis samples from 10 individuals whom we prospectively identified as meeting our study criteria (Table 1). Briefly, leukapheresis samples were collected from five matched healthy volunteers and five patients with newly diagnosed glioblastoma multiforme before resection. T cells were isolated and purified from non-adherent peripheral blood mononuclear cells (analyzed for purity >95% by flow cytometric analysis and validated by real-time PCR; Fig. 1A), from which total RNA was isolated and used in microarrays as described in Materials and Methods.

Following standard quality control analysis and external validation by quantitative real-time RT-PCR, resulting microarray data were filtered on level of gene expression from which significantly different values between control and glioblastoma multiforme T-cell populations were determined by ANOVA ($P < 0.05$). Based on these data, we did an unsupervised analysis on all samples by hierarchical clustering for 7,156 genes in $CD4^+$ cells, 6,546 genes in $CD8^+$ cells, and 6,406 genes in T_{reg} cells (Fig. 1B).[3] The usefulness of this type of analysis is that it helps define coordinate regulation of similarly related

[3] Complete gene lists for this and all analyses described are available at http://data.cgt.duke.edu/Learn2.php.

genes, often termed "gene trees," within a group or population. Coordinate regulation of various "branches" of these gene trees was found to be very similar within either the healthy or glioblastoma multiforme groups but was very different between these two groups, especially within the $CD4^+$ and T_{reg} cell compartments. Overall patterns of expression were highly reproducible between samples within a group. These data support the notion that T-cell expression profiles in healthy volunteers and patients with newly diagnosed glioblastoma multiforme exhibit fundamental and intrinsic differences at the level of transcription that are specific to each group.

To further investigate the expression profiles generated from the microarray analysis of $CD4^+$ and $CD8^+$ T cells and T_{reg} cells, scatter analysis was done on gene expression in these cells from

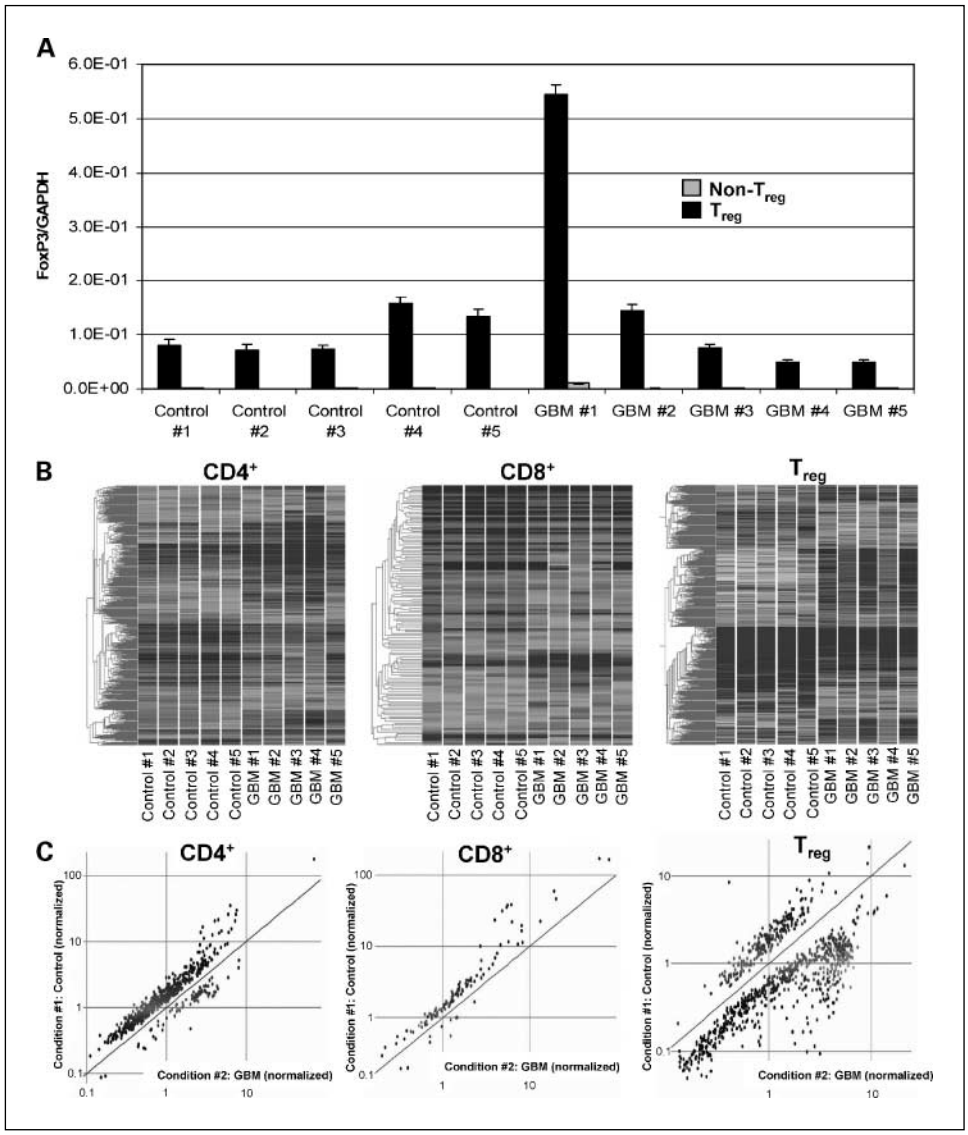

Fig. 1. Hierarchical clustering and scatter profiles of T-cell gene expression in healthy volunteers and patients with newly diagnosed primary glioblastoma multiforme (*GBM*). $CD4^+$ or $CD8^+$ T cells or $CD4^+CD25^+CD45RO^+FoxP3^+$ T_{regs} were purified from non-adherent peripheral blood mononuclear cells and total RNA was isolated and arrayed as described in Materials and Methods. *A,* validation of T_{reg} versus non-T_{reg} cells by real-time RT-PCR. Total RNA from T_{reg} or non-T_{reg} cells (five healthy volunteers and five glioblastoma multiforme patients) isolated by differential fluorescence-activated cell sorting of $CD4^+CD25^+CD45RO^+$ (T_{reg}) versus $CD4^+CD25^-CD45RO^-$ (non-T_{reg}) were transcribed into first-strand cDNA using a Superscript III 1st-Strand cDNA Synthesis System for RT-PCR. The human *FoxP3* gene was relatively quantified by real-time PCR using Taqman gene expression assays (Applied Biosystems) between the 9 and 10 exon boundary of the gene, producing a 107-bp PCR product (reference sequencing no. NM.0140009), which was then normalized to human GAPDH (Applied Biosystems). Real-time PCR data was generated on an ABI7900 thermal cycler (Applied Biosystems), and relative expression of FoxP3 to GAPDH was calculated. *B,* cluster analyses of $CD4^+$ and $CD8^+$ T cells and T_{reg} cells from controls and glioblastoma multiforme patients produces gene trees (*gray connecting lines on left*), which show coordinate regulation of groups of similarly related genes. Each gene is represented by a single row, and each sample is represented by a single column. The color heat map represents genes in a graded fashion along a spectrum of activation, extending from strongly up-regulated genes (*light gray*) to down-regulated genes (*dark gray*). Complete gene lists for this and all analyses described are available at http://data.cgt.duke.edu/Learn2.php. *C,* scatter profiles of significantly different gene expression for $CD4^+$ and $CD8^+$ T cells from controls and glioblastoma multiforme patients, as determined by ANOVA ($P < 0.05$), show substantial decreases in mRNA expression in T cells from patients with glioblastoma multiforme. Specifically, in $CD4^+$ cells, 520 transcripts are up-regulated above reference in controls, whereas only 83 transcripts are increased above reference in glioblastoma multiforme. Similarly, in $CD8^+$ cells, 111 transcripts are up-regulated above reference in controls, whereas only 11 transcripts are increased above reference in glioblastoma multiforme. In contrast, scatter profiles of significantly different gene expression for T_{regs} from controls and glioblastoma multiforme patients, as determined by ANOVA ($P < 0.05$), show substantial increases in mRNA expression in patients with glioblastoma multiforme. In T_{reg}, 221 transcripts are upregulated above reference in controls, whereas 684 transcripts are increased above reference in glioblastoma multiforme.

healthy volunteers or patients with glioblastoma multiforme. Scatter analysis for $CD4^+$ and $CD8^+$ T cells shows substantial decreases in specific mRNA transcripts in $CD4^+$ and $CD8^+$ T cells from patients with glioblastoma multiforme, as determined by ANOVA ($P < 0.05$; Fig. 1C). In $CD4^+$ cells, a total of 520 transcripts are increased or up-regulated above reference RNA levels in controls, whereas only 83 transcripts are increased above reference levels in T cells from patients with glioblastoma multiforme. Furthermore, we found that a total of 78 transcripts are down-regulated ≥2-fold in $CD4^+$ cells from patients with glioblastoma multiforme when compared with the levels of these same transcripts in controls, whereas only 15 transcripts are down-regulated ≥2-fold in controls when compared with levels in patients with glioblastoma multiforme. Similarly, in $CD8^+$ cells, 111 transcripts are up-regulated above reference RNA levels in controls, whereas only 11 transcripts are increased above reference in glioblastoma multiforme. Within these same populations, we observed that a total of 16 transcripts are down-regulated ≥2-fold in patients with glioblastoma multiforme when compared with levels in controls, whereas only two transcripts are down-regulated ≥2-fold in controls when compared with levels in glioblastoma multiforme. Specifically, the majority of these differentially expressed genes are found to be involved in T-cell activation, such as CD150, T-cell receptor (TCR) variable regions, TCR accessory signaling proteins, and β_2-microglobulin. On average, a 6-9 fold decrease in these specific transcripts is found in patients with glioblastoma multiforme compared with levels in controls.

In sharp contrast to the profiles observed in $CD4^+$ and $CD8^+$ populations, the immunosuppressive T_{reg} cell population up-regulated 684 transcripts above reference RNA levels in patients with glioblastoma multiforme, compared with 221 transcripts up-regulated above reference in controls. Specifically, 455 transcripts are up-regulated ≥2-fold in patients with glioblastoma multiforme when compared with levels in controls, whereas only 82 transcripts are up-regulated ≥2-fold in controls when compared with levels in glioblastoma multiforme. Analysis of these data yields on average a 4.25-fold increase in specific transcripts in patients with glioblastoma multiforme compared with levels in controls. Differentially expressed genes here include decorin, macrophage migration inhibitory factor, natural killer cell inhibitory receptor, and interleukin-1 receptor antagonist. These data uncover a coordinated inverse relationship for gene expression within non-T_{reg} $CD4^+$ and $CD8^+$ populations and that of T_{regs} between healthy volunteers and patients with glioblastoma multiforme.

Genetic profiles of T cells in healthy volunteers and patients with glioblastoma multiforme segregate independently. Principal components analysis is a decomposition technique that produces a set of expression patterns known as principal components. This technique simplifies a data set by reducing multidimensional data sets to lower dimensions for the purposes of analysis. Specifically, principal components analysis is a linear transformation of the data to a new coordinate system such that the greatest variance by any projection of the data comes to lie on the first coordinate (i.e., the first principal component), the second greatest variance on the second coordinate, and continues as such for the number of components generated from the analysis. Principal components analysis is often used for dimensionality reduction in a data set, while retaining those characteristics of the data set that contribute most to its variance, by keeping lower-order principal components and ignoring higher-order ones. Such low-order components are thought to contain the most important aspects of the data. For our purposes, gene expression values determined by ANOVA ($P < 0.05$) produce principal components for $CD4^+$ and $CD8^+$ T cells and T_{reg} cells from controls or patients with glioblastoma multiforme, based on the first three principal components statistically generated by Genespring Software analysis (Fig. 2). We observe relative relatedness among samples within the T-cell compartments of controls or patients with glioblastoma multiforme and distinct separation of the control and glioblastoma multiforme groups. This type of behavior suggests that T-cell compartments within control and glioblastoma multiforme populations are characteristically different and segregate independently of one another in a computational model based on statistically validated expression signatures.

Binary regression analyses of T cells from patients with glioblastoma multiforme propose training sets of genes for characterizing and validating prospective glioblastoma multiforme T-cell profiles. The prevalence of significantly down-regulated genes relevant to immunologic function in T cells from patients with glioblastoma multiforme provides evidence for intrinsic suppression of the immunologic transcriptome. To further characterize this observation, we did Bayesian binary regression analyses (23) to develop models that could define expression profiles of T cells investigated in these studies. This type of analysis helps to discern patterns of gene expression representing the underlying structure present in the data. Discriminator genes identified from binary regression analysis, also known as the metagene, form the predictive basis for this type of analysis. To test whether or not the discriminator genes determined by binary regression could accurately predict each sample within each T cell group, we did a cross-validation hold-one-out analysis in which one sample is removed from the test group, and the remaining samples are then used to regenerate expression patterns for prediction. As described by Potti et al. (22), the removed sample is then analyzed to determine if the collective remaining samples can accurately predict its classification. The values on the horizontal axis are estimates of the signature score from the regression, and the values on the vertical axis are estimated classification probabilities with the corresponding 95% probability intervals marked to indicate the uncertainty about these estimated values.

As shown in Fig. 3, the expression patterns generated from each T-cell compartment are able to reproducibly distinguish T-cell signatures from patients with glioblastoma multiforme. For $CD4^+$ cells, a total of 50 discriminator genes determined by binary regression of statistically validated expression profiles were able to produce a metagene that accurately predicted whether the T-cell sample was from a patient with glioblastoma multiforme or from a healthy volunteer. Similarly, for $CD8^+$ T cells and T_{reg} cells, a total of 100 discriminator genes for each cell type were able to do the same. Collectively, these data provide evidence that gene profiles identified here are statistically valid, useful as a training set for characterizing T-cell phenotypes in patients with glioblastoma multiforme, and could help to identify therapeutic targets for reversing the immunosuppressed phenotype in these patients.

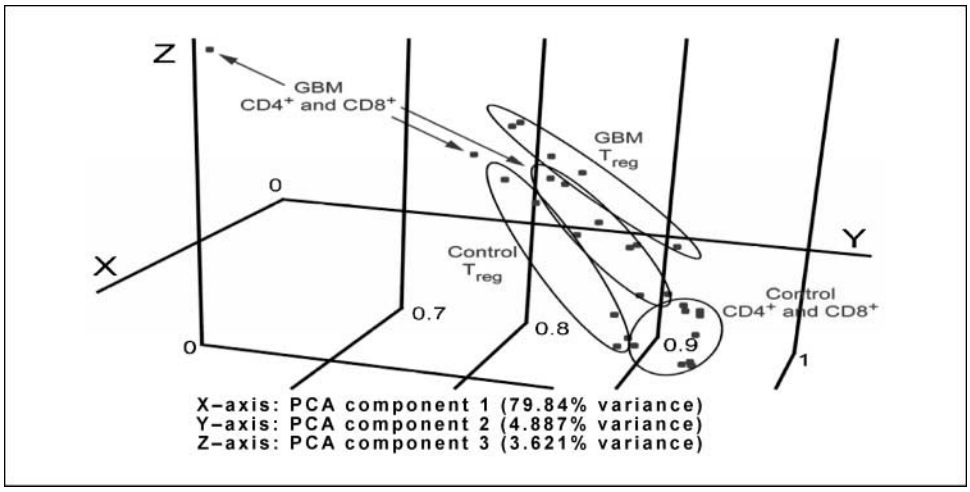

Fig. 2. Principal components analysis for T cells from normal healthy control volunteers and patients with newly diagnosed primary glioblastoma multiforme. For this analysis, gene expression values for each signature are extracted from all experimental samples and mean centered. Single-value decomposition analysis is then applied across all samples, from which linear combinations of these patterns are then assembled to represent the behavior of all of the genes in a given sample, characterizing the most abundant themes or building blocks that reoccur in many genes in a sample. Samples from controls or samples from patients with glioblastoma multiforme are circled and labeled.

Specific immune genes are differentially expressed in the T-cell compartments of patients with glioblastoma multiforme compared with healthy volunteers. Based on the data presented thus far, we next investigated specific pathways and the numbers of genes involved in each pathway that uniquely produce the differential expression profiles we observe for the various T-cell compartments in controls and patients with glioblastoma multiforme. To begin, gene ontology pathway analyses in

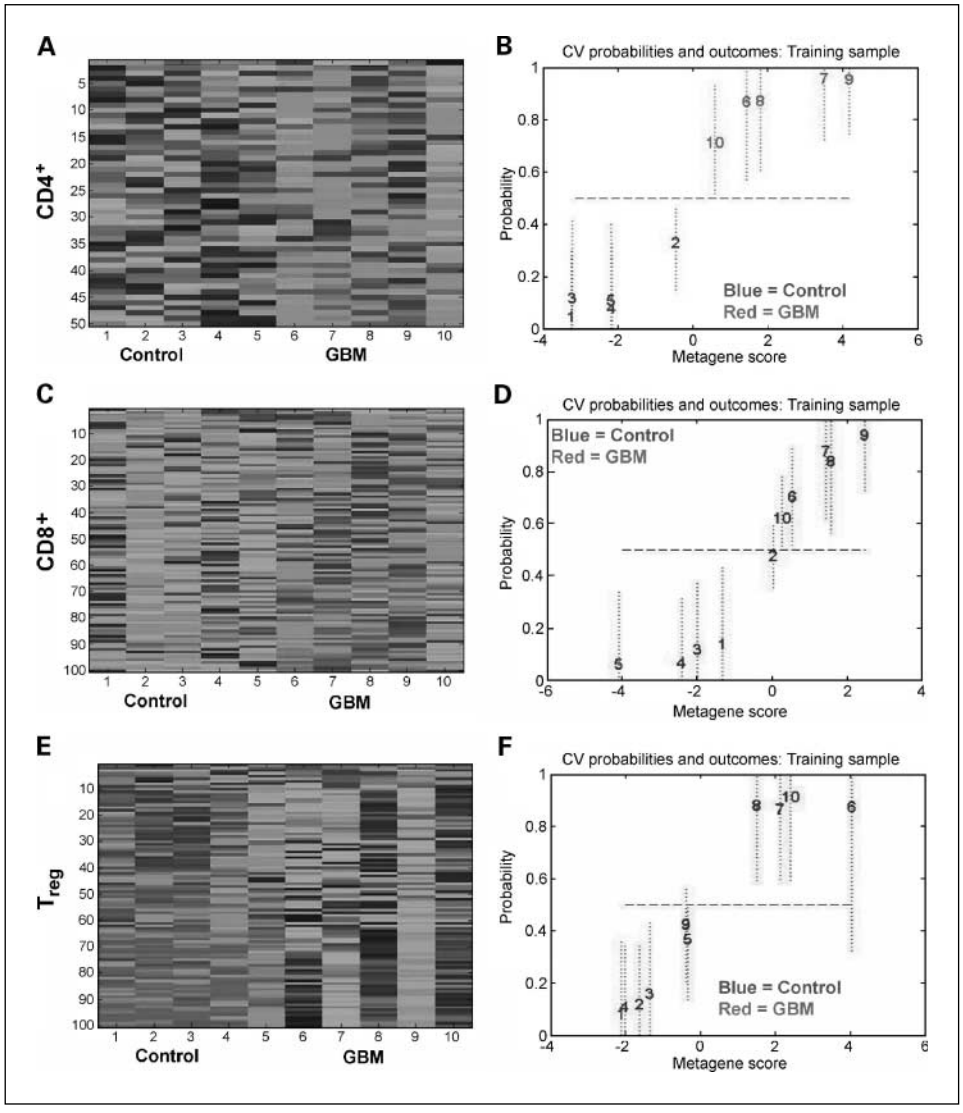

Fig. 3. Binary regression and cross-validation analyses of T cells from normal healthy control volunteers versus patients with newly diagnosed primary glioblastoma multiforme. Statistical analyses of genes expressed at levels above reference were done and determined to be significant by ANOVA. Leave-one-out cross-validation (*CV*) probabilities of individual samples in a training cohort ($n = 5$) of normal controls (*bottom*) and patients with newly diagnosed primary glioblastoma multiforme (*top*) were done as described in Materials and Methods. The values on the horizontal axis are estimates of the overall metagene score in the regression analysis. The corresponding values on the vertical axis are estimated classification probabilities with corresponding 95% probability intervals marked to indicate uncertainty about these estimated values. The horizontal dashed line represents an arbitrary cutoff value to show the accuracy of predictions for any given probability of the conditions being compared. Discriminator genes identified from binary regression analysis (*A, C*, and *E*). Cross-validation analyses (*B, D*, and *F*). Complete gene lists for each training set for $CD4^+$ and $CD8^+$ T cells and T_{reg} cells are available at http://data.cgt.duke.edu/Learn2.php.

Table 2. Gene ontology pathway analyses of T cells from normal healthy control volunteers versus patients with newly diagnosed primary glioblastoma multiforme

Biological process (no. genes in list, $P < 0.05$)	Control			GBM		
	$CD4^+$	$CD8^+$	T_{reg}	$CD4^+$	$CD8^+$	T_{reg}
Cell communication (2,452)	23 (0.94%)	10 (0.41%)	7 (0.29%)	6 (0.24%)	1 (0.04%)	43 (1.75%)
Cell growth and/or maintenance (4,262)	92 (2.16%)	15 (0.35%)	32 (0.75%)	4 (0.09%)	1 (0.02%)	99 (2.32%)
Binding (2,937)	73 (2.49%)	10 (0.34%)	21 (0.72%)	6 (0.20%)	1 (0.03%)	52 (1.77%)
Catalytic activity (3,998)	63 (1.58%)	16 (0.40%)	17 (0.43%)	7 (0.18%)	4 (0.10%)	98 (2.45%)
Defense immunity protein activity (1,076)	12 (1.12%)	2 (0.19%)	3 (0.28%)	3 (0.28%)	0 (0%)	21 (1.95%)
Signal transducer activity (2,131)	11 (0.52%)	10 (0.47%)	3 (0.23%)	5 (0.23%)	0 (0%)	19 (0.89%)

NOTE: Enumeration of genes involved in specific biological processes in normal healthy control volunteer T cells versus T cells from patients with newly diagnosed primary glioblastoma multiforme shows differential gene expression in specific pathways. Total gene numbers that populate a specific biological process list are included in parentheses to the left, whereas total percent composition for a particular cell population within a specific process list is noted in parentheses to the right. These pathways were generated based on statistical analysis of genes expressed at levels above reference and determined to be significant by ANOVA. Gene ontology pathway analyses were done and described using Genespring Software (v.7.2) from Agilent Technologies, Inc. (Santa Clara, CA).
Abbreviation: GBM, glioblastoma multiforme.

which we analyzed the number of genes involved in annotated biological processes (Table 2), based on ANOVA. Using this type of analysis, we find there are substantial decreases in gene expression along specific cellular pathways that represent the underlying biology of $CD4^+$ and $CD8^+$ T cells of patients with glioblastoma multiforme when compared with controls. In particular, the largest decreases occur in genes involved in cell growth (*STAT4* and *BRAP1*), receptor binding (*calcium/calmodulin-dependent protein kinase* and *Rho guanine nucleotide exchange factor 3*), and catalytic activity (*ribosomal protein S6 kinase* and *cyclophilin E*), all processes that are essential to normal T-cell proliferation and function. However, in marked contrast, we find considerable increases in gene expression in these same pathways for immunosuppressive regulatory T cells from patients with glioblastoma multiforme when compared with controls.

To further investigate the composition of the differential expression profiles observed, we determined the genes that notably defined the profile in each T-cell compartment for controls and patients with glioblastoma multiforme. To do so, we began by identifying genes that were the most up-regulated or down-regulated in a population or were found to be similarly regulated and belonging to a group of genes that are involved in a particular biological process. Our analysis was striking in that the largest cohort of genes identified, which fit both these criteria, were genes related specifically to T-cell regulation and function. For the complete analyses, a total of 78 transcripts were down-regulated ≥2-fold in $CD4^+$ cells from patients with glioblastoma multiforme, whereas only 15 transcripts were down-regulated ≥2-fold in controls. Similarly, in $CD8^+$ cells, a total of 16 transcripts were down-regulated ≥2-fold in patients with glioblastoma multiforme, whereas only two transcripts were down-regulated ≥2-fold in controls. However, in marked contrast to this, a total of 455 transcripts were up-regulated ≥2-fold in T_{regs} from patients with glioblastoma multiforme, whereas 82 transcripts were up-regulated ≥2-fold in controls.[4] Table 3 summarizes the notable findings from these lists.

In Table 3A, we identified specific genes involved in the processes of TCR antigen binding, activation, and intracellular signaling to be down-regulated 2-5 fold in $CD4^+$ cells of patients with glioblastoma multiforme in comparison with levels in controls. Specifically, genes for the TCR variable region, TCR-mediated fyn signal transduction, MHC class I β_2-microglobulin, and TCR accessory molecules were uncovered in this analysis. Similarly, in Table 3B, we again find genes involved in these same processes to be down-regulated 2.4- to 6.5-fold in $CD8^+$ cells of patients with glioblastoma multiforme in comparison with levels in controls. Of note, we observed decreases in transcript levels for TCR variable regions and genes involved in TCR-mediated signaling. Consistent with these observations of dysregulated immune gene expression in glioblastoma multiforme, we observe in Table 3C that numerous transcripts involved in immune down-regulation or inhibition-related processes are up-regulated, and some substantially so, in T_{regs} of patients with glioblastoma multiforme in comparison with levels in controls. Notably, *decorin, migration inhibitory factor, natural killer cell inhibitory receptor, interleukin-1 receptor antagonist,* and genes induced by transforming growth factor-β are all increased. In contrast, Table 3D lists genes that are down-regulated ≥2-fold in T_{regs} from patients with glioblastoma multiforme compared with controls. Finally, we observe that numerous immunomodulatory genes are coexpressed at increased levels in both $CD4^+$ and $CD8^+$ T cells of healthy controls compared with patients with glioblastoma multiforme, suggesting a central and/or coordinated expression (data not shown).

For the studies described here, we find reduced expression of immunomodulatory genes, whereas expression of immunoinhibitory genes is increased in immunosuppressive T_{regs} of patients with primary glioblastoma multiforme. Collectively, these mechanisms may be working in concert and could, at least in part, promote the conditions of immunosuppression and lymphopenia commonly observed in patients with glioblastoma multiforme. Invariably, the overall significance and importance of the individual expression signatures we have identified may only be relevant or meaningful in the context of coordinate T-cell immunity. We conclude that T cells from patients with glioblastoma multiforme display a consistent,

[4] Complete lists for these analyses are available at http://data.cgt.duke.edu/Learn2.php.

intrinsic and specific dysregulation of gene function at the level of mRNA. Based on this, we propose that dysregulation of T-cell immunity in glioblastoma multiforme is bipartite yet inextricably linked.

Discussion

Patients with glioblastoma multiforme typically exhibit a comprehensive and severe suppression of their immune system, which serves to dysregulate T-cell number, phenotype, or both (24). Cutaneous anergy, lymphopenia, impaired antibody production, reduced lymphocyte protein synthesis, and diminished lymphocyte responsiveness have all been reported (25–33). Although invariably present, B- and T-cell lymphopenia is not thought to sufficiently explain these functional defects (12).

The purpose of these studies was to determine if the genetic expression profiles of T cells at the level of mRNA provided any insight into the mechanisms of the T-cell deficiencies in patients with glioblastoma multiforme and to establish and

Table 3. Changes in immunoregulatory gene expression in T cells from patients with newly diagnosed primary glioblastoma multiforme when compared with normal healthy control volunteers

Operon Oligo ID	*P*	Fold change	Description
A.			
H300005559	0.0246	−5.247	TCR α chain v region
H300013752	0.0339	−4.386	Fyn-binding protein
H200005324	0.0478	−3.512	Fyn-binding protein
H300013913	0.0455	−2.993	TCR interacting molecule
H200004202	0.0457	−2.986	Signaling lymphocytic activation molecule (cd150)
H300022062	0.000955	−2.734	Forkhead box protein p1
H200006811	0.0113	−2.588	F-box only protein 25
H200006095	0.00225	−2.513	β_2-Microglobulin
H300015245	0.0457	−2.45	HLA class i histocompatibility antigen, α chain g
H300019523	0.0477	−2.4	STAT4
H200005242	0.0109	−2.058	DNA mismatch repair protein MLH1
B.			
H200000072	0.0103	−6.573	Cytohesin binding protein
H300022679	0.043	−6.561	TCR γ chain v region
H300005559	0.0186	−5.987	TCR α chain v region
H200016225	0.0436	−3.729	Apolipoprotein b mRNA editing enzyme
H200016648	0.0134	−3.51	Receptor-interacting serine/threonine protein kinase 3
H300000762	0.0348	−3.141	TCR β chain v region
H300019523	0.0288	−2.418	STAT4
C.			
H300017419	0.000834	+33.59	Decorin precursor
H300021659	0.000185	+24.64	Decorin precursor
H300018456	0.00676	+15.17	Decorin precursor
H300022401	0.000228	+9.863	Fas-activated serine/threonine kinase
H300005694	4.59E−05	+9.694	Serine/threonine protein kinase PLK
H200005865	5.81E−05	+9.423	Macrophage migration inhibitory factor (MIF)
H200015342	0.000968	+6.117	Growth arrest–specific protein 7
H300000805	0.000771	+5.595	Stress-induced phosphoprotein 1 (STI1)
H300021387	0.00563	+4.629	G_1/S–specific cyclin E2
H300017591	0.01	+4.364	MAP kinase activating death domain-containing protein
H200011770	0.00178	+3.2	Sparc
H300015185	0.00332	+2.769	NK inhibitory receptor
H300022783	0.0308	+2.73	HLA class i histocompatibility antigen, α chain g
H200012149	0.029	+1.992	Transforming growth factor-β–induced protein Ig-h3
H200007550	0.0182	+1.986	Phosphatidylinositol 3-kinase
H200006821	0.0329	+1.826	Interleukin-1 receptor antagonist protein
D.			
H200015754	0.0481	−2.942	DNA polymerase τ
H200005290	0.0204	−2.623	EGF-tm7 latrophilin-related protein
H300014672	0.0145	−2.608	Phosphatidylinositol glycan
H200007339	0.0385	−2.427	Prostaglandin D2 receptor
H200012017	0.0215	−2.41	T-cell activation kelch repeat protein
H200005778	0.0471	−2.258	Hedgehog-interacting protein
H200012823	0.024	−2.163	Ubiquitin-conjugating enzyme
H200018962	0.0253	−2	Ras-related protein Rab-8b

NOTE: A. ≥2-fold decreases of immunomodulatory genes in $CD4^+$ cells from patients with glioblastoma multiforme, compared with expression in control $CD4^+$ cells. B. ≥2-fold decreases of immunomodulatory genes in $CD8^+$ cells from patients with glioblastoma multiforme, when compared with expression in control $CD8^+$ cells. C. Increased expression of immunomodulatory genes in T_{reg} cells from patients with glioblastoma multiforme, when compared with expression in control T_{reg} cells. D. Decreased expression of immunomodulatory genes in T_{reg} cells from patients with glioblastoma multiforme, when compared with expression in control T_{reg} cells.
Abbreviations: NK, natural killer; EGF, epidermal growth factor; MAP, mitogen-activated protein.

statistically validate genetic signatures for $CD4^+$ and $CD8^+$ T cells and T_{reg} cell populations in healthy volunteers and patients with glioblastoma multiforme. To our knowledge, this is the first study to investigate mRNA expression profiles of T cells in patients with glioblastoma multiforme. Others have shown that investigation of T-cell signatures in other diseases, such as thrombosis (22), renal cell carcinoma (34), and melanoma (35, 36), is not only highly informative but can also be predictive of clinical outcome and design of interventional therapies (18, 23). Inasmuch, our studies have identified reproducible metagene profiles in the T-cell transcriptomes of patients with glioblastoma multiforme. Specifically, we find deficits and abnormalities in the levels of numerous immunomodulatory transcripts, which provide a genetic fingerprint for the dysregulated T-cell immunity clinically observed in patients with glioblastoma multiforme (8–10). Overall, levels of transcripts for genes involved in normal T-cell function are notably decreased, specifically in the processes of TCR antigen binding, activation, and intracellular signaling. Conversely, expression of numerous genes involved in inhibiting the immune response is significantly up-regulated in T_{regs}. *Decorin*, a gene product known for its potent binding capacity for transforming growth factor-β (37–39), migration inhibitory factor (40–43), and interleukin-1 receptor antagonist (44, 45), is increased in expression in T_{regs} from patients with glioblastoma multiforme, consistent with the premise of active inhibition of immune responses by this population of cells (15). The substantial up-regulation of *decorin* (up to 33-fold) may be of central importance in understanding the role of transforming growth factor-β in T_{reg} induction and activity in these patients (46, 47). Furthermore, it is interesting to note that, although not statistically significant by ANOVA, the *interleukin-2 receptor* was expressed at higher levels in the $CD4^+$ and $CD8^+$ cells of healthy volunteers compared with glioblastoma multiforme patients, whereas this receptor was expressed at lower levels in T_{regs} from healthy volunteers compared with glioblastoma multiforme patients. This finding lends support to the notion that differential regulation of *interleukin-2 receptor* between T-cell subsets is an aspect of high-grade glioma-mediated immunosuppression. Future investigations into the immunologic transcriptomes of T cells in low-grade glioma could help to further characterize this disease and its immunosuppressive mechanisms.

Our data support the notion that clusters of genes identified within these signatures may have central importance for the development and maintenance of these various cellular phenotypes. Cross-validation analyses were able to correctly classify samples tested for each T-cell compartment, in spite of the heterogeneous nature of the populations studied. In addition, whereas developing expression profiles as tools to characterize and classify clinical phenotypes and outcomes are important to this work, they may also help to identify potential target molecules for therapeutic intervention.

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