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Transcriptional Profile of Tuberculosis Antigen–Specific T Cells Reveals Novel Multifunctional Features

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In latent tuberculosis infection (LTBI) spread of the bacteria is contained by a persistent immune response, which includes CD4+ T cells as important contributors. In this study we show that TB-specific CD4+ T cells have a characteristic chemokine expression signature (CCR6+CXCR3+CCR4−), and that the overall number of these cells is significantly increased in LTBI donors compared with healthy subjects. We have comprehensively characterized the transcriptional signature of CCR6+CXCR3+CCR4− cells and found significant differences to conventional Th1, Th17, and Th2 cells, but no major changes between healthy and LTBI donors. CCR6+CXCR3+CCR4− cells display lineage-specific signatures of both Th1 and Th17 cells, but also have a unique gene expression program, including genes associated with susceptibility to TB, enhanced T cell activation, enhanced cell survival, and induction of a cytotoxic program akin to CTLs. Overall, the gene expression signature of CCR6+CXCR3+CCR4− cells reveals characteristics important for controlling latent TB infections. The Journal of Immunology, 2014, 193: 2931–2940.

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The sequences presented in this article have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE56179.

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Materials and Methods

Study subjects

Leukapheresis samples from 12 adults with LTBI and 12 control donors were obtained from the University of California, San Diego Antiviral Research Center clinic (age range, 20–65 y). Subjects had a history of a preferred tuberculosis skin test. LTBI was confirmed by a positive Quantiferon-TB Gold In-Tube test (Celltestis), as well as by a physical examination and/or chest x-ray that was not consistent with active TB. None of the study subjects endorsed vaccination with bacillus Calmette–Guerin (BCG) or had laboratory evidence of HIV or hepatitis B virus. The control donors had a negative tuberculin skin test, as well as a negative Quantiferon-TB test. Research conducted for this study was performed in accordance with approvals from the Institutional Review Board at the La Jolla Institute for Allergy and Immunology (FWA no. 0000032).
All participants provided written informed consent prior to participation in the study.

**PBM C isolation**

PBMCs were obtained by density gradient centrifugation (Ficoll-Hypaque, Amersham Biosciences) from 100 ml leukapheresis sample, according to the manufacturer’s instructions. Cells were suspended in FBS (Gemini Bio-Products) containing 10% dimethyl sulfoxide and cryopreserved in liquid nitrogen.

**Isolation of cells and FACS analysis**

HLA class II tetramers conjugated using PE-labeled streptavidin were provided by the Tetramer Core Laboratory at Benaroya Research Institute. CD4 T cells were purified using the Miltenyi Biotec T cell isolation kit II according to the manufacturer’s instructions. Purified cells were incubated in PBS containing 0.5% BSA and 2 mM EDTA (pH 8.0) (MACS buffer) with a dilution of class II tetramer (10 μl tetramer per 50 × 10^6 CD 4 T cells) for 2 h at room temperature. Cells were then stained for cell surface Ags using anti-CD4-allophycocyanin-εFluor 780 (RPA-T4), anti-CD45RA-εFluor 450 (HI100) (both from Affymetrix/eBioscience), anti-CD3-Alexa Fluor 700 (UCHT1), anti–CXCR3 (CD183)-allophycocyanin or CXC3-Alexa Fluor 488 (IC16/CXC3), anti–CD19-V500 (HIB19), anti–CD14-V500 (M5E2), anti–CD8-V500 (RPA-T8), and anti–CD25-FITC (M-A251), anti–CCR4-PE-Cy7 or CCR4-PE (1G1), and anti–CCR6 (CD196)-biotinylated (11A9) (all from BD Biosciences) followed by streptavidin-BV605 (BioLegend), anti–CCR7 (CD197)-PerCP-Cy5.5 (G0437) (BioLegend), and LiveDead Aqua (Affymetrix/eBioscience) to exclude dead cells. For experiments to evaluate protein expression, anti–CCR2 (CD192)-PE (K0362, BioLegend), anti–CD117 (KIT)-PE (YB5.88), and anti–CD117-FITC (104D2), both from Affymetrix/eBioscience were used. Samples were acquired on a BD FACSAria or LSR II flow cytometer and analyzed using FlowJo software. For phenotyping experiments, cells were stained with surface markers as above without the tetramer. For cell sorting, cells were sorted into six separate populations: tetramer +, CCR6 +CXCR3+CCR4-2 naive (naive) cells (gating strategy in Supplemental Fig. 1).

For RNAseq of activated cells, CCR6+CXCR3+CXC4- naive cells were sorted from purified CD4+ T cells on a BD FACSAria flow cytometer. These and remaining cells (used as APCs) were rested overnight in complete RPMI medium at 37 C in 5% CO2. APCs were stained with CFSE according to the manufacturer’s instructions (Affymetrix/eBioscience). Cells were cultured at a ratio of 2:1 CD4+ T cells/APCs in the presence of 5 μg/ml TB peptide pool or 0.1 μg/ml FMA with 1 μg/ml ionomycin for 6 h in complete RPMI medium at 37 C in 5% CO2. Unstimulated cells were used to assess non-specific/background cytokine production. After 6 h, cells were harvested and stained for cell surface Ags CD4, CD3, CD8, CD19, CD14, CXCR3, CXC4, CD4, and Live/Dead using the same clones and colors as described above. After washing, cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences) and then stained for cytokines using anti–IFN-γ-PerCP-Cy5.5 (4S.B3), anti–TNF-α-εFluor 450 (Mab11), anti–IL-17A-PE-Cy7 (eBio64DEC17), and anti–IL-2-FTTC (Miq-17H12), all from Affymetrix/eBioscience. Samples were acquired on a BD LSR II flow cytometer. The frequency of cells responding to the TB-specific peptides was quantified by determining the total number of gated subset and cytokine cells and background values subtracted (as determined from the medium alone control) using FlowJo software (Treestar). A cut-off of 2-fold the background was used. Combinations of cytokine-producing cells were determined using Boolean gating in FlowJo software.

**Microarray data sequencing**

Total RNA was purified using a miRNAeasy kit (Qiagen) and quantified as described previously (14). Purified total RNA (10–15 ng) was used for poly(A) selection (Poly(A)Purist kit, Life Technologies). Poly(A)-selected RNA was amplified with a whole transcriptome amplification sequencing technology kit (SEQR, Sigma-Aldrich) as per the manufacturer’s recommendations. One microgram of this amplified cDNA was treated with restriction enzyme (SEQR, Sigma-Aldrich) to remove the primer sequences and then purified using AMPure XP beads (Beckman Coulter). Efficiency of removal of SEQR primer sequences was assessed by PCR. From this step, 250 ng purified DNA was diluted with TE buffer to obtain a total volume of 65 μl. Diluted cDNA was sonicated with a Covaris E220 multiplex sonicator to generate 100- to 250-bp DNA fragments. Approximately 250 ng DNA was used for preparing a standard SOLID sequencing library (5500 SOLID fragment 48 library core kit and fragment library barcode adapters 1–96). Following emulsion PCR samples were sequenced on the 5500 SOLID sequencer to obtain 35-bp single end reads (SOLID EZ Bead E120 system Table 1). Whole-transcriptome and sequencing library preparations were performed in a 96-well format, which significantly reduced hands-on time, besides reducing technical and assay-to-assay variability. Multiple quality control steps were included to determine total RNA quality and quantity, optimal poly(A) selection, ligation efficiency, and number of PCR amplification cycles to assess removal of whole-transcriptome PCR amplification adaptors, shearing of amplified cDNA, fragment size selection, and efficiency of ligation of sequencing adaptors and to determine library complexity. Samples that failed quality control were eliminated from further downstream steps.

**RNA sequencing analysis**

The 35-bp single end reads were aligned to human hg19 reference genome using the TopHat alignment program (15) allowing reads to span a splice junction. The alignment results were parsed via the SAMtools package (16) to generate SAM files. To quantitatively analyze the gene expression levels under various cell types and disease types, we only counted the short reads uniquely mapping to an annotated features of the hg19 genome by using htsq-count (17). In this study, we selected the “union” option in htsq-count to deal with reads overlapping more than one feature. After removing absent features (zero counts in all samples), we employed the R/Bioconductor package aroma.light (18) to perform quantile normalization of the samples for each gene set (column) and generate expression. The quantile normalized raw counts were then imported to R/Bioconductor package DESeq (19) to identify differentially expressed genes among distinct cell types. We considered genes differentially expressed between two groups of samples when the DESeq analysis resulted in an adjusted p value <0.05 and in addition when the fold change in gene expression was >2-fold. For genes with low expression close to the detection threshold, normalized counts <3 were set equal to 0 when calculating fold-change values. The sequences used in this article have been submitted to the Gene Expression Omnibus under accession number GSE56179 (http://www.ncbi.nlm.nih.gov/geo/).

**Statistical analysis**

A two-tailed Mann–Whitney U test or a one-tailed t test was used for statistical analysis. Differences with a p value <0.05 was considered significant.

**Results**

**TB epitope-specific memory T cells are predominantly CCR6+CXCR3+CXC4-**

Using the T cell library method and TB lysate and peptide pools, it was previously shown that TB-specific memory T cells are predominantly present in the CCR6+CXCR3+CXC4- memory subset (2, 20). To measure the frequency and distribution of T cells specific for individual TB epitopes, we used a culture filtrate protein of 10 kDa (CFP10)52–66-DRB5*0101 tetramer (CFP1062-66, QAAAVRFEQAANKQK) (21). Epitope-specific CD4+ memory T cells, including CD45RA+CCR7+ central memory, CD45RA+CCR7- T effector memory, and CD45RA+CCR7- T effector memory expressing CD45RA (gating strategy in Supplemental Fig. 1), were detected in five LTBI donors at frequencies ranging from 0.022 to 0.519% (median, 0.09; interquartile range, 0.03–0.33) (Supplemental Fig. 2). These cells were not detected in the CD4+ naive subset or in the CD4+ memory subset from TB uninfected non-BCG vaccinated control donors (HCs) (Supplemental Fig. 2). Next, we investigated the frequency of epitope-specific memory T cells...
in four memory T cell subsets, defined based on chemokine receptor expression pattern (3) (Supplemental Fig. 1): CCR6+CXCR3+CCR4+ (Th1), CCR6+CXCR3+CCR4+ (Th17), and CCR6+CXCR3+CCR4+ (Th2) cells. As expected from our previous data (2), the epitope-specific CD4+ memory T cells were predominantly present in the CCR6+CXCR3+CCR4+ subset, with a median of 92% of tetramer+ cells (Fig. 1A, 1B, gating strategy in Supplemental Fig. 1).

To examine the cytokine profile of these cells, we stimulated CD4+ T cells with TB-specific peptides. These peptides were chosen based on known reactivity in the selected donors, all of which were included in the previously described genome-wide epitope screen (2) (Supplemental Table I). The responding CCR6+CXCR3+CCR4+ cells are multifunctional and produce IFN-γ, TNF-α, and IL-2 but not IL-17 (Fig. 1C, 1D). Most CCR6+CXCR3+CCR4+ cells were IFN-γ+TNF-α+IL-2+ (median 38% of cytokine-producing cells), IFN-γ+TNF-α+ (25%), and TNF-α+ (18%), followed by TNF-α+IL-2+ (12%) and IFN-γ+ (3%) (Fig. 1D), similar to findings in a previous study of TB-specific epitopes (2). This confirms that these cells are a major source of TNF-α, in line with the hypothesis that TNF-α blockers might impede the function of these cells, leading to TB reactivation.

**CCR6+CXCR3+CCR4+ memory T cells are increased in subjects with LTBI**

Because TB-specific memory T cells predominantly fall into the CCR6+CXCR3+CCR4+ memory subset, we hypothesized that this subset of T cells could be selectively expanded in LTBI subjects compared with HCs. To address this we compared the frequency of CCR6+CXCR3+CCR4+ , CCR6+CXCR3+CCR4+ (Th1), CCR6+CXCR3+CCR4+ (Th17), and CCR6+CXCR3+CCR4+ (Th2) memory subsets in LTBI and HC donors. As expected, the fraction of the CCR6+CXCR3+CCR4+ subset was significantly (p = 0.003) increased in LTBI donors, with a median of 10.5% compared with 5.5% in HCs (Fig. 2). All other T cell subsets were seen with similar frequencies in the two cohorts: Th1, 16.5% for LTBI and 16% for HCs; Th2, 8% for LTBI and 8.5% for HCs; and Th17, 5% for LTBI and 5% for HCs (Fig. 2B and gating strategy in Supplemental Fig. 1). These results suggest that the CCR6+CXCR3+CCR4+ subset is preferentially expanded following TB infection, and it is likely to be required for life-long containment of mycobacteria in the host. Furthermore, the presence of this memory subset in healthy subjects with no previous TB exposure or infection indicates that Ag-specific memory T cells to other pathogens may also reside in this subset.

**The transcriptional profile of TB-specific memory T cells is similar to CCR6+CXCR3+CCR4+ memory subset in LTBI and HC donors**

To determine the transcriptional profile of TB-specific memory T cells, we isolated a pure population of CFP10 52–66-DRB5*01:01 tetramer+ cells from five HLA-matched LTBI donors (cell numbers ranging from 10,000 to 70,000). We performed RNA sequencing in these cells and compared them to the broader CCR6+CXCR3+CCR4+ memory subset, as well as to the conventional Th1, Th2, and Th17 memory subsets from the same LTBI donors.
Mapping of short mRNA reads to the genome showed that transcripts of the phenotypic markers used for sorting (CXCR3, CCR6, and CCR4) were detectable, and differences in expression levels were consistent with the sorting strategy (Fig. 3A). Gene expression values were quantile normalized and pairwise comparisons were performed between groups of samples from four to five donors each using DESeq (19). We considered a gene as differentially expressed if the adjusted p was <0.05 and the change in magnitude of expression was at least 2-fold. As expected, we observed increased expression of the two key transcription factors, T-bet and RORC, that are characteristic of Th1 and Th17 cells, respectively, and have both been shown to be expressed in CCR6+CXCR3+CCR4+ cells (Fig. 3B). This confirms that the sorting strategy and RNA profiling analysis should reliably detect differences in the gene expression profiles of different memory subsets.

Next, we compared the overall transcriptional profiles of different CD4 memory subsets and donor groups. A total of 177 genes met our conservative cutoffs for differential expression when comparing Th1 versus Th17 cells in LTBI donors (red dots in Fig. 3B). A similar number of differences were found when comparing CCR6+CXCR3+CCR4+ cells to Th1, Th17, Th2, or tetramer+ cells in LTBI donors (namely 181, 267, and 455, respectively; Fig. 3C). The same held true within HC donors (Fig. 3C). Thus, there are comparable or more differences in the transcriptional program of CCR6+CXCR3+CCR4+ cells compared with any different CD4+ T cells. A total of 1670 genes were differentially expressed in the pairwise comparisons made between different cell types and donor groups shown in Fig. 3C and 3D (for a detailed list of these genes, see the “Differentially expressed genes” table in Gene Expression Omnibus submission GSE56179). Fig. 4A shows a heat map of the expression level of these genes in the different individual samples. When grouping samples based on the similarity of their gene expression pattern by unsupervised clustering, samples from the same cell type fell in separate clusters for Th1, Th2, and Th17 cells, and samples from donors with different disease states were intermixed within those clusters. Samples from CCR6+CXCR3+CCR4+ and tetramer+ cells grouped together in one cluster, but there was a tendency of samples from LTBI donors and HC donors to separate within this cluster and for the tetramer+ samples to be more similar to samples from LTBI donors.

Given that the same donors were the source of tetramer+ and CCR6+CXCR3+CCR4+ LTBI samples, caution has to be applied when interpreting the increased similarity of TB-specific tetramer+ cells with CCR6+CXCR3+CCR4+ cells in TB-infected individuals. Still, given the significant expansion of the CCR6+CXCR3+CCR4+ subset in LTBI donors, it is possible that tetramer+ cells constitute a specialized subset of cells within the CCR6+CXCR3+CCR4+.
CCR6+CXCR3+CCR4⁺ compartment that have a differential expression pattern for a subset of genes.

**CCR6⁺CXCR3⁺CCR4⁻ cells display hallmarks of both Th1 and Th17 transcriptional programs**

A total of 357 genes were differentially expressed in Th1 versus Th17 cells when comparing groups of either HC or LTBI donors. When examining the expression profile of these genes in CCR6⁺CXCR3⁺CCR4⁻ cells, most showed a pattern similar to Th1 cells (172 genes, 48%), a sizeable fraction showed an intermediate expression level (128 genes, 36%), and comparably fewer genes displayed a pattern similar to Th17 cells (57 genes, 16%). Notably, the lineage-specific transcription factor T-bet (TBX21) of Th1 cells was upregulated in both CCR6⁺CXCR3⁺CCR4⁻ and Th1 cells compared with Th17 cells, as well as several cytotoxic factors such as granzymes A and K, perforin (PRF1), and the transcription factor EOMES (Fig. 4B). However, granzymes B and M were exclusively upregulated in Th1 cells and consistently lower in both Th17 and CCR6⁺CXCR3⁺CCR4⁻. Conversely, the lineage-specific Th17 transcription factor RORC was upregulated in both CCR6⁺CXCR3⁺CCR4⁻ and Th17 cells compared with Th1 cells along with other Th17-selective genes such as ADAM12, PTPN13, and IL17RE, the receptor for IL-17C. IKZF2 (Ikaros), however, was upregulated in Th17 (and Th2) cells but not in CCR6⁺CXCR3⁺

![Image](https://example.com/image.png)

**FIGURE 3.** The transcriptional program of TB-specific cells are conserved in the CCR6⁺CXCR3⁺CCR4⁻ compartment. (A) Mapping of short mRNA reads to genes encoding CXCR3, CCR4, and CCR6 in Th2 (red), Th1 (green), Th17 (purple), and 6⁺X3⁺4⁻ (orange) cells is shown. Dot plots show expression for each individual sample tested. Data represents means ± SEM. (B) MA plots comparing gene expression between groups of samples are shown. Geometric mean of expression between the samples (x-axis) is compared with fold change in expression between the samples (y-axis). Differentially expressed genes (red circles) were identified based on having an adjusted p of <0.05 according to the DESeq analysis and when they showed an at least 2-fold change in expression. Genes that did not meet these cutoffs were plotted in gray. (C) Number of differentially expressed genes comparing groups of samples from Th subsets within LTBI and HC donor cohorts. (D) Number of differentially expressed genes when comparing samples from the same subset between HC versus LTBI donors and when comparing tetramer⁺ cells to the different Th subsets.
FIGURE 4. Expression pattern of genes differentially transcribed between sets of samples. (A) Samples from different donors and cell types were clustered based on their similarity in gene expression in the complete set of 1670 differentially expressed genes. Dendrogram showing the sample clustering is shown on the upper right with samples from the same cell type making up the four main clusters. Disease state of the donor of each sample is indicated next to the dendogram (orange, LTBI; blue, HC). The heat map displays expression of each gene (column) as a fold change of the median expression in all samples, with blue indicating lower expression and red indicating higher expression. Selected groups of genes are shown in the heat map: 1) CCR6+CXCR3+CCR4+ signature genes (6+X3+4+2 from LTBI or HC donor being 2-fold higher or 2-fold lower than all other Th subsets from the same donor cohort). 2) Genes that are significantly different in Th1 versus Th17 samples in either LTBI or HC donors (Th1 versus Th17 signature), subdivided into three groups that show either 6+X3+4+ expression similar to Th1, 6+X3+4+ expression similar to Th17, or 6+X3+4+ expression that is intermediate between the two. (B) Mapping of short mRNA reads to genes differentially expressed in 6+X3+4+(orange), Th1 (green), Th17 (purple), Th2 (red), and tetramer+ (brown) cells is shown. Dot plots show expression for each individual sample tested. Data represents means ± SEM.
CCR4− and Th1 (Fig. 4B). Overall, this confirms that CCR6+ CXCR3+CCR4− cells show hallmarks of both Th1 and Th17 expression and that within the signature genes differentiating Th1 cells from Th17 cells, the expression pattern more closely resembles that of Th1 cells.

**CCR6+CXCR3+CCR4− cells selectively express genes associated with TB susceptibility and enhanced T cell persistence**

All previously published analyses of the transcriptional program of CCR6+CXCR3+CCR4− cells have focused on candidate genes of interest, most of which were known to play a role in T cell lineage development, such as RORC and T-bet. In this study, we analyzed in an unbiased fashion which genes are expressed differentially in CCR6+CXCR3+CCR4− cells as compared with the conventional CD4 memory subsets: Th1, Th17 and Th2 cells. We considered a gene to be differentially expressed in CCR6+CXCR3+CCR4− cells when its median expression level was consistently 2-fold higher (or lower) than its median expression level in either Th1, Th17, or Th2 cells. We included genes when they met these criteria in either LTBI or HC donors. A total of 412 genes met these criteria, with 203 of them increased and 209 decreased in the CCR6+CXCR3+CCR4− subset, as compared with the other T cell subsets (Fig. 4). Pathway analysis of CCR6+CXCR3+CCR4−–specific upregulated genes showed enrichment of genes related to cytokine/receptor interactions (i.e., BAFF [CD117, c-Kit], IL12RB2 [CD257, TNFSF13B]) (Fig. 4B). The increased expression of genes involved in cell survival and proliferation (i.e., BAFF, MDR1 (ABCB1), and KIT) suggests that the CCR6+CXCR3+CCR4− cells may represent a highly persistent memory population. Furthermore, other genes with increased expression (CCR2 and IL12RB2) have been linked to TB susceptibility (22, 23), supporting the notion that CCR6+CXCR3+CCR4− cells are important for control of TB infection.

The list of genes downregulated in CCR6+CXCR3+CCR4− cells includes TIGIT (Fig. 4B), a surface protein that has T cell–intrinsic regulatory inhibitory function. Impairment of this function is associated with increased T cell persistence and immunoreactivity (24). Also, CCR6+CXCR3+CCR4− cells have significantly lower expression of ThPOK (Fig. 4B), the loss of which has been described to result in derepression of aspects of the gene expression program of the CD8+ CTL lineage, resulting in cytotoxic activity of CD4+ T cells (25). Overall, CCR6+CXCR3+CCR4− cells display lineage-specific signatures of both Th1 and Th17 cells, but they also have a unique gene expression program, including genes associated with susceptibility to TB, enhanced T cell activation, enhanced cell survival, and induction of a cytokine toxic program akin to CTL cells, suggesting a highly potent and multifunctional T cell subset.

**CCR6+CXCR3+CCR4− cells produce a broad spectrum of cytokines upon activation**

As shown in Fig. 1, upon stimulation with T cell epitopes from TB, CCR6+CXCR3+CCR4− cells from LTBI donors produced IFN-γ, TNF-α, and IL-2. To more broadly examine the functional profile of these cells, we stimulated CCR6+CXCR3+CCR4− cells from three LTBI donors with PMA/ionomycin and compared their transcriptional profile to resting CCR6+CXCR3+CCR4− cells. We specifically examined change of expression for a panel of 52 cytokines including all that have been previously reported to be produced by human T cells in an epitope-specific manner (26). Fig. 5 shows all cytokines in the panel that showed a >3-fold induction of expression after PMA/ionomycin stimulation. As expected, the production of IFNG, TNF, and IL2 could reliably be detected also at the transcript level. Additionally, a large number of cytokines were produced upon stimulation by CCR6+CXCR3+CCR4− cells, namely CSF1/2, CCL3/4, GZMB, IL6/17A/22, CXCL9, and VEGFA. CSF1 (M-CSF) and CSF2 (GM-CSF) play a major role in differentiation, survival, and enhancing the antimicrobial activity of macrophages (27, 28). CSF-1 has also been shown to play a role in the adaptive immune response against TB (29). Interestingly, mutations in the CCL4 (30) and IL22 (31) loci have been associated with increased susceptibility to TB. Overall, these data reinforce that CCR6+CXCR3+CCR4− cells are capable of producing a broad spectrum of cytokines that contribute to their ability to contain LTBI.

**Protein expression pattern of CCR6+CXCR3+CCR4− signature genes**

Next, we determined whether the unique transcriptional profile of CCR6+CXCR3+CCR4− cells was reflected in a similar expression profile at the protein level. CD4+ cells from HC and LTBI donors were examined for the surface expression of CCR2 and KIT. Both markers were expressed at significantly higher levels in CCR6+CXCR3+CCR4− cells (CCR2, 58.4%; KIT, 7.1%) compared with the other CD4 memory subsets: Th1 (CCR2, 58.4%; KIT, 7.1%) compared with the other CD4 memory subsets: Th1 (CCR2, 27.2%; KIT, 1.0%), Th17 (CCR2, 31.7%; KIT, 2.0%), and Th2 (CCR2, 12.4%; KIT, 0.6%) (Fig. 6A). The expression of CCR2 and KIT was also investigated in TB-specific cells by costaining cells with the DRB5*01:01 CFP10 tetramer (Fig. 6B). Interestingly, most TB epitope–specific cells expressed CCR2 (92.0%), but were negative for KIT (94.9%) (Fig. 6C). Selection of CCR2+ cells within CCR6+CXCR3+CCR4− cells results in an ~45% increase of tetramer+ and thus TB-specific cells (Fig. 6D). The exclusion of KIT+ cells had only a minor effect, with a 2.3% increase. Thus, the transcriptional profile pinpointed additional markers expressed by the CCR6+ CXCR3+CCR4− T cell subset and at the same time suggested that
FIGURE 6. The transcriptional profile of the CCR6+CXCR3+CCR4+ subset is reflected by a similar expression profile of proteins. (A) CD4+ T cells were stained for CCR2 and KIT and expression was compared between different subsets. Top panel, CCR6+ versus CCR6−; bottom panel, 6′X3′4+. Th1, Th17, and Th2. Each dot represents one donor; median ± interquartile range is indicated. ⋆p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, unpaired one-tailed t test. (B) CCR2 (top) and KIT (bottom) expression in tetramer+ CCR6+CXCR3+CCR4+ cells (black dots) compared with CCR6−CXCR3−CCR4− (gray dots) is shown. (C) Percentage of tetramer+ CCR6+CXCR3+CCR4+ cells expressing CCR2 and KIT is shown. Data represent median ± interquartile range from three donors. (D) Percentage increase in tetramer+ cells compared with CCR6+CXCR3−CCR4− when CCR2+ and/or KIT+ is included in the staining panel. Data represent median ± interquartile range from three donors.

Discussion

Previous studies of TB-specific transcriptional signatures used whole blood rather than sorted T cell populations (32–37). Although useful for diagnostic purposes, for example, the mechanistic interpretation of the appearance of a new transcriptional signature in whole blood is complicated in that it does not distinguish between changes in gene expression in the same cells from changes in composition of cells in the blood. In this study, we show that, strikingly, the gene expression profile in different T cell subsets is essentially unchanged between healthy and latently infected individuals. However, in latently infected donors there is an expansion of the particular set of memory T cells that are TB specific. Thus, differences in CD4 T cell gene expression between LTBI and healthy donors are primarily a result of a change in the relative frequency of different CD4 subsets.

CD4+ memory T cell subsets are defined by the coordinate expression of selected cytokines, chemokine receptors, and transcription factors. The well-characterized subsets Th1, Th2, and Th17 can be distinguished based on their expression of CXCR3, CCR6, and CCR4 (38). In this study, using these chemokine receptors as markers we have characterized a subset of cells that express CXCR3 and CCR6 but not CCR4. Similar cells had been previously shown to express the hallmark transcription factors of both Th1 and Th17 cells, namely T-bet and RORC (3, 6, 10), and have consequently been referred to as Th1-coexpressing CCR6, Th17.1, Th1Th17, Th17/Th1, and Th1/17 cells (2, 5–9). However, our in-depth analysis of the transcriptional signature of CXCR3+ CCR6+CCR4− cells suggests that they are associated with a characteristic transcriptional profile that sets them apart from both Th1 and Th17 cells.

Our original interest in CCR6+CXCR3+CCR4− cells was triggered by our finding that TB-specific CD4+ T cells in LTBI donors fall nearly exclusively into this subset. These cells are remarkable in that they can easily be detected directly ex vivo owing to their ability to mount a strong multifunctional response to their cognate Ags. Multiple lines of evidence suggest that TB-specific memory cells are necessary for the often life-long containment of TB in latent infection (39–42). Adding to this evidence, we found in the present study that the number of cells in the CCR6+CXCR3+CCR4− subset is greatly increased in LTBI (and by definition asymptomatic) donors. This study was completed in a non–TB-endemic population. Ongoing studies include a larger cohort from different ethnicities, locations, disease states, and BCG vaccination status. This will provide answers pertaining to this subset and transcriptional signatures in patients from an endemic area or with different disease states. Our transcriptional analysis revealed that CCR6+CXCR3+CCR4− cells preferentially express CCR2 and IL-12 receptor and upon activation produce large amounts of CCL4 and IL-22, all of which have been implicated in higher susceptibility to TB infection (22, 23, 30, 31). Understanding the characteristics of CCR6+CXCR3+CCR4− cells that provide them with the ability to contain TB infection should in turn provide better correlates of efficacy for TB vaccine development, which are currently lacking, and might also suggest specific pathways that can be exploited for development of antimycobacterial drugs.

Previous reports on CCR6+CXCR3+CCR4− cells (or cells sorted on some but not all of the markers CXCR3+, CCR6+, and CCR4−) have shown that such cells can produce IL-17 upon in vitro expansion (3, 5, 8). We did not detect IL-17 production of...
TB-specific CCR6+CXCR3+CCR4+ cells upon ex vivo Ag-specific stimulation. The lack of ability to detect TB-specific IL-17–producing CCR6+CXCR3+CCR4+ cells is in agreement with a previous study (3) in which CCR6+CXCR3+CCR4+ cells stimulated with TB protein extract (PPE) for 5 d did not produce IL-17, whereas CCR6+CXCR3+CCR4+ cells stimulated with candida extract under identical conditions did. This suggests that a subset of CCR6+CXCR3+CCR4+ cells is responsible for IL-17 production, and our ability to detect some IL-17 expression in bulk CCR6+CXCR3+CCR4+ cells from LTBI donors after PMA/ionomycin stimulation is in agreement with this conclusion. Given that CCR6+CXCR3+CCR4+ cells showed a slight separation in the gene expression profile between HC and LTBI donors supports the notion that markers could be defined discriminating CCR6+CXCR3+CCR4+ cells that produce IL-17 from those that do not, with the latter being presumably enriched in LTBI donors. However, although there may be some differences between CCR6+CXCR3+CCR4+ cells in HC and LTBI donors, many more commonalities exist, especially in comparison with conventional Th1, Th2, and Th17 cells, suggesting an overarching shared transcriptional program in CCR6+CXCR3+CCR4+ cells.

Within the shared transcriptional program that distinguishes CCR6+CXCR3+CCR4+ cells from other memory subsets, several genes were indicative of CCR6+CXCR3+CCR4+ cells showing increased immune activation after prolonged stimulation. CCR6+CXCR3+CCR4+ cells express significantly higher levels of CCR2, and the TB-specific cells are almost exclusively CCR2+. CCR2 has been described as a marker of terminally differentiated T cells that is the result of multiple Ag encounters (43), in which the case of TB-specific cells is likely the outcome of chronic stimulation in LTBI donors. Furthermore, CCR6+CXCR3+CCR4+ cells lack expression of TIGIT, which has been shown to result in hyper-proliferative T cell responses (24). Finally, CCR6+CXCR3+CCR4+ cells have significantly lower expression of the master transcription factor ThPOK. Downregulation of ThPOK expression in mouse CD4 T cells coincides with chronic activation and results in loss of the Th program and the gain of a gene expression program typical of CTLs (25). CD4+ CTL cells have been described previously in the context of infectious diseases (44–46). Overall, this expression profile is consistent with the hypothesis that CCR6+CXCR3+CCR4+ cells have undergone multiple rounds of Ag stimulation and that in course overcome intrinsic barriers that normally reduce the responsiveness of T cells, making them effective controllers of persistent or recurrent infections.

CCR6+CXCR3+CCR4+ cells are persistently present in LTBI donors in large numbers. This could be the result of increased longevity of these cells, which is consistent with the observed gene expression signature, as CCR6+CXCR3+CCR4+ cells selectively express MDR1. MDR1 is associated with survival and longevity of cells (47, 48). Also, KIT is expressed significantly higher on the CCR6+CXCR3+CCR4+ cells as compared with all other T cell subsets and is associated with enhanced cell survival (5), as is the transcription of BAFF (49). Alternatively, the persistent presence of CCR6+CXCR3+CCR4+ cells could also be the result of them being constantly replenished from a yet-to-be-identified progenitor population.

In reviewing the literature on CCR6+CXCR3+CCR4+ cells and genes associated with their unique transcriptional program, several reports implicated these cells and genes in chronic inflammatory autoimmune diseases such as rheumatoid arthritis and Crohn’s disease (5, 7, 9, 50). The exact properties that make CCR6+CXCR3+CCR4+ cells desirable to control infections may make them particularly harmful as disease-causing cells in autoimmune and other inflammatory diseases. Thus, the genes discovered in the present study as uniquely associated with CCR6+CXCR3+CCR4+ cells may not only be useful to better identify what genes should be induced by vaccination, but also what genes could potentially be targeted to treat autoimmunity and inflammation.

The origin of CCR6+CXCR3+CCR4+ cells remains to be defined. A previous study of T cells enriched in the joints of rheumatoid arthritis patients showed that these cells have a CCR6+CCR4+ phenotype and are derived from conventional Th17 T cells, as assessed by a shared TCR repertoire (7). Furthermore, the authors showed that Th17 cells can be converted in vitro to this phenotype by culturing them in the presence of IL-12, as was also previously shown (9). This suggests that there is a pathway for Th17 cells to differentiate toward the multifunctional CCR6+CXCR3+CCR4+–CTL-like phenotype. Other studies suggest that CCR6+CXCR3+CCR4+ cells may also arise from Th1 cells (8). It therefore remains to be seen whether the CCR6+CXCR3+CCR4+ cells are a separate lineage or similar to the ThPOK+ CD4+ T cells in mice, representing an advanced state of differentiation for cells of different origin, including both Th1 and Th17 cells.

In conclusion, this study describes the transcriptional signature of CCR6+CXCR3+CCR4+ cells to an unprecedented level of detail. Our results suggest that CCR6+CXCR3+CCR4+ cells are important in controlling chronic/laten infections and also play a role in pathogenesis and drug resistance of autoimmune diseases. Furthermore, they represent a persistent human T cell subset and thus may be important to understanding mechanisms of long-term immune memory and vaccine responses. These cells and their transcriptional signature may be exploited to improve diagnosis, characterization, and treatment of not only TB patients but ultimately also patients with inflammatory diseases.

Disclosures
The authors have no financial conflicts of interest.

References


27. 2012. Detectable changes in the blood transcriptome are present after two weeks of antituberculosis therapy. PLoS ONE 7: e46191.


Supplementary figure 1. Gating strategy for phenotyping and cell sorting.

Cells were gated according to schematics. Sorted CD4⁺CD3⁺DUMP CD25⁻ subsets are indicated by numbers; 1) tetramer⁺, 2) naïve (CD45RA⁺CCR7⁺CCR6⁻CXCR3⁻CCR4⁻), 3) CCR6⁺CXCR3⁺CCR4⁻, 4) Th17 (CCR6⁻CXCR3⁻CCR4⁻), 5) Th1 (CCR6⁻CXCR3⁺CCR4⁻), and 6) Th2 (CCR6⁻CXCR3⁻CCR4⁺). DUMP includes CD8, CD14, CD19 and Live/Dead distinguisher.
Supplementary figure 2. Phenotype of TB-epitope-specific CD4 T cells.

Dot plots from each individual donor tested. Plots are gated on CD4$^+$ naïve or CD4$^+$ memory T cells, according to CD45RA and CCR7 expression, and the numbers indicate the percentage of tetramer$^+$ cells from each cell population (A). Scatter plot of the proportion of tetramer$^+$ memory vs. naïve cells in LTBI (n=5) and HC (n=6) donors. Each dot represents one donor, median ± interquartile range is indicated. Mann Whitney test, **, p<0.01 (B).
Supplementary table I. Peptide pool for cytokine production profiling.

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