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Induced Sézary Syndrome PBMCs poorly express immune response genes up-regulated in stimulated memory T cells

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Abstract

Background—Dysfunctions in memory T cells contribute to various inflammatory autoimmune diseases and neoplasms. We hypothesize that investigating the differences of genetic profiles between resting and activated naïve and memory T cells may provide insight into the characterization of abnormal memory T cells in diseases, such as Sézary syndrome (SS), a neoplasm composed of CD4⁺ CD45RO⁺ cells.

Objective—We determined genes distinctively expressed between resting and activated naïve and memory cells. Levels of up-regulated genes in resting and activated memory cells were measured in SS PBMCs, which were largely comprised of CD4⁺ CD45RO⁺ cells, to quantitatively assess how different Sézary cells were from memory cells.

Methods—We compared gene expression profiles using high density oligo-microarrays between resting and activated naïve and memory CD4⁺ T cells. Differentially expressed genes were confirmed by qRT-PCR and immunoblotting. Levels of genes up-regulated in activated and resting memory T cells were determined in SS PBMCs by qRT-PCR.

Results—Activated memory cells expressed greater numbers of immune-mediated genes involved in effector function compared to naïve cells in our microarray analysis and qRT-PCR. Nine out of 14 genes with enhanced levels in activated memory cells had reduced levels in SS PBMCs ($p < 0.05$).

Conclusions—Activation of memory and naïve CD4⁺ T cells revealed a diverging gap in gene expression between these subsets, with memory cells expressing immune-related genes important for effector function. Many of these genes were markedly depressed in SS patients, implying Sézary cells are markedly impaired in mounting immune responses compared to memory cells.

Keywords

T cells; activation; memory; CD4/helper T cells; Sézary Syndrome

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INTRODUCTION

The transformation of naïve to memory T cells leads to the development of a focused antigen-specific response from the immune system. Memory T cells lay the foundation for immunological memory against familiar antigens. Memory cells have distinct advantages over naïve cells because they have lower activation thresholds [1-3], decreased dependence on co-stimulation [4-6], broader cytokine profiles [7-9], and increased effector functions [10-11]. However, when immunological memory goes awry, disease processes develop [12-14]. Investigating differences between memory T cells and naïve T cells may shed light on the pathogenesis of many T cell-mediated diseases. Specifically, identifying gene expression differences between naïve and memory T cells may reveal new characteristics of de-regulated memory T cells, such as those in Sézary syndrome.

Dominated by the presence of Sézary cells, Sézary syndrome (SS) is a leukemic form of cutaneous T cell lymphoma (CTCL). While Sézary cells appears to share phenotypic features of normal memory cells including CD45RO and CD4 expression [15-16], they also appear to deviate from their benign counterparts. We have previously demonstrated that the malignant T cell in these SS patients may display characteristics of regulatory T (T_{reg}) cells because of their inability to induce cytokines when stimulated [17]. The extent of the difference between Sézary and normal memory cells is unknown, and measuring the Sézary cell expression of genes up-regulated in resting and activated memory cells compared to naïve cells would help assess this contrast. Furthermore, looking at these genes whose levels are distinctly enhanced in memory cells in SS patients could yield valuable information about the evolution of a Sézary cell from a memory cell. Potentially, some of these genes could be over-expressed by Sézary cells, represent novel biomarkers for diagnosis, and may be intricately involved in disease pathogenesis.

Gene microarrays have provided new insights into identifying distinct gene expression patterns that highlight differences in cell types and characterize gene expression signatures [18]. Microarrays provide a high through-put approach to profile the entire transcriptome for the discovery of novel genes that are functionally important. Approaches to profile gene expression include cDNA arrays and oligonucleotide arrays, with high density oligonucleotide arrays providing the advantage of analyzing all potential genes in a cell population.

To better understand differences between memory and naïve cells, we employed high density oligonucleotide microarrays (Affymetrix GeneChip Human Genome U133 Plus 2.0 Array) to compare gene expression profiles in resting and stimulated naïve T cells, and resting and stimulated memory T cells. The gene expression pattern may provide insight into the molecular basis for T cell memory. We identified lists of genes that were different by at least five-fold in these subsets of T cells. These gene lists can be clustered based on gene ontology. Furthermore, we sought to confirm their increased expression at the transcriptional and translational level by qRT-PCR and Western blots, respectively. We then measured resting and activated SS peripheral blood mononuclear cell (PBMC) expression of the confirmed up-regulated genes in activated and resting memory cells. While this approach could shed more light into the development of memory cells from naïve cells, it also gives us new insight into the evolution of a malignant Sézary cell from a memory cell.

MATERIALS AND METHODS

Patients

After informed consent was given, blood samples were obtained from 11 normal volunteers, and 7 SS patients, whose characteristics were summarized in Table 1. Normal volunteers were recruited from the American Red Cross, and both normal controls and SS patients were recruited from the Henry Ford Hospital Outpatient Clinics. All provided informed written consent. The research study was approved by the Henry Ford Hospital Institutional Review Board, whose guidelines are set by the Declaration of Helsinki. The diagnosis of Sézary syndrome was given to patients with an absolute Sézary cell count of $>1,000$ cells/mm³, which is an International Society for Cutaneous Lymphoma criterion [19]. The study was approved by the Institutional Review Board of Henry Ford Hospital.

Antibodies

Purified T cell populations were labeled with directly conjugated monoclonal antibodies (Phycoerythrin (PE)-conjugated mouse anti-human chemokine (C-C) motif receptor 6 (CCR6) (BD Pharmingen, San Jose, CA), phycoerythrin cyanin 5 (PE-Cy5)-conjugated mouse anti-human CD4 (Immunotech, Marseille, France), fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD45RA (BD Pharmingen), and PE-conjugated mouse anti-human CD45RO (BD Pharmingen)) for flow cytometry analysis. Protein was labeled with the following primary antibodies for detection by immunoblot analysis: goat anti-human neuroepithelial cell transforming gene 1 (NET1) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-human neuropilin and tolloid-like-2 (NETO2) antibody (R&D systems, Minneapolis, MN), mouse anti-human tumor necrosis factor superfamily, member 11 (TNFSF11) antibody (R&D Systems), goat anti-human tumor necrosis factor receptor superfamily, member 18 (TNFRSF18) antibody (R&D Systems), and goat anti-human actin (Santa Cruz Biotechnology). Secondary antibodies (HRP-conjugated goat-anti-mouse IgG (Pierce Biotechnology, Rockford, IL), and HRP-conjugated mouse anti-goat IgG (Santa Cruz Biotechnology)) were also employed for immunoblot analysis.

Isolation and stimulation of memory and naïve CD4⁺ T cells from peripheral blood

Blood samples underwent Ficoll separation to obtain peripheral blood mononuclear cells (PBMCs), as previously described [17]. Human memory (CD45RO⁺) and naïve (CD45RA⁺) CD4⁺ T cells were isolated from PBMCs of normal volunteers using negative selection as recommended by the manufacturer (StemCell Technologies, Vancouver, BC, Canada). The isolated CD4⁺ CD45RA⁺ and CD45RO⁺ T cell subsets were greater than 90% pure, as determined by FACS analysis using surface molecules. CD4⁺ CD45RO⁺ and CD45RA⁺ T cells were cultured overnight at 37°C in a 5% CO₂ humidified incubator. These cells were stimulated with 250 ng/mL of phorbol 12-myristate 13-acetate (PMA) (#524400, Calbiochem, La Jolla, CA) and 10 µM of the calcium ionophore A23187 (Calbiochem), and cultured for two and six hours. The untreated controls were incubated in RPMI 1640 medium with L-glutamine (Invitrogen, Carlsbad, CA), 10% heat-inactivated fetal calf serum (Sigma, St. Louis, MO), and 1% penicillin/streptomycin (5000 units/mL and 5000 µg/mL, respectively, Invitrogen).

Flow cytometry studies

The purified cells were co-stained with directly-conjugated primary antibodies mentioned above for 30 minutes, spun at 1700 rpm for 5 minutes, and re-suspended in 500 µLs of 1X Hanks buffer. Analysis of flow cytometry samples was performed on BD LSR flow

cytometry instrument (Becton Dickinson, Franklin Lakes, NJ), and interpreted using CellQuest software.

RNA isolation and reverse transcription

Cells were lysed using Trizol (Invitrogen) according to the manufacturer's instructions. 15-20 μ L of DEPC-treated water was added to the precipitated RNA, which was quantified by spectrophotometry. Reverse transcription was performed with 1-3 μ g of RNA, oligo dT primers, and 0.5 μ L of Superscriptase II, according to the manufacturer's directions (Invitrogen).

Microarray analysis

In vitro transcription of cDNA from a total of three pairs of memory and naïve cells from three normal human volunteers produced biotinylated cRNA using GeneChip IVT labeling kit (Affymetrix, Santa Clara, CA). Biotinylated cRNA was fragmented in 5X fragmentation buffer at 94°C for 35 minutes, and hybridized to HG U133 Plus 2.0 GeneChip microarrays (Affymetrix) for 16 hours at 45°C. The hybridized probe array was then washed and stained on the GeneChip Fluidics Station 450 (Affymetrix), and scanned using GeneChip Scanner 3000 (Affymetrix).

Microarray analysis and clustering of data were performed using GeneSpring version 7.3.1 (Agilent Technologies, Santa Clara, CA). We examined 54,675 transcripts that were common to both arrays, and GeneSpring version 7.3.1 produced quantifiable levels of gene expression for these genes based on signal intensities. The raw data were expressed as logs of ratio, with lower limits of 0.01 and upper limits of 100. After the values below 0.01 were converted to 0.01 (floor method), the data were median normalized. Genes whose signals were predominantly absent or under the noise level were not considered for analysis. Two-way ANOVA using the parameters of time (0, 2 and 6 hours) of activation and cell type (naïve or memory) was performed to determine statistically significant differences between T cell subsets. The Benjamini-Hochberg (BH) false discovery rate was utilized as multiple testing correction. A self-organizing map defining 9 clusters was performed using 100,000 iterations and a neighborhood radius of 4. Genes showing differential expression in each memory versus naïve cell sample set of at least five-fold were selected for further analysis of functional enrichment. Hierarchical clustering using Pearson correlation as a similarity measure was executed to group genes and T cell subsets with congruent expression patterns. Semi-supervised hierarchical clustering was performed using GeneSpring version 7.3.1. Gene ontology (GO) annotations were collected using the publicly available DAVID (Database for Annotation, Visualization, and Integrated Discovery) database (<http://david.abcc.ncifcrf.gov>). For further confirmation tests to validate gene expression, candidate genes underwent the following criteria in order to minimize false positive rates: 1) having a raw signal > 1000, and 2) having other probe sites for the same genes expressing raw signals > 1000, and 3) showing differential expression of at least five-fold in the majority of samples. We also excluded estimated sequence tags encoding cDNA clones, hypothetical proteins, and genes encoding immunoglobulins.

Quantitative reverse-transcription-PCR (qRT-PCR)

qRT-PCR analysis was carried out in 30 μ L reactions containing 0.5 μ L of cDNA, 333 μ M forward and reverse primers for CCR6, chemokine (C-X-C motif) receptor 5 (CXCR5), prostaglandin E receptor 2 (EP2), guanine nucleotide binding protein (G protein), alpha 15 (GNA15), interleukin 1 receptor, type I (IL1RI), interleukin 1 receptor, type II (IL1RII), interleukin (IL)-4, IL-5, IL-9, IL-13, IL-17, IL-21, IL-22, *Homo sapiens* v-maf musculoaponeurotic fibrosarcoma oncogene homolog (v-maf), human DNA sequence from clone RP1-108C2 on chromosome 6p12.1-21.1 containing the MCM3 gene, mucolipin-2,

NET1, NETO2, regulator of G-protein signaling 2 (RGS2), TNFSF11, and TNFRSF18 (Supplemental Fig. 1), and SYBR Green 1 master Mix which includes Taq. Applied Biosystems BI7000 machine (Applied Biosystems, Foster City, CA) with the following parameters set at five minutes at 95°C, and then 40 cycles of 95°C for 15 seconds and 60°C for one minute were used to carry out the reactions. C_T (cycle threshold) values were generated and standardized to the normalizer gene β -2-microglobulin (β 2M). C_T values were then converted to relative gene expression using the $2^{-\Delta\Delta C_T}$ formula [20].

Immunoblot analysis

Total protein from at least 10 million T cells was isolated as described previously [21]. 10 μ g of protein was denatured in 2X Laemmli buffer at 95°C, and loaded and separated on a 10% Tris glycine SDS gel. Separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA) in transfer buffer, washed in Tris-buffered saline, blocked with I-block (Tropix, Bedford, MA) for 2 hours, and incubated with the appropriate primary antibody overnight. The membrane was then washed in Tris-buffer saline, incubated with the corresponding secondary antibody for one hour, and washed again in Tris-buffer saline. The membrane was then incubated with developing solution for chemiluminescence (Pierce Biotechnology Inc., Rockford, IL), and autoradiography was performed to detect the proteins.

Statistical analysis

Analyzed data were used from quantitative real time PCR experiments. Statistical significance was determined by the student's t-test, with differences considered significant when $p < 0.05$.

RESULTS

Identification of Genes Higher in Resting Naïve or Activated Memory Cells

Memory and naïve $CD4^+$ T cells were purified from normal healthy volunteers ($n=3$) to investigate gene regulation differences between these cell types. A schematic overview of the experimental design is provided in Fig. 1. The purified naïve and memory $CD4^+$ T cells were activated using PMA/A23187, which by-passes surface signaling to study gene regulation directly. The treated cells were processed for total RNA isolation for microarray analysis (Affymetrix GeneChip Human Genome U133 Plus 2.0 microarrays) where a total of 54,675 transcripts were analyzed. To identify genes expressed differentially, we performed two-way ANOVA (time and cell type) and used the Benjamini-Hochberg (BH) method, which was used to adjust multiple comparisons, and yielded a total of 406 genes using the parameter of cell type and 13,478 genes using the parameter of time ($p < 0.05$ (BH-adjusted)). A self-organized map using a 3×3 grid with 100,000 iterations and neighborhood radius of four was generated to investigate the patterns of the expression of 406 genes found to be significantly different between cell types through two-way ANOVA analysis (Fig. 2a). To identify differentially expressed genes, we filtered gene sets using criteria that the genes were differentially expressed by at least five-fold and ten-fold between resting and activated memory and naïve T cells. Venn diagrams illustrating the overlap of genes up-regulated in the different T cell subsets at zero, two, and six hours of stimulation were produced with genes up-regulated by at least five-fold outnumbering those up-regulated by at least ten-fold by three- to five-fold (Fig. 2b).

We first investigated differences in gene expression between naïve versus memory cells. Our microarray analysis identified a total of 24 different genes that were expressed at least five-fold higher in resting naïve compared to resting memory T cells. Hierarchical clustering was performed on these genes, and yielded the gene tree and accompanying table exhibited

in Fig. 3a. Table 1 highlights the different subsets of genes higher in resting naïve cells as determined by gene ontology (GO) analysis. Specifically, the percentage of genes that clustered into the GO categories “immune system process” and “response to stimulus” and were up-regulated in resting naïve cells versus memory cells was more than 10% lower than the percentage of genes higher in resting memory versus naïve cells (Table 1).

A set of 40 different genes was found to be higher by at least five-fold in resting memory versus resting naïve cells, as summarized by the gene tree and table in Fig. 3b. In classifying the 40 genes higher in resting memory cells, the top five gene categories were identical to those characterizing genes that were up-regulated in resting naïve versus memory cells, and had similar percentages as well (Table 1).

Identification of Genes Higher in Activated Naïve or Activated Memory Cells

We next examined genes that were higher in activated naïve cells compared to memory cells. Eight genes were found to be up-regulated by at least five-fold in naïve T cells activated for two and six hours compared to their memory T cell counterparts by microarray analysis. A gene tree reflecting hierarchical clustering analysis and a table of all genes were depicted in the order of expression in Fig. 3c. Of all comparisons of T cells, genes higher in activated naïve compared to memory cells had the highest percentage of up-regulated genes in the GO category “cellular process” and the lowest percentage of up-regulated genes in the GO categories “metabolic process”, “immune system process”, and “gene expression” (Table 1).

A total of 45 distinct genes had greater than five-fold higher expression in memory T cells activated for two and six hours compared to naïve T cells stimulated for the same time. A gene tree produced from hierarchical clustering of these genes and an accompanying table were shown in Fig. 3d. Comparing activated naïve versus activated memory cells, activated memory cells had more up-regulated genes involved in the “immune system process”, “response to stimulus”, and “metabolic process” by over 10%. For activated memory versus activated naïve cells, “response to stimulus” and “immune system process” had the highest percentage of genes up-regulated compared to all other comparisons of T cell subsets (Table 1). The EASE score, a modified Fisher Exact test and an enriched p-value [22], was significantly less than 0.01 for these categories, suggesting that these genes were more specifically associated with these categories than random chance (Table 1). Upon further classification, compared to all other comparisons of T cell subsets, activated memory versus activated naïve T cells had the highest percentage of genes classified under the GO categories “immune response” (31.1%, EASE=8e-9), “response to external stimulus” (22.2%, EASE=2.7e-6), “response to stress” (17.8%, EASE=0.0044), “defense response” (24.4%, EASE=8.9e-8), “cell communication” (40%, EASE=6.3e-4), “cell activation” (6.7%, EASE=0.08), and “cytokine production” (11.1%, EASE=9e-5) (data not shown).

Confirmation of Up-regulated Genes in Resting Naïve or Memory Cells

A total of 24 and 40 distinct genes were shown to be up-regulated at least five-fold in resting naïve versus resting memory cells, and vice versa, respectively. We utilized the criteria described in the *Materials and Methods* to select candidate genes for verification of gene expression at the translational and transcriptional level. We identified one candidate gene, neuroepithelial cell transforming gene 1 (NET1), that was highly expressed in resting naïve T cells compared to resting memory T cells, and four candidate genes (chemokine (C-C) motif receptor 6 (CCR6), prostaglandin E receptor 2 (EP2), v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian) (v-maf), and regulator of G-protein signaling 2 (RGS2)), that were higher in resting memory T cells compared to resting naïve T cells.

Next we analyzed the expression of candidate genes using qRT-PCR in additional T cell samples for verification. Primers for each of these candidate genes are shown in Supplemental Fig. 1. The qRT-PCR results showed that NET1 was at least five-fold higher at the mRNA level in resting naïve than resting memory cells ($n=7$) ($p<0.05$) (Fig. 4a). Because it is not known whether NET1 is differentially expressed in resting naïve T cells at the protein level, we examined NET1 protein expression in resting and activated naïve and memory T cells. Immunoblot results verified an increased level for NET1 in resting naïve cells (Fig. 4b).

For genes expressed higher in resting memory cells, qRT-PCR analysis from paired samples of resting and activated naïve and memory cells ($n=5-8$) confirmed two out of four genes (CCR6, v-maf) that were higher by at least five-fold ($p<0.05$) (Fig. 5a). To confirm protein expression of up-regulated genes in resting memory T cells, the CCR6 gene was chosen, which has been shown previously to be present on the surface of memory T cells [23]. Flow cytometry analysis from purified resting and activated memory and naïve cells confirmed that CCR6 was higher in resting memory cells (Fig. 5b).

Confirmation of Up-regulated Genes in Activated Naïve or Memory Cells

Activated naïve versus activated memory T cells and vice versa showed at least five-fold increased expression in eight and 45 distinct genes, respectively. While none of the genes that were higher in activated naïve versus activated memory cells fit our criteria delineated in the *Materials and Methods*, there were 18 candidate genes (chemokine (C-X-C motif) receptor 5 (CXCR5), guanine nucleotide binding protein (G protein), alpha 15 (Gq class) (GNA15), interleukin 1 receptor, type I (IL1RI), interleukin 1 receptor, type II (IL1RII), interleukin (IL)-4, IL-5, IL-9, IL-13, IL-17, IL-21, IL-22, v-maf, MCM3, mucolipin 2, neuropilin and tolloid-like 2 (NETO2), regulator of G-protein signaling 2 (RGS2), tumor necrosis factor (ligand) superfamily, member 11 (TNFSF11), and tumor necrosis factor receptor superfamily, member 18 (TNFRSF18)) that were higher in activated memory T cells compared to naïve T cells.

qRT-PCR analysis confirmed 14 out of 18 genes (CXCR5, IL-1RII, IL-4, IL-5, IL-9, IL-13, IL-17, IL-21, IL-22, v-maf, NETO2, RGS2, TNFSF11, and TNFRSF18) that were up-regulated by at least five-fold in activated T cells versus naïve T cells ($n=6-9$) (Fig. 6a, Table 2). These genes showed at least five-fold up-regulation at either two and six hours, two hours only, or six hours only. All showed statistical significance at two hours and/or six hours ($p<0.05$) after stimulation. The highest fold change was seen in IL-22 in activated memory vs. activated naïve T cells. For genes up-regulated in activated memory versus activated naïve cells, we selected the genes TNFRSF11, TNFRSF18, and NETO2, which have not been previously described to be up-regulated in these cells in humans for protein analysis. Immunoblots for TNFRSF18 and NETO2 protein expression in activated memory compared to naïve cells did not show significant differences. However, immunoblot results showed that activated memory cells had higher TNFSF11 expression than activated naïve cells (Fig. 6b).

Assessment of Sézary syndrome PBMCs using genes highly expressed in memory versus naïve cells

Sézary cells express markers of memory T cells [15-16]. To determine differences in gene expression in SS patients compared to normal memory cells, we performed qRT-PCR analysis on PBMCs from seven SS patients, using the list of the 15 confirmed genes that were up-regulated in activated memory versus naïve cells and resting memory versus naïve cells. Flow cytometric analysis yielded over 90% of the SS PBMCs to be CD45RO⁺ (data not shown). The expression of the aforementioned genes was compared to that of purified

memory cells from six to nine normal patients. In comparison to purified and activated normal memory cells, SS PBMCs exhibited decreased expression of at least 2.5-fold differences at two and six hours of activation in eight out of 14 genes (IL-4, IL-9, IL-13, IL-17, IL-21, IL-22, v-maf, NETO2) ($p < 0.05$). The most profound difference in expression was found in IL-22, with SS PBMCs showing 472-fold and 630-fold lower levels than normal memory cells at two and six hours of activation. Another cytokine gene, IL-5, was down-regulated at only six hours of activation in SS PBMCs compared to normal memory cells ($p < 0.05$). Of the two genes (CCR6, v-maf) whose levels were enhanced in resting memory versus naïve cells, only CCR6 showed over four-fold diminished expression in resting SS PBMCs versus normal memory cells at statistically significant levels ($p < 0.05$) (Fig. 7).

DISCUSSION

In this study, we analyzed the transcriptomes of memory and naïve CD4⁺ T cells and identified differential regulation of a set of genes important in immune function in memory cells. Moreover, our investigation is the first to compare activated memory and activated naïve CD4⁺ T cells by oligomicroarray analysis, and the results provide a global gene expression change upon stimulation. We then used these genes up-regulated in resting and activated memory versus naïve cells as standards of measurement in order to compare differences between SS PBMCs, which were predominantly composed of CD4⁺ CD45RO⁺ T cells, and normal memory cells. The marked depression of the majority of these genes in resting and stimulated SS PBMCs compared to normal memory cells revealed a phenotype that may reflect differences between SS T cells and normal memory T cells and the pathogenesis of Sézary syndrome.

Since the vast majority of the genes analyzed did not show statistically significant differences between naïve and memory cells, this suggests that memory and naïve T cells have very similar transcriptomes. Of the potential 54,675 transcripts initially tested, only a small number of genes showed at least a five-fold difference in gene expression change between the two groups of cells at different time periods of stimulation. This finding is consistent with previous experiments looking at these T cell subsets using cDNA microarrays that analyzed a smaller number of genes [24]. From our study, a limited number of genes was differentially expressed between naïve and memory T cells that accounted for the functional phenotype. Furthermore, by stimulation we showed that some of these genes are differentially poised for activation, which may play a crucial role in effector function that is important in the memory T cell response.

Resting memory and naïve cells showed similar genetic profiles based on gene ontology analysis. The top five gene ontology categories were identical. While the percentages of genes up-regulated in the top five categories were similar in each comparison, there were differences in the specific genes between resting naïve and memory T cells, suggesting that only a few genes are important for their functional difference.

Upon stimulation, there was a growing divergence in gene expression between activated naïve and memory cells compared to their resting counterparts, highlighting a more robust immune function for memory cells. Compared to their memory counterparts, activated naïve cells expressed the fewest number of genes up-regulated at least five-fold, and activated memory cells generated over five times as many genes whose expression increased at least five-fold higher than activated naïve cells. Moreover, GO analysis of activated naïve versus activated memory cells and vice versa showed a discrepancy of gene phenotypes. Although the top two gene categories were the same in both comparisons, the two comparisons of cells showed differing characteristics, with activated naïve cells expressing more genes

specializing in “cellular process,” and activated memory cells expressing genes under the “metabolic process,” “immune system process,” and “response to stimulus” categories. This suggests that memory cells are poised to express genes important in effector function.

Activation of memory cells emphasized their ability to induce genes important in the immune response. The difference in the percentage of “immune system process” genes between activated memory versus naïve cells and vice versa was over two-fold higher than that between resting memory versus naïve cells and vice versa. Furthermore, the percentage difference of up-regulated “response to stimulus” genes for activated memory versus naïve cells and vice versa was almost two-fold higher than that between resting memory versus naïve cells and vice versa. Activated memory versus activated naïve cells also showed the highest up-regulation of genes in several subcategories related to immune function, including “immune response,” “response to external stimulus,” “response to stress,” “defense response,” and “cytokine production,” compared to all other comparisons of T cell subsets. Genes higher in memory cells augment immunological functions, and inappropriate activation of these cells may be seen in diseases where memory T cells play a role, such as autoimmune diseases or malignancies of memory T cells.

Of note, activated memory cells were found to express eight different cytokines (IL-4, IL-5, IL-9, IL-13, IL-17, IL-21, IL-22, and IL-26) that were at least five-fold higher than activated naïve cells by microarray analysis. These genes were largely responsible for the up-regulation of “response to stimulus” and “immune system process” genes in activated memory versus naïve cells. Moreover, no cytokine was found to be up-regulated greater than five-fold in the other comparisons of T cell subsets in our study. As these cytokine genes are important in the stimulation of other cells that drive the immune response, they also distinguish activated memory cells from naïve cells. In fact, five of these cytokines, IL-4, IL-9, IL-17, IL-22, and IL-26, were amongst the list of top ten up-regulated genes in activated memory cells compared to naïve cells.

For activated memory versus naïve cells, 14 out of 18 genes were verified to have higher mRNA expression of at least five-fold. The finding that 10 out of the 14 genes confirmed were classified under the “immune system process” and/or “response to stimulus” categories further strengthens the notion that immunological function varies greatly between activated memory and naïve cells. Several of these genes, including IL-17 [25], IL-21 [26], IL-4 [27], IL-5 [27], IL-13 [28], IL-22 [29], and CXCR5 [30], have been previously shown to have increased protein in activated human memory versus naïve cells.

In assessing the characteristics of the PBMCs of SS patients, we assessed the expression of the 14 genes that were distinctively enhanced in activated memory cells. Nine of these 14 genes were diminished in stimulated SS PBMCs at two and/or six hours compared to normal memory cells at statistically significant levels ($p < 0.05$). We had previously demonstrated that PBMCs from several SS patients showed defects in the ability to express multiple T_H1 cytokines, T_H2 cytokines, and T_H17 cytokines [17]. In this current study, as additional cytokines including the T_H2 cytokines IL-9 and IL-21, and T_H1 and T_H17 cytokine IL-22 were sampled, further global suppression of cytokine expression was confirmed in the SS PBMCs. These T cells do not behave as normal memory T cells, and may resemble regulatory T (T_{reg}) cells which do not express high levels of cytokines.

Previous investigations comparing cytokine levels in the PBMCs of SS patients and normal controls have implicated a T_H2 -mediated process [31-33]. However, because SS is a malignancy of $CD4^+ CD45RO^+$ cells, our study focused on cytokine mRNA expression from SS PBMCs and normal $CD4^+ CD45RO^+$ cells, instead of normal PBMCs. The extensive down-regulation of cytokine production seen in SS PBMCs compared to normal

CD4⁺ CD45RO⁺ cells supports the notion that Sézary syndrome may actually be represented by a malignant population of T_{reg} cells. In fact, malignant T cells isolated from SS patients have demonstrated T_{reg} cell-like properties by inhibiting IL-2 production and proliferation of activated T cells [34-35]. Furthermore, the global suppression of cytokine levels may potentially explain the immunosuppression that many SS patients are afflicted with and ultimately succumb to..

The suppression of other resting memory cell markers (CCR6) and activated memory cell markers (v-maf, NETO2) in SS PBMCs also imply a growing schism between the predominant CD45RO⁺ cell population in SS PBMCs and normal memory cells. While the functions of v-maf and NETO2 in T cells are unknown, CCR6 aids in the homing of lymphocytes to areas of inflamed peripheral tissue by mediating lymphocyte adhesion to endothelial cells [36-37] and chemotaxis [23, 38]. The down-regulation in CCR6 expression in these SS PBMCs hampers the ability of lymphocytes to travel to peripheral tissue such as the skin to combat tumor growth and may explain the subdued anti-tumor response in these patients.

When we also compared expression levels of the other five genes that did not show statistically significant differences between activated SS PBMCs and normal memory cells, four out of the five genes (CXCR5, IL-1RII, TNFRSF18, and RGS2) trended downward in SS PBMCs (data not shown). This prevalent downward shift of expression of many of these genes implies suppression of normal memory T cell function in the CD45RO⁺ cell population in SS patients. On the other hand, TNFSF11 levels rose in SS PBMCs compared to normal memory cells, particularly at the resting state, during which we observed a 10-fold up-regulation in TNFSF11 expression in SS PBMCs (data not shown). Levels of TNFSF11, a transmembrane protein that is important in osteoclast differentiation [39], and activates the NF- κ B, and MAPK pathways resulting in inflammatory conditions [40-42], have been noted to be up-regulated in malignancies driven by memory T cells, such as SS [43]. Further exploration by functional studies is warranted to determine whether TNFSF11 is a potential gene target involved in SS.

In summary, we investigated gene expression differences on a dynamic scale between memory and naïve CD4⁺ T cells and employed differentially expressed genes to assess differences between memory T cells and SS PBMCs, which were largely composed of CD4⁺ CD45RO⁺ cells. As microarray analysis determined genes expressed over five-fold higher in resting and activated memory versus naïve cells, and vice versa, qRT-PCR analysis confirmed the vast majority of the genes identified. Resting naïve and resting memory cells showed similar functional gene profiles, as evidenced by their similar gene ontologies. Activation by PMA and A23187 further distinguished these two subsets of T cells by revealing the enhanced immunological potency of memory cells. A reduction in expression of the majority of the genes up-regulated in resting and activated memory T cells was observed in SS PBMCs at statistically significant levels (Fig. 8). Because many of these genes have immunological functions, SS PBMCs differ from normal memory T cells in their phenotype, and these differences may provide new understanding into the development of specific T cells into the malignant Sézary cell.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- [1]. Luqman M, Bottomly K. Activation requirements for CD4+ T cells differing in CD45R expression. *J Immunol.* 1992; 149:2300–6. [PubMed: 1388189]
- [2]. Tanchot C, Lemonnier FA, Perarnau B, Freitas AA, Rocha B. Differential requirements for survival and proliferation of CD8 naive or memory T cells. *Science.* 1997; 276:2057–62. [PubMed: 9197272]
- [3]. London CA, Lodge MP, Abbas AK. Functional responses and costimulator dependence of memory CD4+ T cells. *J Immunol.* 2000; 164:265–72. [PubMed: 10605020]
- [4]. Croft M, Bradley LM, Swain SL. Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells. *J Immunol.* 1994; 152:2675–85. [PubMed: 7908301]
- [5]. Van de Velde H, Lorre K, Bakkus M, Thielemans K, Ceuppens JL, de Boer M. CD45RO+ memory T cells but not CD45RA+ naive T cells can be efficiently activated by remote co-stimulation with B7. *Int Immunol.* 1993; 5:1483–7. [PubMed: 7505109]
- [6]. Croft M. Activation of naive, memory and effector T cells. *Curr Opin Immunol.* 1994; 6:431–7. [PubMed: 7917111]
- [7]. Ehlers S, Smith KA. Differentiation of T cell lymphokine gene expression: the in vitro acquisition of T cell memory. *J Exp Med.* 1991; 173:25–36. [PubMed: 1898663]
- [8]. Kristensson K, Borrebaeck CA, Carlsson R. Human CD4+ T cells expressing CD45RA acquire the lymphokine gene expression of CD45RO+ T-helper cells after activation in vitro. *Immunology.* 1992; 76:103–9. [PubMed: 1352764]
- [9]. Armitage RJ, Macduff BM, Ziegler SF, Grabstein KH. Multiple cytokine secretion by IL-7-stimulated human T cells. *Cytokine.* 1992; 4:461–9. [PubMed: 1292630]
- [10]. Kedl RM, Mescher MF. Qualitative differences between naive and memory T cells make a major contribution to the more rapid and efficient memory CD8+ T cell response. *J Immunol.* 1998; 161:674–83. [PubMed: 9670942]
- [11]. Cho BK, Wang C, Sugawa S, Eisen HN, Chen J. Functional differences between memory and naive CD8 T cells. *Proc Natl Acad Sci U S A.* 1999; 96:2976–81. [PubMed: 10077622]
- [12]. Kuhn A, Sontheimer RD. Cutaneous lupus erythematosus: molecular and cellular basis of clinical findings. *Curr Dir Autoimmun.* 2008; 10:119–40. [PubMed: 18460883]
- [13]. Krathen MS, Fiorentino D, Werth VP. Dermatomyositis. *Curr Dir Autoimmun.* 2008; 10:313–32. [PubMed: 18460893]
- [14]. Nestle FO. Psoriasis. *Curr Dir Autoimmun.* 2008; 10:65–75. [PubMed: 18460881]
- [15]. Reinhold U, Abken H, Kukel S, Moll M, Muller R, Oltermann I, et al. CD7– T cells represent a subset of normal human blood lymphocytes. *J Immunol.* 1993; 150:2081–9. [PubMed: 7679701]
- [16]. Reinhold U, Abken H. CD4+ CD7– T cells: a separate subpopulation of memory T cells? *J Clin Immunol.* 1997; 17:265–71. [PubMed: 9258765]
- [17]. Chong BF, Wilson AJ, Gibson HM, Hafner MS, Luo Y, Hedgcock CJ, et al. Immune function abnormalities in peripheral blood mononuclear cell cytokine expression differentiates stages of cutaneous T-cell lymphoma/mycosis fungoides. *Clin Cancer Res.* 2008; 14:646–53. [PubMed: 18245523]
- [18]. Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science.* 1995; 270:467–70. [PubMed: 7569999]
- [19]. Kim YH, Willemze R, Pimpinelli N, Whittaker S, Olsen EA, Ranki A, et al. TNM classification system for primary cutaneous lymphomas other than mycosis fungoides and Sezary syndrome: a proposal of the International Society for Cutaneous Lymphomas (ISCL) and the cutaneous

lymphoma task force of the European Organization of Research and Treatment of Cancer (EORTC). *Blood*. 2007

- [20]. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻($\Delta\Delta C_T$) Method. *Methods*. 2001; 25:402–8. [PubMed: 11846609]
- [21]. Wong HK, Kammer GM, Dennis G, Tsokos GC. Abnormal NF-kappa B activity in T lymphocytes from patients with systemic lupus erythematosus is associated with decreased p65-RelA protein expression. *J Immunol*. 1999; 163:1682–9. [PubMed: 10415075]
- [22]. Hosack DA, Dennis G Jr, Sherman BT, Lane HC, Lempicki RA. Identifying biological themes within lists of genes with EASE. *Genome biology*. 2003; 4:R70. [PubMed: 14519205]
- [23]. Liao F, Rabin RL, Smith CS, Sharma G, Nutman TB, Farber JM. CC-chemokine receptor 6 is expressed on diverse memory subsets of T cells and determines responsiveness to macrophage inflammatory protein 3 alpha. *J Immunol*. 1999; 162:186–94. [PubMed: 9886385]
- [24]. Liu K, Li Y, Prabhu V, Young L, Becker KG, Munson PJ, et al. Augmentation in expression of activation-induced genes differentiates memory from naive CD4⁺ T cells and is a molecular mechanism for enhanced cellular response of memory CD4⁺ T cells. *J Immunol*. 2001; 166:7335–44. [PubMed: 11390484]
- [25]. Shin HC, Benbernou N, Esnault S, Guenounou M. Expression of IL-17 in human memory CD45RO⁺ T lymphocytes and its regulation by protein kinase A pathway. *Cytokine*. 1999; 11:257–66. [PubMed: 10328864]
- [26]. Asao H. Analysis of gamma-c-dependent cytokines-mediated immunoregulation. *Rinsho byori*. 2007; 55:51–8. [PubMed: 17319491]
- [27]. Brinkmann V, Kristofic C. TCR-stimulated naive human CD4⁺ 45RO[–] T cells develop into effector cells that secrete IL-13, IL-5, and IFN-gamma, but no IL-4, and help efficient IgE production by B cells. *J Immunol*. 1995; 154:3078–87. [PubMed: 7897199]
- [28]. Jones D, O'Hara C, Kraus MD, Perez-Atayde AR, Shahsafaei A, Wu L, et al. Expression pattern of T-cell-associated chemokine receptors and their chemokines correlates with specific subtypes of T-cell non-Hodgkin lymphoma. *Blood*. 2000; 96:685–90. [PubMed: 10887135]
- [29]. Chen Z, Tato CM, Muul L, Laurence A, O'Shea JJ. Distinct regulation of interleukin-17 in human T helper lymphocytes. *Arthritis Rheum*. 2007; 56:2936–46. [PubMed: 17763419]
- [30]. Colantonio L, Recalde H, Sinigaglia F, D'Ambrosio D. Modulation of chemokine receptor expression and chemotactic responsiveness during differentiation of human naive T cells into Th1 or Th2 cells. *Eur J Immunol*. 2002; 32:1264–73. [PubMed: 11981813]
- [31]. Vowels BR, Cassin M, Vonderheid EC, Rook AH. Aberrant cytokine production by Sezary syndrome patients: cytokine secretion pattern resembles murine Th2 cells. *J Invest Dermatol*. 1992; 99:90–4. [PubMed: 1607682]
- [32]. Saed G, Fivenson DP, Naidu Y, Nickoloff BJ. Mycosis fungoides exhibits a Th1-type cell-mediated cytokine profile whereas Sezary syndrome expresses a Th2-type profile. *J Invest Dermatol*. 1994; 103:29–33. [PubMed: 8027577]
- [33]. Rook AH, Kubin M, Cassin M, Vonderheid EC, Vowels BR, Wolfe JT, et al. IL-12 reverses cytokine and immune abnormalities in Sezary syndrome. *J Immunol*. 1995; 154:1491–8. [PubMed: 7822812]
- [34]. Krejsgaard T, Gjerdrum LM, Ralfkiaer E, Lauenborg B, Eriksen KW, Mathiesen AM, et al. Malignant Tregs express low molecular splice forms of FOXP3 in Sezary syndrome. *Leukemia*. 2008; 22:2230–9. [PubMed: 18769452]
- [35]. Heid JB, Schmidt A, Oberle N, Goerdts S, Krammer PH, Suri-Payer E, et al. FOXP3+CD25[–] tumor cells with regulatory function in Sezary syndrome. *J Invest Dermatol*. 2009; 129:2875–85. [PubMed: 19626037]
- [36]. Campbell JJ, Hedrick J, Zlotnik A, Siani MA, Thompson DA, Butcher EC. Chemokines and the arrest of lymphocytes rolling under flow conditions. *Science*. 1998; 279:381–4. [PubMed: 9430588]
- [37]. Fitzhugh DJ, Naik S, Caughman SW, Hwang ST. Cutting edge: C-C chemokine receptor 6 is essential for arrest of a subset of memory T cells on activated dermal microvascular endothelial cells under physiologic flow conditions in vitro. *J Immunol*. 2000; 165:6677–81. [PubMed: 11120783]

- [38]. Homey B, Dieu-Nosjean MC, Wiesenborn A, Massacrier C, Pin JJ, Oldham E, et al. Up-regulation of macrophage inflammatory protein-3 alpha/CCL20 and CC chemokine receptor 6 in psoriasis. *J Immunol.* 2000; 164:6621–32. [PubMed: 10843722]
- [39]. Takayanagi H. Mechanistic insight into osteoclast differentiation in osteoimmunology. *Journal of molecular medicine (Berlin, Germany).* 2005; 83:170–9.
- [40]. Darnay BG, Haridas V, Ni J, Moore PA, Aggarwal BB. Characterization of the intracellular domain of receptor activator of NF-kappaB (RANK). Interaction with tumor necrosis factor receptor-associated factors and activation of NF-kappaB and c-Jun N-terminal kinase. *J Biol Chem.* 1998; 273:20551–5. [PubMed: 9685412]
- [41]. Franchimont N, Reenaers C, Lambert C, Belaiche J, Bours V, Malaise M, et al. Increased expression of receptor activator of NF-kappaB ligand (RANKL), its receptor RANK and its decoy receptor osteoprotegerin in the colon of Crohn's disease patients. *Clin Exp Immunol.* 2004; 138:491–8. [PubMed: 15544627]
- [42]. Lerebours F, Vacher S, Andrieu C, Espie M, Marty M, Lidereau R, et al. NF-kappa B genes have a major role in inflammatory breast cancer. *BMC Cancer.* 2008; 8:41. [PubMed: 18248671]
- [43]. Booken N, Gratchev A, Utikal J, Weiss C, Yu X, Qadoumi M, et al. Sezary syndrome is a unique cutaneous T-cell lymphoma as identified by an expanded gene signature including diagnostic marker molecules CDO1 and DNM3. *Leukemia.* 2008; 22:393–9. [PubMed: 18033314]

Fig. 1.

Schematic overview of steps for T cell purification, microarray analysis, gene expression analysis, and confirmatory testing of resting and activated naïve and memory T cells. Peripheral blood mononuclear cells (PBMCs) isolated from normal patients were purified into CD4⁺ naïve (CD45RA⁺) and memory (CD45RO⁺) T cell populations by negative selection and stimulated with PMA/A23187 for zero, two, and six hours as described in the *Materials and Methods*. Microarray analysis as described in the *Materials and Methods* generated lists of genes up-regulated at least five-fold in resting and activated naïve versus memory cells and vice versa. Candidate genes were selected for qRT-PCR and immunoblot verification.

Fig. 2.

Gene expression analysis by self-organizing map and Venn diagrams of genes that were significantly different between naïve and memory cells through two-way ANOVA analysis. (a) A self-organizing map of nine clusters, 100,000 iterations, and neighborhood radius of four was produced to evaluate gene expression patterns when naïve and memory cells were stimulated with PMA/A23187 for zero, two, and six hours. (b) Venn diagrams were produced to demonstrate the number of genes differentially expressed by at least five or ten-fold between naïve (RA) and memory (RO) cells at zero, two, and six hours of stimulation. All genes had p-values < 0.05 based on two-way ANOVA analysis of variance and Benjamini-Hochberg (BH) method.

Fig. 3. Heat maps of genes expressed differentially by at least five-fold. Heat maps were produced for genes up-regulated in resting naïve (RA) cells compared to resting memory (RO) cells (a), resting RO cells compared to resting RA cells (b), activated RA cells compared to activated RO cells (c), and activated RO cells compared to activated RA cells (d). Each row of the heat map corresponds to the table row containing the gene name, GenBank accession number, mean fold change (denoted as mean fold Δ and represented by ratios of mean expression of naïve versus memory cells (a, c), or memory versus naïve cells (b, d)), and p-value. The blue bars denoted low expression, while the red bars represented high expression. p-values were obtained from two-way ANOVA analysis of variance and Benjamini-Hochberg (BH) method.

Fig. 4.

Confirmatory testing of up-regulated genes in resting naïve versus memory cells. (a) qRT-PCR was performed for neuroepithelial cell transforming gene 1 (NET1) that was up-regulated in resting naïve versus memory cells. Expression of these genes was measured against time of stimulation. Error bars represented standard error of the mean. p-values were also calculated for each time point (*: $p < 0.05$). (b) Immunoblotting analysis evaluated NET1 expression in naïve and memory cells, which were either resting (-) or activated (+) with PMA/A23187 for six hours. Actin was employed as a positive control.

Fig. 5. Confirmatory testing of up-regulated genes in resting memory versus naïve cells. (a) mRNA expression of two candidate genes, chemokine (C-C) motif receptor 6 (CCR6), and v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian) (v-maf), that were up-regulated in resting memory versus naïve cells was measured by qRT-PCR. Expression of these genes was measured against time of stimulation. Error bars represented standard error of the mean. p-values were also calculated for each time point (*: p<0.05, **: p<0.005). (b) Flow cytometry analysis of CCR6 in resting and activated naïve and memory cells was performed.

Fig. 6.

Confirmatory testing of up-regulated genes in activated memory versus activated naïve cells. (a) qRT-PCR was performed to confirm elevated mRNA expression of genes up-regulated in activated memory versus naïve cells. The temporal expressions of interleukin-22 (IL-22), neuropilin and tolloid-like-2 (NETO2), and chemokine (C-X-C motif) receptor 5 (CXCR5) that were up-regulated at both two and six hours, two hours only, and six hours only, respectively, were displayed as linear graphs. Error bars represented standard error of the mean. p-values were also calculated for each time point (*: $p < 0.05$). (b) Western blot analysis of TNFSF11 was performed in naïve and memory cells, which were either resting (-) or activated (+) with PMA/A23187 for six hours (b).

Fig. 7.

Quantitative comparison of SS PBMCs versus normal memory cells. qRT-PCR was performed to evaluate mRNA expression of 14 up-regulated genes in activated and resting memory versus naïve cells in resting and activated SS PBMCs versus normal memory cells. The temporal expressions of nine out of 14 genes that were up-regulated at both two and six hours and six hours only in normal memory cells compared to SS PBMCs at statistically significant differences ($p < 0.05$) were displayed as linear graphs. In addition, CCR6 was up-regulated at all time points of stimulation in normal memory cells compared to SS PBMCs ($p < 0.05$). Error bars represented standard error of the mean. p-values were also calculated for each time point (*: $p < 0.05$, **: $p < 0.005$).

	Resting	Activated
Memory > Naïve T cells	CCR6, maf	CXCR5, IL-1RII, IL-4, IL-5, IL-9, IL-13, IL-17, IL-21, IL-22, maf, NETO2, RGS2, TNFSF11, TNFRSF18
Naïve > Memory T cells	NET1	
Memory T cells > SS PBMCs	CCR6	IL-4, IL-5, IL-9, IL-13, IL-17, IL-21, IL-22, v-maf, NETO2

Fig. 8.

Summary of confirmatory testing of genes differentially regulated in naïve and memory T cells and comparison of these genes in SS PBMCs versus memory T cells. NET1 was up-regulated in resting naïve versus resting memory cells, two out of four genes increased in expression in resting memory versus naïve cells, and 14 out of 18 genes were up-regulated in memory versus naïve cells activated for two and six hours with PMA/A23187. Compared to normal memory cells, activated SS PBMCs showed down-regulation of nine out of 14 genes up-regulated in activated memory versus naïve cells, and resting SS PBMCs had decreased expression in CCR6, which was found to be up-regulated in resting memory versus naïve cells.

Table 1

The largest gene categories for each comparison of T cell subsets, as determined by gene ontology analysis using the DAVID database. Percentages of genes and EASE scores for each group are expressed in parentheses

Resting naïve < memory cells	Resting memory > naïve cells
24 genes	40 genes
Cellular Process (70.8%, 0.23)	Cellular Process (65.0%, 0.19)
Biological Regulation (45.8%, 0.032)	Metabolic Process (35.0%, 0.92)
Metabolic Process (37.5%, 0.90)	Biological Regulation (32.5%, 0.18)
Multicellular Organismal Process (29.2%, 0.13)	Developmental Process (22.5%, 0.20)
Developmental Process (29.2%, 0.22)	Multicellular Organismal Process (22.5%, 0.33)
Gene Expression (20.8%, 0.61)	Response to Stimulus (20%, 0.28)
Biological Adhesion (8.3%, 0.58)	Immune System Process (17.5%, 0.014)
Response to Stimulus (8.3%, 0.98)	Localization (17.5%, 0.49)
Localization (8.3%, 0.98)	Gene Expression (15%, 0.81)

Activated naïve > memory cells	Activated memory > naïve cells
8 genes	45 genes
Cellular Process (87.5%, 0.36)	Cellular Process (57.8%, 0.68)
Biological Regulation (37.5%, 0.66)	Immune System Process (35.6%, 7.6e-10)
Developmental Process (25%, 0.76)	Response to Stimulus (33.3%, 8.7e-4)
Multicellular Organismal Process (25%, 0.81)	Biological Regulation (33.3%, 0.11)
Gene Expression (12.5%, 1)	Metabolic Process (31.1%, 0.98)
Metabolic Process (12.5%, 1)	Developmental Process (28.9%, 0.015)
Localization (12.5%, 1)	Multicellular Organismal Process (26.7%, 0.093)
Response to Stimulus (12.5%, 1)	Gene Expression (17.7%, 0.60)
Biological Adhesion (12.5%, 1)	Localization (11.1%, 0.89)

Table 2

Summary of qRT-PCR results with corresponding standard errors (STerr) and p-values for verification of up-regulated genes in activated memory versus naïve cells

CXCR5	N	RO	STerr	RA	STerr	p-value
0 hrs.	7	3.450	0.644	1.226	0.362	0.014
2 hrs.	7	4.374	1.669	1.847	1.286	0.255
6 hrs.	7	9.101	2.928	0.906	0.237	0.031
IL-1RII						
0 hrs.	7	1.223	0.303	1.194	0.253	0.943
2 hrs.	7	3.830	0.892	2.108	0.499	0.125
6 hrs.	7	6.986	1.926	1.063	0.119	0.022
IL-4						
0 hrs.	8	2.274	0.315	1.596	0.470	0.254
2 hrs.	8	1724.040	490.760	226.017	184.489	0.019
6 hrs.	8	2199.392	546.556	179.582	86.603	0.008
IL-5						
0 hrs.	8	3.203	1.318	1.756	0.798	0.367
2 hrs.	8	60.505	23.327	5.012	1.569	0.049
6 hrs.	8	146.884	43.316	7.843	2.964	0.015
IL-9						
0 hrs.	8	2.456	0.955	1.530	0.328	0.384
2 hrs.	8	1.406	0.422	1.163	0.281	0.639
6 hrs.	8	11.028	3.117	1.545	0.562	0.019
IL-13						
0 hrs.	7	0.437	0.106	3.150	2.285	0.280
2 hrs.	7	2428.185	794.518	122.538	65.693	0.027
6 hrs.	7	1287.007	389.222	77.285	48.979	0.021
IL-17						
0 hrs.	9	2.340	1.003	1.887	0.515	0.695
2 hrs.	9	3271.381	877.112	47.303	13.947	0.006

CXCR5	N	RO	STErr	RA	STErr	p-value
6 hrs.	9	7346.671	1873.388	97.894	31.481	0.005
IL-21						
0 hrs.	9	4.675	1.313	1.644	0.556	0.058
2 hrs.	9	8890.591	1627.479	835.689	210.633	0.001
6 hrs.	9	10693.888	2390.769	2079.919	817.887	0.007
IL-22						
0 hrs.	7	0.755	0.505	2.445	1.200	0.230
2 hrs.	7	4776.517	1322.162	60.451	15.882	0.012
6 hrs.	7	11284.588	3680.340	119.187	29.820	0.023
Maf						
0 hrs.	8	5.929	1.212	1.177	0.231	0.005
2 hrs.	8	10.181	1.832	0.583	0.102	0.001
6 hrs.	8	16.896	2.840	0.819	0.183	0.001
NETO2						
0 hrs.	8	3.330	0.577	1.551	0.584	0.048
2 hrs.	8	10.612	2.319	1.426	0.182	0.005
6 hrs.	8	58.361	17.299	32.324	24.137	0.397
RGS2						
0 hrs.	8	5.159	1.612	1.320	0.398	0.050
2 hrs.	8	28.167	6.029	4.599	1.870	0.005
6 hrs.	8	35.943	12.251	5.491	2.841	0.043
TNFSF11						
0 hrs.	8	2.712	0.645	1.910	0.939	0.495
2 hrs.	8	26.364	7.024	4.319	0.877	0.016
6 hrs.	8	40.024	7.888	5.335	1.595	0.003
TNFRSF18						
0 hrs.	6	1.229	0.191	1.602	0.779	0.659
2 hrs.	6	20.400	4.499	3.764	0.561	0.014
6 hrs.	6	45.300	9.099	4.992	0.910	0.007