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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

Kebin Liu,* Yu Li,* Vinayakumar Prabhu,† Lynn Young,† Kevin G. Becker,‡ Peter J. Munson,† and Nan-ping Weng1*

In an attempt to understand the molecular basis for the immunological memory response, we have used cDNA microarrays to measure gene expression of human memory and naive CD4+ T cells at rest and after activation. Our analysis of 54,768 cDNA clones provides the first glimpse into gene expression patterns of memory and naive CD4+ T cells at the genome-scale and reveals several novel findings. First, memory and naive CD4+ T cells expressed similar numbers of genes at rest and after activation. Second, we have identified 14 cDNA clones that expressed higher levels of transcripts in memory cells than in naive cells. Third, we have identified 135 (130 known genes and 5 expressed sequence tags) up-regulated and 68 (42 known genes and 26 expressed sequence tags) down-regulated cDNA clones in memory CD4+ T after in vitro stimulation with anti-CD3 plus anti-CD28. Interestingly, the increase in mRNA levels of up-regulated genes was greater in memory than in naive CD4+ T cells after in vitro stimulation and was higher with anti-CD3 plus anti-CD28 than with anti-CD3 alone in both memory and naive CD4+ T cells. Finally, the changes in expression of actin and cytokine genes identified by cDNA microarrays were confirmed by Northern and protein analyses. Together, we have identified ~200 cDNA clones whose expression levels changed after activation and suggest that the level of expression of up-regulated genes is a molecular mechanism that differentiates the response of memory from naive CD4+ T cells. The Journal of Immunology, 2001, 166: 7335–7344.

The phenomenon that the immune system can remember the identity of a pathogen and respond to it at an accelerated speed and with greater strength in a successive encounter is known as immunological memory and has been used as the basis for vaccination and/or immunization for over two centuries (1). At the cellular level, immunological memory is carried by memory CD4+ and CD8+ T and B lymphocytes that are derived from Ag-naive lymphocytes after antigenic activation (2, 3). The function of memory CD4+ T cells, unlike memory B (4) and CD8+ T cells (5), is to produce cytokines that in turn influence the functions of effector cells and the outcome of an immune response (6). Recent studies have identified memory and naive CD4+ T cells based on the differential expression of CD45 isoforms (CD45RA and CD45R0 for naive and memory CD4+ T cells, respectively) and other markers (7–10) and have characterized several functional differences between these two subsets of CD4+ T cells (11, 12).

The well-documented differences between human memory and naive CD4+ T cells include the requirements for activation and the magnitude of the subsequent cellular responses (13–16). Ag-naive CD4+ T cells require the engagement not only of TCR as signal 1, but also a costimulatory receptor, such as CD28, as signal 2 for a complete activation leading to proliferation and differentiation into helper T cells (15, 17–19). Stimulation with signal 1 in the absence of signal 2 may lead naive T cells into an unresponsive or anergic state (20). In contrast, memory CD4+ T cells appear to have a lower activation threshold than naive T cells. Depending on the in vitro system, memory CD4+ T cells can be either fully activated (13) or partially activated by signal 1 alone (15, 17, 21, 22). Once activated, memory and naive CD4+ T cells display quite different cellular responses (6, 23). Memory CD4+ T cells produce a broader spectrum and a greater quantity of cytokines including IL-1α, IL-2, IL-4, IL-5, IL-6, IL-17, IFN-γ, TNF-α, and GM-CSF, whereas naive CD4+ T cells produce only IL-1α, IL-2, IFN-γ, and TNF-α (24–29).

At the molecular level, information regarding the difference between memory and naive CD4+ T cell response is limited (30). With the development of DNA microarray technology, the systematic analysis of gene expression has become feasible (31, 32). Two recent reports have analyzed gene expression in murine T cells and human CD4+ T cell clones using DNA arrays (33, 34). Teague et al. (33) studied murine T cells after activation with a superantigen in vivo. They reported that resting T cells express about the same number of genes as activated T cells and that activation via superantigen induced expression of 51–280 genes in murine T cells. Rogge et al. (34) analyzed human CD4+ Th1 and Th2 clones in vitro and showed that Th1 and Th2 clones expressed different sets of genes that could in turn modulate effector functions. However, analysis of the gene expression profile of memory CD4+ T cells and its comparison with naive CD4+ T cells at the genome scale has not been reported.
HERE WE REPORT A GENOME-SCALE ANALYSIS OF GENE EXPRESSION IN HUMAN MEMORY AND NAIVE CD4+ T CELLS AFTER ANALYZING 54,768 UNIQUE CDNA CLONES. WE DEMONSTRATE THAT MEMORY AND NAIVE CD4+ T CELLS HAVE A SIMILAR PATTERN OF GENE EXPRESSION AT REST AND AFTER IN VITRO STIMULATION WITH ANTI-CD3 mAb ALONE OR WITH ANTI-CD3 PLUS ANTI-CD28 (ANTI-CD3/CD28) mAbs. AT THE INDIVIDUAL GENE LEVEL, WE HAVE IDENTIFIED 14 CDNA CLONES THAT WERE HIGHLY EXPRESSED IN MEMORY CD4+ T CELLS RELATIVE TO NAIVE CD4+ T CELLS AND ~200 CDNA CLONES Whose EXPRESSION LEVELS WERE CHANGED IN MEMORY CD4+ T CELLS AFTER IN VITRO ANTI-CD3/CD28 STIMULATION. ALTHOUGH THE mRNA LEVELS OF THE DOWN-REGULATED GENES ARE DIMINISHED RELATIVELY UNIFORMELY, THE LEVELS OF UP-REGULATED GENES ARE HIGHER IN MEMORY THAN IN NAIVE CELLS AND ARE HIGHER AFTER STIMULATIONS WITH ANTI-CD3/CD28 THAN WITH ANTI-CD3 ALONE. TOGETHER, THESE RESULTS PROVIDE A GENERAL ASSESSMENT OF ACTIVATION-IndUCED CHANGES IN GENE EXPRESSION IN MEMORY AND NAIVE CD4+ T CELLS AND SUGGEST THAT THE INCREASED EXPRESSION OF CERTAIN GENES defines MEMORY CD4+ T CELL RESPONSE.

MATERIALS AND METHODS

ISOLATION OF MEMORY AND NAIVE CD4+ T CELLS FROM PERIPHERAL BLOOD

HUMAN MEMORY AND NAIVE CD4+ T CELLS WERE ISOLATED FROM PERIPHERAL BLOOD BY IMMUNOMAGNETIC SEPARATION AS PREVIOUSLY DESCRIBED (12). IN BRIEF, BLOOD WAS OBTAINED FROM NORMAL DONORS OF THE NATIONAL INSTITUTES OF HEALTH BLOOD BANK. PBMC WERE ISOLATED BY FICOLL GRADIENT CEN trifUGATION (ORGANON TEKNIKA, DURHAM, NC), AND THEN WERE INCUBATED WITH A PANEL OF mouse mAbs, INCLUDING mAbs AGAINST CD3 (B9.8), CD19 (FMC63), CD11b (NIH11b-1), CD14 (63D3), CD16 (3G8), MHC CLASS II (IVA12), ERYTHROCYTES (GLYCOPHORIN, 10F7), AND PLATELETS (37FP7); mAbs AGAINST CD45RA (FMC71) OR CD45R0 (UCHL-1) WERE ADDED RECIPROCALLY FOR CD11b (NIH11b-1), CD14 (63D3), CD16 (3G8), MHC CLASS II (IVA12), mouse mAbs, INCLUDING mAbs AGAINST CD8 (B9.8), CD19 (FMC63), CD40, CD80, CD83, AND CD86. T CELLS FROM PERIPHERAL BLOOD WERE CULTURED AT 200,000 CELLS/ML IN RPMI 1640 MEDIUM, AND INCUBATED AT 37°C FOR 10 MIN, FROZEN ON DRY ICE, AND lyophilized TO DRYNESS. THE DRIED mRNA AND oligo(dT) Mixtures WERE DISsolved IN RESERVE TRANSCRIPTION SOLUTION CONTAINING 1X MOLoney murine leukemia virus buffer, 571 μM EACH OF dATP, dGTP, AND dTTP, 40 μg/ML [a32P]dCTP (2000 Ci/mmOL, 10 μCi/μL), AND 240 U OF MOLoney murine leukemia virus reverse transcriptase (Promega, Madison, WI) AND INCUBATED AT 42°C FOR 2 H. TO DEGRADE RNA, 39.5 μL WATER AND 12.5 μL 1 N NaOH WERE ADDED TO THE REACTION MIXTURE AND INCUBATED AT 37°C FOR 10 MIN AND THEN REpITALIZED WITH 12.5 μL 1 M Tris-HCl (pH 8.0) AND 10 μL 1 N HCl. THE UNINCORPORATED NUCLEOTIDES WERE REMOVED FROM THE PROBE BY A G-50 SPIN COLUMN (Bio-Rad, Hercules, CA). THE Labeled cDNA probes WERE HEATED AT 100°C FOR 3 MIN, QUICKLY COOLED ON ICE, AND USED IMMEDIATELY.

cDNA microarray experiments using commercial filters

THE GENE DISCOVERY ARRAY (GDA)2 human filters version 1.3 consisting of 22 × 22-cm AND ONE 11 × 22-cm FILTER WERE PURCHASED FROM INCYTE GENOMICS (ST. LOUIS, MO). THE FILTERS CONTAIN A NONREDUNDANT humAn CDNA CLONES SPOTTED IN DUPLICATE. HYBRIDIZATION WAS CONDUCTED ACCORDING TO MANUFACTURER’S INSTRUCTION. IN BRIEF, FILTERS WERE PREHYBRIDIZED IN 35 ML FOR (22 × 22-cm FILTERS) OR 17.5 ML FOR (11 × 22-cm FILTER) QUICKHYBRID HYBRIDIZATION SOLUTION (STRATAGENE, LA JOLLA, CA) AT 43°C FOR 2 H IN ROLLER BOTTLes AND HYBRIDIZED IN THE PRESENCE OF SHEARED SALMON SPERM DNA (115 μg/ML) (RESEARCH Genetics), Cot1 DNA (0.1 μg/mL) (Life Technologies), AND CDNA PROBES AT 43°C FOR 12 H. CDNA PROBES PREPARED FROM 1.8 AND 0.9 μg mRNA WERE USED FOR THE 22 × 22-cm AND 11 × 22-cm FILTERS, RESPECTIVELY. FILTERS WERE WASHED TWICE WITH 200 ML × 0.2SSC/0.1% SDS AT 22°C FOR 30 MIN, ONCE WITH 1× SSC/0.1% SDS AT 65°C FOR 30 MIN, AND TWICE WITH 0.6× SSC/0.1% SDS AT 65°C FOR 30 MIN. THE FILTERS WERE THEN EXPOSED TO PhosphorImager screens (MOlecular Dynamics, Sunnyvale, CA) FOR 20 H, AND THE IMAGES WERE COLLECTED BY A Phos- phorImager scanner (Storm 860; Molecular Dynamics). THE human Gene Filters (GF200, GF201, AND GF202) CONTAINING A TOTAL OF 16,056 CDNA CLONES IN EACH FILTER WERE PURCHASED FROM RESEARCH Genetics. HYBRIDIZATION WAS CONDUCTED ACCORDING TO THE MANUFACTURER’S INSTRUCTION AND WAS UNDER SIMILAR CONDITIONS TO THOSE DESCRIBED ABOVE. cDNA PROBES PREPARED FROM 0.6 μg mRNA WERE USED FOR EACH HYBRIDIZATION BASED ON THE SIZE OF THE FILTERS.

Custom-made cDNA microarray filters

A TOTAL OF 2,878 CDNA CLONES WERE SELECTED FOR CONSTRUCTING THE CUSTOM-MADE CDNA MICROARRAY. CDNA CLONES WERE OBTAINED FROM INCYTE Genomics AND RESEARCH Genetics AND WERE CLONED IN THIS LABORATORY. Plasmids WERE isolated FROM BACTERIA WITH AN ALKALINE lysis kit (EDGE BioSystems, Gaithersburg, MD). THE INSERT OF CDNA CLONES WERE AMPLIFIED BY PCR USING A PAIR OF COMMON PRIMERS, universal forward (5′-CTGaAGGC GATTAGGTGCCGA-3′) AND universal reverse (5′-TGAgcGGC GTAACAATTTACCCAGGAAACG-3′), PRECIPITATED WITH ETHANOL AND RESUSPENDED IN TRIS-EDTA BUFFER. THE PCR PRODUCTS WERE DENATUREd BY NOAH (0.1 N) AND SPOTTED ONTO NILON MEMBRANE (NyTran SuperCharge membrane; Schleicher & Schull, Keene, NH) FOR GMS 417 Arrayer (Affymetrix, Santa Clara, CA). THE PIN SIZE (Diameter) OF THE ARRAYER IS 300 μM, AND THE SPACE BETWEEN THE CENTERS OF TWO SPOTS IS 665 μM. EACH CDNA CLONE WAS SPOTTED IN DUPLICATE ONTO EACH MEMBRANE. THE PRINTED FILTERS WERE TREATED WITH UV CROSS-LINKING (STRATAGENE) FOR IMMOBILIZING DNA TO THE MEMBRANE.

Analysis of microarray results by P-SCAN

Image files were collected from the PhosphorImager in “gel” format and were used to construct the PhosphorImager software as described (36) AND freely available at http: //abs.cit.nih.gov/pscan. briefly, the HYBRIDIZATION spots on the image of the microarray were located, and the average intensity was determined for the pixels within a circle of fixed size around the spot. THESE NUMERICAL INTENSITIES WERE SAVED IN A FILE ALONG WITH ARray coordinates AND merged WITH the “gene list.” THE analysis steps included a normalization of spot intensity by dividing by the median spot intensity for the entire image AND THE determination of relatively over- and
underexpressed genes between two experimental conditions. For the GDA filters, a 2-fold cutoff was established to select interesting spots whose intensities have changed between two images; one of which must also show an intensity above the background level (approximated by the median intensity). Resulting lists of spots were then reviewed graphically to verify that the apparent spot intensities had, in fact, changed between the two original images.

The custom-made cDNA microarrays consisting of three filters were analyzed using the same methods as above but with the “custom array” option of P-SCAN. Each array contained two spots corresponding to each clone, an internal replicate that was used for analyzing the consistency of the results. A database of microarray data was created using Matlab (version 5.2; Mathworks, Natick, MA) for results of the custom-made microarrays. This database contained a GenBank identifier for each clone, the location on the array of each clone, and the numerical measured intensity for each experiment using these microarrays. It also contained coded information about the experiment type (experiment number, cell type, cell pretreatment, time after treatment, replicate number, internal replicate number, etc.). Using this database, it was possible to quickly test a variety of normalization strategies and to select subsets of clones that met certain criteria. An initial subset was determined by requiring that the median change in normalized intensity of each clone from two independent hybridization experiments was >1.5-fold compared with the unstimulated condition. These selected clones were then presented in a color-coded (red = intensity increase, green = intensity decrease, white = no change) array, and inconsistently colored clones (indicating inconsistent expression ratios) were removed from the study.

**Northern blot analysis**

Northern blotting was conducted as described previously (37). The probes were PCR products of selected cDNA clones and labeled by random priming using Ready to Go DNA labeling beads (Amersham Pharmacia Biotech, Piscataway, NJ) in the presence of [α-32P]dCTP (New England Nuclear, Boston, MA). Prehybridization and hybridization were conducted at 55°C for 1 and 16 h, respectively. The washing includes twice with 2× SSC/0.1% SDS at 22°C for 15 min, once with 1× SSC/0.1% SDS at 65°C for 30 min, and twice with 0.5× SSC/0.1% SDS at 65°C for 30 min. The filters were removed from the study.

**Western blot analysis**

Cells were lysed in Kyrkias lysis buffer (20 mM HEPES, pH 7.4, 50 mM β-glycerophosphate, 10 mM sodium fluoride, 1% Triton X-100, 10% glycerol, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 100 μg/ml [4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride]). Lysates from ~1×10⁷ cells were loaded to each well and separated by SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were blocked with anti-actin Ab (University of Iowa, Iowa City, IA), washed three times, and incubated with donkey anti-rabbit Ig HRP-linked whole Ab (Amersham Pharmacia Biotech) according to manufacturer’s instructions. Signals were detected using the ECLPlus detection system (Amersham Pharmacia Biotech). Prehybridization and hybridization were conducted at 55°C for 1 and 16 h, respectively. The washing includes twice with 2× SSC/0.1% SDS at 22°C for 15 min, once with 1× SSC/0.1% SDS at 65°C for 30 min, and twice with 0.5× SSC/0.1% SDS at 65°C for 30 min. The filters were stripped and probed again with anti-ZAP70 Ab (a gift from Dr. J. F. Camarillo, National Institute on Aging, National Institutes of Health, Baltimore, MD).

**Analysis of F-actin by flow cytometry**

Freshly isolated and stimulated memory and naive CD4⁺ T cells were stained with Alexa Fluor 488 phalloidin (Molecular Probes) and analyzed by flow cytometry.

**Cytokine assays**

Supernatants were collected from in vitro-stimulated memory and naive CD4⁺ T cells. IL-2, IL-4, IL-5, IFN-γ, TNF-α, and TNF-β concentrations were determined using ELISA immunoassay kit (BioSource International, Camarillo, CA, and R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**Results**

**Experimental design for gene expression profiles of human memory and naive CD4⁺ T cells**

Our strategy to assess the transcriptional nature of memory and naive CD4⁺ T cells is shown in Fig. 1. We have applied the cDNA microarray method with custom filters for the initial genome-scale screening and custom-made filters for detailed analysis of activation-induced gene expression changes. We then used conventional methods (Northern and Western blots) for confirmation of array results. The custom filters consisted of a total of 54,768 nonredundant cDNA clones that were obtained from two sources: 1) GDA human filters (GDA version 1.3; Incyte Genomics; 45,968 clones), and 2) GeneFilters (GF200, GF201, and GF202; Research Genetics; 16,056 clones including 7,166 overlapping clones with the GDA filters). The custom-made filter arrays consisted of 2,878 cDNA clones selected from either the commercial filters or genes with known immunological functions that were not on the commercial filters.

Memory and naive CD4⁺ T cells were isolated from peripheral blood of normal donors. The proliferative response of purified memory and naive CD4⁺ T cells under in vitro stimulation conditions was assessed. As expected, anti-CD3 stimulation induced a low degree of proliferation in memory but not in naive CD4⁺ T cells, and anti-CD3/CD28 induced rapid and strong proliferation in both memory and naive CD4⁺ T cells as measured by the incorporation of [3H]thymidine (Fig. 2A). The profiles of the CFSE experiment showed that memory and naive CD4⁺ T cells divided...
a maximum of six times following anti-CD3/CD28 stimulation for 5 days (Fig. 2B). Almost all memory (98.6%) and naive cells (99.8%) entered cell cycle after stimulation. However, memory cells had a greater number of cells in division 5 and 6 than naive cells (Fig. 2B).

The optimal time after in vitro stimulation for gene expression analysis was determined by the level of induced expression of two activation markers (CD69 and CD25) in memory and naive CD4+ T cells. After anti-CD3 stimulation, the percentage of CD69+ memory and naive CD4+ T cells reached plateau around 16–48 h. However, memory cells had a higher percentage of CD69-expressing cells (76.6%) than in naive (50.5%) populations at their respective peak (Fig. 2C). No CD25 expression was detected in either memory or naive CD4+ T cells after anti-CD3 stimulation (data not shown). After anti-CD3/CD28 stimulation, the kinetics of CD69 expression was similar to that after anti-CD3 stimulation, and the percentage of CD25 expressing cells approached a plateau 16 h after stimulation (Fig. 2C). Therefore, we selected 16 h after stimulation for the initial analysis of activation-induced genes in memory CD4+ T cells. Interestingly, although memory and naive CD4+ T cells exhibited a similar percentage of surface expression of CD69 and CD25 after stimulation, the intensity levels of CD69 and CD25 were higher in memory than in naive CD4+ T cells (Fig. 2D).

**Genome-scale gene expression profiles of memory and naive CD4+ T cells at rest and after in vitro stimulation**

At the genome-scale level, memory and naive CD4+ T cells expressed similar numbers of genes at rest (22 and 20%), after stimulation with anti-CD3 alone (28 and 27%), and with anti-CD3/CD28 (19 and 20% of total cDNA clones) based on a conservative estimate that the expressed gene must be at least 2-fold higher than the median intensity of all clones on the filter and clearly visible over the background. The results were derived from two independent experiments with quadruplicated data for each clone and are listed in http://www.grc.nia.nih.gov/branches/li/weng/arraydata.htm. Comparative analyses of gene expression using commercial and subsequently custom-made filters revealed that the difference in gene expression between freshly isolated memory and naive CD4+ T cells was very small and that significant changes of gene expression were found after stimulation in both memory and naive CD4+ T cells.

**Genes highly expressed in memory CD4+ T cells**

After a sequential analysis of gene expression using the commercial and the custom-made filters, we identified 14 cDNA clones (11 known genes and 3 expressed sequence tags (ESTs)) that were expressed at consistently higher levels in memory cells than in naive cells (Fig. 3A). Among the 11 known genes, CD69, cyclin-dependent kinase inhibitor 1B, and natural killer cell protein 4 are involved in T cell activation and cell cycle regulation (38–40). Integrin β5, proteoglycan 1, and annexin A1 function in cell-cell and cell-matrix interaction, which could contribute to lymphocyte migration and activation processes (41–43). The functional importance of the other genes and ESTs in memory cell function is not clear and will require further study. Upon in vitro stimulation with anti-CD3/CD28 for 16 h, the expression of four cDNA clones was up-regulated (proteoglycan 1, protein kinase inhibitor, cytochrome c, and one EST) (Figs. 3B and 4A) and three clones were down-regulated (annexin A1, cyclin-dependent kinase inhibitor 1B, and one EST) (Figs. 3B and 4B) in both memory and naive CD4+ T cells. The remaining seven clones did not change their expression.

**FIGURE 2.** Profiles of cell proliferation and activation marker (CD69 and CD25) expression in memory and naive CD4+ T cells after stimulation with anti-CD3 or with anti-CD3/CD28. A. Profiles of [3H]thymidine incorporation of memory and naive CD4+ T cells after in vitro stimulation. B. Percentages of memory and naive CD4+ T cells at a particular number of divisions after 5 days stimulation with anti-CD3/CD28. Memory and naive CD4+ T cells were stimulated in the presence of CFSE for 5 days. The CFSE profiles were collected by FACS, and analyzed by ModFit. The cell division times are indicated at the top and the percentage of cells that had divided certain times is indicated at the bottom of each figure. C. The percentage of memory and naive CD4+ T cells that express CD69 or CD25 after stimulation. D. Fluorescence intensity (FI) of CD69- or CD25-expressing memory and naive CD4+ T cells. The FI of CD69-expressing cells stimulated with both anti-CD3 and anti-CD3/CD28 for 16 h and CD25-expressing cells stimulated with anti-CD3/CD28 for 16 h are presented. The open area represents the FI of memory CD4+ T cells, and the filled area represents the FI of naive CD4+ T cells. All experiments were conducted with cells from three different donors, and the representative results from one donor are shown.
levels significantly after stimulation in both memory and naive CD4⁺ T cells (Fig. 3B).

General characteristics of activation induced changes in gene expression between memory and naive CD4⁺ T cells

After the sequential analyses using the commercial and custom-made filters, we have identified ~200 cDNA clones whose expressions are changed in memory CD4⁺ T cells after anti-CD3/CD28 stimulation (Fig. 4, A, B, and D). The reproducibility of the changes of these selected clones were analyzed between two independent experiments (Fig. 4C) and were confirmed by Northern analysis from a randomly selected 16 cDNA clones (data not shown). Among the up-regulated cDNA clones, 130 are known genes and 5 are ESTs (Fig. 4A). In general, memory cells expressed higher levels of transcripts of these up-regulated genes than did naive cells, and cells stimulated with anti-CD3/CD28 expressed higher levels of these same transcripts than cells stimulated with anti-CD3 alone (Fig. 4D). The average log-ratios of these up-regulated known genes (±SE) are 0.065 ± 0.005 (anti-CD3-treated naive cells), 0.133 ± 0.005 (anti-CD3-treated memory cells), 0.260 ± 0.007 (anti-CD3/CD28-treated naive cells), and 0.339 ± 0.006 (anti-CD3/CD28-treated memory cells).

Among the 68 activation induced down-regulated cDNA clones, 42 are known genes and 26 are ESTs (Fig. 4, B and D). In contrast to up-regulated genes, the majority of the activation down-regulated genes (70%) had a similar degree of reduction in mRNA levels in both memory and naive CD4⁺ T cells stimulated with either anti-CD3 or anti-CD3/CD28 (Fig. 4D). The average log-ratios (±SE) of these down-regulated genes are −0.219 ± 0.007 (anti-CD3-treated naive cells), −0.206 ± 0.010 (anti-CD3-treated memory cells), −0.295 ± 0.007 (anti-CD3/CD28-treated naive cells), and −0.296 ± 0.008 (anti-CD3/CD28-treated memory cells).

Functional characteristics of up-regulated genes in memory and naive CD4⁺ T cells

To further analyze the function of genes that were up-regulated after anti-CD3/CD28 stimulation, we divided them into five functional groups and a “miscellaneous” group: 1) transcriptional regulation, 2) receptor and signal transduction, 3) cytokines and receptors, 4) cell cycle, and 5) structure and metabolism (Fig. 4A).

There are several distinct factors that contribute to the transcriptional regulation in memory and naive CD4⁺ T cells. The basic transcription factors, including transcription factor 6-like 1, LSF, and SL1, may regulate the basal level of transcription during lymphocyte activation. Histone deacetylase 2 and nonhistone chromosomal proteins HMG14 and HMG17 are known for regulating chromatin structure during transcription (44), which has been reported in the regulation of cytokine gene expression (45). V-Myc, IL enhancer binding factor 2 (NF-ATp45), and NF-xB are involved in mediating signal transduction and cell proliferation (46) as well as in regulating transcription of IL-2, IL-4, and other genes involved in T cell activation and differentiation (47, 48).

The engagement of TCR and costimulatory receptors that activate tyrosine kinases and their downstream targets does not require new gene expression (49). However, a sustained activation event does require transcription of these signaling-related genes. Indeed, many activation up-regulated genes appeared to function in signaling, such as H-RYK receptor-like tyrosine kinase and HAX-1, a protein associated with the Src family tyrosine kinase substrate HS1 that functions in promoting cell survival in activated CD4⁺ T cells (Fig. 4A) (50). In addition, a large group of genes, including RhoC, nuclear GTPase, Ras-related GTP-binding protein RAGA, GTP-binding protein NGB, guanine nucleotide exchange factor, and GTP-binding protein RAB-2, were significantly induced by TCR stimulation. Interestingly, calcium/calmodulin kinase II (CAMKII), which plays a critical role in T cell activation and in generation of memory cells (51), was significantly increased after activation.

Although the majority of memory and naive CD4⁺ T cells have not entered the S phase of cell cycle after 16 h of anti-CD3/CD28 stimulation measured by [³H]thymidine incorporation, genes involved in cell division were already up-regulated when compared with resting cells. These induced genes include cyclins, cyclin-dependent kinases, DNA repair enzymes, and chromosomal proteins involved in DNA replication and chromosome segregation (Fig. 4A). In addition, genes related to cell cytokkeleton, including actin, actin-related protein 3, and β-tubulin, were also significantly increased along with genes involved in basic cellular functions and energy metabolism such as GAPDH, glycogen synthase, ATP synthase, and ribosomal proteins (Fig. 4A).

The ultimate consequence of activation of memory and naive CD4⁺ T cells is the production of cytokines, which influence the outcome of an immune response. Remarkably, we observed that both memory and naive CD4⁺ T cells expressed a large group of cytokine genes, including IL-2, IL-9, IFN-γ, pro-B cell colony-enhancing factor, and lymphotixin α (TNF-β) (Fig. 4A). The expression of IL-4 and IL-5 was extremely low and was detected by Northern blot in activated memory CD4⁺ T cells (see Fig. 6). In addition, we have observed enhanced expression of IL-1R and its accessory protein and IL-15R in both memory and naive CD4⁺ T cells after activation. Because some common cytokine receptors such as CD25 and IL-4R were not on the commercial or custom-made filters, we were not able to assess the expression status of these genes.
FIGURE 4. Gene expression profiles in memory and naive CD4^+ T cells after in vitro stimulation with anti-CD3 or anti-CD3/CD28. Genes that were up-regulated (A) or down-regulated (B) after in vitro stimulation with anti-CD3 alone or with anti-CD3/CD28 for 16 h are shown. Each colored square represents the ratio of stimulated over resting memory or naive CD4^+ T cells from one measurement of one cDNA clone. Two independent hybridization experiments were conducted with a total of six independent replicate measurement of each cDNA clone. The scale of the intensity ratio is –1:1 in logarithm value (10-fold down-regulation to 10-fold up-regulation) with green color indicating a decrease and red indicating an increase. The GenBank accession number and gene names are indicated at the right. C, The scatter plot comparing the reproducibility of up- and down-regulated cDNA clones from two independent hybridization experiments. Each axis represents the mean log-ratio value of each clone (blue dot) in memory CD4^+ T cells at rest and after stimulation with anti-CD3/CD28 from each experiment. The center blue line indicates an identical ratio value for the two independent experiments. The up-regulated clones shown in A plus ESTs (data not shown) are marked in red and the down-regulated clones shown in B plus ESTs (data not shown)
Functional characteristics of activation down-regulated genes in memory and naive CD4+ T cells

The functional roles of genes that are highly expressed in the resting T cells and down-regulated after activation have begun to be understood (52). Their functions range from prohibiting cellular proliferation to preventing apoptosis (33, 53). To facilitate further analysis, we have separated these down-regulated genes into four groups: 1) transcriptional regulation, 2) receptor and signal transduction, 3) antiproliferation, and 4) cell survival with the remaining genes placed in a “miscellaneous” group (Fig. 4B).

We identified several genes that function in prohibiting cellular proliferation, including B cell translocation gene 1 (54), IFN-induced transmembrane protein 1 (55), RhoGDP dissociation inhibitor (56), and latent TGF-β binding protein 1 (57). We also identified genes that are involved in cell survival and antiapoptosis, such as ubiquination factor E4, which is known to maintain cell viability and survival under stress conditions (58), and neuronal apoptosis inhibitory protein, which is capable of protecting motor-neuron from apoptosis (59). The expression of two important IL receptors, IL-7α (60) and IL-10β (61), were decreased as well. In addition to these functionally grouped activation down-regulated genes, a gene involved in DNA recombination and repair (Ku Ag) was also identified (Fig. 4B).

Increase of actin expression in activated memory and naive CD4+ T cells

Actin polymerization plays a critical role in TCR signaling (62). To assess changes in actin expression, we have performed Northern analysis and confirmed that mRNA levels of actin and its upstream regulator, the small guanosine triphosphatase RhoC (63), increased in both memory and naive CD4+ T cells after stimulation (Fig. 5A). To further characterize actin changes at the protein level, we examined the actin monomer and polymers in memory and naive CD4+ T cells after in vitro stimulation. Both monomer and polymerized actin were significantly increased after stimulation with anti-CD3 alone or with anti-CD3/CD28 (Fig. 5, B and C). In agreement with the levels of mRNA, memory cells expressed higher levels of actin protein than naive cells under both stimulation conditions, and anti-CD3/CD28 induced higher levels of actin than did anti-CD3 alone (Fig. 5, B and C). In memory CD4+ T cells, the levels of polymerized actin in cells were measured by FACS analysis. The polymerized actin intensity in resting naive CD4+ T cells is arbitrarily set at 1.

Memory CD4+ T cells secrete higher levels of IL-2, IL-4, IL-5, IFN-γ, TNF-α, and TNFβ proteins than naive CD4+ T cells

The essential function of CD4+ T cells is to produce cytokines and to “help” the effector cells in an immune response. Our cDNA microarray and/or Northern blot analyses showed that several lymphokine genes, including IL-2, IL-4, IL-5, IFN-γ, TNF-α, and TNFβ, were up-regulated differentially in memory and naive CD4+ T cells after stimulation (Figs. 4A and 6A). To test whether changes in the mRNA content resulted in corresponding changes in protein levels, and to determine the kinetics of cytokine production, we measured IL-2, IL-4, IL-5, IFN-γ, TNF-α, and TNFβ proteins in the supernatants of stimulated memory and naive CD4+ T cells over a 5-day period. Consistent with the mRNA expression, IL-2, IL-4, IL-5, IFN-γ, TNF-α, and TNFβ proteins were not detected in freshly isolated lymphocytes but were significantly induced after stimulation (Fig. 6, B and C). The kinetics of secreted IL-2, IL-4, IL-5, IFN-γ, TNF-α, and TNFβ were also quite different between the two subsets of T cells (Fig. 6, B and C). After anti-CD3 stimulation, all six cytokines were undetectable in the supernatant of naive cells, but low levels of IL-2, IFN-γ, TNF-α, and TNFβ were detected in the supernatant of memory cells (Fig. 6B). After anti-CD3/CD28 stimulation, both naive and memory cells secreted high levels of IL-2, IFN-γ, TNF-α, and TNFβ (Fig. 6C). However, memory cells secreted significantly greater amounts of these cytokines than did naive cells over a 5-day period. Interestingly, IL-4 and IL-5 were only detected from memory cells but not naive cells (Fig. 6C). Thus, memory CD4+ T cells should induce a stronger immune response through production of a larger quantity and greater variety of cytokines than naive CD4+ T cells.

Discussion

We have analyzed the gene expression profiles in memory and naive CD4+ T cells at rest and after stimulation using cDNA microarray technology. Here, we report an identification of 14 cDNA clones that are highly expressed in memory CD4+ T cells and of ~200 cDNA clones whose expression was changed after in vitro stimulation with anti-CD3/CD28. We present results showing that...
the expression levels of those up-regulated genes are higher in memory cells than in naive CD4⁺ T cells after stimulation, suggesting that the level of expression of these genes is a molecular mechanism that differentiates the response of memory from naive CD4⁺ T cells.

Despite recent advances in the phenotypical and functional characterization of memory CD4⁺ T cells, information regarding the molecular features of these cells is limited. The comparative analysis in this report has revealed some interesting differences as well as similarities between freshly isolated memory and naive CD4⁺ T cells. Memory cells express several genes including CD69, integrin β₂, proteoglycan 1, annexin A1, and NK cell protein 4, that are involved in activation, cell adhesion, and migration (38, 40–43). These processes are critically important for memory cell functions. In addition, memory cells also express higher levels of CDK inhibitor 1B that prevent cells from entering the cell cycle (39). Thus, these small sets of genes provide a glimpse of the delicate balance between expression of genes in activation/migration and in maintenance of the “resting” status of memory cells. Nevertheless, the difference in gene expression between freshly isolated memory and naive CD4⁺ T cells is small. There are several possible explanations that are not mutually exclusive. First, the difference between memory and naive CD4⁺ T cells is small at the gene transcript level but is significant at the protein (functional) level. Second, the current microarray method is not sensitive enough to detect subtle changes of the transcripts between memory and naive CD4⁺ T cells. It is particularly difficult to detect differences in genes that are expressed at low copy number, such as IL-4 and IL-5 genes. Third, recent studies suggest that memory and naive CD4⁺ T cells isolated based on CD45 isomers can be further divided into functional subsets (64, 65). Analysis of memory cell subsets isolated based on additional cell surface markers, such as the chemokine receptor CCR7, might reveal a greater difference in gene expression between subsets of memory and naive CD4⁺ T cells (28). Further studies with improved cDNA microarray methods are needed to determine the subtle differences in gene expression profiles using subsets of memory CD4⁺ T cells.

Memory and naive CD4⁺ T cells also share significant similarities in gene expression. A number of genes that function in maintenance of the “resting” status of T cells, such as genes involved in antiproliferation and antiapoptosis (53, 66), are expressed in freshly isolated memory and naive CD4⁺ T cells. These genes participate in transcription, signaling, antiproliferation, and cell survival. The expression of these genes was significantly down-regulated after activation, suggesting that the “resting” status of memory and naive CD4⁺ T cells is likely to be actively maintained through regulated expression of these antiproliferation and survival-related genes.

Among the cDNA clones that are highly expressed in memory CD4⁺ T cells vs naive CD4⁺ T cells, four were significantly up-regulated after stimulation with anti-CD3/CD28, suggesting that the higher expression levels of these activation-related genes/ESTs may contribute to the fast and strong response of memory CD4⁺ T cells upon stimulation. The expression kinetics of CD69 in resting and activated CD4⁺ T cells is quite interesting. Freshly isolated memory cells express CD69 mRNA yet have low to undetectable surface expression of CD69 protein. However, 16 h after stimulation, memory cells expressed high levels of CD69 protein, although the mRNA levels did not change. This suggests that CD69 mRNA has a high turnover rate (38) and that there are different mechanisms regulating CD69 mRNA transcription and protein translation.

The consequences of stimulating T cells through the TCR alone or through both TCR and costimulatory receptors have been well documented (3, 67). Here we compared quantitative and qualitative changes in gene expression in memory and naive CD4⁺ T cells stimulated with anti-CD3 alone or with anti-CD3/CD28. After anti-CD3 stimulation, both memory and naive CD4⁺ T cells down-regulated several groups of genes including antiproliferative genes and up-regulated many genes functioning in activation and proliferation. Although memory CD4⁺ T cells responded to anti-CD3 stimulation by up-regulating some activation-related genes including cytokine genes, these changes are not sufficient to induce complete activation and proliferation of memory cells.

In contrast, anti-CD3/CD28 stimulation induced significant changes of gene expression in both memory and naive CD4⁺ T cells. Further comparative analysis indicated that memory and naive CD4⁺ T cells exhibited a similar pattern of gene expression for both up-and down-regulated genes, although expression of the up-regulated genes was significantly higher in memory cells after anti-CD3/CD28 stimulation (Fig. 4D). Thus, the function of co-stimulation via CD28 can be viewed as an amplifier of activation signals that enhance the expression of those up-regulated genes. The consequences of stimulating T cells through the TCR alone or through both TCR and costimulatory receptors have been well documented (3, 67). Here we compared quantitative and qualitative changes in gene expression in memory and naive CD4⁺ T cells stimulated with anti-CD3 alone or with anti-CD3/CD28. After anti-CD3 stimulation, both memory and naive CD4⁺ T cells down-regulated several groups of genes including antiproliferative genes and up-regulated many genes functioning in activation and proliferation. Although memory CD4⁺ T cells responded to anti-CD3 stimulation by up-regulating some activation-related genes including cytokine genes, these changes are not sufficient to induce complete activation and proliferation of memory cells. In contrast, anti-CD3/CD28 stimulation induced significant changes of gene expression in both memory and naive CD4⁺ T cells. Further comparative analysis indicated that memory and naive CD4⁺ T cells exhibited a similar pattern of gene expression for both up- and down-regulated genes, although expression of the up-regulated genes was significantly higher in memory cells after anti-CD3/CD28 stimulation (Fig. 4D). Thus, the function of co-stimulation via CD28 can be viewed as an amplifier of activation signals that enhance the expression of those up-regulated genes.

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fewer or lower levels of these “resting” maintenance genes. These resting memory T cells express antiproliferation and other survival genes to maintain the “resting” status. Compared with naive cells, memory T cells may express fewer or lower levels of these antiproliferation-related genes and higher levels of activation-related genes. Stimulation through the TCR alone (anti-CD3 alone) induces down-regulation of antiproliferation and other genes that are required for maintaining the resting condition and up-regulation of genes that are required for activation. However, the expression level of those up-regulated genes is not high enough to lead to cytokine production and proliferation in naive cells and can only produce low levels of a limited number of cytokines and undergo partial proliferation in memory cells. In contrast, stimulation through the TCR and costimulatory receptor CD28 (anti-CD3/CD28) induces a similar down-regulation of antiproliferation genes but higher expression levels of up-regulated genes that are sufficient to completely activate both memory and naive CD4+ T cells. However, even in the presence of costimulatory stimulation, memory cells express higher levels of those activation up-regulated genes and secrete more cytokines than naive cells. Thus, our model proposes that activation of CD4+ T cells requires two sequential molecular events: down-regulation of genes required for maintenance of the resting status and up-regulation of genes required for activation and proliferation, and that the quantitative difference in expression levels of those activation up-regulated genes is a molecular basis for the qualitative difference between memory and naive CD4+ T cell response (Fig. 7).

This expression dosage gradient model for memory CD4+ T cell response was built on the basis of gene expression profiles of memory and naive CD4+ T cells after 16 h of stimulation. Will the gene expression dosage gradient apply at other time points during activation as well? What are the functional relationships of those genes whose expression changes after activation? Further studies of kinetic changes in gene expression as well as of memory cell subsets will be able to test our model of the memory cell response and lead to a better understanding of memory CD4+ T cells and ultimately the mechanisms of immunological memory.

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