The generation and maintenance of memory T cells is central to the development of protective immunity, as characterized by a rapid and vigorous response after the encounter with a given pathogen or antigen (1, 2). Despite the complexity of the memory T cell populations, recent studies in both mice and humans indicate that the memory T cell pool is composed of two main compartments, central memory and effector memory T cells (TCM and TEM, respectively), which are characterized by distinct homing capacities and effector functions (3, 4).

Through their expression of CC chemokine receptor (CCR) 7 and CD62L, TCM preferentially home to T cell areas of secondary lymphoid organs and display few immediate effector functions; however, they readily proliferate and differentiate to effector cells in response to antigenic stimulation. TEM that have lost the constitutive expression of CCR7 have an increased capacity to proliferate and persist in vitro. Using global gene expression profiling, single cell proteomics, and functional assays, we show that the survival of CD4+ TCM depends, at least in part, on the activation and phosphorylation of signal transducer and activator of transcription 5a (STAT5a) and forkhead box O3a (FOXO3a). TCM showed a significant increase in the levels of phosphorylation of STAT5a compared with TEM in response to both IL-2 (P < 0.04) and IL-7 (P < 0.002); the latter is well known for its capacity to enhance T cell survival. Moreover, ex vivo TCM express higher levels of the transcriptionally inactive phosphorylated forms of FOXO3a and concomitantly lower levels of the proapoptotic FOXO3a target, Bim.

Experiments aimed at blocking FOXO3a phosphorylation confirmed the role of this phosphoprotein in protecting TCM from apoptosis. Our results provide, for the first time in humans, an insight into molecular mechanisms that could be responsible for the longevity and persistence of CD4+ TCM.
express tissue-homing receptors associated with inflammation and more readily display effector functions. The current model proposes that upon reinfection, T_EM rapidly constrain pathogen invasion in inflamed peripheral tissues, whereas T_CM are rapidly activated by DCs in secondary lymphoid organs and generate successive waves of effectors able to completely eliminate the pathogen (2).

Experiments performed in mouse models suggest that T_CM have a better capacity to reconstitute the memory T cell pool and to mediate protective immunity than T_EM because of their greater capacity to proliferate and persist in vivo (5, 6). Studies in primate models show that induction of central memory CD4^+ T cells after SIV challenge correlates with prolonged survival (7), thereby highlighting the importance of gaining a better understanding of the mechanisms underlying T_CM induction and persistence for successful vaccine development. The molecular mechanisms underlying the persistence of these cell subsets are still unknown, and it remains unclear whether T_CM and T_EM use the same mechanisms to persist in the host. The long-term maintenance of memory T cells relies on the survival of individual cells and their level of homeostatic cell division to compensate for their gradual attrition through apoptosis (2, 8). Using in vivo labeling with deuterated glucose to measure the turnover of distinct subsets of CD4^+ T cells in healthy humans, Macallan et al. have shown that T_EM have a more rapid turnover than T_CM, suggesting that T_EM are being replaced at a faster rate than T_CM (9). These different turnover rates might be attributed to intrinsic differences in their susceptibility to apoptosis, although this has never been directly addressed.

The nature of the signals that ensure the persistence of T_CM is under intense investigation. Results obtained in mouse

![Figure 1. Functional and phenotypical characterization of CD4^+ T_CM and T_EM.](image)

(A) CD45RA, CD27, and CCR7 labeling profile and gating strategy for naive, T_CM, and T_EM. Percentages obtained for each population are indicated. The purity of sorted cells was consistently >95%. (B) Perforin and Gr-B expression in ex vivo T_CM and T_EM subsets. Perforin and Gr-B expression were assayed by intracellular staining. The percentages of T_CM and T_EM expressing perforin and Gr-B are indicated in each quadrant. SSC, side scatter. (C) Rab-27a protein levels in ex vivo sorted T_CM and T_EM subsets. Similar results were obtained in three independent experiments. (D) Susceptibility of T_CM and T_EM to Fas-induced apoptosis. T_CM and T_EM were sorted by flow cytometry and treated with 1.25 μg/ml of the anti-Fas antibody CH11 or 100 μg/ml etoposide for 24 h. The percentage of apoptotic cells was assessed by flow cytometry using Annexin V labeling. The results are depicted as a percentage of apoptotic cells ± SD of three independent experiments. (E) Proliferation and persistence of purified T_CM and T_EM. Sorted T_CM and T_EM were co-cultured with mDCs in the presence of superantigen (SEA) for 15 d. After 1–15 d, the proportion of proliferating cells was assessed by staining of anti-TCRV_β22, as V_β22 is known to be a highly SEA-reactive Vβ. Results are represented as the percentage of Vβ22-positive cells.
models have suggested that signaling through TCR and γ chain cytokine receptors is required for long-term survival of memory T cells (10–13). For example, memory CD4 cells persisted for extended periods upon adoptive transfer into intact or lymphopenic recipients but not in IL-7−/− mice (11). Moreover, Kassiotis et al. have demonstrated that the homeostatic expansion capacity of both CD4+ naive and memory cells is dependent on the expression levels of TCR and CD5, a negative regulator of TCR signaling (13). It is possible that TCM and TEM respond distinctively to these signals, thereby influencing their long-term maintenance. In this study, we have used gene and protein expression profiling and functional assays of human CD4+ TCM and TEM to identify the mechanisms underlying their maintenance. Our results provide a molecular basis for the capacity of CD4+ TCM to resist apoptosis and to persist in a stable manner in the host, thereby conferring long-term protective immunity against reinfection.

RESULTS
Functional and phenotypic characterization of CD4+ TCM and TEM
Memory T cell subsets were sorted by flow cytometry from whole PBMCs isolated from 13 healthy donors based on CD45RA, CD27, and CCR7 expression. Naive cells are characterized as CD45RA+, CD27+, and CCR7+; TCM have the phenotypes CD45RA−, CD27+, and CCR7+; TEM are defined by the lack of expression of these three markers (CD45RA−, CD27−, and CCR7−) (Fig. 1 A) (14); and transient memory T cells (TTM) are characterized as CD45RA−, CD27−, and CCR7− (TTM; Fig. 1 A). All TCM (>95%) expressed CD28, CD62L, and CD95. TEM were also homogeneously CD28+ and CD95+, albeit only 30–40% expressed CD62L (unpublished data). As expected, the ex vivo sorted TEM subpopulation expressed the effector molecules granzyme B and perforin, whereas these two molecules were undetectable in TCM (Fig. 1 B). TEM also showed higher

Figure 2. Gene expression array analysis of ex vivo CD4+ TCM and TEM. (A) PCA. TCM (blue) separate well from TEM (red) in this three-dimensional projection of the first three principal components. (B) Two-way hierarchical clustering of CD4+ TCM and TEM expression profiles. The far left column represents sample labels, with TCM donors shown as green squares, and TEM donors shown as orange squares. Each additional column represents a different gene; green indicates lower than median expression (down-regulation) and red indicates higher than median expression (up-regulation). The results of the two-dimensional hierarchical clustering of genes and samples (Pearson correlation similarity measure) are shown as horizontal and vertical dendrograms.
(threefold) expression levels of Rab27a, a molecule involved in degranulation and effector function (15), than TCM (Fig. 1 C). Collectively, these results show that T EM are functionally and phenotypically more differentiated than T CM.

CD4+ T CM are resistant to Fas-induced apoptosis and show enhanced proliferation capacity after stimulation with mature DCs (mDCs)

T CM are long lived and should thus be resistant to cell death signals. We therefore determined their sensitivity to apoptosis as compared with the shorter-lived T EM. Sorted T CM and T EM were cultured in the presence or absence of anti-Fas antibody or etoposide for 24 h (n = 3). Annexin V labeling showed a significant difference (P ≤ 0.007) in the capacity of T CM to resist Fas-mediated apoptosis as compared with T EM (Fig. 1 D). Of note, T CM are less prone to undergo spontaneous apoptosis (i.e., without any apoptotic inducers; P ≤ 0.02) than the T EM subset (Fig. 1 D). Moreover, in response to etoposide, used as a nonspecific apoptotic inducer, both T CM and T EM present similar sensitivity to apoptosis, thereby confirming that the apoptotic machinery is intact in both cell types. We also determined the capacity of purified T CM and T EM to proliferate and persist in a 15-d culture assay after stimulation with staphylococcal enterotoxin superantigen (SEA)–pulsed mDCs. Proliferation was determined by quantifying the expansion of the SEA-responsive TCRVβ2+ T cells. T CM present a better expansion potential and can persist longer than T EM, as demonstrated by a 10-fold increase in the absolute number of SEA-responsive TCRVβ2+ T cells in a 15-d culture period (Fig. 1 E). Similar data were also generated using carboxyfluorescein diacetate succinimidyl ester, whereby T CM undergo several more rounds of proliferation when compared with T EM (unpublished data). Collectively, these results demonstrate that CD4+ T CM and T EM subsets exhibit different capacities to proliferate, persist, and undergo both spontaneous and Fas-induced apoptosis. These observations

| Table I. Differential expression of apoptosis-related genes in CD4+ T CM and T EM |
|-----------------|-----------------|------------|----------------|
| Gene            | Name             | Accession   | p-value| AVG FC | GO annotation |
| MAL             | T cell differentiation protein | AK096093 | 0.02016 | 2.68 | Proapoptotic |
| NGFRAP1         | TNFRSF16-associated protein 1 | CR593909 | 0.01641 | 2.55 | Apoptosis |
| TNFRSF7         | TNF receptor 7   | CR624829 | 0.05641 | 2.37 | Antiapoptotic |
| RELA            | Transcription factor NF-κB3 | BC033522 | 0.01149 | 2.17 | Antiapoptotic |
| TOSO            | Fas apoptotic inhibitory molecule 3 | NM_005449 | 0.07717 | 2.15 | Antiapoptotic |
| PIM2            | Pim-2 oncogene   | NM_006875 | 0.00954 | 1.93 | Antiapoptotic |
| STAT5a          | Signal transducer and activator of transcription | NM_003152 | 0.00543 | 1.72 | Antiapoptotic |
| TNFRSF8         | TNF receptor 8   | AA147604 | 0.00511 | 1.51 | Apoptosis |
| NOTCH3          | In multiple clusters |         | 0.01807 | 1.39 | Antiapoptotic |
| TNF-α           | TNF member 2     | BC028148 | 0.00372 | 1.38 | Proapoptotic |
| EZF1            | EZF transcription factor 1 | BC050369 | 0.03707 | 1.38 | Proapoptotic |
| TNF57           | TNF ligand 7     | BM464627 | 0.00271 | 1.37 | Antiapoptotic |
| BIRC6           | Apollon          | NM_016252 | 0.05593 | 1.34 | Antiapoptotic |
| NGFR            | NGF receptor 16   | AK125088 | 0.00767 | 1.34 | Proapoptotic |
| CASP3           | Caspase 3        | NM_004346 | 0.05753 | –1.33 | Proapoptotic |
| TNFRSF1B        | TNF receptor 1B  | BC052977 | 0.02560 | –1.39 | Pro- or antiapoptotic |
| CASP8           | Caspase 8        | NM_033557 | 0.07165 | –1.44 | Proapoptotic |
| YARS            | Tyrosyl-tRNA synthetase | AK125213 | 0.14050 | –1.46 | Proapoptotic |
| TGF1            | TGF-β–induced factor | NM_170695 | 0.07018 | –1.59 | Proapoptotic |
| IGFBP1          | IGF Factor binding protein 1 | NM_004943 | 0.01336 | –1.65 | FOXO3a target |
| CLU             | Clusterin        | NM_203339 | 0.03113 | –1.84 | Proapoptotic |
| GZMB            | Granzyme B      | BQ052893 | 0.00231 | –1.93 | Proapoptotic |
| LGALS3          | Galectin 3      | AB209391 | 0.13870 | –2.04 | Proapoptotic |
| LGALS1          | Galectin 1      | BF570935 | 0.06611 | –2.49 | Proapoptotic |

Significant genes were selected using an ANOVA t test (P < 0.05 or a fold change >1.7) and associated with an “apoptosis” GO annotation. Each gene on the arrays was spotted in duplicate to avoid false positive signals and to ensure the reproducibility of the data obtained. The fold change values were obtained from the average value of 13 independent hybridizations (AVG FC). The genes upregulated in T EM are bolded. A complete list of 270 genes is available in Table S1.

*p-values were determined by ANOVA, based on an F-test.

Fold change values were calculated from the average value of 13 independent hybridizations by subtracting the mean expression of the log2 ratio obtained in T CM from the log2 ratio obtained in T EM. That value was then converted into fold change.
led us to investigate the cell survival pathways responsible for that resistance to cell death in T_CM and to characterize the differences in these pathways between T_CM and T_EM.

**Gene array analysis of T_CM and T_EM showed differences in gene expression associated with survival pathways**

Sorted T_CM and T_EM were hybridized on two different chips: the custom immune array (~3,000 unique transcripts) and a standardized 19K array (~10,000 unique transcripts). The first level of analysis was performed by submitting 26 samples (13 replicates per class) to unsupervised cluster analysis using principal component analysis (PCA). PCA enables the discrimination and visual clustering of two or more classes, where objects with similar patterns of gene expression are placed next to each other. As shown in Fig. 2 A, T_CM and T_EM visually segregate on a PCA plot. The distance separating T_CM from T_EM reflects differences in overall gene expression between the two subsets. These differences were observed in most samples (10 out of 13 T_CM clustered together with three outliers). Two-way hierarchical clustering of T_CM and T_EM (Fig. 2 B) further demonstrated that T_CM and T_EM subsets group apart and that the gene expression signature of T_CM is considerably different than that of T_EM.

Using single gene searches, we identified the genes that distinguished T_CM from T_EM. Genes selected using analysis of variance (ANOVA), where P < 0.05 or a fold change >1.7 were considered significant. We identified >270 significant genes that distinguished both subsets. Within the selected genes, 6% were related to apoptosis, 9% to cell cycle, and 7% to signaling. These genes also encompassed biological functions, including (a) homing and adhesion, (b) gene expression regulation, (c) immune response, and (d) transport. The complete list of genes can be found in Table S1 (available at http://www.jem.org/cgi/content/full/jem.20061681/DC1). Apoptosis-related genes displaying a different expression profile when comparing T_CM with T_EM are listed in Table I. T_EM expressed higher levels of TOSO, CD27, STAT5a, PIM-2, RelA, and Birc6 (Bruce) mRNA, all belonging to distinct antiapoptotic pathways (16–20), than their T_EM counterparts. In contrast, T_EM showed higher levels of genes involved in the induction of apoptosis, including caspase-8 and caspase-10, as well as several proteins endowed with a proapoptotic function, such as galectin-1 and galectin-3 (21), clusterin (22), YARS (23), and TGIF, a TGF-β–targeted gene (24). This expression profile suggested that T_EM contain an active proapoptotic machinery, thereby explaining their enhanced susceptibility to cell death (Fig. 1 D). On the other hand, several genes that promote cell survival were selectively expressed at high levels in T_CM, rendering them more resistant to apoptosis. Of note, T_EM (CD45RA−, CD27+, and CCR7+) display an intermediary phenotype between T_CM and T_EM. Indeed, their gene expression profiles are very much comparable to T_EM for certain genes (including CD62L, TOSO, and PIM2) and to T_CM for other genes such as Bim, FasL, or IFN-γ (Fig. S1).

In the next set of experiments, we validated and detailed the gene array data by performing real-time RT-PCR on the same donor samples (Table II). The results showed a significant increase in the forhkeh box O3a (FOXO3a) transcriptional target proapoptotic genes, including Bim (25), FasL (26), and genes involved in cell cycle regulation, including GADD45a (27) and p130, a member of the retinoblastoma family (28) in the T_EM subset. Furthermore, the RT-PCR data confirmed the up-regulation of the survival gene CD27 and the antiapoptotic PIM-2 kinase, with the latter being regulated by the STAT5a cascade in T_CM (29). These results validated our gene array data and further suggested the involvement of STAT5a and FOXO3a signaling pathways in mediating the survival of T_CM.

**STAT5a signaling pathway is functionally up-regulated in T_CM**

STAT5a is a downstream effector of γc cytokine receptors (30). We observed differential expressions of PIM-1 and PIM-2, two transcriptional targets of STAT5a in the ex vivo T_CM subset. Indeed, T_CM showed twofold higher expression of both PIM-1 and PIM-2 than T_EM (n = 3; Fig. 3 A). Because of the importance of the STAT5a pathway in the

<table>
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<th>Gene</th>
<th>Fold change T_CM/T_EM</th>
<th>Fold change T_EM/T_CM</th>
<th>Function</th>
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<td></td>
<td>Antiapoptotic</td>
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<tr>
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<td>2.5</td>
<td>Cell cycle</td>
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<td></td>
<td>5.9</td>
<td>AKT inhibitor</td>
<td>NM_01946</td>
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</table>

Total mRNA was isolated from T_CM and T_EM and analyzed by quantitative RT-PCR using a low density array. The bolded genes are up-regulated in T_CM, whereas the other genes are up-regulated in T_EM. Fold changes represent the changes in transcript level in T_EM compared to T_CM for two donors.

*Fold change values were calculated from the average value of two independent experiments using log2 ratio values converted to fold change.*
regulation of T cell survival (30), we evaluated the ability of IL-2 and IL-7 to trigger the STAT5a signaling pathway in CD4+ T cell memory subsets. The phosphorylated form of STAT5a (Y694; pSTAT5a) was quantified by flow cytometry. Basal pSTAT5a levels were similar in TCM and TEM (Fig. 3 B). All TCM and TEM up-regulated pSTAT5a in response to a brief IL-7 treatment (Fig. 3 B). However, the levels of pSTAT5a were significantly higher (30% ± 6.5; P < 0.002) in TCM as compared with TEM (Fig. 3 C). Treatment with IL-2 also induced differential pSTAT5a levels (P < 0.04) between TCM and TEM. Indeed, 90–100% of TCM showed a phosphorylated STAT5a form, compared with 50–60% observed in TEM. Of note, TEM present a bimodal distribution of pSTAT5a in response to IL-2, indicating that TEM are heterogeneous in terms of response to IL-2.

The differences in pSTAT5a levels were not caused by differences in the levels of expression of IL-2 or IL-7 receptors. Indeed, the proportion of cells expressing CD127 (IL-7Rα), CD25 (IL-2Rα), and CD132 (γc chain) on TCM were comparable to those on TEM (Fig. 3 D). Of note, CD122 (IL-2Rβ) was undetectable on ex vivo TCM and TEM (unpublished data). Although IL-2R is expressed on ~20% of TCM as assessed by cytometry, 100% of these cells are able to phosphorylate STAT5a in response to IL-2. This suggests that TCM express levels of IL-2R below the detection limits of our assay that are sufficient to induce STAT5a signaling in response to IL-2. Collectively, these results indicate that the STAT5a pathway, as shown by the levels of pSTAT5a and its downstream effectors (PIM-1 and PIM-2), is differentially regulated between TCM and TEM. The observed differences suggest that TCM display an enhanced capacity to mobilize the STAT5a pathway for their survival as compared with TEM. These results complement previous data obtained by Willinger et al., who reported that CD8+ TCM present a higher ability to phosphorylate STAT5a in response to both IL-2 and IL-7 than CD8+ TEM (31).

Regulation of the FOXO3a pathway in memory CD4+ T cell subsets

FOXO3a transcriptional activity is regulated through direct phosphorylation. Once phosphorylated, FOXO3a is excluded from the nucleus and thus becomes transcriptionally inactive. FOXO3a controls the expression of several genes implicated in apoptosis and cell cycle regulation, including FasL, Bim, Gadd45a, p27kip, and p130 (32, 33). Our gene array analysis and RT-PCR data suggested the specific involvement of the FOXO3a pathway in TCM survival (Table II). Therefore, we analyzed the phosphorylation status of FOXO3a in TCM and TEM. We observed that the levels of pFOXO3a (S315, S253, and T32) were reproducibly (n = 5) more than twofold higher in ex vivo TCM as compared with TEM. It is worth noting that expression levels of total FOXO3a remained similar in the two memory subsets (Fig. 4 A, top). We then determined whether the observed reduction in FOXO3a phosphorylation levels seen in TEM was associated with increased levels of FOXO3a target proteins.

Our results show that TEM expressed threefold higher levels of Bim and p130 proteins and a 1.7-fold higher expression of GADD45a when compared with the TCM compartment.
FasL, whose mRNA was clearly expressed at higher levels in TEM than in CM (Table II), was undetectable in ex vivo TEM and TEM when assayed by Western blot and cytometry (unpublished data). However, upon T cell activation induced by PMA and ionomycine, FasL was selectively up-regulated in TEM (in 30% of the TEM subset), whereas it remained undetectable in CM (Fig. 4 B). Collectively, our data show that a high expression of pFOXO3a observed in TEM is associated with the shutdown of Bim, Gadd45a, and p130 proteins, thereby favoring their long-term survival.

Blocking of AKT and IκB kinase (IKK) activity prevents FOXO3a phosphorylation, leading to TEM cell death

Several kinases, including AKT, IκB-β, casein kinase 1, and DYRK1A, have been reported to directly phosphorylate FOXO3a (34, 35). To identify the kinases involved in the phosphorylation of FOXO3a in CM, we analyzed FOXO3a phosphorylation in CD4+ T cells (CD4+ T cells were used because of the limiting amounts of TEM obtained after sorting) after treatment with specific kinase inhibitors. We used the pharmacological kinase inhibitors AKT-IV and wedelolactone, which inhibit AKT and IKK activities, respectively. We also tested two other kinase inhibitors: STO-609, specific for CamKK and described as an upstream mediator of AKT (36), and the MEK1/2 inhibitor U0126, used as an irrelevant kinase inhibitor. The results (Fig. 5 A) clearly showed that treatment with the AKT and IKK inhibitors led to a specific and significant reduction in the levels of pFOXO3a (S253). The expression levels of pFOXO3a (S253) in CD4+ T cells were eightfold lower in the presence of the AKT inhibitor and 4.5-fold lower in the presence of the IKK inhibitor as compared with untreated cells (Fig. 5 A). It is worth noting that, in total CD4+ T cells, pFOXO3a (S315) is barely detectable (unpublished data). FOXO3a can induce apoptosis through the up-regulation of proapoptotic genes (25, 34, 37, 38). To confirm the importance of the phosphorylation of FOXO3a in memory T cell survival, purified CD4+ T cells were treated with different kinase inhibitors, and apoptosis was assessed using Annexin V labeling. Fig. 5 B shows that the proportion of Annexin V+ cells is increased in a dose-dependent fashion after exposing CD4+ T cells to AKT or IKK inhibitors. Moreover, we observed a significant up-regulation of the levels of the proapoptotic molecule Bim, known to be a FOXO3a target, in cells treated with AKT or IKK inhibitors (three- and eightfold, respectively; Fig. 5 C). Of note, the AKT and IKK inhibitors did not change the levels of FasL expression when assayed by Western blot or cytometry (unpublished data). These results indicated that the dephosphorylation of FOXO3a in CD4+ T cells was associated with Bim up-regulation and apoptosis. None of the other kinase inhibitors tested induced apoptosis in CD4+ T cells (Fig. 5 B), even when used at much higher concentrations (not depicted). Collectively, these results show that among the kinase inhibitors tested, only those able of inducing FOXO3a dephosphorylation have the capacity to activate apoptosis.
to induce CD4+ T cell apoptosis. It is thus likely that activated AKT and IKK promote CD4+ cell survival in part by phosphorylating FOXO3a, thereby repressing its transcriptional activity and leading to the down-regulation of the proapoptotic molecule Bim.

To assess the importance of AKT and IKK in the survival of memory cells, we evaluated the expression of the phosphorylated forms of these proteins in T<sub>CM</sub> and T<sub>EM</sub> subsets. First, pIKKα/β expression was assayed in ex vivo sorted T<sub>CM</sub> and T<sub>EM</sub>. Although pIKKα/β was expressed in T<sub>CM</sub>, it was undetectable in T<sub>EM</sub> (Fig. 5 D). Second, because the phosphorylated form of AKT was undetectable by Western blot on ex vivo sorted cells, we performed a Phosflow analysis on PBMCs treated with H<sub>2</sub>O<sub>2</sub> (which is known to induce phosphorylation of AKT). The results showed that the induction of pAKT (S473) was higher in T<sub>CM</sub> than T<sub>EM</sub> in response to H<sub>2</sub>O<sub>2</sub> (Fig. 5 E and Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20061681/DC1). More importantly, in response to CD28 triggering, T<sub>CM</sub> presented a modest (<11601/20%) but consistent increase (P<0.007) in AKT phosphorylation as compared with the T<sub>EM</sub> (Fig. 5 E). These results suggest that in resting T<sub>CM</sub>, the constitutive activation of IKKα/β could lead to FOXO3a phosphorylation. Moreover, CD28 triggering leads to higher levels of pAKT in T<sub>CM</sub> and could also promote FOXO3a phosphorylation. Collectively, these results suggest that the phosphorylation levels of FOXO3a in T<sub>CM</sub> can be maintained, both in their resting state and upon CD28 triggering, through the activation of IKK and AKT, respectively, thereby promoting T<sub>CM</sub> survival.

The corollary of the results (Fig. 5) is that the lack of FOXO3a phosphorylation could render T<sub>CM</sub> susceptible to signals inducing cell death. To determine the implication of inhibitory CD4+ T cells were treated with 1.6 μM AKT-IV or 100 μM wederolactone for 24 h. Cells were analyzed by Western blotting using Bim specific antibodies. (D and E) Regulation of AKT and IKK phosphorylation in CD4+ memory subsets. (D) pIKKαβ (S176/180) protein levels in ex vivo sorted T<sub>CM</sub> and T<sub>EM</sub>. Prolonged exposure did not reveal any pIKK signal in T<sub>EM</sub>. Similar results were obtained in three independent experiments. (E) PBMCs from healthy donors were treated with 5 mM H<sub>2</sub>O<sub>2</sub> or 2 μg/ml Ig–cross-linked CD28 for 15 min at 37°C and labeled with CD4+, CD27+, CD45RA−, and pAKT (S473)-specific antibodies. The levels of pAKT were assessed by flow cytometry in T<sub>CM</sub>- and T<sub>EM</sub>-gated cell subsets. The results are represented as the mean fold increase ± SD of five independent experiments, calculated as follows: (MFI of stimulated cells/MFI of unstimulated cells). p-values (determined by the two-tailed t test) are shown.
the AKT and IKK signaling pathways in the survival of TCM, we sorted TCM and TEM and exposed them to AKT or IKK inhibitors at their IC50 (1.6 \mu M AKT-IV and 100 \mu M wedelolactone). After 24 h of treatment, the proportion of apoptotic cells was quantified by Annexin V–FITC labeling. (top) Results from a representative individual. Histogram plots show the percentage of Annexin V+ cells in TCM and TEM after a 24-h exposure to AKT or IKK inhibitors. The dashed lines correspond to untreated cells, and the plain lines correspond to cells treated with kinase inhibitors. (bottom) Bar graph representation of the fold increase of apoptosis in TCM and TEM in response to IKK or AKT inhibitors. The fold increase of apoptosis is calculated as the percentage of apoptotic cells in treated cells divided by the percentage of apoptotic cells in untreated cells. Similar results were obtained in two independent experiments.

Figure 7. FOXO3a phosphorylation is dependent on TCR and cytokine engagement. (A) CD4+ T cells were cultured in the presence of 2 \mu g/ml CD3, 2 \mu g/ml CD28, CD3+CD28, 100 U/ml IL-2, 10 ng/ml IL-7, 50 \mu g/ml IFN-\gamma, or 50 ng/ml PMA for 15 min and analyzed by Western blotting for pFOXO3a (S253 and S253) expression levels. (B) CD4+ T cells were cultured in the presence of 100 U/ml IL-2, 10 ng/ml IL-7, or CD3 + CD28 for 30 min and analyzed by Western blotting for pFOXO3a (T32) expression levels. The results are representative of two independent experiments.

Figure 6. Susceptibility of TCM and TEM to apoptosis induced by kinase inhibitors. Sorted TCM and TEM were cultured with or without AKT and IKK inhibitors as indicated. After 24 h, the percentage of apoptotic cells was quantified by Annexin V–FITC labeling. (top) Results from a representative individual. Histogram plots show the percentage of Annexin V+ cells in TCM and TEM after a 24-h exposure to AKT or IKK inhibitors. The dashed lines correspond to untreated cells, and the plain lines correspond to cells treated with kinase inhibitors. (bottom) Bar graph representation of the fold increase of apoptosis in TCM and TEM in response to IKK or AKK inhibitors. The fold increase of apoptosis is calculated as the percentage of apoptotic cells in treated cells divided by the percentage of apoptotic cells in untreated cells. Similar results were obtained in two independent experiments.

FasL expression suggests that cell death induced by these kinase inhibitors could be achieved in part through FOXO3a dephosphorylation and the subsequent increased expression of the proapoptotic molecule Bim.

TCR and cytokine triggering phosphorylate distinct sites on FOXO3a
To identify the signals that trigger FOXO3a phosphorylation in CD4+ T cells, we quantified the levels of pFOXO3a (S253, S315, and T32) in response to CD3 and/or CD28 cross-linking, IL-2, IL-7, IFN-\gamma, and PMA treatment. pFOXO3a (S253) was easily detectable in ex vivo CD4+ T cells, and none of the tested stimuli induced considerable changes in the phosphorylation status of this protein (Fig. 7 A). In contrast, FOXO3a phosphorylation on S315 was significantly induced in response to CD3 + CD28 triggering. It is worth noting that CD3 or CD28 triggering alone did not lead to FOXO3a phosphorylation (S315), indicating that the synergy of both signals is essential for FOXO3a phosphorylation at S315 (lanes 2, 3, and 4; Fig. 7 A, top). None of the other tested inducers (including \gamma c cytokines) led to FOXO3a (S315) phosphorylation. Interestingly, the levels of pFOXO3a at T32 were drastically increased when CD4+ T cells were treated with IL-7 or IL-2. No induction of pFOXO3a (T32) was observed when cells were triggered with CD3 + CD28 (Fig. 7 B). Collectively, these results indicate that TCR and \gamma c cytokines IL-2 and IL-7 triggering induce specific FOXO3a phosphorylation at distinct sites (S315 and T32, respectively).
suggesting that the convergence of both signals is required to induce optimal FOXO3a phosphorylation and the subsequent inhibition of its transcriptional up-regulation of the proapoptotic machinery.

**DISCUSSION**

The involvement of TCR/ligand signaling in the generation and long-term survival of CD4+ TEM has been a matter of much debate. Indeed, although the persistence of both CD8 and CD4+ T cells seems to occur in the absence of MHC antigen (39, 40), it has been demonstrated that repeated exposure to native or cross-reactive epitopes improves memory T cell survival and function (12, 13). Moreover, cytokines such as IL-2 and IL-7 have also been shown to enhance CD4+ TEM survival, although the exact mechanisms leading to this function have yet to be identified (10, 11, 41). In this paper, we focus on ex vivo polyclonal TCM and TEM to identify the molecular pathways involved in the maintenance and survival of memory cells. Using highly purified subsets of TCM and TEM obtained by multiparametric cell sorting, we propose that two converging specific signaling pathways are involved in providing TCM with the capacity to survive and resist apoptotic signals. Our results indicate that TCR and γc cytokine (IL-2 and IL-7) signaling induces FOXO3a phosphorylation, thereby preventing the transcription of proapoptotic molecules such as Fasl and Bim; moreover IL-2 and IL-7 allow the activation of STAT5α and the concomitant induction of several antiapoptotic molecules (PIM-1 and PIM-2). In contrast, protective functions associated with these pathways are less efficient in TEM (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20061681/DC1).

In this study, we first examined the properties of TCM and TEM, based on a previously established classification of CD4+ T cell subsets (3, 4), using transcriptional profiling by cDNA microarrays. Our results show that these two subsets exhibit considerable differences at the gene expression level, including several genes endowed with apoptotic functions. Other reports have investigated the gene expression profiles of human TCM and TEM in both CD8+ (31, 42) and CD4+ populations (43, 44). For the CD8+ T cell subsets, TCM clearly cluster apart from TEM (31). According to the literature and our data, CD4+ TCM and TEM also display different gene expression profiles. Despite the use of different DNA chips and different surface markers to distinguish TCM (CD45RO+ CD27+ [44], CD45RO+ CCR7+ [43], or CD45RA– CD27+ CCR7+ in the present study) from TEM, several common genes were shown to be up-regulated in TEM (ID2, CD63, CCL4, Grz-A, and DUSP6), whereas others were up-regulated in TCM (MAL, CD62L, and CD27) (Tables I and II; and Table S1) (43, 44). This indicates that human TCM display a specific gene signature that distinguished them from TEM. Among the genes differentially expressed by TCM and TEM, we consistently found that TCM express lower levels of Bim, Fasl, Gadd45α, and p130, genes that are all described as FOXO3a target genes (Tables I and II) (25–28).

FOXO3a activity is regulated through phosphorylation. In its phosphorylated form, FOXO3a is retained in the cytoplasm and is functionally inactive. Once dephosphorylated, it is translocated into the nucleus and leads to the expression of proapoptotic targeted genes such as Fasl and Bim (33). Several reports have shown the role of FOXO3a-induced transcriptional activation of Fasl or Bim in the active induction of apoptosis in different models. These include, for Bim, cytokine deprivation in lymphoid cells (38) and paclitaxel induction of apoptosis in breast cancer cells (45), and Jurkat cells and neutrophils for Fasl (37, 46). Of note, none of those reports has investigated the synergistic role of both molecules in FOXO3a-mediated apoptosis. Using mutant forms of FOXO3a, Brunet et al. demonstrated that the localization of FOXO3a is not dependent on a specific phosphorylation site and that increased FOXO3a phosphorylation enhanced its retention in the cytosol. Higher levels of pFOXO3a lead to its increased retention in the cytoplasm (37).

Our results indicate that TCM express a high level of pFOXO3a for most FOXO3a putative phospho-sites, including T32, S253, and S315. In addition, ex vivo TCM present reduced levels of Bim. On the other hand, FOXO3a is significantly less phosphorylated in TEM, thereby promoting its activation. Indeed, we consistently observed a significant up-regulation of FOXO3a transcriptional targets in TEM: Bim, p130, and GADD45a at both mRNA and protein levels. The increased presence of pFOXO3a, primarily in TCM (which are more resistant to apoptosis than TEM), strongly suggests that elevated levels of pFOXO3a could account, in part, for this subset’s resistance to Fas-mediated apoptosis and enhanced survival capacity. On the other hand, the up-regulated expression of Fasl in TEM upon T cell activation suggests that this subset is more sensitive to activation-induced cell death, as shown by the limited survival of these cells upon TCR triggering, as well as by their enhanced sensitivity to Fas-induced cell death (Fig. 1, D and E; and Fig. 4 B).

Several kinases appear to be involved in FOXO3a phosphorylation, including IKK and AKT (32). In this paper, we show that IKKα/β and AKT play critical roles in the phosphorylation of FOXO3a in TCM. Indeed, AKT and IKK inhibitors not only prevent FOXO3a phosphorylation at S253, but also increase the expression of proapoptotic molecules such as Bim, leading to apoptosis. Moreover, we demonstrated that pIKKα/β levels are increased in TCM ex vivo as compared with TEM. This increased expression of activated IKK in TCM could lead to FOXO3a phosphorylation and the inhibition of its transcriptional activity. Because IKK also phosphorylates IκB (a negative regulator of NF-κB), it could also result in the activation of NF-κB, the major transcription factor of several survival genes, and therefore favor TEM cell survival (47). pAKT was undetectable ex vivo in both memory subsets. However, H2O2 or CD28 treatments lead to a more significant up-regulation of pAKT in TCM as compared with TEM. These results strongly suggest that TCM survival ability depends, in part, upon the activation of IKK and/or AKT, whereas reduced activities of AKT and/or IKKα/β in
T<sub>EM</sub> lead to the inhibition of FOXO3a phosphorylation and the subsequent induction of the proapoptotic molecule Bim. These results concur with data obtained in Bim knockout mice showing that the absence of Bim leads to the expansion of memory cells (48).

Our results show that the induction of FOXO3a phosphorylation at S315 required both CD3 and CD28 engagement, whereas T32 phosphorylation is dependent on γc cytokine (IL-2 and IL-7) triggering. Both AKT and IKK could be responsible for the phosphorylation of FOXO3a at S315 upon TCR/CD28 triggering. TCR signaling triggers the activation of NF-κB and IKK (49), and CD28 triggering enhances AKT phosphorylation (50). The phosphorylation of FOXO3a at T32 induced by IL–7 or IL–2 can be regulated by AKT, as Barata et al. shows that the activation of the phosphatidylinositol 3-kinase–AKT axis is mandatory for the IL–7-mediated viability of T cells (51), and IL–2 triggering can also induce AKT activation (52). Moreover, we and others also have shown that IL–4 can induce T32 phosphorylation (unpublished data) (53).

To date, no mediator has been described as a unique inducer of memory CD4<sup>+</sup> T cell survival. As such, it is possible that multiple signals with redundant or partially overlapping functions could be involved to ensure the optimal survival of memory T cells. Our results suggest that cytokines and TCR/CD3 signaling pathways converge to ensure distinct FOXO3a phosphorylation at T32 and S315, respectively. Thus, the combination of both signals could lead to higher levels of FOXO3a phosphorylated forms (quantitatively and qualitatively) and its subsequent inactivation, favoring the long-term survival of T<sub>CM</sub>. Our data provide a molecular mechanism for the observation reported by Seddon et al., who demonstrated that both IL–7 and CD3 engagement are involved in maintaining homeostatic levels of T<sub>CM</sub> (10). The results described here improve our understanding of the molecular mechanisms associated with T<sub>CM</sub> maintenance. The identification of these mechanisms is fundamental for deciphering the dysregulation of memory cells, which is often observed in several chronic diseases, including viral infections or tumors. They also pave the way for the development of strategies aimed at restoring the functionality of these cascades through the generation of immunotherapeutics that target these pathways, thereby promoting the establishment of a long-lived memory T cell pool.

MATERIALS AND METHODS

Reagents and antibodies. Recombinant human IL–2 was obtained through the National Institutes of Health AIDS Reagent Repository. IL–7 and IL–4 were purchased from R&D Systems. The kinase inhibitors AKT-IV, STO-069, U0126, and wortmannin were obtained from Calbiochem. Etoposide was purchased from Sigma-Aldrich; anti-FAS (clone 145-2C11) was obtained from BD Biosciences, except for anti-CD45RA–ECD from Immunotech. All antibodies for flow cytometry were purchased from BD Biosciences, except for anti-CD45RA–ECD from Immunotech. All antibodies for flow cytometry were purchased from BD Biosciences, except for anti-CD45RA–ECD from Immunotech.

RNA isolation, amplification, and microarray hybridization. Sample RNA was extracted using an RNA extraction kit (Qiagen) and amplified using an RNA kit (MessageAmp; Ambion) according to the manufacturer's instructions. The amplified RNA (aRNA) was verified for quality and quantity using a bioanalyzer (model 2100; Agilent Technologies) and measuring the OD. All patient samples were hybridized against amplified universal aRNA at 37°C for 18 h on a custom human immune array. Detailed information on the labeling and hybridization procedures is available at http://www.microarrays.ca. Experimental design, sample description and preparations, hybridizations, data analysis, and annotations meet MIAME compliance.

Microarray data preprocessing. Microarrays were scanned at 16 bits using the ScanArray Express (Packard Instrument Co.). The microarrays were then screened for quality, first by visual inspection of the array with flagging of poor quality spots and then with automated scripts that scanned the quantified output files and measured overall density distribution on each channel and the number of flagged spots. Lowest normalization was then applied on the scanned chips. Microarray data is available in the National Center for Biotechnology Information Gene Expression Omnibus under accession no. GSE4741 and is titled “Convergence of TCR and cytokine signaling leads to FOXO3a phosphorylation and drives the survival of central memory CD4<sup>+</sup> T cells.”

Selection of the top 100 T<sub>CM</sub>/T<sub>EM</sub>-discriminating genes. From the set of 19k microarray genes that passed quality control criteria during preprocessing of microarray data, we retained only the genes that significantly discriminate T<sub>CM</sub> from T<sub>EM</sub> according to the F-test (P < 0.01). From this set, a final subset of 100 genes was further manually selected according to known functions and pathways, including apoptosis, cell cycle, and signaling.

PCA. A data matrix comprising 13 T<sub>CM</sub> and 13 T<sub>EM</sub> samples (rows), and the 100 top T<sub>CM</sub>/T<sub>EM</sub>-discriminating genes (see previous section) was constructed. Using singular value decomposition of the data matrix, a standard PCA of the data's 100 × 100 covariance matrix was computed, with each sample comprising 100 genes. PCA was computed and plotted generated by GeneLinker Platinum software (version 4.6; Improved Outcomes Software).

Two-way hierarchical clustering. Hierarchical clustering was performed over the same set of 26 samples and 100 genes as used for PCA. We used the Pearson correlation as the similarity measure between genes and samples for clustering. Analyses were performed using GeneLinker Platinum software.

Quantitative real-time PCR analysis. Changes in gene expression observed by array analyses were verified by a low density array performed on a detection system (7900 HT; Applied Biosystems). In brief, cDNA was...
synthesized from total RNA (1 μg per sample) in an RT reaction in 20 μl of 1× first-strand synthesis buffer (Invitrogen). Amplification of cDNA (1–20 μl) was performed using SYBR Green PCR buffer (PerkinElmer) containing 0.1 μM of specific primers. Before the samples were analyzed, standard curves of purified, target-specific amplicons were created. The mRNA expression for each gene was determined by comparing it with its respective standard curve. This measurement was controlled for RNA quality, quantity, and RT efficiency by normalizing it to the expression level of the GAPDH gene. Statistical significance was determined by use of normalized fold changes and ANOVA. The p-values were calculated using a two-tailed t test, assuming that the true variances were unknown.

**Induction and quantification of apoptosis.** Sorted cells were cultured in complete RPMI 1640 and treated as indicated in the figure legends. ApoImotopic cells were detected using Annexin V labeling according to the manufacturer’s protocol (Biosource International). The fluorescence signals were measured by flow cytometry using a flow cytometer (LSRII; BD Biosciences). Approximately 50,000 gated events were collected for each sample.

**Western blotting analysis.** TCM and TEM were sorted as described in isolation of CD4+ T cell subpopulations. Cells were washed twice with PBS and resuspended in lyse buffer containing 50 μM NaF and 1 mM sodium pyrophosphate. 10 μg of proteins from total cell extracts were separated on SDS-PAGE and electrotransferred onto polyvinylidene membranes (Roche). Membranes were incubated overnight at 4°C with specific antibodies, as described in the figure legends. Detection of the immune complexes was performed using HRP-conjugated goat anti–mouse (1:2,500) or goat anti–rabbit (1:10,000) antibodies. HRP activity was detected using an enhanced chemiluminescence detection procedure (ECL Plus; GE Healthcare). Membranes were subsequently stripped and reblotted with an antibody against actin (1:10,000).

The expression level of actin was used to control for equal loading. Protein expression levels were expressed as a percentage of the highest signals obtained.

**Intracellular staining.** The cells were labeled with anti-CD4–AmCyan, anti-CD27–PE, and anti-CD45RA–ECD, and then fixed with 2% paraformaldehyde for 15 min at room temperature and −20°C. Intracellular staining was performed using anti-CD4–APCcy7, anti-CD45RA–ECD, anti-CD27–PE, and anti-STAT5a (N694)–Alexa Fluor 647 specific antibodies for 30 min at room temperature. For the analysis, the cells were gated on TCM and TEM. An average of 20,000 gated events was collected on an LSRII flow cytometer.

**Proliferation assay.** Sorted TCM or TEM were co-cultured with mDCs (T cell/DC ratio = 40:1) in the presence of 50 ng/ml SEA. After 1–15 d of co-culture, cells were labeled with anti-CD4 and anti-TCR/V-B22. For the analysis, cells were gated on CD4+ T cells, and ∼150,000-gated events were collected on an LSRII flow cytometer.

**Phosflow analysis of STAT5a and AKT status.** PBMCs were resuspended at 20 million cells/ml in RPMI 1640 and incubated for 30 min in the presence of CCR7–FITC antibodies (20 μl/million cells) at room temperature. The cells were washed and resuspended at a cell concentration of 5 million cells/ml in PBS and stimulated for 15 min at 37°C in the presence of 100 U/ml IL-2 or 10 ng/ml IL-7. After stimulation, the cells were fixed for 10 min at 37°C using Cytofix buffer (BD Biosciences), pelleted, and permeabilized in Perm III buffer (BD Biosciences) for 30 min on ice. The cells were washed twice in staining buffer (BD Biosciences) and rehydrated for 30 min on ice in the staining buffer. Cells were labeled with anti-CD4–APCcy7, anti-CD45RA–ECD, anti-CD27–PE, and anti-phosphoSTAT5a (Y694)–Alexa Fluor 647 specific antibodies for 30 min at room temperature. For the analysis, the cells were gated on TCM and TEM. An average of 20,000 gated events was collected on an LSRII flow cytometer.

For CD28 cross-linking, the cells were resuspended at 10 million cells/ml in the presence of 2 μg/ml CD28 for 30 min on ice. The cells were washed twice in PBS and subsequently stimulated by cross-linking with 20 μg/ml rabbit anti-mouse IgG (Biosource International) in 25 μl of prewarmed medium for 15 min. The cells were fixed and permeabilized as described and labeled with CD4–APCcy7, CD45RA–ECD, CD27–PE, and pAKT S473–Alexa Fluor 488. Flow cytometry analysis was performed on gated TCM and TEM. Approximately 20,000-gated events were collected on an LSRII flow cytometer.

**Online supplemental material.** Table S1 shows a complete list of genes that are differentially expressed in TCM and TEM. Fig. S1 A shows the induction of STAT-5α phosphorylation in TCM. Fig. S1 B shows real-time RT-PCR in TCM, TEM, and TTM subsets. Fig. S2 shows histograms of Phosflow analysis of AKT phosphorylation after treatment with H2O2. Fig. S3 is a proposed model showing how signaling through TCR and cytokines induce survival of TCM. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20061681/DC1.

We thank Eric Massicotte for cell sorting, Marc Vendette for technical assistance, Jean-Pierre Routy and Rachid Boulouzzi for providing blood samples, and Abdelkader Yacheu for administrative support. We also thank Nicolas Chomont, Alain Dumont, Jim Woodgett, and Sylvain Meloche for critically reviewing the manuscript.

This study was supported by funds from the National Institutes of Health, the Canadian Institutes of Health Research, Genome Quebec, Genome Canada, Fonds de Recherche en santé du Québec, and the Canadian Network for Vaccines and Immunotherapeutics. R.-P. Sealy is the Canada Research Chair in Human Immunology.

The authors have no conflicting financial interests.

Submitted: 7 August 2006
Accepted: 29 November 2006

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