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Reversible Senescence in Human CD4+CD45RA+CD27− Memory T Cells

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Persistent viral infections and inflammatory syndromes induce the accumulation of T cells with characteristics of terminal differentiation or senescence. However, the mechanism that regulates the end-stage differentiation of these cells is unclear. Human CD4+ effector memory (EM) T cells (CD27−CD45RA−) and also EM T cells that re-express CD45RA (CD27+CD45RA+; EMRA) have many characteristics of end-stage differentiation. These include the expression of surface KLRG1 and CD57, reduced replicative capacity, decreased survival, and high expression of nuclear γH2AX after TCR activation. A paradoxical observation was that although CD4+ EMRA T cells exhibit defective telomerase activity after activation, they have significantly longer telomeres than central memory (CM)-like (CD27+CD45RA−) and EM (CD27−CD45RA−) CD4+ T cells. This suggested that telomerase activity was actively inhibited in this population. Because proinflammatory cytokines such as TNF-α inhibited telomerase activity in T cells via a p38 MAPK pathway, we investigated the involvement of p38 signaling in CD4+ EMRA T cells. We found that the expression of both total and phosphorylated p38 was highest in the EM and EMRA compared with that of other CD4+ T cell subsets. Furthermore, the inhibition of p38 signaling, especially in CD4+ EMRA T cells, significantly enhanced their telomerase activity and survival after TCR activation. Thus, activation of the p38 MAPK pathway is directly involved in certain senescence characteristics of highly differentiated CD4+ T cells. In particular, CD4+ EMRA T cells have features of telomere-independent senescence that are regulated by active cell signaling pathways that are reversible. The Journal of Immunology, 2011, 187: 2093–2100.

Human T cell memory is mainly maintained throughout life by episodes of proliferation induced by antigenic challenge and to a much lesser extent by continued generation of new cells as the thymus involutes early in life (1). The repeated lifelong stimulation of T cells induces them to differentiate both functionally and phenotypically (1, 2). Since the original use of CD45RA and CD45RO Abs to identify unprimed and primed/memory subsets of T cells, respectively (3), it has become clear that some primed/memory T cells in both CD8 (4, 5) and CD4 (6, 7) compartments can re-express the CD45RA molecule. These cells were subsequently shown to be largelyCCR7−CD27−CD28− and therefore have a highly differentiated effector memory (EM)-like phenotype (EMRA) (4, 8). Although both EM and EMRA T cells have proliferative defects, the latter population can exhibit potent effector functions (5, 7, 9, 10). Highly differentiated populations of EM and EMRA-like CD4+ and CD8+ T cells have been shown to accumulate in older human (7, 11–13) patients with persistent viral infections (14–18) and those with inflammatory syndromes such as rheumatoid arthritis (19–21). In addition, EMRA T cells are the dominant memory population that persists after some forms of vaccination (22). However, the exact nature of these T cells is not clear.

The maintenance memory T cells by proliferation of pre-existing pools of cells may be limited because there is a finite limit to the extent of proliferation that a cell population can experience before growth arrest occurs (23). This constraint is set by the erosion of repeating hexameric sequences of DNA at the ends of chromosomes known as telomeres, which are lost with each replicative cycle (23). In the absence of compensatory factors, telomeres shorten by ~50–100 bases after each round of proliferation until a critical point is reached at which the exposed DNA end of the telomere is recognized as a DNA double-stranded break (24). This recruits a complex of proteins that are involved with DNA repair and is commonly referred to as the DNA damage response (DDR) (23, 25, 26). The development of telomere-dependent senescence is retarded if cells can upregulate the enzyme telomerase, which adds telomeres back to the ends of chromosomes (24). Cellular senescence can also occur when telomeric or nontelomeric DNA is damaged by other means that are independent of telomere shortening (telomere-independent senescence) (23). This includes damage by reactive oxygen species, ionizing radiation, chromatin perturbation, and activation of p53 and stress pathways (23). In most of these situations, a DDR that is virtually identical to that triggered by telomere erosion is induced, and unless the DNA can be repaired, the cells undergo growth arrest. Senescence-related DNA damage foci, irrespective of whether they arise as a result of...
telomere-dependent or telomere-independent DNA damage, can be identified by staining for molecules within the complex such as the histone protein γH2AX (27). When human CD4+ T cells differentiate progressively from a naive to an EM phenotype, they lose their capacity to upregulate telomerase activity, and this is associated with progressive telomere reduction (28). However, the telomere length and telomerase activity of CD4+CD27+CD45RA+ (EMRA) T cells have not been investigated previously.

In this study, we made the unexpected observation that although CD4+ EMRA T cells have many phenotypic and functional characteristics of an end-stage or senescent population, they have significantly longer telomeres than CD27+CD45RA+ (central memory [CM]) and CD27+CD45RA- (EM) cells from the same donor. This is the first report, to our knowledge, that CD4+CD27+telomere length and telomerase activity of CD4+CD27+associated with progressive telomere reduction (28). However, the lose their capacity to upregulate telomerase activity, and this is the inhibitor for 30 min. A solution of 0.1% DMSO was used as control.

Materials and Methods

Blood sample collection and isolation
Heparinized peripheral blood was collected from healthy volunteers between the ages of 26 and 60 y (median age, 39 y), with approval from the Ethics Committee of the Royal Free Hospital. PBMCs were isolated by Ficoll-Hypaque density gradient (Amerham Biosciences, Uppsala, Sweden). CD4+ T cells were purified by positive selection using the VARIOMATIC system (Miltenyi Biotec), according to the manufacturer’s instructions. In some experiments, CD4+ T cells were further sorted into CD45RA/CD27 subsets using a FACSAria flow cytometer (BD Biosciences). Following surface staining for CD45RA, CD27, and CD4, PBMCs were incubated with the protein nuclear acid telomeric probe (C3TA2)3 conjugated to Cy5 (Panagene). After the samples were heated for 10 min at 82˚C, they were left to hybridize. Samples were washed in posthybridization buffer, followed by PBS, and analyzed immediately by flow cytometry.

Cell culture and use of inhibitors

Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, 50 μg/ml gentamicin, and 2 μM 1,2-glutamine (all from Invitrogen) at 37˚C in a humidified 5% CO2 incubator. Purified CD4+ subsets were activated in the presence of anti-CD3 Ab (purified OKT3, 0.5 μg/ml) and PBMCs irradiated with 40-Gy gamma radiation, as a source of multiple costimulatory ligands provided by B cells, dendritic cells, and macrophages found in these populations. In other experiments, where we were investigating the changes in the level of apoptosis the cells were activated by anti-CD3 Ab in the presence of recombinant human (rh)IL-2 (5 ng/ml) (R&D Systems) because irradiation in the APCs induces excessive cell death, which impacts negatively on accurate gating of the appropriate activated populations. In some experiments, the p38 inhibitor BIRB796 was added to the culture at a final concentration of 500 nM (29). Cells were prewarmed with the inhibitor for 30 min. A solution of 0.1% DMSO was used as control.

Flow cytometric analysis of cell phenotype

Isolated T cells were resuspended in PBS containing 1% BSA and 0.1% sodium azide (Sigma-Aldrich) and then stained for 10 min at room temperature with the following anti-human Abs: CD45RA (allophycocyanin, clone MEM56; Abcan); CD27 (PE, clone M-T271; BD Pharmingen); and CD4 (PE-Cy7, clone SK3; BD Pharmingen). KLGR1 (Alexa Fluor 488; a gift from Prof. P. Fischer, University of Freiburg). Intracellular staining was performed for Bcl-2 (PE, clone Bcl-2/100; BD Pharmingen), Ki67 (FITC, clone B56; BD Pharmingen), and p38 (rabbit polyclonal anti-p38 [Cell Signaling Technology]; Alexa Fluor 488 goat anti-rabbit Ig [Invitrogen]). The intracellular staining was performed using the Foxp3 Staining Buffer Set (Miltenyi Biotec), according to the manufacturer’s instructions. Apoptosis was assessed using an Annexin V/Propidium Iodide detection kit (BD Pharmingen). Samples were acquired on a BD LSR II flow cytometer (BD Biosciences) after fixation with 1% formaldehyde (Sigma-Aldrich). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Staining of phosphorylated proteins by flow cytometry

The analysis of p38 (pT180/pY182) was performed directly ex vivo. Following surface staining for CD45RA, CD27, and CD4, PBMCs were fixed with warm Cytofix Buffer (BD Biosciences) at 37˚C for 10 min. Cells were then permeabilized with ice-cold Perm Buffer III (BD Biosciences) at 4˚C for 30 min and incubated with the anti-p38 Ab (pT180/pY182) (Alexa Fluor 488, clone 36; p38; BD Pharmingen) for 30 min at room temperature. Cells were washed in Stain Buffer (BD Pharmingen). For the detection of γH2AX (pSer139) (Alexa Fluor 488, clone 2F3; BioLegend), purified subsets were activated with 0.5 μg/ml immobilized anti-CD3 and 5 ng/ml rhIL-2 for 4 d. Intracellular staining was performed using the BD Phoshflow buffers above mentioned. Samples were acquired on a BD LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Telomere-length measurement by flow-fluorescent in situ hybridization coupled to flow cytometry

Telomere length of CD4+ T cell populations defined by expression of CD45RA and CD27 was assessed by a modified version of the three-color flow-fluorescent in situ hybridization (FISH) method that was described previously (30, 31). In brief, CD4+ cells that were negatively isolated using MACS beads were surface stained, washed in PBS and then fixed in 1 ml BS (Perbio Science). The reaction was quenched with 50 mM Tris (pH 7.2) in PBS. After the cells were washed in PBS followed by hybridization buffer, they were incubated with the protein nuclear acid telomeric probe (C3TA2)3 conjugated to Cy5 (Panagene). After the samples were heated for 10 min at 82˚C, they were left to hybridize. Samples were washed in posthybridization buffer, followed by PBS, and analyzed immediately by flow cytometry. All samples were run in triplicate alongside cryopreserved PBMCs with known telomere characteristics to ensure consistency of results. Kilobase length was determined from mean fluorescence intensity values using a standard curve. The standard curve was constructed using samples of varying telomere length analyzed both by flow-FISH and telomeric restriction fragment analysis (31).

Measurement of telomerase activity

Telomerase activity was determined using a modified version of the telomeric repeat amplification protocol (TRAP) (Oncor, Gaithersburg, MD) as described previously (31). In brief, purified subsets (Supplemental Fig. 1) were activated with anti-CD3 (0.5 μg/ml) and irradiated APCs for 4 d (Supplemental Fig. 3). Telomerase activity is only induced after T cell activation. To ensure that we are measuring a telomerase defect and not a proliferative defect in T cells per se, we measure the proportion of proliferating cells in each isolated T cell subset by staining with the nuclear cell cycle marker Ki67 prior to the telomerase assay. Absolute numbers of CD3+Ki67+ T cells in each sample are counted using Trucount beads, and PCR for telomerase activity is performed with samples adjusted to 500 Ki67+ T cells/reaction. Therefore, we are biasing the results against ourselves. In populations like the EMRA T cells that do not proliferate very well, we make extracts from larger numbers of cells so that we get the same number of proliferating cells in each PCR. We have described this assay previously (32). Cells were lysed from freshly isolated CD4+ T cells used for telomeric elongation, using a [γ-32P] ATP–end–labeled telomerase substrate primer. These samples were then amplified by PCR amplification, using 25–28 cycles of 30 s at 94˚C and 30 s at 59˚C. The PCR products were run on a 12% polyacrylamide (Sigma-Aldrich) gel that was then exposed to an autoradiography film (Hyperfilm MP, Amersham Biosciences). As a negative control, lysis buffer was used in place of cell extract. A control template containing the same sequence as the telomerase substrate primer plus eight telomeric repeats was used as a PCR positive control. We have either used radioactivity or real-time PCR analysis to measure the products of the standard TRAP assay with similar results (data not shown).

RT-PCR analysis of Bcl-2 mRNA

Expression of Bcl-2 mRNA was analyzed by semiquantitative RT-PCR amplification. CD4+ cells were cultured with anti-CD3 (0.5 μg/ml) and rhIL-2 (5 ng/ml) in the presence of BIRB796 for 3 d. Total RNA was isolated using RNeasy kit (Qiagen), and cDNA was synthesized. Bcl-2 expression was evaluated by RT-PCR on an ABI PRISM 7500 (Applied Biosystems) with the following primers: 5′-TTGTTTACGTG-GCCGTGTTTC-3′ (forward) and 5′-GAAGACCTGCAAAGCCAGC-3′ (reverse). The housekeeping 18S mRNA, used as an external standard, was amplified from the same cDNA reaction mixture using specific primers. The level of Bcl-2 was expressed as a ratio to the level of 18S to normalize for differences in the amount of input cDNA. The level of Bcl-2 was then compared with the level of 18S.

Western blot analysis

CD4+ T cells were activated with PMA (0.5 μg/ml; Sigma-Aldrich) and ionomycin (0.5 μg/ml; Sigma-Aldrich) in the presence or absence of
BRB796. Cells were harvested after 30 min of stimulation, and lysates were obtained by sonication in 50 mM Tris-HCl (pH 7.5), 2 mM EGTA, and 0.1% Triton X-100 buffer. Lysates from 2 × 10⁶ cells were fractionated on SDS-polyacrylamide electrophoresis gels and analyzed by immunoblotting with either anti–phospho-p38 (pThr180/pTyr182; Cell Signaling Technology), anti–pJNK (pThr183/pTyr185; BD Biosciences), or anti–β-actin (Abcam) using the ECL Advanced Western blotting Detection kit (Amersham Biosciences), according to the protocol provided by the manufacturer.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA). Data are presented as mean ± SEM. A p value < 0.05 was considered significant.

Results

CD4⁺ CD27⁺ CD45RA⁺ (EMRA) T cells exhibit phenotypic and functional characteristics of cellular senescence

CD4⁺ T cells can be subdivided into four populations on the basis of their relative surface expression of CD27 and CD45RA molecules (Fig. 1A). These subsets are analogous to those identified in other reports in which surface CCR7 together with CD45RA expression were used to distinguish between T cells at different stages of differentiation (4, 33). On the basis of changes in surface receptor expression, functional activity, and telomere length, undifferentiated populations have been shown to express CD27⁺ CD45RA⁺, those that are at an early stage of differentiation are CD27⁺CD45RA⁻, whereas highly differentiated CD4⁺ T cells are CD27⁻ CD45RA⁻ (4, 7). Although the CD4⁺ EMRA T cells also exhibited characteristics of highly differentiated T cells (7, 34), their relative telomere length or telomerase activity has not been investigated previously. We found that CD4⁺ EMRA T cells express high levels of surface KLRG1 that identify senescent T cells (Fig. 1B) (35, 36), and this, together with their elevated expression of CD57 (7), supports the possibility that they may be an end-stage population. The CD27⁻ CD45RA⁻ (EM) population also expresses high levels of both KLRG1 (Fig. 1B) and CD57 (7), indicating that they also have characteristics of senescence. The phosphorylation of the histone H2AX (γH2AX) can be used to identify DDR foci in senescent fibroblasts (27, 37). We found that after TCR activation both of the CD27⁻ CD45RA⁻ (EM) and the CD27⁺ CD45RA⁺ (EMRA) T cell populations expressed significantly higher levels of γH2AX than the other subsets (Fig. 1C). This was not due to the identification of replicating instead of damaged DNA as we excluded proliferating blast T cells from our analysis (Supplemental Fig. 2). We also found that telomerase activity was significantly reduced in the CD27⁻ CD45RA⁺ cells compared with the other populations (Fig. 1D), which is an additional characteristic of highly differentiated T cells that are close to senescence (28, 38). Therefore, both CD27⁻ CD45RA⁻ and CD27⁺ CD45RA⁺ T cells have the characteristics of T cells that are close to senescence; however, these changes were more pronounced in the latter population.

CD4⁺CD27⁻ CD45RA⁺ EMRA T cells have relatively long telomeres

One prediction from the observation of low telomerase activity in CD27⁻ CD45RA⁺ EMRA T cells was that these cells would have very short telomeres. The relatively low numbers of these cells in vivo precluded the use of conventional DNA isolation and electrophoretic methods to analyze their telomere lengths. Instead, we investigated the telomere length of MACS-isolated CD4⁺ T cells by three-color FISH coupled to flow cytometry using CD45RA, CD27, and a fluorescence-labeled telomere probe (flowFISH; Fig. 2A, Supplemental Fig. 3). We confirmed that relatively undifferentiated T cells (CD27⁺CD45RA⁺) have longer telomeres than the early differentiated, CM-like subset (CD27⁻CD45RA⁻), which in turn have significantly longer telomeres than the EM-like
(CD27−CD45RA−) T cell population (Fig. 2B). Surprisingly, however, we found that CD27−CD45RA+ EMRA T cells have significantly longer telomeres than the CM-like CD27+CD45RA+ and the EM-like CD27−CD45RA− memory subsets but shorter telomeres than the undifferentiated/naive CD27+CD45RA+ T cells (Fig. 2B). This suggested that although both CD27−CD45RA− EM and CD27−CD45RA+ EMRA T cells have the characteristics of end-stage populations, the senescence in the latter population was not associated with excessive telomere erosion.

Telomerase inhibition in CD4+ T cells by TNF-α–induced p38 signaling

The relatively long telomeres despite low telomerase activity in CD4+CD27−CD45RA+ EMRA T cells suggested that this enzyme may be actively inhibited in these cells. Previous studies have shown that the cytokine TNF-α can induce loss of CD28 expression and T cell differentiation (39) and inhibit telomerase activity in human CD8+ T human T lymphocytes (40). In addition this cytokine has been shown to induce the activation of p38 MAPK (41–43) that has an essential role in both telomere-dependent and telomere-independent senescence of fibroblasts (27, 44, 45). We found that TNF-α inhibited telomerase activity in TCR-activated CD4+ T cells (Fig. 3A), confirming observations that were made in the CD8+ T cell population. In addition, this cytokine induced p38 activation in CD4+ T cells (Fig. 3B), confirming previous reports (41–43). We also showed that TNF-α–induced telomerase inhibition was directly mediated by p38 signaling by blocking p38 signaling in activated T cells by the addition of BIRB796 (BIRB), a p38 inhibitor that blocks the activation of all four of the isoforms of p38 (46). This inhibitor was specific at the concentration used in our experiments, because it blocked the phosphorylation of p38 (pThr180/pTyr182) but not JNK (pThr183/pTyr185) in activated T cells (Fig. 3C) in accordance with others (46, 47). The addition of BIRB enhanced whereas the addition of TNF-α or BIRB to activated CD4+ T cells that had been treated with TNF-α abrogated the inhibitory effects of this cytokine. Identical results were observed in a second experiment with a different donor (data not shown). This suggested that a p38 signaling pathway may be involved in the inhibition of telomerase activity by TNF-α.

**FIGURE 2.** CD4+27− RA+ T cells do not have the shortest telomeres. A, Comparison of telomere analysis by flow-FISH technique and Southern blotting. A total of 19 PBMC samples were collected from individuals of different ages ranging from umbilical cord blood samples to individuals >70 y old. Cells were cryopreserved in 10% DMSO for telomere-length analysis by flow-FISH and snap frozen as pellets for Southern blotting. B, Telomere length in CD4+ T cells defined by CD45RA and CD27 expression was determined by three-parameter flow-FISH. Each circle represents one individual with the mean telomere length shown as a horizontal bar. Statistical analysis was performed using the two-tailed Student t test (GraphPad Prism). **p < 0.01.

**FIGURE 3.** TNF-α decreases telomerase activity of total CD4+ cells. A, Total CD4+ T cells were activated with anti-CD3 Ab and irradiated autologous APCs with and without TNF-α for 4 d. Cell extracts from equivalent numbers of Ki67+ cells were used to determine telomerase activity. Error bars represent the SE from the mean of three separate experiments (A, right panel). Statistical analysis was performed using the Wilcoxon-matched pairs test (GraphPad Prism). B, Total CD4+ were stimulated for 20 min with TNF-α, and phosphorylated p38 expression was assessed by flow cytometry. A representative plot from three donors is shown. C, Western blot showing the effects of the p38 inhibitor BIRB796 on p38 and JNK phosphorylation. β-Actin was used as a loading control. D, Total CD4+ T cells were activated with anti-CD3 Ab and irradiated APCs with and without TNF-α or BIRB for 4 d. Cell extracts from equivalent numbers of Ki67+ cells were used to determine telomerase activity. The bar graph shows the relative telomerase activity following treatment with TNF-α and/or BIRB. One of two experiments with similar results is shown. *p < 0.05.
a mechanism for telomerase inhibition in highly differentiated T cells. The reason why BirB induced telomerase in TNF-α–untreated cells was probably because of inhibition of endogenous p38 activity because this was a mixed population of CD4+ T cells containing both differentiated as well as undifferentiated T cells.

**CD4+CD27−CD45RA+ EMRA T cells express high levels of p38 MAPK activity**

Although several studies have been performed on the role of p38 in the activation and cytokine secretion of T lymphocytes (41–43), its involvement with lymphocyte differentiation is unclear. When we examined the levels of either the total p38 or the phosphorylated form of this molecule in CD4+ T cell subsets directly ex vivo, we found that the highest expression of both was in the CD27−CD45RA+ EMRA CD4+ T cell population (Fig. 4A, 4B). However, the CD27−CD45RA− EM subset also showed significantly higher levels of this molecule than the naive CD27+CD45RA+ and CD27−CD45RA− CM populations (Fig. 4A, 4B). Therefore, both the CD27−CD45RA− EM and CD27−CD45RA+ EMRA T cells upregulate the p38 MAPK that may be involved in certain senescence characteristics, in particular telomerase downregulation of these cells.

**p38 MAPK signaling regulates senescence-associated functional changes in CD4+CD27−CD45RA+ EMRA T cells**

Previous studies showed that CD4+CD27−CD45RA+ EMRA T cells have diminished capacity to expand in culture and were highly susceptible to apoptosis compared with the other subsets (7). We investigated whether this defect was mediated by p38 signaling. In these particular experiments, we activated the T cell subsets with anti-CD3 Ab and IL-2 instead of anti-CD3 Ab and irradiated autologous PBMCs as APCs to avoid the confusion of subsets with anti-CD3 Ab and IL-2 instead of anti-CD3 Ab (7). We investigated whether this defect was mediated by p38 MAPK signaling because of inhibition induced death in the APCs. We confirmed that CD27−CD45RA+ EMRA T cells were impaired in their ability to expand in culture after TCR and IL-2 activation compared with the other subsets (Fig. 5A). However, the addition of BirB to these cells during activation significantly increased the cell recovery after activation (Fig. 5A). In addition, we showed that the reduced ability of CD27−CD45RA+ EMRA T cells to expand after activation was due to increased levels of apoptosis (Fig. 5B, Supplemental Fig. 4A) and not decreased capacity to enter cell cycle as identified by staining with ki67 Ab (Fig. 5C). The inhibition of apoptosis by p38 blockade was due in part to the upregulation of the antiapoptotic molecule Bcl-2 in these cells (Supplemental Fig. 4B, 4C).

We next questioned whether the low telomerase activity in the CD27−CD45RA+ EMRA T cell population was linked to increased p38 signaling in these cells. We found that the low telomerase activity in the CD27−CD45RA+ EMRA T cell population that was stimulated with anti-CD3 and irradiated autologous APCs was significantly enhanced by up to 3.5-fold by blocking p38 with BirB compared with controls (Fig. 5D, 5E). Although telomerase activity in the CD27−CD45RA− EM population was also increased by blocking p38 signaling, this result was not significant. These results also suggest that the low telomerase activity that has previously been found in highly differentiated human CD4+ T cells (28) is more pronounced in the CD27−CD45RA+ (EMRA) compared with the CD27−CD45RA− (EM) T cell population. Thus, the decreased capacity of CD4+CD27−CD45RA+ T cells to expand in culture and to upregulate telomerase after activation is mediated largely by p38 signaling.

**Discussion**

There has been a long-standing interest in the regulation of human T cell senescence and how this may restrict the maintenance of immune memory (48–53). Previous work has focused either on the role of persistent viruses in shaping the T cell repertoire during aging and how this leads to immune dysfunction (12, 13, 17, 53, 54) or the impact of telomere erosion on restricting the proliferative capacity of memory T cell populations (55). Both avenues of investigation were linked by the observation that persistent viruses such as CMV induce the accumulation of large populations of virus-specific CD8+ and CD4+ T cells that are highly differentiated and have very short telomeres (17, 28, 54). In general, telomere erosion in T cells is accompanied by changes in the cell surface phenotype that mark a progressive increase in differentiation (48, 56). However, in this study, we have shown that the correlation between the end-stage phenotype and excessive telomere erosion does not apply to CD4+ EMRA T cells that appear to be close to senescence yet have relatively long telomeres. CD4+ EMRA T cells upregulate γH2AX, a component of the complex of proteins that make up the senescence-related DDR (57). However, it is not clear at present what initiates the senescence program in this population. We showed previously that highly differentiated CD27−CD28− T cells in both CD4 (28) and CD8 subsets (31) have short telomeres. CD45RA+CD27− T cells are mainly CD27−CD28− in both CD4 and CD8 subpopulations but not all CD27−CD28− T cells express CD45RA (4). Thus, if telomeres in CD28−CD27− subsets are investigated, the characteristics of the smaller EMRA population are missed. We previously showed that within CD8 cells that are identified on the basis of CD45RA and CD27 expression, the EMRA population, especially in younger donors also showed longer telomeres (58), as has been found in the CD4+ population in the current study.

We found that p38 signaling, which is increased in the CD27−CD45RA+ (EMRA) compared with the CD27−CD45RA− (EM) T cell populations, is involved in the induction of increased apoptosis and impaired telomerase activity in these populations after

**FIGURE 4.** CD4+27+RA+ T cells express higher levels of total and phosphorylated p38 ex vivo. The median fluorescence intensity of total p38 (A) and of phosphorylated p38 (B) was assessed ex vivo in PBMCs by gating within total CD4+ T cells and within each of the CD27+CD45RA subsets. Overlays of total p38 (A) and of phospho-p38 (B) within the respective subsets are shown. The values represent the median fluorescence intensity of p38 within each subset. The bar graphs represent the ex vivo mean fluorescence intensity of total p38 (A) and phospho-p38 (B) normalized for the levels of expression in the naive population. Error bars represent the SE (A, n = 7; B, n = 10). Statistical analysis was performed using the two-tailed Student t test (GraphPad Prism). *p < 0.05, **p < 0.01.
activation. It is possible that proinflammatory cytokines such as TNF-α may induce the p38 expression in T cells in vivo. Indirect evidence for this is that anti-TNF-α therapy in patients with rheumatoid arthritis depletes CD8+ EMRA T cells, suggesting that there may be a role for this cytokine in the generation of these cells in vivo (59). However, TNF-α alone did not induce CD45RA expression in CM or EM CD4+ T cells in vitro (N.E. Riddell and A.N. Akbar, unpublished observations). Because the EMRA population has relatively long telomeres, one possibility is that they may be generated directly from the naive T cell pool. This is currently under investigation. In contrast, previous studies have shown that IL-7 can induce CD45RA re-expression in CM CD4+ T cell populations (7, 60); however, it is not known whether this cytokine also induces p38 activation in these cells. It also remains to be determined whether the process of CD45RA re-expression itself is dependent on p38 signaling. The clarification of the interrelationship between cytokine signaling and p38-mediated senescence will be important to define the role of proinflammatory cytokines in inflammatory diseases such as rheumatoid arthritis, where excessive T cell differentiation and senescence occur (20).

It would be of particular interest to determine whether the defective ability of naïve CD4+ T cells in patients with this disease to upregulate telomerase activity is related to increased p38 signaling in these cells (61). In addition, CD4+ EMRA T cells that are specific for particular immunodominant epitopes of persistent viruses such as CMV accumulate in older humans (7, 62, 63). This suggests that CMV infection may either directly or indirectly induce a p38-related senescence program in CMV-specific CD4+ T cells that does not necessarily depend on telomere erosion. Although CD4+ EM and EMRA T cells have senescence-related proliferative defects, they are multifunctional in terms of cytokine secretion and express high levels of granzyme B and perforin (7) and may be important for protection against certain infections in vivo (59). They are therefore distinct from functionally exhausted T cells that have proliferative defects but also progressively lose the ability to secrete cytokines and to mediate cytotoxicity (64–66). Previous studies have shown that T cell exhaustion can be regulated by the ligation of surface inhibitory receptors such as PD-1 and TIM-3 and that by blocking these receptors certain functional responses can be reinstated (reviewed

![Figure 5](http://www.jimmunol.org/)

**Figure 5.** p38 inhibition enhances cell recovery by decreasing apoptosis and increases telomerase activity in CD4+CD45RA+CD27- T cells. A, T cells were activated with anti-CD3 Ab and IL-2 for 4 d in the presence or absence of BIRB. The bar graph represents the number of cells recovered normalized for the initial number of cells placed in culture. Error bars represent the SEM. The dashed line represents the initial cell input (n = 3). B, The bar graph shows the percentage of apoptotic cells (Annexin V+propidium iodide [PI]+) within each subset that was activated as in A, in the presence or absence of BIRB. Error bars represent the SEM (n = 3). C, The bar graph shows the percentage of Ki67+ proliferating cells in the presence and absence of BIRB796 in cells that were activated as in A. The results from five experiments are shown. D, An autoradiography of a TRAP assay acrylamide gel from a representative experiment is shown. Telomerase activity was determined by TRAP assay. Purified subsets were activated with anti-CD3 and irradiated APCs for 4 d in absence or presence of BIRB. The bar graph shows the fold change of telomerase activity following treatment with BIRB. As a negative control, lysis buffer was used instead of cell extract. Results have been normalized for the telomerase activity of each population in absence of the inhibitor. Error bars represent the SE from the mean of four separate experiments. Statistical analysis was performed using the two-tailed Student t test (GraphPad Prism). *p < 0.05.
in fate determination that governs the generation of short-lived possibility is that the induction of p38 signaling has a pivotal role in the p38 pathway to counteract the effects of chronic inflammation that is detrimental during aging (69, 70).

Although p38 signaling has a role in inducing senescence, it is also involved in the production of proinflammatory cytokines such as IL-1β, TNF-α, and IL-6 by T cells (44, 67, 68). An interesting possibility is that the induction of p38 signaling has a pivotal role in fate determination that governs the generation of short-lived cells (reviewed in Ref. 2). We now show that T cell senescence associated with decreased Akt (Ser 473) phosphorylation and proliferative dysfunction of highly differentiated CD8+ T cells.

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References


