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Generation and Growth of CD28nullCD8+ Memory T Cells Mediated by IL-15 and Its Induced Cytokines

Wai Kan Chiu, Monchou Fann, and Nan-ping Weng

Accumulation of CD28nullCD8+ T cells and the defects of these cells in response to antigenic stimulation are the hallmarks of age-associated decline of T cell function. However, the mechanism of these age-associated changes is not fully understood. In this study, we report an analysis of the growth of human CD28null and CD28+CD8+ memory T cells in response to homeostatic cytokine IL-15 in vitro. We showed that 1) there was no proliferative defect of CD28nullCD8+ memory T cells in response to IL-15 compared with their CD28+ counterparts; 2) stable loss of CD28 expression occurred in those actively dividing CD28+CD8+ memory T cells responding to IL-15; 3) the loss of CD28 was in part mediated by TNF-α that was induced by IL-15; and 4) CCL4 (MIP-1β), also induced by IL-15, had a significant inhibitory effect on the growth of CD28null cells, which in turn down-regulated their expression of CCR5 receptor. Together, these findings demonstrate that CD28nullCD8+ memory T cells proliferate normally in response to IL-15 and that IL-15 and its induced cytokines regulate the generation and growth of CD28nullCD8+ T cells, suggesting a possible role of IL-15 in the increase in CD28nullCD8+ T cells that occurs with aging.

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3 Abbreviations used in this paper: γc, common γ-chain; PI, proliferation index.

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Materials and Methods

Isolation of CD28null and CD28+ memory phenotype CD8+ T cells from human peripheral blood

CD28null and CD28+ memory phenotype CD8+ T cells were isolated from peripheral blood of normal volunteers by immunomagnetic separation and by cell sorting as previously described (25). In brief, blood was obtained from healthy adults and aged volunteers of the National Institute on Aging Clinical Research Branch under Institutional Review Board-approved protocols (MR123-05H and GR098-12-28-01a) and mononuclear cells were isolated by Ficoll gradient centrifugation (ICN Biomedicals). Memory phenotype CD8+ T cells were then enriched by removing other types of cells through incubation with a panel of mouse mAbs against CD4, CD24, CD11b, CD14, CD16, MHC class II, erythrocytes, platelets, and CD45RA. Ab-bound cells were subsequently removed by incubation with anti-mouse IgG-conjugated magnetic beads (Qiagen). These enriched memory phenotype CD8+ cells were either separated into CD8+ CD45RA-CD28null and CD8+ CD45RA+CD28+ memory T cells by a cell sorter (MoFlo; Dako-Cytomation) or further purified into CD28+ memory T cells through positive immunomagnetic selection using a human CD8 multisort kit (Miltenyi Biotec). In experiments comparing young (age 19–29) and old (age 76–86) donors, total CD8+ T cells were isolated directly by anti-CD8 beads as described above from PBMC. The purities of both CD8 subsets were over 96%.

Analysis of cell surface receptors and intracellular proteins

Fluorescent dye-labeled Abs against CD8-Tricolor, CD8-PE, CD95-PE, CD95L-PE, Bcl-2-PE, mouse IgG1-control-PE, CD45RA-FITC, and mouse-IgG1-control-FITC were obtained from Caltag Laboratories. Anti-IL-15Rα was purchased from R&D Systems and was FITC labeled by a fluorescein Protein Labeling kit from Fitz. Abs were conjugated to Caltag Anti-IL-15Rα. CD28-PE, CD28-PE, CD122-PE, CD132-PE, and CD45RA-allophycocyanin were purchased from BD Biosciences. Expression of CD28 on CD8 T cells were determined by two different mAbs from Caltag Laboratories and BD Biosciences and similar results were obtained. Also isotype- and fluorescent dye-matched IgG controls were used in FACS staining. For surface marker analysis, freshly purified and IL-15-treated CD8+ memory phenotype cell subsets were incubated with three or four different fluorescent dye-conjugated Abs and prepared for FACS analysis according to manufacturers instructions. For intracellular protein analysis, cells were fixed, washed, and permeabilized by Cytofix and Cytoperm (BD Biosciences), and data were acquired by FACScan or FACScalibur and analyzed by CellQuest Pro software (BD Biosciences).

Proliferation assay

We used a cell division tracking dye, CFSE (Invitrogen Life Technol), to measure the proliferation of cells as previously described (26). In brief, sorted CD28null and CD28+ memory phenotype T cells were incubated with 5 μM CFSE for 10 min at 37°C, washed with RPMI 1640 once, and cultured at 1 × 10⁶ cells/ml in RPMI 1640 supplemented with 10% FBS and penicillin (10 U/ml)/streptomycin (10 μg/ml). In vitro Gen Life Technologies) in the presence of recombinant human IL-15 (50 ng/ml or specified) (PeproTech), anti-CD3 alone, or anti-CD8 plus anti-CD28 (Invitrogen Life Technologies) either on 24-well or 48-well flat-bottom plates (BD Biosciences), dependent on the number of cells available. The proliferative responses of CD28null and CD28+ memory T cells to anti-CD3 or anti-CD28 were analyzed on day 4 and to IL-15 were analyzed on days 5, 10, and 15 by FACScan. The division rate was calculated by software ModFit LT 3.0 Mac (Verity House Software).

RT-PCR

Total RNA were extracted from freshly isolated and IL-15 cultured sorted CD28null and CD28+ memory T cells as well as from anti-CD3/CD28 Ab-stimulated CD28null and CD28+ memory T cells by a standard procedure as previously described (27). In brief, RNA were extracted from cells by Stat 60 (TelTest) based on manufacturers’ protocol and quantified by NanoDrop (NanoDrop Technologies) and reverse transcription (SuperScript II; Invitrogen Life Technologies) based on manufacturer’s instructions. mRNA levels of CD28 (forward: 5′-GGGCTGCTTTGGCTGCTTC AAC-3′ and backward: 5′-ACCACAGTGGCAGACTGCTA-3′), IL-2 (forward: 5′-GAGATGACCGCACTTCTTAC-3′ and backward: 5′-CCACCTAGACCTTAGACTGCA-3′), IL-4 (forward: 5′-CTTCGCTGAGGTCCTTCTC-3′ and backward: 5′-ACGAGAGGGTTCGCTGCAC-3′), IL-7 (forward: 5′-GAGATGACCGCACTTCTTAC-3′ and backward: 5′-CCACCTAGACCTTAGACTGCA-3′), IL-15 (forward: 5′-GAGATGACCGCACTTCTTAC-3′ and backward: 5′-CCACCTAGACCTTAGACTGCA-3′), and GM-CSF (forward: 5′-GAGATGACCGCACTTCTTAC-3′ and backward: 5′-CCACCTAGACCTTAGACTGCA-3′) were determined by real-time quantitative RT-PCR and normalized based on the level of β-actin (forward: 5′-CTTGGCACCAACGC-3′ and backward: 5′-GCGGATCCACACCGAGACT-3′). Specifically, PCR was conducted in 25-μl total volume with 0.1 μM primers using a SyBr Green kit on ABI Prism 7700 (Applied Biosystems) for cycles specified in the figure legends. The specific amplification of RT-PCR products was confirmed by agarose (2.5%) gel electrophoresis. Serial dilutions of cDNA at 1/1, 1/4, and 1/16 were used for quantitation and the images were taken by the Fluor gel imaging system (Alpha Innotech).

TNF-α neutralization and rTNF-α

In the TNF-α neutralization experiments, 10, 20, and 50 μg/ml anti-TNF-α (W6/32 or monoclonal; Centocor) or 20 μg/ml Enbrel (Immunex), a soluble TNF-α receptor, was added to the culture of CD28null and CD28+ memory T cells for the initial screening. Similar results were obtained with Remicade and Enbrel, so we used 20 μg/ml Remicade in the subsequent experiments. Neutralizing Abs to GM-CSF (1 μg/ml), INF-γ (200 ng/ml), IL-5 (100 ng/ml), IL-6 (500 ng/ml), IL-8 (10 μg/ml), and IL-13 (200 ng/ml) (PeproTech), were used according to manufacturers’ instructions and/or our own screening. Species-matched IgG of equal concentrations were used as control. rTNF-α was used at 200 ng/ml based on initial titration (PeproTech). Neutralizing Abs and recombinant cytokines were added on days 0, 7, and 14 to cell culture. In some experiments, Abs and cytokines were replaced twice a week. CD28 expression in cultured memory CD8 T cells was analyzed by FACScan at days 0, 7, 14, and 21.

Neutralization of MIP-1β and rMIP-1β

In MIP-1β-neutralizing experiments, 6 μg/ml polyclonal anti-MIP-1β Ab (PeproTech) was added to the culture of CD28null and CD28+ memory phenotype T cells according to manufacturers’ instructions and/or our own testing. In addition, we also used a monoclonal anti-MIP-1β Ab (6 μg/ml; R&D Systems) to verify the specificity of anti-MIP-1β effects. The results from both Abs were similar and we used the polyclonal anti-MIP-1β Abs in subsequent experiments. A total of 500 ng/ml rMIP-1β (PeproTech) was used based on initial titration. CD28 expression was analyzed as described above. In experiments that examined the MIP-1β effect on CD28null and CD28+ memory T cells, CD8+ memory T cells were purified, cultured for 14 days in the presence of IL-15, and sorted into CD28null and CD28+ subsets. Both subsets were labeled with CFSE and cultured with 50% fresh medium and 50% pooled conditioned medium was collected from IL-15-cultured CD8 memory T cells of 10 donors. Each subset was further divided into two pools: IL-15 culture alone and IL-15 culture with anti-MIP-1β Ab. Cells were cultured for an additional 14 days and their proliferation rates were analyzed by FACScan and software ModFit LT 3.0 Mac.

Measurement of cytokines in culture supernatant and plasma

Culture supernatants of IL-15-treated CD28null and CD28+ memory phenotype T cells with various neutralizing Abs and cytokines were collected at days 7, 14, and 21 and the concentration of 17 cytokines (CSF, GM-CSF, INF-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, TNF-α, MCP-1, and MIP-1β) were measured by BioPlex protein array system (Bio-Rad) according to manufacturers’ instructions. Plasma samples were isolated from whole blood by centrifuging at 1,400 rpm for 20 min and were frozen at −20°C. The samples were thawed the day before measurement and were spun at 14,000 rpm for 5 min to remove precipitated proteins. Cytokines were measured by both BioPlex protein array system (TNF-α and MIP-1β) and LincoPlex Human Cytokine 22-plex kit (shares 16 cytokines with BioPlex and 6 additional cytokines, i.e., eotaxin, IL-1α, IL-15, MIP-1α, INF-γ-inducible protein 10, and RANTES) (Linco Research) according to manufacturers’ instructions.

Statistical analysis

The differences of biological parameters between CD28null and CD28+ memory T cells were analyzed by a two-tailed Student’s t test. The differences of biological parameters between donors with a high percentage of CD28nullCD8+ T cells and donors with a low percentage of CD28nullCD8+ T cells were analyzed by a one-tailed Student’s t test.

Results

The response of CD28nullCD8+ memory T cells to homeostatic cytokine IL-15 is not impaired

A prominent defect of CD28nullCD8+ T cells is its poor proliferative response to antigenic stimulations (4, 28), yet the number of CD28nullCD8+ T cells increases in peripheral blood with age. Because the maintenance of memory CD8+ T cells is dependent on

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homoeostatic cytokines, we wanted to determine whether there was any defect in the proliferation of CD28nullCD8+ T cells to homoeostatic cytokines such as IL-15. We isolated CD28null and CD28+ memory CD8+ T cells from healthy adults (Fig. 1A) and cultured them in the presence of IL-15. To rule out a transient down-regulation of surface expression of CD28 in these CD28nullCD8+ memory T cells, we analyzed the transcripts of CD28 by real-time RT-PCR and confirmed the absence of CD28 mRNA in CD28nullCD8+ memory T cells (Fig. 1B). To further analyze CD28nullCD8+ T cells, we compared the expression levels of IL-15 receptors (IL-15Rα, IL-2/IL-15β, and γc) and apoptosis/survival markers (Fas, Fas ligand, and Bcl2) before and after IL-15 treatment and found that both IL-15 receptors and apoptosis/survival markers were similarly expressed between CD28null and CD28+CD8+ T cells, independent of the age of the donors (Fig. 1, C and D). To rule out the potential function of residual CD28 in CD28nullCD8+ memory T cells, we analyzed the IL-2 expression by real-time RT-PCR and confirmed that our CD28nullCD8+ memory T cells lacked the ability to transcribe IL-2 in response to the stimulation by anti-CD3 plus anti-CD28 Abs (data not shown).

Although CD28null and CD28+ CD8+ memory T cells showed similar celular expressions, the proliferative deficiency in response to the cross-linking of TCR alone (anti-CD3) or the cross-linking of TCR in conjunction with costimulatory receptor CD28 (anti-CD3 plus anti-CD28) was obvious in CD28nullCD8+ memory T cells. The proliferation indexes (PI) were 1.3 and 1.0 for CD28+ and CD28null cells after anti-CD3 stimulation for 4 days, respectively, and were 2.5 and 1.6 for CD28+ and CD28null cells after anti-CD3/CD28 stimulation for 4 days, respectively (p < 0.01, n = 6) (Fig. 2A). In contrast, CD28nullCD8+ memory T cells proliferated at a comparable rate to their CD28+ counterparts in response to IL-15 at 50 ng/ml (Fig. 2A) and at a lower concentration (5 ng/ml) (data not shown) in vitro. The average PI at day 14 after IL-15 culture was 5.6 for CD28− cell and 4.9 CD28null cells (p = 0.6, n = 6). These findings suggest that maintenance of CD28nullCD8+ memory T cells in vivo may be mediated by homoeostatic cytokines such as IL-15. To further determine whether age influences IL-15-induced cell division of CD28nullCD8+ T cells, we compared CD8+ T cells from young and old donors in response to IL-15 in vitro. We found that CD28+ and CD28nullCD8+ T cells from both age groups had similar proliferation rates: PI was 2.72 and 2.53 for CD28+ and CD28nullCD8+ T cells of young donors, respectively, and PI was 2.77 and 2.74 for CD28+ and CD28nullCD8+ T cells of old donors, respectively. This suggests that age does not alter the proliferation rates of CD28nullCD8+ T cells in response to IL-15 (Fig. 2B).

IL-15 induces generation of CD28null cells from CD28+CD8+ memory T cells in vitro

Loss of CD28 expression in CD28+CD8+ T cells has been associated with repeated antigenic stimulation during chronic infection and with aging in vivo, and under mitogenic stimulation in vitro (7, 18, 29). Recently, one report showed that γc sharing cytokines including IL-15 are capable of down-regulating CD28 expression in CD28+CD8+ T cells (18). However, it is unclear whether the cytokine-mediated loss of CD28 expression in CD8+ T cells is transient or stable and what mechanisms underlie such a loss. To address these questions, we analyzed the kinetic expression of CD28 on highly pure CD28+CD8+ memory T cells cultured with IL-15. We found that CD28 expression was relatively stable during the initial few rounds of cell divisions (Fig. 3A); the average ratio of CD28null to CD28+CD8+ memory T cells cultured with IL-15 was 2.5 and 1.6 for CD28null cells after anti-CD3 stimulation for 4 days, respectively (p < 0.01, n = 6) (Fig. 3A). In contrast, CD28nullCD8+ memory T cells proliferated at a comparable rate to their CD28+ counterparts in response to IL-15 at 50 ng/ml (Fig. 3A) and at a lower concentration (5 ng/ml) (data not shown) in vitro. The average PI at day 14 after IL-15 culture was 5.6 for CD28− cell and 4.9 CD28null cells (p = 0.6, n = 6). These findings suggest that maintenance of CD28nullCD8+ memory T cells in vivo may be mediated by homoeostatic cytokines such as IL-15. To further determine whether age influences IL-15-induced cell division of CD28nullCD8+ T cells, we compared CD8+ T cells from young and old donors in response to IL-15 in vitro. We found that CD28+ and CD28nullCD8+ T cells from both age groups had similar proliferation rates: PI was 2.72 and 2.53 for CD28+ and CD28nullCD8+ T cells of young donors, respectively, and PI was 2.77 and 2.74 for CD28+ and CD28nullCD8+ T cells of old donors, respectively. This suggests that age does not alter the proliferation rates of CD28nullCD8+ T cells in response to IL-15 (Fig. 2B).
We then analyzed induction of IL-2 and IL-2R(C). IL-15-treated CD28null and CD28+ memory T cells were prepared by CellQuest Pro. A representative histogram is shown from six independent donors. 

To determine whether loss of CD28 expression was limited to the surface expression or occurred at the transcription level, we sorted CD28nullCD8+ T cells generated after IL-15 treatment and analyzed the transcripts of CD28 by real-time RT-PCR. We found that CD28 mRNA was absent in CD28nullCD8+ memory T cells (Fig. 3C). We then analyzed induction of IL-2 and IL-2Rα (or CD25) expression as the function of CD28 and demonstrated that these CD28nullCD8+ memory T cells transcribed neither IL-2 nor CD25 in response to anti-CD3/CD28 stimulation (Fig. 3D). Furthermore, we found that loss of CD28 expression was stable in IL-15-generated CD28nullCD8+ memory T cells over a month (data not shown). These findings suggest that IL-15-mediated down-regulation of CD28 expression occurred primarily in actively dividing CD8+CD8+ memory T cells and that IL-15-induced loss of CD28 expression in CD8+ memory T cells was stable under continuous IL-15 stimulation.

Generation of CD28null cells is unaffected by IL-15-induced GM-CSF, IFN-γ, IL-5, IL-6, IL-8, and IL-13

Our previous studies showed that IL-15 is capable of inducing production of effector cytokines in CD8+ memory T cells (25, 30), including TNF-α, which has been recently reported to induce down-regulation of CD28 expression in CD4+ T cells (29). To understand the role of IL-15-induced cytokines in regulation of CD28 expression, we measured 17 cytokines in the supernatant of IL-15-treated CD28nullCD8+ memory T cells and found that 8 cytokines were highly induced in the IL-15-treated culture supernatant of CD8+ memory T cells (Table I). Neutralizing Abs specific to each of the eight cytokines were applied individually in the culture. With optimal concentrations of the Abs, we confirmed the neutralization of each cytokine by measurement of Ab-treated supernatants (data not shown) but we did not observe significant change of the percentage of CD28null cells in IL-15-cultured CD8+ memory T cells in the absence of individual cytokine GM-CSF, IFN-γ, IL-5, IL-6, IL-8, and IL-13 (Table I). We did not observe changes of the percentage of CD28null cells in IL-15-cultured...
CD8⁺ memory T cells after blocking all six cytokines simultaneously (data not shown). This suggests that IL-15-induced GM-CSF, IFN-γ, IL-5, IL-6, IL-8, and IL-13 at least did not participate in the generation of CD28nullCD8⁺ T cells in vitro individually.

IL-15-induced TNF-α down-regulates CD28 in CD28⁺CD8⁺ memory T cells

To determine whether TNF-α mediates down-regulation of CD28 expression in CD28⁺CD8⁺ memory T cells, we used neutralizing anti-TNF-α Ab to block the TNF-α in the culture medium and found a significant decline in loss of CD28 expression in anti-TNF-α-Ab-treated cells (24.8%) as compared with the control (30.4%) at day 14 (p = 0.002, n = 12) (Fig. 4, A and B). In the presence of anti-TNF-α Ab, the number of CD28nullCD8⁺ memory T cells among cells was ~15% less than that of the control after 14 days (Table II). To confirm the neutralizing effect of anti-TNF-α Ab, we tested different concentrations of Ab (10, 20, and 50 μg/ml) and measured TNF-α in the supernatant of Ab-treated cells. We found no detectable levels of TNF-α in the anti-TNF-α-Ab-treated culture supernatant (data not shown).

To confirm the effect of TNF-α in down-regulation of CD28 expression in CD8⁺ memory T cells, we tested the effect of added rTNF-α. The percentage of CD28nullCD8⁺ memory T cells in cultures containing IL-15 plus 200 ng/ml rTNF-α was significantly higher than that in control cultures (IL-15 alone) at day 14 (59.2 vs 30.4%) in control cultures with IL-15 alone, p = 7.1 × 10⁻⁴) and day 21 (67.4 vs 44% in control, p = 0.001) (Fig. 4C and Table II). Furthermore, the loss of CD28 expression induced by exogenous GM-CSF, IFN-γ, IL-5, IL-6, IL-8, and IL-13 at least did not participate in the generation of CD28nullCD8⁺ T cells in vitro individually.

### Table II. Effects of TNF-α and MIP-1β on the changes of the percentage of CD28nullCD8⁺ memory T cells under IL-15

<table>
<thead>
<tr>
<th>Condition</th>
<th>Day 7 (%)</th>
<th>Day 14 (%)</th>
<th>Day 21 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-15 + Anti-TNF-α/IL-15</td>
<td>94 ± 6</td>
<td>85 ± 4⁶</td>
<td>90 ± 3⁶</td>
</tr>
<tr>
<td>IL-15 + TNF-α/IL-15</td>
<td>216 ± 2⁶</td>
<td>216 ± 18⁶</td>
<td>185 ± 14⁶</td>
</tr>
<tr>
<td>IL-15 + Anti-MIP-1β/IL-15</td>
<td>83 ± 9</td>
<td>133 ± 10⁶</td>
<td>125 ± 8⁶</td>
</tr>
<tr>
<td>IL-15 + MIP-1β/IL-15</td>
<td>98 ± 5</td>
<td>100 ± 3</td>
<td>90 ± 8</td>
</tr>
<tr>
<td>IL-15 + Anti-TNF-α + MIP-1β/IL-15</td>
<td>98 ± 4</td>
<td>87 ± 2⁷</td>
<td>82 ± 8</td>
</tr>
<tr>
<td>IL-15 + TNF-α + Anti-MIP-1β/IL-15</td>
<td>190 ± 16⁶</td>
<td>214 ± 18⁶</td>
<td>201 ± 22⁶</td>
</tr>
</tbody>
</table>

⁶ Results are statistically significant compared to IL-15 alone by Student’s t test.
CD28null and CD28null data in presented as mean/SEM (n = 10). A, Down-regulation of CCR5 expression in CD28null and CD28null memory T cells was observed at 50 ng/ml rTNF-α, the ratio of CCR5 expression levels between treated and untreated CD28null cells was significantly lower than that between control cultures with IL-15 alone, p = 0.03 (Fig. 4D). We found a significant increase in the number of CD28null cells at day 14 (38.3 vs 30.4% in control cultures with IL-15 alone, p = 0.03) and at day 21 (47.9 vs 44% in control, p = 0.01) (Fig. 4D and Table II). A monoclonal anti-MIP-1β Ab was capable of down-regulating CD8+ CD8+ memory T cells in vitro. Together, these findings indicate that TNF-α is capable of down-regulating CCR5 expression in CD28null and CD28null memory T cells. As neutralization of TNF-α resulted in an ~15% reduction in the number of CD28null cells induced by IL-15, we speculated that other IL-15-induced cytokines might play a role in regulation of CD28 expression and the growth of CD28null and CD28null memory T cells. MIP-1β, a chemokine involved in T cell migration and proliferation (31), was highly induced by IL-15 (Table I). When MIP-1β was blocked by neutralizing polyclonal anti-MIP-1β Ab in IL-15 cultures (Fig. 4D), we found a significant increase in the number of CD28null cells at day 14 (38.3 vs 30.4% in control cultures with IL-15 alone, p = 0.03) and at day 21 (47.9 vs 44% in control, p = 0.01) (Fig. 4D and Table II). A monoclonal anti-MIP-1β blocking Ab had a similar effect (data not shown). When we tested the effect of added rMIP-1β in the culture, we noticed a decreased percentage of frequently dividing cells as compared with the control but they did not reach statistical significance (Fig. 4E and Table II). However, we did not observe an additive

FIGURE 6. Production of TNF-α and MIP-1β by IL-15-treated CD8+ memory T cells in vitro. A. The level of TNF-α is correlated with the level of MIP-1β in the supernatant of CD8+ CD8+ memory T cells. B. MIP-1β negatively regulates level of TNF-α in CD28null CD8+ memory T cells. C. Down-regulation of MIP-1β expression in CD28null CD8+ memory T cells. D. Decrease of CCR5 expression in CD28null CD8+ memory T cells was observed at day 21 (47.9 vs 44% in control, p = 0.01) (Fig. 4D and Table II). A monoclonal anti-MIP-1β blocking Ab had a similar effect (data not shown). When we tested the effect of added rMIP-1β in the culture, we noticed a decreased percentage of frequently dividing cells as compared with the control but they did not reach statistical significance (Fig. 4E and Table II). However, we did not observe an additive
effect of reducing CD28null cells when both anti-TNF-α Ab and rmIP-1β were applied in the IL-15 culture (Table II).

To examine the action of MIP-1β in IL-15-cultured memory CD8 T cells, we isolated CD28null and CD28null cells from IL-15-treated CD28null CD8 T cells that had been cultured for 2 wk and cultured them separately using the MIP-1β-containing conditional medium in the presence or absence of anti-MIP-1β Abs for another 14 days. We found a significant increase in CD28null cell proliferation in the presence of anti-MIP-1β Ab as compared with that of nonspecific Ab control (PI was 2.6 for anti-MIP-1β treated vs 2.0 for the control, p = 0.05, n = 10) (Fig. 5A). Although a slight increase in the proliferation of CD28null cells in the presence of anti-MIP-1β Ab as compared with that of nonspecific Ab control was also observed, it did not reach statistical significance (PI was 2.2 for anti-MIP-1β treated vs 1.9 for the control, p = 0.19, n = 10) (Fig. 5B). To understand the differential effect of MIP-1β on the growth of memory T cell subsets, we analyzed the expression of CCR5, a MIP-1β receptor, and found that exposure of MIP-1β in vitro resulted in down-regulation of CCR5 in both CD28null and CD28null CD8 T memory T cells (Fig. 5C). Furthermore, we found a significantly lower level of CCR5 expression in CD28null than in CD28null memory T cells ex vivo (Fig. 5D). As lower levels of CCR5 were expressed in CD28null CD8 T cells than in their CD28null counterparts, the inhibitory effect of increased MIP-1β on the growth of CD28null CD8 T cells was minimized. These findings suggest that the growth of CD28null CD8 T cells was affected by both positive and negative effects of cytokines, and that the effects of these cytokines were also regulated by the expression of their corresponding receptors.

Discussion

The deficiency of activation-induced proliferation of CD28null CD8 T cells has been considered a key defect in age-associated T cell dysfunction. In this regard, it was somewhat surprising that CD28null and CD28null CD8 T cells had a similar growth rate in response to IL-15 (Fig. 2). This suggests that the IL-15-mediated growth pathway is intact in CD28null CD8 T memory T cells, providing a plausible explanation for the age-associated expansion of CD28null CD8 T cells mediated by homeostatic cytokines. Furthermore, the fact that IL-15 and its induced cytokines are capable of down-regulating CD28 expression points out the prominent role of these cytokines in generation and maintenance of CD28null CD8+ T cells with age.

Despite the identification of homeostatic cytokine in regulation of CD28 expression in CD28null CD8 T cells, the mechanisms of their action are not fully understood. TNF-α, which has been shown to down-regulate CD28 expression in CD8 T cells through alteration of CD28 promoter activity, accounts for ~15% of the CD28null CD8 T memory T cells in culture with IL-15, as indicated by the effect of neutralizing anti-TNF-α Ab (Fig. 4). We did not detect effects on CD28 expression by individual neutralization of other IL-15-induced cytokines (GM-CSF, IFN-γ, IL-5, IL-6, IL-8, and IL-12) alone or in combination (Table I). Although it was somewhat surprising that anti-MIP-1β and TNF-α did not have an additive effect when added to the culture together (Table II), it should be noted that the lack of MIP-1β led to an increase in TNF-α and TNF-α was not the sole factor that determined CD28 down-regulation. Moreover, TNF-α and MIP-1β seem to regulate CD28 expression through different mechanisms; TNF-α directly regulates CD28 down-regulation while MIP-1β regulates cell proliferation. Because IL-15 serves primarily as a growth factor for CD8 memory T cells in this culture system, it is difficult to distinguish its growth function from its potential roles in CD28 expression. Whether the down-regulation of CD28 expression in the
remaining CD8<sup>+</sup> memory T cells is mediated by IL-15 directly or by IL-15-induced cytokines in addition to TNF-α remains to be determined.

The finding that MIP-1β delivers an inhibitory effect on the growth of CD28<sup>+</sup>CD8<sup>+</sup> T cells is unexpected (Fig. 5). It appears that IL-15 exerts a paradoxical effect on CD8<sup>+</sup> memory T cells. IL-15 induces TNF-α, which promotes generation of CD28<sup>+</sup> T cells, while it also induces MIP-1β, a chemokine that inhibits the growth of CD28<sup>+</sup>CD8<sup>+</sup> memory T cells. Strikingly, the level of TNF-α appears to positively regulate the level of MIP-1β while the level of MIP-1β appears to negatively regulate the level of TNF-α (Fig. 6). Furthermore, the observed reduction in expression of CCR5 in CD28<sup>-</sup>CD8<sup>+</sup> memory T cells in vitro and in vivo potentially acts to minimize the negative growth impact of MIP-1β on these cells. Together, these data suggest that a tightly regulated feedback system may operate here to balance the effects of cytokines on the growth of CD8<sup>+</sup> memory T cells. The immune system is constantly adjusting and balancing effects that may be beneficial and detrimental in specific circumstances. In this case, the undesirable effect of IL-15-mediated homeostatic proliferation is downregulation of CD28. By producing MIP-1β, IL-15 indirectly modulates the accumulation of CD28<sup>+</sup> T cells. Further modulating the overall outcome of these events, expression of CCR5 is reduced on CD28<sup>+</sup>CD8<sup>+</sup> memory T cells, making them less susceptible to inhibition by MIP-1β.

The factors that contribute to the accumulation of CD28<sup>+</sup>CD8<sup>+</sup> memory T cells with age are complex. Although we have shown that IL-15-induced TNF-α and MIP-1β are capable of regulating CD28<sup>+</sup>CD8<sup>+</sup> T cells, other cytokines have also been linked to CD28 regulation. IL-12 and IL-4 are able to slow down the loss of CD28 expression in CD8<sup>+</sup> T cells and/or stimulate CD28 expression in CD4<sup>+</sup> T cells to re-express CD28 (32, 33). Additional experiments are needed to determine the interactions and mechanisms among these cytokines in regulation of CD28 expression and CD28<sup>+</sup>CD8<sup>+</sup> T cells. Our findings presented in this report suggest that homeostatic cytokine IL-15 is a cause of the generation and accumulation of CD28<sup>+</sup>CD8<sup>+</sup> memory T cells. Based on previous reports and present findings, we propose a two-step and feedback model for age-associated accumulation of CD28<sup>+</sup>CD8<sup>+</sup> memory T cells (Fig. 8). At the first step, loss of CD28 expression occurs in CD28<sup>+</sup>CD8<sup>+</sup> T cells as a correlate of proliferation induced by either antigenic stimulation and/or homeostatic cytokines such as IL-15. The majority of T cells may re-express CD28 after the removal of such stimulations. Only those T cells with stable loss of CD28 expression, probably influenced by the continuous presence of the stimulators, TNF-α and possible additional cytokines, enter the second phase. During the second phase, the proliferative ability of CD28<sup>+</sup>CD8<sup>+</sup> memory T cells to stimulate Ag is impaired but remains intact to homeostatic cytokines. The cytokine-mediated growth of CD28<sup>+</sup>CD8<sup>+</sup> memory T cells appears to be complex as well. Both positive (IL-15) and negative (MIP-1β) growth factors for CD28<sup>+</sup>CD8<sup>+</sup> memory T cells exist and the positive growth effects of CD28<sup>+</sup>CD8<sup>+</sup> memory T cells eventually prevail with the advance of age. A better understanding of the mechanisms behind the generation and growth of CD28<sup>+</sup>CD8<sup>+</sup> memory T cells may facilitate the development of therapeutic means to improve the immune function in the elderly.

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**Disclosures**

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**References**


9. Monteiro, J., F. Batilwalla, H. Ostert, and P. K. Gregersen. 1996. Shortened telomeres in clonally expanded CD8<sup>+</sup>CD8<sup>+</sup> T cells imply a replicative history that is distinct from their CD8<sup>+</sup>CD8<sup>+</sup> counterparts. J. Immunol. 156: 3587–3590.


