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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 CCL4 receptor CCR5. 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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A n essential part of the adaptive immune response, CD8+ T cells mediate protection against cancer and infection by intracellular pathogens. The function of CD8+ T cells depends on their ability to respond to the engagement of TCRs and costimulatory receptors with their respective ligands on APCs. CD28, a key costimulatory receptor for T cell activation, participates in a range of events from the amplification of the TCR signals to the induction of essential cytokine secretion such as IL-2 (1, 2). Without signals from CD28 generated by binding to B7 family members on APC, the interaction between TCR and the Ag/MHC complex alone is not sufficient to completely activate T cells and therefore an efficient T cell response is not generated.

Loss of CD28 expression on CD8+ T cells is a hallmark of the age-associated decline of T cell function (3–5). Because CD28nullCD8+ T cells are absent in the newborn (3, 6), increase with chronic infection (7, 8), and have shorter telomere length than their CD28+ counterparts (9, 10), it is generally believed that CD28nullCD8+ T cells are derived from CD28+CD8+ T cells. As a consequence of repeated stimulation, CD28nullCD8+ T cells exhibit a reduced proliferative response to TCR cross-linking and mitogen (PHA) (3, 11–15); they are also associated with replicative senescence in long-term culture (16–18). Despite their proliferative capacities, CD28nullCD8+ T cells retain or even enhance their cytotoxic properties (3, 4, 19). However, the mechanisms underlying age-associated accumulation of CD28nullCD8+ memory T cells are not fully understood. As memory T cells are maintained by homeostatic cytokines such as IL-15 in an Ag-independent manner (20–22), it is of great interest to determine the responsiveness of CD28nullCD8+ memory T cells to homeostatic cytokines.

A transient down-regulation of CD28 expression on T cells occurs after antigenic stimulation (23). Recently, down-regulation of CD28 in T cells by cytokines sharing the common γ-chain (γc)3 receptors including IL-15 has been reported (18); however, it is unclear whether such down-regulation is transient or stable and what mechanisms are responsible for these cytokine-mediated down-regulations of CD28. Furthermore, TNF-α, a proinflammatory cytokine secreted by various cells including T cells, has also been shown to down-regulate CD28 expression in CD4+ T cells (24). Whether TNF-α can down-regulate CD28 expression in CD8+ T cells and whether other cytokines are also involved in the regulation of CD28 expression remain to be determined.

In this report, we showed that CD28null and CD28+CD8+ memory T cells exhibited comparable growth under IL-15 stimulation in vitro. Furthermore, we showed that IL-15-mediated proliferation resulted in a stable loss of CD28 expression in CD28+CD8+ memory T cells in part through the induction of TNF-α secretion. Surprisingly, we found that IL-15-induced production of CCL4 had a significant inhibitory effect on the growth of CD28nullCD8+ memory T cells and a less inhibitory effect on that of CD8+ cells. Finally, we showed that peripheral blood of older donors was characterized by high basal levels of TNF-α and an increase in the number of CD28nullCD8+ memory T cells, and that high basal levels of CCL4 in peripheral blood of older donors was associated with the down-regulation of CCR5 (a receptor for CCL4) on these CD28nullCD8+ memory cells. Together, our findings suggest that CD28nullCD8+ memory T cells can be generated by cytokine-mediated down-regulation of CD28 expression in CD28+CD8+ memory T cells and that their accumulation may result from the dynamic interactions among cytokines induced by IL-15.

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3 Abbreviations used in this paper: γc, common γ-chain; PI, proliferation index.
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 (MR1220-05H and GR09-12-28-01a). 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 Abs 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 CD8+ memory T cells through positive immunomagnetic selection by 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, CD28-PE, CD95-PE, CD95L-PE, Bcl2-PE, mouse IgG1-control-PE, CD45RA-FITC, and mouse-IgG1-control-FITC were obtained from Caltag Laboratories. Anti-IL-15Ra was purchased from R&D Systems and was FITC labeled by a Fluorescin Protein Labeling kit from Pierce. Abs against CCR5-FITC, 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 manufacturer’s instructions. For intracellular protein analysis, cells were fixed, washed, and permeabilized by Cytofix and Cytoperm (BD Biosciences), and data were acquired by FACSscan or FACSComp (BD Biosciences).

Proliferation assay

We used a cell division tracking dye, CFSE (Invitrogen Life Technologies), 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 x 10^5 cells/ml in RPMI 1640 supplemented with 10% FBS and penicillin (10 U/ml)/streptomycin (10 μg/ml). cDNA synthesis was confirmed by agarose (2.5%) gel electrophoresis. Serial 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).

TFN-α neutralization and rTNF-α

In the TFN-α neutralization experiments, 10, 20, and 50 μg/ml anti-TNF-α (W6/32; Celtion) or 20 μg/ml Enbrel (Immunex), a soluble TNF-α receptor, was added to the culture of CD28null CD8+ 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), IFN-γ (200 ng/ml), IL-5 (100 ng/ml), IL-6 (500 ng/ml), IL-8 (10 ng/ml), and IL-13 (200 ng/ml) (PeproTech), were used according to manufacturers’ instructions and/or our own screening.

TCRT-PCR

Total RNA were extracted from freshly isolated and IL-15-cultured sorted CD8null and CD28null and CD28+ memory T cells by a standard procedure as previously described (27). In brief, RNA were extracted from cells by Stat 60 (Tel-Test) based on manufacturers’ protocol and quantitated by NanoDrop (NanoDrop Technologies) and RNA integrity was confirmed by real-time RT-PCR analysis and normalized based on the level of β-actin (forward: 5'-GCTGGACACCCGAGCCTCC-3' and backward: 5'-GGGCATCCACACCGGTACT-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).

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 CD8+ memory phenotype T cells according to manufacturers’ instructions and 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+ CD8+ memory T cells, CD8+ memory T cells were cultured, 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 FACSscan and software ModFit LT 3.0 Mac.

Measurement of cytokines in culture supernatant and plasma

Culture supernatants of IL-15-treated CD28null CD8+ memory phenotype T cells with various neutralizing Abs and cytokines were collected at days 7, 14, and 21 and the concentration of 17 cytokines (GM-CSF, IFN-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., cectnin, IL-1α, IL-1β, cytokine-inducible protein 10, and RANTES) (Lincor Research) according to manufacturers’ instructions.

Statistical analysis

The differences of biological parameters between CD28null and CD28+ CD8+ 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 CD28null CD8+ T cells and donors with a low percentage of CD28null CD8+ T cells were analyzed by a one-tailed Student’s t test.

Results

The response of CD28null CD8+ memory T cells to homeostatic cytokine IL-15 is not impaired

A prominent defect of CD28null CD8+ T cells is its poor proliferative response to antigenic stimulations (4, 28), yet the number of CD28null CD8+ T cells increases in peripheral blood with age. Because the maintenance of memory CD8+ T cells is dependent on...
homeostatic cytokines, we wanted to determine whether there was any defect in the proliferation of CD28nullCD8 T cells to homeostatic 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 CD28null/CD8+ memory T cells, we analyzed the transcripts of CD28 by real-time RT-PCR and confirmed the absence of CD28 mRNA in CD28null/CD8+ memory T cells (Fig. 1B). To further analyze CD28null/CD8+ T cells, we compared the expression levels of IL-15 receptors (IL-15Ra, 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 on CD8 T cells, we compared CD8+ memory T cells from both age groups had similar proliferation rates to IL-15 (Fig. 2A).

IL-15 induces generation of CD28null cells from CD28+ 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 was 1.4 (Fig. 3B). In contrast, we found that a significant loss of CD28 expression occurred after the fifth cell division; the average ratio of CD28null to CD28+CD8+ memory T cell was 0.43 for the cells that had undergone fewer than five cell division (n = 11) (Fig. 3B). To
down-regulation of CD28 expression in CD4+ T cells.

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 1). 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 1). 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 CD28−/CD8+ 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 CD28−/CD8+ 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 cultures. 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 CD28−/CD8+ 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

**Figure 4.** IL-15-induced TNF-α and MIP-1β influence the number of CD28− cells in CD28−CD8+ memory T cells. A. Generation of CD28− cells from CD28−CD8+ memory T cells after IL-15 treatment. CD28−CD8+ memory T cells isolated from cell sort were labeled with CFSE and cultured in the presence of IL-15. B. Reduction of CD28− cells by blocking TNF-α with Ab in IL-15-treated CD28−CD8+ memory T cells. Anti-TNF-α treatment (20 μg/ml) was added along with IL-15 to freshly sorted CD28−CD8+ memory T cells. C. Increase of CD28− cells by supplement of TNF-α in IL-15-treated CD28−CD8+ memory T cells. rTNF-α (200 ng/ml) was added along with IL-15 to freshly sorted CD28−CD8+ memory T cells. D. Increase of CD28− cells in the presence of MIP-1β-neutralizing Ab in IL-15-treated CD28−CD8+ memory T cells. Anti-MIP-1β (6 μg/ml) was added along with IL-15 to sorted CD28−CD8+ memory T cells. E. Reduction of CD28− cells in the presence of MIP-1β. MIP-1β (500 ng/ml) was added along with IL-15. In all culture conditions, cells were collected at days 7, 14, and 21 and the rate of cell division and CD8 expression were analyzed by FACS. The representative plots and the mean ± SEM from 5 to 13 independent donors are shown.

**Table I. Summary of highly expressed cytokines in the supernatant of IL-15-cultured CD28−CD8+ memory T cells and their involvement in the regulation of CD28−CD8+ memory T cells**

<table>
<thead>
<tr>
<th>Cytokinea</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Neutralizing Ab Effects</th>
<th>CD28− (200 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>22.2</td>
<td>30.6</td>
<td>28.5</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>161.4</td>
<td>79.3</td>
<td>26.7</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>IL-5</td>
<td>41.2</td>
<td>104.3</td>
<td>37.8</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>IL-6</td>
<td>1037.5</td>
<td>461.6</td>
<td>224.6</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>IL-8</td>
<td>5883.7</td>
<td>1179.6</td>
<td>267.3</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>IL-13</td>
<td>202.6</td>
<td>407.7</td>
<td>233.3</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>238.3</td>
<td>120.4</td>
<td>137.9</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>TNF-α</td>
<td>14.4</td>
<td>14.2</td>
<td>8.4</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

a The concentration of cytokines was picograms per milliliter.

b Data were presented as mean ± SEM (n = 5).

**Table II. Effects of TNF-α and MIP-1β on the changes of the percentage of CD28−/CD8+ 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 + TNF-α/IL-15</td>
<td>94 ± 6</td>
<td>85 ± 4b</td>
<td>90 ± 3b</td>
</tr>
<tr>
<td>IL-15 + TNF-α/IL-15</td>
<td>216 ± 26b</td>
<td>216 ± 18b</td>
<td>185 ± 14b</td>
</tr>
<tr>
<td>IL-15 + Anti-MIP-1β/IL-15</td>
<td>83 ± 9</td>
<td>133 ± 10b</td>
<td>125 ± 8b</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 ± 2b</td>
<td>82 ± 8</td>
</tr>
<tr>
<td>IL-15 + TNF-α + Anti-MIP-1β/IL-15</td>
<td>190 ± 16b</td>
<td>214 ± 18b</td>
<td>201 ± 22b</td>
</tr>
</tbody>
</table>

a CD28−CD8+ memory T cells were cultured with IL-15 (50 ng/ml) in the presence or absence of neutralizing Abs or recombinant TNF-α or MIP-1β or a combination of Ab and recombinant TNF-α or MIP-1β for a given time. The percentage of CD28−CD8+ memory T cells in the culture was determined by FACS analysis. The relative percentages of CD28−CD8+ memory T cells under the various conditions to the IL-15 control are presented as mean ± SEM (n = 5–13).

b Results are statistically significant compared to IL-15 alone by Student’s t test.
TNF-α is dosage dependent, and a significant level of down-regulation of CD28 expression was observed at 50 ng/ml rTNF-α (data not shown). A high concentration of exogenous TNF-α provided at the beginning of culture accelerated CD28 down-regulation as early as 7 days of culture (Fig. 4C and Table II). Together, these findings indicate that TNF-α was capable of down-regulating CD28 expression in CD28null CD8+ memory T cells.

IL-15-induced MIP-1β has a significant negative effect on the proliferation of CD28null but not CD28+ CD8+ 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 CD28+ CD8+ 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% vs 44% in control, 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 7. Levels of TNF-α and MIP-1β in peripheral blood. A. Increased concentration of TNF-α. B. Increase of MIP-1β in blood plasma in old donors. C. Increase of the percentage of CD28nullCD8 T cells with age. Whole blood was collected from young (age < 34, n = 15) and old (age > 70, n = 59) donors. Plasma was isolated and cytokine level was measured by BioPlex and LincoPlex protein array systems, and the percentage of CD28nullCD8 T cells was determined by FACS analysis.

As shown above, it appears that IL-15 induced two antagonist cytokines, TNF-α and MIP-1β, for regulating CD28nullCD8 T cell generation and maintenance. To determine whether TNF-α regulates MIP-1β, we measured MIP-1β in the supernatant from either rTNF-α or anti-TNF-α-treated cultures and found that the levels of MIP-1β in the culture supernatant correlated with the level of TNF-α (Fig. 6A). This suggests that the production of MIP-1β was regulated by the level of TNF-α in the supernatant. To examine whether the level of MIP-1β reciprocally regulates TNF-α, we measured TNF-α in the supernatant from either anti-MIP-1β- or rMIP-1β-treated cultures and found that anti-MIP-1β resulted in an increase of TNF-α level in the supernatant (Fig. 6B). This suggests that the level of MIP-1β negatively regulated the level of TNF-α in IL-15-treated CD8+ memory T cells. To further determine whether the levels of TNF-α correlate with the level of MIP-1β in vivo, we analyzed the concentration of TNF-α, MIP-1β, and the percentage of CD28nullCD8 T cells in the blood of healthy young (age 18–34, n = 15) and old (age over 70, n = 59) donors. We detected significantly higher levels of TNF-α (p = 0.001) (Fig. 7A) correlating with a significantly higher level of MIP-1β (p = 0.01) (Fig. 7B), along with an increase in the percentages of CD28nullCD8 T cells (p = 0.001) (Fig. 7C) in the old-age group. These findings suggest that cytokine production is tightly regulated by feedback mechanisms and that alteration of the balance of these cytokines may explain the accumulation of CD28nullCD8+ T cells with age.

**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 CD28+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 CD28nullCD8+ memory T cells, providing a plausible explanation for the age-associated expansion of CD28nullCD8+ 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 CD28nullCD8+ T cells with age.

Despite the identification of homeostatic cytokine in regulation of CD28 expression in CD28null CD8+ T cells through alteration of CD28 promoter activity, accounts for ~15% of the CD28nullCD8+ 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-13) 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\(^{+}\) memory T cells is mediated by IL-15 directly or by IL-15-induced cytokines in addition to TNF-\(\alpha\) remains to be determined.

The finding that MIP-1\(\beta\) delivers an inhibitory effect on the growth of CD28\(^{null}\)CD8\(^{+}\) T cells is unexpected (Fig. 5). It appears that IL-15 exerts a paradoxical effect on CD8\(^{+}\) memory T cells. IL-15 induces TNF-\(\alpha\), which promotes generation of CD28\(^{null}\) T cells, while it also induces MIP-1\(\beta\), a chemokine that inhibits the growth of CD28\(^{+}\)CD8\(^{+}\) memory T cells. Strikingly, the level of TNF-\(\alpha\) appears to positively regulate the level of MIP-1\(\beta\) while the level of MIP-1\(\beta\) appears to negatively regulate the level of TNF-\(\alpha\) (Fig. 6). Furthermore, the observed reduction in expression of CCR5 in CD28\(^{null}\)CD8\(^{+}\) memory T cells in vitro and in vivo potentially acts to minimize the negative growth impact of MIP-1\(\beta\) 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\(^{+}\) 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 down-regulation of CD28. By producing MIP-1\(\beta\), IL-15 indirectly modulates the accumulation of CD28\(^{null}\) T cells. Further modulating the overall outcome of these events, expression of CCR5 is reduced on CD28\(^{null}\)CD8\(^{+}\) memory T cells, making them less susceptible to inhibition by MIP-1\(\beta\).

The factors that contribute to the accumulation of CD28\(^{null}\)CD8\(^{+}\) memory T cells with age are complex. Although we have shown that IL-15-induced TNF-\(\alpha\) and MIP-1\(\beta\) are capable of regulating CD28\(^{null}\)CD8\(^{+}\) 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\(^{+}\) T cells and/or stimulate CD28\(^{null}\) CD8\(^{+}\) 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\(^{null}\)CD8\(^{+}\) T cells. Our findings presented in this report suggest that homeostatic cytokine IL-15 is a cause of the generation and accumulation of CD28\(^{null}\)CD8\(^{+}\) memory T cells. Based on previous reports and present findings, we propose a two-step and feedback model for age-associated accumulation of CD28\(^{null}\)CD8\(^{+}\) memory T cells (Fig. 8). At the first step, loss of CD28 expression occurs in CD28\(^{+}\) CD8\(^{+}\) 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-\(\alpha\) and possible additional cytokines, enter the second phase. During the second phase, the proliferative ability of CD28\(^{null}\)CD8\(^{+}\) memory T cells to stimulate Ag is impaired but remains intact to homeostatic cytokines. The cytokine-mediated growth of CD28\(^{null}\)CD8\(^{+}\) memory T cells appears to be complex as well. Both positive (IL-15) and negative (MIP-1\(\beta\)) growth factors for CD28\(^{null}\)CD8\(^{+}\) memory T cells exist and the positive growth effects of CD28\(^{null}\)CD8\(^{+}\) memory T cells eventually prevail with the advance of age. A better understanding of the mechanisms behind the generation and growth of CD28\(^{null}\)CD8\(^{+}\) 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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