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Modulation of CD28 Expression: Distinct Regulatory Pathways During Activation and Replicative Senescence

Abbe N. Vallejo, Johann C. Brandes, Cornelia M. Weyand, and Jörg J. Goronzy

The costimulatory molecule CD28 has a restricted tissue distribution and is expressed on T cells and some plasmacytoma cells. Although CD28 is constitutively expressed, its expression is transiently down-regulated following T cell activation and declines progressively with in vitro senescence. In vivo, CD8+ T cells and, less frequently, CD4+ T cells may completely lose CD28 surface expression during chronic infections and with aging. This correlates with changes of nuclear protein-binding activities to two motifs, site α and β, within the CD28 minimal promoter. Both α- and β-bound complexes are found only in lymphoid tissues, in CD8+ T cells, and in some transformed B cells. These complexes are coordinately expressed except during replicative senescence, which is characterized by the down-modulation of site β- but not site α-binding activities. In contrast, T cell activation induces a parallel decline in both site α- and β-binding activities. CD4+ and CD8+ T cells differ in their β-binding profiles, which may explain the more pronounced down-regulation of CD28 in senescent CD8+ T cells. In vivo expanded CD4+CD28null and CD8+CD28null T cells uniformly lack α- and β-bound complexes, resembling the pattern seen in chronically activated cells and not of senescent cells. The Journal of Immunology, 1999, 162: 6572–6579.

The CD28 Ag is the major costimulatory molecule required in the generation of T cell-mediated immune responses (1, 2). Upon interaction with its ligands CD80 and CD86, CD28 transduces activation signals that lead to the expression of anti-apoptotic proteins and enhance the synthesis of several cytokines including IL-2. Hence, T cells should either become anergic or undergo apoptosis in the absence of CD28-mediated signals (3–6). Mice with targeted deletion of the CD28 gene are severely immunocompromised. These gene-knockout animals are unable to

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3 Current address: School of Medicine, Universität München, Munich, Germany.

4 Abbreviations used in this paper: RA, rheumatoid arthritis; EMSA, electrophoretic mobility shift assay.

T cell clones were established by limiting dilution cloning as described previously (12, 21). The clones were derived from PBMC of healthy donors or RA patients, some of whom have participated in previous studies (11, 22–24). Culture conditions for these clones have been described (11).
Short and long term cultures of T cell lines were established from PBMC of healthy donors by stimulation with 1 μg/ml PHA (Sigma Chemical, St. Louis, MO). After 10 days, CD4⁺ and CD8⁺ T cells were separated by negative selection using the VarioMACS system (Miltenyi Biotec, Auburn, CA) or by standard panning protocols. The purity of these lymphocyte populations was confirmed by flow cytometric analysis of cells stained for CD3, CD4, and CD8. Sublines of CD28⁺ and CD28null cells from both CD4⁺ and CD8⁺ T cell lines were established by standard FACS procedures. These cell lines were maintained by a weekly stimulation with γ-irradiated neuraminidase/galactose oxidase-treated EBV-transformed B lymphoblastoid cell lines and 15 U/ml exogenous IL-2.

The EBV-transformed B lymphoblastoid cell lines (BNI40, HT10, MGDAR, Rei, and TAB085) used in this study were established in our laboratory by standard protocols or were obtained from the 11th Histocompatibility Workshop. The P1J cell line (27) was provided by Dr. Paul Leibson (Mayo Clinic, Rochester, MN). These cell lines were maintained in the same culture media used for the plasmacytomas but without the addition of IL-6.

Various cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). These include the B cell line, Ramos; the T cell lines, Jurkat, HUT78, and 6TCEM20; the epithelial carcinoma lines, HeLa and Colo205HSR; the erythroleukemia line, K562; the promyelocytic line U937; and the rhadomyosarcoma line, RD. Cells were propagated according to ATCC recommendations.

Flow cytometry
Phenotypes of cells were periodically examined by standard flow cytometry procedures. This involved triple immunofluorescence staining with Abs to CD3, CD4, and CD8 and analysis by FACSVantage or FACSCalibur cytometers (Becton Dickinson, San Jose, CA). Each experiment included cells incubated with isotype controls (Simultest, Becton Dickinson). Analysis of cell populations were done using the WinMDI program (J. Trotter, Scripps, La Jolla, CA).

Reverse transcriptase-PCR
The presence of CD28 mRNA in the various cells was assessed by standard RT-PCR. Experiments were conducted using amplification primers appropriately designed to detect the different splice forms of CD28 mRNA (28, 29). To amplify CD28 transcripts containing exons 1, 2, and 3, PCR was performed with the primers CTCAGGTGCTCTGCTCCTCGCGCATGGTCACCAGGCTACCC and CAAGGCGGACTCCAAACAC, respectively, paired with the common primer GATAGGCTGCGAAGTCGCGTCGTC and 10 ng/ml human recombinant IL-6 (Genzyme, Cambridge, MA) and 1 ng/ml human recombinant IL-6 (Genzyme, Cambridge, MA).

T cell activation
There were two T cell activation procedures. In one set of experiments, triplicate cultures of 5 x 10⁵ T cells were incubated with autologous, freshly isolated, plastic-adherent monocytes and 5 μg/ml soluble anti-CD3 Ab (OKT3, ATCC). In other studies, T cell cultures were incubated on immobilized OKT3 and the anti-CD28 mAb 29.2 (PharMingen, San Diego, CA). The OKT3 (100 ng/ml) and 28.2 (300 ng/ml) mAbs were immobilized on rabbit anti-mouse Ig-coated plates. These Ab concentrations were determined to induce proliferation of freshly isolated and short term T cell lines.

As system controls, T cell cultures that were stimulated with 50 ng/ml PMA and 10 nM monomycin (Sigma) or T cells were incubated with IgG isotype controls.

Nuclear extracts and electrophoretic mobility shift assay (EMSA)
Surgical tissue specimens were obtained from the Surgical Pathology Laboratories, Mayo Clinic, cut into small pieces, and digested with collagenase by standard techniques. Single-cell suspensions were obtained, and nuclear extracts were prepared as described previously (11, 30). Briefly, between 5 x 10⁶ and 1 x 10⁷ cells were lysed in cold HEPES hypotonic buffer, and the nuclei were isolated by centrifugation. Nuclear proteins were extracted in 50 μl of a high salt buffer (31), and protein concentration was determined using a protein assay kit (Bio-Rad, Richmond, CA). Similar nuclear extract preparations were made with cell lines as indicated.

EMSAs were conducted as described previously (11, 30). Briefly, –20 μg of nuclear extract were combined with 30 μl of binding buffer containing 3 μg of poly(dI-dC) (ICN Pharmaceuticals, Costa Mesa, CA) and 3 μg of nonspecific oligonucleotide. To this mixture, 5 μl of wash buffer were added, and the total reaction volume was adjusted to 50 μl with binding buffer and incubated on ice for 30 min. About 40 fmol of radio-labeled probes were added and incubated for an additional 30 min at room temperature. The sequences of oligonucleotide probes corresponding to the sites α and β motifs were synthesized as indicated in Fig. 1. Protein-DNA complexes were resolved in 6% nondenaturating polyacrylamide gels and autoradiography. Generally, each experiment consisted of replicate reactions for two separate preparations of nuclear extracts of the same cell line. The reproducibility of EMSAs was assessed by repeated experiments as indicated.

As previous work (11), the specificities of α-β-binding activities were validated by three criteria: 1) the higher mobility of α-bound complexes compared with β-bound complexes; 2) the lack of reciprocal competition between the two motifs; and 3) by irrelevant probes. The irrelevant probes (Ets-1, Elk-1, AP3, and NfκB) have been described previously. The SP1 sequence was used as an experimental system control (11, 30).

Results
Expression of CD28-specific transcription factors in CD28⁺ and CD28null lymphoid cells
In previous studies (11), we demonstrated that CD28 expression on CD4⁺ T cells was correlated with nuclear proteins binding to sequence motifs in the CD28 minimal promoter, referred to as sites α and β (Fig. 1). In EMSAs, probes corresponding to site α yielded a single band, while a faster and a slower migrating band were found with probes corresponding to site β. Introduction of mutations into either motif resulted in the inactivation of promoter activity, suggesting that CD28 expression requires the coordinated binding of nuclear proteins to both sequence motifs. To explore the correlation between CD28 expression and CD28-specific transcription factors, we analyzed various lymphoid cells that differ in CD28 expression. Representative flow cytometric analysis of various cell lines is shown in Fig. 2, and the results are summarized in Table I. CD4⁺ and CD8⁺ T cells were sorted for their expression of CD28, and CD28⁺ and CD28null T cells were analyzed separately. The vast majority of peripheral blood CD4⁺ T cells expressed the CD28 molecule. A subset of CD28nullCD4⁺ T cells existed only in some individuals, in whom such cells comprised <5% of the CD4 T cell compartment (11). However, CD8⁺CD28null T cells were common (data not shown). All CD28null T cells lacked CD28 mRNA as determined by RT-PCR (Table I). Cell surface density of CD28 on peripheral CD8⁺CD8⁺ and CD4⁺CD4⁺ T cells was equivalent (Fig. 2).

In contrast to T cells, plasmacytoma cells and some EBV-transformed B lymphoblastoid cell lines had a dim expression of CD28. In Fig. 2, results are shown for the plasmacytoma cell lines
ANBL/6 and KAS6/1. Both cell lines had low levels of CD28 expression as determined by flow cytometry and transcribed CD28 mRNA as determined by RT-PCR. The lymphoblastoid cell line PJI had a minimal cell surface staining for CD28 and expressed CD28 mRNA, whereas the lymphoblastoid B cell line MGAR was negative for CD28 mRNA and lacked cell surface CD28 expression.

Nuclear extracts from all of the cell types shown in Table I were analyzed for proteins binding to sites α and β of the CD28 minimal promoter. Results shown in Fig. 3 demonstrate a correlation between the presence of α-bound complexes (band A) and CD28 expression, irrespective of the cell surface density of the molecule. Among the T and B cell lines examined, the expression of site α-specific proteins was highly correlated with the presence of CD28 mRNA.

The results were more complex for site β. Although all CD28⁺ T and B cells showed site β-binding activities, the mobility patterns of the DNA-protein complexes varied among the different cell types. CD4⁺ CD28⁺ T cells and the CD28⁺ T cell tumor line Jurkat showed two β-bound complexes, a slow mobility B1 band and a faster mobility B2 band (Fig. 3). In contrast, the CD28⁻ CD8⁺ T cells, as well as the CD28⁺ plasmacytoma and EBV-transformed B lymphoblastoid cell lines had a complete loss or significant diminution of the slow migrating B1 complex. The diminution of the B1 complex was apparently not responsible for the low expression of CD28 on B cells. CD8⁺ T cells lacking this band showed a level of CD28 cell surface expression that was equivalent to CD4⁺ T cells (Fig. 2).

Expression of CD28-specific transcription factors is limited to lymphoid tissue

As depicted in Fig. 3, the presence of site α- and β-binding proteins in lymphoid cells was strictly correlated with CD28 expression. Moreover, the presence of these DNA-protein complexes was highly synchronous in that none of the CD28⁺ cells examined expressed only one of these two complexes. Consistent with previous data (11), reciprocal competition assays showed that site α and β probes do not block motif-specific binding activities.

To determine whether α- and β-bound complexes are truly lymphoid-specific and are synchronously expressed, we examined a series of tissues. As shown in Fig. 4, site α- and β-binding activities were found only in the spleen and were uniformly absent in all other tissues examined. Reciprocal competitive EMSAs between the two sequences revealed that the binding activities of splenic extracts were site α- and β-specific (data not shown).

In addition to the surgical tissue specimens shown in Fig. 4, the proerythroid leukemia cell line K562, the promonocytic cell line U937, the rhabdomyosarcoma cell line RD, and the epithelioid carcinoma cell lines HeLa and Colo320HSR were analyzed. These cell lines expressed CD28 at neither the protein nor the mRNA levels (Table I). In EMSAs, these cells also lacked binding activities to site α and β sequences (data not shown).

**Table 1** CD28 expression in various human cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Surface CD28⁺</th>
<th>CD28 mRNA²</th>
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<td></td>
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<tr>
<td>Colo320HSR</td>
<td>Null</td>
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</table>

* Determined by flow cytometry. High refers to ≥1 log magnitude and low refers to <1 log magnitude of CD28 expression over an isotype control. Null refers to CD28 expression equivalent to an isotype control.

* Determined by RT-PCR.

Modulation of CD28 expression following T cell activation

Although CD28 is constitutively expressed on T cells, studies have indicated that activating stimuli induce a transient reduction in the levels of CD28 expression (1, 13). Indeed, T cells cocultured with autologous monocytes in the presence of anti-CD3 mAb elicited the down-regulation of CD28 on the cell surface after 24 h of...
culture (Fig. 5A). We then examined whether this phenomenon may be associated with the specific transcription factors binding to site α and β of the CD28 gene promoter. As depicted in Fig. 5B, T cell stimulation resulted in a parallel reduction in the levels of specific binding activities to both motifs within 24 h after stimulation. These reductions in site α- and β-binding activities were seen in both CD4+ and CD8+ T cells. The activation-induced changes in both the cell surface expression of CD28 and the α-/β-binding activities were transient. The levels of α-/β-binding activities returned to baseline levels after 72 h, whereas the cell surface levels of CD28 returned to preactivation levels between 72 and 96 h (data not shown). In agreement with previous reports (1, 6), stimulation of cells with ionomycin and PMA induced a similar pattern of transient down-regulation of CD28 and in site α-/β-binding activities (data not shown).

Effect of CD28 cross-linking on CD28 expression

In the above experiments, activation of T cells was presumably accomplished by the simultaneous engagement of the TCR-CD3 complex and CD28 by OKT3 and CD80/CD86, respectively, presented on monocytes. To directly assess whether these observations could be attributed to TCR-CD3 and/or CD28 engagement, Ab cross-linking experiments were conducted. T cells were cultured on anti-mouse Ig-coated plates with the addition of anti-CD28 and anti-CD3 (OKT3) mAbs. As shown in Fig. 6A, cross-linking of CD3 with or without co-cross-linking of CD28 induced the down-regulation of CD28 expression. The cross-linking of CD28 by itself did not affect the cell surface density of CD28. An analysis of α-/β-binding profiles of parallel cultures showed that cross-linking of CD3 alone or the simultaneous cross-linking of CD3 and CD28 resulted in significant reductions of α- and β-binding activities (Fig. 6B). In contrast, CD28 cross-linking alone did not affect α-/β-activities.

Modulation of CD28 expression during replicative senescence

Loss of CD28 expression on T lymphocytes has been proposed to be a marker of replicative senescence (14, 15). This is particularly relevant for CD8+ T cells, which show increased frequencies of CD28null cells in patients with chronic infections as well as during normal aging (32–38). In contrast, the emergence of CD28null T
cells within the CD4 compartment appears to be the exception rather than the rule (11). To explore the mechanism(s) of CD28 down-regulation during replicative senescence, CD4+ and CD8+ T cells were purified, activated with immobilized anti-CD3 mAb, and cultured in the presence of exogenous IL-2. Before in vitro activation, the percentage of CD28null cells in the purified CD4+ T cell population was <0.1%. During the culture period of 12 wk, there was a minimal emergence of CD4+CD28null T cells accounting for 0.8% of the total population. In contrast, fresh CD8+ T cells contained between 33% and 48% CD28null T cells. There was also a minimal increase in the frequencies of these cells by an average of 1% during the culture period (data not shown), indicating that a 12-wk culture was not sufficient to allow for the emergence of CD28null T cells. However, this culture period was associated with a clear down-regulation of CD28 expression that was more pronounced on CD8+ T cells compared with that seen on CD4+ T cells. A representative experiment is shown in Fig. 7A with purified CD4+CD28+ and CD8+CD28+ T cells. During the 12-week culture, CD28 expression on CD4+ cells declined from a mean fluorescence channel of 260 to 192. In CD8+ cells, CD28 expression declined within 4 wk with 48% of the cells showing a CD28low phenotype. By 12 wk, all CD8 T cells had a reduced CD28 expression; the mean fluorescence channel had decreased from 225 to 55.

Cultured CD4+ and CD8+ T cells were analyzed for the site α- and β-binding activities (Fig. 7B). In both cell types, there was a dissociated regulation of site α- and β-binding activities. Although nuclear factors binding to site α were maintained in both cell types during the culture period, there was a characteristic pattern of loss in the site β-binding activities. As already shown in Fig. 3, nuclear extracts from freshly isolated CD4+CD28+ T cells showed two β-bound complexes, a slow migrating B1 band, and a faster migrating B2 complex. Cultured CD4+ T cells showed a reduction or the complete loss of the slower migrating B1 band, whereas the faster migrating B2 band was maintained (Fig. 7B). Freshly isolated CD8+CD28+ T cells, on the other hand, already lacked the B1 band (Fig. 3) that was lost in CD4+ T cells during the culture period. The culture of CD8+ T cells resulted in the further decline of the faster migrating B2 band. This loss was already evident after 4 wk of culture, and a significant loss of binding activity was seen after 12 wk.

Discussion

CD28 is the requisite costimulatory molecule in the generation of immune responses. Although it is constitutively expressed on CD4+ T cells, CD28 may be entirely absent on functional T cells (11, 12, 39–41). Variations in the levels of its surface expression are thought to have a major impact on the fine-tuning of immune responses (1, 6, 13, 42–45). Therefore, understanding the exact dynamics of CD28 regulation is of paramount importance.

In previous work (11), we mapped two sequence motifs in the minimal CD28 gene promoter (Fig. 1) that are occupied by two noncompeting nuclear factors. Both motifs, site α and β, are located downstream from the TATA box. The central contributions

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**Figure 6.** CD28 cross-linking does not affect site α- and β-binding activities. Short term CD4+CD28+ T cell lines were cultured on anti-mouse Ig-coated plates in the presence of anti-CD28 Ab (28.2), anti-CD3 (OKT3), or mouse IgG for 24 h. Parallel cultures of the same cell line were monitored for CD28 expression by flow cytometry (A) and for α-β-binding activities by EMSAs as in Fig. 5 (B). EMSAs were conducted with replicate binding reactions from 2 batches of nuclear extracts as depicted. Data shown are representative of 2 independent experiments. Shaded curve – isotype control.

**Figure 7.** Modulation of site β-binding activities in long term T cell cultures. CD4+CD28+ and CD8+CD28+ T cells were isolated from a healthy donor, cultured with immobilized anti-CD3 mAb (OKT3), or mouse IgG for 24 h. Parallel cultures of the same cell line were monitored for CD28 expression (fluorescence intensity) at the indicated time points, parallel cultures were monitored for binding activities by EMSAs as in Fig. 5 (B). EMSAs were conducted with replicate binding reactions from 2 batches of nuclear extracts as depicted. Data are representative of two independent experiments. Solid lines, freshly isolated cells; dashed lines, 4-wk cell lines; dotted lines, 12-wk cell lines; shaded curve, isotype control.
of these sequences in CD28 expression can be inferred from mutational studies that demonstrated the loss of motif-specific binding activities and the inactivation of the minimal promoter. Moreover, a CD28\textsuperscript{null} phenotype among CD4\textsuperscript{+} T cells is associated with the loss of binding activities of both motifs (11). The present study was designed to investigate how sites \( \alpha \)- and \( \beta \)-binding activities are involved in modulating CD28 expression, particularly under conditions known to affect levels of cell surface expression. Data presented herein provide several lines of evidence showing that the dynamics of \( \alpha \)- and \( \beta \)-binding activities are predictive for different patterns of CD28 expression. First, tissue specificity of site \( \alpha \)- and \( \beta \)-bound nuclear factors is indicated by the presence of both nuclear factor complexes exclusively in lymphoid cells (Figs. 2–4, Table I). Second, CD28 expression is directly correlated with the highly coordinated expression of site \( \alpha \)- and \( \beta \)-bound nuclear proteins (Figs. 2–4). Finally, at least two distinct pathways for CD28 down-regulation are indicated by the differential binding activities of \( \alpha \) and \( \beta \) sequences (Figs. 5–7). These pathways occur at conditions known to modulate CD28 expression, i.e., the transient down-regulation of CD28 following T cell activation and the progressive reduction in CD28 expression that is characteristic of T cells during replicative senescence.

The notion that sites \( \alpha \) and \( \beta \) play a role in CD28 expression derives from the data showing the congruence of specific motif-binding activities and CD28 expression (Figs. 2 and 3, Table I). These activities are seen in both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells and in B cells that express CD28. As shown previously, the \( \alpha \)- and \( \beta \)-bound proteins represent two distinct molecules based on their differences in gel mobility and the lack of reciprocal competition the two sequence motifs (11). Preliminary studies on the isolation of the \( \alpha \)- and \( \beta \)-bound proteins reveal that they represent \( \approx 47\)-kDa and \( \approx 39\)-kDa molecules, respectively (A. N. Vallejo et al., unpublished observations). In contrast, various CD28\textsuperscript{null} cells, including T cell lines and clones, lack any detectable \( \alpha \)-/\( \beta \)-binding activities. The absence of both complexes in CD28\textsuperscript{null} T cells indicates a strong correlation between the coordinate expression of both DNA-binding proteins and cell surface expression of CD28. This suggestion is consistent with data from point mutations in either the \( \alpha \) or \( \beta \) motif that show the abrogation of transcriptional activity of the CD28 promoter (11).

Data presented here also show that \( \alpha \)-/\( \beta \)-bound complexes are found only in lymphoid tissues (Fig. 4), indicating that they are both gene- and tissue-specific. This is a rather unexpected finding because it is not uncommon for nuclear factors, whether they are activators of transcripion, scaffold/architectural proteins, or basal factors to be involved in the transcriptional control of different genes in various tissues. Whether this exclusivity of site \( \alpha \)- and \( \beta \)-specific nuclear factors to lymphoid tissue is attributed to single motif-specific binding proteins or larger tissue-specific complexes remains to be examined.

Whereas the relative levels of site \( \alpha \)-binding activities are equivalent in all CD28\textsuperscript{+} cells, there are distinguishable \( \beta \)-binding profiles. CD4\textsuperscript{+}CD28\textsuperscript{+} T cells generally show two \( \beta \)-bound complexes (B1 and B2), CD28\textsuperscript{B} B cells and CD8\textsuperscript{CD28\textsuperscript{T}} T cells show a single band, B2 (Fig. 3). These results raise the possibility that there may be a differential requirement of the \( \beta \) complexes for CD28 transcription in CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells and in CD28\textsuperscript{+} transformed B cells. These differences in \( \beta \)-binding profiles however, did not correlate with CD28 cell surface expression. Transformed B cells have extremely low levels of CD28 expression when compared with T cells (Fig. 2). CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells have equivalent cell surface densities of CD28 that are multifold higher compared to those seen on plasmacytoma cells.

The significance of CD28 expression on B cells is not known, although it has been reported to be a feature of plasmacytomas, but not of primary B cells (46–48). In the present work, we also report that some, but not all, EBV-transformed lymphoblastoid cells express CD28. Some studies suggest that this may provide another survival advantage for immortalized or transformed B cells as indicated by the coexpression of CD80, the counterreceptor for CD28, on the same B cells (48) and its ability to generate a signaling cascade (49). Regardless of its function in B cells, the unusual expression of CD28 on these cells is associated with the acquisition of both \( \alpha \)- and \( \beta \)-binding activities (Figs. 2 and 3, Table I).

The present data also show that the site \( \alpha \) and \( \beta \) binding activities influence the fine-tuning of CD28 expression. Activating stimuli induce a transient reduction in the levels of cell surface expression of CD28 accompanied by the coordinate down-regulation of \( \alpha \)- and \( \beta \)-binding activities (Figs. 5 and 6). Continuous culture of CD4\textsuperscript{+} T cells results in the modulation of \( \beta \)- but not \( \alpha \)-binding activities that is accompanied by a progressive decrease in the cell surface expression of CD28 (Fig. 7A).

Down-regulation of CD28 expression following T cell activation is well documented (1, 6, 13). As demonstrated in Figs. 5 and 6, the engagement of the TCR-CD3 complex, or mitogenic agents such as PMA/ionomycin induce significant reductions in the levels of CD28 cell surface expression. These are accompanied by the down-regulation of both site \( \alpha \)- and \( \beta \)-binding activities. Cross-linking of CD28 alone did not affect CD28 expression or \( \alpha \)-/\( \beta \)-activities, indicating that modulation of CD28 expression is directly influenced by activation signals emanating from the TCR-CD3 complex. These data corroborate previous studies showing activation-induced down-modulation of CD28 gene promoter activity (11). They are also consistent with other studies demonstrating the rapid and selective reduction in the steady state levels of CD28 mRNA following activation (45). Collectively, these observations indicate the existence of a regulatory pathway that specifically targets the down-regulation of CD28 gene transcription. The characteristic reduction in both \( \alpha \)- and \( \beta \)-binding activities following activation strongly support this hypothesis. The functional impact of CD28 down-regulation in T cell-mediated responses remains to be explored. However, receptor down-regulation of the TCR complex, CD4, and CD8 have been reported (50–54). This is thought to effectively reduce the avidity of receptor-ligand interactions, resulting in the modulation of T cell effector functions. Hence, variations in CD28 expression may influence the strength of the costimulatory signal that determines whether T cells undergo proliferation or anergy (1–10). Additionally, reduction in CD28 expression following activation has been shown to correlate with increased susceptibility to Fas-induced apoptosis (10, 45), indicating the importance of receptor modulation in maintaining T cell homeostasis.

While activation-induced down-regulation of CD28 expression equally affected CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, the gradual loss of CD28 expression during continuous culture was more pronounced in the CD8\textsuperscript{+} T cells (Fig. 7). This type of cell culture has been proposed as a model of replicative senescence (14, 15, 55) and mimics the characteristic increase in the frequencies of CD8\textsuperscript{CD28\textsuperscript{null}} T cells in vivo during aging or chronic infections (14, 31–35). In support of this hypothesis, CD8\textsuperscript{+}CD28\textsuperscript{null} T cells have been shown to have significantly shorter telomeres compared with CD8\textsuperscript{CD28\textsuperscript{+}} T cells (56). In contrast to CD8\textsuperscript{+} T cells, CD4\textsuperscript{+}CD28\textsuperscript{null} T cells are infrequent and are found only in some elderly individuals and among RA patients (11, 12), suggesting that either CD8\textsuperscript{+} T cells have a higher turnover compared with
CD4+ T cells or that CD8+ T cells have a high propensity of losing CD28 expression. Data presented here support the later hypothesis. Continuous culture of CD4+ and CD8+ T cells resulted in a more rapid decline in CD28 expression on CD8+ T cells than on CD4+ T cells (Fig. 7A).

The reduction of CD28 expression on T cells during continuous culture is correlated with changes in the β-binding profiles while the α profile is maintained (Figs. 3 and 7B). While freshly isolated CD4+CD28+ T cells have two β-bound complexes, B1 and B2, cultured CD4+CD28+ T cells lose the B1 but not the B2 complex. In contrast, CD8+ T cells, which generally exhibit only the B2 complex, gradually lost this complex during continuous culture. These results show that regulation of CD28 expression in CD4+ and CD8+ T cells can be distinguished by their β-binding profiles and that this difference may be related to the progressive loss of CD28 expression during replicative senescence in vitro.

Although the T cell culture system showed the down-regulation of CD28, a complete loss of CD28 expression and the emergence of CD8+CD28null or CD4+CD28null T cells was not achieved. This is in marked contrast with the in vivo situation where T cells completely lose the expression of CD28, and CD28low T cells are not observed (11, 32–36). As the data show, CD4+CD28null and CD8+CD28null T cells freshly isolated from peripheral blood lack both α- and β-bound complexes (Fig. 3) (11). This is unlike in vitro replicative senescence wherein site β- but not site α-binding activity is down-modulated (Fig. 7B). In contrast, activation results in the down-regulation of both α- and β-binding activities (Fig. 6).

Thus, we propose that continuous activation in vivo, rather than and are modulated by activating stimuli. Under these conditions, motif-specific binding activities are restricted to lymphoid tissues and CD28 promoter correlate with the patterns of expression of CD28. Both activation and replicative senescence modulate CD28 expression and its modulation during continuous culture of CD4+CD28+ T cells.

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