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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 CD28+ 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 costimulatory molecule CD28 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 form germinal centers in response to immunization, and CD28null T cells can initiate, but not sustain, proliferation (7–10). These observations attest to the critical role of CD28 in T cell-mediated immunity.

CD28 is expressed on nearly all human CD4+ T cells. However, CD4+CD28null T cells do emerge to detectable levels in some individuals with aging (11). These unusual T cells are also found in patients with rheumatoid arthritis (RA) (12). Previous studies have shown that this deficiency in CD28 expression is due to a transcriptional block. CD28 gene expression is associated with the presence of nuclear proteins binding to two distinct sequence motifs, site α and β, within the minimal promoter of the CD28 gene (11). Mutations in either sequence motif abrogate CD28 gene promoter activities, suggesting that an interaction between proteins binding to both sequences is required for CD28 expression.

CD4+CD28null T cells lack DNA-binding activities for both sequence motifs. Although CD28 is constitutively expressed on CD4+ T cells, its level of surface expression is not static. At least two mechanisms influencing CD28 expression have been reported, TCR-mediated activation (1, 13) and in vitro replicative senescence (14, 15). Modulation of CD28 cell surface expression is of particular importance because the biological outcome of T cell stimulation is dependent on the interplay of four molecules with very different functions, i.e., CD28 and CD152 with their ligands, CD80 and CD86 (1). CD28 and CD152 are related glycoproteins but differ greatly in their binding properties to CD80 and CD86 (16, 17). They both have fast on/off rates for CD80/CD86; however, CD152 has a 10-fold higher affinity for these molecules than does CD28. Additionally, homodimerization of CD152 has been reported to increase its avidity for CD80 (18–20). Differences in the patterns of cell surface expression of CD28 and CD152 will, therefore, have immediate impact on whether a positive CD28-mediated signal or a negative CD152-mediated signal prevails.

We have now explored the roles of the CD28-associated α- and β-specific complexes in fine-tuning the level of CD28 expression. Surprisingly, these two nuclear protein complexes were detected exclusively in lymphoid cells and, in most instances, their binding activities were coordinated. Upon TCR ligation, transient diminution of both DNA-binding activities was observed accompanied by the down-regulation of CD28 expression. In contrast, there was a progressive loss of CD28 expression during replicative senescence that was associated with a decrease in site β-, but not in site α-, binding activity. These data indicate that CD28 expression is controlled by protein complexes binding to sites α and β. However, distinct mechanisms lead to CD28 down-regulation following T cell activation and during replicative senescence.

Materials and Methods

Cell culture

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 T 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 plasmacytoma cell lines ANBL6, KAS6/1, and KP6 (provided by Dr. Diane Jelinek, Mayo Clinic, Rochester, MN) were normally isolated from patients with multiple myeloma (25, 26). They were maintained in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% FCS (Summit Biotech, Ft. Collins, CO), 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Grand Island, NY), and 1 ng/ml human recombinant IL-6 (Genzyme, Cambridge, MA).

The EBV-transformed B lymphoblastoid cell lines (BIN40, HT10, MGAR, Rei, and TAB085) used in this study were established in our laboratory by standard protocols or were obtained from the 11th Histocompatibility Workshop. The PJP cell line (27) was provided by Dr. Paul Leibson (Mayo Clinic). 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 Co102HSR; 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 done with the primers CTCAGGTGCTCTTGGCTCTC, CGCCAT GCCAGCAAGGCTTACG, and CAAGCCGGCTGCACACACC, respectively, paired with the common primer TATAGGTTGCAAGGCCTGC corresponding to the 3'-end of exon 4. PCR products were fractionated in 2% agarose gels.

T cell activation

There were two T cell activation procedures. In one set of experiments, triplicate cultures of 5 × 10^5 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 28.2 (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 ionomycin (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 × 10^6 and 1 × 10^6 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 was 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 radiolabeled probes were added and incubated for an additional 30 min at room temperature. The sequences of oligonucleotide probes corresponding to the site α and β motifs were synthesized as indicated in Fig. 1. Protein-DNA complexes were resolved in 6% nondenaturing 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 in 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 CD28+CD8+ and CD28+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.
Among the T and B cell lines examined, the expression of CD28 mRNA is highly synchronous in that none of the CD28 null cells uniformly lack CD28 mRNA as determined by RT-PCR. 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 PJL 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 promotor. Results shown in Fig. 3 demonstrate a correlation between the presence of site 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<sup>+</sup> and no CD28<sup>null</sup> cells showed site β-binding activities, the mobility patterns of the DNA-protein complexes varied among the different cell types. CD4<sup>+</sup> CD28<sup>+</sup> T cells and the CD28<sup>+</sup> 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<sup>−</sup> CD8<sup>+</sup> T cells, as well as the CD28<sup>−</sup> 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<sup>+</sup> T cells lacking this band showed a level of CD28 cell surface expression that was equivalent to CD4<sup>+</sup> 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<sup>+</sup> 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 Colo320HR 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).

**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

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

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Surface CD28&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CD28 mRNA&lt;sup&gt;ab&lt;/sup&gt;</th>
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<tr>
<td>Primary CD4&lt;sup&gt;+&lt;/sup&gt; T cell lines</td>
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<td>J28P</td>
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</tr>
<tr>
<td>J28N</td>
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<td>T28P</td>
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<td>+</td>
</tr>
<tr>
<td>T28N</td>
<td>Null</td>
<td>+</td>
</tr>
<tr>
<td>Primary CD8&lt;sup&gt;+&lt;/sup&gt; T cell lines</td>
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<tr>
<td>F32-8N</td>
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<td>+</td>
</tr>
<tr>
<td>DH-8P</td>
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<td>+</td>
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<tr>
<td>DH-8N</td>
<td>Null</td>
<td>+</td>
</tr>
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<tr>
<td>HUT78</td>
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<tr>
<td>EBV-transformed B lymphoblastoid cell lines</td>
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<td></td>
</tr>
<tr>
<td>BIN40</td>
<td>Null</td>
<td>+</td>
</tr>
<tr>
<td>HT10</td>
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<td>+</td>
</tr>
<tr>
<td>MGAR</td>
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<td>+</td>
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<tr>
<td>PJL</td>
<td>Low</td>
<td>+</td>
</tr>
<tr>
<td>Rei</td>
<td>Low</td>
<td>+</td>
</tr>
<tr>
<td>TAB085</td>
<td>Low</td>
<td>+</td>
</tr>
<tr>
<td>B cell lymphomas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ramos</td>
<td>Low</td>
<td>+</td>
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<tr>
<td>Plasmacytomas</td>
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<tr>
<td>ANBL6</td>
<td>Low</td>
<td>+</td>
</tr>
<tr>
<td>KP6</td>
<td>Low</td>
<td>+</td>
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<tr>
<td>KAS6/I</td>
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<td>+</td>
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<td>Proerythroid leukemia cell line</td>
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<td>K562</td>
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<td>Rhabdomyosarcoma</td>
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<td>RD</td>
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<td>+</td>
</tr>
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<td>Epithelioid carcinoma cell line</td>
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</tr>
<tr>
<td>HeLa</td>
<td>Null</td>
<td>+</td>
</tr>
<tr>
<td>Colo320HR</td>
<td>Null</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined by flow cytometry. High refers to $\geq 1$ log magnitude and low refers to $\leq 0.5$ and $<1$ log magnitude of CD28 expression over an isotype control. Null refers to CD28 expression equivalent to an isotype control.

<sup>b</sup> Determined by RT-PCR.
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- and β-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 (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 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 are representative of 2 independent experiments. Shaded curve – isotype control.

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 CD28 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
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 α- and β-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 α- and β-binding activities are predictive for different patterns of CD28 expression. First, tissue specificity of site α- and β-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 α- and β-bound nuclear proteins (Figs. 2–4). Finally, at least two distinct pathways for CD28 down-regulation are indicated by the differential binding activities of α and β 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 α and β 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 α- and β-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 α- and β-bound proteins reveal that they represent ~47-kDa and ~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 α-/β-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 α or β motif that show the abrogation of transcriptional activity of the CD28 promoter (11).

Data presented here also show that α/β-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 transcription, scaffold/architectural proteins, or basal factors to be involved in the transcriptional control of different genes in various tissues. Whether this exclusivity of site α- and β-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 α-binding activities are equivalent in all CD28\textsuperscript{+} cells, there are distinguishable β-binding profiles. CD4\textsuperscript{+}CD28\textsuperscript{+} T cells generally show two β-bound complexes (B1 and B2), CD28\textsuperscript{−} B cells and CD68\textsuperscript{+}CD28\textsuperscript{+} T cells show a single band, B2 (Fig. 3). These results raise the possibility that there may be a differential requirement of the β complexes for CD28 transcription in CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells and in CD28\textsuperscript{−}-transformed B cells. These differences in β-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 B 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 α- and β-binding activities (Figs. 2 and 3, Table I).

The present data also show that the site α and β 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 α- and β-binding activities (Figs. 5 and 6). Continuous culture of CD4\textsuperscript{+} T cells results in the modulation of β- but not α-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 α- and β-binding activities. Cross-linking of CD28 alone did not affect CD28 expression or α-/β-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 α- and β-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+CD8+ T cells have two β-bound complexes, B1 and B2, cultured CD4+CD8+ T cells lose the B1 but not the B2 complex. In contrast, CD8+ T cells, which generally exhibit only the B2 complex, gradually lose 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 CD28null 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 replicative immunosenescence, may account for the emergence of CD28null T cells. CD4+CD28null T cells are highly oligoclonal (57, 58), suggesting that they may be derived from the chronic activation and proliferation of their CD8+CD4+ progenitors. This interpretation is supported by the isolation of T cell clones with identical TCR β-chain sequences in both CD8+ and CD28null subsets (57). The loss of CD28 expression could be a mechanism to reestablish nonresponsiveness of a T cell under conditions of continuous antigenic stimulation. This suggestion is consistent with the high frequencies of CD4+CD28null T cells in the chronic inflammatory disease RA (12, 59).

In summary, data presented here show that the dynamics of the binding profiles of the α and β sequence motifs in the CD28 gene promoter correlate with the patterns of expression of CD28. Both motif-specific binding activities are restricted to lymphoid tissues and are modulated by activating stimuli. Under these conditions, α- and β-bound complexes are coordinately expressed. However, this coordinate expression is not maintained during in vitro replicative senescence. Among CD8+ T cells, continuous culture induces the selective diminution of the β-protein complex with a concomitant decrease in the levels of cell surface expression of CD28. Changes in CD4+ T cells are less pronounced with the selective loss of one of the two β-bound complexes accompanied by a slight, but significant, down-regulation of CD28 expression. The complete disappearance of function in both motifs is correlated with a CD28null phenotype of both CD4+ and CD8+ T cells in vivo. Although both activation and replicative senescence modulate CD28 expression, further studies are needed to determine which mechanism leads to the CD28null endpoint phenotype observed in vivo. Characterization of the molecular nature of the motif-specific complexes will be critical to our further understanding of the mechanisms involved in CD28 expression and its modulation during adaptive immune responses that led to the development of CD28null T cells.

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