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CD28 IS AN INDUCIBLE T CELL SURFACE ANTIGEN THAT TRANSDUCES A PROLIFERATIVE SIGNAL IN CD3+ MATURE THYMOCYTES[1]

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The rearrangement of TCR genes during thymic ontogeny creates a repertoire of T cell specificities that is refined to ensure the deletion of autoreactive clones and the MHC restriction of T cell responses. Signals delivered via the accessory molecules CD2, CD4, and CD8 have a crucial role in this phase of T cell differentiation. Recently, CD28 has been identified as a signal transducing molecule on the surface of most mature T cells. Perturbation of the CD28 molecule stimulates a novel pathway of T cell activation regulating the production of a variety of lymphokines including IL-2. We have studied the expression and function of CD28 during thymic ontogeny, and in resting and activated PBL. A variable percentage of resting thymocytes were CD28+ (3 to 25%, $n = 8$), but it was found in high density only on mature CD3+(bright) CD4/CD8 cells. Both unseparated thymocytes and isolated CD3−CD28−/dull cells proliferated when stimulated with PMA plus IL-2 or PMA plus ionomycin. PMA treatment also rapidly up-regulated CD28 expression in the CD3− subset as these cells became CD3−CD28+(bright). Despite the ability of PMA to induce high density CD28 expression in CD3− cells, CD3− thymocytes did not proliferate in response to PMA plus anti-CD28 mAb, in contrast to unseparated cells. CD3+ thymocytes stimulated with immobilized anti-CD3 mAb also failed to proliferate in culture. However, the addition of either IL-2 or anti-CD28 mAb supported proliferation, suggesting that only CD3+ cells could respond to CD28 signaling. The comitogenic effect of anti-CD3 and anti-CD28 mAb was IL-2 dependent as it was abrogated by an anti-IL-2R mAb. Interestingly, the expression of CD28 on the cell surface of CD3+ cells was also inducible, as flow cytometric analysis demonstrated a 10-fold increase in cell surface CD28 by 24 to 48 h after anti-CD3 stimulation of both CD3+ thymocytes and peripheral blood T cells. This increase was accounted for by a commensurate increase in CD28 mRNA levels. Together, these results suggest that CD28 is an inducible T cell antigen in both CD3− and CD3+ cells. In addition, stimulation of the CD28 pathway can provide a second signal to support the growth of CD3+ thymocytes stimulated through the TCR/CD3 complex, and may therefore represent a mechanism for positive selection during thymic ontogeny.

T cells are important regulators of immune surveillance. The antigen specificity of individual T cells is determined by the TCR, a membrane-bound heterodimer that recognizes nominal Ag complexed to self-MHC molecules (1–3). The α/β subunits which comprise most TCR are expressed on the cell surface in close association with a series of five invariant proteins referred to as CD3, and together they form the TCR/CD3 complex (1–4).

During intrathymic development, most T cells rearrange the TCR-α- and β-genes to generate the diverse repertoire of T cell specificities that are observed in peripheral lymphoid tissue (2). However, during this process 99% of T cells die within the thymus and thus never achieve functional maturity (5). The generation of the mature T cell repertoire is currently believed to be governed by two processes, clonal deletion and positive selection (6, 7). Clonal detection refers to the elimination of T cells with a specificity for self-Ag, i.e., those that are potentially autoreactive. Positive selection results in mature T cells being able to recognize Ag only in an MHC-restricted fashion. The mechanism by which clonal deletion and positive selection take place are poorly understood, but have been proposed to involve positive and negative signaling through surface molecules on the developing T cell (2, 8, 9).

The Ag-specific activation of mature T cells via the TCR/CD3 complex (3) can be mimicked in a polyclonal fashion by antibodies with specificity directed at conserved epitopes on the TCR itself or at epitopes on the closely associated CD3 molecules (10–12). Perturbation of the TCR/CD3 complex by either means leads to the hydrolysis of phosphotidylinositol bis-phosphate to its components diacylglycerol and inositol Tris-phosphate (13, 14). Diacylglycerol induces the translocation and activation of protein kinase C, and inositol Tris-phosphate increases intracellular free calcium (13–15). These later events can be mimicked by PMA, a direct activator of protein kinase C, and the calcium ionophore ionomycin, respectively. Mature T cells can also be activated by perturbation of the CD2 molecule (16, 17). This interaction requires coexpression of the TCR/CD3 complex, and

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apparently involves the same intracellular second messengers (17). Peripheral blood T cells activated through either the TCR/CD3 or CD2 pathway produce IL-2 and respond to it by proliferating (18, 19).

CD28 is a cell surface Ag found on 80% of peripheral blood T cells and on plasma cells (20, 21). We have previously demonstrated that the production of IL-2 by CD3-activated mature T cells is enhanced more than 10-fold by concurrent stimulation of the CD28 pathway with the mAb 9.3 (22). In addition, CD28 stimulation of mature T cells, whereas not directly mitogenic (23), synergizes with submaximal doses of anti-CD3 (24) or anti-CD2 mAb (24) to enhance cell proliferation.

It has been reported that although anti-CD3 mAb are potent stimuli for mature T cells found in the periphery, they appear to transmit a negative regulatory signal to some thymocytes (26), and can even induce cell death (27). In contrast, $CD3^+$ thymocytes can be induced to proliferate by stimulation with anti-CD2 mAb in combination with either PMA or IL-2 (26, 28). Recently, Yang et al. (29) have shown that CD28 stimulation of thymocytes could also synergize with anti-CD2 mAb to deliver a proliferative signal. Neither CD2 nor CD28 stimulation alone was sufficient for IL-2 gene induction, but the combination of the two resulted in IL-2 gene expression and cell proliferation. CD3 stimulation, either alone or in combination with CD2 stimulation, did not activate IL-2 expression or proliferation in thymocytes, but the combination of CD3 and CD28 stimulation was not examined.

Due to the ability of CD28 stimulation to support the proliferation of thymocytes stimulated through the CD2 pathway, to augment proliferation and lymphokine gene expression in mature T cells stimulated via either CD2 or the TCR/CD3 complex, and the uncertainty regarding the role of TCR/CD3 triggering during thymocyte maturation, we studied the expression of CD28 in resting and activated thymocytes and PBL. We now report that a variable percentage of unstimulated thymocytes are $CD28^+$, but that it is found in high density only on the $CD3^{+(bright)}$ CD4/CD8 single positive population. $CD3^-CD28^{-/dull}$ thymocytes were induced to express CD28 on their cell surface when cultured with PMA, but they remained $CD3^-$. These $CD3^-CD28^{+(bright)}$ cells did not proliferate in response to PMA plus anti-CD28 mAb, although they did respond to PMA plus IL-2 or PMA plus ionomycin. In contrast PMA plus anti-CD28 stimulation supported the proliferation of unfractionated cells, suggesting that only $CD3^+$ cells could respond via the CD28 pathway. As previously reported, when $CD3^+$ thymocytes were stimulated in culture with anti-CD3 mAb alone they failed to proliferate (26, 27). However, anti-CD28 stimulation was capable of providing a second signal to trigger their proliferation, which was then inhibitable by IL-2R blockade.

MATERIALS AND METHODS

mAb. The mouse anti-human mAb 9.3 (anti-CD28), G19-4 (anti-CD3), G17-2 (anti-CD4), and G10.1 (anti-CD8) were produced as previously described (30–33). SA36.6G (anti-IL-2R, CD25) was a generous gift from Dr. Edmond Yunis (Dana-Farber Cancer Institute, Boston, MA) (34). FITC- and phycoerythrin-conjugated nonspecific isotype-matched control antibodies, and T11 (anti-CD2) were purchased from Coulter Immunology (Hialeah, FL). FITC-labeled goat anti-mouse IgG2a and IgG1 were purchased from FisherBiotech (Orangeburg, NY).

Reagents. PHA was purchased from Sigma Chemical Co. (St. Louis, MO). Ionomycin and human IL-2 were obtained from Calbiochem (San Diego, CA). The IL-2 was supplied as a solution containing 640 half-maximal U/ml, and lot 801720 was used in these experiments. PMA was purchased from Sigma, and reconstituted as a 1 mg/ml solution in dimethylsulfoxide. Fresh working dilutions of PMA (at 5 μg/ml in PBS) were prepared as needed for each experiment.

Isolation of thymocytes. Thymic tissue was obtained as surgical pathology specimens from children less than 3 y old who underwent routine thymectomy at the time of cardiothoracic surgery. Thymic tissue was gently expressed through a nylon mesh to obtain a single cell suspension, and mononuclear cells were isolated using Ficoll-Paque density gradient centrifugation. $CD3^+$ thymocytes were obtained by the panning technique. Briefly, anti-CD3 antibody was immobilized on plastic culture dishes by adsorbing the mAb G19-4 to the surface of a dish previously coated with a goat anti-mouse mAb as described (35). Unfractionated thymocytes were incubated for 1 h at 37°C, after which the nonadherent cells were removed and the plates washed three times with media. Adherent cells were >95% $CD3^+$ as determined by indirect immunofluorescence. To obtain $CD3^-$ thymocytes, unfractionated cells were incubated with a saturating amount of G19-4 (anti-CD3) for 1 h at 4°C. The cells were washed three times, and incubated with goat anti-mouse Ig-coated magnetic beads (Advanced Magnetics Institute, Cambridge, MA) for 30 min on a rocker at 20°C. The beads were then collected using a magnet and the separation procedure was repeated on the unbound cell fraction. The remaining unbound cells were >95% $CD3^-$ (by indirect immunofluorescence), >99% $CD2^+$ and were all either $CD4^-CD8^-$ or $CD4^+CD8^+$.

Isolation of PBL. All blood donors were healthy volunteers who gave informed consent to venipuncture. PBMC were isolated from venous blood by density gradient centrifugation using Ficoll-Paque. When specified, purified $CD28^+$ T cells were obtained using a protocol previously described (36). Briefly, monocytes were first depleted by plastic adherence. Next, $CD28^+$ cells were negatively selected by incubating the plastic nonadherent cells with saturating amounts on mAb directed at CD11, CD20, CD16, CD14, and HLA-DR followed by separation using goat anti-mouse Ig-coated magnetic beads (Advanced Magnetics Institute). Cell purity was monitored by staining the negatively selected cells with FITC-conjugated mAb directed at CD2 (T11) and CD28 (9.3), and was always >99% and 98% positive, respectively, as compared to staining with a nonspecific control mAb. Monocytes, B cells and large granular lymphocytes were not detectable by flow cytometric analysis in the $CD28^+$ cell preparations.

Cell culture. PBL were cultured in complete medium consisting of RPMI 1640, 10^5 U/liter penicillin, 100 μg/liter streptomycin, 5 mM HEPES, 2 mM L-glutamine, and 10% FCS. All products were purchased from GIBCO Laboratories (Grand Island, NY). Cells were cultured at a density of 1×10^6/ml. When used, anti-CD3 antibody was immobilized on plastic culture dishes (35). Thymocytes were cultured in a similar fashion as PBL except that the medium was supplemented with IL-2 (a 1/10 dilution of the stock solution yielding a final concentration of 64 half-maximal U/ml) unless otherwise stated. The concentration of 9.3 mAb used in cell cultures was always 1 μg/ml. To stimulate PBL, PMA was used at a concentration of 2.5 ng/ml and ionomycin at 125 ng/ml. For thymocyte stimulation, the concentration of PMA was 5 to 10 ng/ml, and ionomycin was used at 0.4 to 0.8 μg/ml.

Proliferative assays. Cells were cultured in complete medium in 96-well round-bottomed microtiter plates at 1×10^5 cells/well in a total volume of 0.2 ml. To determine the proliferation of $CD3^+$ cells, 1×10^5 unseparated thymocytes were incubated in wells coated with anti-CD3 mAb, after 1 h the nonadherent cells were removed and the wells gently washed with media. Proliferation, measured as DNA synthesis, was determined by adding 1 μCi of [^{3}H]thymidine (ICN Radiochemicals, Irvine, CA) to each well for the last 18 h of culture, after which plates were harvested with a PHD 200 Cell Harvesting System (Cambridge Technologies, Cambridge, MA). All assays were performed in quadruplicate.

Cell staining. The 1×10^6 cells were washed twice, mixed with a saturating amount of the appropriate FITC- or phycoerythrin-conjugated antibody, and suspended in 100 μl of a solution of 50% FCS and 50% PBS containing 0.1% sodium azide. Cells were incubated for 45 min at 4°C, washed twice with cold PBS, and resuspended in 0.5 ml PBS with 1% formaldehyde for flow cytometric analysis.

Flow cytometry. Single- and dual-color fluorescent analyses were performed on an Epics C cell sorter (Coulter Electronics) or a FACScan (Becton Dickinson, Mountain View, CA). Positive cells were defined as those with fluorescent intensity beyond a threshold defined by the negative control (cells stained with isotype-matched nonspecific mouse Ig). Dead cells were excluded from analysis using forward and right-angle light scatter gating. Electronic subtraction was used to correct for spectral emission overlap from the green

≥515 nm) into the red (≥590 nm) channel. Cell staining was measured in arbitrary units as the log of fluorescent intensity, and in all cases is displayed on a 3 decade scale.

Northern blot analysis. Cells were harvested from cultures by resuspension and centrifugation, and total cellular RNA was extracted with guanidinium isothiocyanate (37). The samples were equalized for ribosomal RNA, and the equalization was confirmed by ethidium bromide staining of equal amounts of the RNA samples on a nondenaturing 1% agarose gel as previously described (38). These equalized RNA samples (5 to 10 μg) were separated on 1% agarose-formaldehyde gels and transferred to nitrocellulose. Membranes were baked under a vacuum for 2 h and then prehybridized (36). The DNA probes used were labeled by nick translation and hybridized to the membranes as previously described (36), after which the membranes were exposed to x-ray film at −70°C.

DNA probes. The probes used in these experiments were gene-specific inserts isolated from low melting point agarose gels after digesting of the plasmid in which they were propagated with the appropriate restriction endonucleases. The HLA-B7 probe was a 1.4-kb *Pst* 1 fragment isolated from pHLA-B7 (39). The CD28 probe (1.5 kb) was a gift of Drs. Brian Seed and Alessandro Aruffo (Harvard Medical School, Boston, MA) (40).

RESULTS

Only $CD3^{+(bright)}$ thymocytes express high levels of CD28. To define the relative distributions of the CD3 and CD28 Ag on developing thymocytes, we stained lymphocytes from thymic tissue obtained from surgical pathology specimens. Figure 1 is an example of a two-color analysis of resting thymocytes stained for CD3 and CD28, and demonstrates a trimodal population of cells. The majority of cells is either $CD3^+CD28^+$ or $CD3^-CD28^-$. A third group of cells stained with anti-CD3 alone, and these cells represented a distinct population which expressed a low density of the CD3 surface Ag. In this thymus, few cells stained with anti-CD28 alone, and the intensity of staining in the $CD3^-$ cells was less than that in the $CD3^+$ cells. In other instances we observed a greater proportion of $CD3^-CD28^+$ cells, but in all instances these cells expressed a 5- to 10-fold lower density of CD28 relative to that found on $CD3^+CD28^+$ cells. In a total of eight experiments, 44 ± 24% of thymocytes were $CD3^-CD28^-$, 33 ± 18% $CD3^+$ $CD28^+$, 13 ± 6% $CD28^-$, and 10 ± 8% $CD3^-CD28^+$.

CD28 is inducible but does not stimulate proliferation in $CD3^-$ thymocytes. To determine whether CD28 was inducible on $CD3^-$ thymocytes, which expressed either no, or low levels of, CD28 in the unstimulated baseline state (Fig. 1), we cultured $CD3^-$ cells for 48 h in either medium alone, or PMA, and stained with anti-CD28 mAb (Fig. 2*A*). CD28 expression on $CD3^-$ cells cultured in medium decreased somewhat at 48 h (relative to nonspecific staining). In contrast, CD28 expression was augmented 10- to 100-fold on a per cell basis in thymocytes cultured in PMA. Identical results were obtained when cells were cultured in either PMA plus ionomycin or PMA plus IL-2. In all instances the cells remained $CD3^-$ (Fig. 2*B*).

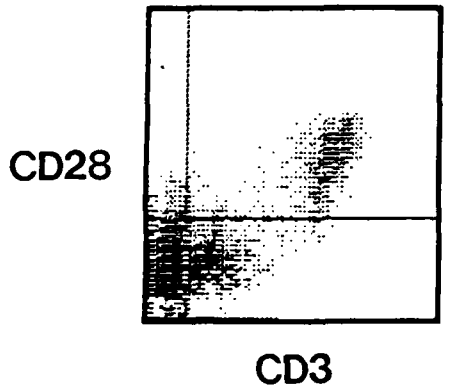

Figure 1. Two-color staining of unstimulated thymocytes with anti-CD3 and anti-CD28 mAb. Unstimulated thymocytes were incubated with the mAb G19-4-FITC (anti-CD3) and 9.3-phycoerythrin (anti-CD28) for 30 min at 4°C, washed three times with PBS, and analyzed by flow cytometry. Fluorescent intensity is displayed on a 3-log scale. The population of thymocytes that is both low density $CD3^+$ and $CD28^-$ is well visualized in this patient.

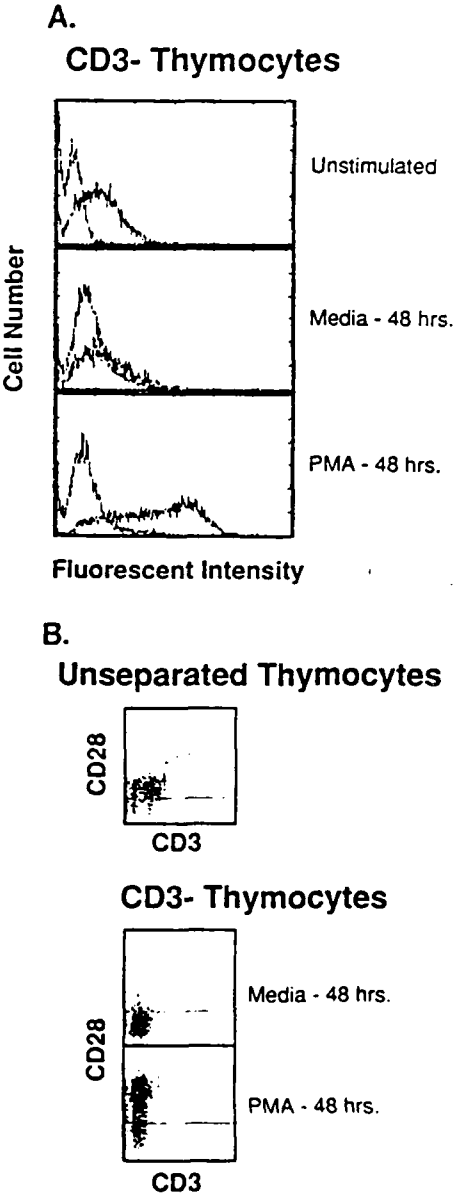

Figure 2. The CD28 surface Ag is inducible on $CD3^-$ thymocytes. *A*. $CD3^-$ thymocytes were negatively selected by immunoadsorbtion using magnetic beads and then cultured with media alone, or media plus PMA (10 ng/ml) for 48 h. Cells were stained with anti-CD28-PE mAb either before culture or after 48 h in culture. Cells stained with PE-labeled nonspecific mouse Ig (Msig) served as negative controls. *B*. $CD3^-$ thymocytes selected and stimulated as above were also analyzed by two-color flow cytometry to verify that they remained $CD3^-$ in culture. Staining of unseparated and cultured cells was done with anti-CD3-FITC plus anti-CD28-PE. Cursor placement was determined by simultaneous staining of cells with FITC- and PE-labeled Msig. In both panels fluorescent intensity is displayed on a 3-log scale.

Despite the fact that $CD3^-$ thymocytes could be induced to express a level of CD28 similar to that observed on unstimulated $CD3^{+(bright)}$ cells, the CD28 pathway did not mediate a proliferative response in the $CD3^-$ cells. Unfractionated or $CD3^-$ thymocytes were cultured with media alone, PMA, or PMA combined with either ionomycin, IL-2, or 9.3 (Table I). Neither cell population proliferated spontaneously or upon stimulation with PMA alone, whereas both unfractionated and $CD3^-$ cells were capable of responding to PMA plus ionomycin or PMA plus IL-2. However, only unfractionated thymocytes proliferated in response to PMA plus anti-CD28 stimulation, although the $CD3^-$ cells expressed a high density of CD28 on their surface (Fig. 2).

$CD3^+CD28^+$ thymocytes proliferate via CD3 pathway in presence of anti-CD28 mAb. We next asked whether CD28 could function to activate thymocytes through the CD3 pathway. Such a possibility was suggested by two findings. First, CD28 stimulation provided a comitogenic signal with PMA for thymocytes only when the cells concomitantly expressed CD3, whereas $CD3^-$ cells responded to PMA plus IL-2. Second, all resting thymocytes that expressed high levels of $CD28^+$ were also $CD3^{+(bright)}$. To examine this hypothesis, $CD3^+$ cells were kept in culture on the anti-CD3-coated plates used for panning. Their proliferate response in 96 h was measured when they were incubated with complete medium alone, or in medium supplemented with 9.3, IL-2, or both (Table II, experiment 2). CD3 stimulation alone resulted in minimal proliferation; however, the addition of 9.3 or IL-2 yielded a strong proliferative response, and no augmentation was observed when 9.3 and IL-2 were combined. A similar pattern was observed with unseparated thymocytes (Table II, experiments 1 and 3). Neither 9.3 nor IL-2 supported thymocyte proliferation in the absence of concurrent CD3 stimulation.

Comitogenic effect of CD3 and CD28 on thymocytes is IL-2 dependent. Inasmuch as CD28 was known to significantly augment IL-2 production in CD3-stimulated PBL (22), and because either anti-CD28 mAb or IL-2 acted synergistically with anti-CD3-stimulated thymocytes (Table II), we postulated that the effect of anti-CD28 was to induce IL-2 production. Thus, SA36.6G, an anti-IL-2R mAb, was added to thymocytes stimulated with either anti-CD3 alone, or in combination with anti-CD28 or IL-2 (Fig. 3). CD3 stimulation alone did not induce thymocyte proliferation, but the addition of either anti-CD28 or IL-2 supported a strong proliferative response that was inhibited by anti-IL-2R blockade in a dose-dependent fashion. Proliferation was completely blocked at the highest dose of anti-IL-2R mAb used (1/100).

CD28 is inducible on $CD3^+$ thymocytes and peripheral blood T cells. The fact that CD28 expression on $CD3^-$ thymocytes was lower than on $CD3^+$ thymocytes, and was inducible on $CD3^-$ cells with PMA, raised that possibility that this increase seen with phorbol ester treatment in vitro was mimicking an event occurring during the natural T cell maturational process. If this were true, then CD28 would not be inducible on either $CD3^+$ thymocytes or on mature PBL, both of which already expressed high and equivalent levels of CD28 (data not shown). However, when we examined the cell surface phenotype of $CD3^+$ thymocytes stimulated to proliferate with anti-CD3 mAb and IL-2, there was a significant increase in cell surface CD28 expression, with peak levels occurring at 72 h (Fig. 4). Furthermore, the increase in CD28 expression upon cell activation was not a unique feature of thymocytes, as CD28 was also induced in PBL, with activated cells expressing up to 10 times as much CD28 Ag as resting cells by 48 h (Fig. 5). This increase began between 12 and 24 h after activation and persisted for at least 7 days, which was the latest time point tested (data not shown).

Increased CD28 surface Ag after cell activation results from increased CD28 mRNA expression. We next sought to determine if increases in CD28 surface Ag were the result of increased CD28 gene expression. $CD28^+$ T cells were stimulated in culture, harvested at various time points, total cellular RNA was extracted, and analyzed for CD28-specific mRNA. CD28 mRNA was constitutively expressed at low levels in resting $CD28^+$ T cells (Fig. 6). Multiple species of mRNA were detected, in agreement with previous observations (40). After stimulation through the TCR/CD3 complex, CD28 mRNA was induced, with peak expression occurring after 6 h, and elevated message levels persisting for at least 36 h (Fig. 6). The changes in steady state mRNA levels we observed were sufficient to account for the increase in CD28 cell surface expression.

Stimulation of T cells through the TCR/CD3 pathway activates protein kinase C and increases intracellular calcium, effects that can be duplicated using the pharmacologic agents PMA and ionomycin, respectively. When $CD28^+$ T cells were stimulated with PMA alone, CD28 was induced with kinetics similar to that observed in TCR/CD3-activated cells (Fig. 7), indicating that activation of protein kinase C is sufficient to induce expression of the CD28 gene. No change in CD28 mRNA was observed in cells treated with ionomycin alone (data not shown).

TABLE I

CD28 pathway does not function in $CD3^-$ thymocytes

Stimulus	cpm (mean ± SD)[a]	
	Unseparated thymocytes	$CD3^-$ thymocytes[b]
None	113 ± 25	198 ± 38
PMA	609 ± 85	323 ± 72
PMA + 9.3	10496 ± 1105	517 ± 124
PMA + Ionomycin	18365 ± 838	10348 ± 2574
PMA + IL-2	16978 ± 3643	14847 ± 2584

[a] Cells were cultured for 96 h in microtiter plates. PMA was used at a concentration of 10 ng/mL, ionomycin at 400 ng/mL, anti-CD28 mAb 9.3 at 1 μg/mL, and IL-2 was used at 64 half-maximal U/ml (see *Materials and Methods*). [^{3}H]TdR was added to the cultures 18 h before harvesting.

[b] $CD3^-$ thymocytes were isolated by panning on tissue culture plates coated with an anti-CD3 antibody.

DISCUSSION

The CD28 molecule appears to be the surface control element of a novel pathway of T cell activation that regulates the production of a variety of lymphokines including the T cell autocrine growth factor IL-2 (22). Approximately 80% of peripheral blood $CD3^+$ T cells are $CD28^+$ (20), but the relationship of CD28 to CD3 in the process of intrathymic T cell ontogeny has not been well characterized. We now report that a variable percentage of unstimulated thymocytes are $CD28^+$, but that it is found in high density only on the $CD3^{+(bright)}$ CD4/CD8 single positive population.

Although $CD3^-$ cells expressed little or no CD28, direct

TABLE II

Proliferation of thymocyte subsets to CD3 stimulation

Cell Fraction[a]	Mitogen[b]	Additional Stimuli[b]	cpm (mean ± SD)		
			Expt 1	Expt 2	Expt 3
Unseparated	None	None	117 ± 21	100 ± 19	168 ± 84
		IL-2	1320 ± 276		
		9.3	154 ± 37		
		IL-2 + 9.3	1828 ± 211		
Unseparated	Anti-CD3 Ab	None	2685 ± 741	3132 ± 1342	352 ± 160
		IL-2	55898 ± 9489	31545 ± 1620	36954 ± 4887
		9.3	66700 ± 3900	24312 ± 709	15359 ± 3610
		IL-2 + 9.3		28532 ± 9309	
$CD3^+$	Anti-CD3 Ab[c]	None		1810 ± 863	
		IL-2		32262 ± 1962	
		9.3		30059 ± 2840	
		IL-2 + 9.3		34080 ± 1162	
$CD3^-$	Anti-CD3 Ab	None			163 ± 72
		IL-2			354 ± 64
		9.3			218 ± 58
		IL-2 + 9.3			ND

[a] $CD3^+$ and $CD3^-$ thymocytes were isolated by panning on tissue culture plates coated with an anti-CD3 antibody. Cells were cultured for 96 h in microtiter plates. [^{3}H]TdR was added to the cultures 18 h before harvesting.

[b] The anti-CD3 mAb G19-4 was immobilized on the culture wells using a 1/1500 dilution of ascites fluid. This concentration of Ab was previously shown to induce maximal proliferation of peripheral blood T cells; 9.3 was used at a concentration of 1 μg/ml; IL-2 was used at 64 half-maximal U/ml.

[c] Due to the positive selection procedure used to obtain $CD3^+$ cells (i.e., an anti-CD3 mAb) we were unable to culture them in the complete absence of mitogenic stimuli.

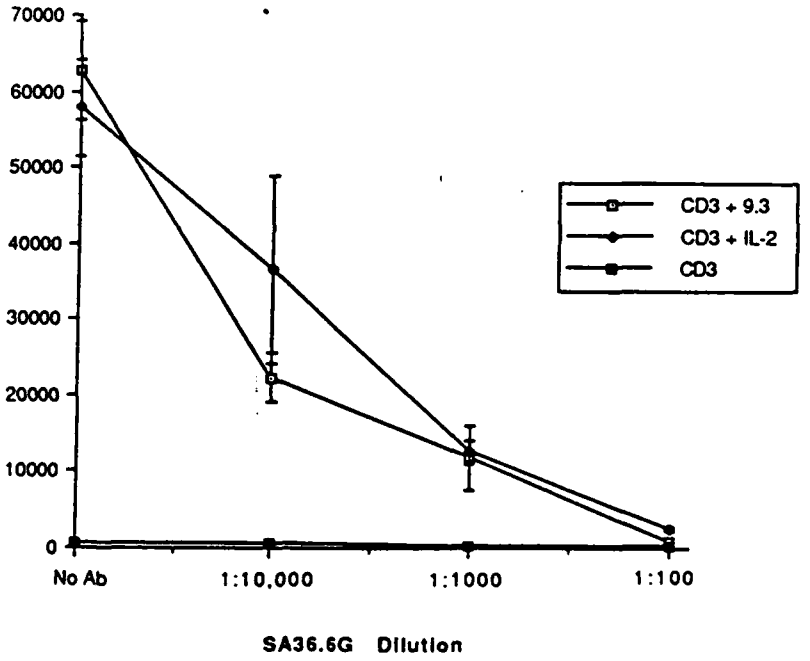

Figure 3. The comitogenic effect of CD3 and CD28 on thymocytes is IL-2 dependent. Thymocytes were cultured on anti-CD3-coated tissue culture plates. Wells either contained medium alone, 9.3 mAb (anti-CD28, 1 μg/ml), or IL-2 (64 half-maximal U/ml). Where indicated, the anti-IL-2R mAb SA36.6G was added as a 1/100, 1/1,000, or 1/10,000 dilution of ascites. Cultures were harvested after 96 h and [^{3}H]dThd (1 μCi/well) was added 18 h before harvest. *Error bars* indicate ± 1 SD.

activation of protein kinase C via phorbol ester treatment induced the cell surface expression of CD28 in high density, although the cells remained $CD3^-$. This population of $CD3^-CD28^{(bright)+}$ cells was unable to proliferate in response to CD28 signaling. Previous work has shown that peripheral blood T cells were able to respond to phorbol ester plus anti-CD28 stimulation even after CD3 had been modulated off their cell surface (23). The observation that $CD3^-$ thymocytes do not respond to the same stimuli suggests that either the CD28 pathway operates through different intracellular mechanisms in this population, or that $CD3^-$ thymocytes have not yet reached the maturational stage at which CD28 is a functional pathway despite the fact that CD28 expression is inducible in these cells. Although we cannot at present discriminate between the two possibilities, we favor the latter interpretation. Previous studies demonstrating that only mature $CD1^-$ thymocytes can respond to anti-CD2 plus anti-CD28 stimulation (23) are consistent with our findings.

Yang et al. (29) have also examined the phenotype of thymocytes with respect to CD3 and CD28, and found generally similar percentages of cell fractions. In contrast to these results, Toribio et al. (28) have reported virtually no CD28 expression in unstimulated mature $CD3^{+(bright)}$ thymocytes, while isolated $CD3^-CD4^-CD8^-$ prothymocytes expressed a low density of CD28. The reason for the discrepancy is unclear, but may be an artifact of their

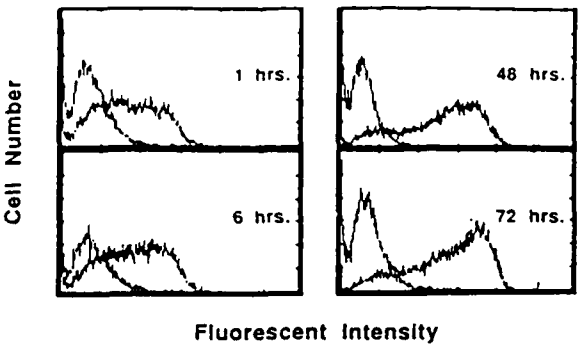

Figure 4. The CD28 surface Ag is inducible on CD3⁺ thymocytes. CD3⁺ thymocytes were positively selected by panning on anti-CD3-coated tissue culture plates. After a 1-h incubation, the nonadherent cells were removed, the plates were washed, and the adherent cells were left on the anti-CD3-coated plates and cultured in complete medium supplemented with IL-2. Cells were harvested at serial time points and stained with anti-CD28-PE mAb or with mouse g-phycoerythrin. Fluorescent intensity is displayed on a 3-log scale.

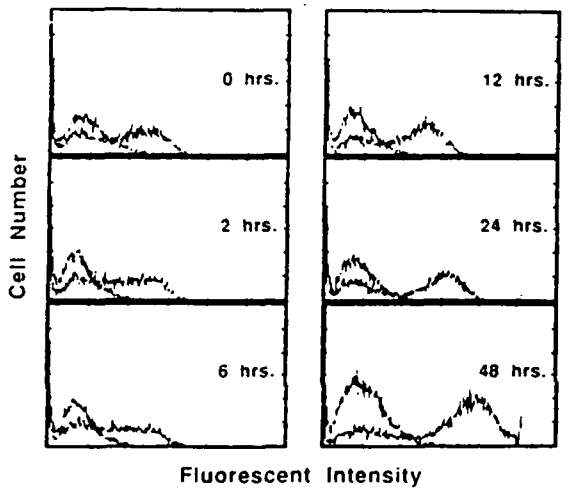

Figure 5. CD28 surface antigen is inducible on PBL stimulated through the TCR/CD3 complex. PBL were stimulated on anti-CD3-coated tissue culture plates, harvested at serial time points, and stained with anti-CD28-PE mAb or with mouse Ig-phycoerythrin. Fluorescent intensity is displayed on a 3-log scale.

cell separation process, as both Yang et al. (29) and our own study analyzed unfractionated cells using two-color immunofluorescence.

Previous studies have suggested that CD3 stimulation of thymocytes either results in the transmission of a negative signal or in cell death (26, 27). This was postulated to be an important mechanism in the deletion of autoreactive clones that encounter their target Ag in the thymus. We and others (28; present report) have now shown that CD3⁺ thymocytes can proliferate in response to CD3 stimulation if a second signal in the form of IL-2 is provided. It has been previously demonstrated that thymocytes can produce and respond to IL-2 when stimulated with a combination of mitogens such as PMA plus anti-CD2 mAb (26) or anti-CD2 plus anti-CD28 mAb (29). However, in the former model, concomitant anti-CD3 stimulation inhibited IL-2 gene expression and cell proliferation. Thus, although CD3-activated thymocytes could respond to exogenous IL-2, the physiologic relevance of this finding has been unclear as the availability of IL-2 to CD3-stimulated thymocytes remained unestablished. Our data now show that CD28 stimulation of CD3-activated thymocytes can directly lead to IL-2-dependent proliferation.

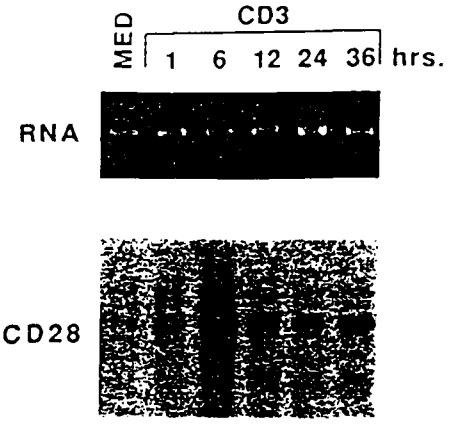

Figure 6. CD28 gene expression is induced by stimulation of the TCR/CD3 pathway. CD28⁺ T cells were cultured on anti-CD3-coated tissue culture plates and harvested at the indicated times. Control cells were cultured on uncoated plates (media). RNA was isolated and equalized for rRNA. The equalization was confirmed by ethidium bromide staining of 10% portions of each RNA sample separated on a non-denaturing 1% agarose gel (*upper panel*). Northern blots were prepared, and the filters were hybridized sequentially with cDNA probes specific for CD28 (*lower panel*).

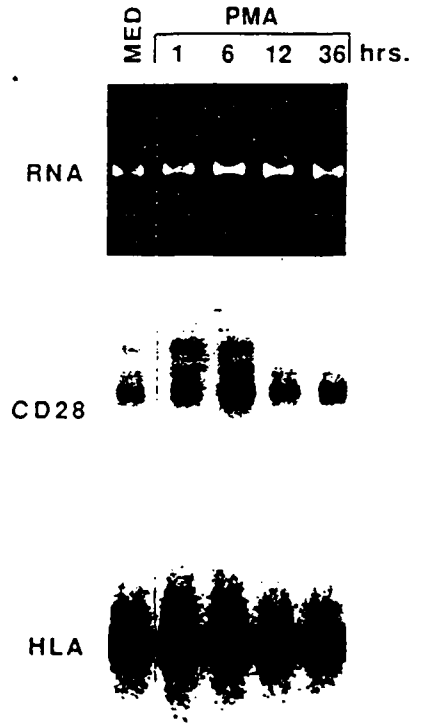

Figure 7. Protein kinase C activation alone is sufficient to induce expression of the CD28 gene. CD28⁺ T cells were cultured with medium alone (MED) or with PMA (3 ng/ml) and harvested at the time points indicated. RNA was isolated and equalized (*upper panel*) as described in the text. Northern blots were prepared and the filters were hybridized sequentially with cDNA probes specific for CD28 (*middle panel*) and HLA (*lower panel*).

Signaling through the CD28 pathway may represent part of a thymic selection mechanism. For example, binding of antibody or Ag to the TCR/CD3 complex of immature thymocytes may induce competence to receive and

respond to a second signal. In the thymic microenvironment, this signal might consist of the natural ligand for CD28. Anti-CD3 stimulation may induce the expression of high affinity IL-2R, which if followed by anti-CD28 stimulation could lead to IL-2 production and clonal proliferation. However, in the absence of anti-CD28 stimulation, a negative or even autolytic signal might be transmitted. A similar model has been demonstrated for the cell line FDC-P1 (41). Stimulation of these cells induces competence to receive a proliferative signal that is supplied by IL-3; however, competent cells die if deprived of IL-3.

Our results also indicate that the CD28 molecule is itself an activation Ag, which is up-regulated both at the mRNA and protein level in response to physiologic stimulation (via the TCR/CD3 complex) of both thymic and peripheral blood T cells. Furthermore, this increase in CD28 expression is a sustained event, as reflected by the continued increase in CD28 Ag levels on the surface of activated cells for at least 6 days. Previously reported variations in CD28 expression upon stimulation of T cells may reflect different methods of cell activation (42). For example, when we examined surface CD28 expression in PHA-activated PBL, we found that within 6 h, CD28 and CD3 were comodulated off the cell surface (data not shown). The modulation of cell surface molecules such as CD3 has been previously reported to occur upon stimulation with PHA, a plant lectin that binds to cell surface glycoproteins (43). Subsequently, we found an increase in CD28 surface Ag, similar to that seen with anti-CD3-activated T cells; however, this increase was not seen until 48 to 96 h after activation. These findings are consistent with the data of Lesslauer et al. (44) who reported an increase in CD28 surface Ag expression by day 3 in PHA-activated PBL by using immunoprecipitation to quantify CD28 protein.

The natural ligand for CD28 remains unknown despite searches for both soluble factors and cell surface molecules that might serve this function. The location of the natural ligand (soluble vs. membrane bound) will be important in helping elucidate the physiologic role of the CD28 pathway, because some effects ascribed to 9.3 occur using the bivalent antibody alone, whereas others require additional cross-linking on beads or with a second antibody (45, 46). Nevertheless, the data presented above suggest that the CD28 surface molecule may play an important physiologic role not only in regulation of lymphokine production, but also in the maturation and selection of the T cell repertoire within the thymus.

Note added in proof. We have recently identified a natural ligand of CD28 as the B7/BB-1 molecule expressed on activated B cells.

(P. S. Linsley, E. A. Clark, and J. A. Ledbetter. The T cell antigen CD28, mediates adhesion with B cells by interaction with the activation antigen B7/BB-1. *submitted for publication*).

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