CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines

Craig B. Thompson*†‡, Tullia Lindsten†, Jeffrey A. Ledbetter§, Steven L. Kunkel§, Howard A. Young†, Stephen G. Emerson§, Jeffrey M. Leiden*†‡, and Carl H. June**

*Howard Hughes Medical Institute and Departments of Medicine, Pathology, and Microbiology/Immunology, University of Michigan, Ann Arbor, MI 48109; †Oncogen Corporation, Seattle, WA 98121; ‡Laboratory of Molecular Immunoregulation, National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD 21701; and **Naval Medical Research Institute, Bethesda, MD 20814

Communicated by J. L. Oncley, November 9, 1988 (received for review August 4, 1988)

ABSTRACT CD28 is a 44-kDa glycoprotein expressed as a homodimer on the surface of a major subset of human T cells. Previous studies have demonstrated that the binding of monoclonal antibodies to the CD28 surface antigen can augment the proliferation of purified human T cells stimulated with suboptimal doses of mitogens or anti-T-cell receptor/CD3 complex antibodies. In this report, we show that CD28 stimulation augments T-cell immune responses by specifically inducing a 5- to 50-fold enhancement in the expression and secretion of interleukin 2, tumor necrosis factor type α, lymphotoxin, interferon γ, and granulocyte–macrophage colony-stimulating factor in normal human T cells stimulated to proliferate by crosslinking of the T-cell receptor/CD3 complex. This CD28-mediated induction of lymphokine/cytokine gene expression occurred even in T cells stimulated with optimal concentrations of mitogens or anti-T-cell receptor/CD3 antibodies, although under these conditions CD28 activation failed to enhance the proliferative response. The activation pathway induced by stimulation of CD28 is distinct from other biochemical pathways that induce lymphokines/cytokines because CD28 stimulation can induce lymphokine/cytokine gene expression in the presence of the immunosuppressant cyclosporine. Together these data suggest that the CD28 cell surface molecule is part of a distinct activation pathway that specifically modulates the expression of multiple lymphokine/cytokine genes.

T cells are important regulators of in vivo immune responses. The specificity of a T-cell-initiated immune response is mediated by the T-cell receptor/CD3 (TCR/CD3) complex (1). Interaction of the TCR/CD3 complex with a foreign antigen presented in association with a major histocompatibility complex protein results in T-cell proliferation and in induction of T-cell effector functions, such as lymphokine production and cytolytic (2). Although antigen-specific T-cell activation is initiated through the TCR/CD3 complex, a number of additional cell surface molecules appear to be involved in regulating T-cell immune responses (3, 4).

We have shown that stimulation of the CD28 surface antigen, a 44-kDa homodimeric glycoprotein (5, 6) present on ≈80% of peripheral blood T cells, leads to enhanced proliferation and interleukin 2 (IL-2) production by CD28+ T cells that have been activated by crosslinking of the TCR/CD3 receptor (7). A basic question that remains unanswered is whether or not the CD28 activation pathway acts primarily to increase the percentage of cells that are induced to proliferate in response to crosslinking of the TCR/CD3 receptor complex and thus increase the number of cells in culture producing IL-2. Alternatively, the CD28 pathway may enhance IL-2 gene expression per cell and thus increase T-cell proliferation by inducing greater amounts of IL-2, a growth factor that is required by T cells to maintain proliferation. T cells activated by stimulation of the TCR/CD3 complex have been shown to produce a number of secreted molecules, in addition to IL-2, that serve to modulate the functional state of a wide variety of cells (8). Thus, it was also of interest to study whether the effect of CD28 stimulation is specific for IL-2 gene induction or whether CD28 might play a more generalized role in the induction of lymphokine/cytokine production.

In the present study we have investigated the role of CD28 activation in regulating lymphokine/cytokine production by normal human T cells. CD28 activation alone does not induce lymphokine expression in resting T cells. However, the CD28 activation pathway significantly enhances the production of multiple lymphokines, including IL-2, interferon γ (IFN-γ), tumor necrosis factor type α (TNF-α), lymphotoxin (LT), and granulocyte–macrophage colony-stimulating factor (GM-CSF) by anti-CD3-activated or phorbol myristate acetate (PMA)-activated CD28+ T cells. This induction of lymphokine production is sufficient to account for the previously observed effect of CD28 activation on T-cell proliferation at suboptimal doses of mitogens or TCR/CD3 antibodies. Compared with other activation pathways that induce lymphokine/cytokine gene expression, CD28 induces lymphokine/cytokine gene expression that is relatively resistant to the immunosuppressive effects of cyclosporine (CSP). The ability of CD28 to sustain the production of multiple lymphokines in activated T cells treated with pharmacological doses of CSP may account for the reported CSP resistance of T-cell proliferation induced by CD28 plus PMA.

MATERIALS AND METHODS

Cells. Approximately 2.5 × 10⁶ human peripheral blood lymphocytes were isolated from buffy coats obtained by leukopheresis of healthy donors aged 21–31 years by using density gradient centrifugation. The CD28+ subset of T cells was then isolated from the peripheral blood lymphocytes by negative selection using immunoadsorption. This strategy takes advantage of the reciprocal and nonoverlapping distribution of the CD11 and CD28 surface antigens on T lymphocytes as described (7).

Monoclonal Antibodies (mAbs). Anti-CD28 mAb 9.3 (IgG2a) was purified on protein A-Sepharose, dialyzed against isotonic phosphate-buffered saline, filtered through a 0.22-µm sterile filter, and cleared of aggregates by centrifugation (100,000 × g for 45 min) prior to use in functional assays (9). Anti-CD3 mAb G19-4 (IgG1) was produced and purified as described (9). mAb G19-4 was used after first

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adsorbing the antibody to the surface of plastic tissue culture plates as described (10).

Thymidine/Uridine Incorporation. CD28+ T cells were cultured at 10^5 cells per well in the presence of medium, phophoehagglutinin (PHA) at 10 μg/ml, PMA at 3 ng/ml, anti-CD28 mAb 9.3 at 100 ng/ml, or immobilized anti-CD3 mAb G19-4 at 200 ng per well. Cells were cultured in quadruplicate samples in flat-bottomed 96-well microtiter plates in RPMI 1640 medium containing 5% (vol/vol) heat-inactivated fetal calf serum. The means and standard deviations of incorporated [3H]thymidine or [3H]uridine were determined by liquid scintillation counting after cells were collected on glass fiber filters. The failure of cells in these cultures to proliferate in response to PHA is the result of rigorous depletion of accessory cells as described above. The binding of isotype-matched mAbs to other T-cell surface antigens (CD2, CD4, CD6, CD7, CD8, or CD45) failed to mimic the effects observed with anti-CD28 (data not shown).

RNA (Northern) Blot Analysis. CD28+ T cells were cultured at 2 x 10^5 cells per ml under a variety of conditions. The cells were harvested; total cellular RNA was isolated and equalized for rRNA as described (11). Northern blots were prepared and hybridized sequentially with [32P]-labeled nick-translated gene-specific probes. After hybridization, blots were washed and exposed for autoradiography at −70°C. Quantitation of band intensities was performed by densitometry (12).

DNA Probes. The IL-2 probe was a 1.0-kilobase (kb) PstI I cDNA fragment (8), the IFN-γ probe was a 1.0-kb PstI I cDNA fragment (13), the GM-CSF probe was a 700-base-pair EcoRI–HindIII fragment cDNA (14), the 4F2 probe was a 1.85-kb EcoRI cDNA fragment (12), the interleukin 4 probe was a 0.9-kb Xho I cDNA fragment (15), and the HLA probe was a 1.4-kb Pst I fragment from the HLA-B7 gene (12). TNF-α and LT specific probes were synthesized as gene-specific 30-nucleotide oligomers (16, 17).

Lymphokine Assays. CD28+ T cells were cultured at 10^5 cells per well in the presence of various combinations of stimulators. Culture supernatants were harvested at 24 hr and serial dilutions were assayed for IL-2 by using a bioassay as described (18). One unit was defined as the amount of IL-2 needed to induce half-maximal proliferation of the human cytotoxic T-cell line CTL-L2 at 24 hr of culture. In separate experiments the relative levels of IL-2 for each of the culture conditions above were independently confirmed using a commercially available ELISA (Genzyme). TNF-α/LT levels were measured by using a semiautomated L929 fibroblast lytic assay as described (19). Units of TNF-α/LT were defined using an internal standard for TNF-α (Genzyme). The independent presence of both TNF-α and LT was confirmed by the ability of mAb specific for each cytokine to partially inhibit cell lysis mediated by the supernatant from cells costimulated with anti-CD3 plus anti-CD28. IFN-γ was measured by radioimmunoassay with a commercially available kit (Centocor, Malvern, PA). Units for IFN-γ were determined from a standard curve using 125I-labeled human IFN-γ provided in the test kit. GM-CSF was detected by stimulation of proliferation of the human GM-CSF-dependent cell line AML-193 (20) in the presence of neutralizing mAbs to TNF-α and LT. The [3H]thymidine uptake induced by purified GM-CSF at 10 ng/ml was defined as 100 units.

RESULTS

CD28 Stimulation Augments Uridine But Not Thymidine Incorporation in T Cells Stimulated with Optimal Doses of Anti-CD3. Previous reports have demonstrated that anti-CD28 stimulation can augment proliferation of T cells stimulated with mitogens or TCR/CD3 antibodies (21–25). In experiments using highly purified CD28+ T cells, we have found that CD28 augmentation of the anti-CD3-induced proliferative response is only apparent when low levels of anti-CD3 were used to initiate the response (26). When we used >200 ng of anti-CD3 in a microtiter well, we no longer observed any enhancement of T-cell proliferation (as measured by thymidine incorporation) as a result of CD28 stimulation. The results of one such experiment are shown in Fig. 1. Anti-CD28 did not augment the thymidine incorporation of accessory-depleted populations of resting CD28+ T cells stimulated with a concentration of anti-CD3 titrated to induce the maximal proliferative response. However, as reported, anti-CD28 was able to elicit a proliferative response in the same cell population when cells were stimulated with anti-CD28 and PMA. Neither PMA nor anti-CD28 alone was able to induce proliferation in resting CD28+ T cells.

Despite the inability of anti-CD28 to augment thymidine incorporation in T-cell cultures stimulated with optimal concentrations of anti-CD3, we did find that anti-CD28 consistently augmented the rate of uridine incorporation of these same anti-CD3-treated T-cell cultures (Fig. 1). CD28 stimulation alone did not enhance uridine incorporation of resting T cells. The CD28-mediated increase in uridine incorporation of anti-CD3 treated cells was observed as early as 6 hr after stimulation and persisted throughout the 72-hr culture period at all doses of anti-CD3 tested (data not shown). These results demonstrate that CD28 stimulation can enhance metabolic activity of anti-CD3-stimulated T cells in the absence of a demonstrable effect on anti-CD3-induced proliferation.

Fig. 1. Effects of anti-CD28 stimulation on DNA and RNA synthesis of anti-CD3-activated T cells. CD28+ T cells were cultured at 10^5 cells per well in the presence of medium (MED), PHA, PMA, anti-CD3 mAb 9.3 (aCD28), or immobilized anti-CD3 mAb G19-4 (aCD3). Equal aliquots of cells were cultured for 18 hr and then pulse-labeled for 6 hr with [3H]uridine at 1 μCi per well or were cultured for 72 hr and then pulse-labeled for 6 hr with [3H]thymidine at 1 μCi per well. The data are presented as mean ± 1 SD for incorporated [3H]thymidine (Upper) and [3H]uridine (Lower). The data presented are representative of five experiments.
CD28 Activation Specifically Enhances the mRNA Expression of Multiple Lymphokine Genes. To address whether CD28 augmentation of T-cell metabolic activity might be due to induction of a specific T-cell effector function, we next investigated the effect of CD28-mediated activation on the production of the soluble immunoregulatory molecules IL-2, TNF-α, LT, IFN-γ, and GM-CSF. Purified resting T cells were activated with an optimal concentration of anti-CD3 in the presence or absence of the anti-CD28 mAb 9.3. RNA was extracted from cells at various times after stimulation and analyzed for the expression of IL-2, TNF-α, LT, IFN-γ, and GM-CSF by Northern blot analysis (Fig. 2). Stimulation with anti-CD3 alone resulted in small but significant increases in steady-state mRNA levels of all five lymphokines/cytokines examined. In contrast, 5- to 50-fold higher peak levels of mRNA expression of all five of the lymphokines/cytokines were observed when cells were costimulated with anti-CD3 and anti-CD28. Maximal mRNA expression for all of the examined lymphokines/cytokines was seen within 6 hr of stimulation of the cells with either anti-CD3 alone or anti-CD3 plus anti-CD28. However, the CD28-costimulated population maintained detectable levels of mRNA expression throughout the proliferative response. Similar enhancement of lymphokine mRNA expression by anti-CD28 was seen in cells stimulated by anti-CD3 concentrations from 4 to 200 ng per well (data not shown).

The increased mRNA expression of these T-cell secretory genes was not simply the result of a generalized increase in the steady-state mRNA expression of all T-cell activation-associated genes. The mRNA level for the heavy-chain gene of the activation-associated 4F2 surface antigen, which is induced after anti-CD3 activation of resting T cells (12), was not further augmented by costimulation of cells with anti-CD28. Moreover, the levels of HLA class I mRNA, while slightly induced by anti-CD3 stimulation, were not further induced by costimulation of cells with anti-CD3 plus anti-CD28. In addition, not all T-cell lymphokines were induced in CD28+ T cells. We were unable to detect interleukin 4 gene expression within 24 hr after anti-CD3, anti-CD3 plus PMA, or anti-CD3 plus anti-CD28 stimulation of CD28+ T cells (data not shown). The increases in lymphokine/cytokine mRNA expression that were observed in CD28-stimulated cells could not be accounted for by an increase in the percentage of proliferating cells in the culture. At 48 hr after stimulation, 41.2% of the cells stimulated with anti-CD3 alone and 46.1% of the cells stimulated with both anti-CD3 and anti-CD28 were found in the S, G2, or M phases of the cell cycle. More than 90% of cells in both populations entered G1 as demonstrated by cell volume increases.

Enhancement of Lymphokine/Cytokine Gene Expression by CD28 Activation Leads to Increases in Lymphokine/Cytokine Secretion. To confirm that the observed increases in steady-state lymphokine/cytokine mRNA levels lead to parallel increases in secretion of these molecules, we assayed the culture medium supernatants of cells stimulated through the CD28-activation pathway for the presence of these same lymphokines/cytokines (Table 1). T cells incubated in medium alone or stimulated with PMA, anti-CD28, or the calcium ionophore ionomycin produce little or no soluble IL-2, TNF-α/LT, IFN-γ, or GM-CSF. Crosslinking of cells with mAbs directed against the TCR/CD3 complex led to the production of low levels of IL-2, TNF-α/LT, IFN-γ, and GM-CSF. In contrast, when cells were stimulated with anti-CD3 plus anti-CD28, there was a substantial augmentation in the secretion of all lymphokines/cytokines tested (Table 1). However, costimulation with PMA plus ionomycin did not induce expression of any lymphokine gene tested (data not shown). The data presented are representative of five experiments.

activation of the TCR/CD3 complex can be mimicked by activation of these pathways with PMA and the calcium ionophore ionomycin, respectively (1, 28). Treatment of purified CD28+ T cells with either of these agents alone did not lead to significant production of any of the lymphokines/ cytokines tested (Table 1). However, costimulation with PMA plus ionomycin did induce a low level of secretion of IL-2, TNF-α/LT, and IFN-γ (GM-CSF was not tested). The addition of anti-CD28 mAb to PMA-plus ionomycin-treated cells significantly enhanced the expression of all of the lymphokines/cytokines examined. Again, no significant in-
TCR/CD3 activation resistant to lymphokine/cytokine completely suppressed T-cell anti-CD3 cell proliferation for the expression stimulated enhancement in or anti-CD3, GM-CSF and expression CSP to amine whether CD28 cycle was partially suppressed CSP while secretion lymphokine costimulated with addition In anti-CD28 mAb increase in the presence of CSP. Costimulation of resting T cells with anti-CD28 mAb plus PMA induced T-cell proliferation (Fig. 1) and also led to high concentrations of TNF-α/LT, IFN-γ, and IL-2 in the cell supernatant (Table 1). This result suggests that CD28 can act as a costimulus with PMA to lead to both T-cell proliferation and lymphokine/cytokine production. Costimulation of resting T cells with ionomycin plus anti-CD28 mAb failed to induce proliferation or lymphokine production (data not shown).

**CD28 Stimulation Can Induce Lymphokine Expression and Secretion in the Presence of CSP.** A characteristic feature of the CD28 activation pathway is that in contrast to the TCR/CD3 activation pathway, T-cell proliferation induced by anti-CD28 plus PMA is resistant to the immunosuppres- sant CSP (7). This effect is specific to the CD28 signal since cell proliferation induced by anti-CD3 plus anti-CD28 is only partially suppressed by CSP while proliferation induced by anti-CD3 plus PMA is completely suppressed (26). To ex- amine whether CD28 also led to CSP-resistant lymphokine/cytokine expression and secretion, we tested the ability of CSP to suppress the induction of IL-2, TNF-α, LT, IFN-γ, and GM-CSF after stimulation with PMA plus anti-CD28, anti-CD3, or anti-CD3 plus anti-CD28. By Northern blot analysis, stimulation with PMA plus anti-CD28 led to lymphokine/cytokine gene expression that was relatively resistant to suppression by CSP (Fig. 3). In contrast, gene expression stimulated by way of the CD3 pathway was completely suppressed in the presence of CSP. Costimulation with anti-CD3 plus anti-CD28 again led to significant enhancement in the expression of all five lymphokine/cytokine genes in comparison to cells stimulated with anti-CD3 alone. In addition the lymphokine/cytokine production of cells costimulated with anti-CD3 plus anti-CD28 was partially resistant to suppression by CSP.

Similar results were obtained when lymphokine levels were measured in culture supernatants of cells stimulated in the presence of CSP (Table 1). CSP was able to completely suppress lymphokine production induced by anti-CD3 or

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<th>Stimulus</th>
<th>IL-2, units/ml</th>
<th>TNF-α/LT, units/ml</th>
<th>IFN-γ, units/ml</th>
<th>GM-CSF, units/ml</th>
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**DISCUSSION**

Our results suggest that stimulation of the CD28 surface antigen induces an intracellular activation pathway that is distinct from those induced by stimulation of the TCR/CD3...
complex. The CD28 pathway functions to specifically augment the expression and secretion of a set of lymphokines/cytokines produced by T cells. The lymphokines produced by CD28\(^*\) T cells and augmented by CD28 stimulation include: IL-2, which can facilitate the cell-cycle progression of T cells; TNF-\(\alpha\) and LT, cytokines which have been shown to be involved in the lysis of tumor cells in vitro; IFN-\(\gamma\), which displays a wide variety of anti-viral and anti-tumor effects; and GM-CSF, which functions as a multilineage hematopoietic growth factor. This group of lymphokines has been reported to be produced in the mouse by the T\(_{H4}\) subset of helper T cells (8). Based on our observations, it is possible that the CD28 surface antigen defines a similar helper-cell subset in man. Consistent with this hypothesis, we have been unable to induce expression of the T\(_{H2}\)-specific lymphokine interleukin 4 (8) in CD28\(^*\) T cells by stimulation with anti-CD3, anti-CD3 plus anti-CD28, or anti-CD28 plus PMA.

The CD28 activation pathway does not induce lymphokine/cytokine production on its own, but rather requires the permissive effect of at least one additional pathway provided by treatment with either anti-CD3 or PMA. Anti-CD28 plus PMA act synergistically to induce lymphokine/cytokine production and T-cell proliferation, while CD28 costimulation of cells treated with PMA plus ionomycin, or cells stimulated through crosslinking of their TCR/CD3 complex, enhances their expression of lymphokines/cytokines without significant augmentation in the percentage of cells that enter and traverse the cell cycle. The difference in the effects on cell proliferation between these costimulatory experiments may be caused by the failure of PMA to induce IL-2 expression without anti-CD28 costimulation. The addition of anti-CD28 mAb to PMA-activated cells leads to IL-2 gene expression, which may in turn stimulate T-cell proliferation. In contrast, TCR/CD3 crosslinking even in the absence of anti-CD28 stimulation induces sufficient IL-2 production to induce autocrine proliferation (refs. 7 and 29 and Table 1), but activation of this pathway alone is insufficient to lead to significant levels of IL-2 in culture supernatants. Use of optimal doses of ionomycin plus PMA to mimic the intracellular effects of TCR/CD3 crosslinking can significantly enhance the production of IL-2, but even under these conditions anti-CD28 can induce a nearly 10-fold enhancement in IL-2 gene expression (Table 1).

Our results have several important basic scientific and clinical implications. First, the results suggest that the production of secretory immunoregulatory molecules by normal peripheral blood T cells can be induced by mechanisms that are distinct from those that primarily regulate the proliferation and clonal expansion of such cells. The CD28 signal may function to regulate the transition of lymphokine/cytokine production by T cells in vivo from an autocrine to a paracrine level. This could represent a biochemical mechanism for the induction of the multiple immunological effects of T-cell help. Stimulation of the CD28 pathway in vivo could also explain the reported (30) independence of some immune responses to CSP suppression. In addition, a variety of syndromes, including septic shock and tumor-induced cachexia, may involve activation of the CD28 pathway and augmented production of potentially toxic levels of lymphokines/cytokines. Finally, the ability of the CD28 pathway to specifically augment lymphokine/cytokine production of antigen receptor-activated T cells suggests that it may be possible to specifically augment in vivo immune responses by pharmacologic manipulation of this pathway. While the structure of CD28 suggests that it is a cell surface receptor (6), at present it is not known if there is a naturally occurring ligand for the CD28 surface molecule. Despite this, the ability to stimulate the CD28 activation pathway with mAbs should contribute to our understanding of the coordinate regulation of the expression of various T-cell secretory molecules.

We thank our colleagues David Thomas, Michael Imperiale, Wayne McCormack, June C. H. Thompson, and Gregory Stella for their critical review of the manuscript; Nancy Craighead, D. S. Smoot, and Jeanelle Pickett for expert technical assistance; and Jeannelle Pickett for assistance in preparing the manuscript. We also thank one of the referees for suggesting the experiments with anti-CD3 plus PMA and PMA plus ionomycin. These studies were funded in part by funds provided by the Naval Medical Research and Development Command. T.L. is a Leukemia Society of America Special Fellow.