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CD28 Costimulation Augments IL-2 Secretion of Activated Lamina Propria T Cells by Increasing mRNA Stability Without Enhancing IL-2 Gene Transactivation

Rivkah Gonsky,* Richard L. Deem,* Doo Han Lee,† Alice Chen,* and Stephan R. Targan2*

The pathways leading to activation in lamina propria (LP) T cells are different from peripheral T cells. LP T cells exhibit enhanced IL-2 secretion when activated through the CD2 pathway. Costimulation of CD28 leads to synergistic enhancement of IL-2 secretion. Previous studies have characterized the CD28 augmentation of TCR-mediated signaling in peripheral blood T cells through transcriptional activation of an IL-2 promoter CD28 response element (CD28RE), along with enhanced mRNA stability. This study characterized molecular events involved in CD28 costimulation of IL-2 production in LP mononuclear cells (LPMC). LPMC exhibited increased IL-2 production in response to CD28 costimulation, compared with cells activated through CD2 alone. IL-2 secretion was paralleled by increased expression of IL-2 mRNA, resulting from enhanced IL-2 mRNA stability. In contrast to transcriptional activation in PBMC, EMSA revealed that CD28 coligation of CD2-activated LPMC does not result in increased binding of trans-factors to the CD28RE, nor did Western blots detect changes in IκBα or IκBβ levels following CD28 coligation. Furthermore, CD28 coligation fails to enhance IL-2 promoter-reporter or RE/AP construct expression in CD2-activated LPMC. The results reported herein indicate that the molecular mechanisms involved in CD28 cosignaling and regulation of IL-2 secretion in LP T cells are unique to that compartment and differ from those seen in peripheral blood T cells. These observations suggest a biological significance for different mechanisms of IL-2 activation in initiation and maintenance of the cytokine repertoire found in the mucosa. The Journal of Immunology, 1999, 162: 6621–6629.

IL-2 is secreted by activated T cells and plays an important role in initiation and regulation of cellular immune responses. Evidence indicates that regulation of IL-2 expression by PBMC takes place largely at the level of transcriptional regulation. Transcriptional regulation of the IL-2 promoter has been extensively studied in PBMC and T cell lines following TCR activation (1). The 300-bp promoter region of the IL-2 gene upstream of the transcriptional start site contains multiple binding sites for transcriptional activating factors including NF-AT, NF-κB, AP-1 and OCT-binding proteins (2). Cooperative binding of these trans-activating factors appears to determine the balance of transcriptional activation. In addition to transcriptional regulation of IL-2 mRNA levels, an inducible degradation mechanism has been reported (3, 4). Production of IL-2 can be enhanced markedly by treatment with translational inhibitors such as cycloheximide without directly affecting the rate of IL-2 transcription (5).

Maximal activation of T cells requires two signals, the first of which is generated by engagement of the TCR, with a second signal provided by a costimulatory molecule. One major costimulatory T cell surface molecule is CD28 (6, 7). IL-2 expression by CD28 costimulated peripheral blood (PB)3 lymphocytes is mediated transcriptionally as well as posttranscriptionally. CD28 costimulation activates transcription of IL-2 through the binding of κB-like transcription factors to a promoter motif defined as the CD28 response element (CD28RE) (8). The Rel family of proteins, p50, p65, and c-Rel, are components of the transcriptional complex binding to the CD28RE in PBMC (9). A major component of regulation of κB-like transcription factors is the control of their intercellular localization. κB-like transcription factors are normally shielded within the cytoplasm through their physical interaction with the IκB inhibitory protein (10, 11). T cell activation results in the rapid and transient decrease of IκB, thereby releasing the transcriptionally active κB factors and increasing translocation to the nucleus (10, 11). CD28-mediated IL-2 gene expression in PBMC is regulated likewise by stabilization of IL-2 mRNA. The IL-2 mRNA possesses AU-rich sequences within the 3′ untranslated region identified as the target signal for rapid mRNA degradation (12). While CD28 costimulation enhances IL-2 mRNA stability, as well as the stability of other cytokines, no preferential enhancement in the stability of c-myc or c-fos was detected, notwithstanding the presence of AU-rich motifs in the mRNA (13). Thus, regulation of IL-2 RNA levels appears to be complex, involving specific additional pathways that may be sensitive to the events initiating T cell activation (14).

The activation pathways of PB T cells are different from those of lamina propria (LP) T cells (15, 16). LP T cells do not respond well to activation via the TCR/CD3 receptor. However, they do exhibit increased proliferation and cytokine production when activated via the CD2 pathway (16, 17). CD28 coligation further enhances the activation of LP T cells. LP T cells are generally

3 Abbreviations used in this paper: PB, peripheral blood; LP, lamina propria; LPMC, LP mononuclear cells; UC, ulcerative colitis; CD, Crohn’s disease; CD28RE, CD28 response element.

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thought to achieve a more intense activation state than PB T cells. The intensity can be amplified further as has been demonstrated in conditions of dysregulated inflammation such as Crohn’s disease (CD) and ulcerative colitis (UC). These disorders are characterized by widespread intestinal inflammation and an enhanced T cell activation state with increased production of inflammatory mediators including IL-2 (18).

We have developed a model of LP-like T cells by coculturing PB T cells in the presence of irradiated Daudi B cells and IL-2, which mimics a phenotype of CD2 pathway-dominant cytokine secretion similar to that seen in lamina propria lymphocytes. Previous studies have shown that the LP-like T cell model is validated by its functional equivalency to LP T cells, not only with regard to CD2 pathway dominance, but with identical phosphorylation patterns upon CD2 pathway stimulation as well (16). Furthermore, recent studies have provided evidence that the LP-like T cell activation model is most representative of the activated state of T cells present in inflamed CD mucosa, as evidenced by CD2-activated up-regulation of AP1-transactivating factors with similar kinetic profiles (19). The LP-like T cell model has very similar activation properties to those expressed by LP mononuclear cells (LPMMC) from inflamed CD mucosa, and allows investigations of the mechanism of cytokine gene regulation to be performed initially on a more easily manipulated experimental system.

Little is known regarding the underlying mechanisms regulating IL-2 secretion in LP T cells. Recent studies have suggested that regulation of IL-2 production in LPMMC following CD2 activation may involve a distinct transcriptional regulatory mechanism different from that observed in T cell lines (19). The purpose of this study was to determine whether enhanced IL-2 secretion by CD2 and CD28 costimulation was the product of unique events that may involve a distinct transcriptional regulatory mechanism different from that observed in T cell lines (19).

Materials and Methods

Target cells and culture media

The murine T cell line, CTLL-2, was obtained from American Type Culture Collection (Manassas, VA) and was maintained at 37°C in a humidified atmosphere of 5% CO2 in RPMI 1640 supplemented with 10% FCS with 10 U/ml rIL-2 (R&D Systems, Minneapolis, MN). The human B cell line, Daudi, was obtained from American Type Culture Collection and maintained at 37°C in a humidified atmosphere of 5% CO2 in RPMI 1640 supplemented with 10% FCS.

Monoclonal Abs

Anti-CD2 mAbs (clones CB6 and GD10) were a gift from Chris Benjamin (Princeton, NJ). Anti-CD28 Abs (clones I, Sigma, St. Louis, MO), 0.1 μg/ml DNase I (Sigma), 50 μg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml fungizone, with the solution changed every 30 min until the supernatant was free of epithelial cells. The remaining LP was minced into 1- to 2-mm pieces and digested for 10 min in RPMI 1640 containing 10% FCS, 0.5 mg/ml collagenase B (Boehringer Mannheim, Indianapolis, IN), 1 mg/ml hyaluronidase (Sigma, St. Louis, MO), 0.1 mg/ml DNase I (Sigma), 50 μg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml fungizone in shaker water bath (100 rpm). The supernatant was collected, filtered through 110-μm nylon mesh (Spectrum Laboratory Products, Houston, TX), and centrifuged at 500 g for 5 min. The cell pellet was resuspended in 15 ml and centrifuged at 30 × g for 5 min to remove epithelial and other large cells. The supernatant was removed and lymphocytes were isolated by separation on Ficol-Hypaque gradients. The cells were then washed three times with HBSS and resuspended in RPMI 1640 containing 10% FCS. PBMC were isolated from normal healthy volunteers by separation on Ficol-Hypaque gradients.

Induction of LP-like T cells

Mononuclear cells from Ficol-Hypaque gradients were cultured in RPMI 1640 with 10% FCS with a 1:5 ratio Daudi:lymphocytes plus 10 U/ml rIL-2 for 5 days (16). Daudi were irradiated with 3000 rad and washed three times in HBSS before addition to cultures. Following a 5-day culture, LP-like cells were washed with HBSS to remove dead Daudi cells (at this point, there were virtually no live Daudi cells in the culture as determined by flow cytometry).

Stimulation of mononuclear cells

For stimulation through the CD2 receptor, LPMMC and LP-like T cells were stimulated with 0.1 μg anti-CD2 Abs (both CB6 and GD10 clones)/10^6 cells at 37°C for the times indicated for each experiment. CD28 costimulation was carried out with 0.1 μg anti-CD28 Ab. Stimulation of T cells with anti-CD2 Abs did not require further cross-linking, since the combination of two anti-CD2 Abs directed against different epitopes was sufficient to induce activation.

IL-2 bioassay

IL-2 activity was determined using a CTLL-2 bioassay (21). rIL-2 standards and dilutions of samples were added to 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) containing 5 × 10^4 CTLL-2/well and incubated for 48 h at 37°C. Wells were then pulsed with 2 μCi/well [3H]thymidine for 4 h. Wells were harvested using a Harvester 96 (Tomtec, Orange, CT) microplate harvester and counted using a Microbeta 1450 liquid scintillation counter (Wallac, Gaithersburg, MD). Data acquisition and analysis was performed using the ELISA Master program for Macintosh computers, developed by R. L. Deem.

Northern blot analysis

Total cellular RNA was extracted using the Quagen RNeasy kit (Chatsworth, CA). RNA was electrophoretically separated on a denaturing 1% agarose gel containing 7% formaldehyde. Gels were transferred to nylon membrane (Amer sham, Arlington Heights, IL) and hybridized to 32P-labeled DNA probe. Isolated cDNA was labeled by random priming and used at 10^6 cpm/ml of hybridization buffer. Blots were prehydrized (50% formamide, 0.75 M NaCl, 75 mM sodium citrate, 1× Denhardt solution, 25 mM sodium phosphate (pH 6.5), and 100 μg/ml of sheared salmon sperm DNA) at 42°C for 2 h and hybridized overnight in prehybridization solution containing labeled probe and 10% dextran sulfate.

Nuclear run-on

A total of 5 × 10^5 LPMMC were stimulated and nuclei isolated as previously described (22). In vitro transcription was carried out at 26°C for 20 min in transcription buffer (50 mM HEPES (pH 7.9), 100 mM KCl, 2 mM DTT, 30 μM EDTA, 1 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 2 mM MnCl2, 35 mM (NH4)2SO4, 8.8 mM creatine phosphate, 40 μg/ml creatine phosphokinase), and 100 μCi of [α-32P]UTP. Labeled mRNA transcripts were purified using Quagen RNeasy kit for liquid samples and hybridized to 2 μg of cDNA insert immobilized on a nylon membrane.

Preparation of nuclear protein extracts

Nuclear protein extractions were carried out with 5 to 10 × 10^6 LPMMC or LP-like T cells. Following activation, cells were centrifuged, washed in...
cold PBS, and kept on ice for subsequent extraction steps. The cell pellet was resuspended in 0.9 ml of RSB (10 mM Tris (pH 7.4), 10 mM NaCl, 5 mM MgCl₂, 0.5 mM DTT, 2 μM leupeptin, 1 μg/ml aprotinin, 1 mM PMSF, and 0.1 mM EGTA), and 0.1 ml of 5% NP-40 was added. Samples were mixed by gentle inversion and kept on ice for 10 min followed by centrifugation. The pellet was resuspended in 25–60 μl (volume is dependent on the starting number of cells) of cold buffer C (20 mM HEPES (pH 7.4), 0.42 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% v/v glycerol, 0.5 mM DTT, 20 μM leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF). Samples were incubated on ice for 30–40 min during which time they were pipetted twice. Cellular debris was removed by centrifugation and nuclear proteins were diluted with an equal volume of buffer D (20 mM HEPES (pH 7.4), 50 mM KCl, 0.2 mM EDTA, 20% v/v glycerol, 0.5 mM DTT, 20 μM leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF). Protein concentrations were determined by Coomassie Plus assay (Pierce, Rockford, IL).

**EMSA**

Double-stranded oligonucleotide was end-labeled with [γ-32P]ATP and T4 polynucleotide kinase. A total of 3–6 μg of nuclear extract protein was incubated at 25°C with 0.25 mg/ml poly(dI-dC), in 20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, and 50 mM Tris (pH 7.5) for 10 min. The oligonucleotide was then added (20,000 cpm) and the binding reactions were incubated for an additional 30 min. Specificity was determined by the addition of 100-fold excess unlabeled oligonucleotide as competitor. The DNA-protein complexes were separated from unbound probe on a prerun native 5% polyacrylamide gel in low ionic strength buffer (22.3 mM Tris (pH 7.4), 22.3 mM borate, and 0.5 mM EDTA (pH 8.0)). After 2 h, the gel was dried under vacuum and exposed to x-ray film. The oligonucleotide used was the composite IL-2-CD28/RE/AP site: (5'-GGTTAAAAGAAATCCCAAAGGTGATCAGC-3'). Nonspecific competitor oligonucleotide used was: (5'-GAGCCTGATTTCCCCGAATGA TGACG-3').

**DNA constructs**

The human IL-2 cDNA clone and β-actin were obtained from American Type Culture Collection. A human IL-2 luciferase reporter plasmid containing a 600-bp fragment of the IL-2 promoter subcloned immediately 5' to the luciferase gene has been described previously (23). The human RE/AP and mutant RE/AP promoter-reporter constructs have been previously described (24) and were a generous gift from Virginia Smith-Shapiro and Arthur Weiss (University of California, San Francisco, CA). The plasmid TRE2 luciferase, used to determine AP-1-dependent trans-activation, was generated by subcloning three copies of the collagenase AP-1 binding sites into a luciferase reporter plasmid (a gift from M. Karin) (25).

**Transfection**

Freshly isolated LPMC and PBMC were primed for transfection competence by culturing for 16 or 20 h, respectively, in RPMI 1640 medium containing 10% FCS, 50 mM 2-ME, and 1 μg/ml PHA-L (Sigma) as previously described (19, 23). Cells were then washed and resuspended in 250 μl fresh medium at 2 × 10⁷ cells/ml and electroporated in the presence of 50 μg of reporter construct (250 V, 2250 microfarads, 48 ohms) using 4-mm (gap width) cuvettes in a BTX Electro Cell Manipulator (Genetron-Tween-20 and analyzed for immunoreactivity with antisera to IκBα and 1αβB (Santa Cruz Biotechnology, Santa Cruz, CA) with an enhanced chemiluminescence detection system (Pierce).

**Results**

Costimulation by CD28 augments IL-2 secretion and increases mRNA accumulation in LPMC and LP-like T cells activated via the CD2 pathway

We have described previously a model developed using in vitro-activated PB T cells, known as LP-like T cells, which reproduce the CD2-driven cytokine secretion phenotype seen in LP T cells (16, 19). LP T cells and LP-like T cells are significantly more responsive than PB T cells to activation via the CD2 pathway than the CD3 pathway, as exhibited by the secretion of IL-2 and other cytokines (16). Coligation of CD2, but not CD3, with CD28 resulted in a marked increase of IL-2 secretion in LPMC and LP-like T cells (16).

To assess the molecular mechanisms of CD28-enhanced IL-2 secretion, we first examined the kinetics of IL-2 production following activation of LPMC and LP-like T cells via the CD2 pathway in the presence or absence of CD28 costimulation. As shown in Fig. 1A, measurable amounts of IL-2 were detected in supernatants of LPMC isolated from inflamed mucosa as early as 3 h following CD2 activation. IL-2 levels continued to increase over 24 h. CD28 costimulation resulted in a striking increase of IL-2 production at all time points measured. CD28 coligation of LP-like T cells likewise resulted in enhancement of IL-2 secretion at all time points tested (Fig. 1B).

To determine the molecular events associated with CD28-augmented IL-2 production, mRNA was measured at time points following CD2 or CD2 + CD28 activation of LPMC and LP-like T cells. Northern blot analysis of IL-2 mRNA from LPMC revealed that following activation by CD2, cytokine secretion was preceded by an increased expression of IL-2 mRNA. IL-2 mRNA was detectable within 1 h, peaked at 4 h, and then declined to barely detectable levels by 6 h (Fig. 2A). CD28 costimulation resulted in a striking elevation of IL-2 mRNA expression, as well as a prolonged period of detectability. The early kinetics of IL-2 mRNA accumulation appeared similar to that seen with CD2 activation alone. IL-2 mRNA was detectable at 1 h following activation and peaked at 4 h. However, while no IL-2 mRNA was detectable from the CD2-activated cells at 24 h following activation, IL-2 mRNA was still detectable from cells costimulated with CD28.

Figs. 1B and 2B demonstrate that IL-2 secretion and mRNA levels in the LP-like T cell model activated by Abs to CD2 or...
CD2 + CD28 are similar to those seen in LPMC from inflamed mucosa. Activation of LP-like T cells via the CD2 pathway resulted in a steady increase in IL-2 secretion within 3 h. The increase in IL-2 persisted throughout 24 h. IL-2 mRNA was detectable within 2 h, and persisted over 6 h, but declined by 10 h (Fig. 2B). In contrast, the levels of IL-2 mRNA expressed in LP-like T cells costimulated with CD28 were significantly elevated compared with those stimulated with CD2 alone. Increased IL-2 mRNA expression in CD2- and CD28-costimulated LP-like T cells was still detectable at 10 h and remained elevated at the 24-h time point. CD2 alone was unable to induce cytokine production or expression of IL-2 mRNA (data not shown). Thus, CD28 costimulation of the CD2 activation pathway leads to an increase in IL-2 secretion that is paralleled by enhanced level and duration of IL-2 mRNA expression in both LPMC and LP-like T cells.

Costimulation by CD28 enhances mRNA stability in LPMC and LP-like T cell activated via the CD2 pathway

The increased IL-2 mRNA accumulation induced by CD2 and CD28 coligation could be related to an increase in mRNA transcription, processing, and/or enhanced mRNA stability. To determine whether this augmentation was related to mRNA stability, LPMC from inflamed mucosa were preactivated by CD2 or CD2 and CD28 for 2 h (maximum for mRNA expression in LPMC is 2–4 h). Actinomycin D was added to prevent further transcription, and mRNA decay was monitored for the time periods indicated in Fig. 3A. CD28 coligation extended the t1/2 of IL-2 mRNA from 70 to 180 min. A strikingly similar result was obtained with LP-like T cells. LP-like T cells were preactivated by CD2 or CD2 and CD28 for 5 h (to maximize mRNA expression in LP-like T cells). As seen in Fig. 3B, the t1/2 of IL-2 mRNA from CD2-activated LP-like T cells was 60 min compared with over 200 min following CD28 costimulation. Thus, CD28 costimulation increases the stability of IL-2 mRNA in both LPMC and LP-like T cells activated via the CD2 pathway.

CD2 costimulation of CD2-activated LPMC does not affect the transcriptional rate of IL-2 gene expression

Previous studies carried out with activated PBMC have reported increased transcription after CD28 costimulation of the CD3 pathway (26). To determine the contribution of CD28 signaling on initiation of IL-2 gene transcription in LPMC, run-on assays were performed. Nuclei were isolated from LPMC activated by CD2 in the presence or absence of CD28 costimulation. As seen in Fig. 4, while IL-2 gene transcription was undetectable in unstimulated LPMC, following CD2 activation induction of IL-2 transcription was evident. CD28 costimulation, however, did not result in an increased transcriptional rate above that observed by CD2 alone (Fig. 4). Under all conditions, close to equivalent levels of β-actin were detected. No increase in signal was detected hybridizing to the plasmid vector alone (Fig. 4B). To further study transcriptional activation, a wild-type IL-2 promoter reporter luciferase construct encompassing the CD28RE, known to be involved in CD28 enhanced gene transcription in PBMC, was transfected into LPMC activated by CD2, and LPMC activated by CD2 and CD28. As reported recently, CD2 signaling in LPMC is transmitted in part by induction of AP-1-transactivating factors (19). Fig. 6 demonstrates that while CD2 ligation results in transactivation of a multimeric AP-1-binding TRE2 reporter construct in LPMC from normal or inflamed mucosa, coligation of CD28 was unable to further enhance promoter activity. These results indicate that while there is

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Effect of costimulation by anti-CD28 on IL-2 mRNA accumulation in LPMC and LP-like T cells activated via the CD2 pathway. EtBr represents ethidium bromide staining of the ribosomal RNA from equalized RNA samples used to prepare the Northern blot. A, LPMC from inflamed mucosa stimulated with 1 μg anti-CD2/10^6 cells or 1 μg anti-CD2/10^6 cells + 1 μg anti-CD28/10^6 cells. B, PBL cultured 5 days in IL-2 with irradiated Daudi cells (LP-like T cells) prior to stimulation with anti-CD2 or anti-CD2 + anti-CD28. Representative of three experiments with similar results is shown.
an increase in the IL-2 promoter activity following CD2 pathway activation of LPMC, costimulation in the presence of CD28 does not further increase transcriptional activity.

Activation of LPMC via the CD28 pathway does not enhance binding of nuclear proteins to the CD28RE, or decrease I-κB function in LPMC and LP-like T cells

Previous studies have identified a CD28RE within the IL-2 promoter region that is required for transcriptional induction of IL-2 gene expression by CD28 costimulated PBMC and T cell lines (8, 9, 26). It is believed that the complexes binding to the CD28RE are members of the NF-κB/Rel family, the function of which is regulated by sequestration of binding of inhibitory I-κB proteins (10, 11). The results depicted in Fig. 5 suggest that while the IL-2 promoter containing the CD28RE responds to CD2 activation, the CD28RE is not the site at which transactivation is enhanced following CD28 coligation. To assess the effect of CD28 coligation on functional regulation of the CD28RE in our system, LP-like T cells were activated with CD2 in the presence or absence of CD28 costimulation and nuclear proteins were analyzed for binding to the composite CD28RE/AP-1 cis regulatory element by EMSA. As shown in Fig. 7, a very modest increase in binding of complexes to the CD28RE/AP-1 element was detected following CD2 activation. No additional alteration in the binding of this complex was detectable following CD28 costimulation. Furthermore, Western

FIGURE 3. Half-life of IL-2 mRNA following anti-CD2 activation. LPMC from inflamed mucosa (A) or LP-like T cells (B) were preactivated by 1 μg anti-CD2/10^6 cells or 1 μg anti-CD2/10^6 cells + 1 μg anti-CD28/10^6 cells for 2 h or 5 h, respectively, followed by addition of actinomycin D (10 μg/ml) to prevent further transcription. Northern blots were prepared and the amount of mRNA was determined at indicated time points by scanning densitometry (left panel). EtBr represents ethidium bromide staining of the ribosomal RNA from equalized RNA samples used to prepare the Northern blot. Representative of three experiments with similar results is shown.

FIGURE 4. Nuclear run-on analysis of IL-2 mRNA. LPMC were pre-activated by 1 μg anti-CD2/10^6 cells or 1 μg anti-CD2/10^6 cells + 1 μg anti-CD28/10^6 cells for 2 h and nuclei were isolated. A, Run-on assays were performed and transcripts were hybridized to filter containing 2 μg of plasmid inserts specific for human IL-2 or β-actin. B, Densitometric readings of run-on in A. Densitometric units were calculated from β-actin and used to correct the readings for IL-2 mRNA and empty vector. Representative of two experiments with similar results is shown.
stimulated cpm was 380,000. Means (as the ratio of cpm stimulated divided by cpm unstimulated. Mean un-counter with coincidence counting turned off. Fold increase was calculated counted on a six detector Wallac 1450 Microbeta liquid scintillation 4 h. Luminescence was measured using a Promega luciferase assay kit, counted on a six detector Wal-lac 1450 Microbeta liquid scintillation counter (Wallac, Gaithersburg, MD) with coincidence counting turned off. Fold increase was calculated as the ratio of cpm stimulated divided by cpm unstimulated. Unstimulated cpm was 41. Representative of four experiments with similar results is shown.

Activation of LPMC via the CD28 pathway does not significantly enhance transcriptional expression of composite CD28/AP promoter-reporter construct

Previous studies carried out in T cell lines have demonstrated that transactivation of IL-2 gene expression following CD28 oligation is conferred through a composite element, RE/AP, consisting of the synergistic interaction of both the CD28RE and the adjacent nonconsensus AP-1-binding site (24). Mutation of either one of these components was sufficient for abolishing the CD28 response of RE/AP promoter-reporter constructs transfected into T cell lines. The data presented above supported the hypothesis that following CD28 costimulation of LPMC no transcriptional up-regulation of the IL-2 promoter could be detected over that observed in the presence of CD2 alone. However, most of the information regarding our understanding of transcriptional regulation of IL-2 expression has been performed in T cell line systems rather than primary T cells. Therefore, it seemed possible that the failure to detect transcriptional up-regulation following CD28 coactivation of LPMC might not be unique for mucosal T cells but, rather, might be due to unique transcriptional regulatory mechanisms related to primary T cells compared with T cell lines. In order to investigate this possibility, PBMC were transfected with multimerized RE/AP and mutant RE/AP reporter constructs, and expression compared with that of LPMC following CD2 and CD2 + CD28 costimulation. Fig. 9 shows that there is approximately a 10-fold activation of the composite RE/AP construct in response to CD2 stimulation in both peripheral T cells and LPMC. Moreover,

FIGURE 5. Transfection of LPMC with an IL-2 promoter-luciferase se-quence. Freshly isolated LPMC were cultured in the presence of 1 μg/ml PHA-L for 20 h and electroporated with the IL-2 promoter construct. Elect-roporated cells were rested for 1 h at 37°C and then stimulated with either 20 g/ml PMA plus 400 nM calcium ionophore. 1 μg anti-CD2/106 cells plus or minus 1 μg anti-CD28/106 cells for 4 h. Luminescence was measured using a Promega luciferase assay kit, counted on a six detector Wallac 1450 Microbeta liquid scintillation counter (Wallac, Gaithersburg, MD) with coincidence counting turned off. Fold increase was calculated as the ratio of cpm stimulated divided by cpm unstimulated. Unstimulated cpm was 380,000. Means (±SEM) of three experiments is shown.

FIGURE 6. Transfection of LPMC with an IL-2 TRE2 promoter-lucif-erase sequence. Freshly isolated LPMC were cultured in the presence of 1 μg/ml PHA-L for 20 h and electroporated with the TRE2 promoter con-struct. Electroporated cells were rested for 1 h at 37°C and then stimulated with either 20 g/ml PMA plus 400 nM calcium ionophore, 1 μg anti-CD2/106 cells plus or minus 1 μg anti-CD28/106 cells for 4 h. Luminescence was measured using a Promega luciferase assay kit, counted on a six detector Wallac 1450 Microbeta liquid scintillation counter (Wallac, Gaithersburg, MD) with coincidence counting turned off. Fold increase was calculated as the ratio of cpm stimulated divided by cpm unstimulated. Unstimulated cpm was 380,000. Means (±SEM) of three experiments is shown.

FIGURE 7. EMSA analysis of 5 μg nuclear protein isolated from LPMC binding to the CD28RE/AP-1 element following stimulation with by 1 μg anti-CD2/106 cells or 1 μg anti-CD2/106 cells + 1 μg anti-CD28/ 106 cells. Lanes: C, competition with 100-fold excess CD28RE oligo; NS, competition with 100-fold excess nonspecific oligonucleotide. Representa-tive of three experiments with similar results is shown.

FIGURE 8. Kinetics of IκBα (A) and IκBβ (B) binding following CD2 activa-tion. PBL were cultured 5 days in IL-2 with irradiated Daudi cells (LP-like T cells) and stimulated with anti-CD2 or anti-CD2 + anti-CD28. Western blot was used to determine the presence of IκBα and IκBβ nuclear proteins. Representative of three experiments with similar results is shown.
leads to up-regulation of NF-κB elements support the conclusion that CD2 signaling in LPMC are distinct from those observed in peripheral T cells transfectected with the RE/AP reporter construct following CD28 costimulation, there is enhanced promoter transactivation (Fig. 9), from 14- to 300-fold; however, in contrast to peripheral T cells, in LPMC from normal mucosa following CD2 + CD28 coligation, only a marginal 2-fold increase in RE/AP reporter activation was noted over that induced by CD2 alone. CD28 stimulation alone did not activate promoter-reporter constructs in all cells tested (data not shown). Likewise, mutation of either the CD28RE or adjacent nonconsensus AP-1 site abolished CD2 responsiveness. These results indicate that the regulatory mechanisms involved in IL-2 gene expression following CD28 pathway stimulation in LPMC are distinct from those observed in peripheral T cell and T cell lines.

Discussion
In this study, we examined molecular events and mechanisms involved in the regulation of IL-2 production in CD2-activated LPMC and LP-like T cells costimulated with CD28. IL-2 gene expression in LPMC and LP-like T cells is highly sensitive to CD2 activation (16). Coligation of the CD28 molecule leads to a synergistic effect, further enhancing IL-2 secretion in these cell populations (16). Our data show that, in LPMC and LP-like T cells, CD28 costimulation enhanced mRNA stability, but did not increase the rate of transcriptional activation. Nuclear run-on data as well as transfection of a 600-bp IL-2 promoter-reporter construct into LPMC revealed significant promoter activity following CD2 activation, but failed to exhibit augmented transcriptional activation following CD2 + CD28 costimulation above that observed with CD2 alone. Likewise, expression of the IL-2 RE/AP composite element was responsive to CD2 stimulation but failed to exhibit enhanced activity following CD28 costimulation. EMSA and Western blot analysis of the CD28RE cis and trans-acting elements support the conclusion that CD2 signaling in LPMC leads to up-regulation of NF-κB-like factors paralleled by rapid decrease of IkBα. Costimulation with CD28 does not alter this pattern of expression. These results highlight again the utility of the LP-like T cell model as a system with analogous activation properties to those expressed by LPMC, which allows investigations of mucosal cytokine gene regulation to be performed initially on a more easily manipulated experimental system.

Numerous studies have been carried out in PBMC and tumor T cell lines activated through the TCR and CD28 costimulation, which indicated that CD28 synergism was due to both increased IL-2 mRNA transcription as well as enhanced mRNA stability (8, 13, 27, 28). CTLA-4 expression is likewise enhanced in a synergistic fashion following TCR and CD28 costimulation involving a mechanism contributed by both transcriptional and posttranscriptional components (29). However, studies performed with murine T cell clones suggest that in these cells increased IL-2 mRNA production is mediated by a more complex series of events (30).

Regulation on a nuclear level does not involve modification of transcriptional activity of the IL-2 enhancer, but rather is the result of increased expression of unspliced IL-2 pre-mRNA (30). A similar mechanism of posttranscriptional regulation of IL-2 expression, at the level of pre-mRNA splicing and processing, has been reported in PBMC activated with PHA but without CD28 costimulation (5). Thus, it remains uncertain as to whether the nuclear effects of CD28 costimulation involve transcriptional activation of the IL-2 promoter in different cell systems.

The vast majority of information acquired regarding transcriptional activation of the IL-2 promoter by CD28 costimulation has been obtained from experiments using human and murine T cell lines. It should be noted that, although primary T cells must be suboptimally activated with PHA in order to achieve transfection competency, no transcription is detectable in the absence of stimulation (19). Recent studies have indicated that regulation of IL-2 gene expression in primary T cells differs from that observed in tumor T cell lines. For example, NF-AT sites are much less important in primary T cells than in tumor T cell lines, while the proximal AP-1 and NF-κB sites are of critical importance (23). In addition, expression of an IL-2 promoter construct mutated at the CD28RE site and transfected into primary T cells resulted in reduced expression following CD28 costimulation; however, mutation of this site did not affect expression following CD2 activation. Moreover, the appearance of components of cis-regulatory factors binding to the CD28RE, particularly c-rel, has been reported to be closely linked with CD28 costimulation in tumor T cell lines, which has not been the case in primary T cells (31). In PBMC following PMA stimulation alone, c-rel binding to the CD28RE was detected in the absence of IL-2 secretion. While costimulation by CD28 did result in enhanced c-rel binding, the presence of CD28 cis-binding factors in cells not actively expressing IL-2 indicate that binding to the CD28RE is not exclusively a response to CD28 costimulation (31). A similar finding of CD28-independent transcriptional activation was reported following CD3 plus PMA costimulation and reporter gene transactivation of a CD28RE promoter-reporter construct (32).

The CD28RE has been reported to bind κB-like proteins, and CD28 costimulation results in the rapid down-regulation of IκBα and concordant translocation of c-rel to the nucleus in PBMC and T cell lines (33). These reports conflict with our observations of LPMC costimulated with CD28. While CD2 activation modestly increases expression of factors binding to the CD28RE, CD28 costimulation did not further enhance this effect. Likewise, a rapid decrease in the level of IκBα was detected following CD2 activation, however, no further or sustained decrease was detected following CD28 costimulation.

Recent evidence suggests that the CD28RE does not function as a discrete element but rather is part of a composite RE/AP site,
observed in PBMC. CD28 costimulation of LPMC increased IL-2 expression in LPMC and LP-like T cells differs from that of transcriptional mechanisms other than the AUUUA motifs regulate IL-2 mRNA stability. Nonetheless, while CD28 costimulation in LPMC, albeit unlikely considering that in PBMC, transcription of the IL-2 promoter requires both the CD28RE and the adjacent AP-1 regions (35). These findings suggest that the CD28RE might be functionally redundant and thereby mediate more than one signaling pathway. Our data suggest that, at least in regard to the composite RE/AP, peripheral T cells behave in a manner similar to that of T cell lines while transactivation of LP T cell entails unique cis-regulatory elements. It is quite likely that the cell system as well as the mode of T cell activation may influence regulation of IL-2 gene transcription.

Interpretation of the data obtained through functional expression of transfected IL-2 promoter-reporter construct are subject to an important bias in that they are reflective of the sequences selected, while elements residing outside of the chosen promoter region would not be detected. In this study, transfection experiments were carried out utilizing a 600-bp IL-2 promoter construct that encompasses the highly conserved region defined as the critical IL-2 promoter element. However, DNase I hypersensitive sites have been identified within 3 kb upstream of the IL-2 transcriptional start site (36, 37). Additionally, the EMSA analyses were conducted utilizing the well-studied CD28RE/AP-1 composite element. Likewise, transcriptional run-on assays, although the methods of choice in analyzing fluctuations in the rate of transcription initiation are susceptible to attenuation through DNA sequences within a gene (37). It is possible that sequences other than those studied might play a role in transcriptional regulation following CD28 costimulation in LPMC, albeit unlikely considering that inhibitory elements have been reported residing upstream of the 600-bp promoter element. Studies are underway to identify other regions that might respond to CD28 costimulation and regulate transcriptional activation in LPMC.

An increase in mRNA stability in PBMC and T cell tumor cell lines following CD28 costimulation has been reported for numerous cytokines, including IL-2, IFN-γ, GM-CSF, and TNF-α (13). Little is known about posttranscriptional regulation of IL-2 expression, however, several cytokines and transiently expressed proto-oncogenes contain one or more highly conserved AUUUUA motifs in the 3’-UT region of their mRNA (12). These consensus elements are determinants for rapid degradation, probably through interaction with specific proteins that can be cross-linked to these AU-rich domains (38). Nonetheless, while CD28 costimulation results in the stabilization of IL-2 mRNA, the proto-oncogenes c-fos, and c-myc were not further stabilized, despite the fact that the mRNAs of these factors possess multiple AUUUUA sequences (13). Recent studies of regulation of IL-2 mRNA stability in T cell lines indicate that multiple mechanisms regulate IL-2 mRNA stability and suggest that both 5′ as well as 3′ sequences within the untranslated region of the IL-2 mRNA are critical for stabilization (39). Furthermore, in activated T cell lines, activation of the c-jun amino-terminal kinase is involved in IL-2 mRNA transcription and stabilization. Thus, it would appear that multiple posttranscriptional mechanisms other than the AUUUUA motifs regulate IL-2 mRNA stability.

In summary, the studies presented here show that regulation of IL-2 expression in LPMC and LP-like T cells differs from that observed in PBMC. CD28 costimulation of LPMC increased IL-2 production paralleled by increased expression of IL-2 mRNA due in part to enhanced IL-2 mRNA stability. However, in contrast to CD28-mediated transcriptional activation in PBMC, EMASA analysis revealed that CD28 coligation of LPMC did not augment binding of trans-acting factors to the CD28RE or alterations in IkBα or IkBβ levels. Although CD2-enhanced IL-2 secretion is reflected by transactivation of IL-2 promoter reporter constructs transfected into LPMC, there was no enhanced CD28-mediated promoter activation following coligation. In addition, these studies represent the first reports of transcriptional activation of the RE/AP composite element in response to CD2 stimulation. These observations suggest a biological significance for different mechanisms of IL-2 gene activation in initiation and maintenance of cytokine production in the mucosa. Furthermore, these results strengthen the use of LP-like T cells as a model for LP T cell activation and the analysis of mucosal cytokine gene regulation.

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References

10. Henkel, T., T. Machleidt, I. Alkalay, M. Kronke, Y. Ben-Neriah, and S. C. Meuer. 1990. Differential responsiveness of κB binding to the nonconsensus AP-1 sequence immediately adjacent to the consensus AP-1 sequence increased, but more importantly, the
AP-1 binding to the IL-2 promoter, resulting in messenger RNA transcription on IL-2 secretion. J. Immunol. 160:4914.


