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*J Immunol* 1998; 160:4914-4922; ;
http://www.jimmunol.org/content/160/10/4914

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Activation of the CD2 Pathway in Lamina Propria T Cells
Up-Regulates Functionally Active AP-1 Binding to the IL-2 Promoter, Resulting in Messenger RNA Transcription and IL-2 Secretion

Rivkah Gonsky,* Richard L. Deem,* Christopher C. W. Hughes,† and Stephan R. Targan²*

The aim of this study was to identify molecular mechanisms involved in transcriptional regulation of IL-2 expression following CD2 and CD3 activation in lamina propria (LP) T cells. Studies used T cells from normal, ulcerative colitis, and Crohn’s disease mucosa and freshly isolated PBMC, PBMC stimulated with IL-2 alone, and PBMC stimulated with IL-2 and cocultured with B cell lines (LP-like T cells). Electrophoretic mobility shift assays were performed with nuclear extracts from cells activated with either anti-CD2 or anti-CD3 Abs. CD2 signaling in LPMC and LP-like T cells led to a pattern of sustained up-regulation of AP-1-binding complexes, whereas CD3 activation resulted in only transient up-regulation. While the pattern of regulation of AP-1 binding observed in normal, uninfamed, or inflamed Crohn’s disease LPMC is similar, differences in intensity of AP-1 binding were observed. Activation of LP-like T cells mimics the up-regulation of AP-1 with a kinetic profile similar to that observed with freshly isolated LPMC from Crohn’s disease-inflamed tissue. The AP-1 complex formed following CD2 activation is composed of \( \text{jun/fos} \) heterodimers. The CD2-enhanced responsiveness is reflected in functional analysis experiments utilizing transfection of both multimeric-TRE or IL-2 promoter-luciferase constructs directly into normal, ulcerative colitis, or Crohn’s disease LPMC. Our data suggest that activation of LP T cells from normal, ulcerative colitis, or Crohn’s disease mucosa through the CD2 pathway leads to induction of AP-1 complexes that bind to the IL-2 promoter, and may play a pivotal role in modulating IL-2 production in the gut.


Activated T cells have been implicated in the pathogenesis of mucosal inflammatory diseases. Although the precise relationship between T cell activation and inflammation is unknown, T cell–derived cytokines are believed to play a central role in intestinal injury (1, 2). Recently, increased T cell–associated cytokine transcripts have been identified in mucosal biopsies from both ulcerative colitis (UC) (IL-4 and IL-10) and Crohn’s disease (IFN-\( \gamma \), IL-2, and TNF-\( \alpha \)), suggesting \textit{in situ} cytokine production from these cells (3). The finding that PHA activation of T cells in organ culture can cause severe epithelial damage suggests an important role of T cell–released cytokines in intestinal injury (1, 4). We have demonstrated previously that stimulation of isolated normal human lamina propria (LP) T cells induces secretion of IFN-\( \gamma \) and TNF-\( \alpha \) that kill human epithelial cell lines by induction of apoptosis (5, 6). These results are consistent with a direct role for T cell–derived cytokines in epithelial cell injury.

Review of the current work with animal models of inflammatory bowel disease (IBD) highlights the central role played by activated T cells and resultant cytokine secretion in intestinal inflammation (7). Immunologically altered rodents have been used to corroborate the findings in human IBD (8–14). Mice with targeted mutations in specific cytokine genes develop colitis and have provided further evidence for the importance of mucosal T cell regulation for the maintenance of a state of constant inflammatory control in the intestine (15–18). The TCR-\( \alpha \)-depleted colitis-developing animals have T cells as well as B cells within the intestinal mucosa. Although these mice exhibit autoantibodies as well as an immune response to food Ags, the precise pathway utilized to activate disease-causing T cells remains unknown (19).

Previous studies revealed that activation of the CD2, but not CD3, pathway is able to induce cell proliferation of normal LP T cells (20, 21). Research in our laboratory has shown that the pathways leading to T cell activation in LP T cells are different from peripheral T cells (22). LP T cells have enhanced cytokine induction, including IL-2 secretion through activation of the CD2 pathway (22, 23). Triggering of CD2 results in tyrosine phosphorylation of a unique 72-kDa substrate, an event not seen following other modes of T cell activation (22). The CD2-dominant pattern of IL-2 secretion as well as the unique tyrosine phosphorylation pattern could be mimicked in PBMC by coculturing isolated PBMC in the presence of irradiated B cells (22). The recent finding that activation via the CD2, but not CD3, pathway is required for IFN-\( \gamma \) release from mucosal T cells derived from active Crohn’s disease lesions emphasizes the importance of this pathway in disease-related T cell activation (24).

The IL-2 gene is not actively transcribed in resting T cells. Although little is known about the signaling pathways subsequent to...
CD2 ligation, the regulatory sequences required for IL-2 production have been studied extensively following phorbol ester stimulation or activation of calcineurin-mediated Ca$$^{2+}$$ signaling. A number of functionally important regions have been defined within the first 300 bp of the promoter (25). Numerous discrete transcriptional response elements have been defined, including those binding NF-AT, Oct-1 and Oct-2, NF-κB, and SP1 (26). The cooperative binding of AP-1 nuclear proteins has been demonstrated to be both necessary and sufficient for gene transactivation of a number of these discrete IL-2 enhancer elements (27). Binding of AP-1 to NF-AT sites aids in the stabilization of the NF-AT complex and is a prerequisite for transactivation. AP-1 also cooperates with NF-κB and Oct proteins, aiding in the stable binding of these proteins to their DNA cis-acting elements. Many analyses of transcriptional regulation of this cytokine have been performed using tumor T cell lines. Recent studies, however, have emphasized the necessity of studying transcriptional regulation of IL-2 in nontumor T cell lines to accurately understand cytokine gene regulation in normal peripheral T cells. Hughes and Pober have shown that the cis-regulatory elements in PB T cells differ from those observed in Jurkat T cells, with the proximal AP-1 site and the NF-κB site being most important, and the NF-AT sites being much less important (28). Furthermore, it appears that different costimulatory signals may converge on the same cis elements of the IL-2 promoter (29).

The purpose of this study was to determine whether the predominance of IL-2 secretion in LPMC and LP-like T cells induced by the CD2 vs CD3 activation pathway was reflected by unique nuclear events that regulate transactivation of the IL-2 gene. The data in this study support the hypothesis that following CD2 activation there is induction of functional AP-1 nuclear protein complexes binding to the IL-2 promoter in LP T cells from both normal, UC, or Crohn’s disease mucosa, which leads to IL-2 gene transcription, which may play a pivotal role in modulating IL-2 production in the gut.

Materials and Methods
Target cells and culture media
The murine T cell line, CTLL-2, was obtained from American Type Culture Collection (ATCC, Rockville, MD) 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 ATCC 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 (Biogen, Cambridge, MA). Anti-CD3 (IgGl) mAb was affinity purified from culture supernatant of OKT3 hybridoma (ATCC).

Purification of LPMC and PBMC
Intestinal specimens were obtained from patients undergoing surgical resection of the colon (with colon carcinoma or treatment-resistant Crohn’s disease or UC) at Cedars-Sinai Medical Center (Los Angeles, CA). Approval for the use of human subjects was obtained from Institutional Review Board at Cedars-Sinai Medical Center. In this study, all tissue specimens were taken from an uninvolved area of resected colon from patients with colonic carcinoma (normal), involved areas of patients with UC, as well as from uninvolved and involved areas of patients with Crohn’s disease. LPMC were isolated using a technique modified from that described previously (30). Briefly, the intestinal specimen was washed with HBSS, and the mucosa were dissected away from the underlying layers. The mucosal layer was incubated in a shaking water bath (100 rpm) in calcium- and magnesium-deficient HBSS, containing 1 mM EDTA, 50 μg/ml gentamicin, 100 μg/ml penicillin, 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 deoxyribonuclease 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 Ficoll-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 Ficoll-Hypaque gradients.

Induction of LP-like T cells
Mononuclear cells from Ficoll-Hypaque gradients were cultured in RPMI 1640 with 10% FCS with a 1:5 ratio of Daudi:lymphocytes plus 10 U/ml rIL-2 for 5 days (22). 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, LPMC, LP-like T cells, and PBMC were stimulated with 0.1 μg anti-CD2 Abs (both CB6 and GD10 clones)/106 cells at 37°C for the times indicated for each experiment. For stimulation through the CD3 receptor, cross-linking of the CD3 receptor was conducted by incubating cells for 10 min with 0.1 μg OKT3/106 cells at 4°C followed by the addition of 1 μg F(ab)2, goat anti-mouse IgG Fc (Jackson ImmunoResearch, West Grove, PA)/106 cells for an additional 10 min at 4°C. 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 instigate activation. Further cross-linking of anti-CD2 Abs with anti-mouse IgG had no effect upon activation (22). Following this incubation, cells were warmed to 37°C for the required stimulation period.

IL-2 bioassay
IL-2 activity was determined using a CTLL-2 bioassay (31). rIL-2 standards and dilutions of samples were added to 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) containing 5 × 103 CTLL-2 cells 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 (Tomec, Orange, CT) microplate harvester and counted using a Microbeta 1450 liquid scintillation counter (Wallac, Gaithersburg, MD).

Northern blot analysis
Total cellular RNA was extracted using the Qiagen RNeasy kit (Chatsworth, CA). RNA was separated electrophoretically on a denaturing 1% agarose gel containing 7% formaldehyde. Gels were transferred to nylon membrane (Amersham, Arlington Heights, IL) and hybridized to 32P-labeled DNA probe. Isolated cDNA insert was labeled by random priming and used at 105 cpm/ml of hybridization buffer. Blots were prehybridized (50% formamide, 0.75 M NaCl, 75 mM sodium citrate, 1× Denhardt solution, 25 mM sodium phosphate, pH 6.5, and 100 μg/ml sheared salmon sperm DNA) at 42°C for 2 h and hybridized overnight in prehybridization solution containing labeled probe and 10% dextran sulfate. Uniformity of RNA loading was determined by hybridization to rRNA.

Preparation of nuclear protein extracts
Nuclear protein extractions were conducted with 5 to 10 × 106 LPMC 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, 3 mM MgCl2, 0.5 mM dTT, 2 μM leupeptin, 1 μM/ml aprotinin, 1 mM PMSF, and 0.1 mM EGTA), and 0.1 ml of 5% Nonidet P-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 to 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 μM/ml aprotinin, and 1 mM PMSF). Samples were incubated on ice for 30 to 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 KC1, 0.2 mM EDTA, 20% v/v glycerol, 0.5 mM...
DNA constructs

The human IL-2 cDNA clone was obtained from ATCC. 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 (28). The plasmid TRE2 luciferase used to determine AP-1-dependent transactivation was generated by subcloning three copies of the collagenase AP-1 binding sites into a luciferase reporter plasmid (a gift from M. Karin, University of California, San Diego) (32).

Transfection

Freshly isolated LPMC were primed for transfection competence by culturing for 16 to 20 h in RPMI 1640 medium containing 10% FCS, 50 mM 2-ME, and 1 mg/ml PHA-L (Sigma), as described for PBMC (28). Cells were then washed and resuspended in 250 µl fresh medium at 2 x 10^7 cells/ml and electroporated in the presence of 50 µg of reporter construct (250 V, 960 microfarads, 45–50 ms) using 4-mm (gap width) cuvettes in a strength buffer (22.3 mM Tris, pH 7.4, 22.3 mM borate, and 0.5 mM EDTA, pH 8). After 2 h, the gel was dried under vacuum and exposed to x-ray film. Gel supershift assays were conducted as described above with the addition of Abs (Santa Cruz Biotechnology, Santa Cruz, CA) to specific transcription factors included in the binding mixture before the addition of labeled oligonucleotide. The oligonucleotides used were the IL-2-proximal AP-1 site and two mutants: IL-2-proximal AP-1 wild-type, (5'-TCCAAA GAGTCATCAGAAGA-3'); IL-2-proximal AP-1 mutant, 1 (5'-TCCAAA GAGGTCAGAAGA-3'); IL-2-proximal AP-1 mutant, 2 (5'-TCCAAA GAGCAGAAGA-3').

The other oligonucleotides used were the AP-1 consensus and NF-kB consensus oligonucleotide site (Promega, Madison, WI).

Results

Enhanced activation of the CD2 pathway in LPMC and LP-like T cell IL-2 secretion is reflected in mRNA levels

We have reported previously on a model developed using PB T cells, known as LP-like T cells, which reproduce the CD2-driven cytokine secretion phenotype seen in LP T cells (22). LP T cells and LP-like T cells are significantly more responsive to activation via the CD2 pathway than IL-2-activated PB T cells, as exhibited by the secretion of IL-2, IFN-γ, TNF-α, and IL-4 (22). This enhanced T cell responsiveness was associated with the selective phosphorylation of a 72-kDa protein substrate. We first examined functional regulation of IL-2 mRNA expression following activation of LPMC and LP-like T cells via the CD2 pathway to investigate the upstream events involved in cytokine regulation. Northern blot analysis of LPMC from inflamed mucosa and LP-like T cells were stimulated for various periods of time either with 1 µg anti-CD2/10^6 cells or by cross-linking the CD3 receptor with 1 µg anti-CD3/10^6 cells, followed by the addition of 1 µg F(ab')2 goat anti-mouse IgG Fc. Five micrograms of mRNA were isolated at indicated time periods and analyzed on Northern blots for expression of IL-2 transcripts. To control for uneven loading, the relative amounts of RNA in each lane were determined by hybridization to 28S rRNA. A, IL-2 mRNA expression from LPMC. B, Densitometric readings of Northern blot in A. Densitometric units were calculated from 28S rRNA. A, IL-2 mRNA expression from LPMC. B, Densitometric readings of Northern blot in A. Densitometric units were calculated from 28S rRNA and used to correct the readings for IL-2 mRNA. C, IL-2 mRNA expression from LP-like T cells. D, Corrected densitometric readings of Northern blot in C. Symbols used: (△), cells stimulated with anti-CD3; (○), cells stimulated with anti-CD2. Representative results of four experiments with similar results are shown.
following activation via CD2 parallel IL-2 protein secretion (data not shown). CD3 ligation, however, does not induce detectable levels of IL-2 mRNA (Fig. 1, A and B). Figure 1, C and D, demonstrates that IL-2 mRNA levels in the LP-like model are similar to that seen in LPMC from inflamed UC mucosa. Activation of LP-like T cells via the CD2 pathway results in a steady up-regulation of IL-2 mRNA detectable within 2 h, which increases steadily over 6 h, but declines by 10 h. By contrast, the levels of IL-2 mRNA in LP-like T cells following CD3 activation were barely detectable. Thus, the predominance of the CD2 activation pathway leading to cytokine secretion is reflected by expression of specific cytokine mRNA in both LPMC and LP-like T cells.

**CD2 ligation leads to a sustained increase in AP-1-binding activity in LP-like T cells**

The finding of increased IL-2 mRNA levels following activation of LPMC and LP-like T cells via the CD2 pathway suggested that modulation may occur at the level of transactivating factors that regulate IL-2 transcription. Previous studies have identified two AP-1 binding sites within the IL-2 promoter region and have shown that an increase in the level of AP-1 binding correlated with transcriptional activation of the IL-2 gene in human T cell lines (27, 33). To begin to evaluate the nuclear events that mediate IL-2 expression following CD2 pathway activation, electromobility shift assays (EMSA) were performed comparing nuclear protein extracts from LP-like T cells with those from freshly isolated PBMC, and PBMC stimulated for 5 days with IL-2 alone. Cultures were activated with Abs to CD2 or by cross-linking with CD3 mAbs. We analyzed nuclear protein binding to a consensus oligonucleotide for AP-1. Figure 2 demonstrates the marked increase in the amount and duration of AP-1 nuclear binding following CD2 ligation in LP-like T cells (Fig. 2A) compared with control CD2-activated T cells (Fig. 2B). Moreover, although CD3 ligation resulted in a transient up-regulation of AP-1 complexes, the changes in the magnitude and duration of AP-1-binding complexes were far less striking when comparing LP-like T cells to control T cells. By contrast, there was no detectable binding of AP-1 observed following either CD2 or CD3 ligation in freshly isolated PBMC T cells (data not shown). Addition of unlabeled AP-1 oligonucleotides (competitor) attests to the specificity of AP-1 binding (far right lane, Fig. 2A). T cell activation by coligation of both CD2 and CD3 did not lead to synergistic up-regulation of AP-1 complexes (data not shown). These results indicate that following CD2 pathway activation, there is a rapid and sustained up-regulation of nuclear protein complexes binding to an AP-1 regulatory element that precedes transactivation. This pattern of up-regulation observed with the LP-like T cell model is distinct from that observed in freshly isolated PBMC or PBMC cultures with IL-2.

**Persistent up-regulation of AP-1-binding activity following CD2 activation of LPMC isolated from normal mucosa**

Much evidence suggests that LP-like T cells reproduce a CD2-driven cytokine response profile and is a paradigm for the activated state of LP T cells. To determine whether nuclear events reflected this similarity, we examined the pattern of induction of transactivating factors from LPMC directly. LPMC were isolated from normal mucosa and then activated with Abs to CD2 or CD3, and nuclear proteins were extracted at times of peak induction previously determined for LP-like T cells. Figure 3 shows EMSA analysis using a consensus oligonucleotide for AP-1. Two distinct patterns of AP-1 binding were observed in the untreated nuclear extracts from normal LPMC. One pattern is characterized by a low level of AP-1 binding in untreated cells, followed by a persistent up-regulation of AP-1 binding in CD2-activated cells (Fig. 3A), CD3 pathway activation resulted in a more transient response (compare the 120-min time point following CD2 and CD3 activation). Another pattern features relatively high levels of AP-1 binding in untreated nuclear extracts with sustained AP-1 binding following CD2 activation, but a marked down-regulation following CD3 activation (Fig. 3B). Thus, although the levels of AP-1 binding in normal untreated cell extracts differed, there was a parallel persistent up-regulation of AP-1 binding following CD2 activation, and a more transient AP-1 binding following CD3 activation irrespective of the initial level of AP-1 binding detected in the unstimulated cell extracts.

**CD2, but not CD3, ligation up-regulates AP-1-binding activity in LPMC isolated from inflamed mucosa of Crohn’s disease patients**

Isolated LPMC are composed of a variety of cell types with various numbers of activated T cells. Since the pattern of AP-1 regulation in normal LPMC was similar to that observed in LP-like T cells, however, much less pronounced, we hypothesized that the number of activated T cells or possibly the state of T cell activation...
in the mucosa may determine the ability to measure the pattern of AP-1 modulation using standard nuclear protein extraction and EMSA techniques. Considering that there is evidence for increased numbers of activated T cells in the mucosa of Crohn’s disease patients (34), the pattern of AP-1 regulation was studied further in LPMC isolated from inflamed and adjacent uninflamed mucosa of Crohn’s disease patients. LPMC were activated with Abs to CD2 and CD3, then nuclear proteins were extracted at times of peak induction seen in LP-like T cells. Nuclear extracts from CD2- or CD3-activated LPMC from uninflamed compared with inflamed mucosa from the same patient, possibly reflecting a difference in the number of activated T cells present in inflamed mucosa. However, in contrast to nuclear extracts from uninflamed mucosa, in inflamed mucosa there was a high level of AP-1 protein detected that was up-regulated following CD2 ligation and markedly down-regulated following CD3 ligation. Thus, LPMC isolated from inflamed mucosa contained T cells that, when activated through the CD2 pathway, result in the up-regulation of AP-1 nuclear binding complexes, whereas no up-regulation occurred when cells were activated through the CD3 pathway. These results demonstrate that while there is a consistent pattern of regulation of AP-1 binding following T cell activation seen in all LPMC, whether from normal or Crohn’s disease mucosa, the distinct up-regulation observed in the LP-like T cell model following CD2 activation can be measured more readily in activated T cells from inflamed mucosa of Crohn’s disease patients.

**FIGURE 3.** EMSA analysis of 5 μg nuclear protein binding to consensus AP-1 sequences following stimulation of normal LPMC (freshly isolated from the colon of carcinoma patients) either with 1 μg anti-CD2/10⁶ cells or by cross-linking the CD3 receptor with 1 μg anti-CD3/10⁶ cells, followed by the addition of 1 μg F(ab’)_2 goat anti-mouse IgG Fc. Representative results of six experiments with similar results are shown.

**FIGURE 4.** A, EMSA analysis, and B, densitometric readings of 5 μg nuclear protein binding to consensus AP-1 sequences following stimulation of LPMC (freshly isolated from small bowel tissue of a Crohn’s disease patient) either with 1 μg anti-CD2/10⁶ cells or by cross-linking the CD3 receptor with 1 μg anti-CD3/10⁶ cells, followed by the addition of 1 μg F(ab’)_2 goat anti-mouse IgG Fc. Symbols for A: C, unstimulated control cells; Comp, unlabeled competitor probe added to cells from inflamed tissue stimulated with anti-CD2. Symbols for B: □, unstimulated LPMC; ■, LPMC stimulated with anti-CD2 Ab; ▼, LPMC stimulated with anti-CD3 Ab and cross-linked with goat anti-mouse IgG Fc. Representative results of four experiments with similar results are shown.

**CD2 ligation of LPMC from inflamed Crohn’s disease mucosa and LP-like T cells leads to a sustained increase in nuclear factors able to bind to the proximal AP-1 site of the IL-2 promoter**

The experiments reported above were performed using a consensus AP-1 oligonucleotide. Gel-shift experiments with oligonucleotides corresponding to the proximal AP-1 site of the human IL-2 promoter were performed to determine whether up-regulation AP-1 binding could also be demonstrated in an IL-2-specific AP-1 cis element. Since alterations in AP-1 binding detected by EMSA analysis were more apparent using activated T cells from Crohn’s disease mucosa, we performed kinetic analysis in both LP-like T cells and LPMC from Crohn’s disease mucosa to compare the pattern of expression of proteins binding with the proximal AP-1 region at time points following CD2 activation. Nuclear extracts were isolated from LP-like T cells at the indicated periods of time
following CD2 or CD3 activation, as shown in Figure 5A. Following CD2 activation, there is a rapid increase in proteins binding to the proximal AP-1 region, which is sustained up to 120 min. Kinetic studies conducted with nuclear extracts from LPMC from an inflamed area of Crohn’s disease mucosa demonstrate a striking similarity to that seen for LP-like T cells (Fig. 5B). Following CD2 activation, there is a rapid and sustained up-regulation of AP-1 protein complexes, while CD3 activation leads to a rapid and transient response. These results suggest that the LP-like model system corroborates the findings in LPMC, i.e., the activation pattern and the kinetics of induction of AP-1 complexes binding to the proximal IL-2 AP-1 cis-acting element are the same as that seen in LP T cells from Crohn’s disease mucosa. In both systems, activation via CD2 results in a sustained up-regulation of AP-1 transactivating factors, while CD3 activation leads to a rapid but transient response.

Specificity of the AP-1 nuclear binding complex

The specificity of AP-1 binding was confirmed through competition experiments utilizing excess oligonucleotides comprising both the wild-type as well as two different mutant oligonucleotides containing base pair substitutions within the proximal AP-1 binding site of the IL-2 promoter region. As seen in Figure 6, nuclear factors bound to the wild-type AP-1 probe and were inhibited by excess wild-type probe, but not by mutant or a nonspecific probe.

Characterization of the AP-1-binding proteins in LPMC and LP-like T cells

To characterize the composition of the AP-1 complex that is present in the nucleus of LPMC and LP-like T cells activated through the CD2 pathway, we performed supershift assays using nuclear extracts from 60 min (peak induction time seen Fig. 2) following CD2 ligation. Nuclear extracts were incubated with specific Abs to all members of the fos or Jun family of proteins before the addition of AP-1 oligonucleotide and EMSA were performed. The supershift data in Figure 7, A and B, show that the AP-1 complexes from both LPMC and LP-like T cells contain both Jun as well as fos proteins, while nonspecific Ab (NF-κB) was unable to elicit a supershift.

Transfection of LPMC suggests the presence of a functional CD2 response element in the IL-2 promoter

Functional analysis utilizing transfection of promoter-reporter constructs into LPMC isolated from normal, UC, and Crohn’s disease mucosa was conducted to determine whether the preferential induction of AP-1 complexes following CD2 ligation on LP T cells resulted in activation of the transfected IL-2 promoter construct in LPMC. As seen in Figure 8, stimulation through CD2 does indeed up-regulate reporter gene transcripts in LPMC from normal, UC, and Crohn’s disease mucosa, confirming previous reports that CD2 signaling targets the IL-2 promoter. However, in contrast to previous studies, CD3 ligation failed to induce reporter gene expression, even though PMA/Ca ionophore was capable of up-regulation of IL-2 promoter activity. Moreover, CD2 ligation resulted in the selected transactivation of a multimeric AP-1-binding TRE2 reporter construct similar to that seen following PMA/Ca ionophore activation in LPMC from normal, UC, and Crohn’s disease mucosa (Fig. 9). CD3 ligation, however, only marginally activated the promoter. These results indicate that an increase in the IL-2 promoter activity following CD2 pathway activation of LPMC from normal as well as UC and Crohn’s disease mucosa is associated with an enhancement of functionally active AP-1 nuclear binding complexes.

Discussion

In this study, we have examined molecular events and mechanisms involved in the regulation of IL-2 production in LPMC from normal, UC, or Crohn’s disease mucosa, and have demonstrated some of the unique properties of LP T cells. IL-2 gene expression in
LPMC is preceded by selected induction of AP-1 nuclear binding factors that results in transcriptional transactivation of the IL-2 gene and mRNA synthesis. Transfection experiments of LPMC from normal, UC, and Crohn’s disease mucosa reveal a marked response of TRE or IL-2 promoter constructs following CD2 activation, but fail to generate a similar response following CD3 ligation. EMSA analysis supports the conclusion that CD2 signaling in LPMC from normal and Crohn’s disease mucosa is transmitted in part by induction of AP-1 transactivating factors comprised of heterodimers of jun/fos. Up-regulation of AP-1 nuclear protein complexes following CD2 activation is most notably reflected in extracts from the inflamed mucosa of Crohn’s disease patients, while the adjacent uninfamed mucosa fail to reveal up-regulation of AP-1 under similar activation conditions. These findings are in direct contrast to those events that follow ligation of the CD3 receptor. CD3 activation of LP T cells results in only poor cytokine expression. The downstream events examined in this study reveal that following CD3 ligation, AP-1 up-regulation is rapid and transient and fails to generate transcription of IL-2 mRNA. This is the first example of up-regulation of AP-1 following activation of the CD2 pathway in gut T cells. Transfection experiments utilizing a TRE reporter construct as well as an IL-2 reporter construct, suggest that up-regulation of AP-1 following CD2 activation has functional significance enabling promoter activation, resulting in IL-2 cytokine expression. In addition, these studies represent the first reports of transfection studies conducted directly in LPMC.

Findings from studies using activated LPMC were compared with those using the LP-like T cell model. Previous studies have shown that the LP-like T cell model is validated by its functional
equivalency to LP T cells with regard to 1) CD2 pathway dominance with relatively low surface expression of CD2 (22) and 2) identical phosphorylation patterns upon CD2 pathway stimulation (22). The data presented in this study further support the LP-like T cell activation model and reveal that it is most representative of the activated state of T cells present in inflamed Crohn’s disease mucosa, as evidenced by CD2-activated up-regulation of AP-1 transactivating factors with similar kinetic profiles. The LP-like T cell model, as it has very similar activation properties to those expressed by LPMC from inflamed Crohn’s disease mucosa, allows investigations of the mechanism of cytokine gene regulation to be performed initially on a more easily manipulated experimental system.

It is interesting to note that while the LP-like T cell model is most reflective of LPMC from inflamed Crohn’s disease mucosa, transfection experiments with the multimeric TRE2 or IL-2 promoter-reporter constructs support the conclusion that regardless of the source of the LPMC (i.e., inflamed or normal mucosa), CD2 activation is followed by sustained up-regulation of functional AP-1 binding.

These results seem at first glance to be contradictory to the results from EMSA analysis. However, EMSA analysis is based on nuclear protein extracted from the total pool of mucosal LPMC. The state of activation and, more importantly, the number of activated cells in normal LPMC are lower than that in extracts from Crohn’s disease mucosa samples (34). Indeed, it is conceivable that the variability in AP-1 binding in extracts from unstimulated normal mucosa may be a result of the number of activated T cells present. Thus, the percentage of the total nuclear extract derived from activated T cells in normal LPMC would be much lower than from inflamed Crohn’s disease mucosa. Since equal concentrations of total nuclear extract are loaded onto gels in EMSA assays, the small percentage contributed by activated T cells in nuclear extracts from normal LPMC is probably too low for accurate detection in this assay. In contrast, the transfection of TRE or IL-2 promoter-reporter constructs directly measures AP-1 or IL-2 promoter-directed transactivation following various stimuli. While the number of activated T cells might well be less in LPMC from normal mucosal, the number of cells is sufficient and the technique is sensitive enough to accurately measure the response of these activated T cells.

The activation state of LP T cells is characterized by a relatively anergic, unresponsive TCR pathway with increased responsiveness to CD2. It is interesting to note the recent studies identifying an AP-1 DNA cis-binding element within the IL-2 promoter region as well as the identification of a defect in AP-1 transactivation suggest that regulation of AP-1 is directly involved in the establishment of anergy (35). These features make the CD2 pathway within the mucosal environment particularly interesting as a candidate for a surface molecule that participates in the maintenance of a population of responsive, activated T cells in the mucosa.

It has been difficult to define a unique role for CD2 in T cell biology. Disruption of the murine CD2 gene results in a healthy mouse with an apparently normal immune system (36). Many studies have shown, however, that activation of the CD2 pathway can profoundly regulate T cell responses. The question that remains is, to what extent is the response physiologic. One implication of the lack of phenotype in the CD2 knockout mouse is that CD2 may not have a unique function, or that other molecules can compensate for its loss (36). Alternatively, absence of CD2 may produce a phenotype that would elude identification unless its loss is coupled with additional perturbation of the immune system (i.e., different genetic backgrounds or induction of inflammation). Recent work by Boussuotis et al. has produced important information related to mucosal T cell biology (37). Studies have demonstrated that CD2 is unique in its ability to participate in the reversal of T cell anergy (37). CD2 is unique in that other ligands, such as CD28 and CTLA-4, are critical for prevention of anergy/tolerance; however, coligation of these ligands cannot reverse the state of T cell anergy.

The induction of AP-1 and its functional consequences are important in activation and cytokine production in LP T cells. The AP-1 protein family is known to play a central role in the transcriptional regulation of IL-2 expression (27). A number of AP-1 binding sites have been identified within the IL-2 promoter/enhancer region. The DNA-binding activity of AP-1, while necessary for transactivation of the IL-2 gene, is not sufficient to insure the successful transcription of the IL-2 mRNA. Following T cell activation, there is the establishment of a complex of multiple transacting factors binding to numerous cis-acting elements critical for gene expression (29). It is the assembly or disassembly of this nuclear protein complex that is believed to be regulated by the activation pathway of the T cell. Our transfection studies establish a role for the CD2 activation pathway in gut T cells, leading to up-regulation of AP-1, a critical transacting factor regulating IL-2 cytokine expression. The composition as well as the phosphorylated state of the Jun/Fos heterodimers will determine whether AP-1 will form an active complex enabling transcriptional activation. Further studies are ongoing to identify the composition changes of the AP-1 complex following CD2 activation in gut T cells, their phosphorylated state, and the role of other transacting factors contributing to IL-2 gene expression following CD2 activation.

Recent reports have emphasized the role DNA-binding factors play in regulation of T cell function in disease. A recent study looking at diminished T cell response in patients with the autoimmune disease Sjogren’s syndrome has identified a functional defect in the Oct-1 protein. This defect was correlated with the inability of T cells to proliferate following activation in patient with the disease (38). Additionally, in the mouse experimental colitis model, administration of antisense oligonucleotides to the transacting factor NF-κB has been reported to lower the level of cytokine expression, thereby tempering intestinal inflammation (39). Activated mucosal T cells are implicated in the pathogenesis of Crohn’s disease. There is an increased number of activated Th1 cells in the mucosa of Crohn’s disease patients. The mucosal inflammation in Crohn’s disease represents a perturbation of what in the intestine is a perpetual state of appropriately controlled inflammatory responses to environmental factors (40, 41). Crohn’s disease patients treated with anti-TNF-α have experienced dramatic and prolonged symptom improvement that parallels marked down-regulation of T cell activation and Th1 (TNF-α, IFN-γ) cytokine production, and the same is true for Crohn’s disease patients treated with IL-10 (42, 43). This down-regulation of cytokine expression in the mucosa is selective and specific to activation of the CD2 pathway (44). Our findings emphasize the importance in understanding downstream events regulating the CD2 activation pathway in LP T cell cytokine production. These observations support the hypothesis that activated T cells and the CD2 pathway are likely to be central to the pathogenesis of IBD. Recent studies have shown that there is diminished proliferative as well as cytokine expression in T cells isolated from active mucosal inflammation in response to activation of the CD3/TCR pathway, while the CD2/CD28 pathway remains responsive (24). In our study, this observation was further characterized in the response of LP T cells isolated from inflamed tissue, which show a distinct disparity in their ability to respond to activation via the CD2 compared with the CD3 pathway. Therefore, cytokine induction through the CD2 pathway in inflamed tissue is preceded by induction of transacting factors comprising the Jun/Fos AP-1 complex, which is capable of
transactivation of IL-2 gene expression. These same cells do not respond to TCR/CD3 stimulation in this mucosal compartment. One possible explanation for this may be that the active lesion may represent a heightened activation state or a change in the number of activated T cells as measured by surface markers. Our transfection data suggest that activated T cells present in the normal mucosal environment exhibit a similar disparity in their response to activation via the CD2 compared with the CD3 pathway.

In summary, the studies presented in this work show that CD2 activation of both normal as well as inflamed LPMC results in the up-regulation of a critical transacting factor, AP-1, and activation of IL-2 tran...