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Regulation of IL-15-Stimulated TNF-α Production by Rolipram[1]

Chitta S. Kasyapa,*† Carrie L. Stentz,* Michael P. Davey,*† and Daniel W. Carr[2]*†

Agents that increase intracellular cAMP have been shown to reduce joint inflammation in experimental arthritis, presumably by lowering the release of proinflammatory cytokines, such as TNF-α. Recent studies suggest that, in joints of patients with rheumatoid arthritis, TNF-α release from macrophages is triggered by their interaction with IL-15-stimulated T lymphocytes. In this report, we analyze the effect of rolipram, a cAMP-specific phosphodiesterase inhibitor, on TNF-α production in this experimental system. Cocultures of U937 cells with IL-15-stimulated T cells, but not control T cells, resulted in increased release of TNF-α. Pretreatment of T cells with rolipram or cAMP analogues inhibited the IL-15-stimulated increases in proliferation, expression of cell surface molecules CD69, ICAM-1, and LFA-1, and release of TNF-α from macrophages. Addition of PMA to T cells dramatically increased the expression of cell surface molecules, but had little or no effect on TNF-α release from either T cells or from cocultures, suggesting that other surface molecules must also be involved in T cell/macrophage contact-mediated production of TNF-α. Addition of PMA synergistically increased the proliferation of IL-15-stimulated T cells and the secretion of TNF-α from IL-15-stimulated T cell/macrophage cocultures. Rolipram and 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) blocked these increases. Measurement of protein kinase A (PKA) activity and the use of inhibitory cAMP analogues (RpCPT-cAMP) confirmed that rolipram worked by stimulating PKA. These data suggest that PKA-activating agents, such as rolipram, can block secretion of TNF-α from macrophages by inhibiting T cell activation and expression of surface molecules. ***The Journal of Immunology,*** **1999, 163: 2836–2843.**

Tumor necrosis factor-α mediates several phases of the pathogenesis of rheumatoid arthritis (RA),[3] including endothelial activation, angiogenesis, synovial fibroblast proliferation, and the destruction of cartilage and bone (1–3). In addition, TNF-α induces the production of other proinflammatory cytokines, such as IL-1, IL-6, IL-8, and GM-CSF (4). It has recently been shown that blocking TNF-α with a "biologic" therapy (soluble receptors that bind TNF-α) is effective treatment for RA (5, 6). Even though monocytes/macrophages are believed to be the main cell type responsible for the production of TNF-α in RA (7, 8), the association of disease severity with human leukocyte Ag HLA-DR (9), the fact that some animal arthritis models are T cell-dependent (10), and clinical success of therapies directed against T cells in RA support the hypothesis that T cells are equally critical for the onset and perpetuation of RA. In vitro cell contact between activated T cells and macrophages induces the production of many cytokines, including TNF-α, IL-1β, IL-10, and IL-12 (11, 12). An in vivo observation of close contact between T cells and macrophages in the RA synovial lining supports this observation (13).

Recently, IL-15 was found to be present at high concentrations in RA synovium (14). A product of synovial macrophages and fibroblasts, IL-15 acts as a chemoattractant (15) and growth factor for T lymphocytes, resembling IL-2 in many of its biological functions (16). Other major effects of IL-15 on T cells include: 1) inducing adhesion molecule redistribution (17), 2) promoting migration of T cells into the RA synovium (14, 18), and 3) stimulation of synovial T cells to express various activation and adhesion markers, such as CD69, ICAM-1, and LFA-1 (17, 19).

It has been proposed that a cognate interaction between IL-15-stimulated T cells and synovial macrophages results in the increased production of TNF-α (20). McInnes et al. (20) demonstrated that expression of surface molecules, such as CD69, ICAM-1, and LFA-1, on IL-15-stimulated T cells plays a critical role in T cell-mediated production of TNF-α by macrophages. Interestingly, PMA, a protein kinase C (PKC) activator, also triggers the expression of all these molecules (21). Therefore, one goal of these studies was to determine whether PMA-treated T cells were capable of inducing TNF-α production from macrophages. PMA has also been shown to induce the expression of IL-15R α-chain (22), suggesting it might augment the activation of T cells by IL-15. A recent report shows that collagen-induced arthritis can be prevented by the administration of soluble IL-15R α-chain, specific for only IL-15 binding (23). This observation further supports the hypothesis that T cells play an important role in the onset and perpetuation of pathogenic events in RA and suggests that pharmacological agents that block the IL-15-mediated stimulation of T cells may be of clinical use in treating RA.

Rolipram, a cAMP-specific phosphodiesterase inhibitor (24), has been shown to ameliorate collagen-induced arthritis in animal models and is under consideration as a possible therapeutic agent for RA (25). Several lines of evidence suggest that rolipram suppresses arthritis in animal models by reducing the levels of TNF-α in the joints (26). Serum levels of TNF-α are shown to be downregulated by an i.p. injection of rolipram in animal models (25,

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[3] Abbreviations used in this paper: RA, rheumatoid arthritis; PKC, protein kinase C; PKA, protein kinase A; CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; 7-AAD, 7-amino actinomycin D.

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27). Also, there are reports that in vitro LPS-induced production of TNF-α by macrophages is inhibited by rolipram in a cAMP-dependent manner (28, 29). However, the mechanism of action and target cell population of rolipram in the synovium has not been elucidated. Rolipram is a specific inhibitor of type IV phosphodiesterase, an enzyme that catalyzes the breakdown of cAMP. Therefore, addition of rolipram to cells causes an increase in intracellular cAMP, which presumably enhances the activity of cAMP-dependent protein kinase A (PKA). Compared with pentoxifylline, another phosphodiesterase inhibitor, rolipram is 500-fold more potent at suppressing TNF-α synthesis (30) and has been suggested to be a better alternative to pentoxifylline (1). Moreover, rolipram synergizes with prostanoids both in elevating cAMP concentrations and in suppressing TNF-α synthesis (31, 32).

In the present report, we analyze the role of IL-15- and PMA-mediated activation of T cells in the production of TNF-α by T cell/macrophage cocultures. We also test the hypothesis that rolipram inhibits the production of TNF-α by inhibiting the IL-15 activation of T lymphocytes, resulting in the inability of T cells to interact with macrophages. Results demonstrate that PMA-induced expression of cell surface molecules on T cells are not sufficient for production of TNF-α, but PMA does augment the effect of IL-15 on TNF-α production and T cell proliferation. Results also demonstrate that rolipram inhibits activation of T lymphocytes and the expression of cell surface molecules resulting in the failure of T cells to induce TNF-α production by macrophages.

Materials and Methods

Materials

IL-15, TNF-α, and a matched pair of Abs for ELISA were purchased from R&D Systems (Minneapolis, MN). FITC-conjugated Abs for CD69, LFA-1, and ICAM-1 are from Ancell (Bayport, MN), and their isotype controls, as well as anti-human CD3, CD14, and CD20, were obtained from Sigma (St. Louis, MO). RPMI 1640, rolipram, kemptide, and PMA were also from Sigma. H-89 and 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) were obtained from Calbiochem (San Diego, CA) and RpCPT-cAMPS from BIOLOG LifeSciences Institute (Hayward, CA). 7-amino actinomycin D (7-AAD) is a product of Molecular Probes (Eugene, OR). The 96-well plates are Falcon (Lincoln Park, NJ) products, and high-binding ELISA plates are from Costar (Cambridge, MA). [^{3}H]thymidine and [^{32}P]ATP were procured from NEN Life Sciences Products (Boston, MA).

Preparation of cells

Mononuclear cells were isolated from heparinized venous blood of healthy donors by Ficoll-paque density gradient centrifugation. Cell cultures were maintained in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate (complete medium). Monocyte depletion was performed by a 1-h incubation of cells (2×10^6 cells/ml) on plastic petri dishes at 37°C. The resultant nonadherent cells were subjected to another 16-h incubation on petri dishes at 37°C for further depletion of adherent cells. The "T cell-enriched population" thus obtained was analyzed for purity by flow cytometry, and was observed to contain >90% CD3-positive cells, <3% CD19-positive cells, and 1% CD14-positive cells. Throughout this report, the T cell-enriched population will be referred to as "T cells." To determine whether any of the agents being added to the T cells were toxic, the viability of cells following various treatments was monitored by both visual inspection of trypan blue uptake and flow cytometry analysis of 7-AAD uptake. No adverse effect of any agent (including 100 μM rolipram) was observed during the incubation period (data not shown).

T lymphocyte activation and proliferation assay

T cells were cultured at 2×10^5 cells/200 μl in 96-well tissue culture plates with or without various modulators. IL-15 (100 ng/ml) and PMA (1 nM) were used as T cell stimulants. CPT-cAMP and rolipram were used at 100-μM final concentrations. A dose response for all the agents was done before fixing the final concentrations used in all the experiments (the IC_{50} for inhibition of TNF-α production in T cell/macrophage cocultures are 10 μM and 60 μM for CPT-cAMP and rolipram, respectively. The dose-response for inhibition of proliferation is shown in Fig. 4). For proliferation studies, the cultures were maintained for 72 h, and [^{3}H]thymidine was added at 1 μCi/well for the last 6 h of the 72-h culture period. Cells were harvested, and radioactive incorporation was measured using a β-plate reader (Wallac, Gaithersburg, MD).

T cell-macrophage coculture

Coculture experiments were performed as previously described (20). Briefly, T cells were cultured for 3 days in complete medium in the presence or absence of T cell activators and cAMP modulators. Cells were then washed three times and fixed for 1 h with 1% paraformaldehyde in PBS at 4°C with gentle agitation. These paraformaldehyde-fixed T cells were washed thrice and then cocultured with U937 cells at a ratio of 7:1 in round bottom 96-well plates, as reported earlier (33). After 48 h, supernatants from these cocultures were harvested and used for the measurement of TNF-α by sandwich ELISA.

TNF-α measurement

ELISA high-binding plates were coated with 100 μl mouse anti-human TNF-α mAb (as per protocol from R&D Systems), diluted to 2 μg/ml in sterile PBS, and incubated overnight at 4°C. The plates were washed four times with TTBS (10 mM TBS, 150 mM NaCl, 0.05% Tween 20) and blocked with BSA (3% w/v in TTBS) for 4 h at room temperature. After additional washes with TTBS, 100 μl TNF-α standards and unknown samples were added to the plates and incubated overnight at 4°C. The plates were washed with TTBS, incubated for 2 h with 100 μl biotinylated goat anti-human polyclonal TNF-α Ab (diluted to 200 ng/ml in TTBS + 0.1% BSA; R&D Systems), washed again, and incubated an additional 30 min at room temperature with 100 μl avidin peroxidase (1:5000 in TTBS). The plates were washed again and developed with an *o*-phenylenediamine peroxidase substrate (Sigma FAST *o*-phenylenediamine dichloride tablet set), according to the manufacturer's instructions. TNF-α was measured colorimetrically at 450 nm and quantified by interpolation from the standard curve constructed from known concentrations of recombinant human TNF-α (R&D Systems). The detection limit of this assay is 5 pg/ml.

FACS analysis of T lymphocytes

T cells, after stimulation with various agents for 72 h, were washed two times with PBS containing 2% FCS and 0.01% sodium azide. Incubation with the FITC-labeled Ab was done on ice for 30 min, and the cells were again washed with PBS containing 2% FCS and 0.01% azide and suspended in PBS containing 10 μg/ml of 7-AAD to stain for dead cells. The stained cells were analyzed on a Becton Dickinson (Mountain View, CA) FACScan analyzer. Results obtained with various Abs were always compared with their respective isotype control Abs.

PKA assay

PKA activity was measured by quantitating the transfer of ^{32}P from [γ-^{32}P]ATP to the synthetic peptide substrate, kemptide. Briefly, an equal number of human peripheral blood T cells was treated with the indicated reagents for 10 min. At the end of the incubation time, 5×10^5 cells were pelleted at $1000 \times g$ and the supernatants decanted. The cells were washed twice in cold PBS. The cells were lysed by the addition of 50 μl of cold lysis buffer (5 mM Tris-HCl (pH 6.8), 5 mM EGTA (pH 8.0), 250 mM sucrose, 0.1% Triton X-100, 0.1 mM DTT, 1 mM PMSF, and 10 μg/ml each of aprotinin, leupeptin, and trypsin inhibitor), followed by vortexing. The PKA assay was performed at 30°C in a 96-well plate. Each treatment was performed in duplicate and then assayed plus and minus 10 μM cAMP in duplicate, yielding eight wells per treatment. The assay was initiated by the addition of 10 μl reaction mixture to 15 μl sample extract, for a total reaction volume of 25 μl. The reaction mixture contained 50 mM MOPS (pH 7.0), 10 mM $MgCl_2$, 0.25 mg/ml BSA, 20 mM Na_3VO_4, 0.25 mM kemptide, and 100 μM ATP supplemented with 0.02 mCi/ml [γ-^{32}P]ATP, (3000 Ci/mmol; DuPont NEN, Boston, MA). Blanks were made by adding 10 μl of reaction mixture without kemptide to 15 μl of cell lysate and MilliQ water. Reactions were terminated after 2 min by quenching a 10-μl aliquot of the ongoing reaction in 15 μl of 1 M HCl. A total of 20 μl of this quenched reaction was spotted on Whatman (Clifton, NJ) P81 phosphocellulose paper and allowed to dry completely before washing three times for 15 min each in excess 75 mM phosphoric acid. Peptide-bound radioactivity was subsequently measured by Cerenkov counting in a Beckman (Fullerton, CA) LS 3801 scintillation counter. Percent activated PKA was calculated as: [(cpm (without cAMP)) − (blank)/(cpm (with cAMP)) − (blank)] × 100.

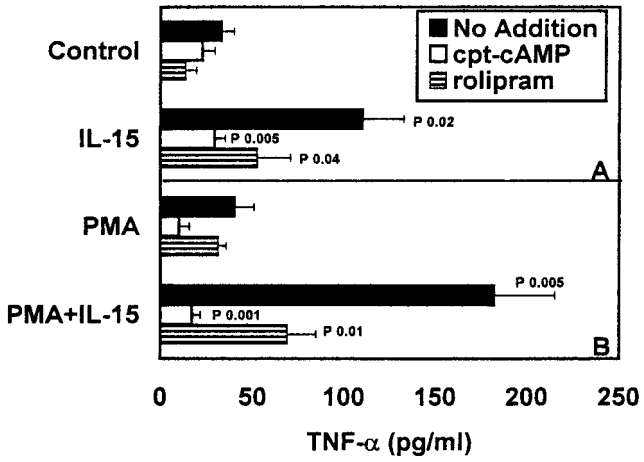

FIGURE 1. Rolipram and CPT-cAMP inhibit the production of TNF-α by T cells stimulated with IL-15 and/or PMA. T cells were stimulated with IL-15 (100 ng/ml) and/or PMA (1 nM) for 72 h in presence and absence of rolipram (100 μM) or CPT-cAMP (100 μM), as indicated on the *y*-axis. At 72 h, cell supernatants were analyzed for TNF-α released using ELISA (see *Materials and Methods*). Values expressed are mean ± SEM of triplicate samples from four experiments. Statistical analysis was performed using the Student's *t* test. For this analysis, samples stimulated with IL-15, PMA, or PMA-IL-15 (filled bars) were compared with the no addition control (also filled bar), and samples with CPT-cAMP and rolipram (open or hatched bars) were compared with the stimulated controls (filled bars) within the same grouping. Only *p* values < 0.05 are shown.

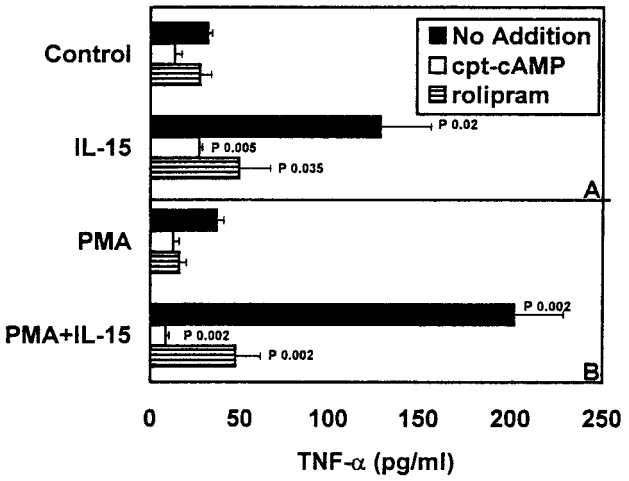

FIGURE 2. Rolipram and CPT-cAMP inhibit the production of TNF-α from the cocultures of U937 cells with T cells stimulated with IL-15 and/or PMA. T cells were stimulated with IL-15 (100 ng/ml) and/or PMA (1 nM) for 72 h in presence and absence of rolipram (100 μM) or CPT-cAMP (100 μM), as indicated on the *y*-axis. The T cells were then fixed with paraformaldehyde and cocultured with U937 cells at a 7:1 ratio for 48 h. Supernatants were then collected and assayed for TNF-α. Values expressed are mean ± SEM of triplicate samples from five experiments. Statistical analysis was similar to Fig. 1.

Statistical analysis

Statistical significance was calculated by Student's *t* test (Excel; Microsoft, Redmond, WA).

Results

Activation of cAMP/PKA pathway in T cells is sufficient to block IL-15 stimulation of TNF-α production from both T cells and T cell/macrophage cocultures

Initial experiments involved an assessment of the effect of rolipram and CPT-cAMP on TNF-α production by T cells alone (Fig. 1) or T cells cocultured with U937 cells (Fig. 2). To determine the effect of cAMP modulators on IL-15-induced production of TNF-α, supernatants from T cells cultured for 72 h in the presence of IL-15 along with CPT-cAMP or rolipram were collected and assayed for TNF-α by ELISA and compared with supernatants from control cultures with no IL-15 (Fig. 1*A*). Since rolipram is a phosphodiesterase inhibitor that increases intracellular levels of cAMP, all studies were also performed in the presence of exogenous CPT-cAMP, a cell-permeable analogue of cAMP. It is expected that rolipram and CPT-cAMP-treated cells would behave similarly. Addition of IL-15 to T cells caused a significant increase in TNF-α production ($p < 0.05$) (Fig. 1*A*, compare filled bars). Addition of CPT-cAMP (open bars) or rolipram (hatched bars) caused a statistically significant reduction in TNF-α release. These results demonstrate that IL-15-stimulated T lymphocytes produce TNF-α that is sensitive to the action of cAMP analogues or cAMP-elevating agents.

TNF-α production by T cell/U937 cocultures was measured using supernatants collected after 48 h of incubation. To ensure that the production of TNF-α in the coculture was solely from U937 cells, T cells were fixed with paraformaldehyde before mixing with U937 cells at a ratio of 7:1 (33). Supernatants from U937 cells incubated with IL-15-stimulated and paraformaldehyde-fixed T cells contained significantly higher amounts of TNF-α ($p < 0.05$) than U937 cells incubated with unstimulated control T cells (Fig. 2*A*, compare filled bars). Supernatants from cocultures containing T cells treated for 72 h with IL-15 in presence of either rolipram (hatched bars) or CPT-cAMP (open bars) and then incubated with U937 cells for 48 h showed significantly reduced amounts of TNF-α. Two additional controls were performed. IL-15-stimulated and paraformaldehyde-fixed T cells cultured alone for 48 h did not release any detectable TNF-α into the supernatant (data not shown). Also, U937 cells cultured in the absence of T cells, with or without IL-15, did not secrete any detectable TNF-α (data not shown). These results indicate that activation of the cAMP/PKA pathway in IL-15-stimulated T cells is sufficient to block TNF-α secretion from both T cells and T cell/macrophage cocultures.

PMA enhances the ability of IL-15 to induce TNF-α production either from T cells or from T cell/U937 cocultures

PMA is an activator of PKC and, when used in combination with either PHA or calcium ionophores, is a potent activator of T cells. However, addition of PMA alone is known to produce a limited or partial activation of T lymphocytes. This partial activation of T cells produces a phenotype with increased expression of surface molecules, such as CD69, LFA-1, and ICAM-1 (21, 34), but does not induce a high rate of proliferation. PMA has also been shown to up-regulate the IL-15 receptor (22). Therefore, to determine the effect of PMA on the ability of IL-15 to stimulate TNF-α production, PMA was added to T cells in the presence and absence of IL-15, and the secretion of TNF-α was assayed from supernatants of either T cells (Fig. 1*B*) or T cell/U937 cell cocultures (Fig. 2*B*). Addition of PMA (1 nM) produced no significant change in TNF-α production either from T cells or from T cell/U937 cultures (Figs. 1 and 2, compare top filled bar in *B* with top filled bar in *A*), as compared with unstimulated controls. However, when used in combination with IL-15, PMA synergistically enhanced the production of TNF-α from T cells by 72 h (Fig. 1*B*, bottom filled bar). Also, T cells stimulated with IL-15 and PMA for 72 h, followed by coculture with U937 cells, increased secretion of TNF-α, as compared with that produced in presence of IL-15 alone (Fig. 2, compare bottom filled bar in *B* with bottom filled bar in *A*). These

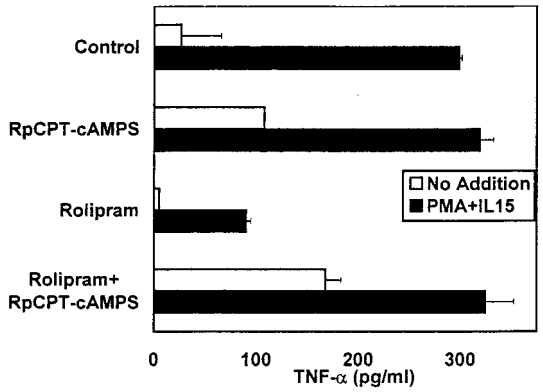

FIGURE 3. The effect of rolipram on TNF-α secretion is blocked by inhibitors of PKA. T cells were simultaneously treated for 72 h with rolipram and RpCPT-cAMP (500 μM) in presence or absence of PMA + IL-15. As described in *Materials and Methods*, T cells were washed and cocultured with U937 cells for 48 h. The cocultures do not contain either RpCPT-cAMP or rolipram. Open bars are control cultures with no addition of PMA + IL-15, while filled bars indicate cultures stimulated with PMA + IL-15. Values expressed are mean ± SEM of triplicate cultures from three experiments.

results show that PMA enhances the ability of IL-15 to induce TNF-α production either from T cells or from T cell/U937 cocultures and suggests that maximal expression of TNF-α requires the combined activation of signaling pathways induced by both PMA and IL-15. Addition of either rolipram (hatched bars) or CPT-cAMP (open bars) to T cells stimulated with PMA and IL-15 blocked the production of TNF-α, demonstrating that these PKA-activating reagents are potent inhibitors of IL-15-induced TNF-α production, even in the presence of PMA.

Inhibitors of PKA block the effects of rolipram

If the effect of rolipram on TNF-α secretion is mediated via the activation of PKA, then PKA inhibitors should block the effect of rolipram. To test this hypothesis, Rp-CPT-cAMP (500 μM), an analogue of cAMP that blocks the activation of PKA, was added to T cells that were either unstimulated or stimulated with PMA plus IL-15. These T cells were then cocultured for 72 h with U937 cells. When T cells were cultured with rolipram and RpCPT-cAMP and then cocultured with U937 cells, the inhibition in TNF-α secretion caused by rolipram was eliminated (Fig. 3, compare bottom two open and closed bars). These data suggest that activation of PKA is required for rolipram to inhibit TNF-α production.

IL-15 and PMA-induced T cell proliferation is inhibited by rolipram and CPT-cAMP

Incubation of human T cells with IL-15 activates T cells and enables them to interact with and stimulate macrophages to produce TNF-α. Rolipram and CPT-cAMP block this activation of T cells (Fig. 2). To determine which parameters of IL-15 induced activation of T cells are being blocked by rolipram and CPT-cAMP, changes in proliferation (measured by thymidine incorporation) and cell surface molecules (measured by flow cytometry) were monitored in the presence and absence of IL-15 (Figs. 4-6). T cells cultured for 72 h with 100 ng/ml IL-15 incorporated ~20 fold more [^{3}H]-thymidine than unstimulated control cells (Fig. 4, *A* and *B*). Addition of either CPT-cAMP or rolipram inhibited T cell proliferation in a dose-dependent manner (Fig. 4, *A* and *B*). Rolipram was less potent than CPT-cAMP with IC_{50} of 10 μM and 5 μM, respectively. These results indicate that both rolipram and

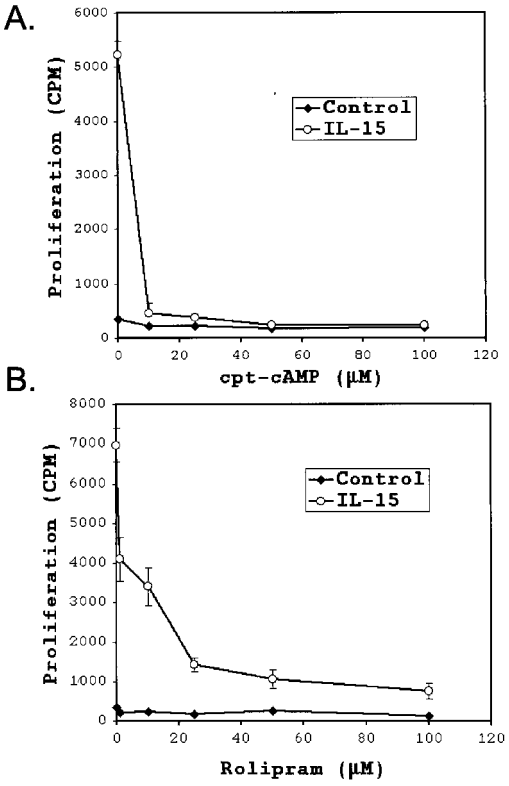

FIGURE 4. IL-15-stimulated T cell proliferation is inhibited by rolipram and CPT-cAMP in a dose-dependent manner. Proliferative response was measured by [^{3}H]thymidine incorporation of Control (♦) or IL-15 (100 ng/ml)-stimulated (○) T cells in presence of increasing concentrations of either CPT-cAMP (*A*) or rolipram (*B*). Values expressed are mean ± SEM of triplicate samples from two different experiments.

CPT-cAMP can effectively suppress IL-15-induced T cell proliferation.

We next tested the additive effect of IL-15 plus PMA on T cell proliferation. PMA (1 nM) by itself did not enhance T cell proliferation (Fig. 5, compare stippled bar with open bar in Control

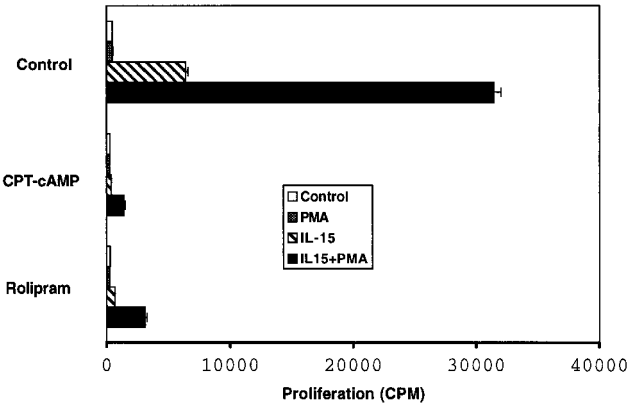

FIGURE 5. Rolipram and CPT-cAMP inhibit PMA and PMA/IL-15-stimulated T cell proliferation. T cell cultures were incubated with PMA (1 nM), IL-15 (100 ng/ml), or PMA + IL-15 (as indicated in legend) for 72 h in the presence or absence of CPT-cAMP (100 μM) or rolipram (100 μM) (as indicated on *y*-axis). [^{3}H]thymidine was added 4 h before the termination of cultures. Radioactive incorporation was measured using a β-plate reader. Values expressed are mean ± SEM of triplicate cultures. One of three independent experiments is presented.

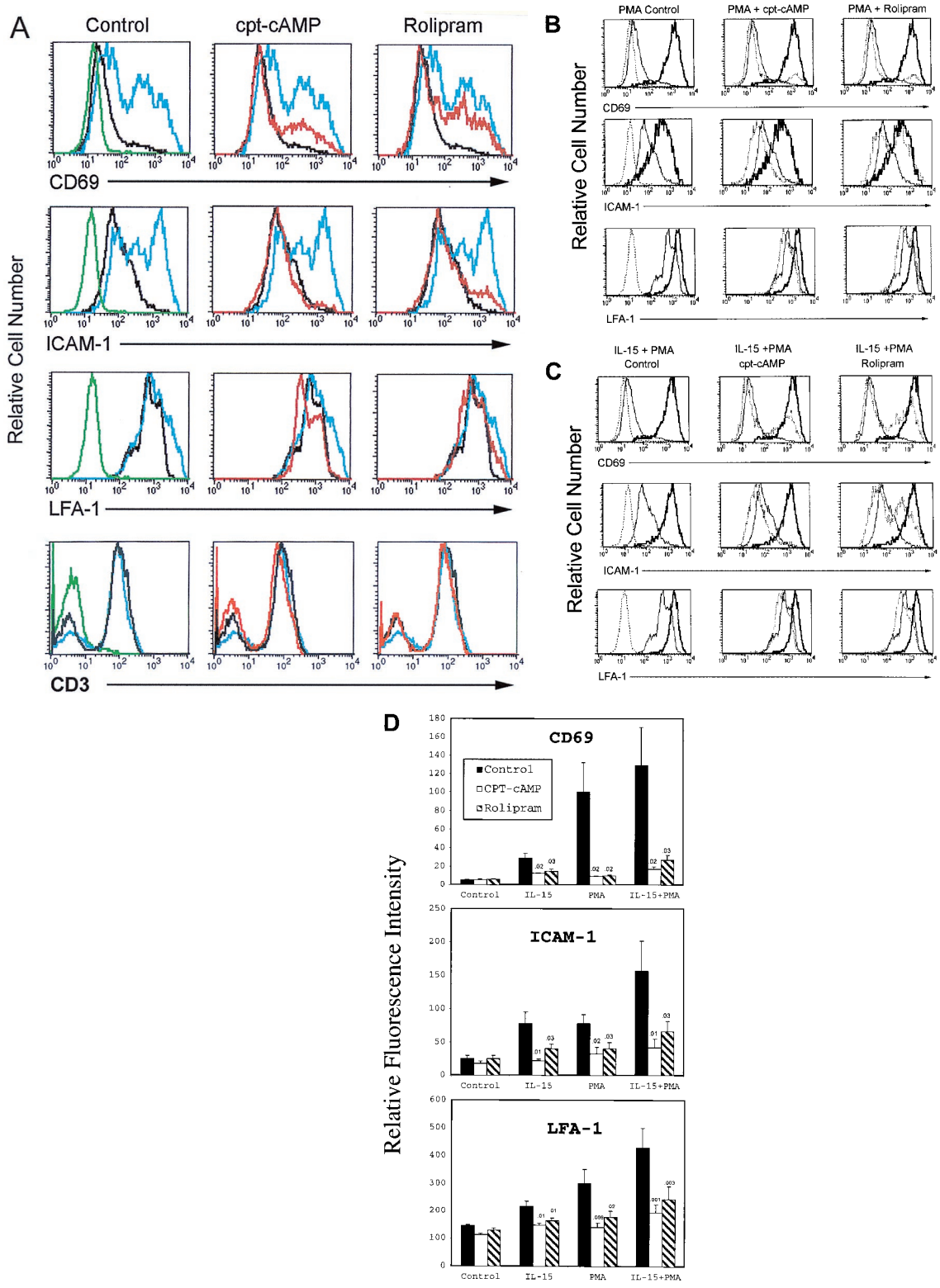

FIGURE 6. Expression of CD69, ICAM-1, and LFA-1 on IL-15, PMA, or PMA/IL-15-stimulated T cells is sensitive to the action of rolipram and CPT-cAMP. FACS analysis of T cells treated with vehicle, IL-15 (100 ng/ml) (*A*), PMA (1 nM) (*B*), or PMA/IL-15 (*C*) plus or minus CPT-cAMP (100 μM) or rolipram (100 μM) (as indicated at top of panel) was performed after 72 h of incubation. Cells were treated with FITC-conjugated Ab against CD69, ICAM-1, LFA-1, or CD3 for 30 min on ice (as described in *Materials and Methods*) and were analyzed on a Becton Dickinson FACScan. *A*, Black lines represent untreated control cultures, while blue lines represent T cells treated with IL-15 (the blue and black lines are also repeated in all three panels to allow for easy comparison with cells treated with CPT-cAMP and rolipram). CPT-cAMP or rolipram treatments (middle and right columns, respectively) are indicated by red lines. The isotype-matched controls (green line, left column) did not change with treatments. *B* and *C*, Light solid lines represent control cultures without any agent, while dark solid lines are for T cells treated with IL-15 (left column and repeated in middle and right column). CPT-cAMP or rolipram treatments (middle and right columns, respectively) are indicated by closely spaced dotted lines. The loosely spaced dotted lines are isotype controls (left column). A representative of four experiments is shown. A summary of data from all four experiments is shown in *D*. The bars represent the means ± SEM of the geometric means from all four experiments. The numbers above the bars are the *p* values calculated from Student's *t* test analysis, comparing the samples treated with either CPT-cAMP or rolipram with the stimulated control from the same group.

grouping), but PMA in combination with IL-15 synergistically enhanced [^{3}H]thymidine incorporation into T cells, as compared with that observed in presence of IL-15 alone (Fig. 5, compare filled bar to hatched bar in Control grouping). Both rolipram and CPT-cAMP inhibited the proliferation induced by IL-15 and PMA. These results indicate that cAMP analogues and cAMP-elevating agents, such as rolipram, can suppress T cell activation induced by IL-15 either in presence or absence of PMA.

Expression of CD69, LFA-1, and ICAM-1 on IL-15- and PMA-stimulated T cells is sensitive to the action of rolipram and CPT-cAMP

Cell contact between macrophages and IL-15-treated T cells leads to TNF-α production by macrophages. Previous studies have shown that IL-15 increases expression of T cell surface molecules, such as CD69, LFA-1, and ICAM-1 (19), and that these molecules facilitate T cell/macrophage interaction (20). To determine whether rolipram and CPT-cAMP effect the expression of these molecules on IL-15-stimulated T cells, the surface concentration of these molecules was monitored by flow cytometry. T cells were stimulated with IL-15 (100 ng/ml) for 72 h and stained with FITC-conjugated Abs, as described in *Materials and Methods*. IL-15 dramatically enhanced the expression of CD69 and ICAM-1 (compare blue lines with black lines in Fig. 6*A*, Control column). The IL-15-induced increase in LFA-1 was modest compared with CD69 or ICAM-1, possibly because basal levels of LFA-1 were already quite high (compare black line with green line in Control column). Addition of CPT-cAMP (100 μM) inhibited the IL-15-induced expression of CD69, ICAM-1, and LFA-1 (compare red line with blue line in Fig. 6, middle column). Likewise, rolipram inhibited the expression of CD69, ICAM-1, and LFA-1 (compare red line with blue line in Fig. 6*A*, right column), although the inhibition caused by rolipram is less potent compared with CPT-cAMP. As a control, we also examined the effect of CPT-cAMP and rolipram on CD3, a cell surface molecule not known to be affected by IL-15. Neither agent had any effect on CD3 expression, suggesting the these agents selectively inhibit the expression only of cell surface markers that are up-regulated during activation.

PMA also enhanced the expression CD69, ICAM-1, and LFA-1 on T cells (compare dark solid lines with light solid lines in Figs. 6*B*). In fact, PMA was more potent than IL-15 at increasing the expression of CD69 (compare blue lines in left column of Fig. 6*A* with dark lines in left column of Fig. 6*B*). Both CPT-cAMP and rolipram inhibited the PMA stimulation of expression of these molecules (compare dotted line with dark solid line in Fig. 6*B*, middle and right columns). A combination of IL-15 and PMA induced a further enhancement in the expression of ICAM-1, as compared with that in presence of either agent alone (compare left columns in Fig. 6, *B* and *C*). Even under these conditions, rolipram and CPT-cAMP effectively inhibited the expression of all three molecules (compare dotted line with dark solid line in Fig. 6*C*, middle and right columns). A summary of the inhibition of expression of CD69, ICAM-1, and LFA-1 by CPT-cAMP and rolipram from four separate experiments is shown in Fig. 6*D*. These results clearly show that PKA-activating agents, such as rolipram and CPT-cAMP, effectively inhibit the expression of cell surface molecules known to participate in T cell/macrophage interaction.

PKA activity of IL-15-stimulated T cells is enhanced by rolipram or CPT-cAMP

To determine the effect of CPT-cAMP and rolipram on PKA activity in IL-15-stimulated T cells, T cells, either control or IL-15-stimulated, were treated with no addition, CPT-cAMP, or rolipram for 10 min. PKA activity was then measured in presence or absence of cAMP in the reaction mixture to determine the percent activation level (Fig. 7). Addition of rolipram increased PKA activity by ~50% in both control and IL-15-stimulated cells (compare striped bars with filled bars). Addition of CPT-cAMP maximally increased PKA activity (~5-fold, compare open bars with filled bars) in both control and IL-15-stimulated cells. Addition of RpCPT-cAMP to the cells completely blocked the activation of PKA by either rolipram or CPT-cAMP (data not shown). These results show that, while both rolipram and CPT-cAMP significantly increase PKA activity in T cells, CPT-cAMP is a significantly more potent activator than rolipram.

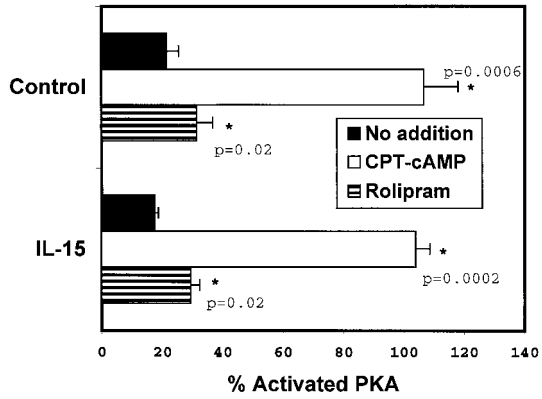

FIGURE 7. Rolipram and CPT-cAMP enhance PKA activity in T cells stimulated with IL-15. PKA activity of T cells, either control or stimulated with IL-15, were treated with no addition (filled bars), CPT-cAMP (100 μM, open bars), or rolipram (100 μM, striped bars) for 10 min at 37°C. PKA activity was measured with and without cAMP, and the ratio is reported as percent activated PKA (see *Materials and Methods* for details). Values expressed are mean ± SEM from two experiments. Asterisks indicate significance difference from control (no addition) to $p < 0.05$.

Discussion

The present paper demonstrates that rolipram inhibits IL-15-mediated T cell activation and, consequently, the ability of T cells to trigger production of TNF-α by macrophages in T cell-macrophage cocultures. TNF-α has been shown to be a critical proinflammatory cytokine found in the synovium of patients with RA (35–38). The principal source of TNF-α in the rheumatoid synovium is macrophages (7, 8). However, recent reports have suggested that synovial T cells may play a critical role in the pathogenesis of RA by stimulating macrophages to secrete TNF-α (10, 39). Rolipram, a phosphodiesterase inhibitor, has been shown to ameliorate collagen-induced arthritis in mice (25, 26). One aim of the present report was to determine whether rolipram was capable of inhibiting this T cell-mediated production of TNF-α by macrophages. To accomplish this, we developed a model system that allowed us to analyze the contribution of each partner in the T cell/macrophage coculture. Normal human healthy PBL were used as a T cell source, and U937 cells as a macrophage source. U937 cells are a macrophage-derived cell line that produces TNF-α upon stimulation with PMA and LPS. U937 cells were selected for these studies instead of PBMC because U937 cells do not respond to IL-15 by producing TNF-α, whereas PBMC have been reported to show autocrine activation by IL-15, resulting in the production of TNF-α (40). Thus, the U937 cells can be cocultured with IL-15-stimulated T cells without the possibility that the macrophages will be affected by IL-15. Furthermore, because the T cells stimulated with IL-15 were fixed with paraformaldehyde before coculturing

with U937 cells, the production of TNF-α can be attributed only to the U937 cells.

IL-15-activated T cells produced a 3-fold enhancement in TNF-α secretion, as compared with T cells cultured without any stimulant. The optimal concentration of IL-15 required for the production of TNF-α was observed to be 100 ng/ml. IL-15-activated T cells, when cocultured with U937 cells, triggered a 4- to 5-fold enhancement in TNF-α production, as compared with controls in which U937 cells are cocultured with unstimulated T cells. High levels of both IL-15 and TNF-α are characteristic of RA synovial fluid (2, 14). Taken together, these data suggest that T cells may contribute to the production of TNF-α in RA synovium in two ways: 1) by directly secreting TNF-α, and 2) by activating macrophages to secrete TNF-α.

Both rolipram and CPT-cAMP are effective in blocking the production of TNF-α by T lymphocytes, as well as U937 that have been cocultured with IL-15-stimulated T cells (Fig. 1). The observation that T cells incubated with IL-15 in presence of rolipram or CPT-cAMP fail to induce the production of TNF-α by U937 cells clearly demonstrates that the effect of rolipram is on the T cells. Further, we have shown that T cells treated with a combination of PMA plus IL-15 produce a synergistic increase in TNF-α production by both T cells alone and T cell/macrophage cocultures. These results suggest that optimal secretion of TNF-α requires the activation of several signaling pathways. An inhibition of PMA + IL-15-stimulated TNF-α secretion by rolipram and CPT-cAMP shows that cAMP signaling can down-regulate many of the pathways that lead to the secretion of TNF-α. Rolipram is known to function by inhibiting cAMP-specific phosphodiesterase, which enhances the intracellular cAMP, and thus increases PKA activity. The observation that RpCPT-cAMPS, an analogue of cAMP that inhibits PKA activation, blocked the effect of rolipram on TNF-α production in cocultures, confirms that rolipram is indeed working via the cAMP/PKA pathway. Further, PKA activity of IL-15-treated T cells was enhanced in presence of rolipram showing a direct evidence that the inhibition of TNF-α secretion by rolipram is due to its ability to enhance intracellular PKA activity.

The inhibitory effect of CPT-cAMP is consistently more potent than rolipram in blocking T cell activation, as measured by TNF-α production, proliferation, or expression of cell surface markers. The reason for this could be that CPT-cAMP is a membrane-permeable, stable analogue of cAMP, while rolipram increases intracellular cAMP by inhibiting phosphodiesterase, an enzyme that breaks down cAMP but has no effect on adenylyl cyclase, the enzyme that produces cAMP. Thus, the intracellular concentration of cAMP is likely to be higher following addition of CPT-cAMP than rolipram. This hypothesis is supported by the observation that addition of CPT-cAMP to T cells produces a significantly greater increase in PKA activity than rolipram (Fig. 7).

To understand the mechanism of action of rolipram on T lymphocytes, we have measured the effect of rolipram on T cell proliferation and expression of various cell surface molecules that are known to play a role in IL-15-induced production of TNF-α. Preincubation of T cells with either rolipram or CPT-cAMP inhibited the proliferation of T cells stimulated with IL-15. Inhibition of T cell proliferation by cAMP analogues in vitro is well known (41). The present data show IL-15-stimulated T cell proliferation is sensitive to cAMP.

Previous studies have shown that IL-15 treatment of T cells results in an increase in several surface molecules, including CD69, LFA-1, and ICAM-1 (19). The up-regulation of these molecules has been shown to facilitate the T cell/macrophage interaction that results in the production of TNF-α (20). mAbs against these molecules are known to decrease the production of TNF-α in coculture (20). Both rolipram and CPT-cAMP inhibit the IL-15-stimulated up-regulation of these key surface molecules. The decreased expression of these molecules on T cells treated with rolipram or CPT-cAMP likely accounts for the inability of the T cells to interact with and stimulate the production of TNF-α from U937 cells.

Increased expression of CD69, LFA-1, and ICAM-1 was also observed when T cells were treated with either PMA alone or PMA plus IL-15. CPT-cAMP, and, to a lesser extent, rolipram, were capable of reducing the increased expression of these surface molecules (Fig. 6); however, there were clear differences in the response of each surface molecule to the activating agents and subsequent inhibition. Interestingly, even though PMA treatment increased expression of CD69, LFA-1, and ICAM-1 at least as well as IL-15, it failed to induce TNF-α production either from T cells or from U937 cells cocultured with PMA-stimulated T cells. This observation clearly indicates that increased expression of these three molecules on T cells is critical but not sufficient to increase production of TNF-α in T cell/macrophage coculture. These data suggest that IL-15, but not PMA, induces a surface molecule or molecules in addition to CD69, LFA-1, and ICAM-1 that is critical for T cell/macrophage interaction leading to secretion of TNF-α. This critical factor is probably not IL-15R, as PMA alone can enhance the expression of IL-15R (22). Further work is in progress to identify this critical factor that is associated with T cell/macrophage contact-mediated production of TNF-α.

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