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CD28 Costimulation Induces δ Opioid Receptor Expression During Anti-CD3 Activation of T Cells[1]

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Previous studies have demonstrated that naive splenic mouse T cells express no or only very low levels of the δ-type opioid receptor (δOR), but stimulation of mouse splenocytes with Con A results in induction of δOR mRNA and protein. In this report we have shown that stimulation of highly purified populations of naive mouse T cells with anti-CD3 mAb alone results in T cell activation, as evidenced by sustained IL-2 secretion and cell proliferation, but fails to elicit δOR expression. However, δOR expression is induced by costimulation of these very pure T cells with anti-CD3 and anti-CD28 mAbs. The δOR induction by anti-CD3 and anti-CD28 costimulation was completely blocked by inhibition of phosphatidylinositol 3-kinase with wortmannin. Because phosphatidylinositol 3-kinase activation in T cells is linked to costimulation, these results suggest that induction of δOR expression during T cell activation is strictly dependent on costimulation. It also appears that costimulatory receptors other than CD28 can provide the signaling required for δOR expression because δOR mRNA was induced by Con A stimulation of splenocytes from CD28-deficient mice. *The Journal of Immunology,* **2002, 168: 4440–4445.**

The expression of opioid receptors on cells of the immune system (recently reviewed in Refs. 1 and 2) provides a mechanism for direct modulation of immune function by opioids. Use of opiates, such as heroin and morphine, and the exacerbated release of endogenous opioid peptides during stress have long been correlated with altered immune function and immunosuppression. Although there is no doubt that many of these opioid-associated effects result indirectly from altered adrenal regulation of cortical hormones (3–5), there is clear evidence that opiates and opioid peptides can directly modify immune cell function as well. The addition of opiates and opioid peptides to lymphocyte cell lines and to isolated lymphocytes has been shown to alter lymphocyte proliferation, T cell rosetting, Ab and cytokine secretion, and chemotaxis (recent reviews include Refs. 6–9).

The conditions under which immune cells express opioid receptors, and therefore would be expected to be sensitive to direct opioid modulation, have not been investigated extensively. δ-type opioid receptor (δOR)[3] expression has been found in unfractionated lymphocytes, primary T cells, and T cell-derived cell lines (10–20). We have previously reported that naive murine splenic T cells do not express δOR (10, 18). While another laboratory has reported δOR expression in naive T cells, the number of transcripts detected is extremely low and expression is detected in only a small percentage of the T cells in splenocytes from pathogen-free mice (17, 19, 21). Although it is not clear whether this minor difference results from animal differences or differences in cell purification, it is apparent that, at most, only a small subpopulation of naive T cells expresses δOR mRNA or protein.

In contrast to naive T cells, δOR mRNA (10) and protein (18) are clearly induced in both $CD4^+$ and $CD8^+$ mouse splenic T cells stimulated with Con A. The induction of T cell δOR expression by Con A is dependent on the presence of non-T cell splenocytes during activation; Con A was unable to induce δOR expression in cultures of purified T cells. This result is consistent with the observation that although lectins can bind directly to T cells they do not activate highly purified T cells in the absence of APCs (22). Furthermore, this result suggested that cell to cell interactions with APC might be involved in regulating δOR expression.

A number of T cell costimulatory receptors have been identified which can alter the pathway and outcome of T cell activation (23, 24). CD28, which is expressed on nearly all $CD4^+$ and $CD8^+$ T cells in mice (25), is one of the most important coreceptors identified thus far in T cell activation in response to numerous stimuli (26–28). During Ag presentation, CD28 is engaged by B7 family ligands expressed on macrophages and dendritic cells, providing an additional activation signal. CD28 costimulation results in increased T cell expression of specific lymphokines, including IL-2, IL-4, IL-5, IL-13, IFN-γ, TNF-α, and GM-CSF, through both transcriptional and translational mechanisms (26–35). CD28 costimulation also increases expression of other factors involved in T cell proliferation and survival, including the IL-2R α- and β-chains (29) and the antiapoptotic factor bcl-x (36, 37). A critical role for CD28 in lectin-induced activation of T cells was suggested by the impaired response of lymphocytes from CD28 knockout mice to Con A but not to PMA plus calcium ionophore (38).

In this report, we have investigated the role of costimulation in induction of T cell δOR expression, focusing on the CD28 T cell costimulatory receptor. Adherent Abs to the CD3ε component of the TCR and to the CD28 coreceptor were used to deliver primary and costimulatory T cell activation signals to naive mouse splenic T cells, respectively. While stimulation with anti-CD3 mAb alone induced apparent T cell activation, δOR expression was not induced in the absence of CD28 costimulation in cultures of highly purified T cells. However, T cell δOR mRNA was induced by Con A stimulation of splenocytes from CD28-deficient mice, suggesting that other costimulatory receptors also can provide signaling for δOR expression.

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[3] Abbreviations used in this paper: δOR, δ-type opioid receptor; PI3-K, phosphatidylinositol 3-kinase; CD40L, CD40 ligand; MAPK, mitogen-activated protein kinase; DIG, digoxigenin.

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Materials and Methods

Mice

Pathogen-free C57BL/6 and B6.12952-*Cd28*^{tm1Mak} male mice were obtained from The Jackson Laboratory (Bar Harbor, ME). They were maintained in a restricted-access clean facility with sterile bedding, food, and water and used between 6 and 12 wk of age.

Splenic CD4$^+$ and CD8$^+$ T cell purification and culture

Single cell splenocyte suspensions were prepared by forcing the tissue through sterile nylon mesh. The recovered cells were washed with HBSS supplemented with 5 mM HEPES, 4.2 mM $NaHCO_3$, 0.03% BSA, and 3 μM oleic acid. For most experiments, CD4$^+$ and CD8$^+$ T cells were selected by fluorescence-based cell sorting to >97% purity. Splenocyte suspensions were stained with fluorochrome-labeled anti-mouse CD8a and CD3 Abs in the presence of Fc Block (BD PharMingen, San Diego, CA) and purified rat IgG (10 μg/ml; Sigma-Aldrich, St. Louis, MO) to block nonspecific staining. Sorting was done on a FACStar instrument (BD Biosciences, Mountain View, CA) using a tightly gated forward/side scatter window for lymphocytes. Doubly labeled lymphocytes were taken as CD8$^+$ T cells while CD3$^+$CD8$^-$ lymphocytes were taken as CD4$^+$ T cells. This identification was confirmed by staining with anti-mouse CD4 Abs. Fluorochrome-labeled Abs and Fc Block all were from BD PharMingen. The CD40 ligand (CD40L) Ab MR1 was a generous gift from Dr. R. Noelle (Dartmouth Medical School, Lebanon, NH). In some instances indicated in the text, CD4$^+$ T cells were selected on magnetic columns (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity of these populations ranged from 85 to 92% as determined by subsequent staining with fluorochrome-labeled anti-CD4 and anti-CD3 Abs.

Purified T cells were cultured in flat-bottom tissue culture wells in one of two serum-free culture mediums: RPMI 1640 supplemented with glutamine to a final concentration of 4 mM, 5 mM HEPES, 1 mM sodium pyruvate, 50 μM 2-ME, 100 U/ml penicillin G, 100 mg/ml streptomycin (RPMI culture medium) and either 1) 1% Nutridoma-NS (Roche Molecular Biochemicals, Indianapolis, IN), 50 μM oleic acid, and 0.5% BSA or 2) 250 μg/ml apotransferrin, 0.5 μg/ml insulin (both from Life Technologies, Rockville, MD), 1× MEM nonessential amino acids (Sigma-Aldrich), and 1% BSA. Gentamycin (10 mg/ml) also was included in some experiments. For activation, the cells were seeded in wells coated with purified anti-CD3 (2C11 hybridoma; provided by J. Bluestone, University of Chicago, Chicago, IL) and/or anti-CD28 (BD PharMingen) Abs. For Con A activation of splenocytes, the cells were cultured at ~2 million cells/ml in RPMI culture medium containing 10% low endotoxin FBS (Atlanta Biologicals, Norcross, GA) and 2.5 μg/ml Con A (Sigma-Aldrich).

IL-2 ELISA

IL-2 accumulation in the culture supernatant was determined by ELISA using IL-2 capture (clone JES6-1A12) and biotinylated (clone JES6-5H4) Abs from BD PharMingen according to the manufacturer's instructions. Streptavidin-HRP was from Genzyme (Cambridge, MA) and TMB substrate was from Sigma-Aldrich.

Western blotting

Cells were washed, suspended in 10 mM Tris-Cl (pH 7.8) lysis buffer containing 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and protease inhibitors (10 μg/ml E64, 7.5 μg/ml pepstatin A, 40 μg/ml 3,4-dichloroisocoumarin, 5 μg/ml benzamidine, 20 μg/ml aprotinin, and 50 μg/ml PMSF), and lysed by repeated freeze-thawing. Equal amounts of total protein were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose in pH 9.9 carbonate buffer (39). Anti-δOR antiserum was from Incstar (Stillwater, MN). The ECL Western Blotting Analysis System from Amersham Pharmacia Biotech (Piscataway, NJ) was used for detection.

RT-PCR

Total RNA was isolated by acid guanidinium isothiocyanate-phenol-chloroform extraction (40) and reverse transcribed with Superscript II (Life Technologies) using random hexamers for priming. The primers for actin cDNA amplification and the nested amplification of δOR cDNA were those described previously (10). To permit detection at low amplification cycles, digoxigenin (DIG)-labeled nucleotides (PCR DIG Labeling Mix) were directly incorporated into actin RT-PCR products. The products were separated on agarose gels and blotted to nylon membranes, incubated with an alkaline phosphatase-labeled anti-DIG Ab, and detected by disodium 3-(4-methoxyspiro(1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan)-4-yl) phenyl phosphate chemiluminescence. δOR RT-PCR products were detected by Southern blotting using a DIG-labeled hybridization probe corresponding to δOR_{78-627}. The RT-PCR products were quantified by densitometry. All reagents for DIG labeling and detection were from Roche Molecular Biochemicals.

Results

Costimulation with anti-CD28 and anti-CD3 mAbs resulted in more rapid and marked induction of δOR protein expression than stimulation with anti-CD3 mAb alone in cultures of CD4$^+$ T cells enriched by magnetic Ab selection (Fig. 1). In addition to the ~62-kDa molecular mass protein band which is predicted for the mature, membrane-associated δOR protein, a second protein band that migrates with an apparently lower molecular mass and that appears to result from receptor endocytosis is detected by Western blotting of whole cell lysates (18). Both the 62- and 49-kDa δOR protein bands could be detected after 24 h in costimulated cultures but not in cultures stimulated with anti-CD3 mAb only. Also, accumulation of δOR protein in costimulated cultures was greater than in cultures activated with anti-CD3 mAb alone for at least 3 days of culture. Cell proliferation was not increased in costimulated cultures compared with those stimulated with anti-CD3 mAb alone at the time of the greatest increase in δOR protein expression. [^{3}H]Thymidine incorporation in triplicate wells was 7332 ± 662 and 7914 ± 460 cpm/well in costimulated and anti-CD3 mAb-only stimulated cultures, respectively, measured from 48 to 72 h of culture.

CD4$^+$ (and CD8$^+$) T cell populations enriched by selection on magnetic columns typically contain 5–10% non-T cells as determined by staining with fluorochrome-labeled anti-CD4 and anti-CD8 mAbs. To address the possibility that these contaminating cells were providing signals that contributed to the lower levels of δOR induction seen with anti-CD3 stimulation alone, CD4$^+$ and CD8$^+$ T cells purified to >97% homogeneity by FACS selection were used for subsequent experiments. The CD4$^+$ T cells were isolated without exposure to anti-CD4 mAb, i.e., they were selected as CD3$^+$CD8$^-$ lymphocytes to avoid perturbing the CD4 receptor signaling system. In preliminary studies, δOR induction

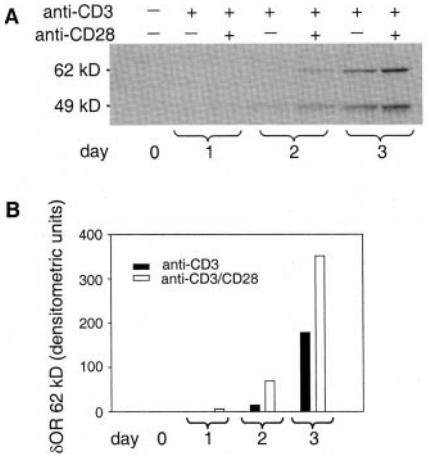

FIGURE 1. Anti-CD3 and anti-CD28 mAb costimulated induction of δOR protein. Cell lysates from freshly isolated CD4$^+$ T cells (day 0) or CD4$^+$ T cells cultured for 1–3 days in wells with adherent anti-CD3 mAb only or adherent anti-CD3 and anti-CD28 mAbs were separated on a 10% SDS-PAGE gel, blotted to nitrocellulose, and detected by chemiluminescent Western blotting using an Ab against an N-terminal peptide of mouse δOR (*A*). Quantitation of the 62-kDa protein band by densitometry is shown in *B*; quantitation of the 49-kDa protein band yielded nearly identical results. Equal amounts of protein were loaded in each lane. CD4$^+$ T cells were enriched to ~85% by selection on magnetic columns.

by anti-CD3 and anti-CD28 mAb costimulation was observed to be decreased somewhat by exposure of $CD3^+CD8^-$ lymphocytes to anti-CD4 mAb.

To determine the conditions resulting in maximal CD3-mediated activation and CD28 costimulation of these highly purified T cells, IL-2 accumulation was measured in cultures of $CD8^+$ and $CD4^+$ T cells (0.1 million cells per 0.2 ml medium per well in 96-well tissue culture plates) stimulated with varying concentrations of adherent anti-CD3 and anti-CD28 mAb. The lowest concentration of anti-CD3 mAb tested for each cell type had been determined in previous work to stimulate near maximal proliferation of $CD4^+$ and $CD8^+$ T cells purified by negative selection techniques (41). In $CD8^+$ T cell cultures stimulated with 150 ng/well of anti-CD3 mAb alone, the mean value of IL-2 accumulation in duplicate cultures was 108 and 188 ng/ml after 1 and 2 days of culture, respectively, and was not increased by doubling the anti-CD3 mAb concentration. IL-2 was 110 and 196 ng/ml, respectively, in these cultures. Costimulation with 50 ng/well anti-CD28 mAb and 150 ng/well anti-CD3 mAb did increase IL-2 accumulation, which was 212 ng/ml after 1 day of culture and 224 ng/ml after 2 days. Doubling the anti-CD28 mAb concentration resulted in no further increase. In $CD4^+$ T cells, IL-2 accumulation was increased only slightly in cultures stimulated with 100 ng/well anti-CD3 mAb compared with those stimulated with 50 ng/well. The values were 80 and 90 ng/ml after day 1 and 87 and 112 ng/ml after day 2 of culture with 50 and 100 ng of anti-CD3 mAb, respectively. Costimulation with 50 ng/well each anti-CD28 and anti-CD3 mAbs more than doubled IL-2 accumulation, which was 224 ng/ml after both 1 and 2 days of culture. No further increase resulted from doubling the anti-CD28 mAb concentration. Therefore, in subsequent experiments anti-CD3 mAb was used at 150 ng per 0.32-cm^2 well area for $CD8^+$ T cells and 50 ng per 0.32-cm^2 well area for $CD4^+$ T cells. Anti-CD28 mAb was used at 50 ng per 0.32 cm^2 for both T cell types.

Because of the smaller cell numbers that could be obtained readily by FACS selection in comparison to magnetic Ab selection, δOR expression was followed by RT-PCR detection of δOR mRNA rather than by Western blotting in these highly purified cell populations. To insure that RT-PCR quantitation was not compromised by differences in mRNA content expected to occur with T cells in different activation states, the RT-PCR conditions used in these studies were chosen to yield nearly linear responses over a 10-fold range of input RNA. For example, using 0.02 and 0.2 μg of total RNA from Con A-activated splenocytes per reverse transcription reaction, the actin RT-PCR products were 131 and 1190 densitometry units, respectively, after 18 cycles and the δOR RT-PCR products were 10 and 89 densitometry units, respectively, after 26 cycles.

The time course of δOR mRNA induction was essentially identical in FACS-purified $CD8^+$ and $CD4^+$ T cells costimulated with anti-CD3 and anti-CD28 mAbs (Fig. 2). δOR mRNA, normalized to actin mRNA, increased from day 1 to day 2 of stimulation and then decreased by day 3 in both $CD8^+$ and $CD4^+$ costimulated cultures. The δOR mRNA detected following 1 day of stimulation is assumed to represent an increase from initially undetectable levels because we routinely do not detect δOR mRNA in freshly isolated T cells.

Although costimulation induced δOR mRNA expression, stimulation with anti-CD3 mAb alone was not sufficient to induce δOR mRNA in $CD4^+$ or $CD8^+$ T cells that had been highly purified by FACS selection. In representative experiments shown, δOR RT-PCR product was readily detected in RNA from $CD4^+$ (Fig. 3*A*) and $CD8^+$ (Fig. 4*A*) T cells stimulated with anti-CD3 and anti-CD28 mAbs, but no RT-PCR product was detected with anti-CD3

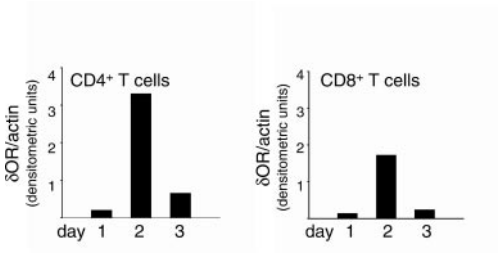

FIGURE 2. Time course of δOR mRNA induction by anti-CD3 and anti-CD28 mAb costimulation $CD4^+$ and $CD8^+$ T cells purified to >95% homogeneity by FACS selection were cultured for the indicated times in wells containing adherent anti-CD3 and anti-CD28 mAbs. Total RNA was isolated and reverse transcribed and PCR amplification and detection were conducted as described in *Materials and Methods*. The data presented are the ratios of δOR RT-PCR products to actin RT-PCR products. For nested amplification of δOR, equal portions of the RT-PCR following 18 amplification cycles with the outer δOR primer pair were amplified for 28 cycles with the inner δOR primer pair. Actin RT-PCR products were amplified for 20 cycles.

mAb stimulation alone, even after much longer exposure times than those shown. Detection of actin mRNA from the same reverse transcribed samples (Figs. 3*B* and 4*B*) provided a positive control.

Inclusion of wortmannin, an inhibitor of the CD28-activated phosphatidylinositol 3-kinase (PI3-K) pathway, during culture completely blocked the appearance of δOR mRNA in both $CD8^+$ (Fig. 4*A*) and $CD4^+$ (Fig. 4*C*) T cells. Wortmannin did not inhibit actin mRNA expression in costimulated cultures (Fig. 4, *B* and *D*).

To confirm the role of the CD28 receptor in the induction of δOR expression in costimulated cultures, splenic T cells purified from CD28-deficient mice were examined. As predicted, no δOR RT-PCR product was detected even after 32 cycles of amplification in either $CD4^+$ or $CD8^+$ T cells purified from CD28-deficient mice and costimulated with anti-CD3 and anti-CD28 mAbs for 2 days (data not shown). Actin RT-PCR products were readily detected after 14 amplification cycles, confirming successful reverse transcription of all of the samples.

However, δOR mRNA was expressed in Con A-activated splenocytes from CD28-deficient mice, suggesting that costimulatory receptors other than CD28 also must be able to provide the signaling required for induction of δOR expression. To investigate the potential role of CD40/CD40L interactions in this induction of δOR expression, the CD40L Ab MR1 was added at the initiation

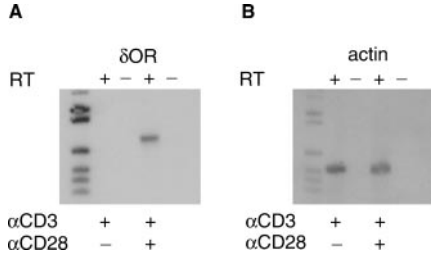

FIGURE 3. Anti-CD3 mAb stimulation alone does not induce δOR mRNA expression in FACS-purified $CD4^+$ T cells. $CD4^+$ T cells that had been purified by FACS selection were cultured for 2 days in wells containing adherent anti-CD3 mAb only or anti-CD3 and anti-CD28 mAbs. Total RNA was isolated and reverse transcribed, and PCR amplification and detection of δOR mRNA (*A*) and actin mRNA (*B*) were performed as described in *Materials and Methods*, except that only the outer δOR primer pair rather than nested amplification was used. RNA samples treated identically, but without reverse transcriptase, are indicated with a minus sign above the corresponding lane.

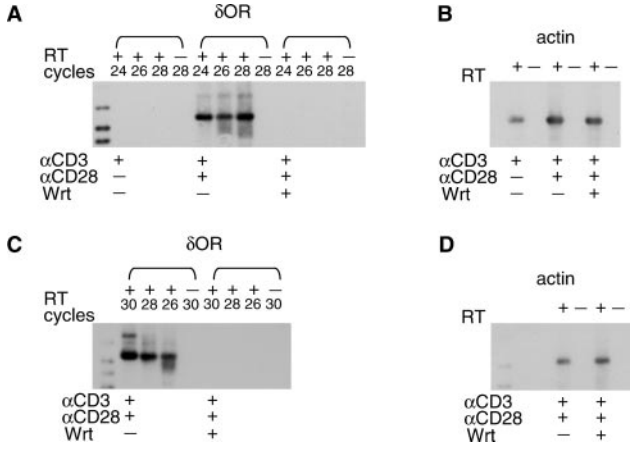

FIGURE 4. Wortmannin inhibits δOR mRNA expression in anti-CD3 and anti-CD28 costimulated T cells. $CD8^+$ (*A* and *B*) and $CD4^+$ (*C* and *D*) T cells purified by FACS selection were cultured for 2 days in wells containing adherent anti-CD3 mAb only or anti-CD3 and anti-CD28 mAbs. Wortmannin (Wrt) was added at the initiation of culture where indicated. Total RNA was isolated and reverse transcribed, and PCR amplification and detection were performed as described in *Materials and Methods*.

of Con A stimulation. MR1 is reported to inhibit costimulatory signaling through CD40L expressed on $CD4^+$ T cells (42). MR1 did not block the induction of δOR mRNA in $CD4^+$ T cells enriched by magnetic selection from Con A-activated splenocytes from either normal C57BL/6 or CD28-deficient mice (Fig. 5). However, MR1 did decrease the number of lymphocyte blasts from 32 to 7% of the total cell population in the Con A-activated splenocyte cultures from normal C57BL/6 mice as determined by FACS analysis of forward/side scatter profiles (data not shown), confirming its activity in this assay. Consistent with their impaired response to Con A stimulation (38), the number of blasts was low in activated splenocyte cultures from the CD28-deficient mice without MR1 (14%) and was scarcely affected by addition of MR1 (11%).

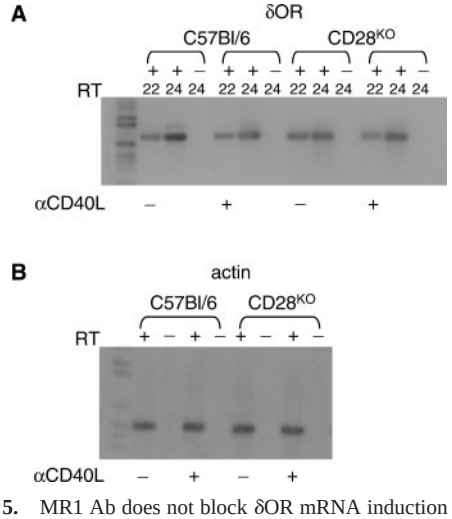

FIGURE 5. MR1 Ab does not block δOR mRNA induction in $CD4^+$ T cells purified from Con A-activated splenocytes. Splenocytes from C57BL/6 (B6) and from CD28-deficient (CD28 KO) mice were activated by culturing for 2 days with Con A (2.5 μg/ml). $CD4^+$ T cells from the activated cultures were enriched by selection on magnetic columns. Total RNA was isolated and reverse transcribed, and PCR amplification and detection of δOR mRNA (*A*) and actin mRNA (*B*) were performed as described in *Materials and Methods*.

Discussion

Stimulation with mAbs to the CD3 component of the TCR complex is widely used as a model of T cell activation. There are many reports showing that when suboptimal anti-CD3 mAb concentrations are used CD28 costimulation clearly enhances T cell activation. However, with optimal CD3 stimulation it is not clear whether CD28 costimulation results in distinct differences in T cell differentiation or simply increases the number of cells undergoing activation. Strong TCR signaling such as that provided by specific Ab stimulation has been suggested in several studies to override the requirement for costimulation (35, 43, 44).

In the studies reported herein, maximally stimulating concentrations of anti-CD3 mAb alone resulted in sustained IL-2 expression in cultures of highly purified $CD4^+$ and $CD8^+$ T cells, a hallmark of T cell activation. However, no induction of δOR mRNA was observed in the absence of CD28 costimulation in either $CD4^+$ and $CD8^+$ T cells that had been purified to near homogeneity by FACS selection. In cultures of enriched $CD4^+$ T cells that contained ≥5% contaminating non-T cells, δOR protein was induced with anti-CD3 stimulation alone, but, with costimulation, δOR protein induction was detected earlier and was greatly enhanced throughout 3 days of culture in comparison to those activated with anti-CD3 mAb only. Importantly, proliferation was not increased in costimulated cultures compared with controls during the time of maximal δOR expression. Thus, the induction of δOR expression by CD28 appears to be distinct from that of T cell activation as defined by IL-2 secretion and proliferation.

The complete inhibition of δOR mRNA induction by the PI3-K inhibitor wortmannin provides further evidence that costimulation is required for δOR expression in anti-CD3-stimulated T cells. Activation of PI3-K and release of phosphatidylinositol phosphates is one of the early signaling events detected following CD28 stimulation. The signal cascade initiated by PI3-K may be responsible for most of the effects of CD28 because wortmannin inhibits the majority of downstream CD28-mediated events (45, 46). In contrast, many CD3-stimulated events have been shown to be wortmannin insensitive. Anti-CD3 mAb-induced proliferation of murine $CD4^+$ T cells was not blocked by wortmannin (47). Also, while CD28-induced activation of Tec family protein kinases is sensitive to wortmannin, CD3-induced activation is resistant (48). Thus, the very early calcium signaling pathways of CD3 and CD28 stimulation are distinguished by their sensitivity to inhibition by wortmannin.

Although CD28 is thought to provide the most critical costimulatory signaling for both naive $CD4^+$ and $CD8^+$ T cells during activation by APC, the ability of CD28-deficient mice to mount effective responses to some types of T cell-dependent immune challenge has been used to suggest that there are alternative costimulatory pathways for many CD28-induced responses (38). Other receptors that provide costimulatory signaling for T cell responses upon interacting with their cognate ligands include CD40L, CD2, LFA-1, and inducible costimulator, a member of the CD28 superfamily (49). Cytokines and chemokines also can provide costimulatory signaling, and many of these costimulatory molecules and receptors have been shown, like CD28, to activate PI3-K (50). It is likely that one or more of these alternate costimulatory pathways is responsible for the induction of δOR by Con A stimulation of splenocytes from CD28-deficient mice and may also contribute to the induction of δOR expression during normal T cell activation. The relative importance of CD28 and other costimulatory receptors for the induction of δOR expression in other models of T cell activation remains to be determined.

During the course of these studies it was reported that T cell δOR expression is induced by anti-CD3 mAb stimulation of splenocyte cultures, and a lower level of induction was observed during culture without deliberate activation (19). The nonstimulated induction was positively correlated with cell culture density, consistent with the suggestion that cellular interactions contribute to the induction of T cell δOR expression. The inability of CD3 stimulation alone to induce T cell δOR mRNA expression in purified T cells in comparison to splenocyte cultures is likely to result from costimulatory signaling being provided by non-T cells in the latter during anti-CD3 stimulation. The ability of non-T cells to provide costimulatory signaling during anti-CD3 stimulation of T cells also is suggested in our studies by the observation that anti-CD3 mAb stimulation alone was able to induce some δOR expression in enriched T cell cultures (Fig. 1) but not in highly purified T cell populations selected by FACS.

The $CD4^+$ T cells used in the experiments reported in this work were isolated without exposure to anti-CD4 mAb to avoid perturbing the CD4 receptor signaling system. Stimulation of the CD4 receptor has been reported variably to contribute both to T cell activation and to induction of anergy (51). In this laboratory it has been observed that incubating $CD4^+$ T cells that had been purified by selection as $CD3^+CD8^-$ lymphocytes with anti-CD4 mAb before stimulation decreases the subsequent induction of δOR expression by anti-CD3/anti-CD28 costimulation (our unpublished results).

It was anticipated by several laboratories that IL-6 would play an important role in inducing opioid receptors during immune cell activation based on the presence of NF-IL6-like binding sites in the promoter regions of the μ, δ, and κ opioid receptors (52). However, IL-6 did not increase the expression of reporter constructs containing these putative NF-IL6 binding sites, which were transiently transfected into the U266 myeloma cell line and into the Raw 264.7 macrophage cell line. Thus, to our knowledge, the CD28 coreceptor is the first specific extracellular signal responsible for T cell δOR expression to be identified.

In T cells, the mitogen-activated family of protein kinases (MAPK) plays a central role in coordinating the response to extracellular signals that lead to T cell differentiation, proliferation, and apoptosis (53). Recently, p38 MAPK was suggested to mediate signal integration during TCR and CD28 costimulation of primary mouse T cells (54). Costimulation of naive mouse splenic T cells with anti-CD3 and anti-CD28 mAbs resulted in synergistic activation of p38 MAPK but not extracellular signal-regulated kinase or c-Jun N-terminal kinase. Wortmannin inhibition of PI3-kinase has been shown to block downstream activation of p38 MAPK during Ag stimulation of a mast cell line (55). Also, in mouse peritoneal macrophages wortmannin blocked p38 MAPK activation by zymosan but not bacterial stimulation (56). We are currently investigating whether p38 MAPK activation mediates CD28 induction of T cell δOR expression.

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