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Regulation of Mu Opioid Receptor Expression in Developing T Cells

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Abstract

We have previously reported that functionally active μ -opioid receptors (MOR) are constitutively expressed at relatively low levels by developing T cells in the thymus. However, very little is known about the regulation of MOR expression by immature T cells. In this report, we first attempted to determine the effect of T cell receptor-induced T cell activation on the expression of MOR. We activated T cells with either the combination of anti-CD3 and CD28, or with superantigen, and observed a substantial increase in MOR transcript expression. We also chose to examine the effect of cytokine-mediated T cell activation on the expression of this opioid receptor. We selected certain cytokines that play a role in T cell development and are known to be present at functional levels in the thymus gland. Our results show that interferon γ (IFN γ), IL-1 β , and IL-2, and in particular transforming growth factor- β (TGF β), all induced significant increases in MOR transcript expression. On the other hand, both TNF α and IL-7 exhibited much weaker effects on MOR expression. These results show that MOR expression by developing T cells is strongly regulated by several cytokines involved in T cell development in the thymus gland.

Introduction

Both endogenous and exogenous μ -, κ - and δ -opioid compounds exert broad immunomodulatory activity, and this includes inhibition of antibody responses (Johnson et al. 1982; Heijnen et al. 1986; Taub et al. 1991; Bussiere et al. 1992; Bussiere et al. 1993; Vassou et al. 2008), phagocytic cell function (Rojavin et al. 1993; Casellas et al. 1991;

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Singh et al. 2007; Wang et al. 2008; Tomassini et al. 2003), and natural killer cell activity (Weber and Pert 1989; Bayer et al. 1990; Gaveriaux-Ruff et al. 1998). Studies from a number of laboratories have established, using reverse transcriptase-polymerase chain reaction (RT-PCR)-based techniques, that leukocytes express the μ -, κ -, and δ -opioid receptors (Chuang et al. 1994; Chuang et al. 1995; Belkowski et al. 1995b; Alicea et al. 1998). Furthermore, the sequences of these receptor transcripts are essentially identical to sequences obtained from cDNA clones acquired from neuronal cells.

It has been suggested that opioids modulate the immune system primarily by regulating the inflammatory response (Eisenstein and Hilburger 1998; Rogers et al. 2003), and this is consistent with the presence of elevated levels of endogenous opioids at sites of inflammation (Mousa et al. 2001; Mousa et al. 2002; Cabot et al. 2001). However, little is known about the homeostatic role of opioids in the function of the immune system. There is evidence that opioid receptors modulate the developmental activity of T cells in the thymus (Sei et al. 1991; Fuchs and Pruett 1993; Frier and Fuchs, 1993; McCarthy et al. 2004; Guan et al. 1997). This is consistent with the finding that opioid receptors are constitutively expressed by developing T cells in the thymus (McCarthy et al. 2001; McCarthy et al. 2004; Belkowski et al. 1995a). We have previously demonstrated that the μ -opioid receptors (MOR) expressed by developing T cells are functionally active, based on the ability of these cells to exhibit a chemotactic response to MOR agonists in vitro (McCarthy et al. 2001). It is well established that morphine administration results in a significant depletion of thymocytes, and this appears to be due to both direct and indirect effects on the immature T cells (Fuchs and Pruett 1993; Sei et al. 1991). Both KOR and DOR are also expressed by developing T cells in the thymus, based on the demonstration of receptor transcripts, or the response of thymocytes to highly selective KOR or DOR agonist treatments in vitro (Belkowski et al. 1995b; Belkowski et al. 1995a; Alicea et al. 1998; Linner et al. 1995; Guan et al. 1998; Zhang and Rogers 2000). Finally, our previous studies have shown that both KOR and DOR participate in the developmental processes that are a part of positive and negative selection of T cells in the thymus (McCarthy et al. 2004; Guan et al. 1997). In contrast, much less is known at this time about the direct role of MOR in the development of T cells.

Little is known about the regulation of opioid receptor expression during T cell maturation in the thymus. The expression of MOR is particularly important since this opioid receptor is the primary receptor that is activated following morphine administration. In the present report, we attempted to determine the effect of T cell activation on the expression of MOR by thymocytes, since activation through the T cell receptor is reported to induce MOR expression in mature T cells (Borner et al. 2008). Moreover, activation of developing T cells via the T cell receptor is an essential component of T cell development in the thymus gland. We also chose to examine the influence of certain cytokines that play a role in T cell development and are known to be present in the thymus during T cell maturation, on MOR expression. We analyzed the effect of IL-1 β , IL-2, IL-7, Interferon γ (IFN γ), Tumor Necrosis Factor α (TNF α), and Transforming Growth Factor β (TGF β) on MOR transcript expression. Our results suggest that MOR expression is under the control of regulatory influences provided during T cell differentiation within the microenvironment in the thymus gland.

Methods

Cell culture

Highly viable single-cell suspensions of thymocytes were obtained from C57Bl/6J mice by standard procedures as described previously (Guan et al. 1994). The DPK cell line was obtained from Dr. Jonathan Kaye of the Scripps Research Institute (Kaye and Ellenberger

1992), and was derived from a spontaneous thymic lymphoma of the T cell receptor transgenic mouse strain AND-TCR. The DPK cells express both CD4 and CD8, and were maintained in Click's medium supplemented with 10% FCS, 2 mM L-glutamine, 25 mM HEPES, 50 μ M β -mercaptoethanol, 0.15% sodium bicarbonate, and gentamicin. The D1F4 cell line fails to express either CD4 or CD8, but expresses the T cell receptor/CD3 complex. This line was originally derived from a p53 knockout mouse (Khaled et al. 1999), was obtained from Dr. Scott Durum (National Cancer Institute), and is dependent on IL-7 for growth and survival. This line was grown in RPMI-1640 supplemented with 10% FCS, 2 mM L-glutamine, 25 ng/ml IL-7 and gentamicin.

Treatment of T cells

Cells were treated with the cytokines IL-1 β , IL-2, IL-7, IFN γ , TNF α , or TGF β (R & D Systems, Inc.; Minneapolis, MN) at the designated concentrations. Thymic T cells were activated via the T cell receptor and CD28, using anti-CD3 and anti-CD28, by a standard protocol. Anti-CD3 (Pharmingen, San Diego, CA) in PBS was added to coat culture wells at a concentration of 2.5 g/ml, and incubated overnight at 4°C. The anti-CD3/PBS mixture was aspirated, and the culture wells were washed gently with cold PBS to remove unbound antibody. Anti-CD28 (Pharmingen) at a final concentration of 2.5 g/ml was then added together with cells at a cell density of 1×10^6 cells/ml in a volume of 5 ml. Alternatively, thymocytes were activated with staphylococcal enterotoxin B (Sigma) (10 μ g/ml) and incubated for the designated times. The plates were incubated at 37°C with 5% CO₂ for the designated period of time, and the cells were harvested and RNA was extracted for mRNA analysis.

RNA isolation

Total RNA was isolated using the RNazol B protocol (Tel-Test Inc., Friendswood, TX) according to the manufacturer's instructions. Briefly, cells were harvested, treated with RNazol B, the cell lysate was centrifuged, and the supernatant was subjected to chloroform extraction. The aqueous phase was extracted with isopropanol, and the RNA was then pelleted at 5400 g for 15 minutes at 4°C. The RNA was washed once with ethanol and stored at -70 °C for later use. The concentration and purity was assessed by spectrophotometry, and the integrity of the RNA determined using electrophoresis in diethyl pyrocarbonate agarose.

RT-PCR

Reverse transcription was carried out using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). An RNA/primer mix was prepared using random hexamers (0.5 μ g/ml) and 1mM dNTP; 1 μ g of RNA was added, incubated for 5 min at 65° C followed by incubation on ice for 1 min. This reaction was diluted with buffer containing 40 units of RNaseOUT recombinant ribonuclease inhibitor, incubated at ambient temperature for 2 min, and followed by addition of 50 units of SuperScript II. These samples were incubated at ambient temperature for 10 min, followed by incubation at 42°C for 50 min, and finally the reactions were terminated at 70° for 15 min. RNase H was then added followed by incubation at 37° C for 20 min. These samples were used for PCR in a buffer containing Thermophilic DNA buffer (Promega), 3mM MgCl₂ (Promega), 0.2 mM dNTP mix (Promega), 0.4 M of the respective primers, 1 unit of *Taq* DNA polymerase (Promega), and cDNA. PCR amplification was carried out in a DNA thermal cycler, GeneAmp PCR System 2400 (Perkin Elmer). The conditions of the PCR were as follows: 95°C for 1 minute, with an annealing temperature of 60°C for G3PDH or 57°C for MOR for 1 minute, and 72°C for 3 minutes for 25–35 cycles (25 for G3PDH and 35 for MOR). Each reaction was preceded with a 5 minute incubation at 94°C and terminated at the end of the cycling with a 72°C 15 minute extension and a 4°C hold. The primers for these experiments were:

G3PDHs- 5' TGA AGG TCG GTG TGA AAC GGA TTT GGC 3'; G3PDHs-5' CAT GTA GGC CAT GAG GTC CAC CAC 3'; MOR87s-5' GCA GAG GAG AAT ATC GGA CGC 3'; and MOR652as- 5' CCC ATC AGG TAG TTA ACA CTC TG 3'. The MOR primers were designed to allow for amplification of a PCR fragment which spans an intron-exon junction between exon 1 and exon 2, corresponding to position -196 to +370 relative to the ATG start site (Min et al. 1994).

Southern blot analysis of RT-PCR products

Both the G3PDH and MOR RT-PCR products were cloned into pGEM-T (Promega) using standard cloning procedures, and these clones were verified by sequencing (The Nucleic Acid/Protein Research Core Facility at the Joseph Stokes Jr. Research Institute, The Children's Hospital of Philadelphia, Philadelphia, PA). The cloned fragment was isolated from these plasmids using the appropriate restriction sites within pGEM-T as a source of probe DNA for the Southern blot analysis (HindIII and PstI for G3PDH, and HindIII for MOR). DNA probes were prepared using the Multiprime DNA labeling system (Amersham International plc., Amersham, United Kingdom) according to the manufacturer's protocol. Briefly, 16 ng of probe DNA was added to initiate a reaction by adding 2.5 µl primer solution, 2.5 µl α-³²PdCTP, and DNA polymerase 1 ('Klenow fragment') followed by incubation at 37°C for 90 minutes. Purification of the probe was carried out using a Pharmacia Nick Column (Amersham Pharmacia Biotech., Uppsala, Sweden). The probe was denatured at 95–100°C for 2 minutes prior to use in the Southern blotting.

The RT-PCR products from each of the cell culture experiments was subjected to electrophoresis, and the gels were transferred to positively charged nylon membranes (Ambion, Austin, TX) using the standard capillary action technique, using a chromatography paper wick, and a transfer buffer composed of 5X SSC/400mM NaOH. The DNA was transferred for 2–3 hours, and the DNA in the nylon membranes was immobilized by UV cross-linking, followed by treatment with a pre-hybridization solution (25mM KPO₄, pH 7.4, 5X SSC, 5X Denhardt's solution, 50 µg/ml salmon sperm DNA (Gibco/BRL), 50% formamide (Fluka, Milwaukee, WI) and incubated at 42 °C with constant shaking for 6 hours. The membranes were then probed with 500,000 cpm of the probe/ml in a hybridization solution (pre-hybridization solution plus 10% dextran sulfate), and incubation of the membranes overnight at 48°C. The membranes were washed 3 times at 20-minute intervals with 2X SSC/ 10% sodium dodecyl sulfate (SDS) at room temperature followed by two 20-minute washes with 0.1X SSC/ 10% SDS at 52°C. The membranes were dried, and autoradiography was carried out in x-ray cassettes containing intensifying screens at -70 °C overnight or for desired exposure time and then developed. Gels were also exposed to Bio-Rad phosphorimaging screens and phosphorimaging analysis was performed using the Bio-Rad G-25 imager.

cAMP analysis

Thymocytes were cultured for 24 hr with or without TGFβ (10U) as described above, washed, and returned to culture in the RPMI-1640 without serum. These cells were then treated with 1mM 3-isobutyl-1-methylxanthine (IBMX) for 15 in at 37°C, and the medium was then replaced with medium containing 1 mM IBMX, 30 µM forskolin, and varying concentrations of DAMGO for 10 min at 37°C. Cells were then harvested, lysed, and the intracellular cAMP expression was determined by ELISA using the cAMP XP Assay Kit (Cell Signaling Technology, Danvers, MA) following the manufacturer's protocol using to determine cAMP levels in 50% of the total cell lysate. Results are reported as pmol/ng protein of cAMP.

Statistical analysis

Data were subjected to assessment of statistical significance using Mann-Whitney nonparametric analysis where significance is considered as $P < 0.05$.

Results

T cell activation induces MOR expression by immature T cells

We previously found that thymocytes exhibit functional MOR activity, based on the ability of these cells to manifest a significant chemotactic response toward a MOR-selective agonist (McCarthy et al. 2001). Based on these findings, we examined the expression of MOR transcripts in resting thymocytes, and we consistently observed a measurable level of MOR expression (Figure 1A). Previous reports have shown that activation of T cells via the T cell receptor induces MOR expression by mature T cells (Borner et al. 2008). We carried out experiments in which we activated thymic T cells with staphylococcal enterotoxin B (SEB) and determined the level of MOR expression. Our results (Figure 1A) show that superantigen-mediated T cell activation induces substantial MOR expression. Superantigens induce T cell activation primarily through the T cell receptor, but these microbial agents also bind the major histocompatibility complex class II dimers with moderate affinity, and may induce a secondary signal as a consequence of this binding activity (Taub et al. 1992). As a consequence, we chose to extend our studies using a more defined method of T cell activation. We treated thymocytes with anti-CD3 or anti-CD28, or the combination, and assessed MOR expression after 24 hours. The results show (Figure 1B) that stimulation of thymocytes by the combination of anti-CD3 and anti-CD28 induces MOR expression. It was interesting that stimulation of these cells by either anti-CD3 or anti-CD28 alone failed to induce substantial MOR expression. We verified these results using two murine immature T cell lines, DPK and D1F4, and the results show (Figure 1C & D) that anti-CD3 with anti-CD28 induces MOR expression in these cell lines as well. These experiments suggest that the induction of MOR expression is a down-stream product of a T cell receptor activation pathway.

Additional experiments show (Figure 1E) that the activation of MOR expression following anti-CD3/CD28 treatment reaches a maximum between 4 and 8 hours in thymocytes. We observed similar kinetics with both the DPK and D1F4 immature T cell lines (data not shown). Moreover, MOR induction consistently declined by 24 hours in both the primary thymocytes and the T cell lines.

Induction of MOR expression in thymocytes following cytokine administration

We chose to extend these studies by examining the effect of several cytokines that are known to be produced within the thymus gland on the expression of MOR. We treated thymocytes with IL-1 β , IL-2, IL-7, IFN γ , TGF β , or TNF α , and examined the level of MOR transcript expression. The results (Figure 2A) show that while each of these cytokines induced MOR expression, the greatest increase in MOR transcript levels was achieved with TGF β (Figure 2B). We also observed substantial induction of MOR expression with IFN γ , IL-2, and IL-1 β , while the effects of TNF α and IL-7 were consistently weaker. Additional analysis (Figures 3A–D) showed that the effects of these cytokines exhibited similar dose-dependent patterns, where the highest doses exhibited substantially weaker effects on MOR expression. These results show that the optimal doses of these cytokines varied between 5U/ml (TGF β), 50U/ml (IFN γ , IL-2, TNF α) or 100U/ml (IL-1 β , IL-7).

In an effort to verify the results obtained by analysis of transcript levels using RT-PCR, we evaluated the effect of TGF β on the expression of functional MOR. We treated thymocytes with or without TGF β (5U/ml) for 24 hr, and then determined the capacity of DAMGO to

induce inhibition of a forskolin-stimulated cAMP response. The results show (Figure 3E) that the TGF β -treatment resulted in a significant enhancement of the DAMGO-induced inhibition of the cAMP response. For example, the cAMP response of cells which did not receive the TGF β treatment was 80 ± 18 pmol, while the TGF β -treated cells exhibited a significantly reduced cAMP response (37 ± 10 pmol). These results are consistent with the data described above which demonstrate an up-regulation of MOR expression following TGF β treatment.

Discussion

The development of mature T cells from immature T cell precursors within the thymus gland requires complex processes that are mediated through the T cell receptor and result in either positive or negative selection. Previous studies by Borner et al. (Borner et al. 2008) have shown that T cell activation results in an up-regulation of MOR transcript expression. However, the regulation of MOR during T cell development has received little attention, even though there are functional data that show thymocytes express functioning MOR (McCarthy et al. 2001). We examined the impact of T cell receptor-mediated activation on MOR expression using two distinct approaches. First, we tested the ability of the commonly employed combination of anti-CD3 together with anti-CD28 to activate the T cell receptor. Second, we employed superantigen14 mediated T cell activation, a process which involves MHC class II-dependent triggering of the T cell receptor. The latter process is somewhat more physiological, since normal antigen-driven activation of T cells is also dependent on the presentation of antigen in the context of the MHC complex. However, we report here that in both cases, the activation of the T cell receptor resulted in a substantial induction of MOR transcript expression.

It is apparent that T cell development is also highly dependent on regulatory influences that are provided by various cytokines. Thymic epithelial cells (TECs) are the major, but not sole, source of cytokines in the thymus (Yarilin et al. 2004). We chose to study cytokines that are produced primarily by the TECs (IL-1 β , TNF α , IL-7 and TGF β), as well as cytokines that are produced by developing or mature T cells (IL-2 and IFN γ), or by both (IL-7, TGF β and TNF α). These cytokines are produced in the thymus gland as a part of the normal processes associated with support of T cell development, including the spontaneous production of IL-1 β , TNF α , and IL-7 by TECs (Wolf et al. 1992). It is also apparent that TECs can be induced to up-regulate cytokine production in response to cell contact with immature T cells (Maeurer and Lotze 1998). On the other hand, these and other T cell products can be strongly upregulated following T cell receptor-driven T cell activation (Mingari et al. 1996; Zhang and Rogers 2000). Finally, there is considerable cross-regulation of cytokine production by other cytokines, since these T cells express a diverse array of cytokine receptors (Yarilin et al. 2004).

We observed the strongest upregulation of MOR expression following TGF β administration. This result was somewhat surprising given the immunosuppressive activity of this cytokine for mature T cells. This cytokine impairs T cell development at an early stage (CD44+CD25+ to CD44-CD25+) in thymic organ cultures (Plum et al. 1995; Suda et al. 1992), and inhibits the proliferation of thymocytes (Mossalayi et al. 1995). The induction of MOR expression by TGF β in the present studies may suggest that this cytokine directs MOR expression at the expense of T cell growth in the thymus. The effects of TGF β on T cell development are diverse, and results in the literature suggest that this cytokine has a pleiotropic role in the development of T cells. Studies to assess the role of this cytokine in T cell development have relied largely on the use of TGF β -knockout mice. There are mixed results from these studies, but Christ et al. (1994) have reported that these mice exhibit hyperplasia of lymphocytes in the thymus medulla, but reduced cellularity in the thymus

cortex. Moreover, Boivin et al. (1995) have reported that TGF β -deficient mice have reduced CD4+8+ cells, but an increase in both the more immature developing T cells, and the more mature CD4-single positive cells. It has been suggested that TGF β may function to promote the development of CD8+ T cells during the course of normal T cell differentiation in the thymus (Christ et al., 1994; Licona-Limon and Soldevila, 2007). Additional work to examine the level of expression of MOR in conditional TGF β -deficient mice might clarify the role of this cytokine in MOR expression.

We also observed substantial upregulation of MOR expression in response to the T cell products IL-2 and IFN γ . While there is some suggestion that IL-2 promotes developing T cell growth (Varas et al. 1997; Tentori et al. 1988), recent evidence shows that IL-2 is required for the development of T regulatory cells (Malek et al. 2008), and there is ample evidence that this cytokine contributes to negative T cell selection in the thymus (Bassiri et al. 2001; Porter et al. 2000). Similarly, IFN γ also promotes apoptosis, and anti-IFN γ antibody reverses this effect, suggesting that these two cytokines have a suppressive effect on developing T cells (Groux et al. 1993). Our results with IFN γ are in contrast to the data reported by Kraus et al. (Kraus et al. 2006) who showed that IFN γ inhibits the expression of MOR in neuronal and mature T cells. The difference between our results and the report from Kraus et al. likely reflects the dissimilarity between signaling pathways in immature and mature T cells.

The capacity of three cytokines (TGF β , IL-2, and IFN γ) with inhibitory and/or proapoptotic activity to up-regulate MOR expression suggests the possibility that MOR expression may participate in the negative selection of developing T cells in the thymus. This result would be consistent with studies we have previously reported which showed that DOR activation resulted in a significant reduction in the maturation of CD4+ mature thymic T cells (McCarthy et al. 2004). The latter studies also showed that DOR activation promotes superantigen-driven negative selection in thymic organ culture, and negative selection is depressed in thymic T cells from DOR-knockout mice. The results in the present report suggest the possibility that both MOR and DOR participate as a component of the negative-selection process for developing T cells in the thymus.

We also observed that IL-1 β and TNF α upregulated MOR expression. These cytokines have mixed effects on T cell development. Neither cytokine alone strongly regulates growth of immature T cells, but when used in combination with additional cytokines both enhance proliferation and differentiation of immature thymocytes (Zlotnik et al. 1995; Zuniga-Pflucker et al. 1995). However, TNF α has the capacity to induce apoptosis of immature developing thymic T cells, an effect that is probably mediated through TNF receptor type 2 (Baseta et al. 2000; Pezzano et al. 2001). Finally, the weakest induction of MOR expression was induced by IL-7, a cytokine with a very well established ability to promote differentiation, growth and survival of immature T cells (Ma et al. 2006). Indeed, IL-7 is required at an early stage of T cell development, and the emergence of CD4/CD8 double-positive thymocytes is blocked in IL-7 receptor defective mice (Nakajima et al. 2000). The relatively weak induction of MOR by IL-7 suggests that MOR is unlikely to play a major role in the functional consequences of IL-7 in thymocyte maturation.

Finally, our present results suggest that while MOR is expressed constitutively by developing T cells, this receptor is substantially up-regulated following activation through either the T cell receptor or through certain cytokine receptors. The consequences of MOR expression on T cell differentiation are not clear at this time, but studies on the role of DOR suggest the possibility that opioid receptors may influence negative selection and/or apoptotic processes that are essential for T cell maturation. This hypothesis would be consistent with our previous studies on KOR, which showed that the development of the

DPK (CD4/CD8 double-positive) T cell line into the CD4+CD8- lineage is inhibited following KOR agonist treatment (Guan et al. 1998). Moreover, we have also shown that superantigen-induced T cell IL-2 production, and both IL-7 receptor and IL-2 receptor α -chain (CD25) expression, are inhibited following KOR agonist administration (Guan et al. 1997; Guan et al. 1998; Zhang and Rogers 2000). More work will be required in order to clarify the precise role of MOR in the development of T cells in the thymus.

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Fig. 1.

RT-PCR analysis of MOR expression in resting and activated primary thymocytes. Panel A. Thymocyte mRNA was subjected to PCR for either MOR (Panel A) or G3PDH (Panel B) was conducted with (Lanes 2 and 4) or without (Lanes 1 and 3) treatment with RT using resting (Lanes 1 and 2) or SEB-activated thymocytes (Lanes 3 and 4). Activated cells were cultured for 24 hr with SEB (10 μ g/ml). The PCR products were then subjected to Southern blot analysis. Results are representative of 5 experiments. Panel B. RT-PCR analysis of MOR and G3PDH expression in untreated thymocytes (Lane 1), or cells treated for 8 hr with anti-CD3 alone (Lane 2), anti-CD28 (Lane 3), or the combination of anti-CD3 and CD28 (Lane 4). RT-PCR was also carried out using DPK cells (Panel C) or D1F4 cells (Panel D)

for MOR expression in untreated cells (Lane 1), or cells cultured with the combination of anti-CD3 anti-CD28 (Lane 2). Results are representative of 6 experiments. Panel E. Time course of MOR expression in thymocytes treated with the combination of anti-CD3 and anti-CD28 for 0 (Lane 1), 2 (Lane 2), 4 (Lane 3), 8 (Lane 4), or 24 hr (Lane 5) as described in Figure 2. Results are representative of 3 experiments.

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Fig. 2.

Effect of treatment with various cytokines on MOR expression in thymocytes. Panel A. Cells were cultured without (Lane 1), or for 24 hr with anti-CD3/CD28 (Lane 2), IFN γ (50U/ml; Lane 3), IL-2 (50U/ml; Lane 4), TNF α (50U/ml; Lane 5), IL-1 β (100U/ml; Lane 6), TGF β (5U/ml; Lane 7), or IL-7 (100U/ml; Lane 8) as described in Figure 2. Results are representative of 6 experiments. Panel B. RT-PCR was carried out with thymocytes treated with the designated cytokines, and the level MOR expression was normalized relative to the expression of G3PDH from the same mRNA extract. Results are expressed as MOR Relative Units (percentage of G3PDH expression). Results are presented as the mean ($\pm$ SE) of at least 3 experiments. All bars are statistically significant ($P < 0.05$).

Fig. 3.

Analysis of the effects of cytokines on MOR expression in thymocytes. Panel A. Cells were treated with IL-1 β at 0, 10, 50, 100, 200, or 400 U/ml (Lanes 1–6). Panel B. Cells were treated with TGF β at 0, 0.02, 0.25, 2.5, 5, or 10 U/ml (Lanes 1–6). Panel C. Cells were treated with IFN γ at 0, 1, 10, 50, 100, 200 U/ml (Lanes 1–6). Following treatment with the designated doses of cytokine for 24 hr, the expression of MOR and G3PDH were determined. Results are representative of at least 4 experiments. Panel D. Cells were treated with the designated doses of cytokine for 24 hr, and the normalized MOR expression was determined. Results are presented as the mean ($\pm$ SE) of at least 4 experiments. All data points are statistically significant (compared with 0 U/ml) except for IL-1 at 10 U/ml. Panel

E. Increased inhibition of forskolin-stimulated cAMP accumulation by DAMGO administration in TGF β -treated thymocytes. Thymocytes were cultured with or without TGF β (5U/ml) for 24 hr and then subjected to analysis of the inhibition of forskolin-induced cAMP levels with graded doses of DAMGO. The level of cAMP in cell lysates was determined by ELISA as described in the Materials and Methods section. Results are presented as the mean ($\pm$ SE) of 3 experiments. Data points for DAMGO doses above 1 nM are statistically significant ($P < 0.05$) comparing the TGF β -treated and non-treated groups.

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