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T lymphocyte proliferation is suppressed by the opioid growth factor ([Met^5]-enkephalin)–opioid growth factor receptor axis: Implication for the treatment of autoimmune diseases

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ABSTRACT

Opioid peptides function as immunomodulatory molecules. Reports have linked the opioid growth factor (OGF), [Met^5]-enkephalin, and its receptor OGFr to autoimmune diseases. OGF repressed the incidence and magnitude of myelin oligodendrocyte-induced experimental autoimmune encephalomyelitis in mice. Given the extensive connection between the immune system and autoimmune diseases, the present study was conducted to examine the relationship of the OGF–OGFr axis and T lymphocyte proliferation. Splenic-derived mouse lymphocytes were stimulated with phytohemagglutin (PHA). All non-stimulated and PHA-stimulated T lymphocytes had immunoreactivity for OGF-like enkephalin and OGFr. OGF markedly suppressed T lymphocyte number in a dose-dependent manner. However, PHA-stimulated T lymphocytes were not altered in cell number by a variety of natural and synthetic opioid-related compounds, some specific for μ, δ, and κ opioid receptors. Persistent blockade of opioid receptors with the general opioid antagonist naltrexone (NTX), as well as antibody neutralization of OGF-like peptides, had no effect on cell number. Non-stimulated T lymphocytes exhibited no change in cell number when subjected to OGF or NTX. Treatment of T lymphocytes with siRNAs for μ, δ, or κ opioid receptors did not affect cell number, and the addition of OGF to these siRNA-exposed cultures depressed the population of cells. T lymphocytes treated with OGFr siRNA also had a comparable number of cells to control cultures, but the addition of OGF did not alter cell number. DNA synthesis in PHA-stimulated T lymphocytes exposed to OGF was markedly decreased from PHA-stimulated cultures receiving vehicle, but the number of cells undergoing apoptosis or necrosis in these cultures was similar to control levels. T lymphocytes subjected to siRNA for p16 and/or p21 had a comparable number of cells compared to controls, and treatment with OGF did not depress cell number in preparations transfected with both p16 and p21 siRNA. These data reveal that the OGF–OGFr axis is present in T lymphocytes and is capable of suppressing cell proliferation. However, T lymphocytes are not dependent on the regulation of cell proliferation by this system. The results showing that the OGF–OGFr axis is an immunosuppressant, offers explanation for reports that autoimmune diseases can be modulated by this system.

Introduction

Opiates and endogenous opioid peptides (referred to collectively as opioids) function as immunomodulatory molecules within both the immune system and the brain (Carr et al. 1996; McCarthy et al. 2001; Peterson et al. 1998; Salzet and Tasiemski 2001; Sharp et al. 1998). The three classical opioid receptors, μ (MOR), δ (DOR), and κ (KOR), as well as nonclassical opioid receptors (e.g., nociceptin/ophanin FQ) have been found on immune cells in multiple studies using pharmacological and cellular/molecular approaches (Carr et al. 1996; McCarthy et al. 2001; Sharp et al. 1998; Sharp 2004; Chuang et al. 1994, 1995). Opioid peptide gene and protein expression for the three families of endogenous opioids: pro-opiomelanocortin, proenkephalin, and prodynorphin have been reported for cells of the immune system (Roth et al. 1989; Linner et al. 1995; Kamphuis et al. 1998; Lolait et al. 1986; Martin et al. 1987; Smith and Blalock 1981). The picture emerging about the role of the endogenous opioids often indicates complex and divergent effects of these peptides that may be dependent or independent on opioid receptors.

[Met^5]-enkephalin is an endogenous opioid peptide derived from preproenkephalin (Akil et al. 1984; Noda et al. 1982). This

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peptide was originally found to be related only to neural cells, and to serve as a neuromodulatory element (Akil et al. 1984). Subsequent studies revealed that [Met^5]-enkephalin is a regulator of neural and non-neural cell proliferation (Zagon et al. 2002). This peptide has been termed the opioid growth factor (OGF) to signify its distinct distribution and biological role (Zagon et al. 2002). OGF action is mediated by the OGF receptor (OGFr). Although OGF–OGFr has the same pharmacological properties of opioid peptides that interact with classical opioid receptors (e.g., blockade by naloxone and stereospecificity), OGFr has nucleotide and protein sequences that are entirely different from that of classical opioid receptors (Zagon et al. 2002). OGF–OGFr interactions inhibit cell proliferation in a tonically active fashion, and rigorously maintain homeostasis of cellular renewal and restorative processes (e.g., wound healing) (Zagon et al. 1997, 1998; Wilson et al. 2000; Blebea et al. 2000; McLaughlin et al. 2005). The OGF–OGFr axis upregulates the cyclin dependent inhibitory kinase pathway, specifically p16 and p21 (Cheng et al. 2007a,b, 2009a), and does not induce apoptosis or necrosis (Zagon and McLaughlin 2003), or differentiation (Zagon and McLaughlin 2005). OGFr is localized on the outer nuclear envelope, and the OGF–OGFr complex undergoes nucleocytoplasmic transport using nuclear localization signals encoded on OGFr for guidance by karyopherin β and Ran through the nuclear pore (Zagon et al. 2005a; Cheng et al. 2009b, 2010a). Blockade of OGF from OGFr by opioid antagonists, antisense strategies, or siRNA technology, as well as neutralization of OGF by antibodies, accelerates the pace of cell proliferation (Zagon et al. 2005b; Donahue et al. 2009).

A number of reports have linked the OGF–OGFr system to autoimmune diseases (Zagon et al. 2009a, 2010). In a study by Zagon et al. (2010), OGF repressed the incidence and magnitude of myelin oligodendrocyte-induced experimental autoimmune encephalomyelitis (EAE) in mice. Splenocytes obtained from normal mice and stimulated by phytohemagglutin (PHA) were found to be depressed in cell number by OGF. Activation of the OGF–OGFr axis also is known to decrease tumor incidence, delay tumor growth, and extend survival in mice with xenografts of human cancer (McLaughlin et al. 2008; Zagon et al. 2008a). In view of the connection between the immune system and autoimmune diseases, as well as neoplasia, the present study was conducted to investigate fully the relationship of the OGF–OGFr axis and T lymphocyte proliferation.

Materials and methods

Mice

Four to six-week-old C57BL/6 male and female mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in-house. All mice were housed in a controlled-temperature room (22–25 °C) with a 12–12 h light/dark cycle (lights on 07:00–19:00). Standard rodent diet (Harlan Teklad, catalog number 2018) and water were available *ad libitum*.

Isolation of lymphocytes

Lymphocytes were isolated from the spleens of adult mice that were euthanized by cervical dislocation. Spleens were removed, and mechanically dissociated with 60-mesh stainless steel screens (Sigma–Aldrich, St. Louis, MO). Cells were collected and the red blood cells lysed in a hypotonic saline solution (17 mM Tris, 0.14 mM NH_4Cl, pH 7.65) for 5 min at 37 °C. The resulting lymphocytes were counted by trypan blue dye exclusion and suspended in Iscove's modified Dulbecco's media (IMDM) supplemented with 10% (v/v) fetal bovine serum (Gibco, Carlsbad, CA), 0.075% (w/v) sodium bicarbonate, 0.00035% (v/v) β-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate. The splenic-derived lymphocytes were either seeded onto slides for immunohistochemical studies or cultured in 24- or 96-well flat-bottom plates for proliferation studies. For all experiments, cells were incubated in a humidified atmosphere at 37 °C with 5% CO_2.

Immunohistochemistry

For immunohistochemical studies, 5×10^5 splenic-derived lymphocytes were added in 500 μl supplemented IMDM in 24-well plates, and were either left as non-stimulated, or were stimulated with 10 μg/ml of PHA (Sigma–Aldrich) for 72 h. Subsequently, cells were harvested, counted, and resuspended at 1×10^6 cells/ml in supplemented IMDM. Two hundred μl of the cell suspension was placed on glass slides and allowed to dry overnight. Preparations were fixed and permeabilized with 95% (v/v) ice-cold ethanol and 100% acetone, and processed for immunohistochemistry using antibodies to OGF, OGFr, and CD3 according to published procedures (Donahue et al. 2009). Polyclonal antibodies to OGF and OGFr were generated in our laboratory and have been fully characterized (Zagon and McLaughlin 1993), while rat anti-mouse CD3 was purchased from eBioscience (clone 17A2, San Diego, CA). Some cells were incubated with secondary antibodies only (rabbit anti-rat IgG, FITC, ab6730D, Abcam, Cambridge, MA; goat anti-rabbit IgG, TRITC, A11011, InVitrogen, Carlsbad, CA) and served as negative controls. All cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize nuclei. At least 4 slides (containing at least 500 cells/slide) per condition were examined.

Mitogen-induced lymphocyte proliferation

For lymphocyte proliferation assays, 5×10^5 splenic-derived lymphocytes were cultured in 500 μl supplemented IMDM in 24-well plates, and were either non-stimulated or were stimulated with 5 or 10 μg/ml of PHA for 72 h. Immediately upon the addition of PHA, cells were treated daily with compounds; media and compounds were not replaced. All compounds were prepared in sterile water and dilutions represent final concentrations. An equivalent volume of sterile water was added to control wells. At designated times, cells were collected, stained with trypan blue, and counted using a hemacytometer. At least two aliquots per well and at least 2 wells/treatment/time point were counted.

Analysis of lymphocyte cell surface markers

Flow cytometric analysis of lymphocyte cell surface markers was determined as described previously (Nair and Bonneau 2006). Briefly, lymphocytes were either non-stimulated, or stimulated with 10 μg/ml PHA for 72 h. CD16/CD32 Fcγ receptors were blocked with an antibody obtained from 2.4G2 hybridoma cell culture supernatants supplemented with 10% mouse serum (Sigma–Aldrich). Cell surface-expression of CD4, CD8, and B220 were detected using anti-CD4 FITC antibody (clone GK1.5; eBioscience), anti-CD8a FITC antibody (clone 53-6.7; eBioscience), and anti-CD45R FITC antibody (clone RA3-6B2, eBioscience), respectively. Following washes with FACS buffer (Hank's buffered saline solution supplemented with 1% (w/v) BSA), cells were resuspended in FACS buffer and immediately analyzed by flow cytometry.

Specificity of endogenous OGF

The specificity of endogenous OGF for lymphocyte growth was evaluated by treating PHA (10 μg/ml)-stimulated lymphocytes with a rabbit polyclonal antibody to OGF (1:200; Co172) (Zagon and McLaughlin 1993); pre-immune rabbit serum (1:200; IgG)

served as a control. Cells were treated with antibody, serum, or sterile water daily without replenishing media, and cell viability was determined following 72 h of treatment, with at least two aliquots/well and at least 2 wells/treatment counted.

Specificity of receptors: knockdown with MOR, DOR, KOR, or OGFr siRNA

MOR, DOR, and KOR targeted siRNAs, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The OGFr-targeted siRNA (antisense, 5′-uagaaacucagguuuggcg-3′; sense, 5′-cgccaaaccugaguuucua-3′) was designed and obtained as a ready-annealed, purified duplex probe from Ambion (Austin, TX). 5×10^5 cells/well were seeded in 24-well plates containing 500 μl/well of supplemented IMDM without antibiotics. Non-stimulated or PHA-stimulated cells were immediately transfected with 20 nM of MOR, DOR, KOR, OGFr, or scrambled siRNA solutions with Oligofectamine reagent (Invitrogen). Cells were incubated for 4 h at 37 °C before the addition of 10^{-6} M OGF, 10^{-5} M NTX, or an equivalent volume of sterile water. Cultures were incubated for an additional 72 h, with OGF, NTX, or sterile water re-administered daily. Cells were collected and either counted or utilized for protein isolation.

Assessment of DNA synthesis, apoptosis, and necrosis

The effect of OGF or NTX on DNA synthesis, apoptosis, and necrosis of PHA-stimulated lymphocytes was evaluated. For [^{3}H]-thymidine proliferation assays, 80,000 cells/well were seeded into 96-well plates in 80 μl/well of supplemented IMDM containing 10 μg/ml PHA and either 10^{-6} M OGF, 10^{-5} M NTX, or an equivalent volume of sterile distilled water. A 1 μCi aliquot of [^{3}H]-thymidine was added to each well and incubated for 4 h. Cells were collected at 24 h onto Whatman glass fiber discs using an automated cell harvester, and radioactivity was determined by liquid scintillation analysis.

For apoptosis and necrosis measurements, cells were stimulated with 10 μg/ml PHA as above, and treated daily with either 10^{-6} M OGF, 10^{-5} M NTX, or an equivalent volume of sterile water. Cells were harvested at 12, 24, 48, and 72 h for flow cytometric analysis. Apoptosis was detected using a fluorescent Annexin V FITC-conjugated antibody (catalog number 556420, Becton Dickinson Co, Franklin Lakes, NJ) that binds with high affinity to phosphatidylserine, which is translocated from the inner (cytoplasmic) to outer (cell surface) leaflet soon after the induction of apoptosis. Cells undergoing necrosis were detected with flow cytometry using 7-amino-actinomycin D (7AAD) (catalog number 00-6993-50, e-Bioscience).

siRNA-mediated knockdown of p16 and p21

Non-stimulated or PHA-stimulated (10 μg/ml) lymphocytes were transfected with 20 nM of p16 and/or p21 siRNA (Santa Cruz Biotechnology), or scrambled siRNA (Ambion) with Oligofectamine reagent (Invitrogen) in antibiotic-free media for 4 h at 37 °C before the addition of OGF (10^{-6} M) or an equivalent volume of sterile water. Cultures were incubated for an additional 72 h with OGF or sterile water administered daily. At 72 h after the start of the transfection, cells were collected and either counted or used for protein isolation.

Western immunoblotting

To determine the level of protein knockdown resulting from siRNA transfections, cells were collected and solubilized in 200 μl RIPA buffer (1× PBS, 10 μM IGEPAL, 1 mg/ml SDS, 5 mg/ml deoxycholic acid), containing a cocktail of protease and phosphatase inhibitors (Roche, Boulder, CO). Total protein concentrations were measured using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Equal amounts of protein (60 μg) were subjected to 15% (w/v) SDS-PAGE followed by transfer of proteins onto nitrocellulose using standard protocols. The following antibodies were purchased from commercial sources: MOR (MOR-1 [H-80] rabbit IgG, catalog number SC-15310), DOR (DOR-1 [E-20], goat IgG, catalog number SC-7490), KOR (KOR-1 [H-70], rabbit IgG, catalog number SC-9112), p16 (F-12 mouse IgG (catalog number SC-1661), Santa Cruz Biotechnology); p21 (clone 6B6, mouse IgG (catalog number 550833), BD PharMingen, San Diego, CA); and β-actin (clone AC-15, Sigma–Aldrich). Anti-OGFr was made in our laboratory and has been fully characterized (Zagon and McLaughlin 1993). Membranes were probed with the above primary antibodies (1:200), followed by appropriate secondary anti-rabbit (Millipore, Billerica, MA; 1:5000), anti-mouse (GE Healthcare-Amersham Biosciences, Piscataway, NJ; 1:5000), or anti-goat (Santa Cruz; 1:2000) horseradish peroxidase-conjugated antibodies, and developed using a chemiluminescence Western blotting detection system (Amersham ECL, GE Healthcare). To determine equal loading of total protein, blots were stripped with stripping buffer (62.5 mM Tris–HCl and 100 mM β-mercaptoethanol/2% (w/v) SDS, pH 6.7) at 50 °C, and reprobed with a monoclonal antibody to β-actin (1:5000).

The optical density of each band was determined by densitometry (QuickOne, Bio-Rad Laboratories) and each value was normalized to β-actin from the same blot. The percentage of protein knockdown was calculated by dividing the normalized value of the transfected samples by the normalized value of the non-transfected samples. Means and SE were determined from at least 2 independent Western blots.

Fluorescent flow cytometery

Flow cytometric analysis was conducted with a FACSCanto® flow cytometer (Becton Dickinson, San Diego, CA). Using forward-angle light scatter and 90° light scatter profiles, electronic gates were set around the live cells and at least 50,000 events were collected per sample. Dot plots and histograms were analyzed using FlowJo® software (TreeStar, Inc., Ashland, OR).

Chemicals

The following compounds were obtained from the indicated sources: [Met^5]-enkephalin (OGF), [Leu^5]-enkephalin (Leu Enk), [D-Pen^{2,5}]-enkephalin (DPDPE), [D-Ala^2,MePhe^4,Glyol^5]-enkephalin (DAMGO), β endorphin (β-End), NTX, dynorphin A1-8 (Dyn), morphine sulfate, endomorphin 1 (Endo-1), endomorphin 2 (Endo-2), Sigma–Aldrich; U69,583, Upjohn Diagnostics (Kalamazoo, MI).

Statistical analysis

All data were analyzed (GraphPad Prism software GraphPad Software Inc., La Jolla, CA) using one-way analysis of variance, with subsequent comparisons made using Newman–Keuls tests.

Results

OGF and OGFr are present in splenic-derived T lymphocytes

Immunoreactivity for OGF (Fig. 1A) and OGFr (Fig. 1B) was localized to the cytoplasm of all non-stimulated, as well as all PHA (10 μg/ml)-stimulated, splenic-derived T lymphocytes

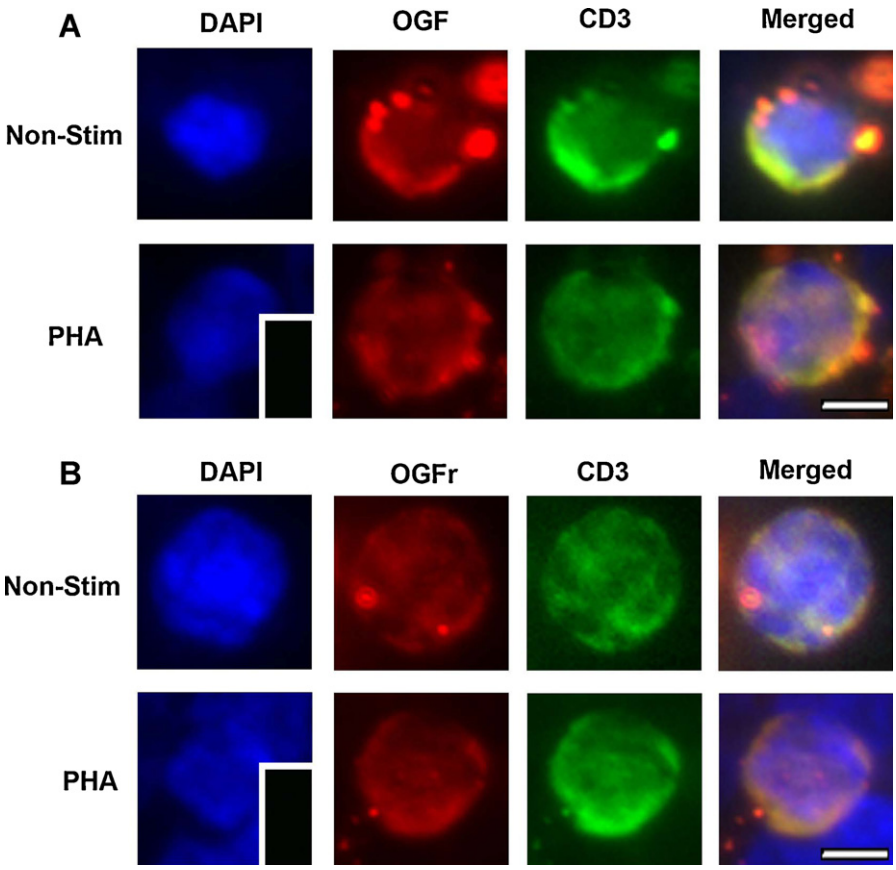

Fig. 1. The presence and distribution of OGF, OGFr, and CD3 in non-stimulated and PHA-stimulated lymphocytes. Photomicrographs of lymphocytes immunostained with polyclonal, ammonium-sulfate purified antibodies to OGF (1:100), OGFr (1:100), or CD3 (1:400). Anti rabbit TRITC-conjugated IgG (1:1000) served as the secondary antibody for OGF and OGFr, while anti rat FITC-conjugated IgG (1:1000) served as the secondary antibody for CD3. Nuclei were visualized with DAPI. Immunoreactivity of OGF (A) and OGFr (B) were associated with the cytoplasm, and a speckling of stain was noted in cell nuclei of these CD3-positive cells. Immunostaining was not detected in cell preparations incubated with secondary antibodies only (insets). Scale bar = 10 μm.

(CD3-positive) following 72 h of incubation; a speckling of immunoreactivity also was noted in the nucleus. No staining was recorded in specimens processed with secondary antibodies only (Fig. 1A and B).

PHA stimulates T cell proliferation

Splenic-derived lymphocytes grown in culture for 72 h in the absence of mitogenic stimulation (non-stimulated) were comprised of 93.4% B cells and 6.6% T cells (3.8% $CD4^+$, 2.8% $CD8^+$) (Fig. 2A). Cultures stimulated for 72 h with 10 μg/ml PHA (a T cell mitogen) were comprised of 50.3% B cells, and 49.7% T cells (6.9% $CD4^+$, 42.8% $CD8^+$); a decrease of 46% in B cells and an increase of 650% in T cells.

Exposure of splenic-derived lymphocytes to PHA showed a concentration-dependent effect on the number of cells (Fig. 2B). At 72 h of exposure to 5 μg/ml, cell number was increased by 39% in comparison to non-stimulated cultures; using 10 μg/ml of PHA, this increase was 62%. Thus, although both 5 and 10 μg/ml of PHA markedly stimulated lymphocytes, statistical comparison between these concentrations of PHA revealed that cultures subjected to 10 μg/ml had a significantly greater number of cells than preparations receiving 5 μg/ml ($p < 0.01$).

OGF suppresses the proliferation of splenic-derived lymphocytes stimulated with PHA

The number of non-stimulated lymphocytes was similar for the initial 24 h, but decreased 29% and 54% at 48 and 72 h, respectively (Fig. 3). Although the number of lymphocytes in non-stimulated and stimulated (10 μg/ml PHA) cultures was comparable at 24 h, at 48 and 72 h the stimulated cultures contained 66% and 262% more cells, respectively (Fig. 3A). Treatment of PHA-stimulated cells with 10^{-6} M OGF resulted in 26% fewer cells than those PHA-stimulated cultures treated with vehicle after 72 h; no differences were noted at 24 and 48 h (Fig. 3A). Based on the composition of these cultures (see Fig. 2A), the number of T lymphocytes at 72 h was estimated to be decreased by 54% from their respective controls.

To further document the inhibitory effects of OGF, OGF in concentrations ranging from 10^{-4} M to 10^{-10} M were added to PHA-stimulated (10 μg/ml) lymphocytes for 72 h, and a dose-dependent inhibitory effect was recorded (Fig. 3B). Dosages ranging from 10^{-4} M to 10^{-7} M reduced the number of lymphocytes by 17–33%. Based on the proportion of T and B cells in our 72 h cultures (see Fig. 2A), cultures treated with 10^{-4} M to 10^{-7} M OGF revealed a reduction of 33–66% T lymphocytes from respective control cultures.

OGF is the singular opioid peptide that inhibits proliferation of PHA-stimulated lymphocytes

To determine whether opioids other than OGF modulate the proliferation of PHA-stimulated (10 μg/ml) lymphocytes, cultures were treated daily for 72 h with 10^{-6} M concentrations of a variety of natural and synthetic opioid-related compounds, some of which were specific for μ, δ, and κ opioid receptors (Fig. 4A). Under the same conditions and concentration (i.e., 10^{-6} M) whereby OGF decreased cell number by 30%, these opioid-related peptides had

no effect on the proliferation of PHA-stimulated splenic-derived lymphocytes.

Blockade of the OGF–OGFr axis with naltrexone does not alter the proliferation of PHA-stimulated lymphocytes

Persistent blockade of classical opioid receptors, as well as the non-classical opioid receptor OGFr, with the general opioid receptor antagonist naltrexone (NTX, 10^{-5} M) also was evaluated for its effect on proliferation of splenic-derived lymphocytes stimulated with PHA at 10 μg/ml. NTX had no effect on cell number in cultures exposed to 10 μg/ml PHA (Fig. 4A).

To determine whether the lack of stimulatory effect seen with NTX was due to a threshold level of mitogenic stimulation, cultures were subjected to a lower concentration (5 μg/ml) of PHA that was previously shown to increase cell number to a lesser extent than 10 μg/ml PHA (Fig. 2B). Using concentrations of NTX ranging from 10^{-4} M to 10^{-10} M in PHA-stimulated (5 μg/ml) cultures at 72 h, the number of cells recorded was comparable to those in control cultures (Fig. 4B).

OGF and NTX do alter non-stimulated T lymphocytes

T lymphocyte preparations that were not stimulated by PHA exhibited no change in cell number after being subjected to 10^{-6} M OGF or 10^{-5} M NTX (data not shown).

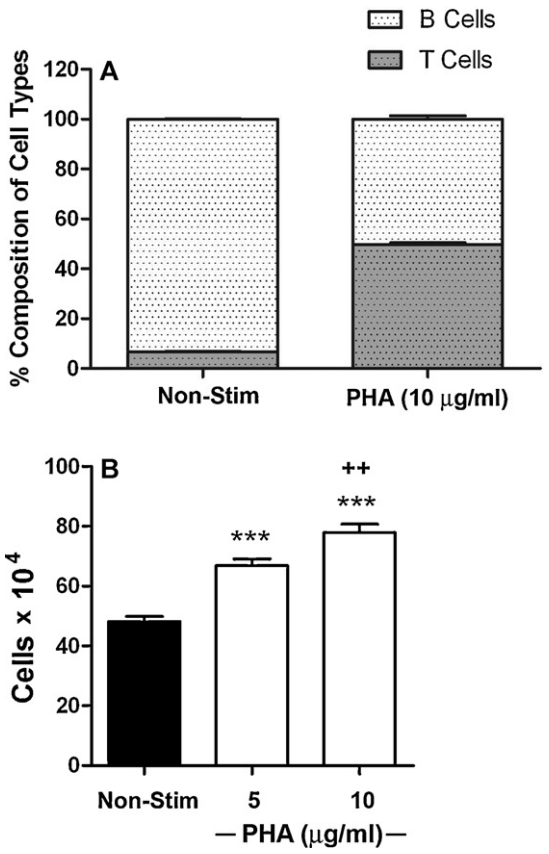

Fig. 2. PHA stimulation increases the number of T lymphocytes. (A) The percentage of T ($CD4^+$ and $CD8^+$) and B ($B220^+$) lymphocytes at 72 h in cultures that were non-stimulated or stimulated with 10 μg/ml PHA. Data represent mean percentage (%) of live cells ± SE of 3 independent cultures. (B) The number of lymphocytes at 72 h. Cultures at 72 h were either non-stimulated or stimulated with 5 or 10 μg/ml PHA. Data represent mean cell counts ± SE for at least 2 aliquots/well from at least 2 wells/group. Significantly different from 72 h non-stimulated cultures at $p < 0.001$ (***), and from 5 μg/ml PHA-stimulated cultures at $p < 0.01$ (++).

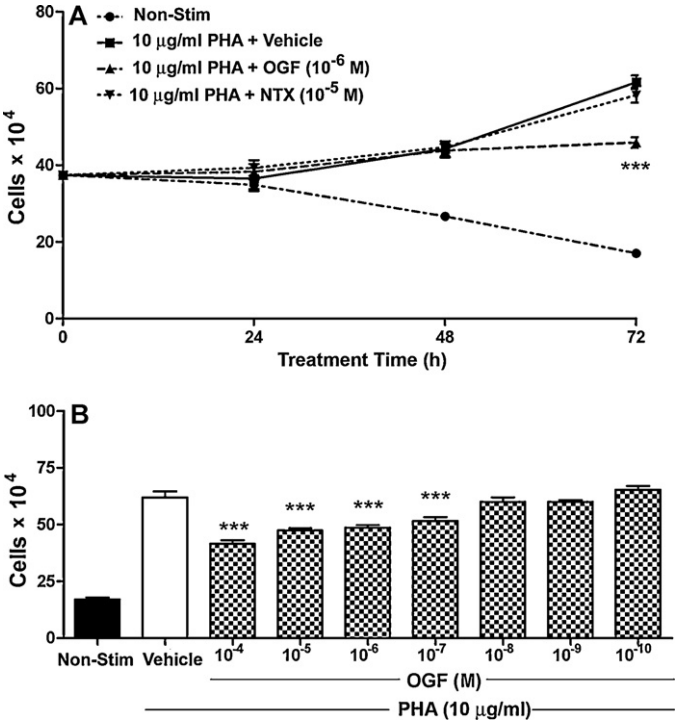

Fig. 3. OGF inhibits the growth of PHA-stimulated lymphocytes in a temporal and dose-dependent manner. (A) Lymphocytes stimulated with 10 μg/ml PHA and subjected to 10^{-6} M OGF, 10^{-5} M NTX, or an equivalent volume of sterile water (vehicle) extending for a 72 h time period. (B) Lymphocytes stimulated with 10 μg/ml PHA and subjected to various concentrations of OGF for 72 h. For all experiments, compounds were added immediately (0 h) after cells were seeded at 500,000 cells/well; compounds were added daily without replacing media. Data represent mean cell counts ± SE for at least 2 aliquots/well from at least 2 wells/group. Significantly different from sterile water PHA-stimulated cells at $p < 0.001$ (***). All stimulated cultures differed from non-stimulated cultures at $p < 0.001$ at 48 and 72 h.

Endogenous OGF does not regulate the proliferation of PHA-stimulated lymphocytes

Given that an OGF-like peptide was detected by immunohistochemistry in T lymphocytes (see Fig. 1A), the action of an endogenous OGF-like peptide on cell proliferation was investigated by antibody neutralization of native OGF-like peptide(s). A concentration of antibody known to block endogenous OGF activity in cancer cells (Donahue et al. 2009) was utilized. Lymphocyte cultures stimulated with 10 μg/ml PHA and treated with an anti-OGF antibody were comparable in cell number relative to cultures receiving pre-immune rabbit serum (IgG), or preparations treated with vehicle (Fig. 4C).

Silencing of OGFr, but not classical opioid receptors, in PHA-stimulated lymphocytes blocks the inhibitory action of exogenous OGF

The requirement of classical and/or non-classical opioid receptors for the ability of OGF to inhibit cell proliferation was evaluated at the molecular level using siRNA technology. MOR, DOR, KOR, or OGFr siRNA-transfected PHA (10 μg/ml)-stimulated cultures had 47%, 57%, 57%, and 85%, respectively, less MOR (Fig. 5A), DOR (Fig. 5B), KOR (Fig. 5C), and OGFr (Fig. 5D) protein levels relative to PHA-stimulated cells that were not transfected. PHA-stimulated cultures treated with scrambled siRNA had protein levels of MOR, DOR, KOR, and OGFr that were comparable to those in non-transfected cells (Fig. 5A–D). Cultures stimulated with PHA (10 μg/ml) and transfected with MOR, DOR, KOR, or OGFr siRNA had an equivalent number of cells relative to cultures not transfected,

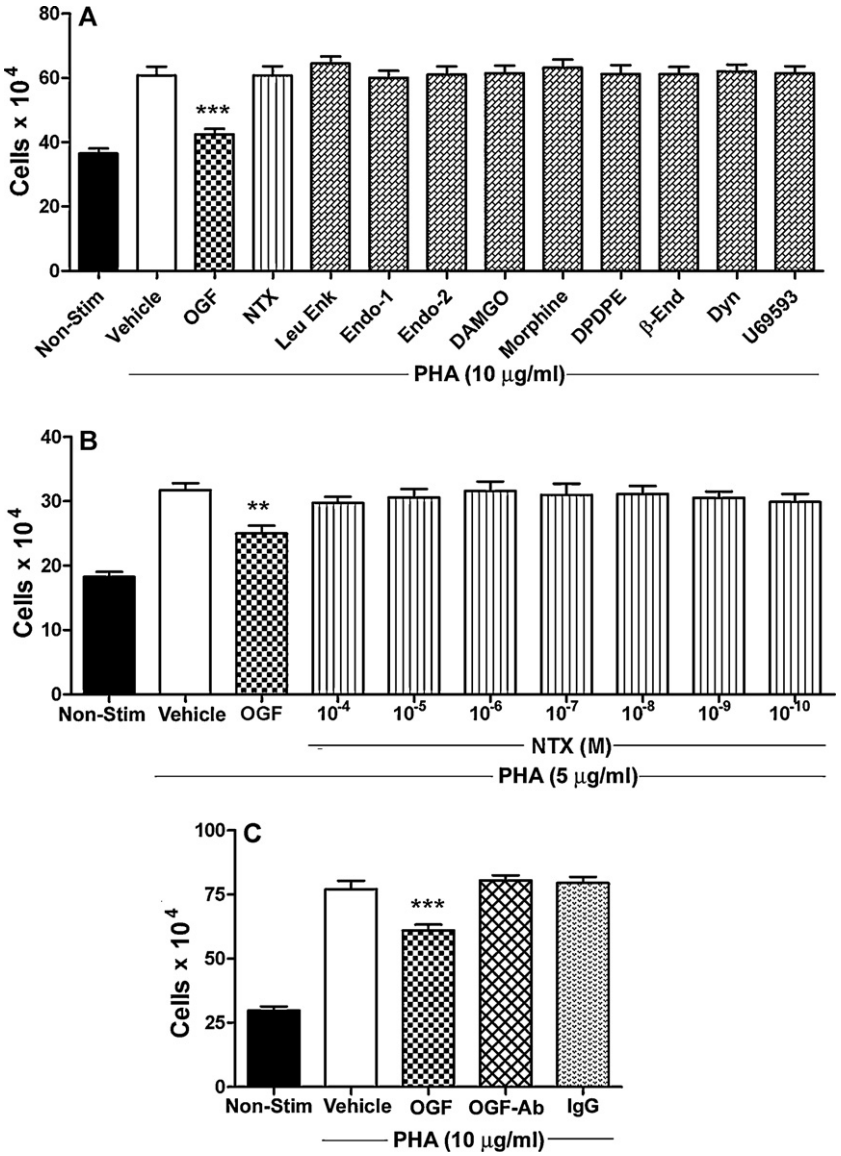

Fig. 4. OGF is the specific opioid peptide involved in the growth inhibition of PHA-stimulated lymphocytes. (A) The effects of various endogenous and exogenous opioids on PHA (10 μg/ml)-stimulated lymphocyte cell number. Cells were seeded at 500,000/well into 24-well plates; all compounds were added every day beginning at 0 h without replacing media. Cultures were treated with 10^{-6} M concentrations of a variety of opioids or 10^{-5} M concentration of the opioid receptor antagonist naltrexone (NTX) for 72 h; controls received an equivalent volume of sterile water (vehicle). (B) Cultures subjected to NTX at concentrations ranging from 10^{-4} to 10^{-10} M administered daily for 72 h to lymphocytes stimulated with 5 μg/ml PHA, had a comparable number cells to those receiving sterile water (vehicle). Cultures receiving OGF (10^{-6} M) were significantly decreased from PHA-stimulated vehicle ($p < 0.01$, **) and by at least $p < 0.05$ from preparations receiving NTX. (C) PHA-stimulated lymphocytes (10 μg/ml) were treated with a polyclonal antibody specific for OGF (Co172), pre-immune serum (IgG), or exogenous OGF (10^{-6} M); antibodies and peptide were administered daily without changing media. The number of cells was assessed at 72 h. Data represent mean cell counts ± SE for at least 2 aliquots/well from at least 2 wells/group. PHA-stimulated cultures receiving OGF, but not OGF antibody (OGF-Ab) or IgG, were markedly depressed in cell number relative to PHA-stimulated cultures exposed to sterile water (vehicle). Significantly different from controls at $p < 0.001$ (***).

as well as compared to cultures transfected with scrambled siRNA (Fig. 5E). The addition of exogenous OGF significantly inhibited cell number in cultures stimulated with 10 μg/ml PHA and transfected with MOR, DOR, or KOR siRNA by 43%, 44%, and 39%, respectively, in comparison to cultures transfected with MOR, DOR, or KOR siRNA and treated with sterile water. However, the addition of exogenous OGF to PHA-stimulated cultures transfected with OGFr siRNA had a comparable number of cells to cultures treated with OGFr siRNA and sterile water.

OGF alters DNA synthesis but does not induce apoptosis or necrosis

To evaluate the mechanism by which OGF inhibits PHA-stimulated lymphocytes, DNA synthesis of cultures exposed to OGF, NTX, or sterile water was determined (Fig. 6). At the 24 h time point, the quantity of [^{3}H]-thymidine incorporation in PHA-stimulated cultures exposed to OGF was decreased by 39% compared to cultures stimulated with PHA and receiving sterile water. The incorporation of [^{3}H]-thymidine in PHA-stimulated cultures subjected to NTX, however, was not altered relative to cultures stimulated with PHA that received sterile water.

Examination of apoptosis by Annexin V staining revealed that cultures stimulated with PHA (10 μg/ml) and receiving OGF or NTX had levels of apoptosis that were similar to stimulated preparations receiving sterile water at 12 h (52%), 24 h (42%), 48 h (25%), and 72 h (6%). Likewise, the number of necrotic cells as measured by 7AAD incorporation in lymphocytes stimulated with PHA (10 μg/ml) and treated with OGF or NTX was similar to PHA-stimulated cultures

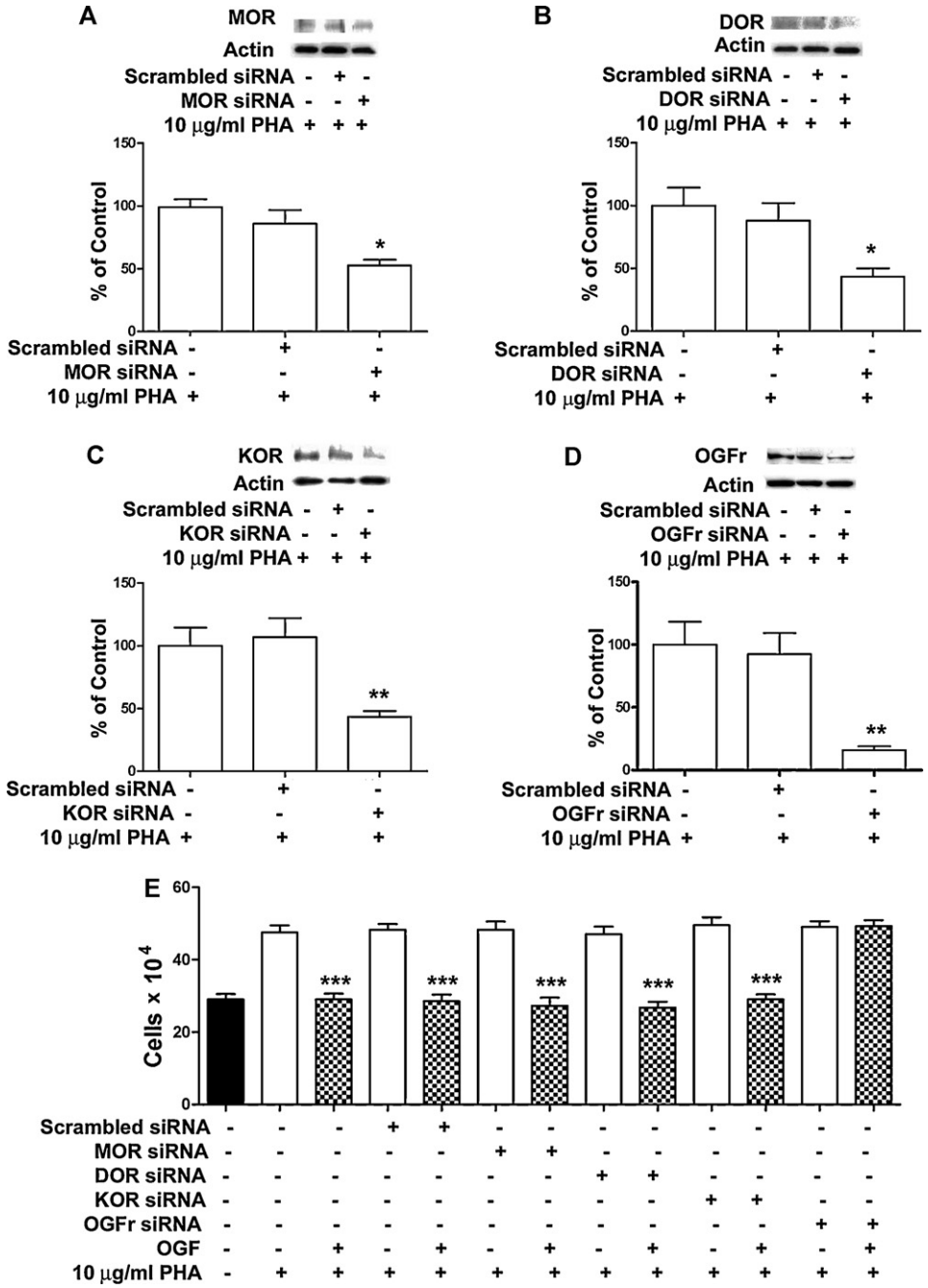

Fig. 5. OGFr is the opioid receptor required for the inhibitory action of OGF on the proliferation of PHA-stimulated lymphocytes. (A–D) Western blot analysis and quantitative densitometry demonstrating the specificity and level of (A) MOR, (B) DOR, (C) KOR, and (D) OGFr knockdown in PHA (10 μg/ml)-stimulated lymphocytes. PHA-stimulated cells were transfected with either MOR, DOR, KOR, OGFr, or scrambled siRNA. Seventy-two hours after the start of transfection, cells were harvested and protein isolated. Data (percentage of MOR/actin, DOR/actin, KOR/actin, or OGFr/actin ratio) represent means ± SE for 2 independent samples. Significantly different from non-transfected and scrambled-siRNA transfected cultures at $p < 0.05$ (*) and $p < 0.01$ (**). (E) Growth of PHA (10 μg/ml)-stimulated lymphocyte cultures transfected with MOR, DOR, KOR, OGFr or scrambled siRNA and treated for 72 h with either OGF (10^{-6} M) or an equivalent volume of sterile water. OGF or sterile water were added daily without changing media, and values represent mean cell counts ± SE for at least 2 aliquots/well and least 2 wells/treatment. Significantly different at $p < 0.001$ (***) from PHA-treated cultures that were not transfected, as well as PHA-exposed cultures that were transfected with scrambled siRNA.

receiving sterile water at 12 h (58%), 24 h (75%), 48 h (63%), and 72 h (56%).

The cyclin-dependent inhibitory kinases p16 or p21 are required for OGF-induced growth inhibition in splenic-derived lymphocytes stimulated with PHA

To test whether the cyclin-dependent inhibitory pathway is involved in the effects of OGF on T lymphocytes, experiments were performed to determine if p16 and/or p21 is(are) required. Lymphocytes stimulated with PHA (10 μg/ml) were transfected with p16, p21, both p16 and p21, or scrambled siRNA. Cells transfected with p16 or p21 siRNA had protein levels that were reduced by 40% and 49%, respectively, from values of PHA-stimulated cultures exposed to sterile water at 72 h (Fig. 7A–C). Protein levels of PHA-stimulated cultures transfected with both p16 and p21 siRNAs reduced p16 and p21 expression by 36% and 57%, respectively; these reductions in protein values did not differ from groups

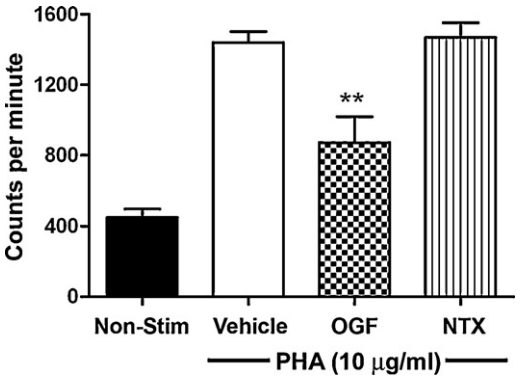

Fig. 6. The inhibitory effect of OGF on DNA synthesis. Cultures were stimulated with PHA (10 μg/ml) and treated with OGF (10^{-6} M), NTX (10^{-5} M), or an equivalent volume of sterile water for 24 h. At 20 h, cells were pulsed with 1 μCi ^{3}H-thymidine for 4 h, collected, and the amount of radioactivity measured. Data represent mean counts per minute of incorporated ^{3}H-thymidine ± SE of three independent wells/treatment group. Significantly different from PHA-stimulated cultures receiving sterile water (vehicle) at $p < 0.01$ (**).

transfected with either p16 or p21 siRNAs alone. However, PHA-stimulated cells exposed to scrambled siRNA were comparable in protein levels of p16 and p21 to PHA-treated non-transfected cells. PHA-stimulated cultures transfected with scrambled siRNA, p16 siRNA, or p21 siRNA and treated with OGF (10^{-6} M) for 72 h had reductions in cell number from 22% to 27% compared to PHA-stimulated cells subjected to sterile water (Fig. 7D). However, cultures stimulated with PHA and transfected with siRNA against p16 and p21, and treated with OGF, were comparable in cell number to PHA-stimulated preparations not transfected and receiving sterile water (Fig. 7D).

Discussion

These data are the first to show that the OGF–OGFr axis has the capability of suppressing T lymphocyte proliferation. Employing a strategy of stimulating splenic-derived T lymphocytes with PHA, the evidence presented to arrive at this conclusion includes the: (1) presence of both OGF and OGFr immunoreactivity in T lymphocytes, (2) depression of T lymphocyte proliferation by OGF in a dose-dependent manner, (3) inability of other natural and synthetic

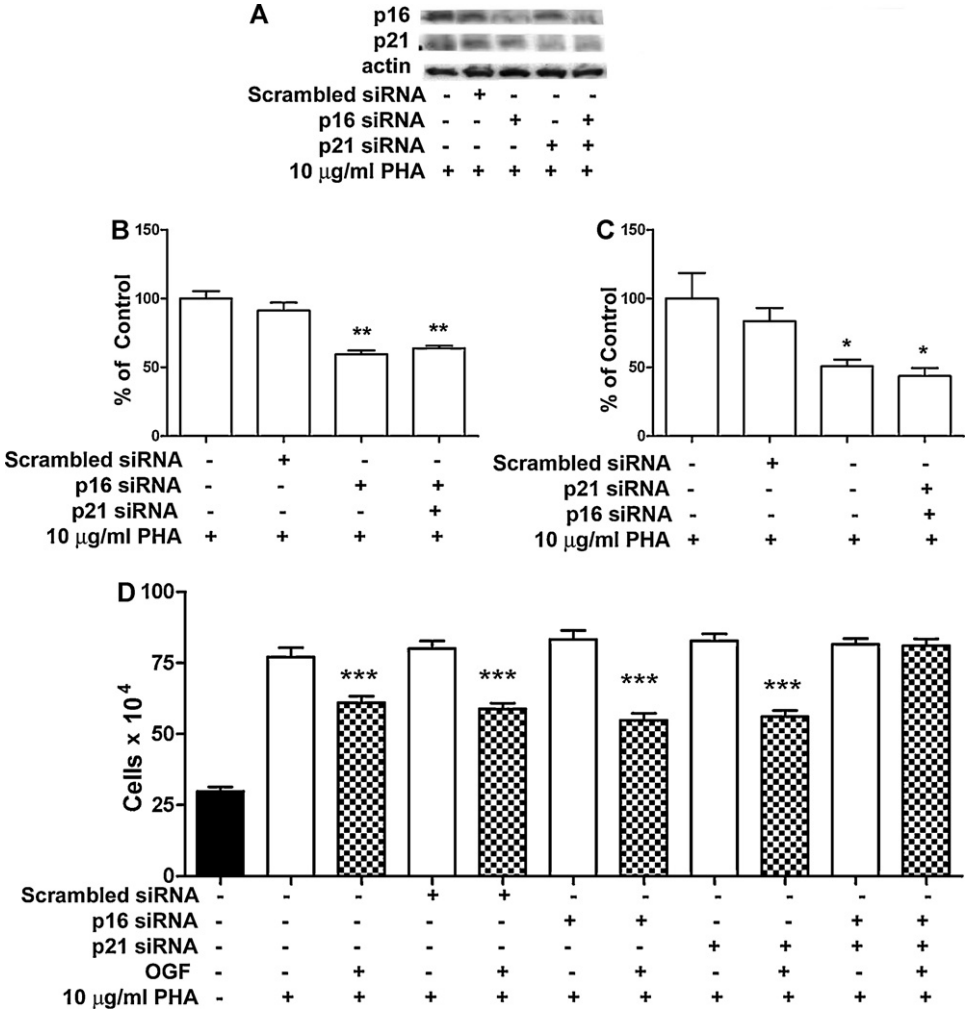

Fig. 7. The OGF–OGFr axis in PHA-stimulated lymphocytes requires either the p16 or p21 cyclin-dependent inhibitory kinase pathway. PHA (10 μg/ml)-stimulated cells were transfected with either p16, p21, p16 and p21, or scrambled siRNA and assessed 72 h after the start of transfection. (A) Western blot analysis demonstrating p16 and/or p21 knockdown. (B and C) Quantitative densitometry monitoring the level of p16 (B) and p21(C) knockdown in PHA (10 μg/ml)-stimulated lymphocyte cultures. Data (percentage of p16/actin and p21/actin ratio) represent means ± SE for 2 independent samples. Significantly different from PHA-treated non-transfected and scrambled siRNA transfected cultures at $p < 0.05$ (*) and $p < 0.01$ (**). (D) Growth of PHA (10 μg/ml)-stimulated lymphocyte cultures transfected with p16, p21, p16 and p21, or scrambled siRNA and treated with OGF (10^{-6} M) or an equivalent volume of sterile water for 72 h. Compounds were added daily without changing media. Values represent mean cell counts ± SE for at least 2 aliquots/well and least 2 wells/treatment. Significantly different at $p < 0.001$ (***) from cultures stimulated with PHA that were not transfected or transfected with scrambled siRNA.

opioids, some of which are specific to μ, δ, and κ opioid receptors, to alter T lymphocyte replication, (4) absence of OGF action in T lymphocytes with a knockdown of OGFr, (5) efficacy of exogenous OGF to decrease T lymphocyte number in preparations with a knockdown of μ, δ, or κ opioid receptors, (6) targeting of exogenous OGF's inhibitory activity on DNA synthesis in T lymphocytes, but no influence on apoptosis or necrosis, and (7) dependency of OGF's suppression of T lymphocyte proliferative processes on p16 and p21 cyclin dependent inhibitory kinase pathways. OGF treatment did not have any effect on cultures that were not stimulated with PHA, indicating that cells had to be activated and proliferating in order for this peptide to influence cellular proliferation. Therefore, the OGF–OGFr axis is an endogenous biological pathway that can be utilized to depress T lymphocyte proliferation.

Despite the presence of OGF-like peptide and OGFr in T lymphocytes, and its ability to suppress cell proliferation as evoked by the addition of exogenous OGF, unstimulated or PHA-stimulated T lymphocytes are not dependent on an autocrine regulation of cell replication at least under the conditions of these studies. The evidence for this conclusion is based on three observations. First, obstruction of endogenous opioids from opioid receptors (including OGF and OGFr) by the opioid antagonist NTX, at concentrations ranging from 10^{-4} to 10^{-10} M, did not alter the population of either stimulated or non-stimulated T lymphocytes. In fact, even when there was a reduction in the concentration of PHA in order to diminish the extent of stimulation and allow greater opportunity for a potential increase in cell number, no change in T lymphocyte number was recorded with the addition of NTX. Thus, our results showing the absence of effect on T lymphocyte proliferation by interfering with OGF–OGFr interfacing support earlier studies with NTX or naloxone that showed a similar lack of change (van den Bergh et al., 1991; Ni et al. 1999; Gilman et al. 1982; Mandler et al. 1986; Jaume et al. 2007). Second, diminishing OGFr by siRNA technology documented no alteration in T lymphocyte number from PHA-stimulated cultures transfected with scrambled siRNA. These data indicate that minimizing OGFr, and thus interfering with peptide-receptor interaction, does not change the course of cell proliferation. Third, antibody neutralization of OGF-like peptides did not modulate cell number. Therefore, reducing OGF does not increase cell proliferation as would be anticipated if the OGF–OGFr axis was continually regulating cell replication.

The absence of a dependence on OGF–OGFr interfacing to maintain the pace of cell proliferation recorded herein represents an exception to the numerous reports indicating that the OGF–OGFr axis is a tonically active system in maintaining homeostasis with respect to cell proliferation (Zagon et al. 2002; Donahue et al. 2009). A number of factors can be offered to explain these findings. First, unlike previous results (e.g., neoplasia and development) with respect to the OGF–OGFr axis, T lymphocytes undergo no homeostatic renewal but require stimulation to initiate DNA synthesis and replication. Thus, the absence of persistent activity between peptide and receptor may act to conserve biological processes, and continued OGF–OGFr interaction is not a requirement in these cells. The fact that administration of OGF can decrease T lymphocyte proliferation, and does so by OGFr mediation, suggests that this axis is indeed functional. OGF could be elevated from paracrine (e.g., monocytes) or endocrine sources under certain conditions wherein T lymphocytes are stimulated, and thereby alter T lymphocyte replication. Because T lymphocyte response is known to require other cell populations, it may be that one or more of these cell types produce OGF. Therefore, future investigations in animals, with and without stimulation, are required, as well as defining the relationship of the OGF–OGFr axis to other cell types (e.g., B cells, NK cells). A second factor that is consistent with the data indicating that interference with OGF–OGFr interfacing does not increase cell proliferation is that although activated T lymphocytes express preproenkephalin mRNA, it is processed into high-molecular weight peptides that do not have opioid activity. Only small amounts of preproenkephalin-derived peptides, which require dibasic cleavage for formation of smaller peptides (e.g., OGF) which are the opioid-active forms, have been recorded in T lymphocytes (Roth et al. 1989; Kuis et al. 1991). These results may explain why the endogenous OGF–OGFr system is not tonically active in T lymphocytes, even though some enkephalin-like immunoreactivity can be observed. This could be due to either the limited amount of OGF available and/or a cross-reaction with larger peptides of proenkephalin. Whether under *in vivo* conditions, in either homeostasis or situations that elicit an immune response, endogenous proenkephalin machinery undergoes a complete posttranslational processing to OGF requires further elucidation.

The effects of opioid peptides on T lymphocytes have been explored by numerous investigators with conflicting results (Carr et al. 1996; McCarthy et al. 2001; Peterson et al. 1998; Salzet and Tasiemski 2001; Sharp et al. 1998). Some of the differences in these studies include whether the peptides are natural or synthetic, the concentration and/or class of peptide being examined, whether cells are stimulated or not stimulated, the time of treatment relative to the time of stimulation, the use of mitogens and the concentration of these mitogens for stimulation, the inclusion or exclusion of serum, and the type of assays utilized to detect cell proliferation (e.g., DNA synthesis and cell counting). Nonetheless, it is important to point out the diverse effects reported for β-endorphin and OGF ([Met^5]-enkephalin), two opioid peptides that have received the most focus on their effects on T lymphocyte proliferation. In the case of β endorphin, studies report an increase (Hemmick and Bidlack 1990; Gilmore and Weiner 1989; Kusnecov et al. 1987) and decrease (Marchini et al. 1995; Hough et al. 1990; Shahabi et al. 1991) in T lymphocyte response. With regard to [Met^5]-enkephalin (OGF), investigators found an increase (Kowalski 1998a,b; Hucklebridge et al. 1990, 1989; Plotnikoff and Miller 1983), decrease (Kamphuis et al. 1998; Shahabi and Sharp 1995; Ohmori et al. 2009; Marić and Janković 1987), or no effect (Gilman et al. 1982; Wybran 1985; Owen et al. 1998; Munn and Lum 1989; Zunich and Kirkpatrick 1988; van den Bergh et al. 1993; Prete et al. 1986; Kusnecov et al. 1989), on T lymphocytes. The results of the present study reveal that only exogenously delivered OGF at concentrations ranging from 10^{-4} to 10^{-7} M had an effect on altering T lymphocyte proliferation, and only if the cultures were stimulated. These greater than physiological concentrations of peptide may have been necessary to reveal OGF action because of the rapid degradative processes known to occur with enkephalins (Dass and Mahalakshmi 1996). Other opioid peptides tested, both natural and synthetic, at a concentration of 10^{-6} M, a dose selected because of the efficacy of OGF at that concentration, did not influence the proliferation of T lymphocytes. Whether other concentrations of these peptides or a delivery system that delays the processing of these peptides can influence T lymphocyte replication is unclear.

Classical (McCarthy et al. 2001; Sharp et al. 1998; Sharp 2004) and non-classical (e.g., OGFr) (Zagon et al. 2010) opioid receptors have been reported to be associated with immune cells, including T lymphocytes. A review of the literature indicates that the effects of β-endorphin on T lymphocytes are not related to opioid receptors (van den Bergh et al. 1991; Hemmick and Bidlack 1990; Gilmore and Weiner 1989; Shahabi et al. 1991), whereas the effects of dynorphins on T lymphocytes have been shown to be blocked by a specific κ opioid receptor antagonist (Ni et al. 1999; Kowalski 1998a,b). Shahabi and Sharp (1995) have used two synthetic compounds, deltorphin and [D-Ala^2]-met-enkephalinamide (DAME) and report that they are inhibitory to T lymphocyte proliferation, and that this antiproliferative activity could be blocked

by the δ receptor antagonist, naltrindole. However, these results were only achieved under certain conditions. First, only pretreatment with deltorphin or DAME would alter the effect of naltrindole, but treatment initiated at the time of stimulation was not effective. Second, naltrindole blocked the effect of deltorphin, but naltrindole alone also depressed DNA synthesis. Third, the investigators used serum free media for their experiments, raising the question as to the shock experienced by the cells when placed from *in vivo* conditions to a serum-free environment. Fourth, anti-CD3-ε stimulated the T cell preparations, yet cells exposed to another T cell mitogen, concanavalin A, were unresponsive. Fifth, the effects of [Met^5]-enkephalin (OGF) were never examined. In the present study, we utilized the native peptide, [Met^5]-enkephalin itself, rather than compounds that are known to utilize apoptotic pathways (Tegeder and Geisslinger 2004). We discovered that this peptide could depress T lymphocyte proliferation when administered at the time of stimulation. Importantly, knockdown of the δ opioid receptor, as well as μ and κ opioid receptors, by siRNA technology did not eliminate the effects of OGF. However, knockdown of the OGF receptor with OGFr siRNA diminished the depressive effects of OGF. Finally, we demonstrate that OGF action has no effect on apoptosis or necrosis, but only on DNA synthesis. Furthermore, we have determined that the molecular target of OGF is the cyclin dependent kinase inhibitory kinase pathway.

The results of these investigations reveal that T lymphocyte proliferation is not dependent on an autocrine mechanism involving the OGF–OGFr axis for regulation. Whether sufficient quantities of paracrine and/or endocrine OGF are available to control T lymphocyte proliferation remains to be clarified. However, this axis can be modulated by exogenous OGF. Thus, the OGF–OGFr system can serve as a means for inducing immunosuppression, and is reliant on native biological components and physiological processes. Based on earlier studies, a more comprehensive picture can be deciphered about the cascade of events that lead to an inhibition of T lymphocyte proliferation by the OGF–OGFr axis. T lymphocytes are activated through the T cell receptor and costimulatory pathways are predominately mediated by the cell surface receptor CD28. Exogenous (and perhaps endogenous) OGF enters these cells by a process of clathrin-mediated endocytosis (Cheng et al. 2010b) and interacts with the OGF receptor on the outer nuclear envelope. The OGF–OGFr complex undergoes nucleocytoplasmic transport with guidance from nuclear localization signals encoded in OGFr (Cheng et al. 2009b). This process utilizes karyopherin β and Ran to shuttle the OGF–OGFr complex to the nucleus (Zagon et al. 2005a; Cheng et al. 2010a). The OGF–OGFr complex increases p16 and/or p21 expression in the cyclin-dependent inhibitory kinase signaling pathway, and delays the G_1-S phase of the cell cycle (Cheng et al. 2007a,b, 2009a). This delay could therefore diminish subsequent events related to T cell proliferation (e.g., inflammation).

The implications of these findings to the clinical setting are profound. OGF has been shown to depress the expression of EAE (Zagon et al. 2010), as well as human neoplasias (McLaughlin et al. 2008; Zagon et al. 2008a, 2009b). Indeed, OGF has been successful in both Phase I and Phase II clinical trials for extending survival and improving the quality of life of patients with advanced pancreatic cancer (Smith et al. 2004, 2010). A low dose of NTX (LDN) also utilizes the OGF–OGFr axis, and LDN has been reported to be safe and effective in patients with active Crohn's disease (Smith et al. 2007). Moreover, the imidazoquinoline, imiquimod (Aldara®, R-837, S-26308), a drug highly efficacious in the treatment of skin disorders including cancer (Miller et al. 1999; Tyring et al. 2002), is known to upregulate OGFr expression (Urosevic et al. 2004) and be dependent on the OGF–OGFr pathway (Zagon et al. 2008b). These findings raise the suggestion of new treatments for autoimmune diseases and other disorders such as neoplasia by modulating the OGF–OGFr system with exogenous OGF (McLaughlin et al. 2008; Zagon et al. 2008a), LDN (Hytrek et al. 1996), or imiquimod (Zagon et al. 2008b; McLaughlin et al. in press), thereby invoking a natural immunosuppressive pathway that is non-toxic and of high efficacy.

In summary, this study provides new insights into the effects of an immunomodulatory agent, OGF, on T lymphocyte proliferation by way of a native biological axis: OGF–OGFr. This opioid system functions to suppress cell proliferative events and targets the cyclin-dependent kinase inhibitory pathway, thereby delaying the G_1-S phase of the cell cycle. Exogenous OGF only could affect T lymphocytes that were stimulated with PHA, indicating that cells had to be activated and proliferating in order for this peptide-receptor axis to influence cellular replication. The data reveal that T lymphocyte proliferation is not dependent on an autocrine mechanism involving the OGF–OGFr axis for regulation. The clinical implications of these findings are profound, as T lymphocytes play a central role in autoimmune diseases. OGF is known to depress the expression of autoimmune diseases and, based on the present results, we would anticipate that exogenous OGF interfaces with OGFr and diminishes subsequent events related to T cell proliferation (e.g., inflammation). Thus, exogenous application of OGF can invoke a natural immunosuppressive pathway through mediation by OGFr to attenuate the cascade of reactions related to T lymphocyte activation and proliferation.

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