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B lymphocyte proliferation is suppressed by the opioid growth factor–opioid growth factor receptor axis: Implication for the treatment of autoimmune diseases

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ABSTRACT

Endogenous opioids are known to repress the incidence and progression of autoimmune diseases. One native opioid peptide, [Met^5]-enkephalin, termed the opioid gowth factor (OGF), interacts with the OGF receptor (OGFr) to suppress the expression of experimental autoimmune encephalomyelitis. The present study examined the role of the OGF-OGFr axis in the regulation of B lymphocyte proliferation. Murine B lymphocytes were stimulated with lipopolysaccharide. Both OGF and OGFr were present in all B lymphocytes. OGF had a dose-dependent effect on growth, with cell number inhibited by up to 43% at 72 h; no other synthetic or native opioid altered cell proliferation. Exogenous OGF depressed cell number in cultures treated with siRNAs for the classical opioid receptors, MOR (μ), DOR (δ), and KOR (κ), however this peptide had no effect in preparations exposed to siRNA for OGFr. The decrease in cell number by exogenous OGF was dependent on p16 or p21 cyclin-dependent inhibitory kinase pathways. Exposure to the opioid antagonist, naltrexone, did not change cell number from control levels. These results suggest that the OGF-OGFr axis is present and functional in B lymphocytes, but this system is not an autocrine regulator of cell proliferation. Thus, at least exogenous OGF and perhaps endogenous OGF by paracrine/endocrine sources, can be an immunosuppressant. Modulation of the OGF-OGFr axis may be a novel paradigm for the treatment of autoimmune diseases.

Introduction

Evidence is accumulating that B lymphocytes are a component in many T lymphocyte-mediated diseases (Dörner et al. 2009; Pescovitz et al. 2009; Dornair et al. 2009). B lymphocytes can play a crucial role as antigen-presenting cells (Noorchashm et al. 1999; Rivera et al. 2001), expressing high levels of class II major-histocompatibility-complex antigens (Lapointe et al. 2003; Serrez and Silveira 2003) and generating cryptic peptides to which T lymphocytes are not tolerant (Davidson and Diamond 2001). Morever, B cells provide T cell costimulation, synthesize cytokines, fix complement, and produce autoantibodies (Adamus 2009; Fraussen et al. 2009; Tsirogianni et al. 2009). After encountering antigen and T cell help in follicles of secondary lymphoid organs, mature naive B cells undergo germinal center reactions leading to their clonal expansion and eventually mature into either memory B cells or Ig-secreting plasma cells. Elucidating the mechanism(s) underlying proliferation of B cells has become important in designing B cell targeted therapies (Dörner et al. 2009; Shlomchik 2009; Balague et al. 2009).

Endogenous opioids are 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). One native opioid peptide that has received considerable attention in this regard has been [Met^5]-enkephalin, an endogenous opioid peptide derived from preproenkephalin (Akil et al. 1984; Noda et al. 1982). 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). To signify its unique distribution and biological role, this peptide has been termed the opioid growth factor (OGF) (Zagon et al. 2002). OGF action is mediated by the OGF receptor (OGFr). Although the OGF-OGFr system has the same pharmacological properties of opioid peptides that interact with classical opioid receptors (e.g., blockade by naloxone, stereospecificity), OGFr has nucleotide and protein sequences that are entirely different from that of classical opioid receptors (Zagon et al. 2002). OGF-OGFr interactions

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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; Wilson et al. 2000; Blebea et al. 2000). The OGF-OGFr axis does not alter differentiation (Zagon and McLaughlin 2005) or migration (Zagon et al. 2007), or induce apoptosis or necrosis (Zagon and McLaughlin 2003), but rather upregulates the cyclin-dependent inhibitory kinase pathway, specifically p16 and p21 (Cheng et al. 2009b, 2007a,b). OGFr is localized on the outer nuclear envelope, and the OGF-OGFr complex undergoes nucleocytoplasmic transport using nuclear localization signals encoded in OGFr for guidance by karyopherin β/Ran through the nuclear pore (Zagon et al. 2005a; Cheng et al. 2009a). Blockade of OGF from OGFr by opioid antagonists or antisense strategies, as well as neutralization of OGF by antibodies, accelerates the pace of cell proliferation (Donahue et al. 2009; Zagon et al. 2005b).

A number of reports have linked the OGF-OGFr system to autoimmune diseases (Zagon et al. 2009b, 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. Given the extensive connection between the immune system and autoimmune diseases, the present study was conducted to investigate the relationship of the OGF-OGFr axis and regulation of B lymphocyte proliferation *in vitro*.

Materials and methods

Mice

Four to 6-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, Indianapolis, IN; 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 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

To determine whether B lymphocytes express OGF and/or OGFr, 5×10^5 splenic-derived lymphocytes were added into 24-well plates containing 500 μl of supplemented IMDM, and were either left as non-stimulated or were stimulated with 10 μg/ml of the B cell mitogen lipopolysaccharide (LPS) (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 CD19 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 CD19 was purchased from eBioscience (clone 1D3, 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 four slides (containing at least 500 cells/slide) per condition were examined.

Receptor binding assays

Receptor binding assays for OGFr were performed using freshly isolated lymphocytes and custom synthesized [^{3}H]-[Met^5]-enkephalin (Perkin Elmer-New England Nuclear; 52.7 Ci/mmol) according to previously published procedures (Donahue et al. 2009). Splenocytes from three animals were pooled for each assay; independent assays were performed at least three times.

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 stimulated with 10 μg/ml (unless otherwise specified) of LPS for 72 h; in some studies cells were stimulated with 5 μg/ml LPS. Immediately upon the addition of LPS, cells were treated daily with compounds; neither media nor compounds were 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 two wells/treatment/timepoint were evaluated.

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 LPS for 72 h. CD16/CD32 Fcγ receptors were blocked with an antibody obtained from 2.4G2 hybridoma cell culture supernatants (a gift from Dr. Bonneau) supplemented with 10% mouse serum (Sigma Aldrich). Cell surface expression of CD4, CD8, and B220 were detected using anti-CD4 FITC (clone GK1.5), anti-CD8a FITC (clone 53-6.7; eBioscience), and anti-CD45R FITC (clone RA3-6B2) antibodies, respectively; all antibodies were obtained from eBioscience. 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 LPS-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 number was determined following 72 h of treatment, with at least two aliquots/well and at least two wells/treatment counted.

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

To determine whether classical opioid receptor(s) are involved with OGF action, siRNAs for MOR (mu [μ]), DOR (delta [δ]), and KOR (kappa [κ]) opioid receptors were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). For the non-classical opioid receptor OGFr, 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 LPS-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 and NTX on DNA synthesis, apoptosis, and necrosis of LPS-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 LPS and either 10^{-6} M OGF, 10^{-5} M NTX, or an equivalent volume of sterile 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, LPS-stimulated cultures were 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, eBioscience).

siRNA-mediated knockdown of p16 and p21

Non-stimulated or LPS-stimulated 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 the 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 antibodies were produced in our laboratory (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 S.E. were determined from at least two independent Western blots.

Fluorescent flow cytometry

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; U69,593, 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 B lymphocytes

Immunoreactivity for OGF (Fig. 1A) and OGFr (Fig. 1B) was localized to the cytoplasm of all non-stimulated, as well as all LPS-stimulated, splenic-derived B lymphocytes (CD19 positive) following 72 h of incubation; a speckling of immunoreactivity for peptide and receptor was noted in the nucleus. No staining was recorded in specimens processed with secondary antibodies only (Fig. 1A and B).

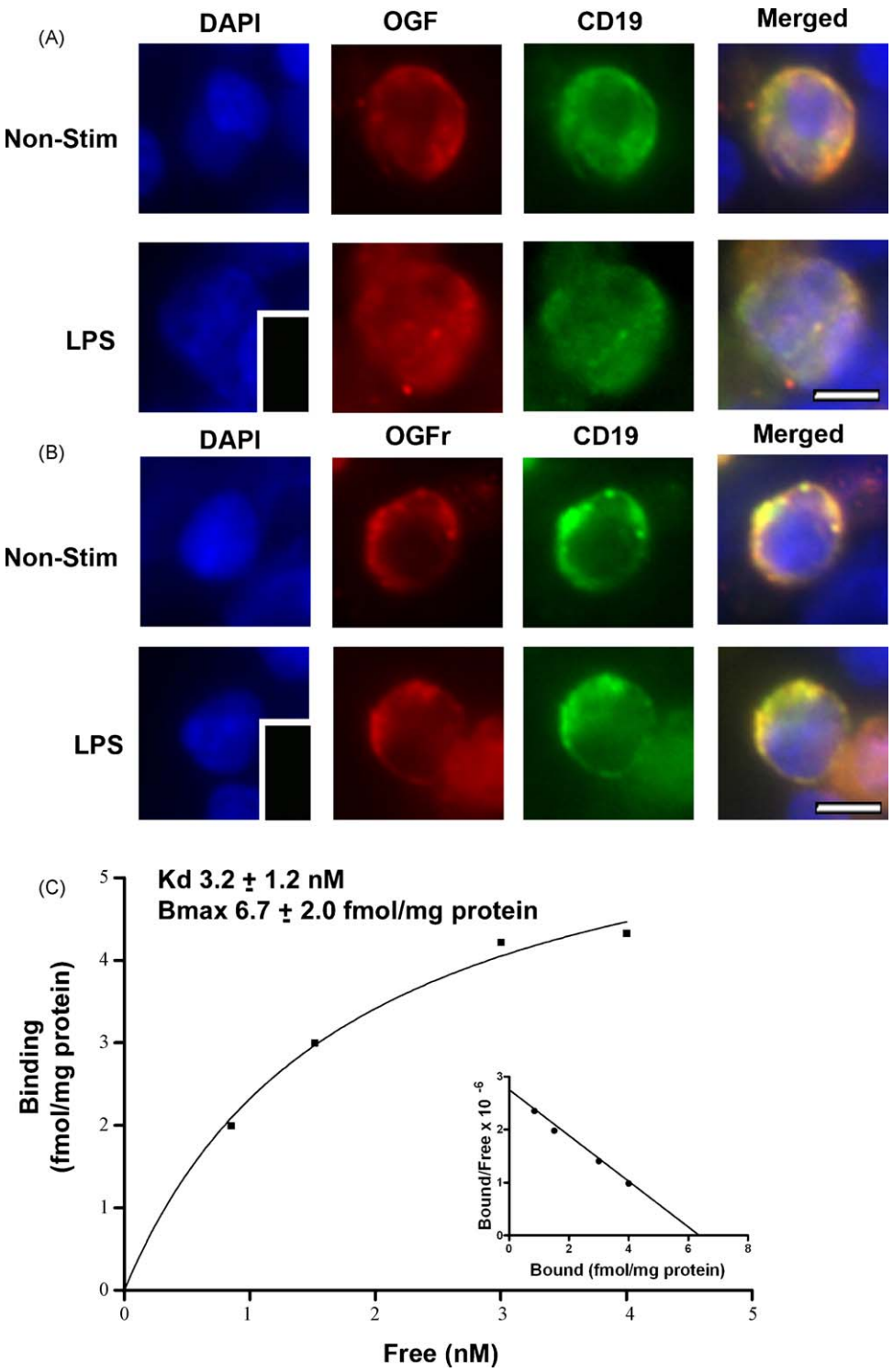

Fig. 1. The presence and distribution of OGF-like peptide (A) and OGFr (B) in CD19-positive non-stimulated and LPS (10 μg/ml)-stimulated lymphocytes. Photomicrographs of lymphocytes immunostained with polyclonal, ammonium-sulfate purified antibodies to OGF (1:100), OGFr (1:100), or CD19 (1:400). Anti-rabbit TRITC-conjugated IgG (1:1000) served as the secondary antibody for OGF and OGFr, while anti-rat FITC-conjugated IgG (1:1000) was utilized as the secondary antibody for CD19. Cell nuclei were visualized with DAPI. OGF-like peptide and OGFr immunoreactivity was associated with the cytoplasm, and a speckling of stain was noted in cell nuclei of CD19-positive cells. Immunostaining was not detected in cell preparations incubated with secondary antibodies only (insets). Scale bar = 10 μm. (C) Representative saturation isotherm of specific binding of [^{3}H]-[Met^5]-enkephalin to nuclear homogenates of freshly isolated lymphocytes. Binding affinity (K_d) and maximal binding capacity (B_{max}) from three independent assays performed in duplicate are expressed as mean ± S.E. Representative Scatchard plot (inset) of specific binding of radiolabeled [Met^5]-enkephalin to lymphocyte proteins revealed a one-site model of binding.

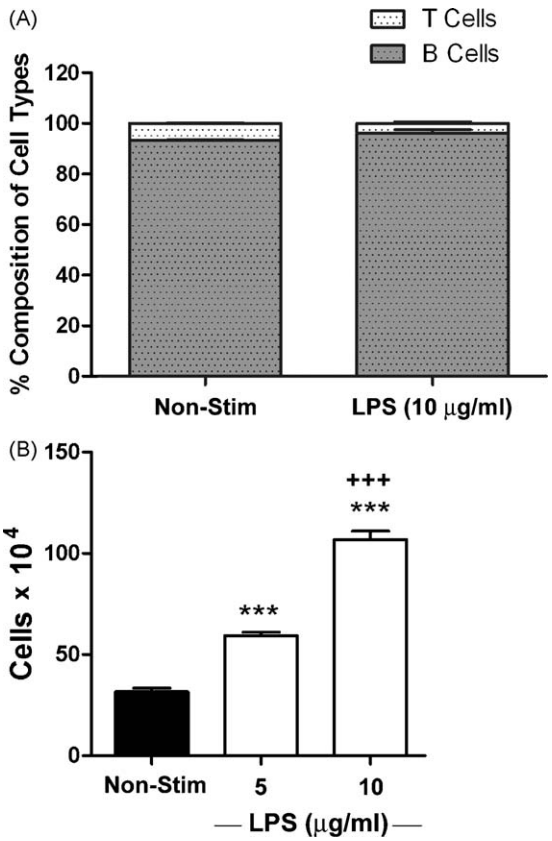

Fig. 2. LPS stimulation increases the number of B lymphocytes. (A) The percentage of B ($B220^+$) and T ($CD4^+$ and $CD8^+$) lymphocytes at 72 h in cultures that were non-stimulated or stimulated with 10 μg/ml LPS. Data represent mean ± S.E. of live cells from three independent cultures. (B) The number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

Receptor binding analysis of nuclear protein from freshly isolated lymphocytes revealed site specific and a one-site model of saturable binding (Fig. 1C). The binding capacity (B_{max}) was 6.7 ± 2.0 fmol/mg protein, and the binding affinity (K_d) was 3.2 ± 1.2 nM.

LPS stimulates B lymphocyte proliferation

Splenic-derived lymphocytes grown in culture for 72 h in the absence of mitogenic stimulation (i.e., non-stimulated) were comprised of 93% B cells and 7% T cells (4% $CD4^+$, 3% $CD8^+$) (Fig. 2A). Cultures stimulated for 72 h with LPS were comprised of 96% B cells, and 4% T cells (2% $CD4^+$, 2% $CD8^+$); an increase of 3% in the proportion of B cells, but a 41% decrease in the proportion of T cells.

Exposure of splenic-derived lymphocytes to LPS showed a concentration-dependent effect on cell proliferation (Fig. 2B). At 72 h of exposure to 5 μg/ml LPS, cell number was increased by 88% in comparison to non-stimulated cultures; using 10 μg/ml of LPS, this increase was 239%. Thus, although both 5 and 10 μg/ml of LPS markedly stimulated lymphocytes, statistical comparison between these two concentrations of LPS revealed that cultures subjected to 10 μg/ml had a significantly greater number of cells than preparations receiving 5 μg/ml ($p < 0.001$).

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

The number of non-stimulated lymphocytes was similar for the initial 24 h period, but decreased 11% and 43% at 48 and 72 h,

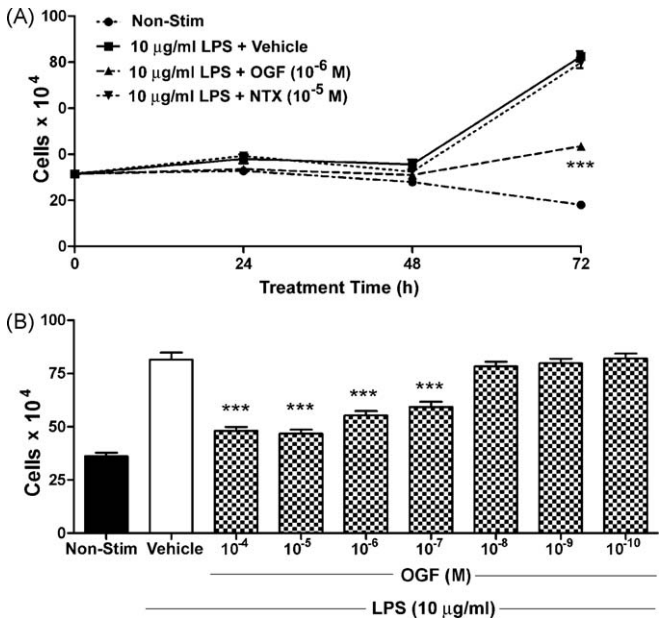

Fig. 3. OGF inhibits the growth of LPS-stimulated lymphocytes in a temporal and dose-dependent manner. (A) Lymphocytes stimulated with 10 μg/ml LPS and subjected to 10^{-6} M OGF, 10^{-5} M NTX, or an equivalent volume of sterile water (vehicle) over a 72 h time period. (B) Lymphocytes stimulated with 10 μg/ml LPS 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 ± S.E. cell counts for at least two aliquots/well and at least two wells/group. Significantly different from sterile water-treated LPS-stimulated cells at $p < 0.001$ (***). Stimulated cultures differed from non-stimulated cultures at $p < 0.001$ at 72 h.

respectively, compared to 0 h (Fig. 3A). Although the number of lymphocytes in non-stimulated and stimulated cultures was comparable at 24 and 48 h, at 72 h the stimulated cultures contained 358% more cells than in non-stimulated preparations (Fig. 3A). Treatment of LPS-stimulated cells with 10^{-6} M OGF resulted in 47% fewer cells than in those LPS-stimulated cultures treated with vehicle after 72 h; no differences were noted at 24 or 48 h (Fig. 3A).

To further document the inhibitory effects of OGF, peptide in concentrations ranging from 10^{-4} to 10^{-10} M were added to LPS-stimulated lymphocytes for 72 h, and a dose-dependent inhibitory effect was recorded (Fig. 3B). Dosages ranging from 10^{-4} to 10^{-7} M reduced the number of lymphocytes by 27–41%.

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

To determine whether opioids other than OGF modulate the proliferation of LPS-stimulated 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, or 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 LPS-stimulated splenic-derived lymphocytes.

Blockade of the OGF-OGFr axis with NTX does not alter the growth of LPS-stimulated lymphocytes

Persistent blockade of classical opioid receptors, as well as the non-classical opioid receptor OGFr, with the general opioid receptor antagonist NTX (10^{-5} M) also was evaluated for its effect on proliferation of splenic-derived lymphocytes stimulated with LPS

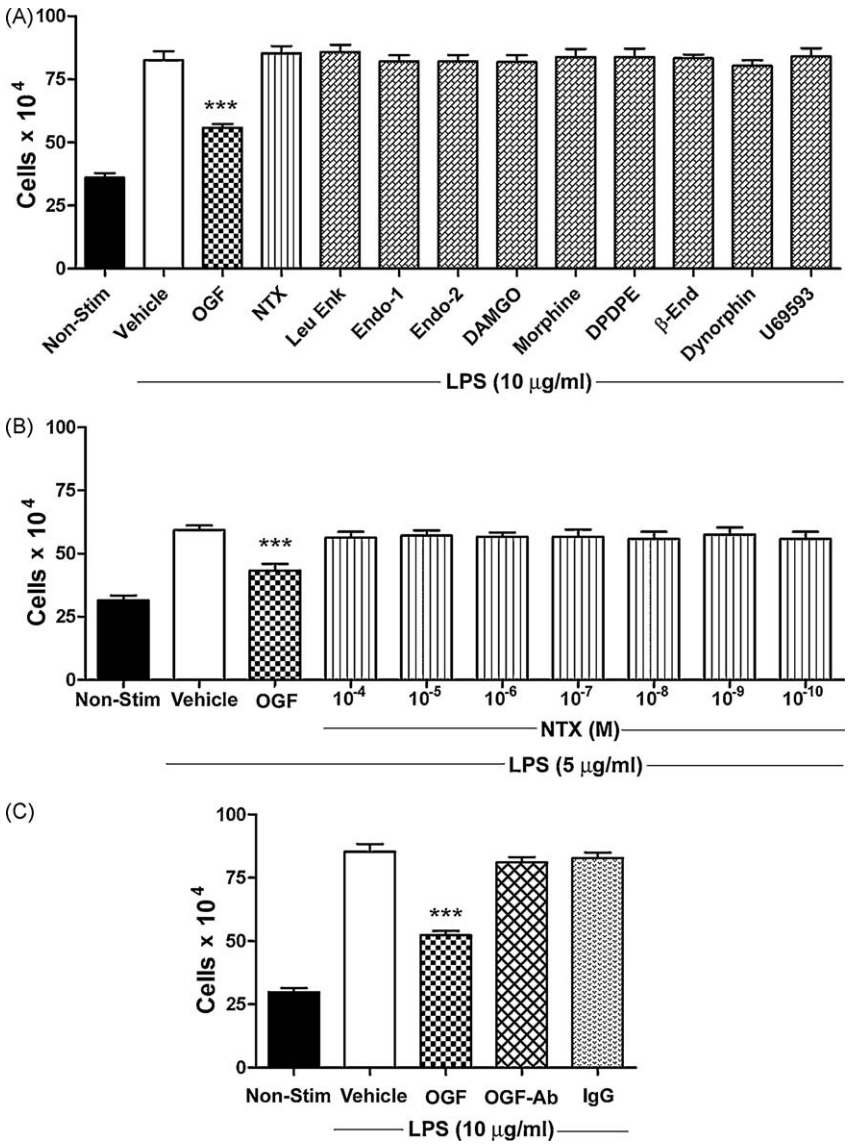

Fig. 4. OGF is the specific opioid peptide involved in the growth inhibition of LPS-stimulated lymphocytes. (A) The effects of various endogenous and exogenous opioids on LPS (10 μg/ml)-stimulated lymphocyte number. Cells were seeded at 500,000/well into 24-well plates; all compounds were added every 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 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 LPS, had a comparable number of cells to those receiving sterile water (Vehicle). Cultures receiving OGF (10^{-6} M) were significantly decreased from LPS-stimulated cells treated with vehicle ($p < 0.001$, ***) and by at least $p < 0.05$ from preparations receiving NTX. (C) LPS-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 ± S.E. cell counts for at least two aliquots/well and at least two wells/group. LPS-stimulated cultures receiving OGF, but not OGF antibody (OGF-Ab) or IgG, were markedly depressed in cell number relative to LPS-stimulated cultures exposed to sterile water (Vehicle). Significantly different from controls at $p < 0.001$ (***).

(10 μg/ml). NTX had no effect on cell number in cultures exposed to this concentration of LPS (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 LPS, previously shown to increase cell number to a lesser extent than 10 μg/ml LPS (Fig. 2B). Using concentrations of NTX ranging from 10^{-4} to 10^{-10} M in LPS-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 not alter non-stimulated B lymphocytes

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

Endogenous OGF does not regulate the growth of LPS-stimulated lymphocytes

Given that an OGF-like peptide was detected by immunohistochemistry in B 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 LPS and treated with an anti-OGF antibody were comparable in cell number relative to cultures receiving pre-immune rabbit serum (IgG) or vehicle (Fig. 4C).

Silencing of OGFr, but not classical opioid receptors, in LPS-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 LPS-stimulated cultures had 51%, 58%, 40%, and 48%, respectively, less MOR (Fig. 5A), DOR (Fig. 5B), KOR (Fig. 5C), and OGFr (Fig. 5D) protein levels relative to LPS-stimulated cells that were not transfected. LPS-stimulated cultures treated with scrambled siRNA had protein levels of MOR, DOR,

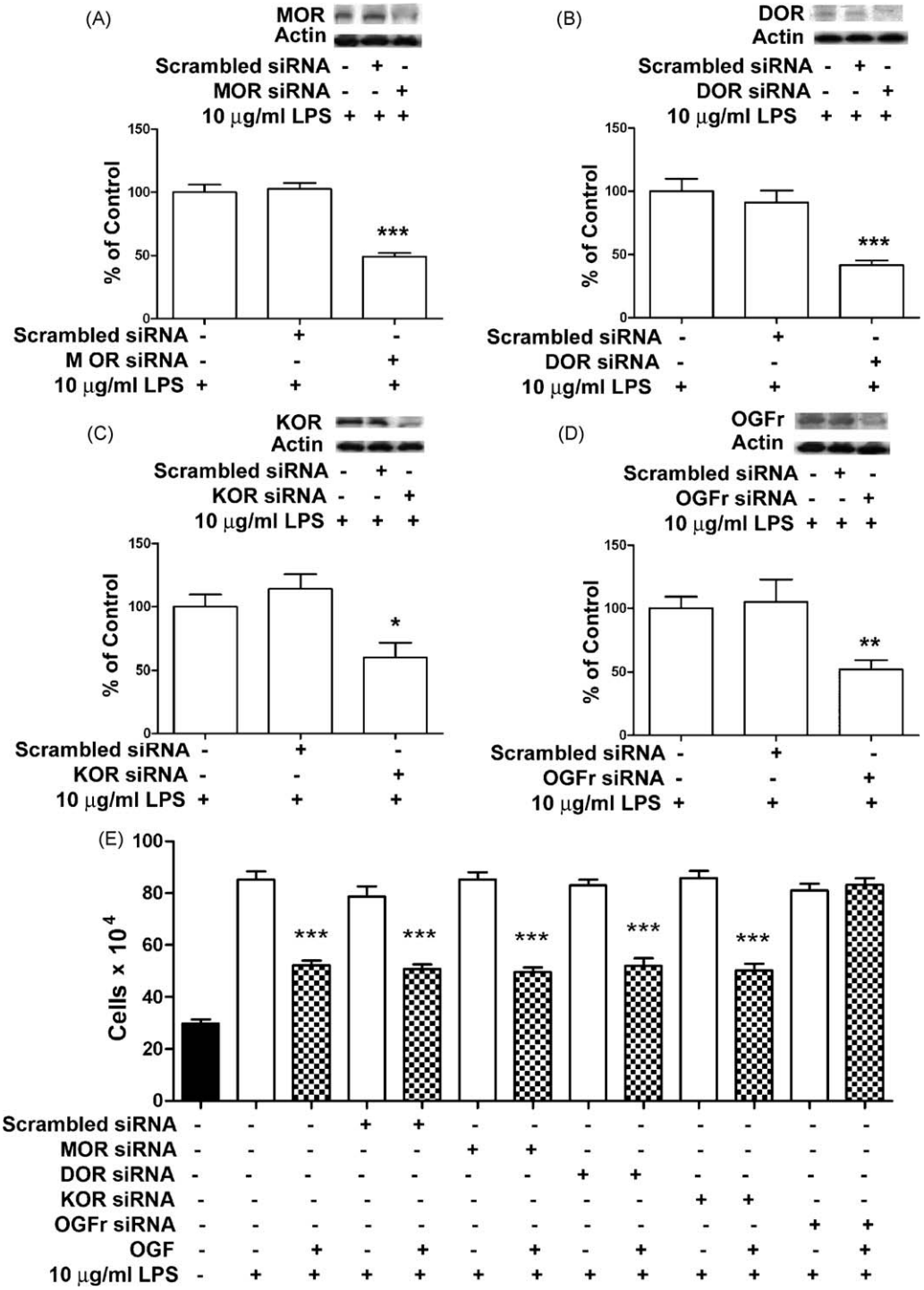

Fig. 5. OGFr is the opioid receptor required for OGF's inhibitory action on cell proliferation in LPS-stimulated lymphocytes. (A–D) Western blot analysis and quantitative densitometry demonstrating the specificity and level of (A) MOR (B) DOR (C) KOR, and (D) OGFr knockdown in LPS (10 μg/ml)-stimulated lymphocytes. LPS-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 mean ± S.E. for two independent preparations. Significantly different from non-transfected and scrambled-siRNA-transfected cultures at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). (E) Cell counts of LPS (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 ± S.E. cell counts for at least two aliquots/well and least two wells/treatment. Significantly different from LPS-treated cultures that were not transfected, as well as LPS-exposed cultures that were transfected with scrambled siRNA at $p < 0.001$ (***).

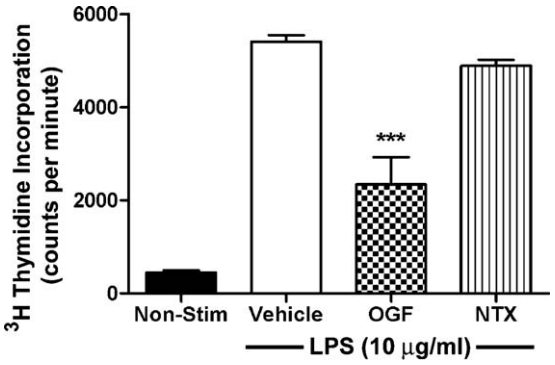

Fig. 6. The inhibitory effect of OGF on DNA synthesis. Cultures were stimulated with LPS (10 μg/ml) and treated with OGF (10^{-6} M), NTX (10^{-5} M), or an equivalent volume of sterile water for 24 h. At 20 h, cells were pulsed with 1 μCi ^{3}H-thymidine for 4 h and the amount of radioactivity measured. Data represent mean ± S.E. counts per minute of incorporated ^{3}H-thymidine from three independent wells/treatment group. Significantly different from LPS-stimulated cultures receiving sterile water (Vehicle) at $p < 0.001$ (***).

KOR, and OGFr that were comparable to those in non-transfected cells (Fig. 5A–D). Cultures stimulated with LPS and transfected with MOR, DOR, KOR, or OGFr siRNA had an equivalent number of cells relative to cultures not transfected, as well as compared to cultures transfected with scrambled siRNA (Fig. 5E). The addition of exogenous OGF (10^{-6} M) significantly inhibited cell number in cultures stimulated with LPS and transfected with MOR, DOR, or KOR siRNA by 42%, 39%, and 41%, respectively, in comparison to cultures transfected with MOR, DOR, or KOR siRNA and treated with sterile water. However, the addition of exogenous OGF (10^{-6} M) to LPS-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 LPS-stimulated lymphocytes, DNA synthesis of cultures exposed to OGF (10^{-6} M), NTX (10^{-5}), or an equivalent volume of sterile water

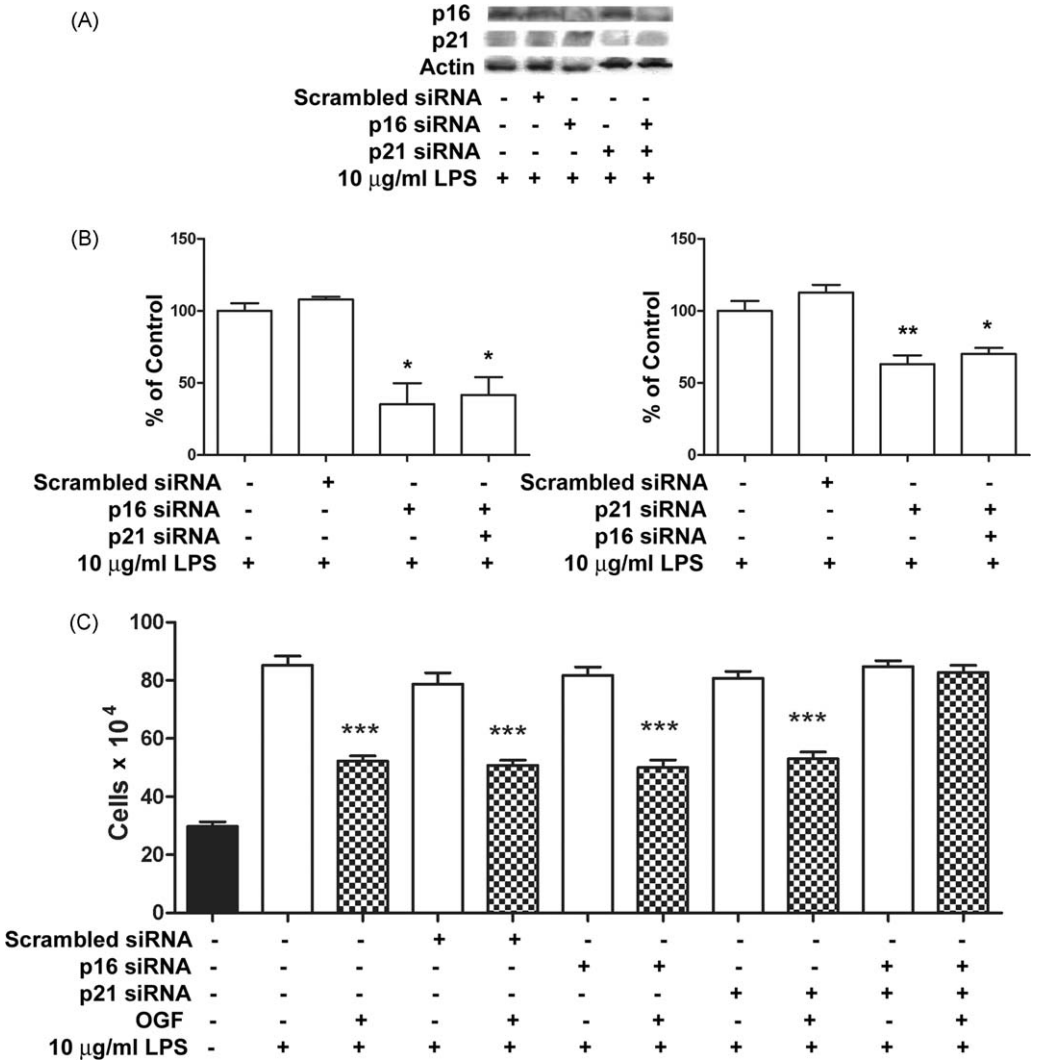

Fig. 7. The OGF-OGFr axis in LPS-stimulated lymphocytes requires either the p16 or p21 cyclin-dependent inhibitory kinase pathway. LPS (10 μg/ml)-stimulated cells were transfected with either p16, p21, p16 and p21, or scrambled siRNA and assessed 72 h after the start of transfection. (A) Western blot analysis demonstrating p16 and/or p21 knockdown. (B) Quantitative densitometry monitoring the level of p16 and p21 knockdown in LPS (10 μg/ml)-stimulated lymphocyte cultures. Data (percentage of p16/actin and p21/actin ratio) represent mean ± S.E. for two independent samples. Significantly different from LPS-treated non-transfected and scrambled siRNA-transfected cultures at $p < 0.05$ (*) and $p < 0.01$ (**). (C) Cell counts of LPS (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 ± S.E. cell counts for at least two aliquots/well and least two wells/treatment. Significantly different at $p < 0.001$ (***) from cultures stimulated with LPS that were not transfected or transfected with scrambled siRNA.

was determined (Fig. 6). At the 24 h timepoint, the quantity of [^{3}H]-thymidine incorporation in LPS-stimulated cultures exposed to OGF was decreased by 56% compared to LPS-stimulated cultures receiving sterile water. The incorporation of [^{3}H]-thymidine in LPS-stimulated cultures subjected to NTX was comparable to cultures stimulated with LPS that received sterile water.

Examination of apoptosis by Annexin V staining revealed that cultures stimulated with LPS and receiving OGF (10^{-6} M) or NTX (10^{-5} M) had levels of apoptosis that were similar to preparations stimulated with LPS and receiving sterile water at 12 h (23%), 24 h (5%), 48 h (4%), and 72 h (6%). Likewise, the number of necrotic cells as measured by 7AAD incorporation in lymphocytes stimulated with LPS and treated with OGF or NTX were similar to LPS-stimulated cultures receiving sterile water at 12 h (34%), 24 h (55%), 48 h (37%), and 72 h (37%).

The cyclin-dependent inhibitory kinases p16 or p21 are required for OGF-induced inhibition of cell proliferation in splenic-derived lymphocytes stimulated with LPS

To test whether the cyclin-dependent inhibitory pathway is involved in the effects of OGF on B lymphocytes, experiments were performed to determine if p16 and/or p21 is(are) required. Lymphocytes stimulated with LPS were transfected with p16, p21, both p16 and p21, or scrambled siRNAs. Cells transfected with p16 or p21 siRNAs had protein levels that were reduced by 64% and 37%, respectively, from values of LPS-stimulated cultures exposed to sterile water at 72 h (Fig. 7A and B). Protein levels of LPS-stimulated cultures transfected with both p16 and p21 siRNAs reduced p16 and p21 expression by 58% and 29%, respectively; these reductions in protein values did not differ from groups transfected with either p16 or p21 siRNAs alone. However, LPS-stimulated cells exposed to scrambled siRNA were comparable in protein levels of p16 and p21 to LPS-treated non-transfected cells. LPS-stimulated cultures that were 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 38% to 41% compared to LPS-stimulated cells subjected to sterile water (Fig. 7C). However, cultures stimulated with LPS and transfected with siRNA against both p16 and p21, and treated with OGF, were comparable in cell number to LPS-stimulated preparations not transfected and receiving sterile water (Fig. 7C).

Discussion

This report is the first to demonstrate that the OGF-OGFr axis is capable of suppressing B lymphocyte proliferation. Using a paradigm of stimulating splenic-derived B lymphocytes with LPS, a number of pieces of evidence are presented to arrive at this conclusion. First, immunoreactivity for both OGF-like peptide and OGFr can be detected in B lymphocytes. Second, B cells contain specific and saturable binding of OGFr for OGF. Third, exogenous OGF depresses B lymphocyte proliferation in a dose-dependent manner. Fourth, other natural and synthetic opioids, some of which are specific to μ, δ, and κ opioid receptors, do not alter B lymphocyte replication. Fifth, exogenous OGF has no effect on B lymphocyte cultures that have a knockdown of OGFr. Sixth, exogenous OGF does depress cell proliferation in cultures with a knockdown of classical opioid receptors (μ, δ, κ). Seventh, exogenous OGF inhibits DNA synthesis in B lymphocytes, but does not influence apoptosis or necrosis. Eighth, OGF's suppression of B lymphocyte proliferative processes is dependent on p16 and p21 cyclin-dependent inhibitory kinase pathways. Moreover, OGF treatment did not have any effect on cultures that were not stimulated with LPS, indicating that cells had to be activated and proliferating in order for this peptide to influence cellular proliferation. Thus, the OGF-OGFr axis is a native and established biological pathway that can be harnessed to depress B lymphocyte proliferation.

Although the OGF-OGFr axis is present in B lymphocytes, and can function to depress cell proliferation as demonstrated by the addition of exogenous OGF, at least under the conditions of these studies, unstimulated or LPS-stimulated B lymphocytes are not dependent on an autocrine regulation of cell replication by the OGF-OGFr system. The evidence for this conclusion emanates from three observations. First, blockade of native opioids from opioid receptors, including OGF and OGFr, in either stimulated or non-stimulated B lymphocyte preparations by 10^{-4} to 10^{-10} M concentrations of the opioid antagonist NTX, did not alter the population of B lymphocytes. Moreover, even when the concentration of LPS was decreased to reduce the magnitude of stimulation and allow a greater window for a potential increase in cell number, exposure to NTX did not lead to a greater number of B lymphocytes. These results documenting an absence of effect on B lymphocyte proliferation by interfering with OGF-OGFr interfacing support previous studies with naloxone that showed a similar lack of change (Gabrilova and Marotti 2000; Kowalski 1998). Second, knockdown of OGFr with OGFr siRNA revealed no alteration in B lymphocyte number compared to LPS-stimulated cultures transfected with scrambled siRNA. These data speak to the fact that attenuating OGFr, and thus obstructing peptide-receptor interaction, does not increase cell proliferation. Third, neutralization of OGF by specific antibodies did not alter cell number. Once again, reducing the peptide, OGF, does not elevate cell proliferation as would be expected if the OGF-OGFr axis was continually tonically regulating cell replication.

These discoveries concerning the absence of a dependence on OGF-OGFr interfacing to maintain the pace of cell proliferation represent an exception to the numerous reports demonstrating 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 may explain these findings. First, unlike any other system studied to date (e.g., neoplasia, cellular renewal, development) regarding the OGF-OGFr axis, B lymphocytes do not undergo homeostatic renewal but rather require stimulation to initiate DNA synthesis and replication. Perhaps the absence of tonic activity between peptide and receptor is a means of conservation of a biological process, and persistent OGF-OGFr interfacing is not a constant requirement in these cells. However, the fact that exogenous OGF can depress B lymphocyte proliferation, and does so with mediation by OGFr, suggests that this axis is viable. As such, OGF from paracrine (e.g., monocytes) or endocrine sources may be capable of altering B lymphocyte replication when one or both are invoked under certain situations wherein B lymphocytes are stimulated. In fact, because the manifestation of B lymphocyte response is known to require other cell populations, it may be that one or more of these cell types produce(s) OGF. Therefore, follow-up studies in animals, with and without stimulation, are mandated regarding B lymphocytes, as well as establishing the relationship of the OGF-OGFr axis to other cell types (e.g., T cells, NK cells). A second factor that may be related to the observation that interference with OGF-OGFr interactions does not elevate cell proliferation is that although activated B lymphocytes express preproenkephalin mRNA (Rosen et al. 1989; Behar et al. 1991), and immune reactive enkephalins have been recorded (Rosen et al. 1989), the exact nature of the enkephalin-containing peptides produced by the lymphoid system is not known. In the case of T lymphocytes, detection of only small amounts of preproenkephalin-derived peptides, which require dibasic cleavage for formation of smaller peptides (e.g., OGF) and are the opioid-active forms, have been recorded (Roth et al. 1989; Kuis et al. 1991) despite the presence of preproenkephalin mRNA. If this is the case in B lymphocytes, it would be understandable why the endogenous OGF-OGFr system is not

physiologically active, even though we detect some enkephalin-like immunoreactivity.

The effects of endogenous opioid peptides on the proliferation of B lymphocytes have been explored by a few investigators, with most reports focused on [Met^5]-enkephalin or β-endorphin. Because of differences in methodologies (e.g., *in vitro* vs. *in vivo,* use of LPS), it is difficult to formulate a consensus of function. In the case of [Met^5]-enkephalin, Soder and Hellstrom (1987) and Kowalski (1998) have reported an increase in B cell proliferation. Das et al. (1997) and Morgan et al. (1990), however, did not observe any change in B lymphocyte proliferation with exposure to this opioid peptide. Furthermore, Gabrilova and Marotti (2000) have found gender-related differences in B lymphocyte proliferation following exposure to [Met^5]-enkephalin. With regard to other endogenous opioid peptides, β-endorphin appears to have no effect on B cell proliferation (Morgan et al. 1990; Gilman et al. 1982; van den Bergh et al. 1993). 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 B lymphocyte proliferation, and only if the cultures were stimulated. These greater than physiological concentrations of peptide may have been necessary to elicit 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 B lymphocytes. Whether other concentrations of these peptides or a delivery system that delays the processing of these peptides can influence B lymphocyte replication is unclear.

The humoral immune response is achieved by antibodies secreted by B lymphocytes. When naive B cells "see" their antigen in the secondary lymphoid tissue in an appropriate environment, which includes T cells and dendritic cells, they become activated and proliferate. The results of these investigations reveal that B 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 B 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 B lymphocyte proliferation by the OGF-OGFr axis. Exogenous (and perhaps endogenous) OGF enters these cells by a process of active transport (Cheng and Zagon, personnel communication) and interacts with the OGF receptor on the outer nuclear envelope. The OGF-OGFr complex translocates to the nucleus with guidance from nuclear localization signals encoded in OGFr (Cheng et al. 2009a), and utilizes karyopherin β/Ran to shuttle to the nucleus (Zagon et al. 2005a). The OGF-OGFr complex upregulates p16 and/or p21 in the cyclin-dependent inhibitory kinase pathway, thereby delaying the G_1-S phase of the cell cycle (Cheng et al. 2009b, 2007a,b). This delay would lead to a diminution in all of the subsequent events related to B cell proliferation (e.g., antibody formation). Little is known about the effects of endogenous opioids and antibody response. Johnson and colleagues (Johnson et al. 1982) reported that [Met^5]-enkephalin and [Leu^5]-enkephalin, but not β-endorphin, depressed sheep red blood cell formation, and Jankovic and Maric (Jankovic and Maric 1987) observed that humoral responses were compromised in rats injected with [Met^5]-enkephalin. Thus, although one would predict that repressed B lymphocyte proliferation as seen herein would lead to a reduction in antibody formation, and this observation is consistent with Johnson et al. (1982) and Jankovic and Maric (1987), further studies under *in vivo* conditions are required.

The clinical implications of these findings are profound. OGF has been shown to depress the expression of EAE (Zagon et al. 2010), as well as the progression of human neoplasias (Zagon et al. 2008b, 2009a; McLaughlin et al., 2005). 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 naltrexone (LDN) also utilizes the OGF-OGFr axis, and this agent is both safe and effective in the treatment of Crohn's disease (Smith et al. 2007). 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 (Zagon et al. 2008b, 2009a; McLaughlin et al. 2008), LDN (Hytrek et al. 1996), or imiquimod (Zagon et al. 2008a), thereby invoking a natural immunosuppressive pathway that is non-toxic and of high efficacy. B lymphocyte targeted therapies (e.g., rituximab) are already in clinical practice for some autoimmune diseases (Dörner et al. 2009; Pescovitz et al. 2009). Therapies directed to the OGF-OGFr axis (e.g., LDN, imiquimod, OGF) would represent an entirely new direction for agents that modulate native biological processes in a non-toxic, highly efficacious, rapid, and relatively inexpensive manner that has minimal side-effects.

Disclosures

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