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Gemfibrozil ameliorates relapsing-remitting experimental autoimmune encephalomyelitis independent of PPAR-α

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Running title: Gemfibrozil inhibits EAE

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Abbreviations: MS, multiple sclerosis; EAE, experimental allergic encephalomyelitis; PPAR, peroxisome prolieferator-activated receptor; iNOS, inducible nitric oxide synthase; Th, T-helper; H&E, Hematoxylin-Eosin; LFB, luxol fast blue; BBB, blood-brain barrier

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Abstract

The present study underlines the importance of gemfibrozil, a lipid-lowering drug and an activator of peroxisome proliferator-activated receptor-α (PPAR-α), in inhibiting the disease process of adoptively-transferred experimental allergic encephalomyelitis (EAE), an animal model of relapsing-remitting multiple sclerosis (RR-MS). Clinical symptoms of EAE, infiltration of mononuclear cells and demyelination were significantly lower in SJL/J female mice receiving gemfibrozil through food chow than those without gemfibrozil. Interestingly, the drug was equally effective in treating EAE in PPAR-α wild type as well as knockout mice. Gemfibrozil also inhibited the encephalitogenicity of MBP-primed T cells and switched the immune response from a Th1 to a Th2 profile independent of PPAR-α. Consistently, gemfibrozil inhibited the expression and DNA-binding activity of T-bet, a key regulator of IFN-γ expression, while stimulating the expression and DNA-binding activity of GATA3, a key regulator of IL-4. Gemfibrozil treatment decreased the number of T-bet positive T cells and increased the number of GATA3-positive T cells in spleen of donor mice. The histological and immunohistochemical analyses also demonstrate the inhibitory effect of gemfibrozil on the invasion of T-bet positive T cells into the spinal cord of EAE mice. Furthermore, we demonstrate that the differential effect of gemfibrozil on the expression of T-bet and GATA3 was due to its inhibitory effect on NO production. While excess NO favored the expression of T-bet, scavenging of NO stimulated the expression of GATA-3. Taken together, our results suggest gemfibrozil, an approved drug for hyperlipidemia in humans, may further find its therapeutic use in MS.

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Introduction

Multiple sclerosis (MS) is one of the most common neurological diseases affecting young adults. Although the etiology of MS is not completely understood, studies of MS patients suggest that the observed demyelination in the CNS is a result of a T cell-mediated autoimmune response (Martin et al., 1992). Experimental allergic encephalomyelitis (EAE) serves as an animal model for MS (Tuohy et al., 1988; Benveniste 1997; Du et al., 2001). Studies using the adoptively transferred EAE model strongly support the view that activated neuroantigen-specific T cells cross the blood-brain barrier (BBB), infiltrate the CNS parenchyma and initiate an inflammatory response (Tuohy et al., 1988; Benveniste 1997). The identification of wide range of proinflammatory cytokines, cell adhesion molecules, chemokines, proinflammatory enzymes like inducible nitric oxide synthase (iNOS) and cyclooxygenase in CNS lesions of MS patients and EAE animals (Martin et al., 1992; Benveniste 1997) suggests that the inflammatory process initiated by activated T cells eventually becomes a broad-spectrum one. Therefore, analysis of molecular mechanisms for the regulation of this broad-spectrum inflammatory process in EAE should help decipher the mechanisms of the disease process in MS/EAE and further the possibility of developing effective therapeutic strategies for MS patients.

Peroxisome proliferator-activated receptors (PPARs), members of the nuclear hormone receptor superfamily, have been implicated in a variety of human disorders. Three isotypes have been described to date, PPAR-α, PPAR-β and PPAR-γ (Lemberger et al., 1996). Activation of PPAR-α mainly leads to the induction of a variety of genes such as those coding for the enzymes for β- and ω-oxidation of fatty acids (Dreyer et al., 1992). Gemfibrozil, an activator of PPAR-α, has been often prescribed in patients to lower the level of triglycerides (Hsu et al., 2001; Bloomfield et al., 2001). This drug decreases the risk of coronary heart disease by increasing the level of

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high-density lipoprotein (HDL) cholesterol and decreasing the level of low-density lipoprotein (LDL) cholesterol (Hsu et al., 2001; Bloomfield et al., 2001). Earlier we (Pahan et al., 2002) have shown that gemfibrozil inhibits the expression of inducible nitric oxide synthase (iNOS) in human astrocytes suggesting that this drug may ameliorate neuroinflammatory disorders. Consistently, Racke and colleagues (Xu et al., 2005; Lovett-Racke et al., 2004) have found that gemfibrozil and other fibrate drugs suppress the induction of NO production in microglia and attenuate the disease process in an actively-immunized primary progressive EAE model in B10.PL mice, one of the animal models of primary progressive MS. Considering the facts that majority of (85 percent) MS patients at the time of original diagnosis suffer from the relapsing-remitting disease, that B10.PL mouse does not have the relevant locus for relapsing-remitting EAE (RR-EAE), and that gemfibrozil is a known agonist of PPAR-α, it is important to determine whether gemfibrozil is capable of suppressing the disease process of RR-EAE and whether anti-neuroimmune effect of gemfibrozil depends on PPAR-α.

Here we demonstrate that gemfibrozil inhibits the disease process of RR-EAE in an adoptively-transferred EAE model in female SJL/J mice by shifting the encephalitogenic immune response from Th1 to Th2 mode without the involvement of PPAR-α. Interestingly, we also present evidence that gemfibrozil increases the expression of GATA-3, a key regulator of Th2 cytokines, and decreases the expression of T-bet, a key regulator of Th1 cytokines, through the inhibition of NO production.

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Materials and Methods

Reagents: Fetal bovine serum (FBS) and RPMI 1640 were from Invitrogen (Carlsbad, CA). Gemfibrozil was purchased from Sigma (St. Louis, MO). L-N^6-(1-Iminoethyl)-lysine (L-NIL) and carboxy PTIO were obtained from Biomol (Plymouth Meeting, PA). Oligonucleotide probes for T-bet and GATA and antibodies against T-bet and GATA3 were purchased from SantaCruz Biotechnology (SantaCruz, CA). Rat anti-mouse pan macrophage marker (moma-2) was purchased from Biosource International (Camarillo, CA). Rabbit anti-mouse iNOS antibody was obtained from Calbiochem. [γ-^{32}P] ATP was purchased from NEN (Boston, MA).

Animals: Female and male SJL/J mice (4-6 weeks old) were purchased from Harlan Sprague Dawley (Indianapolis, IN). The PPAR-α wild type (B6.129) and knock out (B6.129) mice were purchased from Jackson Laboratory and bred with SJL/J mice in the institutional animal care facility under germ-free condition. F1 littermates were bred among themselves and resulting pups were screened by PCR. Female SJL/J X PPAR-α (-/-) B6.129 and SJL/J X PPAR-α (+/+) B6.129 mice were used for adoptive transfer experiments. Animal maintenance and experimental procedures were in accordance with National Institute of Health guidelines and were approved by the Institutional Animal Care and Use committee (IACUC).

Isolation of MBP-primed T cells: Female SJL/J, SJL/J X PPAR-α (+/+) and SJL/J X PPAR-α (-/-) were immunized s.c. with 400 μg bovine MBP (Invitrogen) and 60 μg *Mycobacterium tuberculosis* (H37 RA; Difco Labs, Detroit, MI) in IFA (Calbiochem). Lymph nodes were collected from these mice on day 10 post immunization and single cell suspension was prepared in RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 50 μM 2-ME, 100 U/ml penicillin and 100 μg/ ml streptomycin (Dasgupta et al., 2003, 2004). Lymph node cells (LNC) cultured at a concentration of 4-5 X 10^6 cells/ml in six-well plates were incubated with 50 μg/ ml of MBP for

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4 days. MBP reactivity of LNC was measured by [^{3}H]thymidine (NEN) incorporation assay of parallel microplate cultures. The non adherent LNC were harvested at 500 g and resuspended in RPMI 1640-FBS. Viability of the cells was checked by Trypan blue exclusion. These MBP-primed lymph node T cells were used to induce EAE in mice.

Induction of relapsing-remitting EAE (RR-EAE): The RR-EAE in mice was induced by adoptive transfer of MBP primed T cells via i.v. route as described earlier (Dasgupta et al., 2003, 2004). Briefly, 2 X 10^7 viable MBP-primed T cells suspended in a volume of 200 µl HBSS was injected to naïve mice through tail vein. Pertussis toxin (150 ng/mouse; Sigma, MO) was injected once via i.p route on 0 day. Animals were observed daily for clinical symptoms. Experimental animals were scored by a masked investigator as follows: 0, no clinical disease; 0.5 piloerection, 1.0 tail weakness; 1.5 tail paralysis; 2.0, hind limb weakness; 3.0, hind limb paralysis; 3.5, fore limb weakness; 4.0, fore limb paralysis; 5 moribund or death.

Treatment of mice with gemfibrozil: Mice were treated with gemfibrozil through food chow. Gemfibrozil was solubilized in ethanol and the resulting solution was mixed with chow followed by drying in air. Similarly, control chow received only ethanol as the vehicle. For treatment, mice were given chow containing various doses of gemfibrozil (0.1% - 0.3% w/w) from 0 dpt. In some experiments, mice were allowed to take chow containing 0.2% w/w gemfibrozil in different phases of the disease. Statistical analysis was determined by the RS/1 multicomparison procedure using a one-way ANOVA and Dunnett's test for multiple comparisons with a common control group. Differences between means were considered significant when p values were < 0.05.

Semi-quantitative RT- PCR analysis: It was carried out using a kit from Clontech as described earlier (Dasgupta et al., 2004; Saha and Pahan, 2006). Briefly, total RNA was isolated from

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cerebellar tissues using Ultraspec-II RNA reagent (Biotecx Laboratories, Inc.) and 1 μg of DNase-digested RNA was reverse transcribed using oligo$(dT)_{12\text{-}18}$ as primer and MMLV reverse transcriptase in a 20 μl reaction mixture. The resulting cDNA was appropriately diluted, and diluted cDNA was amplified using Titanium Taq DNA polymerase and following primers.

iNOS: Sense: 5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3'

Antisense: 5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'

IL-1β: Sense: 5'-ATGGCAACTGTTCCTGAACTCAACT-3'

Antisense: 5'-CAGGACAGGTATAGATTCTTTCCTTT-3'

TNF-α: Sense: 5'-TTCTGTCTACTGAACTTCGGGGTGATCGGTCC-3'

Antisense: 5'-GTATGAGATAGCAAATCGGCTGACGGTGTGGG-3'

IL-6: Sense: 5'-TGGAGTCACAGAAGGAGTGGCTAAG-3'

Antisense: 5'-TCTGACCACAGTGAGGAATGTCCAC-3'

PPAR-α: Sense: 5'-CAG AGC AAC CAT CCA GAT GA-3'

Antisense: 5'-AAA CGC AAC GTA GAG TGC TG -3'

PPAR-γ: Sense: 5'- ATG CCA AAA ATA TCC CTG GTT TC -3'

Anti-sense: 5'-GGA GGC CAG CAT GGT GTA GA -3'

PPAR-β: Sense: 5'-ATG GAA CAG CCA CAG GAG GAG ACC C-3'

Anti-sense: 5'-GGC ACT TCT GGA AGC GGC AGT ACT G-3'

GAPDH: Sense: 5'-GGTGAAGGTCGGTGTGAACG-3'

Antisense: 5'-TTGGCTCCACCCTTCAAGTG-3'

Amplified products were electrophoresed on a 1.8% agarose gels and visualized by ethidium bromide staining. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used to ascertain that an equivalent amount of cDNA was synthesized from different samples.

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Quantification of gemfibrozil by HPLC: The concentration of gemfibrozil in mouse brain was measured as described earlier by Kadenatsi et al (1995) with some modifications. Briefly, 100 mg brain tissue of each mice was homogenized in 1 ml chloroform: methanol: perchloric acid (2:1:0.05) and the homogenate was centrifuged at 10,000 x g for 10 min at room temperature. Pravastatin was added as internal standard before homogenization. The organic layer (upper) was taken out carefully and dried up in a Centrivap concentrator (Labconco) followed by resuspension in 50 μl acetonitrile. Ten microliter sample was then analyzed in Waters 2695 separation module HPLC system using the Phenomenex Luna C18 separation column (250 X 4.6 mm). Samples were analyzed with an isocratic gradient consisted of acetonitrile : 0.01 M phosphoric acid (1:1), at the flow rate of 0.5 ml/min at room temperature. The amount of gemfibrozil was quantified by monitoring different concentrations of standards (0.1, 1, 10, 50, and 100 μg/ml).

The same separation column was used to analyze the concentration of gemfibrozil in plasma. Pravastatin was added before vortexing and centrifugation at 9,500 g for 15 min. Clear supernatants were filtered through a 0.2 μm syringe filter and 10 μl filtered supernatants were directly injected into the HPLC system for analysis.

Immunofluorescence microscopy: On 19 dpt (acute phase), six mice from each of the following groups (control, vehicle-treated EAE, and EAE mice receiving gemfibrozil through chow from 8 dpt) were anaesthetized. Following perfusion spinal cord was dissected out from each mouse as described earlier (Dasgupta et al., 2003, 2004). One half of a spinal cord was processed for immunofluorescence microscopy and the other half was used for Hematoxylin-Eosin (H&E) staining. Briefly, for immunofluorescence microscopy, tissues were incubated in PBS containing Tween 20 (PBST), 10% sucrose for 3h, and then 30% sucrose overnight at 4^0C.

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The spinal cord tissues were then embedded in OCT (Tissue-Tek, Elkhart, IN) at -80 ^{0}C and processed for conventional cryosectioning. The frozen sections (8 μm) were treated with cold ethanol (-20^{0}C) followed by two rinses in PBS. Samples were blocked with 3% BSA in PBST for an hour, washed and incubated in PBST-BSA and goat anti-CD3 (1: 50) along with either rabbit anti-T-bet (1: 200) or rabbit anti-GATA3 (1: 200) antibody for double labeling tissue sections. For iNOS and moma-2 colocalization studies, splenic tissue sections were incubated with rabbit anti-iNOS (1:50) and rat anti-mouse pan macrophage marker moma-2 (1:25). After three consecutive washes in PBST, sections were further incubated with Cy2 and Cy5 (Jackson ImmunoResearch Laboratories, West Grove, PA). A set of sections was incubated under similar conditions without primary antibodies. In some experiments, spleen tissue sections were also analyzed by immunofluorescence. The samples were mounted and observed under a BioRad MRC1024ES confocal laser scanning microscope.

Histology: The other part of the tissue was processed for routine histology to obtain perivascular cuffing and morphological details of spinal cord tissues of EAE mice as described earlier (Dasgupta et al., 2003, 2004). The paraformaldehyde-fixed tissues were embedded in paraffin, and serial sections (4 μm) were cut. Sections were stained with conventional H&E staining method. Digital images were collected under bright field setting using a 40X objective.

Staining for myelin: Serial longitudinal sections of paraformaldehyde-fixed spinal cords were stained with Luxol fast blue (LFB) for myelin as described elsewhere.

ELISA: Lymph node cells (1 X 10^6/ml) in each well of a 96 well plate were treated in the presence or absence of MBP and gemfibrozil for a period of 72 h. Supernatants in each well was collected and the presence of Th1 cytokine IFN-γ and Th2 cytokine IL-4 was assayed using high-sensitive ELISA kits (BD Biosciences, Mountain View, CA).

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Assay for NO synthesis: Synthesis of NO was determined by assay of culture supernatants for nitrite, a stable reaction product of NO with molecular oxygen. Briefly, 400 µl of culture supernatant was allowed to react with 200µl of Griess reagent and incubated at room temperature for 15 min. The optical density of the assay samples was measured spectrophotometrically at 570 nm. Fresh culture media served as the blank in all experiments. Nitrite concentrations were calculated from a standard curve derived from the reaction of $NaNO_2$ in the assay.

EMSA: Nuclear extracts were prepared as described earlier (Dasgupta et al., 2004; Pahan and Schmid, 2000) with slight modifications. Briefly, spleens were homogenized in buffer A (10mM HEPES pH 7.5, 10mM KCL, 0.1 mM EGTA, 1.0 mM DTT, 1mM PMSF and 10 µg/ml each of leupeptin, antipain, and pepstatin A), incubated on ice for 15 min and treated with 1.0% Nonidet P40. The extract was then vortexed for 15 s and centrifuged at 14,000 X g for 30 s. The pelleted nuclei were resuspended in buffer B (25% glycerol, 0.4 M NaCl, 20mM HEPES pH 7.5, 1 mM EGTA, 1.0 mM DTT, 1 mM PMSF and protease inhibitors), vortexed, sonicated briefly and incubated on ice for 30 min. The lysates were then centrifuged at 14,000 X g for 10 min. Supernatants containing the nuclear extract were collected and used for EMSA using ^{32}P-labeled double stranded T-bet and GATA oligonucleotide probes (Santa Cruz). The corresponding mutated probes were used at the same time to verify the specificity of T-bet or GATA binding to DNA. The supershift assay was performed by incubating the nuclear extract with 2 µg of either T-bet or GATA3 antibody for a period of 30 min at 4^0C prior to incubation with corresponding oligonucleotides for DNA-binding assay.

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Results

Effect of gemfibrozil on clinical symptoms and disease severity of relapsing-remitting EAE (RR-EAE) in an adoptively-transferred model: To understand the therapeutic efficacy of gemfibrozil against RR-EAE, we examined the effect of this drug on clinical symptoms and disease severity of adoptively-transferred EAE. By adoptive transfer, we have achieved 100% incidence of EAE in female SJL/J mice displaying an acute phase of clinical signs peaking at 19 dpt and subsequently, a pattern of relapsing-remitting signs in the chronic phase (Table-1 & Fig. 1). To determine the appropriate dose of gemfibrozil, mice (n=6 in each group) were treated with different doses of gemfibrozil via food chow from day 0 of transfer of MBP-primed T cells. Control mice received chow containing vehicle only. Clinical symptoms were monitored daily until 30 dpt. We have found that food chow containing 0.1% gemfibrozil (w/w) inhibited the clinical symptoms and the maximum inhibition was observed with food chow containing 0.2 or 0.3% gemfibrozil (w/w) (Fig. 1A). Therefore, mice were treated with chow containing 0.2% gemfibrozil (w/w) in further experiments. The results summarized in Table-1 demonstrate that chow containing 0.2% (w/w) gemfibrozil could significantly ($p < 0.001$) reduce both the incidence and the clinical signs of EAE.

Next we examined whether after oral feeding, gemfibrozil enters into the brain. As mentioned under “Materials and Methods”, the level of gemfibrozil was quantified in plasma and brain tissues of mice (n=4) after 7 d of feeding of chow containing 0.2% gemfibrozil. The level of gemfibrozil in plasma was 56.82 $\pm$ 17.38 μg/ml. Kim et al (2006) have shown that 2 h after an oral dose of 300 mg of gemfibrozil, the plasma concentration of gemfibrozil in healthy male volunteers goes up to approximately 10 μg/ml. Because the usual dose of gemfibrozil in adult patients with hyperlipidemia is 1200 mg/d, the plasma level of this drug may go up to 40 μg/ml,

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which is comparable to the plasma concentration we found in mice. On the other hand, in the brain, the level reached to 17.2 $\pm$ 5.09 μg/gm tissue. However, we did not detect any gemfibrozil in either plasma or brain of mice receiving normal chow containing only the vehicle. These results suggest that gemfibrozil is capable of entering into the brain.

Gemfibrozil inhibits the progression of RR-EAE in an adoptively-transferred model: Next we investigated whether gemfibrozil could be used to prevent the progression of RR-EAE in the adoptively-transferred model. To achieve this goal, mice (n=6) were allowed to take chow containing 0.2% (w/w) gemfibrozil from the onset of acute phase (8 dpt). It is clearly evident from Figure 1B that gemfibrozil was capable of inhibiting EAE clinical symptoms within 5 days of treatment (from 13 dpt). However, there was further marked inhibition on subsequent days of treatment and this inhibition was maintained throughout the duration of the experiment (Fig. 1B). These results clearly suggest that gemfibrozil can control the ongoing relapsing-remitting EAE.

Gemfibrozil inhibits the infiltration of inflammatory cells into the CNS of RR-EAE mice: H & E staining of longitudinal sections of spinal cord was carried out to determine whether the diminished clinical disease in gemfibrozil-treated mice correlated with reduced CNS infiltration of blood mononuclear cells. Blood vessels of the spinal cord of mock control mice were completely devoid of inflammatory infiltrates, while substantial perivascular cuffing of mononuclear cells was observed in the spinal cord of EAE mice at the peak (19 dpt) of the acute phase (Fig. 1C). In contrast, very few immune cell infiltrates were found in the spinal cord of mice receiving gemfibrozil from the onset (8 dpt) of the acute phase (Fig. 1C). These results suggest that gemfibrozil may act in part by either preventing activated myelin-specific T cells and/or inflammatory cells from entering into the CNS parenchyma or from being retained in the CNS.

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Gemfibrozil inhibits the expression of proinflammatory molecules in the CNS of RR-EAE mice: Earlier we (Pahan et al., 2002) and others (Xu et al., 2005) have observed that gemfibrozil attenuates the induction of proinflammatory molecules in glial cells. Therefore, we next examined whether gemfibrozil was capable of inhibiting the expression of proinflammatory molecules *in vivo* in the CNS of EAE mice. Marked expression of pro-inflammatory molecules like iNOS, IL-1β, IL-6, and TNF-α was observed in cerebellum of EAE mice during the peak of the acute phase compared to control mice (Fig. 1D). However, gemfibrozil treatment through food chow dramatically reduced the expression of these pro-inflammatory molecules in the CNS of EAE mice (Fig. 1D).

Gemfibrozil suppresses demyelination in the CNS of RR-EAE mice: It is believed that infiltration of blood mononuclear cells and associated neuroinflammation plays an important role in CNS demyelination observed in MS patients and EAE animals. Therefore, we examined whether gemfibrozil protected EAE mice from demyelination. We stained longitudinal sections of spinal cord by luxol fast blue (LFB) for myelin and observed widespread demyelination zones in the white matter of the spinal cord of EAE mice (Fig. 1C, left column). However, gemfibrozil treatment via chow remarkably restored myelin level in the spinal cord of RR-EAE mice (Fig. 1C, left column).

Level of different PPARs in brain and spleen of mice with RR-EAE: Because gemfibrozil is a known activator of PPAR-α (Lemberger et al., 1996; Dreyer et al., 1992), a member of the nuclear hormone receptor super family, we analyzed the level of PPAR-α and two other PPARs (PPAR-β and PPAR-γ) in cerebellum and spleen of EAE mice at the peak of the acute phase. PPAR-α mRNA that was detected in both cerebellum and spleen of control mice was strongly inhibited after the induction of EAE (Fig. 2). However, gemfibrozil treatment restored the level

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of PPAR-α in both cerebellum and spleen of EAE mice (Fig. 2). On the other hand, as compared to normal mice, the level of PPAR-β increased in cerebellum of EAE mice and decreased in cerebellum of gemfibrozil-treated EAE mice. Surprisingly, either induction of EAE or treatment with gemfibrozil was unable to modulate the level of PPAR-β in the spleen (Fig. 2). Similar to the regulation of PPAR-β in cerebellum, the expression of PPAR-γ also increased in cerebellum of EAE mice as compared to control mice (Fig. 2). However, in contrast to the inhibition of PPAR-β, gemfibrozil was unable to suppress the expression of PPAR-γ in cerebellum of EAE mice. On the other hand, similar to PPAR-α, the expression of PPAR-γ decreased in the spleen of EAE mice but not in the spleen of gemfibrozil-treated EAE mice (Fig. 2). These results suggest that gemfibrozil differentially regulate the expression of different PPARs in brain and spleen of EAE mice.

Does gemfibrozil prevent adoptive transfer of RR-EAE independent of PPAR-α? PPAR-α (-/-) mice were employed to address the question whether gemfibrozil suppresses clinical symptoms of EAE via PPAR-α. PPAR-α (-/-) mice are based on B6.129 background, therefore, we examined if RR-EAE could be induced in this background strain by adoptive transfer. However, in contrast to that observed in female SJL/J mice (Fig. 1A), female B6.129 mice did not develop RR-EAE after adoptive transfer of MBP-primed T cells (Fig. 3A). Only piloerection was observed as the highest clinical symptom in these mice. Therefore, to maintain the RR-EAE-positive loci, we used the F1 cross between B6.129 and SJL/J to induce adoptively-transferred RR-EAE. As observed in Figure 3A, it was possible to induce RR-EAE in female B6.129 X SJL/J mice. Next we examined the effect of gemfibrozil on adoptively-transferred RR-EAE in female (B6.129 X SJL/J) PPAR-α wild type and knockout mice. Four groups (PPAR-α wild type EAE positive control, PPAR-α wild type gemfibrozil-treated EAE, PPAR-α knockout EAE

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positive control, and PPAR-α knockout gemfibrozil-treated EAE) of mice (n=6 in each group) were included in this study. The treatment with gemfibrozil-containing chow (0.2% w/w) in both wild type and knockout groups began from 0 dpt. Upon adoptive transfer, EAE was developed in PPAR-α wild type and knockout mice that attained a peak mean clinical score of around 2.2 at 18 - 19 dpt in both the cases (Fig. 2) followed by a relapsing-remitting course as seen in female SJL/J mice (Fig. 1). Interestingly, gemfibrozil suppressed clinical symptoms of EAE with similar potency in both PPAR-α (+/+) and PPAR-α (-/-) mice (Fig. 2). The results summarized in Table-2 demonstrate that chow containing 0.2% (w/w) gemfibrozil could significantly ($p < 0.001$) reduce incidence and clinical signs of EAE in both PPAR-α (+/+) (A) and PPAR-α (-/-) (B) mice. These results suggest that gemfibrozil ameliorates clinical symptoms of RR-EAE independent of PPAR-α.

Therefore, next we examined if other agonist of PPAR-α also exerts anti-EAE effect independent of PPAR-α. WY14643, a synthetic drug, is considered as a potent agonist of PPAR-α. When tested the effect of this drug on RR-EAE in female (B6.129 X SJL/J) PPAR-α wild type and knockout mice (n=5), we found that similar to gemfibrozil, WY14643 also inhibited clinical symptoms of EAE in both PPAR-α (+/+) and PPAR-α (-/-) mice (Fig. 3B).

Gemfibrozil treatment of donor mice inhibits the generation of encephalitogenic T cells: Because gemfibrozil treatment of recipient mice suppressed the clinical symptoms of RR-EAE, we examined if gemfibrozil treatment of donor mice inhibits the generation of encephalitogenic T cells. SJL/J mice were immunized with MBP/IFA/*M. tuberculosis* as described above, and from the 0 day of immunization, donor mice received chow containing 0.2% (w/w) gemfibrozil. On day 10, the draining lymph nodes were removed and LNCs were activated with 50 μg/ml of MBP for four days. 2×10^7 viable cells were adoptively transferred into naive SJL/J recipients

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and on 0 day post transfer (dpt) of cells, 150 ng of pertussis toxin was injected once via i.p. route. In contrast to MBP-primed T cells isolated from mice receiving vehicle-containing chow, MBP-primed T cells isolated from mice receiving gemfibrozil-containing chow were much less efficient in transferring the disease to recipient mice as judged by a reduction in disease severity (Fig. 4).

Next we were prompted to investigate if gemfibrozil treatment of donor mice inhibits the generation of encephalitogenic T cells via PPAR-α. Therefore, female (B6.129 X SJL/J) PPAR-α (-/-) and wild type mice were immunized and treated with gemfibrozil, and LNC were prepared from draining lymph nodes as described above. Then 2×10^7 viable LNC were adoptively transferred into naive SJL/J recipients followed by treatment with 150 ng of pertussis toxin on 0 dpt. It is clearly evident from Figure 4 that MBP-primed T cells isolated from vehicle-treated B6.129 X SJL/J PPAR-α wild type and knockout mice transferred EAE to recipient SJL/J mice. There was no significant difference in the degree of transfer from PPAR-α wild type and knockout mice (Fig. 4). However, MBP-primed T cells isolated from gemfibrozil-treated B6.129 X SJL/J PPAR-α wild type and knockout mice failed to transfer EAE to recipient SJL/J mice (Fig. 4). In this instance as well, the absence of PPAR-α did not influence the anti-encephalitogenic effect of gemfibrozil. These results clearly suggest that gemfibrozil inhibits encephalitogenicity of neuroantigen-primed T cells independent of PPAR-α.

Gemfibrozil switches the differentiation of MBP-primed T cells from Th1 to Th2 mode independent of PPAR-α: While encephalitogenic IFN-γ producing Th1 cells play an important role in the initiation and progression of EAE, IL-4 producing Th2 cells suppresses the disease process. Therefore, we examined if amelioration of the EAE disease process and suppression of encephalitogenicity of neuroantigen-primed T cells by gemfibrozil is accompanied by inhibition

of the differentiation of myelin-specific T cells into effector Th1 cells, and/or switching towards Th2. Splenocytes isolated from MBP-immunized female SJL/J mice were stimulated with MBP in the presence or absence of gemfibrozil. Figure 5A shows that gemfibrozil dose-dependently inhibited the ability of splenic MBP-primed T cells to produce IFN-γ with the maximum inhibition observed at 100 μM or higher concentration. However, in contrast, gemfibrozil markedly stimulated the production of IL-4 by MBP-primed T cells (Fig. 5B). These results suggest that gemfibrozil is capable of shifting the immune response from a Th1 to a Th2 pattern. This is consistent with earlier report by Lovett-Racke et al (2004) that also demonstrated similar immunomodulatory effect of gemfibrozil in neuroantigen-primed T cells from B10.PL mice. Next we investigated if gemfibrozil required the involvement of PPAR-α to switch the differentiation of MBP-primed T cells from Th1 to Th2. For this, we treated a group of MBP immunized female (B6.129 X SJL/J) PPAR-α (-/-) and wild type mice with either vehicle-treated food chow or food chow containing 0.2% gemfibrozil from the 0 day of immunization. After 10 days of immunization, splenocytes were isolated and stimulated with different doses of MBP for 48h. Results in Figure 5C clearly show that the production of IFNγ increases with increasing concentration of MBP and such increase is found in splenocytes isolated from both female (B6.129 X SJL/J) PPAR-α wild type and knockout mice. However, splenocytes isolated from gemfibrozil-treated both female (B6.129 X SJL/J) PPAR-α wild type and knockout mice produced much less IFN-γ compared to vehicle-treated mice. On the other hand, gemfibrozil treatment stimulated the ability of splenocytes to produce higher levels of IL-4 in both female (B6.129 X SJL/J) PPAR-α wild type and knockout mice compared to vehicle-treated mice (Fig. 5D). Taken together, these results suggest that gemfibrozil does not require PPAR-α to shift the immune response from Th1 to Th2.

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Effect of gemfibrozil on T-bet and GATA3 in MBP-primed splenocytes: We extended our studies further to determine underlying mechanisms behind gemfibrozil-mediated switching of Th cells. While T-bet transcription factor regulates the expression of IFN-γ gene in Th1 cells, GATA3 is known to control the expression of IL-4 in Th2 cells (Szabo et al., 2000; Ouyang et al., 2000). Therefore, we were prompted to determine whether gemfibrozil has any effect on activation and expression of these two transcription factors. Activation of T-bet and GATA3 was monitored by DNA-binding activity. Splenocytes from MBP-immunized female SJL/J mice were incubated with MBP (50 μg/ml) and at different time period of stimulation, non adherent cells were harvested and nuclear extracts were analyzed for DNA-binding activity of T-bet and GATA-3 by electrophoretic mobility shift assay (EMSA). DNA binding activities of T-bet and GATA3 were evaluated by the formation of a distinct and specific complex in gel shift assay. Within 2 h of MBP stimulation, significant amount of T-bet DNA-binding activity was observed that peaked at 12 h (Fig. 6A). This gel shift assay detected a specific band in response to MBP-priming that was not found in case of mutated double-stranded oligonucleotides (Fig. 6A). To further characterize the gel shift band, we performed supershift analysis using antibodies against T-bet. As represented in Figure 6B, the notable DNA-protein band was completely eliminated by pre-incubation of the binding mixture with anti-T-bet antibodies. Although after 24 h of MBP stimulation, the DNA-binding activity of T-bet decreased, certain amount of activity was detected until the duration (72 h) of the experiment (Fig. 6A).

Similarly, MBP-priming also induced the DNA-binding activity of GATA (Fig. 6C). The specific band in response to MBP-priming was observed only in case of the double-stranded wild type oligonucleotides but not in case of the mutated ones (Fig. 6C). By supershift assay, we were able to eliminate this band by anti-GATA3 antibodies (Fig. 6D) suggesting that the observed

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DNA-binding activity was due to GATA-3. Similar to T-bet, the DNA-binding activity of GATA3 was also visible within 2 h of priming and maximum after 12 h (Fig. 6C). However, the DNA-binding activity decreased markedly after 24 h of priming (Fig. 6C).

Next we examined the effect of gemfibrozil on DNA-binding activities of T-bet and GATA3. Splenocytes isolated from MBP-immunized mice were stimulated with 50 μg/ml MBP in the presence or absence of gemfibrozil. After 12 h of stimulation, DNA-binding activities of T-bet and GATA3 were monitored in nuclear extracts. Consistent to the inhibitory effect of gemfibrozil on the release of Th1 cytokine IFN-γ (Fig. 5) in MBP-immunized splenocytes, this drug dose-dependently inhibited the DNA-binding activity of T-bet (Fig. 7A, upper panel). On the other hand, gemfibrozil increased the DNA-binding activity of GATA3 (Fig. 7A, lower panel) that is also consistent to the stimulatory effect of gemfibrozil on Th2 cytokine IL-4. Next we examined the effect of gemfibrozil on the expression of T-bet and GATA3 proteins in MBP-primed splenocytes. While gemfibrozil dose-dependently inhibited the protein expression of T-bet, this drug stimulated the protein expression of GATA3 in a dose-dependent manner (Fig. 7B). These results also suggest that the alteration in DNA-binding activity of T-bet and GATA3 by gemfibrozil is due to altered protein expression of T-bet and GATA3.

Regulation of T-bet and GATA3 by gemfibrozil in vivo in the spleen of donor mice: Because gemfibrozil differentially regulated T-bet and GATA3 in MBP-primed splenocytes, next we examined if gemfibrozil was capable of doing so *in vivo* in the spleen of donor mice. Mice were fed gemfibrozil-containing chow from the 0 day of MBP immunization and on 10^{th} d of immunization, spleens were harvested and EMSA was performed in nuclear extracts to examine the activation of T-bet and GATA3. DNA-binding activity of neither T-bet nor GATA3 was observed in spleens of normal mice (Fig. 8A). However, strong DNA-binding activity of T-bet

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was seen in spleens of MBP-immunized donor mice (Fig. 8A, left panel). Similar to that observed in MBP-primed LNC, gemfibrozil was capable of inhibiting the activation of T-bet *in vivo* in the spleen of donor mice (Fig. 8A, left panel). On the other hand, the DNA-binding activity of GATA3 was observed in spleens of gemfibrozil-treated donor mice but not in untreated donor mice (Fig. 8A, right panel). In another set of parallel experiments, we examined the protein level of T-bet and GATA3 in spleen by double-label immunofluorescence using T cell marker CD3. As expected, numerous CD3 positive cells were found in the spleen of normal mice but those CD3 positive cells did not express T-bet (Fig. 8B). However, marked increase in T-bet:CD3 co-localization was observed in the spleen of MBP-immunized donor mice (Fig. 8B). Again gemfibrozil treatment led to the inhibition of T-bet expression but not CD3 in spleen of donor mice (Fig. 8B). On the other hand, marked GATA3:CD3 co-localization was observed in spleen of gemfibrozil-treated donor mice but not in untreated donor mice (Fig. 8C). Taken together, these results suggest that gemfibrozil is capable of regulating the expression of T-bet and GATA3 differentially both *ex vivo* and *in vivo*.

Regulation of T-bet and GATA3 by gemfibrozil in vivo in the spinal cord of recipient mice: Neuroantigen-primed T cells migrate into the CNS due to their activation status and antigen specificity. Because gemfibrozil inhibited the expression of T-bet and stimulated the expression of GATA3 in LNC and spleen of MBP-immunized donor mice, we examined if gemfibrozil treatment was also capable of doing so *in vivo* in the spinal cord of EAE recipient mice. EAE mice receiving gemfibrozil-containing chow from 8 dpt were sacrificed on 19 dpt (at the peak of the acute phase) and longitudinal sections of spinal cord were double-labeled for CD3 and T-bet. Significant co-localization of T-bet and CD3 was observed in spinal cord sections of EAE mice (Fig. 9) suggesting that T-bet-positive MBP-primed Th1 cells enter into the CNS. However, T-

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bet+ and CD3+ cells were not found in spinal cord sections of gemfibrozil-treated EAE mice. Because gemfibrozil treatment enriched GATA3-potive T cells in spleen of donor mice (Fig. 8C), we also double-labeled spinal cord sections for GATA3 and CD3. Despite several attempts, we were unable to detect any GATA3+ and CD3+ T cells in spinal cord sections of either EAE or gemfibrozil-treated EAE mice (data not shown). It appeared that gemfibrozil treatment inhibited the infiltration of any T cells into the CNS (Fig. 9) which is also consistent to H&E results that demonstrates inhibition of blood mononuclear cell infiltration by gemfibrozil (Fig. 1).

How does gemfibrozil differentially regulate the expression of T-bet and GATA3 in T cells? Earlier we (Pahan et al., 2002) have demonstrated that gemfibrozil inhibits the production of NO and the expression of iNOS in human astrocytes. Recently Xu et al (2005) have also demonstrated similar inhibitory effect of gemfibrozil in microglia. We therefore, hypothesized any possible role NO in gemfibrozil-mediated differential regulation of T-bet and GATA3. At first, we examined if gemfibrozil was capable of inhibiting the expression of iNOS *in vivo* in the spleen, the organ that plays a crucial role antigen presentation and Th cell differentiation. Interestingly, the expression of iNOS in splenic macrophages (Fig. 10) paralleled to the expression of T-bet in splenic T cells (Fig. 8). On the other hand, the expression of GATA3 in T cells (Fig. 8) was inversely proportional to the expression of iNOS in macrophages (Fig. 10). For example, marked expression of iNOS in moma2-positive macrophages was observed in spleen of MBP-immunized donor mice (Fig. 10A). However, gemfibrozil treatment markedly inhibited the expression of iNOS in moma2-positive macrophages (Fig. 10A). Similarly, dose-dependent inhibition of NO production by gemfibrozil was also observed in MBP-primed splenocytes (Fig. 10B).

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Next we investigated if NO was directly involved in gemfibrozil-mediated regulation of T-bet and GATA3. Splenocytes isolated from MBP-immunized mice were treated with different doses of GSNO (an NO donor) in the presence or absence of gemfibrozil during priming with MBP. Gemfibrozil inhibited the expression of T-bet but GSNO dose-dependently reversed the inhibitory effect of gemfibrozil on T-bet (Fig. 11A, upper panel). Complete reversal was observed when GSNO was used at a dose of 750 μM (Fig. 11A, upper panel). On the other hand, gemfibrozil stimulated the expression of GATA3 and GSNO dose-dependently blocked the stimulatory effect of gemfibrozil on the expression of GATA3 (Fig. 11A, lower panel) with complete blockade observed at 500 μM or higher concentration of GSNO. To attest these finding from another angle, splenocytes isolated from MBP-immunized mice were treated with different doses of PTIO (an NO scavenger) during priming with MBP. While scavenging of NO by PTIO suppressed the expression of T-bet (Fig. 11B, upper panel), dose-dependent stimulation of GATA3 expression was observed by PTIO alone (Fig. 11B, lower panel). These novel results strongly suggest that NO is a key regulator of T-bet and GATA3 and that gemfibrozil suppresses the expression of T-bet and increases the expression of GATA3 via inhibiting the expression of iNOS and production of NO.

Earlier, Cunard et al (2002) and Lovett-Racke et al (2004) have also demonstrated that gemfibrozil directs Th2 bias and that biasness is mediated by elevated level of IL-4 as found in gemfibrozil-treated MBP primed T cells. We therefore, examined if NO was capable of regulating IL-4 in MBP-primed splenocytes. It is apparent from Figure 12 that scavenging of NO alone by PTIO increased the production of IL-4 (B) and that GSNO (an NO donor) suppressed gemfibrozil-mediated increased production of IL-4 (A). These results suggest that similar to

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GATA3, IL-4 production is also negatively regulated by NO and that gemfibrozil regulates the release the IL-4 via NO.

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Discussion

Adoptive transfer model of experimental allergic encephalomyelitis (EAE) in female SJL/J mice has long been recognized as a relapsing remitting model for T cell-mediated autoimmune disease MS. The model is proved to be very much useful in determining new therapeutic strategies and testing efficacy of new drugs for MS. Several lines of evidences presented in this work clearly establish that gemfibrozil, an FDA-approved drug for hyperlipidemia in human, inhibits the disease process of relapsing-remitting EAE (RR-EAE) in female SJL/J mice. Our conclusion is based on the following. *First,* adoptively transferred MBP-primed T cells were unable to induce the clinical symptoms of EAE in mice having gemfibrozil-containing chow. *Second,* gemfibrozil was also able to inhibit the progression of EAE when administered after early onset. *Third,* clinical treatment of EAE animals with gemfibrozil was capable of inhibiting the invasion of mononuclear cells into the spinal cord. *Fourth,* gemfibrozil inhibited the ability of myelin-specific T cells to differentiate into Th1 effector cells. On the other hand, gemfibrozil stimulated the differentiation of myelin-specific T cells into Th2 phenotype. Consistently, gemfibrozil also inhibited the encephalitogenicity of myelin-specific T cells.

Similar to other fibrate drugs, gemfibrozil is a known activator of PPAR-α. However, our results suggest that gemfibrozil as well as WY14643, another potent agonist of PPAR-α, do not require PPAR-α to suppress the disease process of EAE. It is known that MS is a Th1-mediated autoimmune disease and switching of Th1 to Th2 phenotype is a way to ameliorate the disease. Our results suggest that gemfibrozil switched the differentiation of MBP-primed T cells from Th1 to Th2 mode without the involvement of PPAR-α. Therefore, it appears that the anti-EAE activity of gemfibrozil does not depend on PPAR-α. Because NO produced from iNOS also plays a role in the disease process of EAE and MS (Bo et al., 1994; Ding et al., 1998), these

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results are consistent to our earlier report that gemfibrozil inhibits the expression of iNOS in human astroglia independent of PPAR-α (Pahan et al., 2002). Recently, we have found that gemfibrozil inhibits the expression of proinflammatory molecules in primary microglia isolated from both PPAR-α wild type and knockout mice (Jana and Pahan, unpublished observation). A study by Xu et al (2005) has indicated the possible involvement of PPAR-α in gemfibrozil-mediated inhibition of microglial iNOS. However, that study did not attempt to examine the effect of gemfibrozil in PPAR-α knockout microglia.

Although our study does not reveal any receptor-centric mechanism(s) behind this anti-encephalitogenic effect of gemfibrozil, we have uncovered an important cellular mechanism that is responsible for gemfibrozil-mediated differential regulation of Th1 and Th2 cells. While the role of T-bet and GATA3 in the regulation of Th cells is well established, signaling mechanisms that regulate T-bet and GATA3 in T cells are poorly understood. Inhibition of DNA binding activity and expression of T-bet but stimulation of DNA-binding activity and expression of GATA3 by gemfibrozil clearly suggest that gemfibrozil-mediated differential regulation of Th1 and Th2 cells is due to differential control of T-bet and GATA3. Because gemfibrozil is capable of inhibiting the expression of iNOS and production of NO, we were prompted to investigate if NO was playing a role in the regulation of Th cells. Here we have found that NO is a key regulator of T-bet and GATA3 in neuroantigen-primed T cells. First, while iNOS:macrophage co-localization was directly proportional to T-bet:T cell co-localization, GATA3:T cell co-localization inversely correlated to iNOS:macrophage in spleen. For example, gemfibrozil inhibited the expression of iNOS leading to stimulation of GATA3 and suppression of T-bet in spleen. Second, gemfibrozil was unable to suppress T-bet and stimulate GATA3 in MBP-primed splenocytes when NO was added during antigen priming. Third, while NO alone did not further

significantly stimulate the expression of T-bet in MBP-primed splenocytes, the expression of GATA3 was suppressed by NO alone. Fourth, the differential effect of gemfibrozil on T-bet and GATA3 could be replicated by adding a NO scavenger alone during antigen priming. For example, PTIO, a NO donor, inhibited the expression of T-bet but stimulated the expression of GATA3 in MBP-primed splenocytes. Because gemfibrozil inhibits the expression of iNOS independent of PPAR-α (Pahan et al., 2002) and NO is the key controller of T-bet and GATA3, PPAR-α was not required for gemfibrozil-mediated differential regulation of T-bet and GATA3 and hence Th1 and Th2. It has been shown that in PPAR-α (-/-) mice, the transcription of IL-2 mRNA is terminated much earlier than in the wild type mice (Jones et al., 2002). On the other hand, the expression of T-bet and IFN-γ starts early following T cell activation and increase with time in PPAR-α (-/-) mice compared to wild type mice (Jones et al., 2002, 2003). But it is to be noted that these studies were performed neither in a strain with RR-EAE relevant loci nor with an EAE-relevant neuroantigen.

NO, a short-lived and diffusible free radical, plays many roles as a signaling and effector molecule in diverse biological systems; it is a neuronal messenger and is involved in vasodilation as well as in antimicrobial and antitumor activities (Nathan 1992). On the other hand, NO has also been implicated in several CNS disorders, including inflammatory, infectious, traumatic, and degenerative diseases (Brosnan et al., 1994; Merrill et al., 1993; Mitrovic et al., 1994; Akama et al., 1998; Samdani et al., 1997). There are considerable evidences for the transcriptional induction of iNOS (the high-output isoform of NOS) in the CNS that is associated with autoimmune reactions, acute infection, and degenerative brain injury (Merrill et al., 1993; Samdani et al., 1997). NO is potentially toxic to neurons and oligodendrocytes that may mediate toxicity through the formation of iron-NO complexes of iron-containing enzyme systems

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(Drapier and Hibbs 1988), oxidation of protein sulfhydryl groups, nitration of proteins, and nitrosylation of nucleic acids and DNA strand breaks (Radi et al., 1991). Here we demonstrate that NO is a key player in regulating the expression of T-bet and GATA3, in which NO increases the expression of T-bet and suppresses the expression of GATA3 in neuroantigen-primed T cells. Therefore, specific targeting of NO either by iNOS inhibitors or NO scavengers could be an important step to stimulate GATA3 and hence switching towards Th2 resulting in suppression of EAE and MS.

Although there is no effective therapy against MS, different forms of interferon-β (IFN-β) have been currently used to treat this disease. However gemfibrozil has several advantages over IFN-β. *First,* IFN-β has a number of side effects including flu-like symptoms, menstrual disorders in women, decrease in neutrophil count and white blood cell count, increase in AST and ALT levels, and development of neutralizing antibodies to IFN-β (Miller 1997; Connelly 1994). However, gemfibrozil is fairly nontoxic. It has been well tolerated in human and animal studies. Being known as 'Lopid' in the pharmacy, it is a commonly used lipid-lowering drug in human since FDA approval in 1981. The Veterans Affairs High-Density Lipoprotein Intervention Trial (VA-HIT) has reported that coronary heart disease events are significantly reduced by gemfibrozil in patients when the predominant lipid abnormality was low HDL-C (Robins et al., 2001). In a double-blind, randomized, placebo-controlled trial, this drug has been shown to reduce small low-density lipoprotein more in normolipemic subjects classified as low-density lipoprotein pattern B compared with pattern A (Superko et al., 2005). Another recent trial has shown that LDL and HDL particle subclasses are favorably changed by gemfibrozil therapy (Otvos et al., 2006). *Second,* MS patients are treated with IFN-β through painful injections which often lead to injection site reactions such as skin necrosis. However, gemfibrozil is usually taken

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orally, the least painful route. Our HPLC results with brain samples also demonstrate that orally-administered gemfibrozil can enter into the naïve CNS.

In summary, we have demonstrated that gemfibrozil, an FDA-approved lipid-lowering drug in human, stimulates GATA3 and suppresses T-bet through the inhibition of NO production, and blocks the disease process of EAE independent of its prototype receptor, PPAR-α. Although the *ex vivo* and *in vivo* situation of mouse spleen and lymph nodes and its treatment with neuroantigen and gemfibrozil may not truly resemble the *in vivo* immunological situation in patients with MS, our results identify gemfibrozil as a possible therapeutic agent to suppress RR-MS via differential modulation of T-bet and GATA3.

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Footnotes:

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Table-1. **Effect of gemfibrozil on clinical symptoms of EAE in female SJL/J mice**

Treatment	Incidence	Mean Peak Clinical Score	Suppression of EAE (%)	
			Incidence	Score
Vehicle	12/12	3.1		
Gemfibrozil (food chow; 0.2% w/w) from 0 dpt	3/6	1.16	50	62.6

EAE was induced in female SJL/J mice through adoptive transfer of MBP-primed T cells. A group of mice receiving MBP-primed T cells was treated with gemfibrozil from 0 dpt. A clinical score of 1 was considered as the incidence of EAE in mice. Differences are significant ($p<0.001$).

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Table-2. **Effect of gemfibrozil on clinical symptoms of EAE in (B6.129 X SJL/J) PPAR-α wild type and knockout female mice**

A. (B6.129 X SJL/J) PPAR-α (+/+) mice

Treatment	Incidence	Mean Peak Clinical Score	Suppression of EAE (%)	
			Incidence	Score
Vehicle	6/6	2.16		
Gemfibrozil (food chow; 0.2% w/w) from 0 dpt	3/6	0.83	50	61.6

B. (B6.129 X SJL/J) PPAR-α (-/-) mice

Treatment	Incidence	Mean Peak Clinical Score	Suppression of EAE (%)	
			Incidence	Score
Vehicle	6/6	2.33		
Gemfibrozil (food chow; 0.2% w/w) from 0 dpt	3/6	0.91	50	60.9

EAE was induced in female (B6.129 X SJL/J) PPAR-α (+/+) (A) and PPAR-α (-/-) (B) mice through adoptive transfer of MBP-primed T cells. A group of mice receiving MBP-primed T cells was treated with gemfibrozil from 0 dpt. A clinical score of 1 was considered as the incidence of EAE in mice. Differences are significant ($p<0.001$).

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Legends to figures

Fig. 1. **Gemfibrozil ameliorates clinical symptoms of EAE and inhibits inflammation in the CNS of EAE mice.** A) Female SJL/J mice were induced EAE by adoptive transfer of MBP-primed T cells. Mice were treated with different doses of gemfibrozil mixed with food chow starting from 0 dpt. Six mice were included in each group. Mice were examined for clinical symptoms everyday until 30 dpt. B) One group of EAE mice (n=6) were treated with gemfibrozil-containing chow (0.2% w/w) from the onset of acute disease (8 dpt). Mice were examined for clinical symptoms everyday until 38 dpt. C) EAE animals receiving either vehicle-containing food chow or gemfibrozil-containing food chow from 8 dpt were sacrificed on 19 dpt. Longitudinal sections of spinal cord isolated from EAE and gemfibrozil-treated EAE animals were stained with either H&E or LFB. Digital images were collected under bright-field setting using a 40x objective. D) On 19 dpt, cerebellar samples were analyzed for iNOS, IL-1β, IL-6, and TNF-α mRNAs by semi-quantitative RT-PCR. Results represent three independent experiments.

Fig. 2. **Effect of gemfibrozil on the expression of different PPARs in EAE mice.** A) EAE animals receiving either vehicle-containing food chow or gemfibrozil-containing food chow from 8 dpt were sacrificed on 19 dpt. Brain (cerebellum) and spleen were analyzed for the expression of PPAR-α, PPAR-β and PPAR-γ by semi-quantitative RT-PCR. Results represent three independent experiments.

Fig. 3. **Effect of gemfibrozil and WY14643 on adoptive transfer of EAE in PPAR-α wild type and knockout mice.** A) Female SJL/J mice were immunized with MBP and MBP-primed T cells were prepared as described under “Materials and Methods”. EAE was induced in female

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(B6.129 X SJL/J) PPAR-α wild type and knockout recipient mice by adoptive transfer of the MBP-primed T cells. A) Recipient mice (n=6) were treated with gemfibrozil-containing chow (0.2% w/w) starting from 0 dpt. B) Recipient mice (n=5) were treated with WY14643-containing chow (0.2% w/w) starting from 0 dpt. Mice were examined for clinical symptoms daily.

Fig. 4. **Gemfibrozil inhibits encephalitogenicity of MBP-primed T cells independent of PPAR-α.** Female (B6.129 X SJL/J) PPAR-α wild type and knockout mice (n=6) were immunized with MBP, IFA and *M. tuberculosis*. From the 0 day of immunization, donor mice were treated with gemfibrozil-containing food chow (0.2% w/w) and on 12 d of immunization, mice were sacrificed and total LNC were further primed with MBP (50 μg/ml) for 4 d. Viable MBP-primed T cells (2 X 10^7) were adoptively transferred to naïve female SJL/J mice. Six mice were used in each recipient group. Mice were examined for clinical symptoms daily.

Fig. 5. **Effect of gemfibrozil on the induction of Th1 and Th2 cytokines in MBP-primed splenocytes.** Splenocytes isolated from MBP-immunized female SJL/J mice were stimulated with MBP in the presence or absence of gemfibrozil. After 72 h of stimulation, induction of IFN-γ (A) and IL-4 (B) was assayed in supernatants. Female (B6.129 X SJL/J) PPAR-α wild type and knockout mice were immunized with MBP and from the 0 day of immunization, mice were treated with gemfibrozil (0.2% w/w) via food chow. After 10 d of immunization, splenocytes were collected and stimulated with MBP in the absence of gemfibrozil. After 72 h of stimulation, induction of IFN-γ (C) and IL-4 (D) was assayed in supernatants. Results are mean ± SD of three different experiments.

Fig. 6. **DNA-binding activities of T-bet and GATA3 in MBP primed T cells.** (A) Splenocytes of MBP-immunized SJL/J mice were incubated *in vitro* in the presence of MBP (50 μg/ml). At different time periods, cells were harvested, nuclear extracts were prepared and electrophoretic

mobility shift assay (EMSA) was performed to detect DNA-binding activities of T-bet (A) and GATA3 (C) using consensus oligonucleotides for T-bet and GATA3. Supershift assays were performed to confirm DNA-binding activities of T-bet (B) and GATA3 (D). Results represent three independent experiments.

Fig. 7. **Effect of gemfibrozil on DNA-binding activities and protein expression of T-bet and GATA3.** Splenocytes isolated from MBP-immunized female SJL/J mice were stimulated with 50 μg/ml MBP in the presence of different doses of gemfibrozil. After 12 h of stimulation, nuclear extracts were prepared and EMSA was performed to determine DNA-binding activities of T-bet and GATA3 (A). The expression of T-bet and GATA3 proteins was monitored by western blot analysis (B). Results represent three independent experiments.

Fig. 8. **Effect of gemfibrozil on DNA-binding activities and protein expression of T-bet and GATA3 *in vivo* in spleen.** Female SJL/J mice were immunized with MBP and from the 0 day of immunization, mice were treated with gemfibrozil via food chow. After 10 d of immunization, spleens were harvested and DNA-binding activities of T-bet and GATA3 were examined by EMSA (A). Splenic cross sections were double-labeled with either CD3 and T-bet (B) or CD3 and GATA3 (C). Results represent three independent experiments.

Fig. 9. **Gemfibrozil inhibits the entry of T-bet-positive T cells into the spinal cord of adoptively-transferred EAE mice.** EAE mice receiving gemfibrozil-containing chow from 8 dpt were sacrificed on 19 dpt. Longitudinal spinal cord sections were double immunolabelled with CD3 and T-bet. Results represent three independent experiments.

Fig. 10. **Effect of gemfibrozil on the expression of iNOS in spleen of donor mice.** Female SJL/J donor mice were treated with gemfibrozil via chow from the 0 day of immunization. After 10 d of immunization, splenic cross sections were double-labeled for iNOS and moma2 (A).

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Splenocytes were stimulated with MBP in the presence of different concentrations of gemfibrozil. After 24 h of stimulation, nitrite concentrations were measured in supernatants (B). Results are mean ± SD of three different experiments.

Fig. 11. **Effect of GSNO and PTIO on the expression of T-bet and GATA3 in MBP-primed splenocytes.** Splenocytes isolated from MBP-immunized mice were treated with 50 μg/ml MBP and different doses of GSNO in the presence or absence of 200 μM gemfibrozil. After 24 h of stimulation, the expression of T-bet and GATA3 proteins was monitored by western blot analysis (A). Splenocytes were stimulated with MBP in the presence or absence of different concentrations of PTIO. After 24 h of stimulation, the expression of T-bet and GATA3 proteins was monitored by western blot analysis (B). Results represent three independent experiments.

Fig. 12. **Effect of GSNO and PTIO on the production of IL-4 in MBP-primed splenocytes.** Splenocytes isolated from MBP-immunized mice were treated with 50 μg/ml MBP and different doses of GSNO (A) and PTIO (B) in the presence or absence of 200 μM gemfibrozil. After 72 h of stimulation, the release of IL-4 was measured in supernatants by ELISA. Results are mean ± SD of three different experiments.

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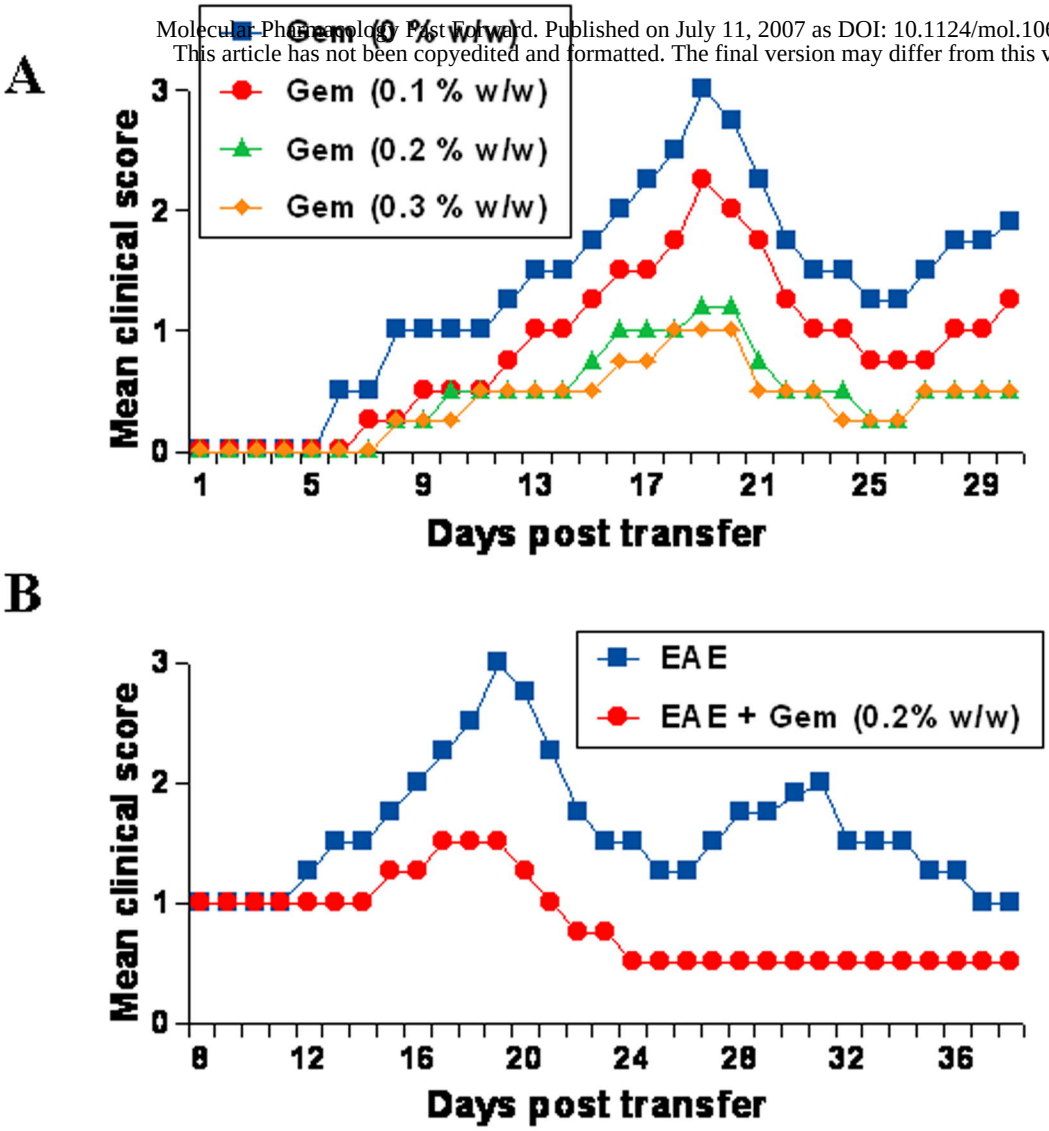


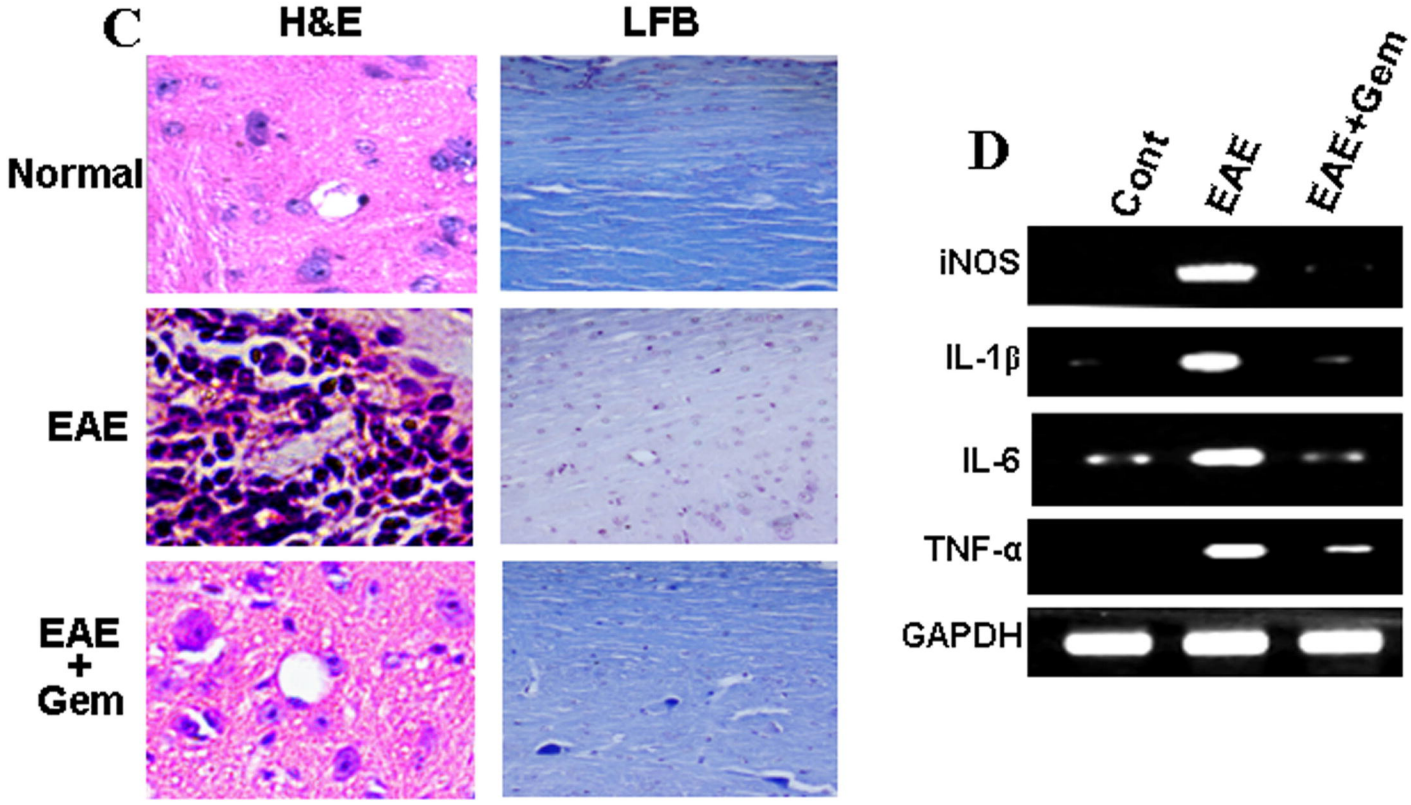
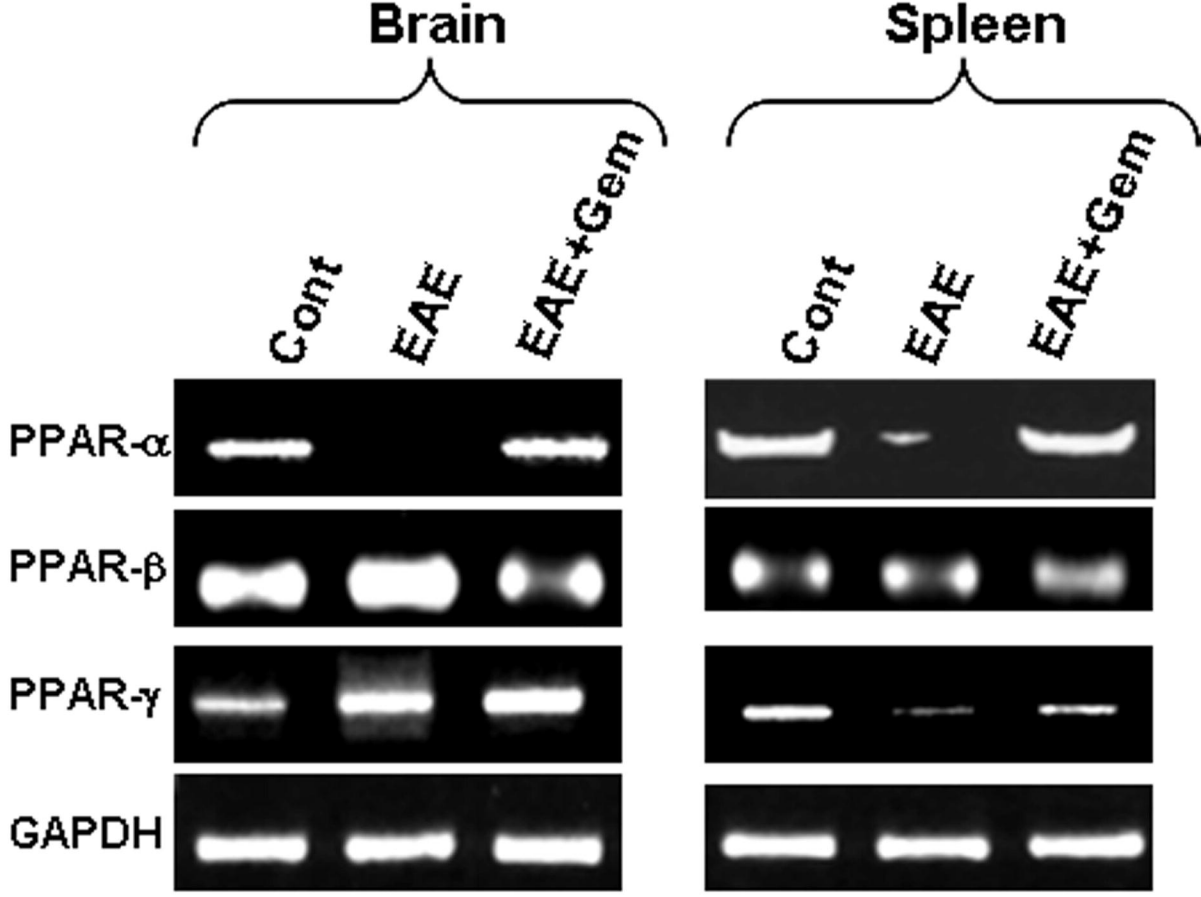


Fig. 1


Brain
Spleen
Cont
EAE
EAE+Gem
Cont
EAE
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PPAR-α
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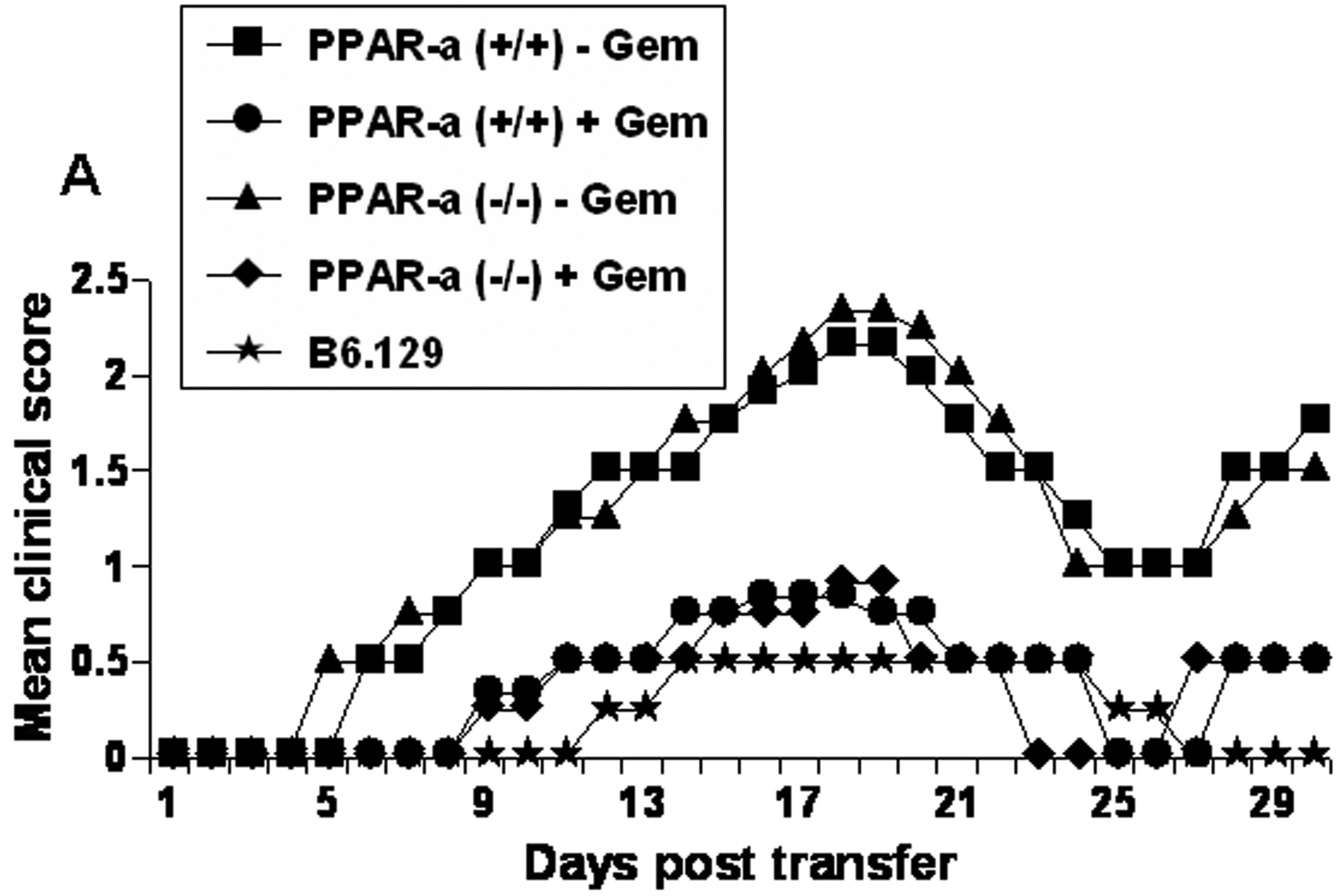
PPAR-γ
GAPDH

Fig. 2



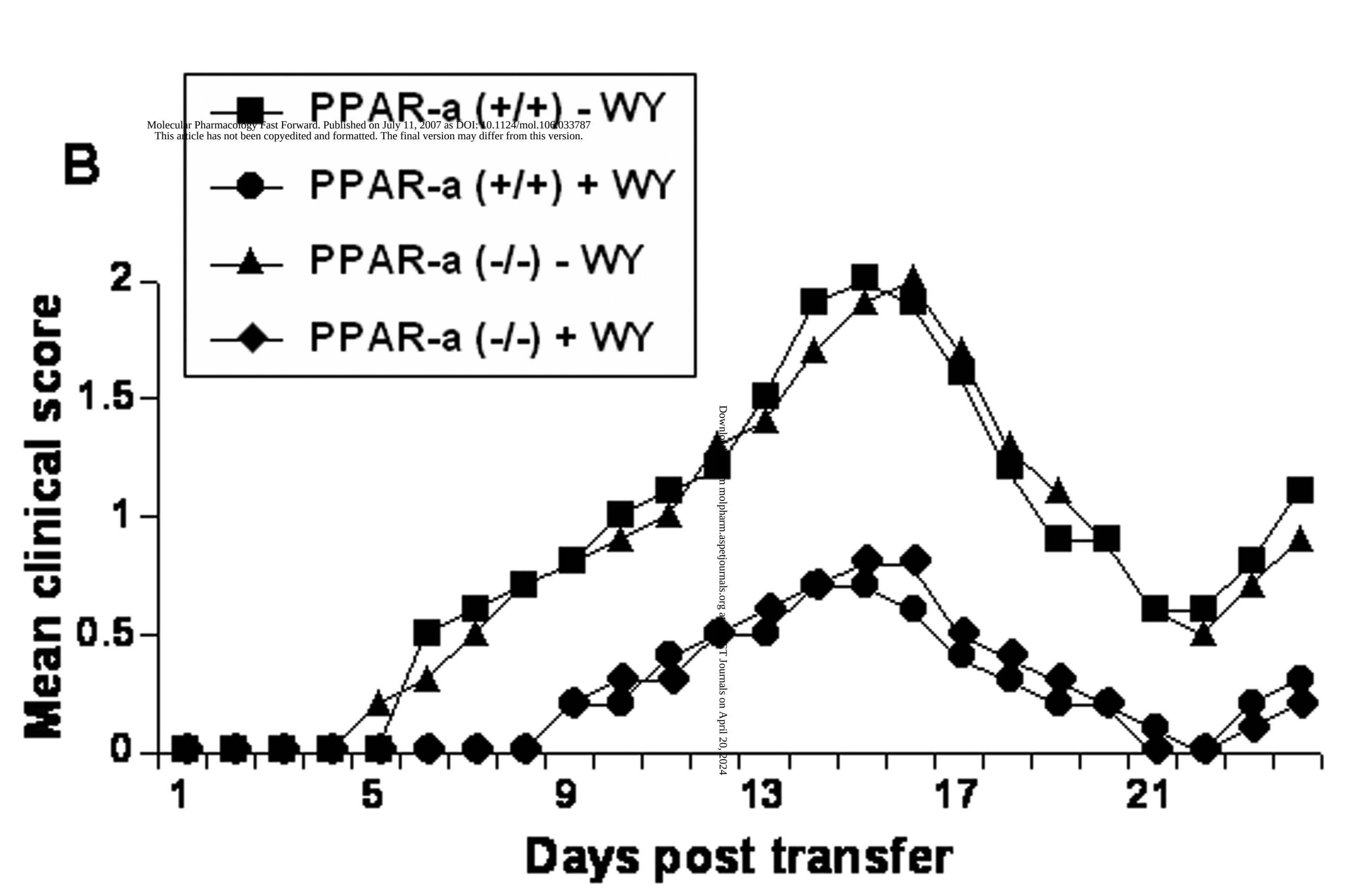


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Fig. 3

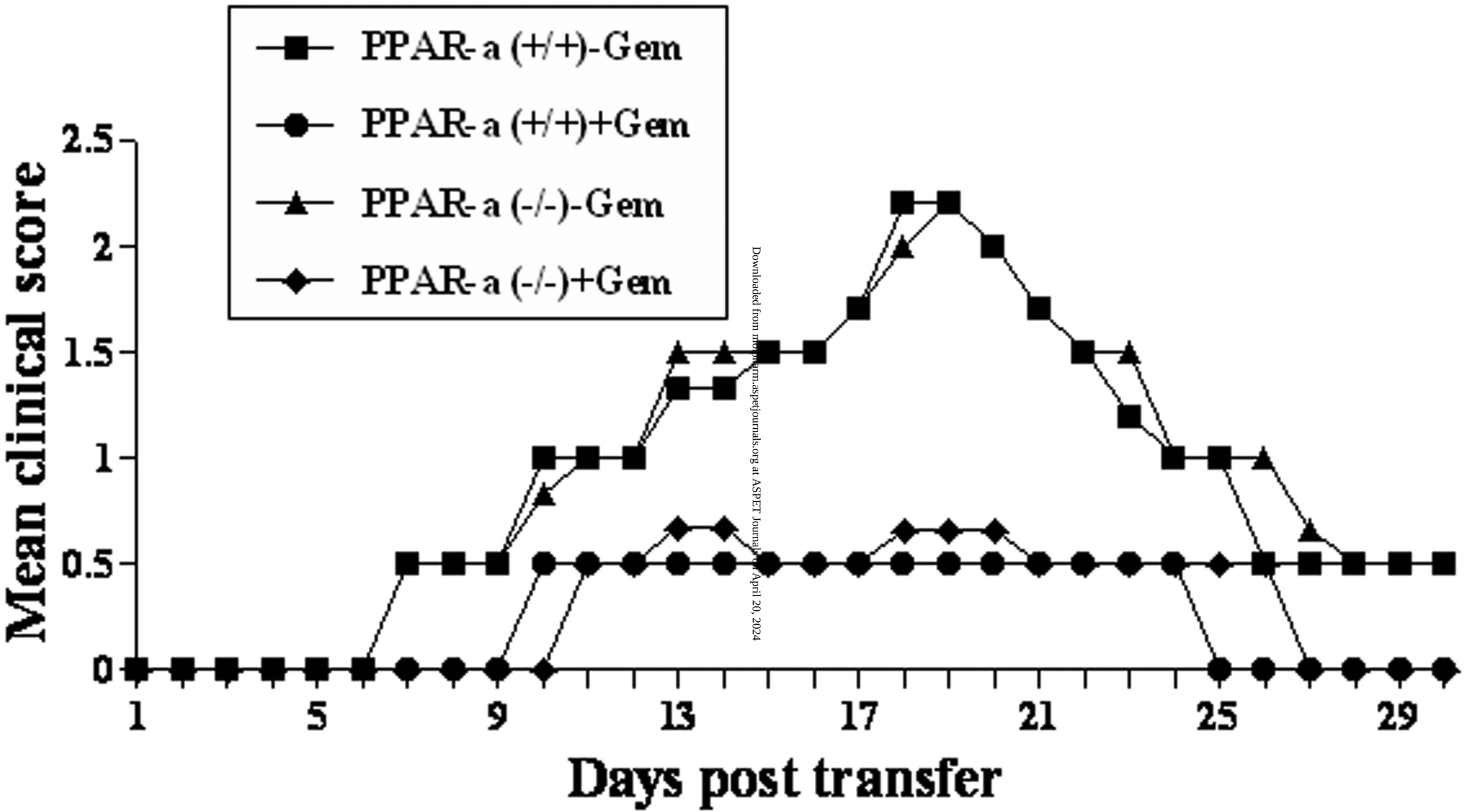
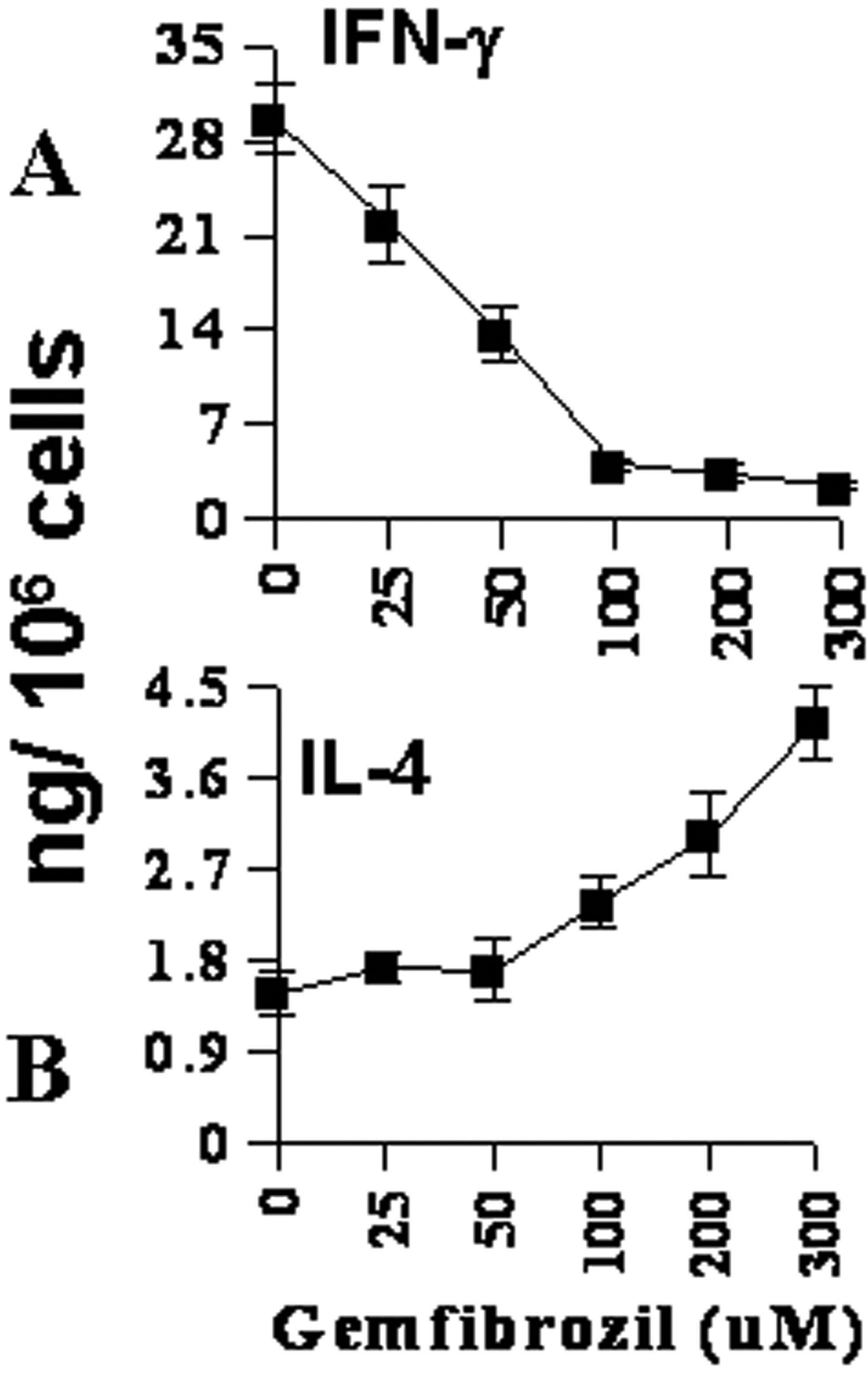
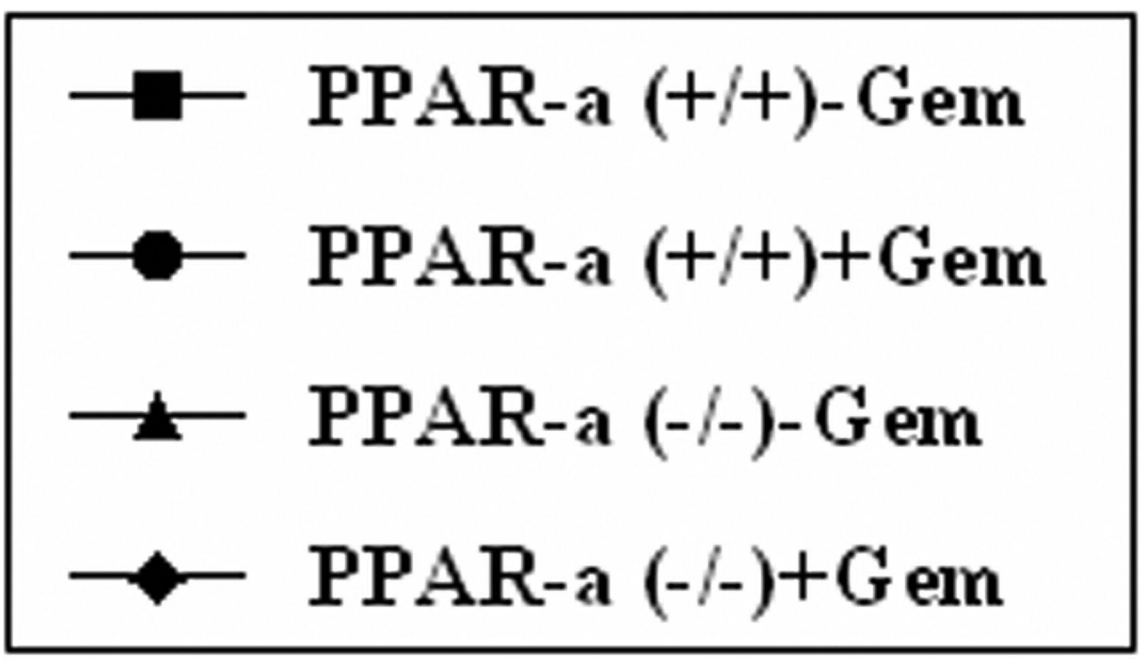


Fig. 4





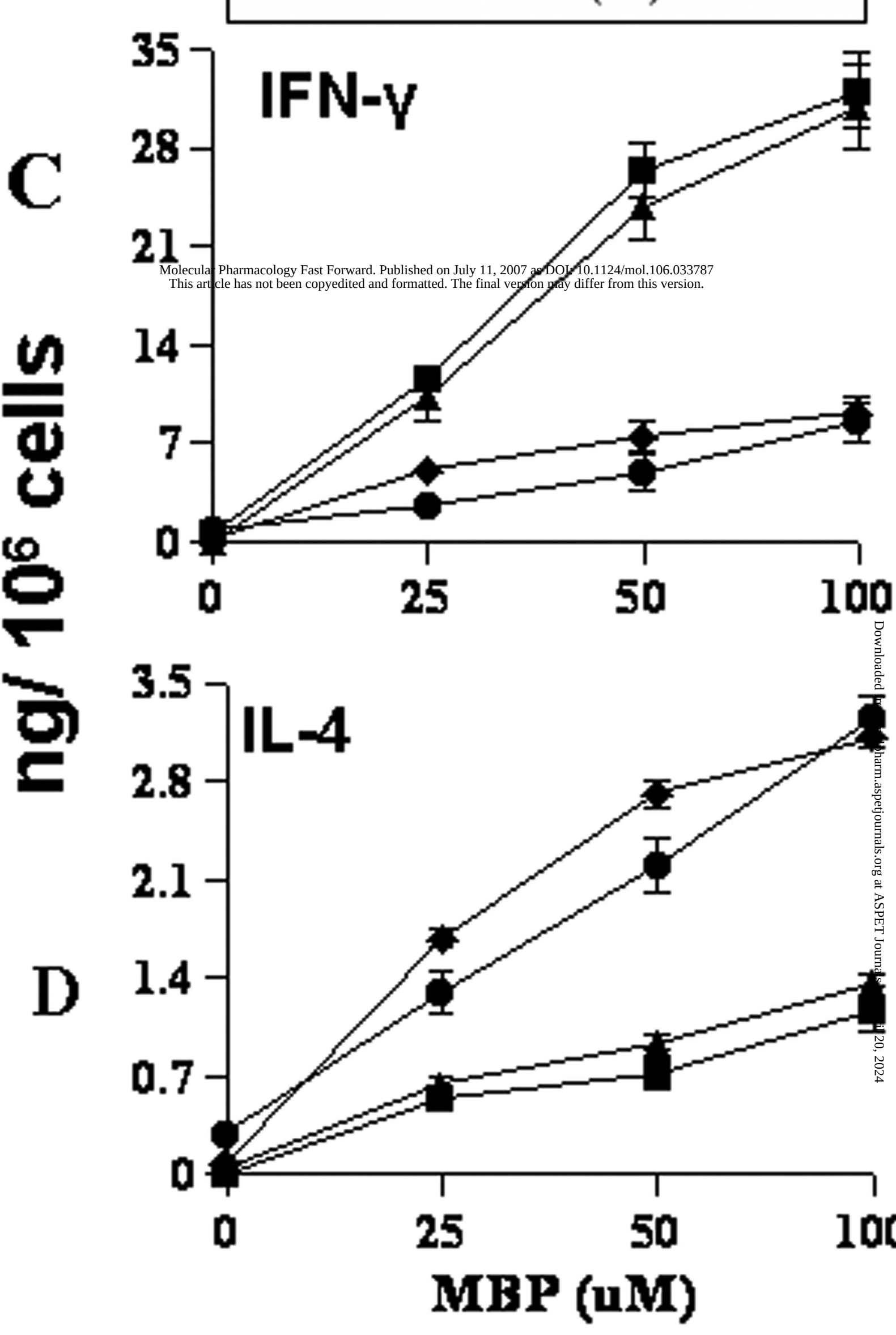


Fig. 5

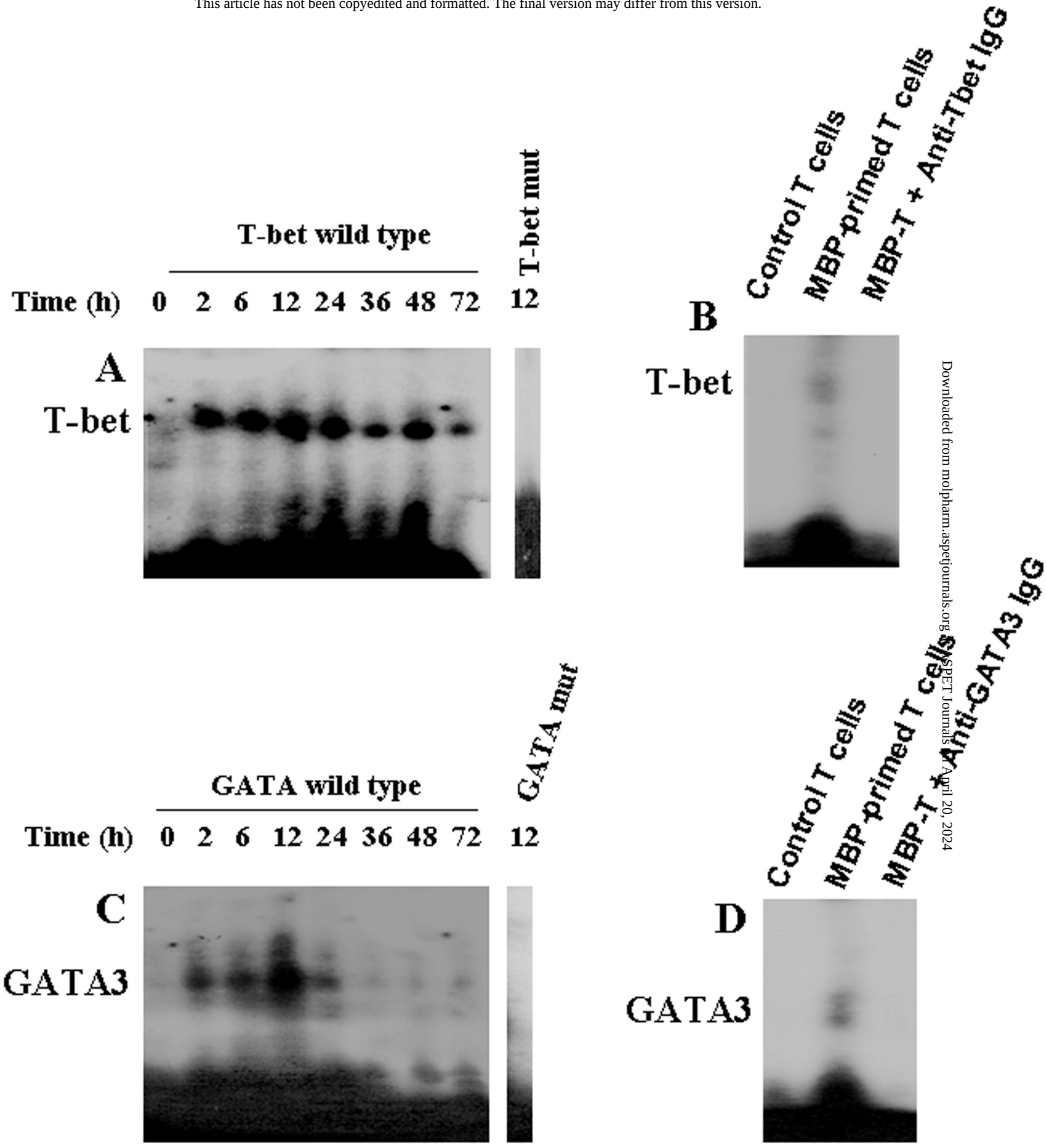


Fig. 6

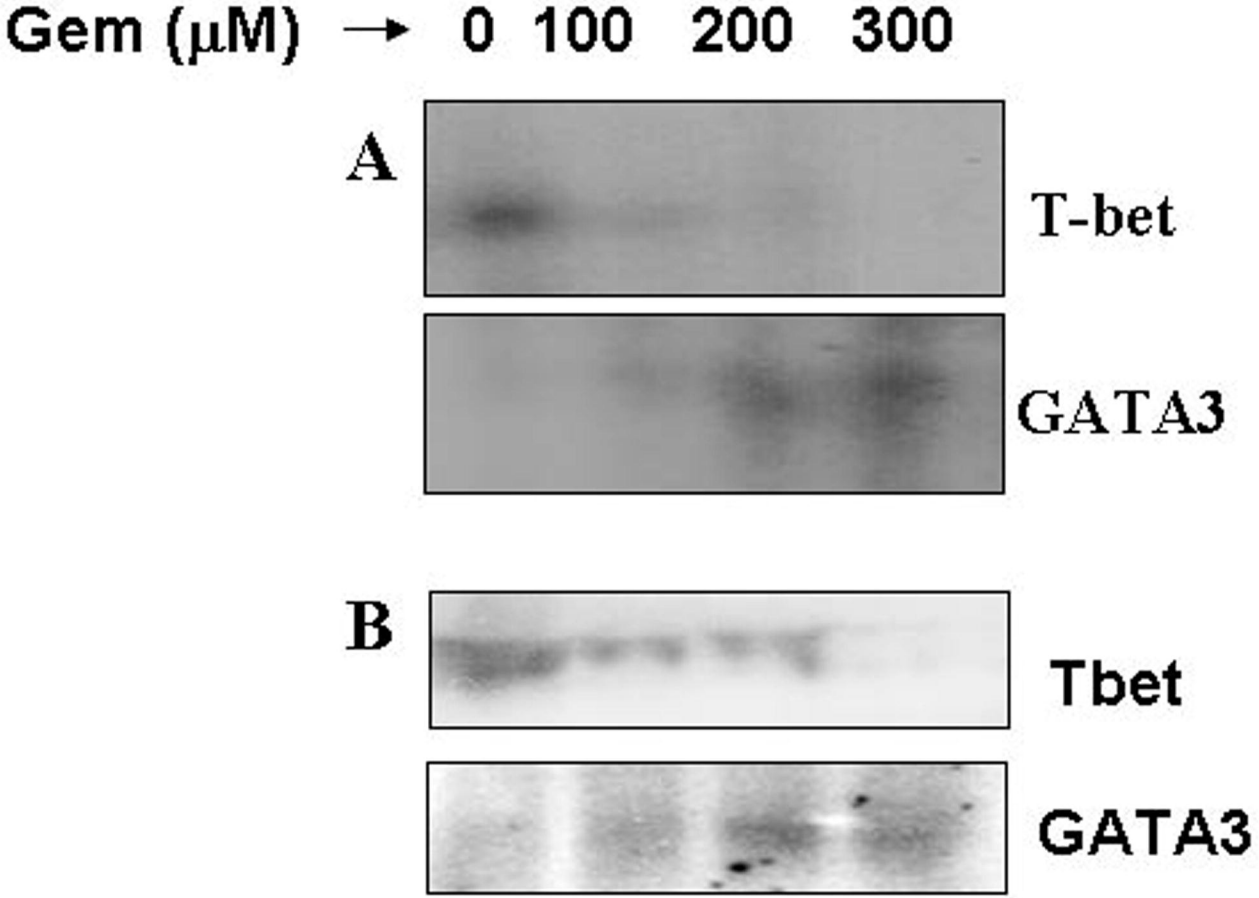
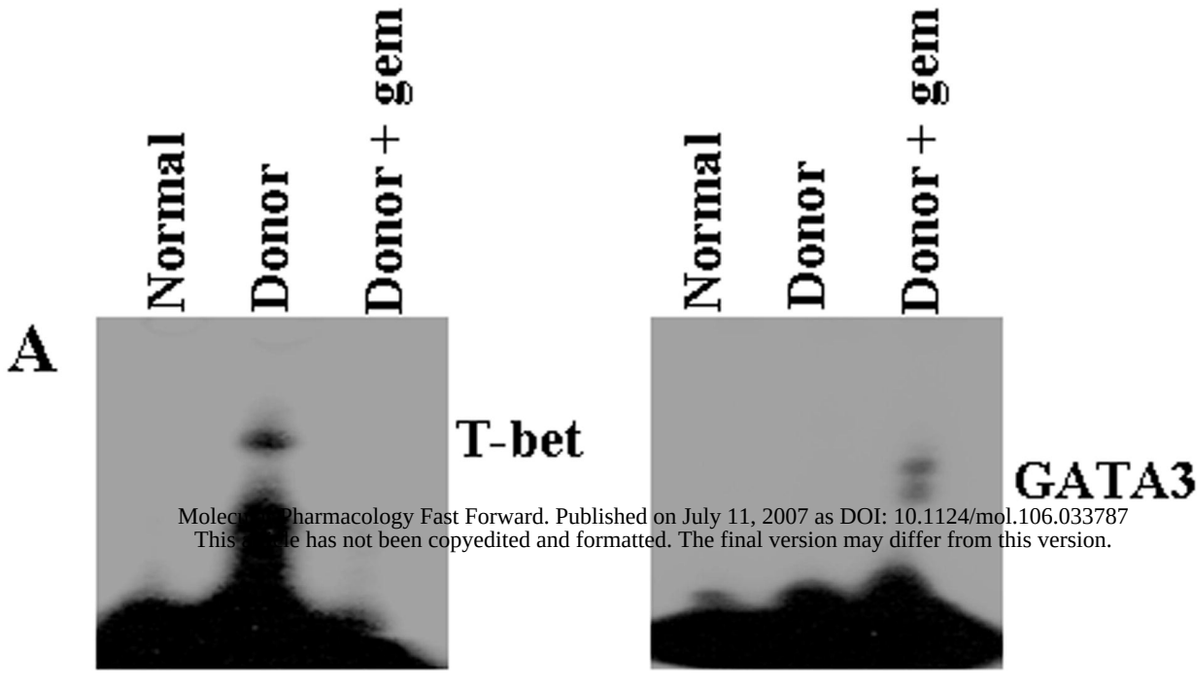
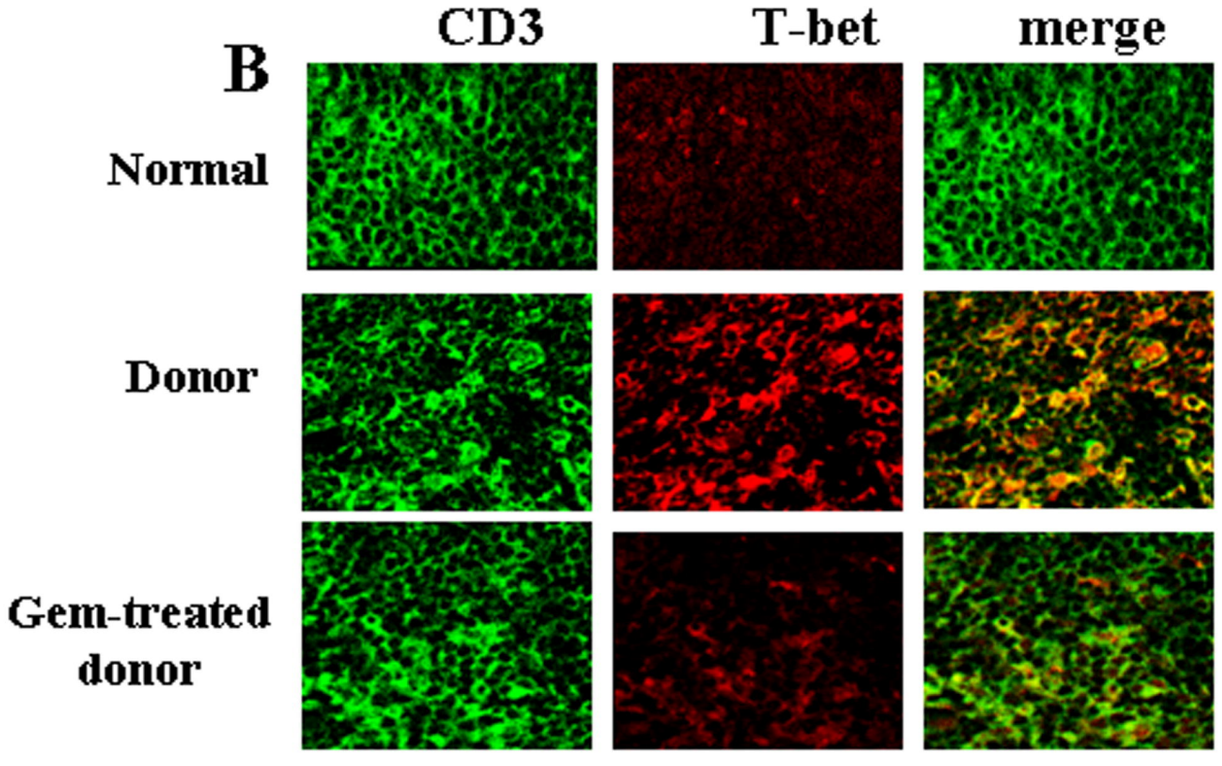


Fig. 7





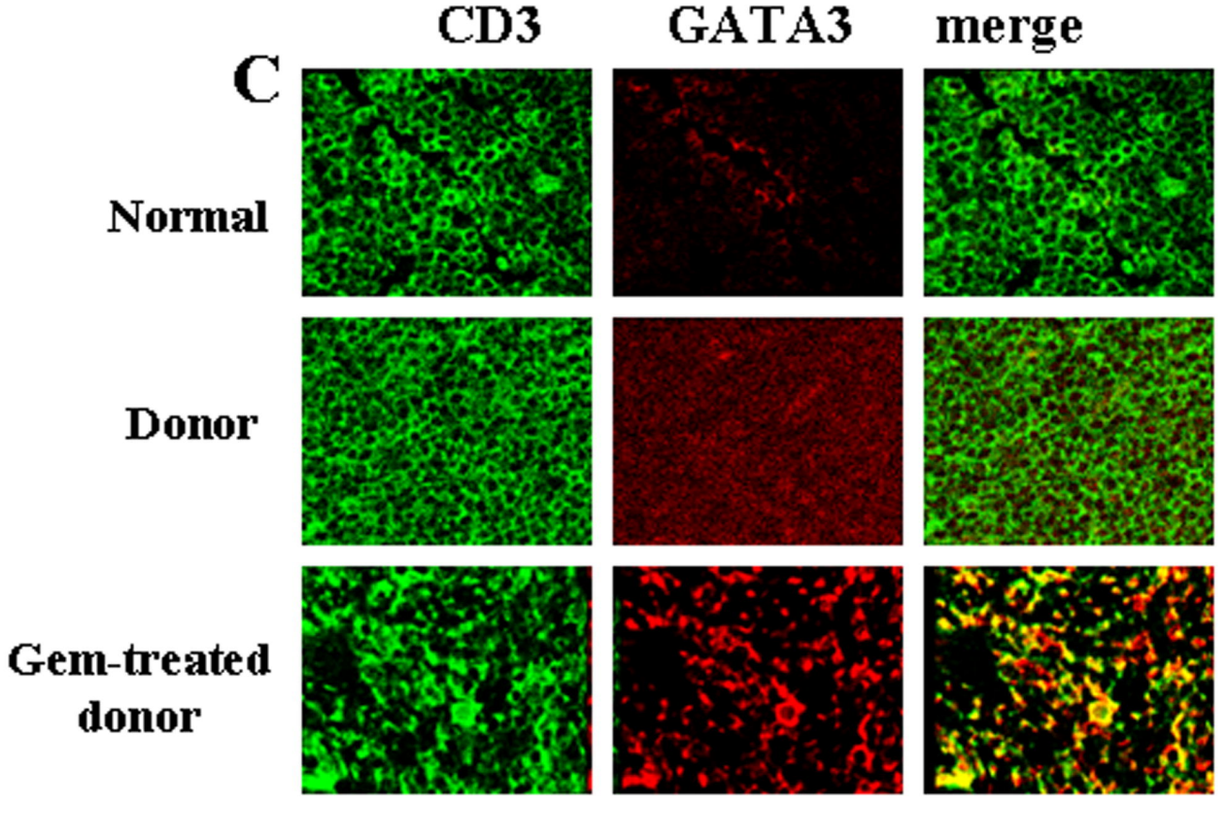


Fig. 8

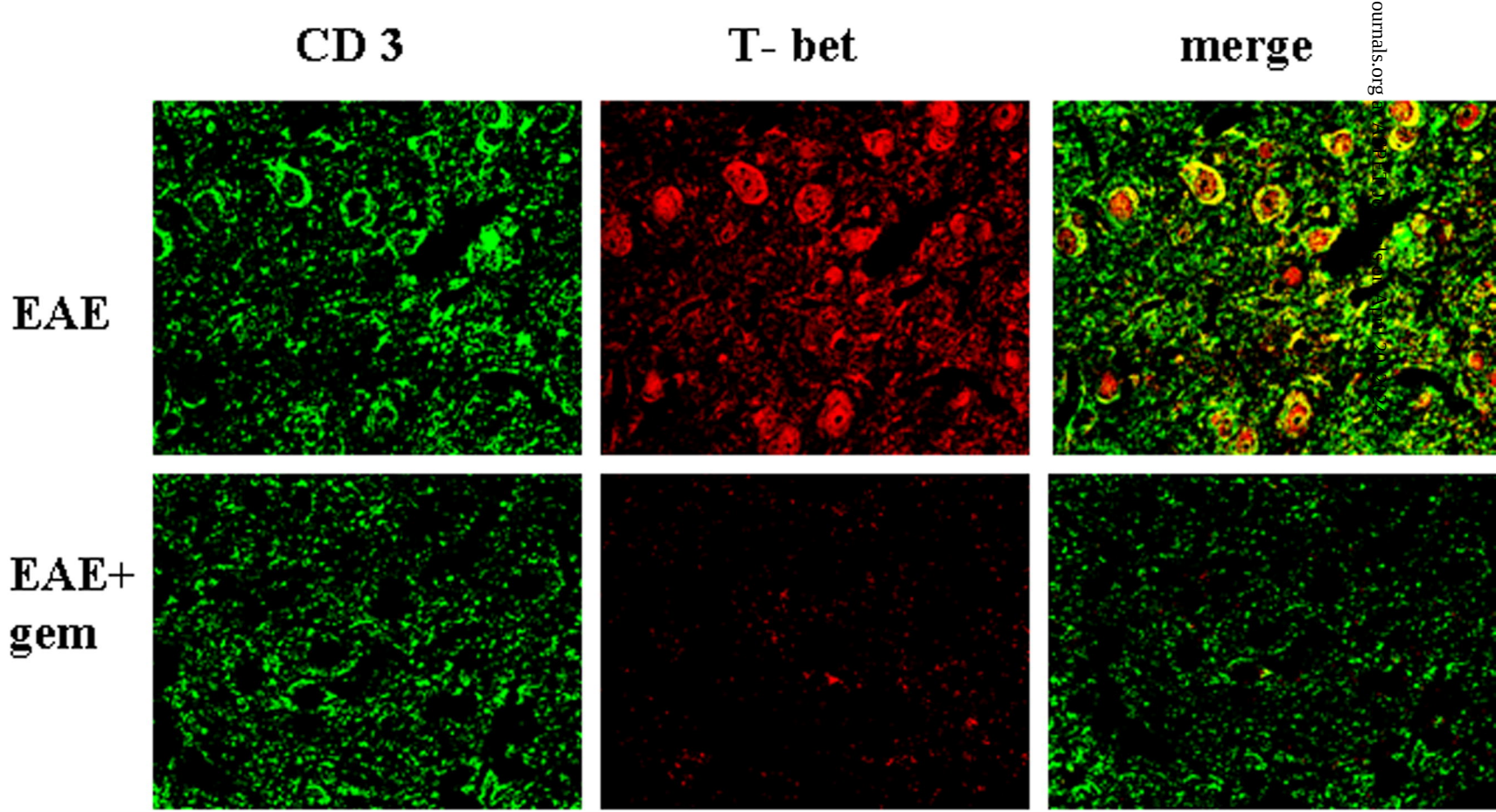
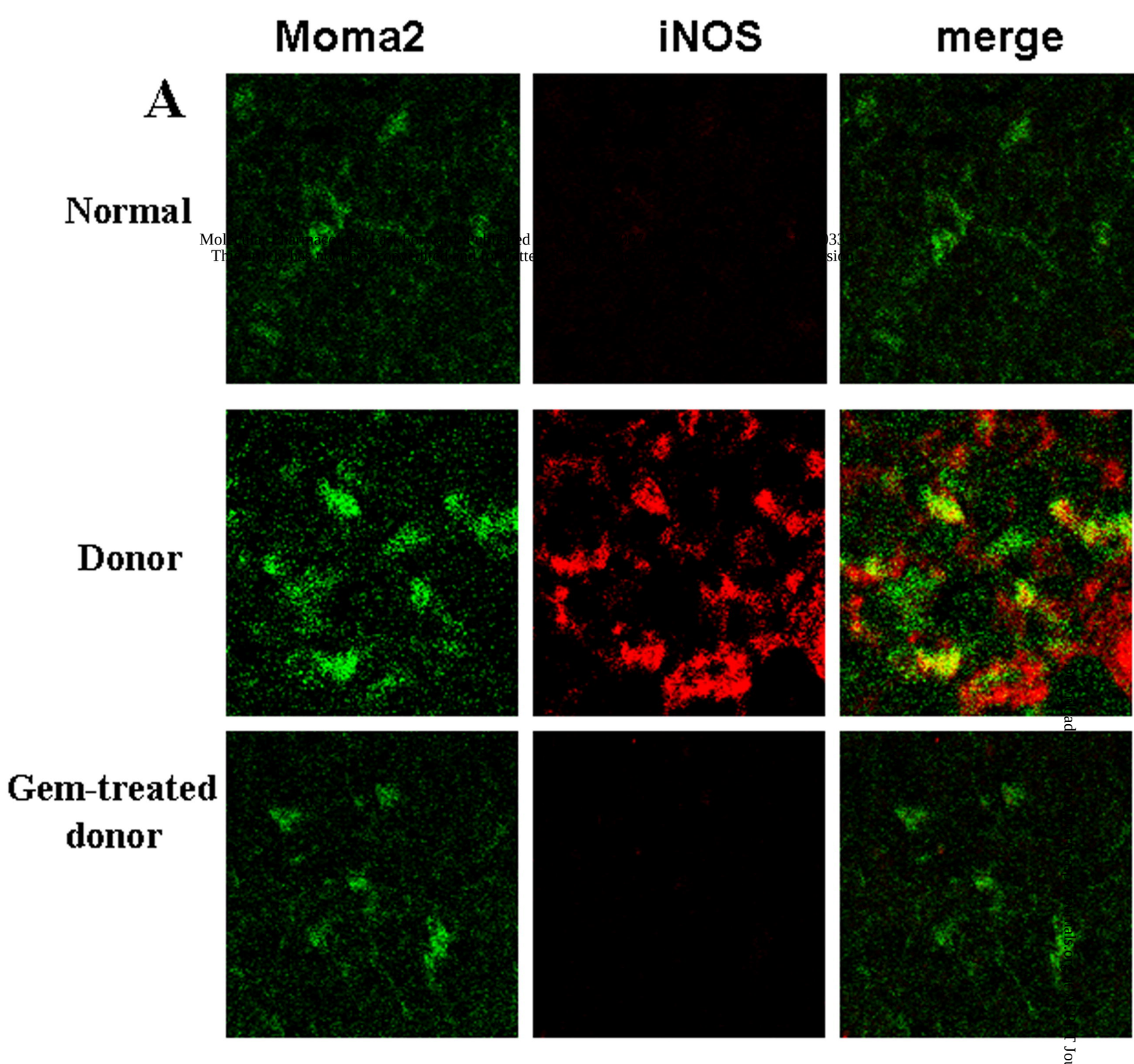
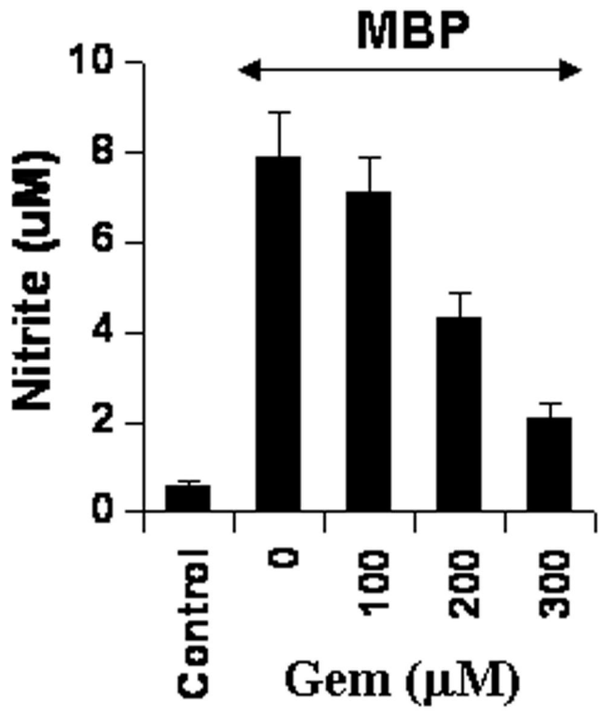


Fig. 9





B

Fig. 10

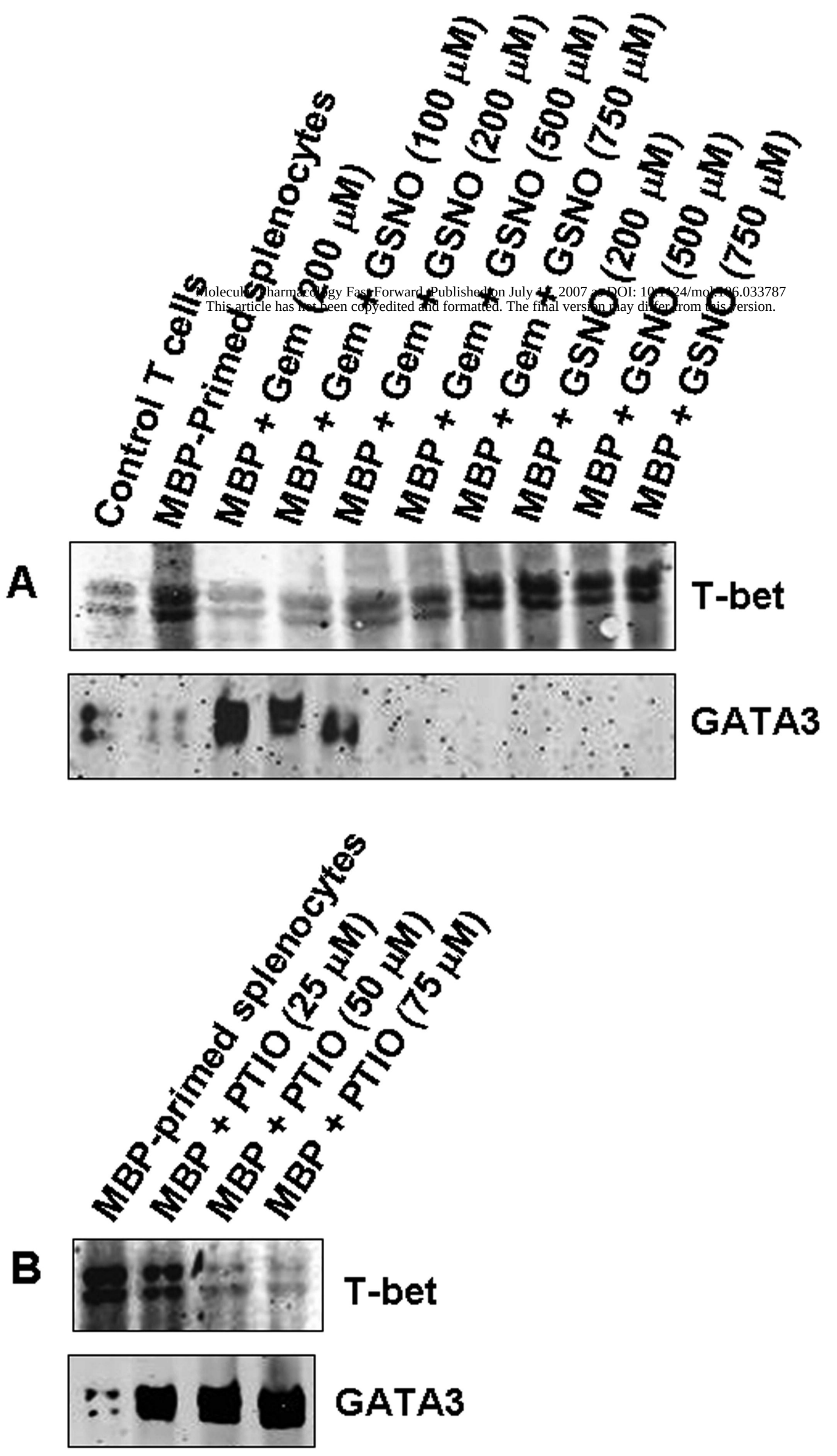
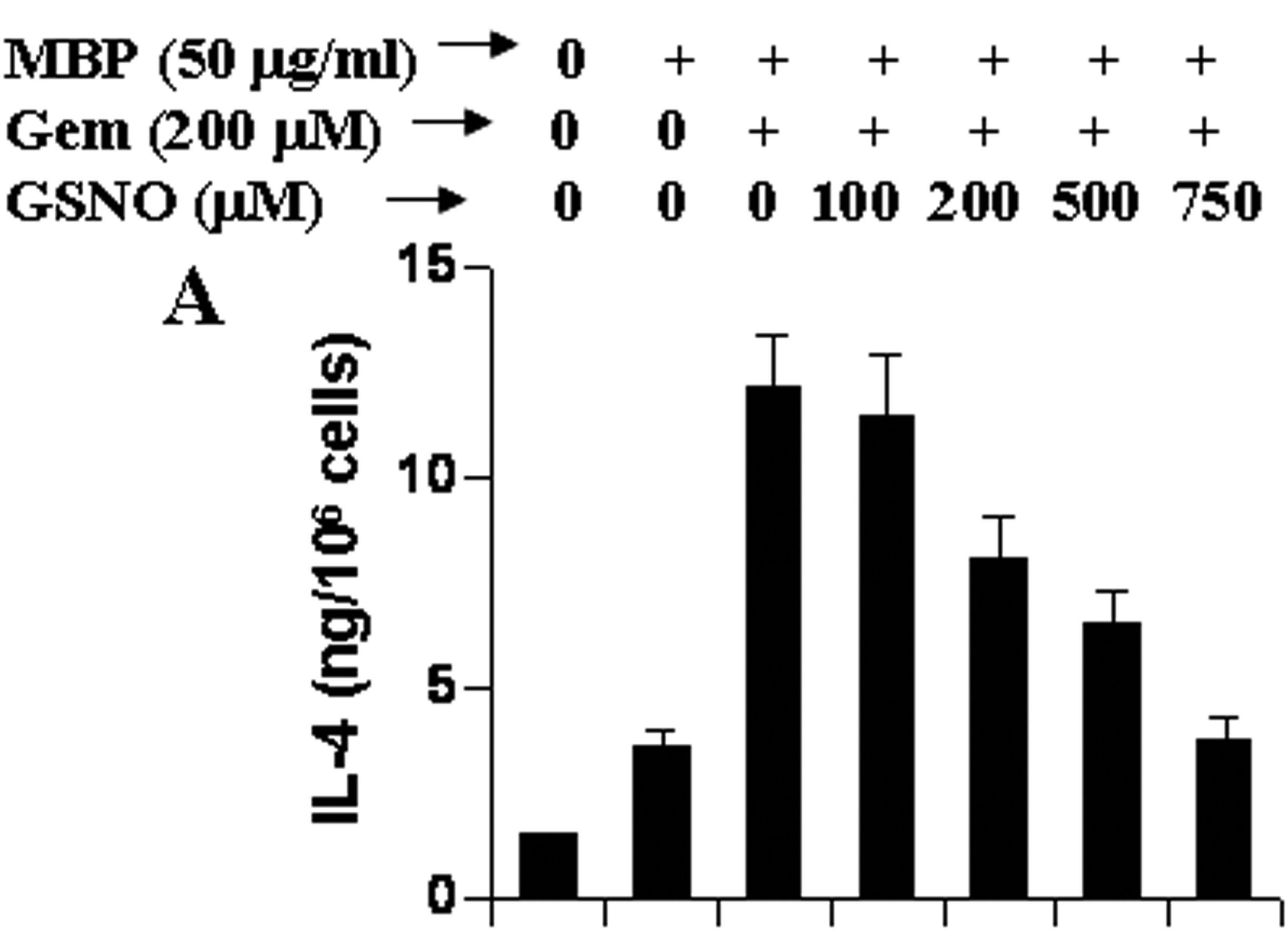


Fig. 11



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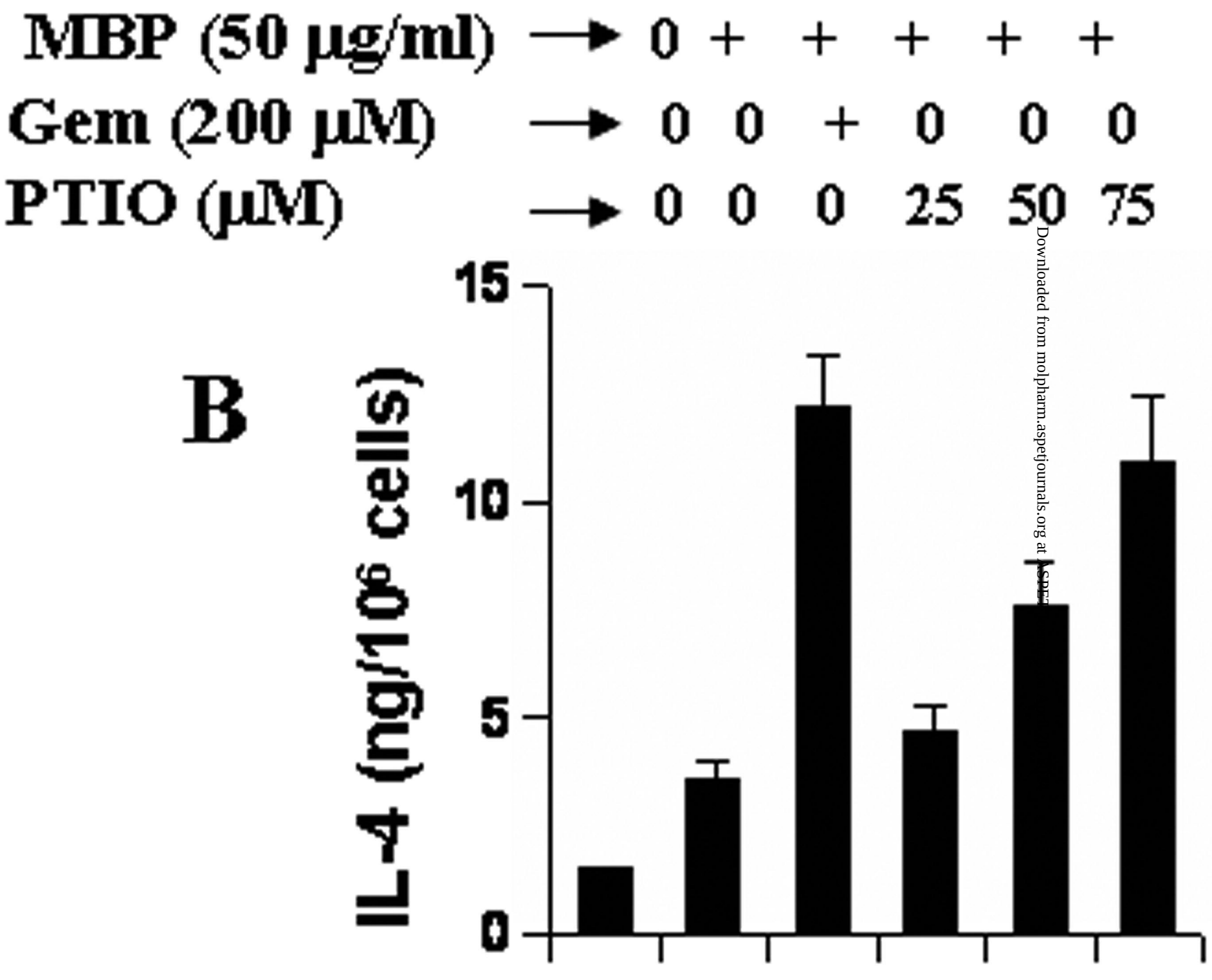

Fig. 12