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Cysteinyl Leukotriene-1 receptor activation in a human bronchial epithelial cell line leads to STAT-1 mediated eosinophil adhesion

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Running title: LTD_4 enhances eosinophil adhesion *via* STAT-1

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Abstract

We studied the effect of leukotriene D_4 (LTD_4) on a human bronchial epithelial cell line (16HBE) overexpressing the $CysLT_1$ receptor (HBE$CysLT_1$R), looking at the associated signal transduction mechanisms as well as at effects on inflammatory cell adhesion. The results obtained showed that LTD_4 increases the phosphorylation of extracellular signal-regulated protein kinase (ERK1/2) and of the signal transducer and activator of transcription 1 (STAT-1) in serine 727 (STAT-1Ser^{727}), resulting in increased eosinophil adhesion to HBE$CysLT_1$R, associated to enhanced surface expression of intercellular adhesion molecule 1 (ICAM-1). Pretreatment with a $CysLT_1$R selective antagonist, or with a selective inhibitor of protein kinase C (PKC), or with a selective inhibitor of the mytogen-activated protein kinase kinase (MEK) successfully suppressed both LTD_4-induced STAT-1Ser^{727} phosphorylation and the associated increase in eosinophil adhesion. The use of the MEK inhibitor and of the selective $CysLT_1$R antagonist in EMSA experiments showed that LTD_4 promotes the nuclear translocation of STAT-1 through the activation of ERK1/2 pathway. The key role of STAT-1 in leukotriene D_4 transduction signalling was confirmed by RNA interference experiments, where silencing of STAT-1 expression abolished the effect of leukotriene D_4 on eosinophil adhesion. In conclusion, for the first time we provide evidence of the involvement of STAT-1 in the signal transduction mechanism of the $CysLT_1$ receptor; phosphorylation of STAT-1, through PKC and ERK1/2 activation, causes enhanced ICAM-1 surface expression and eosinophil adhesion. Effective $CysLT_1$R antagonism may therefore contribute to the control of the chronic inflammatory condition that characterizes human airways in asthma.

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Introduction

Cysteinyl leukotrienes (CysLTs) play an important role in the pathogenesis of airway inflammation and remodeling in asthma (Drazen, 1998; Bisgaard, 2000; Henderson et al., 2002). The biological effects of CysLTs are mediated by at least two G protein-coupled receptors (GPCR), namely cysteinyl leukotrienes receptor 1 ($CysLT_1R$) and 2 ($CysLT_2R$), and the $CysLT_1R$ is known to be involved in most of the biological effects in the lung (Nicosia et al., 1999; Dahlen, 2000). $CysLT_1R$ is expressed in smooth muscle cells and lung macrophages (Lynch et al., 1999), and is widely distributed in human eosinophils, monocytes and neutrophils (Figueroa et al., 2001; Mita et al., 2001); little is known about the expression and the responses of $CysLT_1R$ in the airway epithelium, but recent evidences showed that LTC_4 may elicit TGFβ-release via the activation of p38 Kinase pathway in human airway epithelial cells (Perng et al., 2006).

Leukotriene D_4 (LTD_4) has been reported to activate the mitogen-activated protein kinases p38 (p38MAPK) and the Extracellular Signal-Regulated Kinase1/2 (ERK1/2), through Phosphatidyl Inositol 3-Kinase (PI 3-Kinase) and Protein Kinase C (PKC) activation in human renal mesangial cells (McMahon et al., 2000); furthermore activation of the ERK1/2 through a PKCα-Raf-1-dependent pathway has also been reported in a human monocytic leukemia cell line (Hoshino et al., 1998). Studies performed in intestinal epithelial cells demonstrated that LTD_4 activates the proliferative response through a distinct Ras-independent and PKCε-dependent ERK1/2 activation (Paruchuri et al., 2002), migration through a PI 3-Kinase and Rac-dependent mechanism (Paruchuri et al., 2005), and stress fibers formation by a RhoA and PKC-dependent mechanism (Massoumi et al., 2002).

The signal transducer and activator of transcription 1 (STAT-1) pathway has been associated to the pathogenesis of asthma (Quarcoo et al., 2004); (Chen et al., 2004), and it is

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known to be involved in Interferon-γ (IFN-γ) signalling pathway (Darnell, 1997). Within the sequence of STAT, the C-terminal serine 727 (Ser^{727}), located within a potential MAPK consensus PMSP motif, is phosphorylated by an unknown kinase, and this event increases the transcription factor activity of STAT-1 (Wen et al., 1995; Darnell, 1997). Several GPCRs, such as angiotensin II AT1, and thrombin receptors, can regulate STAT-1 activity through the interaction between ERK1/2 activity and STAT-1 serine phosphorylation (Schindler and Darnell, 1995), suggesting that LTD_4 may also have this activity.

In order to test the possible involvement of STAT-1 in the transduction mechanisms associated with the activation of the $CysLT_1$ receptor, we investigated the effects of LTD_4 in a transformed human bronchial epithelial cell line (16HBE) overexpressing the $CysLT_1$ receptor ($HBECysLT_1R$), reporting that LTD_4 increases the PKC-dependent activation of ERK1/2 and STAT-1 pathways leading to increased intracellular adhesion molecule 1 (ICAM-1) expression and eosinophil adhesion.

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Methods

Transfection and transinfection of pBH-CysLT$_1$R construct in epithelial cells

The SV40 large T antigen-transformed human airway epithelial cell line (16HBE) was used for these studies. 16HBE cell line was cultured as adherent monolayer in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum + 100 U/ml penicillin and 100 mg/ml streptomycin. 16HBE cells have previously been used to study the functional properties of bronchial epithelial cells in inflammation (Merendino et al., 2006). Since 16HBE cells have been showing a weak and variable expression of cysLT$_1$R, we enhanced and normalized its expression by transfection and transinfection with a pBH-CysLT$_1$R construct. CysLT$_1$R c-DNA was obtained from pcDNA 3.1 plasmid (Merck & Co., Inc. Research Laboratories, West Point, PA) (Lynch et al., 1999) using a PCR reaction with the primers LT1 Eco RI 5'GGA ATT CAC CAT GGA TGA AAC AGG AAA TCT GAC AG-3'and LT1 Sal I 5'ACG CGT CGA CCT ATA CTT TAC ATA TTT CTT CTC CTT TTT-3' (Invitrogen, Carlsbad, CA). The PCR product, after Eco RI and Sal I digestion, was subcloned into the Eco RI Sal I site of the retroviral vector pBH to allow stable expression in infected cells.

Phoenix cells were plated at 3-3.5 million cells per 100 mm plate. After 24 hrs 10 µg of DNA (pBH-CysLT$_1$R) were added following a $CaCl_2$ transfection protocol (Graham and van der Eb, 1973). The following day the viral supernatant was filtered and 5 ml were added to 16HBE plates. 48 hrs post-transinfection hygromicin (50 µg/ml) was added to isolate resistent cells (HBECysLT$_1$R cells). The expression of the cysLT$_1$R was routinely checked by western blot analysis using a commercially available antibody (Cayman Chem), showing a sustained and reproducible expression (data not shown).

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Stimulation of HBECysLT$_1$R

0.5 x 10^6 viable HBECysLT$_1$R cells were plated into 75 cm^2 flasks with RPMI 10% FBS for 72 hrs; confluent cells were maintained for 24 hrs in RPMI without FBS and were stimulated with LTD$_4$ (0.01 μM to 1 μM) (Sigma Aldrich, Milan Italy), in the presence or absence of Montelukast (Merck & Co., Inc. Research Laboratories, West Point, PA; 0.1-1μM), added 1 hr before LTD$_4$ stimulation.

LTD$_4$ stimulation was also carried out in the presence of IFN-γ (R&D systems, Inc, MN) using the cells treated with LTD$_4$ (0.1 μM) for 24 hrs (adhesion tests) or 15 minutes (signalling) in the presence or absence of IFN-γ (50 ng/ml), added 1 hr before LTD$_4$ stimulation.

The involvement of specific intracellular signalling pathways was evaluated pre-treating the cells with GF 109203X (a PKC inhibitor, 10 μM; Sigma), or PD 98059 (a MEK inhibitor, 25 μM; Sigma).

Eosinophil separation and adhesion assay

Peripheral blood eosinophils were prepared from normal subjects with the use of dextrane sedimentation and centrifugation over Ficoll cushions, as previously described, followed by negative immunomagnetic selection (Profita et al., 2003).

Eosinophil adhesion was performed as previously described with minor modifications (Zeidler et al., 2000). Purified eosinophils were resuspended in PBS (10^6 cells/ml), labelled for 45 min at 37°C with 50 µg/ml of the fluorochromic dye SFDA (Molecular Probes), washed and resuspended in PBS (0.4 x 10^6 cells/ml). Immediately before addition of eosinophils, medium was removed from the HBECysLT$_1$R cultures (70,000 HBECysLT$_1$R cells/well) grown to confluence in standard 24-well culture plates in complete medium, and cells were washed with warm PBS. Labelled eosinophils (0.2 x 10^6 cells/well) were added

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in a final volume of 0.5 ml. The plates were incubated at 37°C for 25 min and total fluorescence was evaluated using an excitation wavelength of 485 nm and monitoring emission at 530 nm in a Wallac 1420 Victor multilabel counter (PerkinElmer, Finland). Subsequently non-adherent cells were removed by washing, and fluorescence was measured to evaluate bound cells. Adhesion was expressed as percentage of the fluorescence ratio of bound cells on total cells. All test points were performed in triplicate.

Identification of ICAM-1 expression.

The expression of ICAM-1 on the surface of HBECysLT$_1$R cells was determined by direct label immunofluorescence using a FACStar Plus (Becton Dickinson, Mountain View, CA) analyzer. A conjugated mouse anti-human ICAM-1 (anti-CD54, clone 6.5B5)(Dako LSAB, Glostrup, Denmark) that react with a domain I nearest the N-terminal of the molecule of ICAM-1 was used. Negative controls were performed using a FITC-conjugated mouse IgG1 (Dako LSAB). Data are expressed as fluorescence intensity (arbitrary units).

Western Blotting

Total proteins were extracted from HBECysLT$_1$R using RIPA buffer (PBS 1X, Nonidet P-40 1%, sodium deoxycholate 0,5%, SDS 0,1%, Na_3VO_4 100 mM, PMSF 10 μg/ml), and the phosphorylation of p38MAPK, of ERK1/2 (Cell Signaling Technology, Beverly, MA), and of STAT-1 (Ser^{727} and Tyr^{710}), as well as the total amount of p91/STAT-1α (Santa Cruz Biotechnology, Inc., CA) was evaluated using specific antibodies. β-actin (Sigma St. Louis, MO) was used as a housekeeping protein to control the total amount of protein in each samples.

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Cell extracts were transferred in microcentrifuge tubes, left on ice for 45 minutes and centrifuged at 15,000 *g* for 20 min at 4°C. 60 γ of proteins were subjected to SDS-PAGE on 4%-12% gradient gels and blotted onto nitrocellulose membranes. After blocking with PBS containing 5% milk and 0.1% Tween 20, membranes were probed with specific antibodies, washed and incubated with peroxidase-conjugated secondary antibodies. Detection was performed with an enhanced chemiluminescence system (Ambion Inc., Austin, TX) followed by autoradiography. Gel images were aquired using an EPSON GT-6000 scanner and then imported into the National Institutes of Health Image analysis 1.61 program to determine band intensity. Data are expressed as arbitrary densitometric units corrected against the density of β-actin bands.

Preparation of nuclear extracts and STAT-1 binding assay

Cytosolic and nuclear extracts were prepared from stimulated cells using a nuclear cytoplasm extraction kit (Pierce Biotechnology, Rockford, IL). Protein concentration was assessed using the Bradford method.

Binding of STAT-1 was assessed on nuclear extracts using a kit for Lightshift Chemiluminescent EMSA following the protocol provided by the manufacturer (Pierce Biotechnology) with minor modifications. A double-stranded oligonucleotide containing a STAT-1α consensus sequence was labelled on the 3' end with biotin. Briefly, binding reaction mixtures containing 5 μg of nuclear protein, 10 mM Tris, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM $MgCl_2$, 0.05% Nonidet P-40, 50 ng Poly (dI dC) and 40 fmol of oligonucleotide probe were incubated for 20 min at room temperature. Specific binding was confirmed by using a 100- to 400-fold excess of unlabeled probe as specific competitor. Protein-DNA complexes were separated using a 6% nondenaturing acrylamide gel electrophoresis. Complexes were transferred to positively charged nylon membranes

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and uncrosslinked. Gel shifts were visualized with a streptavidin-horseradish peroxidase followed by chemiluminescent detection.

RNA interference of STAT-1 pathway

In order to confirm that the increase in eosinophil adhesion to HBECysLT$_1$R epithelial cells was causally linked to LTD$_4$-dependent STAT-1 activation, (Sledz and Williams, 2005) we tested the effect of p91/STAT-1α silencing, using specific siRNA transfection. Cells were plated in 24 well tissue culture plates and grown in medium containing 10% FBS without the use of antibiotic until 60-80% of confluency. Subsequently, p91/STAT-1α siRNA (10 μM; Santa Cruz Biotechnology, INC) was added to 40 μl of siRNA transfection medium, and the reaction was performed according to the manufacturer instructions until complete transfection of cells (30 hrs at 37°C). For optimal siRNA transfection efficiency control siRNA (10 μM; Santa Cruz Biotechnology, INC) was used containing a scrambled sequence that did not lead to the specific degradation of any known cellular mRNA. Finally, cells were stimulated with LTD$_4$ for 18 hrs and eosinophil adhesion evaluated. The silencing efficacy of the RNA Interference for p91/STAT-1α was checked by western blot analysis.

Statistical analysis

The data were expressed as median±SD of the results of each experiment, unless otherwise stated. Eosinophil adhesion was analysed using ANOVA test with Fisher's test correction.

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Results

LTD_4 increased eosinophil adhesion to $HBECysLT_1R$ cells, reaching a maximal response at concentrations ≥ 0.1 µM (Figure 1A). As expected, the specific $CysLT_1R$ antagonist Montelukast (0.1 to 10 µM) inhibited in a concentration-dependent fashion the effect of LTD_4 (0.1 µM) with a maximal response at concentrations ≥ 1 µM (Figure 1B). The increase in eosinophil adhesion was accompanied by a statistically significant increase in the expression of ICAM-1 on the surface of $HBECysLT_1R$ cells (Figure 2), as shown by FACS analysis of cells incubated with increasing concentrations of LTD_4; as expected, also this effect was abolished by pretreating epithelial cells with the $CysLT_1R$ antagonist Montelukast (1 µM; data not shown).

The treatment of $HBECysLT_1R$ with LTD_4 (0.1 µM) activated ERK1/2 in a time dependent manner, as shown by the increase in the phosphorylated protein. Phospho ERK1/2 was detectable by 5 min, maximal at 10–15 min, and returned to basal values after 30 min (Figure 3). LTD_4 did not appear to activate p38MAPK, but the analysis of total protein lysates showed a significant activation of STAT-1 pathway, as shown by the time-dependent phosporylation of STAT-1 in Ser^{727} in the presence of unchanged amounts of p91/STAT-1α. Interestingly, the stimulation of $HBECysLT_1R$ with LTD_4 did not activate the phosphorylation of STAT-1 in Tyr^{701} (Figure 4A, second lane).

As previously reported, treatment with IFN-γ (50 ng/ml) activated ERK1/2 phosphorylation, but at variance with the results observed with LTD_4, significant phosporylation of STAT-1 both in Ser^{727} and in Tyr^{701} was observed; the amounts of p91/STAT-1α was not increased by the incubation with IFN-γ (Figure 4A). Co-stimulation of $HBECysLT_1R$ with IFN-γ (50 ng/ml) and LTD_4 (0.1 µM) resulted in a modest increase in ERK1/2 as well as in STAT-1Ser^{727} phosphorylation with respect to the effects observed

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with LTD_4 or IFN-γ alone, while STAT-1 Tyr^{701} phosporylation remained at the level observed with IFN-γ alone (Figure 4A). In agreement with the results observed on ERK1/2 and STAT-1 activation, the coadministration of LTD_4 and IFN-γ only caused a small increase in the number of adhering eosinophils when compared to IFN-γ alone (Figure 4B, lower panel). Pretreatment with Montelukast (1 μM) abolished the LTD_4-dependent phosphorylation of ERK1/2 and STAT-1Ser^{727} (Fig 4B. upper panels), returning the number of adhering eosinophils back to the level observed in control (Figure 4B, lower panel).

Pretreatment with either the bisindolylmaleimide PKC inhibitor GF 109203X (10 μM), or with the MEK inhibitor PD 98059 (25 μM, 30 min) at concentrations known to be effective in bronchial epithelial cells (Catley et al., 2004; Chang et al., 2005) inhibited both ERK1/2 and STAT-1Ser^{727} phosphorylation in response to LTD_4 (Figure 5A and 5B), suggesting the involvement of PKC in the phosphorylation of ERK1/2 and the following activation of STAT-1.

Again, in agreement with the observed effect on ERK1/2 activation, the pre-treatment of HBECysLT_1R with either the inhibitor of PKC GF 109203X, or the MEK inhibitor PD 98059 before the stimulation with LTD_4, also inhibited eosinophil adhesion (Figure 6A and 6B), providing evidence that this important inflammatory event is strongly associated to PKC and ERK1/2 intracellular signal pathway activation.

EMSA analysis showed that LTD_4 increased the binding of STAT-1 to the DNA, an effect that was blocked by Montelukast (Figure 7A) and by the MEK inhibitor PD 98059 (Figure 7B).

Temporary transfection of HBECysLT_1R cells with siRNA for p91/STAT-1α caused a significant decrease in the synthesis of STAT-1 protein (Figure 8A). LTD_4 stimulation

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failed to increase eosinophil adhesion to the silenced p91/STAT-1α HBECysLT$_1$R but the response to LTD$_4$ was not affected in cells transfected with control siRNA (10 μM) containing a scrambled sequence (Figure 8B), confirming the newly described role of the STAT-1 activation in the transduction pathway leading to the enhanced eosinophil adhesion in LTD$_4$-treated epithelial cells.

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Discussion

We provide evidence that the increase in eosinophil adhesion to a human bronchial epithelial cell line expressing $CysLT_1R$ ($HBECysLT_1R$) observed in response to LTD_4 is associated to an increased expression of ICAM-1 to the surface of the cells, to ERK1/2 phosphorylation and to STAT-1 phosphorylation in Ser^{727} (but not in Tyr^{701}). IFN-γ dependent eosinopil adhesion also resulted associated to ERK1/2 activation, but STAT-1 phosphorylation occurred both in Ser^{727} and Tyr^{701}. Co-challenge with LTD_4 and IFN-γ yielded a small but consistent additional increase when compared to LTD_4 or IFN-γ alone, either in terms of signal transduction mechanisms activation or eosinophil adhesion. Pretreatment with either the specific $CysLT_1R$ antagonist Montelukast, or the PKC inhibitor GF 109203X (Toullec et al., 1991), or the MEK inhibitor PD 98059 prevented both the effects on signal transduction pathways associated to LTD_4-dependent cellular activation, and the increase in eosinophil adhesion.

The inhibition of the nuclear translocation of p91/STAT-1α by Montelukast or by the MEK inhibitor observed in LTD_4-activated epithelial cells, supported the hypothesis that the nuclear translocation of STAT-1 occurs through the $CysLT_1R$-dependent activation of ERK1/2 pathway. Finally, silencing of STAT-1 protein expression in $HBECysLT_1R$ cells completely suppressed the increase in eosinophil adhesion associated to challenge with LTD_4, providing evidence for the first time of a key contribution of STAT-1 activation to the activity of CysLTs in epithelial cells.

The airway epithelium represents a target as well as a source of inflammatory mediators, and, through the expression of adhesion molecules, may contribute to the recruitment of inflammatory cells ultimately leading to the pathophysiological changes typical of the asthmatic airways (Hamilton et al., 2001). IFN-γ may contribute to this phenomenon

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increasing intracellular adhesion molecule-1 (ICAM-1) expression (Look et al., 1992), a response typically associated to intracellular transduction signals leading to transcription and translation of specific genes (Look et al., 1992). Indeed, in airway epithelial cells IFN-γ inducible gene expression is associated to STAT-1 dependent pathway activation (Look et al., 1992). STAT-1 is selectively activated in airway epithelium of asthmatic subjects and correlates with ICAM-1 expression of airway epithelium of asthmatic subjects (Sampath et al., 1999), while the inhibition of STAT-1 pathway attenuates airway inflammation and hyperreactivity (Chen et al., 2004), suggesting that STAT-1 activation may play an important role in asthma (Quarcoo et al., 2004). The STAT-1 protein is a family of latent transcription factor that are activated by a wide range of cytokines. Upon engagement, STATs become tyrosine phosphorylated, translocate to the nucleus, and induce expression of target genes. At the same time STAT-1 is phosphorylated on Ser^{727}, independently of tyrosine phosphorylation (Wen et al., 1995) and, while tyrosine phosphorylation is required for cytokine-induced STAT-1 dimerization, nuclear translocation and DNA binding, full transcriptional activity of STAT-1 is substantially related to serine phosphorylation. Indeed, activation of STAT-1 through Ser^{727} phosphorylation independent from Tyr^{701} phosphorylation has been reported, and may be involved in the induction of gene transcription regulating apoptosis and Fas Receptor/Fas ligand expression (Stephanou et al., 2001). The mechanism of STAT-1 phosphorylation in Ser^{727} is not yet well understood, although the presence of a potential MAPK consensus PMSP motif suggests the involvement of ERK1/2 (Wen et al., 1995; Darnell, 1997). Indeed, in addition to IFN-γ (Blanchette et al., 2003), several GPCRs, such as angiotensin II AT1, and thrombin receptors, can regulate STAT-1 activity through the interaction between ERK1/2 activity and STAT-1 serine phosphorylation (Schindler and Darnell, 1995).

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It has been reported that $CysLT_1R$ mainly couples with pertussis-toxin insensitive G-protein, although reports exists of coupling with $G_{i/o}$ in human cells (Capra et al., 2004; Capra et al., 2007). For the first time, we provide evidence that CysLTs activation of $HBECysLT_1R$ epithelial cells causes activation of ERK1/2 leading to STAT-1 phosphorylation, nuclear translocation, ICAM-1 surface expression, and enhanced eosinophil adhesion; the significant inhibition of the CysLTs activity observed using a selective PKC inhibitor (Toullec et al., 1991) also suggest the involvement of a PKC-dependent phosphorylation leading to the activation of ERK1/2, possibly through the activation of Raf-1 (Hoshino et al., 1998; Paruchuri and Sjolander, 2003). Additional studies are necessary to fully elucidate the involvement of PKC in the trasduction signalling of $CysLT_1R$ in epithelial cells.

The intercellular adhesion molecule-1 (ICAM-1; CD54) binds to two integrins belonging the to β_2 subfamily, CD11a/CD18 (LFA-1) and CD11b/CD18 (MAC-1), both expressed by leukocytes, resulting in adhesion and transendothelial migration of leukocytes from the bloodstream. Similar processes control leukocytes adhesion to lung airway epithelial cells and may contribute to the damage of these cells observed in asthma (Bloemen et al., 1997). Airway inflammation resulting from viral infection is indeed accompanied by marked leukocytes trafficking and production of CysLTs and IFN-γ (Van Shaik et al., 2000). IFN-γ has also been reported to increase the expression of $CysLT_1R$ in myocytes (Amrani et al., 2001), to promote the release of CysLTs from human eosinophils (Saito et al., 1988), and may have a role in the induction of airway hyperresponsiveness in ovalbumin-challenged experimental animals (Hessel et al., 1997).

Taken together, these evidences support a potential association between ICAM expression, eosinophilic infiltration, CysLTs or IFN-γ production and airway

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inflammation in response to antigen and during asthma exacerbation. Within this line of evidences, our data suggest that IFN-γ and LTD_4 may both contribute to the recruitment of eosinophils in the airway of asthmatic subjects via the activation of airway epithelium, leading to sustained bronchoconstriction and airway hyperreactivity. Studies performed on the intestinal epithelial cells have demonstrated that LTD_4 regulates cell proliferation (Paruchuri et al., 2002), and activates the β_1-integrin dependent adhesion (Massoumi et al., 2002) via PKC-dependent stimulation of ERK1/2. Similarly, in airway smooth muscle cells, it has been shown that $CysLT_1R$ activation induces PKC translocation (Accomazzo et al., 2001), as well as ERK1/2 activation through a G_i-dependent mechanism (Ravasi et al., 2006).

The results obtained in our studies, both using EMSA as well as STAT-1 silencing, for the first time provide evidence of the involvement of STAT-1 in the signal transduction mechanism associated to $CysLT_1R$ activation, supporting the hypothesis that it may represent a key transduction pathway leading to the enhanced eosinophil adhesiveness observed in response to CysLTs activation. The inhibitory effect of Montelukast provides additional support to the potential antiinflammatory activity of $CysLT_1R$ receptor antagonists.

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Footnotes

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Legends for Figures

Figure 1: Effect of LTD_4 on eosinophil adhesion to epithelial cells. (A) $HBECysLT_1R$ cells were stimulated with different concentration of LTD_4 (0.01 to 1 μM) for 24 hrs. (B) $HBECysLT_1R$ cells were stimulated with LTD_4 (0.1 μM) for 24 hrs after 30 minutes of pre-incubation with different concentration of Montelukast (0.1 to 10 μM). Adhering cells were analyzed using fluorimetric analysis as described in Methods. Results were expressed as median and SD of six independent experiments. Statistical analysis was performed using ANOVA test with Fisher's test correction.

*$p<0.05$ vs untreated cells

#$p<0.05$ *vs* LTD_4-treated cells

Figure 2: Expression of ICAM-1 in response to LTD_4. $HBECysLT_1R$ cells were stimulated with different concentrations of LTD_4 (0.01-1 μM) for 24 hrs. At the end of the stimulation, the expression of ICAM-1 on the surface of $HBECysLT_1R$ was determined by direct label immunofluorescence as described in Methods. Results were expressed as geometric mean ± SD of the fluorescence intensity (arbitrary units).

* $p<0.05$ *vs* untreated cells

Figure 3: Kinetics of p38MAPK, ERK1/2 and STAT-1 pathways activation by LTD_4 in epithelial cells. $HBECysLT_1R$ cells were stimulated with LTD_4 0.1 μM for 15, 30, 60 and 120 minutes. Cells extracts were separated with 10% SDS-PAGE, and western blot analyses were carried out sequentially with antiphospho-ERK1/2, antiphospho-p38MAPK, antiphospho-STAT-1Ser^{727}, anti-p91/STAT-1α, and anti-β-actin antibodies. The top panel represents the results of the densitometric analysis of three independent experiments, carried out as described in Methods.

* $p<0.05$ *vs* untreated cells

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Figure 4: Effects of LTD_4 and IFN-γ on signal transduction pathway activation and eosinophil adhesion in epithelial cells. HBECysLT_1R cells were stimulated with IFN-γ 50 ng/ml and/or LTD_4 0.1 μM for 15 minutes (Panel A). Cell lysates were prepared and immunoblotted with antiphospho-ERK1/2, antiphospho-STAT-1Ser^{727}, antiphospho-STAT-1Tyr^{701}, and anti-p91/STAT-1α specific antibodies. HBECysLT_1R cells were stimulated with IFN-γ 50 ng/ml and/or LTD_4 0.1 μM for 15 minutes (for western blot analysis) or 24 hrs.

Similarly HBECysLT_1R cells were stimulated with IFN-γ 50 ng/ml and/or LTD_4 0.1 μM for 15 minutes (western blot analysis) or 24 hrs (eosinophil adhesion) and the specificity of the signal observed with LTD_4 was tested upon preincubation with Montelukast (1 μM, 1 hr before epithelial cell stimulation)(Panel B). The adhesion of eosinophils was evaluated using fluorimetric analysis as described in Methods, and cell lysates were prepared and immunoblotted with antiphospho-ERK1/2, and antiphospho-STAT-1Ser^{727}, and anti-p91/STAT-1α specific antibodies.

Adhesion results (Panel B, bottom) were expressed as median and SD of six independent experiments. Statistical analysis was performed using ANOVA test with Fisher's test correction

The top panels of A and B represents the results of the densitometric analysis of three independent experiments.

*$p<0.05$ *vs* untreated cells, # $p<0.05$ *vs* LTD_4-treated cells

Figure 5: Effect of PKC and MEK inhibition on LTD_4-dependent signal transduction pathways activation in epithelial cells. HBECysLT_1R cells were stimulated

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with LTD_4 0.1 µM for 15 min in absence or presence of GF 109203 (10 µM, 30 min before activation of epithelial cells)(Panel A), or PD 98059 (25 µM, 30 min before activation of epithelial cells)(Panel B). Cell lysates were prepared and immunoblotted with antiphospho-ERK1/2, antiphospho-STAT-1Ser^{727}, and anti-p91/STAT-1α specific antibodies. The data are representative of three independent experiments.

The top panels of A and B represents the results of the densitometric analysis of three independent experiments.

*$p<0.05$ *vs* untreated cells, # $p<0.05$ *vs* LTD_4-treated cells

Figure 6: Effects of PKC and MEK inhibition on LTD_4-induced eosinophil adhesion in epithelial cells. HBECysLT$_1$R cells were stimulated with LTD_4 0.1 µM for 24 hrs in the presence or absence of GF 109203X (10 µM, 30 min before epithelial cell stimulation)(Panel A), or PD 98059 (25 µM, 30 min before epithelial cell stimulation) (Panel B). The adhesion of eosinophils was evaluated using fluorimetric analysis as described in Methods. Results were expressed as median and SD of six independent experiments. Statistical analysis was performed using ANOVA test with Fisher's test correction

*$p<0.05$ *vs* untreated cells

Figure 7: STAT-1 binding to DNA in LTD_4-treated epithelial cells. STAT-1 binding to DNA was evaluated by EMSA on nuclear extracts obtained from HBECysLT$_1$R cells treated with LTD_4 (0.1 µM, 30 minutes) in the presence or absence of Montelukast (1 µM, 1 hr before epithelial cell stimulation)(A), or in the presence or absence of the specific MEK inhibitor PD 98059 (25 µM, 30 min)(B). EMSA was performed with STAT-1 oligonucleotide probes as described in Methods.

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Figure 8: p91/STAT-1α silencing and LTD_4-stimulated eosinophil adhesion to epithelial cells. Temporary transfection of HBECysLT_1R cells with siRNA for p91/STAT-1α was carried out as described in Methods. Expression of p91/STAT-1α protein in control cells and cells transfected with scrambled siRNA or siRNA for p91/STAT-1α (Panel A). Eosinophil adhesion to control cells and cells transfected with scrambled siRNA or siRNA for p91/STAT-1α in response to LTD_4 treatment (0.1 μM, 24 hrs)(Panel B).

p91/STAT-1α was analyzed by western blot using anti-p91/STAT-1α antibodies. Adhering cells were analyzed using fluorimetric analysis as described in Methods. Results were expressed as median and SD of six independent experiments. Statistical analysis was performed by using ANOVA test with Fisher's test correction.

*$p<0.05$ *vs* the respective untreated cells

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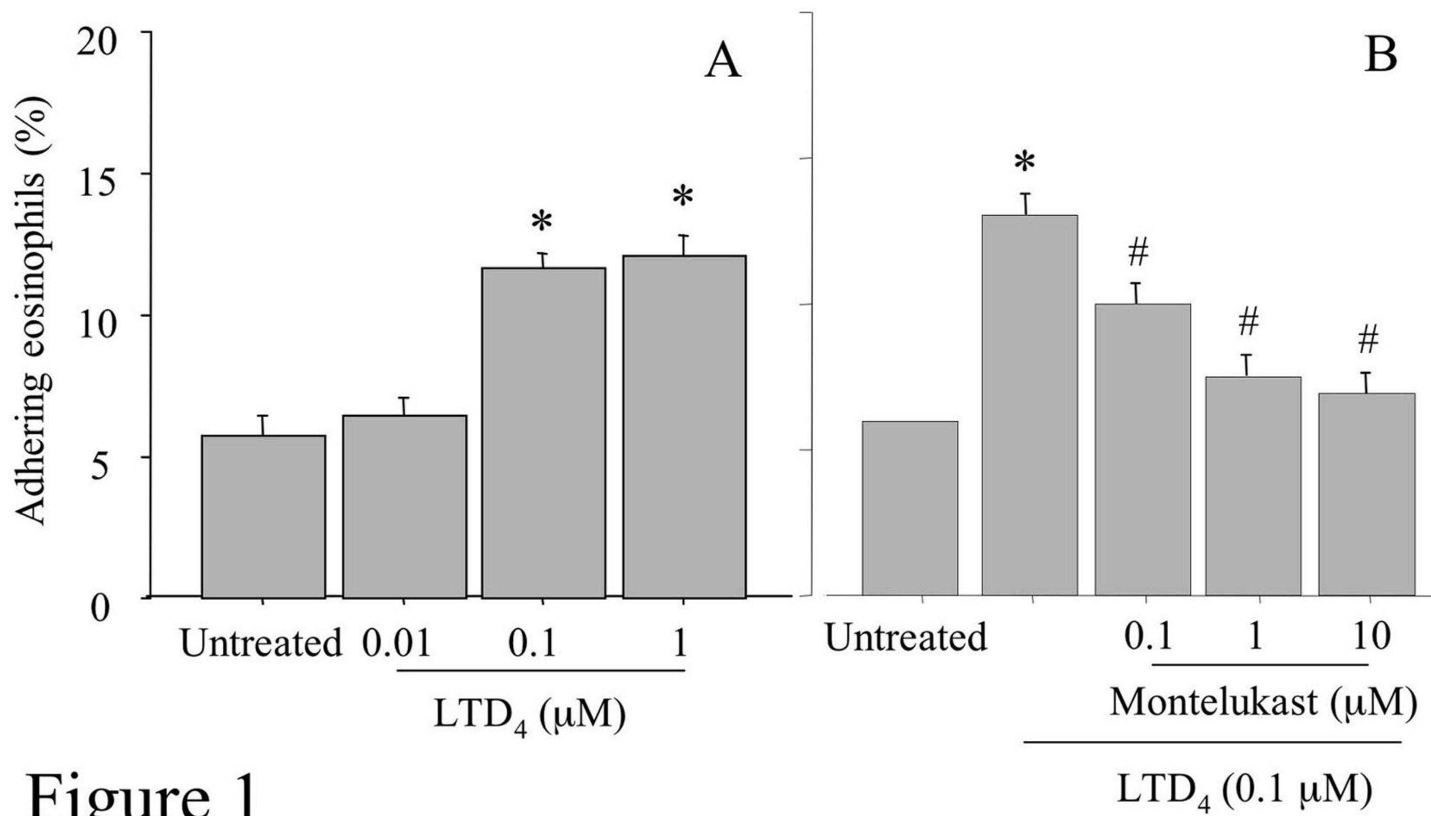

Figure 1

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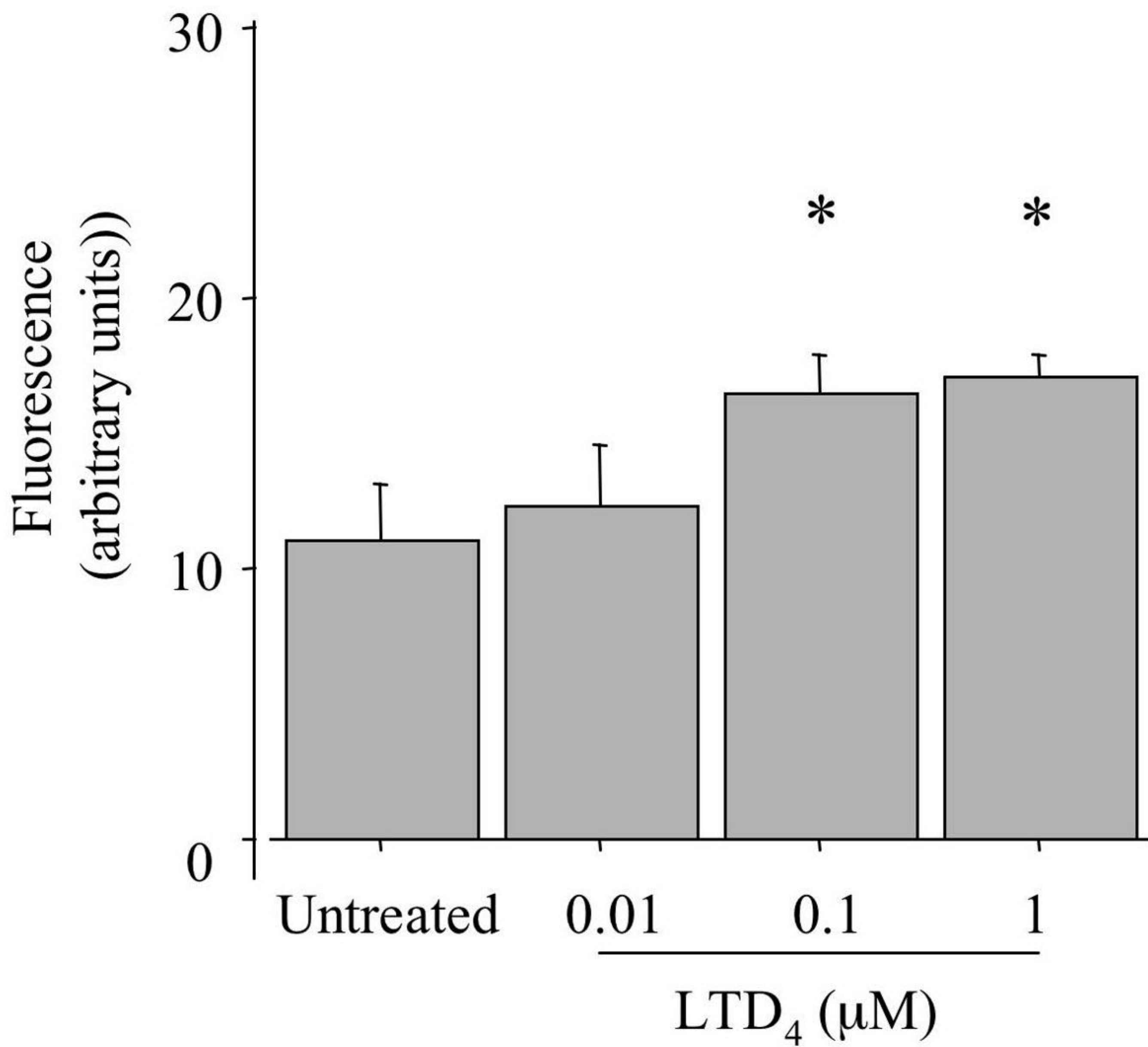

Figure 2

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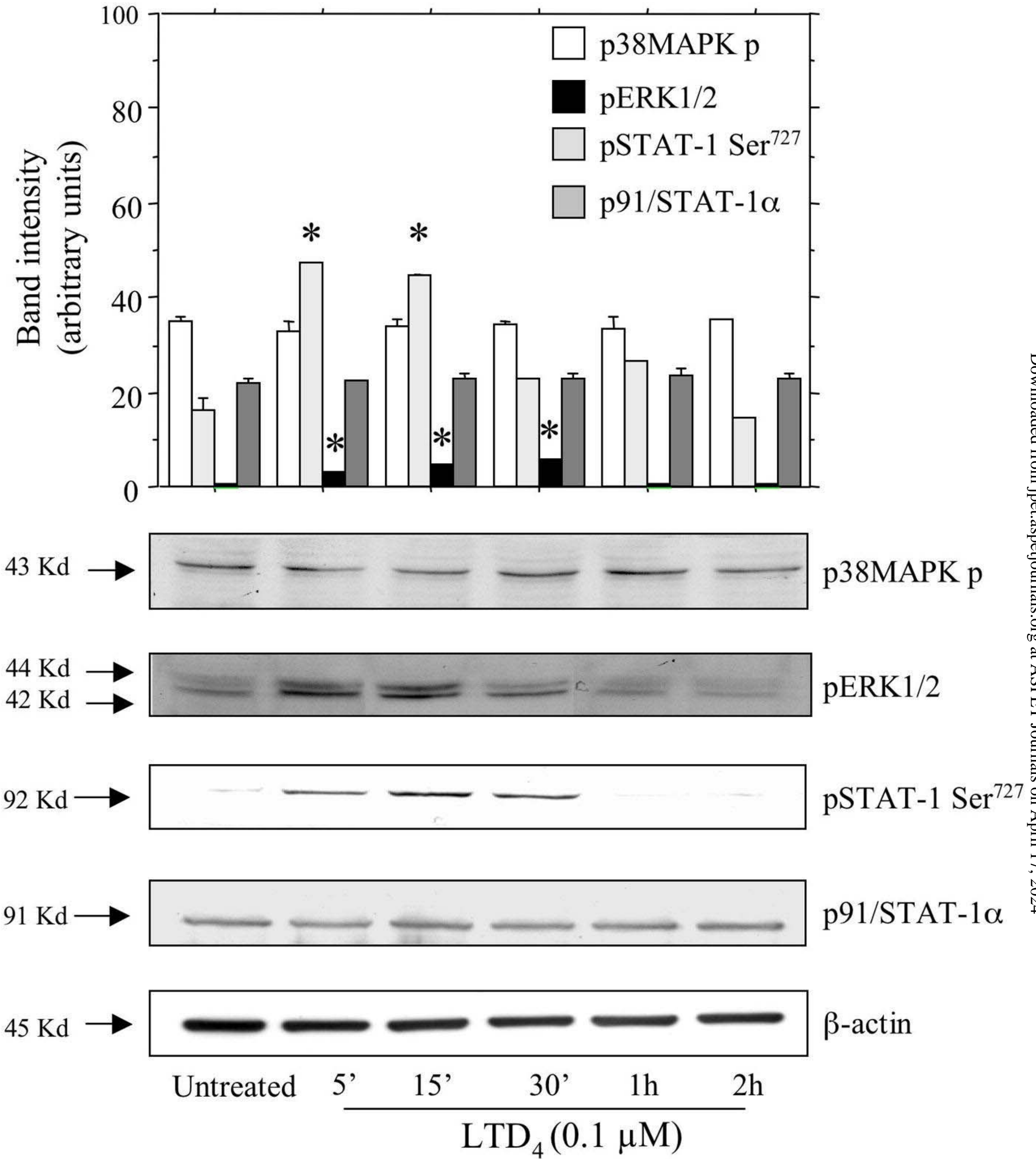

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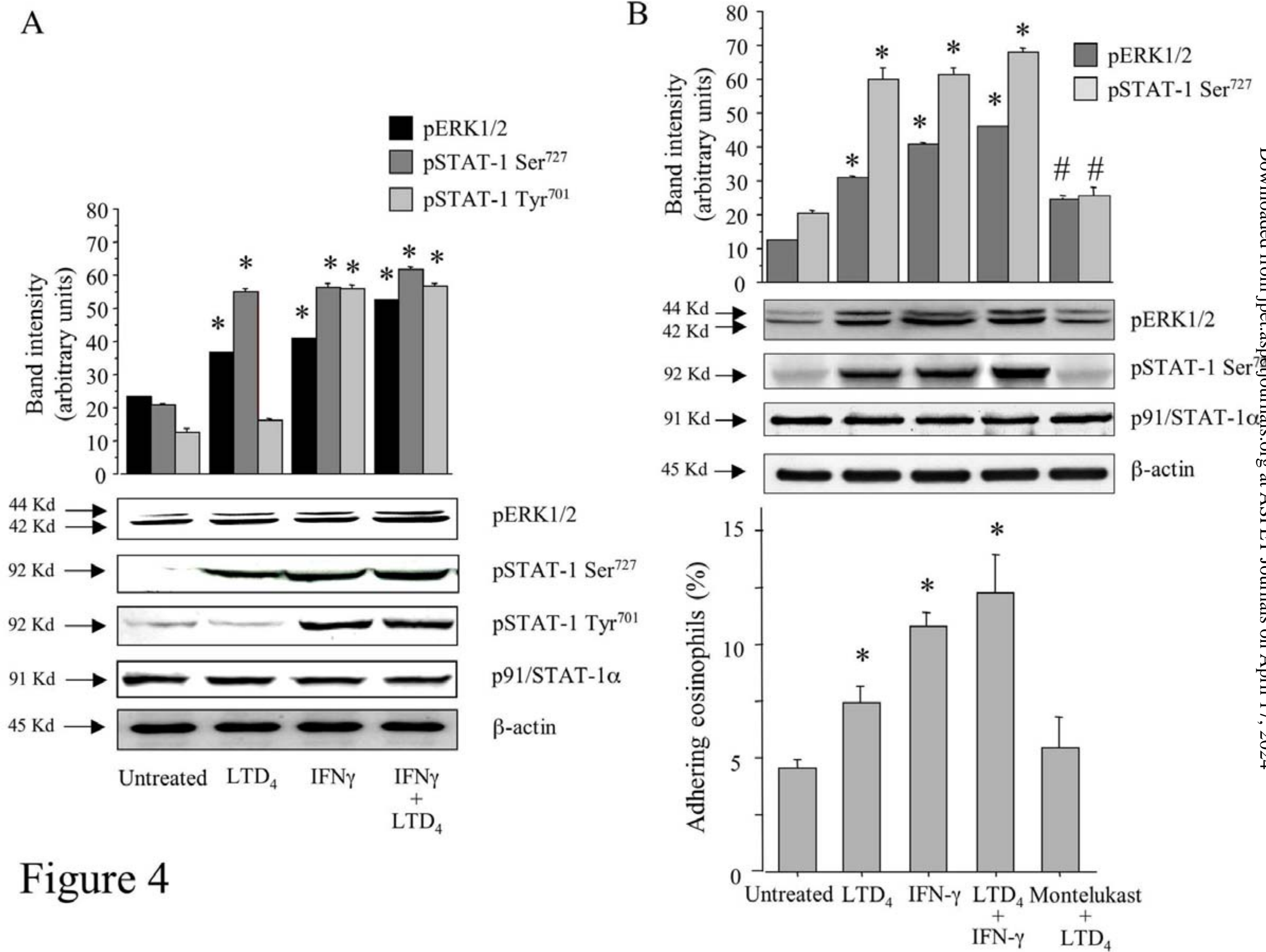

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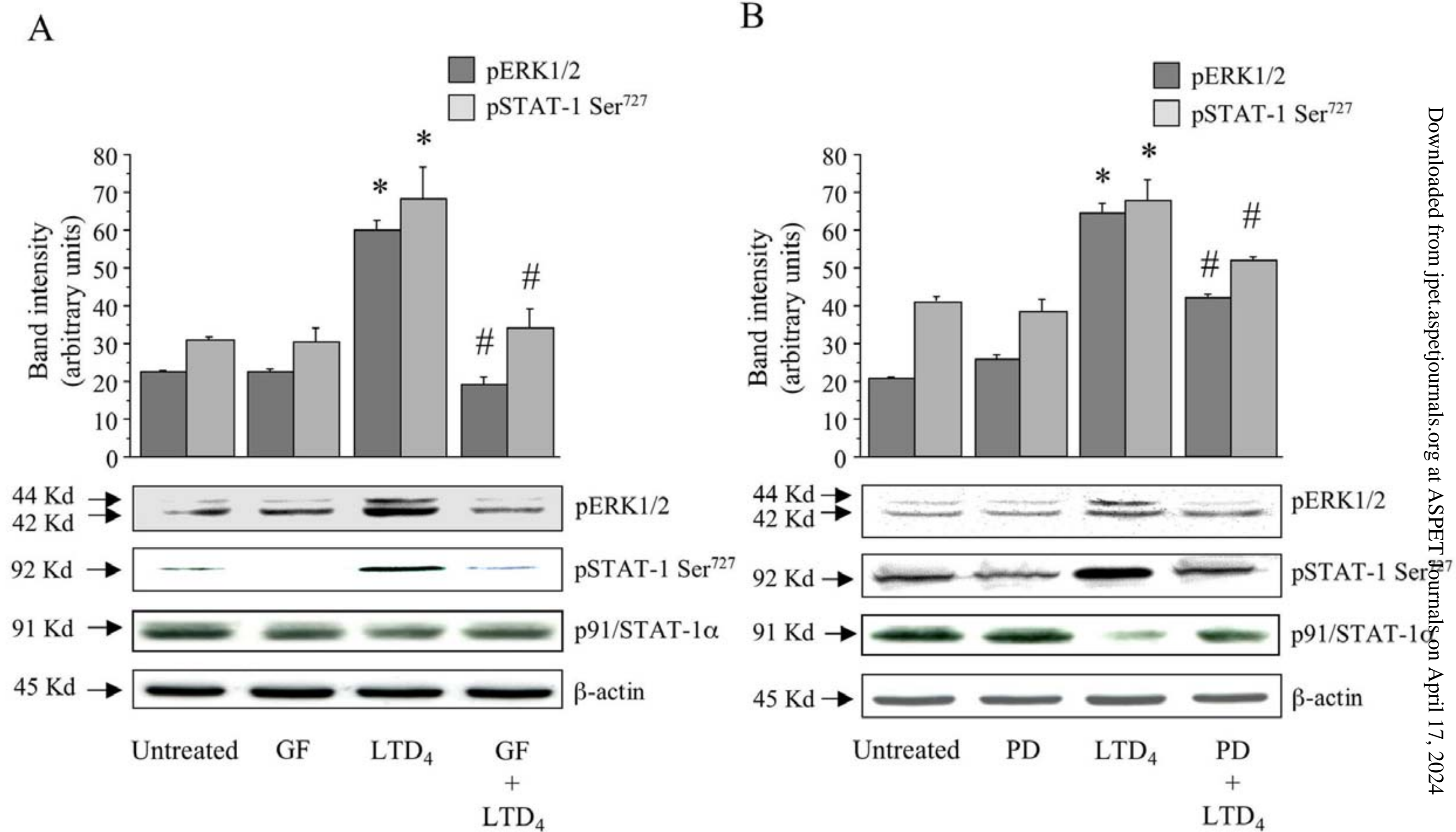

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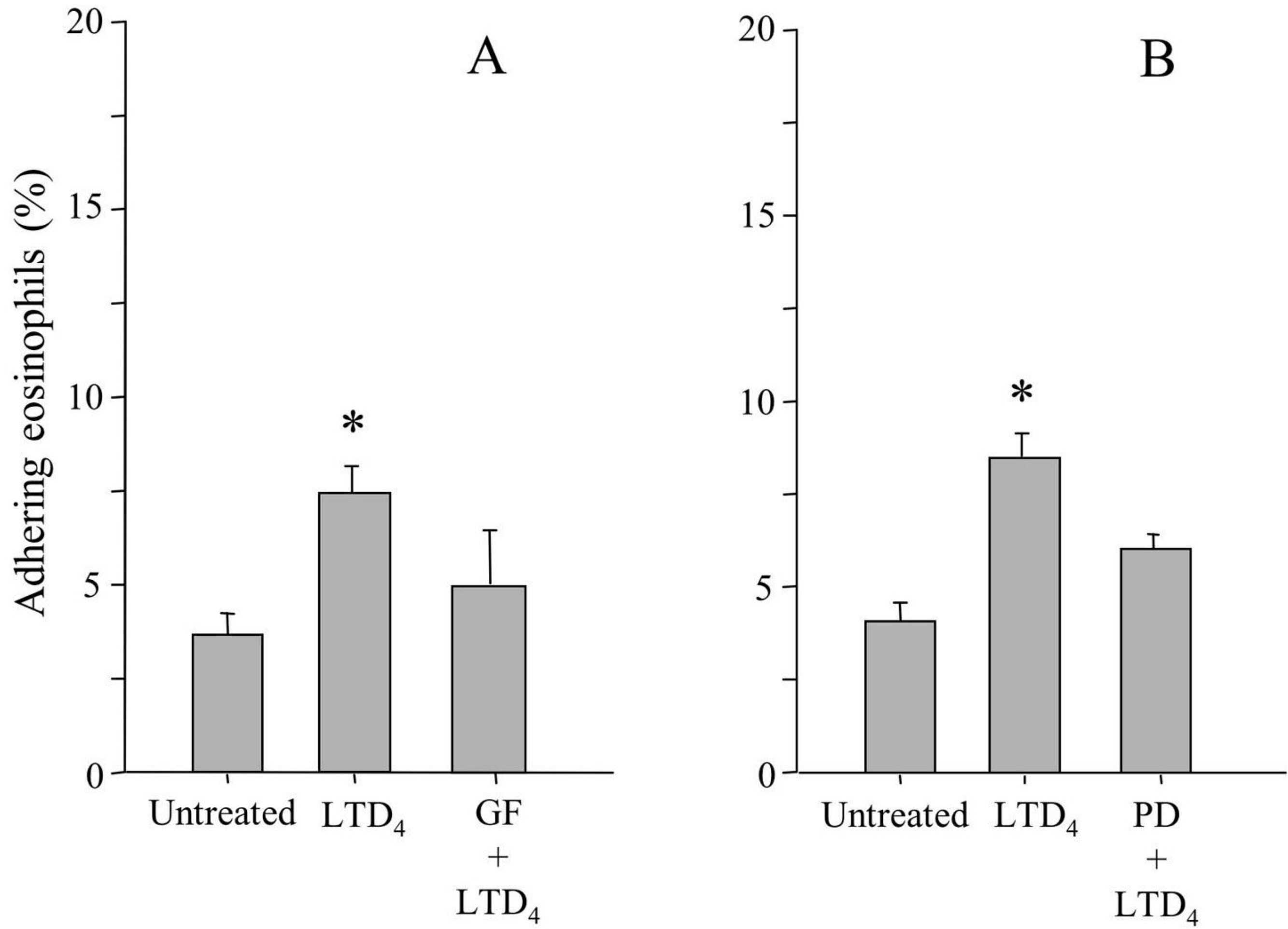

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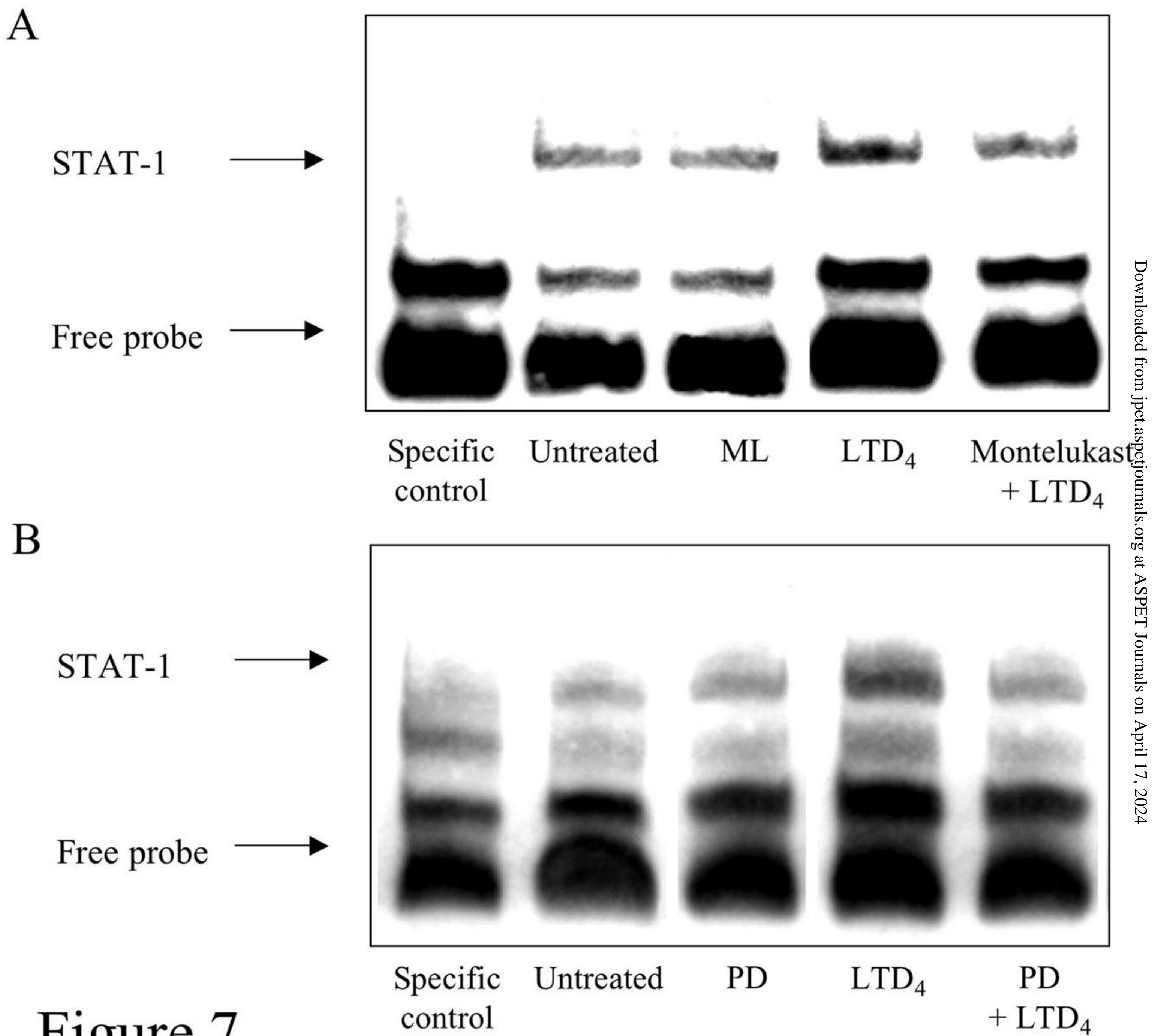

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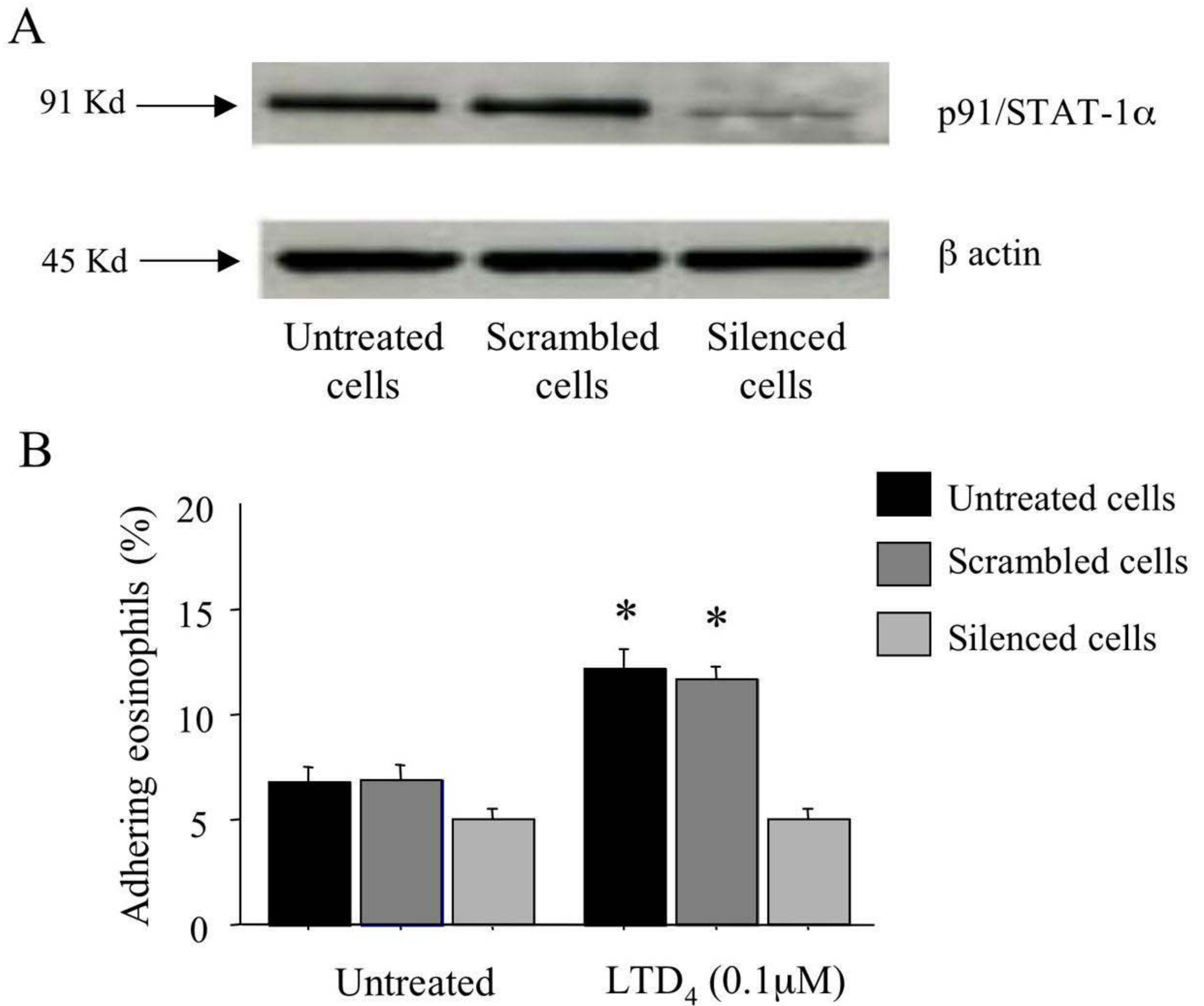

Figure 8

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