

**Identification of the receptor for augmenter of liver regeneration (ALR) and its function in
Kupffer cells**

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Short title: ALR actions on Kupffer cells

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Abbreviations: ALR, augmenter of liver regeneration; ERV1, essential for respiration and vegetative growth; NO, nitric oxide; rrALR, recombinant rat ALR; TNF, tumor necrosis factor.

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SUMMARY

Augmenter of liver regeneration (ALR), a protein produced and released by hepatocytes is mitogenic for hepatocytes *in vivo* but not *in vitro* suggesting that the effect is mediated by nonparenchymal cells. Since mediators produced by Kupffer cells are implicated in hepatic regeneration, we investigated receptor for ALR and its functions in rat Kupffer cells. Radioligand receptor binding, and ALR-induced GTP/G-protein association, and protein, NO and TNF- α synthesis were determined. High affinity receptor for ALR, belonging to the G-protein family, with Kd of 1.25 ± 0.18 nM and Bmax of 0.26 ± 0.02 fmol/ μ g DNA was identified. ALR increased mRNA and protein expression of inducible nitric oxide (NO) synthase, stimulated NO and protein synthesis, and increased the mRNA expression and release of TNF- α . These effects of ALR were mediated via cholera toxin-sensitive G-protein. ALR stimulated p38-MAPK activity and nuclear translocation of NF κ B. While inhibitor of NF κ B (MG132) inhibited ALR-induced NO and protein synthesis, MG132 and p38-MAPK inhibitor (SB203580) blocked ALR-induced TNF- α synthesis. Finally, ALR was found to prevent Kupffer cell-induced inhibition of DNA synthesis in hepatocytes and to promote hepatic regeneration in rats following 40% hepatectomy. These results demonstrate for the first time specific G-protein coupled receptor for ALR and its function in Kupffer cells, and suggest that mediators produced by the ALR-stimulated Kupffer cells may elicit physiologically important effects on hepatocytes.

INTRODUCTION

Hepatic regeneration that follows a variety of chemical, microbial, physical and viral injuries is a remarkable phenomenon that has been investigated extensively in the animal models of liver injury and partial hepatectomy. Multiple endogenously produced and humoral mediators orchestrate hepatic regeneration. The search for the molecules involved in hepatocyte replication led to the identification of a novel protein named augmenter of liver regeneration (ALR) in the soluble fractions of the hypertrophic rodent and canine livers (1-3). ALR protein was purified from the extracts of weanling rat liver (4,5), and its gene cloned in rat, mouse and human (6,7). The ALR sequence is highly homologous among various mammalian species. It also exhibits high homology, at the gene and protein level, with ERV1 (essential for respiration and vegetative growth), which is required for the growth and survival of *Saccharomyces cerevisiae* (6-9).

The native ALR isolated from the weanling and regenerating animal livers, but not from the unmodified adult liver, and cloned ALR were found to be mitogenic and antiatrophic in partially hepatectomized rats, and in dogs after portacaval shunt (1-7). However, presence of equivalent amounts of ALR mRNA and protein (38-42 kDa) in hepatocytes of hypertrophic and resting rat livers (10) suggested that ALR in quiescent hepatocytes is not mitogenic. We recently showed that ALR is a survival factor for hepatocytes, and inhibition of its synthesis via antisense mRNA transfection causes mitochondrial dysfunction and death via apoptosis/necrosis (11). However, ALR is constitutively released by hepatocytes, and is present in significant level in the serum that increases shortly after partial hepatectomy (10). These observations suggest that ALR may exert actions on nonparenchymal cell types. This hypothesis is supported by the findings that rat hepatocytes lack ALR receptor (10,11), and both native (from the hyperplastic rat liver) and the recombinant rat ALR (rrALR) do not stimulate mitosis of cultured rat hepatocytes but do

so upon administration *in vivo* (3,10,11). There is strong evidence that mediators produced by Kupffer cells play a critical role in hepatic regeneration (12-16). Therefore, we investigated if ALR elicits actions on Kupffer cells via specific receptors. The results show high affinity G-protein-coupled receptor for ALR on rat Kupffer cells, activation of which causes stimulation of NO, protein and TNF- α synthesis.

MATERIALS AND METHODS

Preparation of Kupffer cells and hepatocytes. The protocols were approved by the IACUC, University of Pittsburgh in accordance with the NIH regulations. Kupffer cells were prepared essentially as described previously (17) by collagenase/protease digestion of the liver and purification by Metrizamide density gradient centrifugation, followed by centrifugal elutriation. The cells were suspended in Williams' medium E containing 10% fetal calf serum, 250 units/ml penicillin, 250 μ g/ml streptomycin, and plated at a density of 0.5×10^6 cells/cm². The medium was renewed after 3h, and the following day and cells were used on the third day. Purity of the cells as determined by immunostaining for ED2 (Kupffer cells), desmin (stellate cells) and SE-1 (endothelial cells) was greater than 95% (17).

Hepatocytes were prepared as described previously (10,11), and suspended (0.25×10^6 cells/ml) in William's medium E supplemented with 2 mM L-glutamine, penicillin/streptomycin, 10% FBS, and 10^{-6} M insulin. Cells were plated at a density of 0.063×10^6 /cm², the medium renewed 3h later, and the cells used following overnight incubation.

Determination of ALR receptor. Recombinant rat ALR (rrALR), prepared as described previously (7), was radioiodinated by lactoperoxidase procedure (18). For ALR binding assay, the cells were washed and placed in Hank's balanced salt solution (20 mM HEPES, pH 7.0, 1.3

mM CaCl₂, 0.5 mM MgCl₂) containing 0.1% BSA (HBSS/BSA) for 30 min. The medium was then replaced with HBSS/BSA containing 12.5-3200 pM [¹²⁵I]ALR (Specific Activity 1400-1500 µCi/mmol) ± 5 µM unlabeled rrALR (0.3 ml final volume). After 3h of incubation at 25°C, the cells were washed with ice-cold PBS (4X), digested with 0.75 N NaOH, and cell-associated radioactivity was determined. Specific binding was calculated as the difference between cell-associated radioactivity in the presence and absence of unlabeled rrALR.

To determine whether the ALR receptor belongs to the G-protein superfamily of receptors, Kupffer cell membranes were prepared by homogenization with a Polytron homogenizer (Ten 5-sec bursts at 20,000 rpm) in ice-cold 20 mM Tris, pH 7.4, containing 0.5 mM phenylmethylsulfonylfluoride (PMSF) and protease inhibitor cocktail (Sigma #P-8340). The homogenate was centrifuged at 6,000g for 5 min followed by centrifugation of the supernatant at 43,000g for 30 min. The pellet was suspended in 20 mM Tris, pH 7.4, containing protease inhibitors and 5 mM MgCl₂ (Buffer A) at a protein concentration of 1 µg/µl, and frozen at -80°C until use. The binding assay was performed in 100 µl of final volume containing buffer A, 10 µg membrane protein, 3 µM GDP, 0.05 pM [³⁵S]GTPγS, and rrALR. Nonspecific binding was determined in the presence of excess (10 µM) GTPγS. The reaction was initiated by adding membranes, continued at 30°C for specified time, and terminated by adding 3 ml ice-cold buffer A followed by rapid filtration on GF/B glass microfiber membranes (Whatman) presoaked in buffer A. The membrane was washed (3X) and associated radioactivity was determined.

Nitric oxide, protein and cytokine synthesis: Kupffer cells were washed and placed in William's medium E containing 0.1% BSA and indicated concentrations of rrALR. After 24h of incubation at 37°C, the medium was aspirated for determination of NO₂⁻, an end product of NO metabolism by Griess method (19). The medium was also analyzed by ELISA with kits specific

for TNF- α (Pierce Biotechnology, Rockford, IL), TGF- α (Peninsula Laboratories, Belmont, CA) and TGF- β (R&D Systems, Minneapolis, MN).

To determine protein synthesis, the cells were washed and placed in leucine-free MEM containing 1 μ Ci/ml [3 H]leucine. After 4h, the medium was aspirated and cells washed twice with ice-cold HBSS, treated with ice-cold 10% trichloroacetic acid (TCA) for 10 min, and washed once with TCA followed by 95% ethanol. Cells were digested with 5% SDS to measure radioactivity. Total DNA was determined in additional wells using Hoechst reagent (20).

mRNA expression. The mRNA expression of TNF- α and iNOS were determined by semiquantitative reverse transcriptase polymerase chain reaction as described previously (19). Briefly, RNA prepared with a ToTALLY RNA isolation kit (Ambion Inc, Austin, TX), was used for the preparation of complementary DNA (cDNA) using Superscript II cDNA synthesis kit (Life Technologies). For PCR, cDNA equivalent of 50-150 ng of total RNA was used; the expression of β -actin mRNA were determined to normalize the data. The following sequences of primers were used in the PCR reaction: TNF- α - 5'CACGCTCTTCTGTCTACTGA3' (F) and 5'GGACTCCGTGATGTCTAAGT3' (R); iNOS- 5'AGAATGTTCCAGAATCCCTCCCTGGACA3' (F) and 5'GAGTGAGCTGGTAGGTTCTGTTG3' (R); and β -actin- 5'TTCTACAATGAGCTGCGTGTG3' (F) and 5'TTCATGGATGCCACAGGATTC3' (R). PCR amplification was performed by initial denaturation at 94°C for 5 min, followed by 30 cycles (β -actin) or 35 cycles (TNF- α and iNOS) of denaturation at 94°C for 1 min; annealing for 1 min at 55°C (TNF- α and β -actin) or 60°C (iNOS); and extension at 72°C for 1 min for TNF- α and β -actin, and 2 min for iNOS; and finally 10 min of final extension at 72°C. The PCR products were resolved in a 1.5% agarose gel and stained with 1X SYBR Green I (FMC

Biproduct, Rockland, ME). The gels were scanned under blue fluorescence light using a phosphorimager and the band intensity was quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Western blot analysis: The cells were washed twice with PBS, and lysed for 30 min in ice-cold RIPA buffer (Santa Cruz Biotech) containing 0.5 mM PMSF, 25 μ l/ml of Sigma protease inhibitor cocktail and 1mM sodium orthovanadate. The lysate was centrifuged at 15,000g (10 min/4°C). The supernatants containing 10-20 μ g protein were subjected to electrophoresis on sodium dodecylsulfate polyacrylamide gels. The separated proteins were transferred on to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were incubated for 2h at room temperature in 1% BSA in TBS/0.1% Tween-20 to block nonspecific binding, incubated with the primary antibodies (1:1,000 dilution) for 2h at room temperature, washed (4X), incubated in appropriate secondary antibodies (1:50,000 dilution) for 2h at room temperature and washed (4X). Detection was achieved using an ECL chemiluminescence kit (Amersham-Pharmacia). To confirm equal loading, the membranes were stripped to assess the expression of actin.

To determine nuclear translocation of NF κ B, nuclear and cytoplasmic extracts were prepared using extraction reagents from Pierce-Endogen (Rockford, IL). Nuclear (5 μ g) and cytoplasmic (20 μ g) proteins were separated by 10% SDS-PAGE. Western blotting was performed using anti-NF κ B p65 antibody (Santa Cruz; 1:1000) and secondary antibody (1:20,000).

Partial hepatectomy: Male Lewis (LEW, RT.1¹) rats (8-10 weeks old) (Harlan Sprague Dawley, Inc., Indianapolis, IN) were maintained in laminar flow, specific-pathogen-free atmosphere with a standard diet and water supplied *ad libitum*. 40% hepatectomy was performed under isoflurane

anesthesia as described previously (10), and animals were sacrificed at 24h. Animals were administered 50 mg/kg 5-bromo-2'-deoxyuridine (ip) 1h before sacrifice. The number of hepatocytes in S-phase was determined by counting BrDU-labeled nuclei in randomly selected regions around four portal triads in 4 power fields per each portal area (10 portal areas per section) as described previously (21).

RESULTS

ALR receptor in Kupffer cells: Figure 1 shows the results of saturation binding assay (A) and the Scatchard plot derived from these data (B). Bmax was calculated to be 0.26 ± 0.02 fmol/ μ g DNA and a Kd of 1.25 ± 0.18 nM. [35 S]GTP γ S binding assay demonstrated that ALR, time- and concentration-dependently stimulated association of the radiolabeled nucleotide with G-protein in Kupffer cell membranes (Figure 2A, 2B). The association was maximal at 1h and half-maximal at ALR concentration of 5 ± 2 nM.

Effect of ALR on NO, protein and TNF- α synthesis: Since NO synthesis via iNOS is an important step in several biological processes including liver regeneration (22), we determined the effect of ALR on NO production as determined by the release of NO end product NO₂ in the culture medium. ALR stimulated NO synthesis/release in a concentration-dependent manner, with half maximal activity at 4 ± 1 nM (Figure 3A). Consistent with this effect, ALR increased the expression of iNOS mRNA (Figure 3B) and protein (Figure 3C) in Kupffer cells.

ALR caused concentration-dependent stimulation of cellular protein synthesis in Kupffer cells, which was nearly 70% greater than the basal value at 10 ng/ml ALR (Figure 4), and half maximal activity occurred at 2.0 ± 0.3 nM ALR.

TNF- α and TGF- β are major cytokines produced by Kupffer cells under inflammatory conditions and during liver regeneration (23,24). While TNF- α can support hepatic regeneration, and is also apoptotic for hepatocytes under certain conditions, TGF- β is a potent inhibitor of hepatocyte replication as well as a proapoptotic factor. Kupffer cells also synthesize TGF- α , a potent hepatocyte mitogen. Since hepatic TNF- α , TGF- α and TGF- β increase significantly after the increase in ALR protein following portacaval shunt surgery in rats (21), we hypothesized that ALR might affect their synthesis in Kupffer cells. ALR stimulated TNF- α synthesis concentration-dependently with half maximal activity at 1.2 ± 0.4 nM (Figure 5A), and increased its mRNA expression (Figure 5B). However, no change in the TGF- β or TGF- α mRNA or protein was observed in ALR-stimulated Kupffer cells (results not shown).

Since rrALR was expressed and purified from bacterial system (10), and bacterial lipopolysaccharide is known to exert the effects described above in Kupffer cells, in all experiments polymixin B (an inhibitor of LPS action) was included. No difference in the effects of ALR in the absence or presence of polymixin B was observed. Additionally, proteins from empty vector-transfected *E. coli* were extracted in an identical way, and the final eluate was used to stimulate Kupffer cells, which also had no effect.

Effect of G-protein inhibition on ALR-induced effects: Since ALR stimulated specific association of [35 S]GTP γ S with the G-protein, we ascertained if inhibition of G-protein, by the well-established inhibitors pertussis toxin and cholera toxin (27), modulates ALR's effects. Cholera toxin inhibited ALR-stimulated association of [35 S]GTP γ S with the G-protein much strongly as compared to the pertussis toxin (Figure 6A). Cholera toxin but not pertussis toxin abrogated ALR-stimulated NO, protein and TNF- α synthesis (Figure 6B, C and D).

Effect of ALR on NF κ B and MAP kinases: Many extracellular stimuli elicit a variety of cellular responses, including the generation of inflammatory mediators, via nuclear translocation of NF κ B and activation of JNK-, ERK- and p38-MAPK. To examine which of these signaling pathways are involved in ALR's effects, Kupffer cells were pretreated with specific inhibitors prior to stimulation with ALR. NF κ B inhibition by MG132 blocked ALR-induced NO (Figure 7A), protein (Figure 7B) and TNF- α synthesis (Figure 7C). Identical results were also observed with another NF κ B inhibitor pyrrolidine dithiocarbamate (not shown). Inhibitors of JNK- and ERK kinases did not affect ALR's effects, and inhibition of p38 kinase only blocked ALR-induced TNF- α synthesis (Figure 7). Consistent with these observations, ALR stimulated nuclear translocation of NF κ B and activation of p38-MAPK in Kupffer cells (Figure 7D).

Effect of ALR on hepatocytes via Kupffer cells and *in vivo* after partial hepatectomy: In order to elucidate the physiological relevance of ALR's effects on Kupffer cells, we determined hepatocyte DNA synthesis in incubations containing medium conditioned by Kupffer cells in the presence of increasing concentrations of ALR. Additionally, effect of exogenously administered rrALR on hepatic regeneration after 40% hepatectomy in rats was ascertained. As shown in Figure 8A, medium conditioned by Kupffer cells inhibited DNA synthesis in hepatocytes. However, when the medium was conditioned in the presence of ALR, there was concentration-dependent prevention of such inhibition. This effect was not due to TNF- α as this cytokine at 1 ng/ml (the highest concentration observed in the medium of ALR-stimulated Kupffer cells) did not affect hepatocyte DNA synthesis (not shown). ALR administration to rats that have undergone 40% hepatectomy caused increase in BrDU incorporation in hepatocytes (Figure 8B) indicating ALR's potential in promoting hepatic regeneration.

Next, we determined if ALR-conditioned Kupffer cell medium influences DNA synthesis in hepatocytes challenged with TGF- β or TGF- α . Medium conditioned by Kupffer cells in the absence or presence of ALR did not affect TGF- β -induced inhibition of hepatocyte DNA synthesis (Figure 8C). Medium conditioned by Kupffer cells in the absence of ALR completely prevented TGF- α -induced stimulation of hepatocyte DNA synthesis but the effect was partial when the medium was conditioned in ALR's presence (Figure 8C).

DISCUSSION

The present investigation demonstrates presence of high affinity G-protein-coupled ALR receptor and its function in Kupffer cells. Kupffer cells were studied as a potential ALR target because of their ability to influence hepatic regeneration (14-18). Here, we found that ALR stimulates synthesis of NO and TNF- α , but not that of TGF- α or TGF- β , in Kupffer cells. The receptor for ALR was found coupled to the G-protein superfamily, and sensitive to cholera toxin. This specificity was confirmed by inhibition of ALR-induced G-protein activation as well as the synthesis of NO, protein and TNF- α in the presence of cholera toxin. Pertussis toxin, which targets another class of G-proteins caused modest inhibition of GTP/G-protein association without affecting ALR-induced responses. This raises the possibility of a pertussis toxin-sensitive component, which might be coupled to other unidentified ALR-stimulated processes in Kupffer cells.

NO is known to play significant role in the hemodynamic and metabolic regulation in physiology and pathology of the liver. At high concentrations, NO is cytotoxic and inhibits protein synthesis (26), gluconeogenesis (27) and mitochondrial respiration (28) in hepatocytes. Attenuation of the LPS-induced mortality in iNOS-deficient mice (29) supports the notion that

excessive NO production has detrimental consequences (30). However, there are several instances in which lower concentrations of NO were found to be antiapoptotic (31,32) and to play a major role in several critical physiological processes (32-34). Thus the modest stimulation of NO synthesis by ALR in Kupffer cells observed in our experiments suggests ALR's hepatoprotective role.

Interestingly, ALR also stimulated protein synthesis, and mRNA expression and the release of TNF- α , an important signaling polypeptide in the overall hepatic metabolism. TNF- α produced by macrophages induces local effects, including vascular permeability, enhanced microbial killing, and increased migration of neutrophils and macrophages (35,36). TNF- α is not a true mitogen for isolated hepatocytes (23,24), but plays significant role as a priming agent in the early signaling during hepatic regeneration (37). Stimulation of hepatic DNA synthesis upon infusion of TNF- α *in vivo* (38,39), and TNF- α -induced transient activation of NF κ B and STAT3 and a 4-fold increase in the mitogenic response to HGF and TGF- α (37) indicate its importance in liver regeneration. However, it is also known that under conditions of transcriptional inhibition, TNF- α causes death of hepatocytes via apoptosis (31). In pathological conditions involving sepsis and liver failure, TNF- α has been predicted to be an important mediator of LPS-induced toxicity (40,41). In fact, the magnitude of LPS-stimulated TNF- α release was about 3-4 times greater than that by ALR (not shown). These findings suggest that high level of TNF- α produced by LPS-stimulated Kupffer cells reflects pathological situation while significantly less stimulation of its synthesis by ALR may be beneficial in hepatic biology. In this regard, increased hepatic ALR release with concomitant increase in serum ALR were observed after partial hepatectomy in rats (10). This observation, in combination with increased hepatic TNF- α post partial hepatectomy suggests a role of ALR/ TNF- α pathway in liver regeneration.

It has been established that the NF κ B and p38-MAPK activation are common signaling pathways responsible for the final responses to a variety of extracellular stimuli involved in hepatic regeneration, inflammation and failure. LPS-induced TNF- α synthesis in Kupffer cells is mediated by NF κ B and p38-MAPK (42). In Kupffer cells ALR-stimulated TNF- α synthesis is also mediated by p38-MAPK; NF κ B but not p38-MAPK inhibition blocked protein and NO synthesis. This suggests the possibility that ALR, via NF κ B, stimulates synthesis of other proteins that might affect hepatic regeneration. Thus, identification of the additional specific proteins synthesized by ALR-stimulated Kupffer cells will reveal its other roles in overall hepatic metabolism and regeneration.

Earlier research provided evidence that ALR is mitogenic *in vivo* but not *in vitro* for hepatocytes (1-7,10). These observations were confirmed in the present study with rrALR. The physiological implications of these observations are evident from the abrogation of the production of inhibitor(s) of hepatocyte DNA synthesis by ALR in Kupffer cells. This effect cannot be attributed to ALR-induced synthesis of TNF- α , which at the concentration released by ALR-stimulated Kupffer cells, does not affect hepatocyte DNA synthesis, and to TGF- α or TGF- β whose expressions are not affected by ALR. NO is a short-lived molecule and thus it is unlikely to be present in Kupffer cell-conditioned medium added to hepatocyte culture ruling out the possibility that it might be a factor to counter Kupffer cells' inhibitory effect on hepatocytes. However, ALR-conditioned Kupffer cell medium did not reverse TGF- β -induced inhibition of hepatocyte DNA synthesis, and only modestly reversed the complete inhibition of TGF- α -stimulated DNA synthesis caused by Kupffer cell-medium conditioned without ALR. Thus, the significantly increased hepatocyte replication by ALR *in vivo* following partial hepatectomy suggests its complex interaction with other nonparenchymal cells in addition to Kupffer cells. In

this regard, we observed the presence of ALR receptor, similar to that in Kupffer cells, in hepatic stellate cells (Unpublished observation).

In summary, the results of this study provide the first evidence that ALR, via G-protein-coupled receptor, may play an important role in hepatic metabolism by causing NO, protein and TNF- α synthesis in Kupffer cells. Given the important role of Kupffer cells in the regulation of the overall hepatic metabolism via secretory molecules, ALR's effects observed here could be an important part of liver pathophysiology.

FIGURE LEGENDS

Figure 1. Binding of ALR to Kupffer cells. The assay was performed as described in the Methods section. The values are means of duplicate determinations from a representative experiment of 3 repeats. A. Saturation binding. B. Scatchard plot of the data shown in A.

Figure 2. Binding of GTP to Kupffer cell membranes. Membranes were prepared and incubated with the GTP-binding assay mixture containing (A) 1 μ M ALR for indicated time points or (B) indicated concentrations of ALR for 1h. The reaction was stimulated by addition of 10 μ g membrane proteins. Details are described in the Methods section.

Figure 3. Effect of ALR on NO synthesis and iNOS expression. Kupffer cells were incubated with indicated concentrations of ALR (A) or 100 ng/ml ALR (B,C) for 24h in serum-free condition. (A) The values (NO_2 concentration in the culture medium) are means of 3 independent determinations \pm S.D. $*p < 0.005$ vs "0". (B and C) The medium was aspirated and RNA extracts and protein lysates were prepared from the cells. RT-PCR and Western analysis were performed to determine iNOS expression. Representative gels show the mRNA expression of iNOS and β -actin (B), and protein expression of iNOS and actin (C). Bar graphs show relative expression of iNOS mRNA or protein versus that of actin. $*p < 0.05$ vs control

Figure 4. Effect of ALR on protein synthesis. Kupffer cells were incubated with indicated concentrations of ALR for 24h in serum-free condition, and protein synthesis was measured as described in Methods. The values are means of 3 independent determinations \pm S.D. $*p < 0.05$.

Figure 5. Release of TNF- α and its mRNA expression in ALR-stimulated Kupffer cells. (A) Kupffer cells were stimulated with indicated concentrations of ALR for 24h, and TNF- α concentration in the culture medium was determined. $*p < 0.05$ vs control; $**p < 0.01$ vs control. (B) After 24h stimulation with 100 ng/ml ALR, cellular RNA was extracted and TNF- α mRNA

expression was determined by RT-PCR. The bar graph shows relative expression of TNF- α vs that of β -actin. * p <0.05 vs control.

Figure 6. Effect of G-protein inhibition on ALR-induced effects. (A) Kupffer cell membranes were incubated with the G-protein association mixture in the presence of 1 μ M ALR without or with 50 ng/ml pertussis toxin (PX) or 2.5 μ g/ml cholera toxin (CX) for 2h, the reaction was terminated and membrane associated radioactivity was determined. Values (specific [35 S]GTP γ S association) are differences between the binding in the absence and presence of excess unlabeled GTP γ S. Other details are provided in the Methods section. (B-D) Kupffer cells were pre-incubated for 15 min in the absence or presence of 50 ng/ml pertussis toxin or 2.5 μ g/ml cholera toxin, then stimulated with 100 ng/ml ALR. After 24h, NO, protein and TNF- α levels were measured. * p <0.05 vs control; ** p <0.001 vs control; *** p <0.01 vs control.

Figure 7. Involvement of NF κ B and MAPK in ALR-induced NO, protein and TNF- α synthesis. Kupffer cells were pre-incubated with 10 μ M SB283520 (p38 kinase inhibitor), PD98059 (ERK1/2 kinase inhibitor), SP600125 (JNK inhibitor), or MG132 (NF κ B inhibitor) for 30 min before addition of 100 ng/ml ALR. At 24h, (A) NO, (B) protein and (C) TNF- α synthesis were determined. * p <0.05 vs control; ** p <0.01 vs control; # p <0.005 vs ALR; ## p <0.01 vs ALR. (D) Kupffer cells were incubated for 3h with 100 ng/ml ALR, after which nuclear and cytosolic extracts or whole cell lysates were prepared for Western analysis. Cytosolic (Cyt) and Nuclear (Nuc) expression of p65-NF κ B, and total and phosphorylated p38 in the whole cell lysate are shown. Actin expression shows equal loading.

Figure 8: (A) Effect of ALR-conditioned Kupffer cell medium on hepatocyte DNA synthesis. Kupffer cells were incubated with indicated concentrations of ALR for 24h. The medium (KCtoHC) was then transferred to the overnight culture of hepatocytes, and at 24h DNA

synthesis was measured via [³H]thymidine incorporation assay. Results are means of triplicate determinations from a representative experiment performed 3 times with similar results. **p*<0.01 vs HC; ***p*<0.05 vs “0” KCtoHC; ****p*<0.01 vs “0” KCtoHC. **(B) Effect of ALR on hepatic regeneration after 40% hepatectomy.** Rats (200-240g) underwent 40% hepatectomy. 15 min prior to and every 6h after PH, ALR (50 ng/kg) was administered. At 23h, 50 ng/kg BrDU was injected and at 24h, rats were sacrificed, their livers preserved in formalin, and immunostaining for BrDU was performed. Lower panel shows BrDU labeled cells/power field ± S.D. **p*<0.05 vs saline control. **(C) Effect of ALR-conditioned Kupffer cell medium on DNA synthesis in TGF-β- or TGF-α-challenged hepatocytes.** Hepatocytes were placed in unconditioned or Kupffer cell-conditioned medium in the absence or presence of 100 ng/ml ALR. TGF-β or TGF-α (both 2 ng/ml) was added and at 24h, DNA synthesis was determined via [³H]thymidine incorporation assay. Values are means ± S.D. from triplicate determinations. **p*<0.05 vs HC or HC/ALR; ***p*<0.01 vs control; #*p*<0.05 vs KCtoHC control; ****p*<0.001 vs control; ##*p*<0.05 vs KCtoHC+TGF-α.

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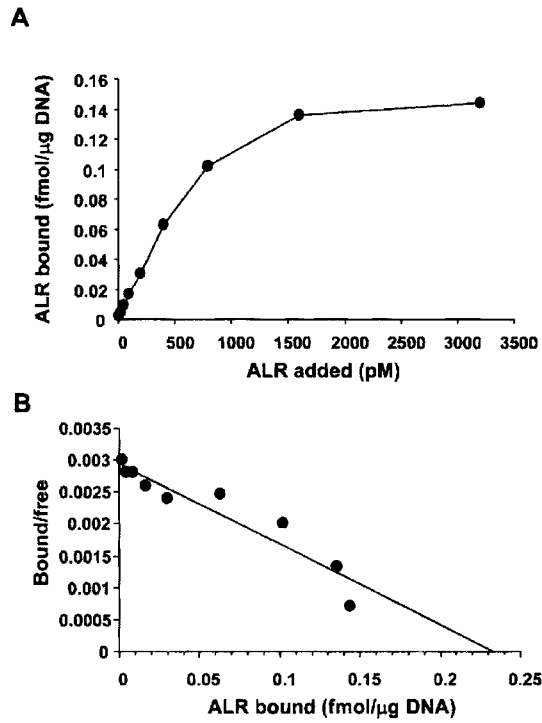
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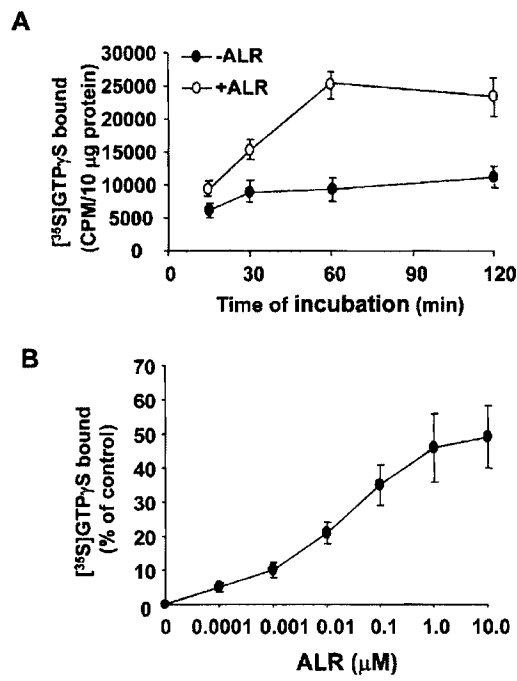
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Figure 1



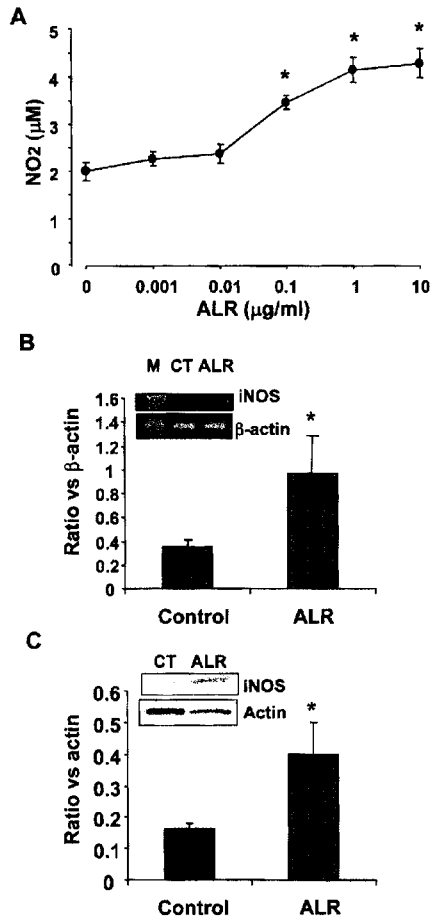
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Figure 2



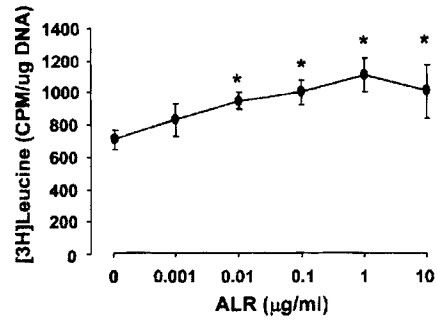
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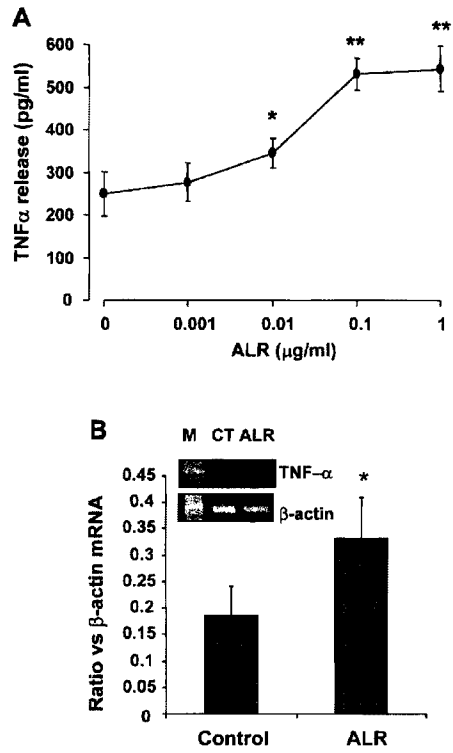
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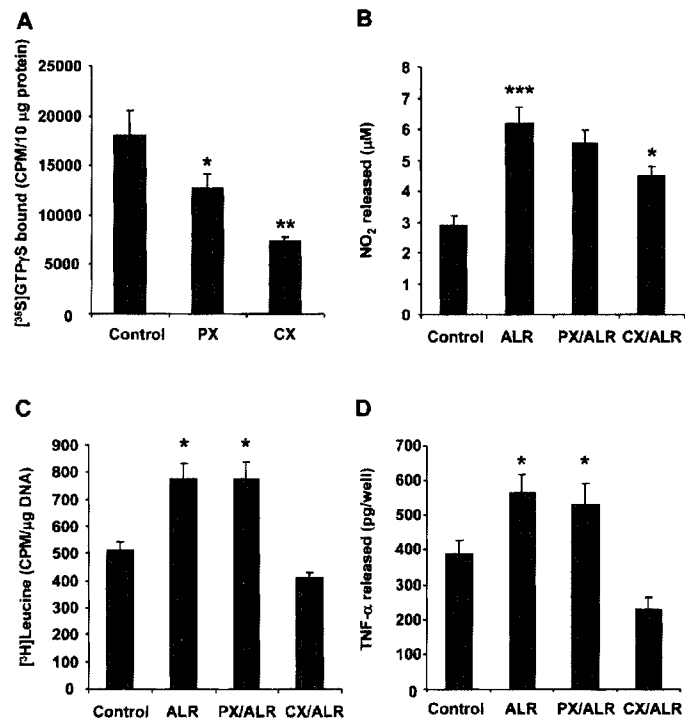
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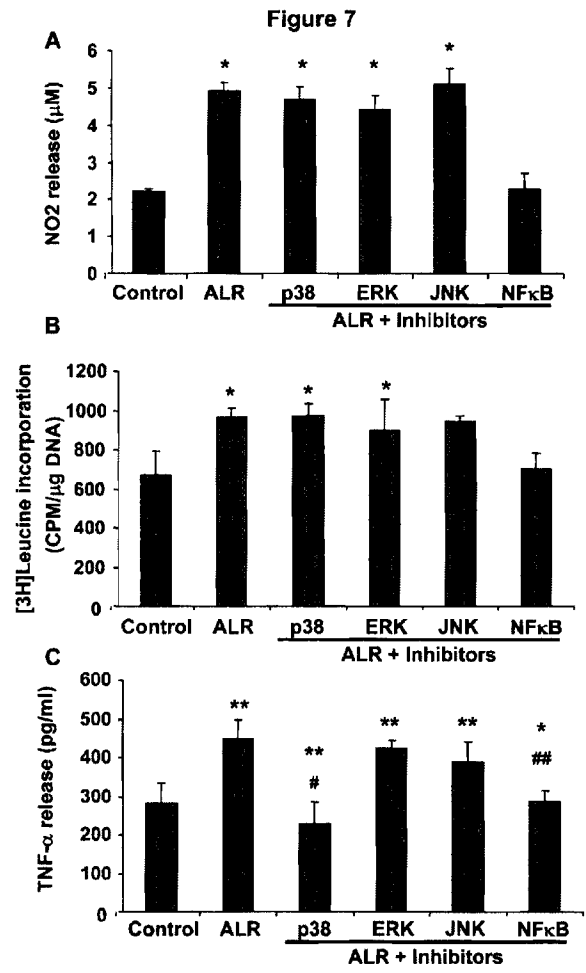


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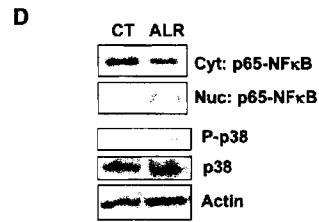


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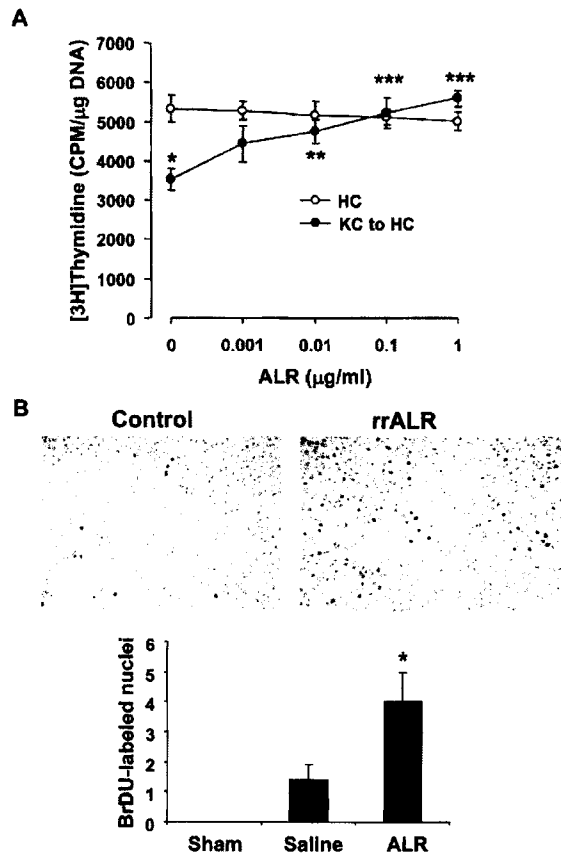
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Figure 7



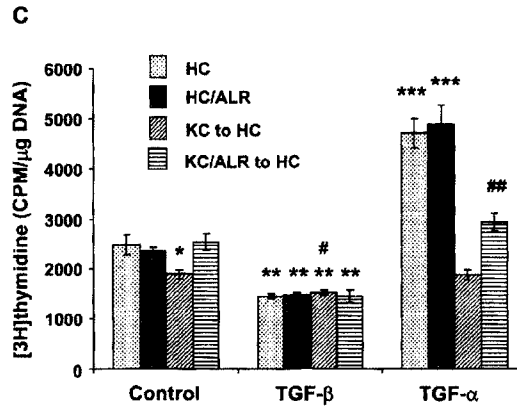
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Figure 8



186x241mm (300 x 300 DPI)

Figure 8



186x241mm (300 x 300 DPI)