Published in final edited form as:
J Cell Physiol. 2010 February ; 222(2): 365–373. doi:10.1002/jcp.21957.

Cholera Toxin-Sensitive GTP-Binding Protein-Coupled Activation of Augmenter of Liver Regeneration (ALR) Receptor and Its Function in Rat Kupffer Cells

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Abstract

Mitogenic effect of augmenter of liver regeneration (ALR), a protein produced and released by hepatocytes, on hepatocytes in vivo but not in vitro suggests 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. Kupffer cells were isolated from rat liver by enzymatic digestion and centrifugal elutriation. Radioligand ([^{125}I] ALR) receptor binding, ALR-induced GTP/G-protein association, and nitric oxide (NO), tumor necrosis factor (TNF)-α, and interleukin-6 (IL-6) synthesis were determined. High-affinity receptor for ALR, belonging to the G-protein family, with K_d of 1.25 ± 0.18 nM and B_{max} of 0.26 ± 0.02 fmol/μg DNA was identified. ALR stimulated NO, TNF-α, and IL-6 synthesis via cholera toxin-sensitive G-protein, as well as p38-MAPK activity and nuclear translocation of NFκB. While inhibitor of NFκB (MG132) inhibited ALR-induced NO synthesis, MG132 and p38-MAPK inhibitor (SB203580) abrogated ALR-induced TNF-α and IL-6 synthesis. ALR also prevented the release of mediator(s) from Kupffer cells that cause inhibition of DNA synthesis in hepatocytes. Administration of ALR to 40% partially hepatectomized rats increased expression of TNF-α, IL-6, and inducible nitric oxide synthase (iNOS) and caused augmentation of hepatic regeneration. These results demonstrate specific G-protein coupled binding of ALR and its function in Kupffer cells and suggest that mediators produced by ALR-stimulated Kupffer cells may elicit physiologically important effects on hepatocytes.

Hepatic regeneration that follows chemical, microbial, physical, and viral injuries is orchestrated by multiple endogenous and humoral mediators. The search for the molecules involved in hepatocyte replication led to the identification of a novel protein “augmenter of liver regeneration (ALR)” in the soluble fractions of hyperplastic livers (LaBrecque and Pesch, 1975; Starzl et al., 1979; Francavilla et al., 1994). ALR protein was purified from the extracts of weanling rat liver (Francavilla et al., 1987, 1991), and its gene cloned in rat, mouse, and human (Hagiya et al., 1994; Giorda et al., 1996). The ALR sequence is highly homologous (>90%) among mammalian species and exhibits high homology (about 40%) with ERVI (essential for respiration and vegetative growth), which is required for the growth and survival of *Saccharomyces cerevisiae* (Lisowsky, 1992; Hagiya et al., 1994;

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Lisowsky et al., 1995; Giorda et al., 1996). The carboxy-terminal (about 15 kDa) fragment of ERVI and ALR contains flavin-linked sulfhydryl oxidase activity that catalyzes oxidation of thiol groups in the protein substrates and plays an essential role in the maintenance of intact mitochondrial membrane and a normal mitochondrial morphology (Becher et al., 1999). The loss of viability of the yeast caused by excision of carboxy-terminal peptide sequence of ERVI can be prevented by insertion of carboxy-terminal sequence of human ALR (Hofhaus et al., 1999) suggesting preservation of the function of the ALR/ERVI gene among various species.

Presence of equivalent amounts of ALR mRNA and protein in hepatocytes of regenerating and resting rat livers (Gandhi et al., 1999) suggests that ALR in quiescent hepatocytes is not mitogenic. Indeed, cloned ALR and the native ALR isolated from hypertrophic animal livers, but not the unmodified adult liver, stimulate hepatocyte replication and prevent portacaval shunt-induced hepatic atrophy in dogs (Francavilla et al., 1994; Hagiya et al., 1994; Giorda et al., 1996). However, rat hepatocytes lack ALR receptor (Gandhi et al., 1999; Thirunavukkarasu et al., 2008), and both native ALR (from hyperplastic liver) and recombinant rat ALR (rrALR) stimulate mitosis of rat hepatocytes in vivo but not in vitro (Francavilla et al., 1994; Gandhi et al., 1999; Thirunavukkarasu et al., 2008). These observations suggested that the growth-promoting effect of ALR in vivo is indirect, elicited via mediators released by nonparenchymal cells (NPCs). It has been shown that Kupffer cells produce mediators that play critical roles in hepatic regeneration (Rai et al., 1996, 1997; Suzuki et al., 1996; Rikiyama et al., 1999; Meijer et al., 2000). We tested the hypothesis that Kupffer cells possess specific receptors for ALR, activation of which stimulates synthesis of the mediators of hepatic regeneration. The results show cholera toxin-sensitive G-protein-coupled high affinity receptor for ALR in rat Kupffer cells, activation of which stimulates nitric oxide (NO), tumor necrosis factor (TNF)-α, and interleukin-6 (IL-6) synthesis, the molecules that support hepatic regeneration (Fausto et al., 1995; Michalopoulos and DeFrances, 1997; Hortelano et al., 2007).

Materials and Methods

Partial hepatectomy

All protocols were approved by the Institutional Animal Care and Use Committee, University of Pittsburgh in accordance with NIH guidelines. Forty percent hepatectomy was performed in male Lewis (LEW, RT. I^{I}) rats (8–10 weeks old) as described previously (Gandhi et al., 1999). Animals were injected 50 ng/kg rrALR (prepared as described in Giorda et al., 1996) in saline or saline (i.v.) at 15 min before, and 6, 12, and 18 h following the surgery. They were then administered 50 mg/kg 5-bromo-2′-deoxyuridine (i.p.) at 23 h and sacrificed at 24 h, when the peak of DNA synthesis is reached (Michalopoulos, 2007). The liver tissue was fixed in 10% buffered formalin or snap-frozen in liquid nitrogen. BrDU-labeled hepatocytes in formalin-preserved portions were counted in randomly selected regions around four portal triads in four power fields per each portal area (10 portal areas per section) as described (Gandhi et al., 2002).

iNOS immunostaining

Sections of the formalin-fixed liver tissue were deparaffinized, washed with phosphate-buffered saline (PBS), and treated with 3% hydrogen peroxide followed by avidin and biotin. The sections were incubated in serum and then with rabbit anti-inducible nitric oxide synthase (iNOS) antibody (BD Transduction Laboratories, San Jose, CA) (1:20) for 16 h at 4°C. The sections were washed and treated with biotinylated secondary goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) (1:200), stained with chromogen and

counterstained with hematoxylin. Parallel sections were immunostained using anti-ED2 antibody to identify Kupffer cells.

Preparation of Kupffer cells and hepatocytes

Kupffer cells were prepared from male Sprague–Dawley rats (200–250g) by collagenase and protease digestion of the liver and purified by Metrizamide gradient centrifugation, followed by centrifugal elutriation as described previously (Yang et al., 2003). Purity of the cells as determined by immunostaining for ED2 (Kupffer cells), desmin (stellate cells), and SE-I (endothelial cells) was greater than 95% (Yang et al., 2003). The cells were plated at a density of 0.5×10^6 cells/cm^2 in Williams' medium E containing 10% fetal calf serum, penicillin/streptomycin, and were used on the third day.

Hepatocytes were prepared from the livers of male Sprague–Dawley rats (200–250 g) by collagenase digestion, low-speed centrifugation, and purification on Percoll gradient as described previously (Gandhi et al., 1999; Thirunavukkarasu et al., 2008). Hepatocytes were plated at a density of 0.063×10^6/cm^2 in William's medium E supplemented with 2 mM L-glutamine, penicillin/streptomycin, 10% fetal bovine serum (FBS), and 10^{-6} M insulin, and were used the following day.

Determination of ALR binding

rrALR was radioiodinated by lactoperoxidase procedure (Bayse et al., 1972). 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 $CaCI_2$, 0.5 mM $MgCI_2$) containing 0.1% bovine serum albumin (HBSS/BSA) for 30 min. The medium was replaced with HBSS/BSA containing 10 pM [^{125}I]ALR (specific activity 1,400–1,500 μCi/mmol) and increasing concentrations of unlabeled rrALR (competition binding analysis) or with HBSS/BSA containing 12.5–3200 pM [^{125}I]ALR ± 5 μM unlabeled rrALR (saturation binding analysis) (0.3 ml final volume). After 3 h incubation at 25°C, the cells were washed with ice-cold PBS (4×), and cell-associated radioactivity was determined. Specific binding in saturation binding analysis was calculated as the difference between cell-associated radioactivity in the absence and presence of unlabeled rrALR.

Determination of G-protein association of ALR receptor

Kupffer cell membranes were prepared by homogenization with a Polytron homogenizer (10 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, St. Louis, MO). 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 $MgCI_2$ (buffer A) at a protein concentration of 1 μg/μl. The binding assay was performed at 30°C in 100μl of final volume containing buffer A, 10 μg membrane protein, 3 μM guanosine diphosphate (GDP), 0.05 pM [^{35}S]GTPγS, and rrALR. Nonspecific binding was determined in the presence of excess (10 μM) GTPγS. The reaction was initiated by adding membranes and terminated by adding 3 ml ice-cold buffer A followed by rapid filtration through GF/B glass microfiber membranes (Whatman, Piscataway, NJ) presoaked in buffer A. The membranes were washed (3×) and associated radioactivity was determined.

Nitric oxide and cytokine synthesis

Kupffer cells were washed and placed in William's medium E containing 0.1% BSA and indicated concentrations of rrALR. After 24 h of incubation at 37°C, the medium was aspirated for the determination of TNF-α and IL-6 (Pierce Biotechnology, Rockford, IL),

TGF-α (Peninsula Laboratories, Belmont, CA), and TGF-β (R&D Systems, Minneapolis, MN) by ELISA, and of NO by Griess method (Uemura and Gandhi, 2001).

mRNA determination

Total RNA was extracted from the liver tissues using the TRIzol reagent (Life Technologies, Inc., Carlsbad, CA), and cDNA was prepared as described previously (Uemura and Gandhi, 2001; Neto et al., 2006). TNF-α, IL-6, and iNOS mRNA expression was quantified by SYBR Green two-step real-time reverse transcriptase polymerase chain reaction (RT-PCR) as previously described (Neto et al., 2006). The expression of each gene was normalized to glyceraldehyde 3-phosphate dehydrogenase mRNA content and calculated relative to sham-operated control. The mRNA expressions of TNF-α, IL-6, and iNOS were also determined by semi-quantitative RT-PCR as described previously (Uemura and Gandhi, 2001). The PCR products resolved in a 1.5% agarose gel were stained with 1 × SYBR Green 1, and the band intensity was quantified in a phosphorimager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The expression of the individual gene was compared with that of β-actin for quantification.

Western blot analysis

The cells were washed with PBS and lysed in ice-cold RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA) containing 0.5 mM PMSF, 25 μl/ml of Sigma protease inhibitor cocktail, and I mM sodium orthovanadate. The cell lysates were centrifuged at 15,000g (10 min/4°C), and supernatants (10–20 μg protein) were subjected to SDS–PAGE. Separated proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were incubated for 2 h in 1% BSA in TBS/0.1% Tween-20 to block nonspecific binding, then incubated with the primary antibodies (1:1,000 dilution) for 2 h. After washing (4×), the membranes were incubated in appropriate secondary antibodies (1:50,000 dilution) for 2 h and washed (4×). ECL chemiluminescence kit (Amersham-Pharmacia, Piscataway, NJ) was used to detect antigen–antibody complex.

For NFκB determination, nuclear and cytoplasmic extracts were prepared using extraction reagents from Pierce-Endogen (Rockford, IL). Nuclear (5 μg) and cytoplasmic (20 μg) proteins were separated by SDS–PAGE. Western blotting was performed using anti-NFκB-p65 antibody (Santa Cruz Biotechnology; 1:1,000) and secondary antibody (1:20,000).

Statistical analysis

Values shown for experiments with cultured cells are means of triplicate determinations ± SD of at least three repeats, unless indicated otherwise. Values for experiments involving in vivo treatments (partial hepatectomy) are means ± SD of measurements from four to six independent tissue samples for each condition. Statistical significance between the groups was determined by one-way ANOVA followed by a test for linear trend. A *P*-value of <0.05 was considered statistically significant.

Results

Effect of ALR on hepatocytes in vivo after partial hepatectomy

ALR administration to rats that had undergone 40% hepatectomy increased BrDU incorporation in hepatocytes (Fig. 1A) indicating ALR's potential in promoting hepatic regeneration. No effect of exogenous ALR on hepatic regeneration was observed in 70% partially hepatectomized rats (not shown) presumably because the regenerative response was already maximal due to endogenously released factors. We have previously shown that as compared to 40% hepatectomy, the release of hepatic ALR was much greater after 70% hepatectomy (Gandhi et al., 1999), and this concentration of endogenously released ALR

may be sufficient to elicit maximal augmentation effect on hepatic regeneration. The mRNA expression of TNF-α, IL-6, and iNOS was significantly greater in ALR-treatment group as compared to the vehicle-treated group at 2 h following partial hepatectomy (Fig. 1B). Although hepatic mRNA expression of IL-6 and iNOS remained elevated in ALR-treated rats relative to vehicle-treated rats at 24 h that of TNF-α was similar (Fig. 1B). Immunohistochemical analysis of the liver sections demonstrated uniformly similar iNOS expression in NPCs throughout the liver tissue following partial hepatectomy. However, the number of NPCs expressing iNOS was greater in the ALR-treated rats than in vehicle-treated rats (29 ± 9 NPCs/100 hepatocytes vs. 17 ± *7* NPCs/100 hepatocytes; $P < 0.05$) (Fig. 1C, upper parts). The number of ED2 (a marker for Kupffer cells)-positive cells was similar in vehicle- and ALR-treated livers (Fig. 1C, bottom parts).

ALR binding to Kupffer cells

Although ALR causes hepatocyte proliferation in vivo after partial hepatectomy, it does not stimulate DNA synthesis in isolated rat hepatocytes (Gandhi et al., 1999; Thirunavukkarasu et al., 2008). Therefore, we examined whether ALR might promote hepatocyte regeneration via Kupffer cells. To that end, we first determined whether Kupffer cells express specific ALR-binding sites. Competition binding assay demonstrated concentration-dependent inhibition of the binding of 10 pM [^{125}I]ALR to Kupffer cells by unlabeled ALR with half maximal effect between 100 and 200 pM (Fig. 2). From these data, and the results of saturation binding (not shown) and Scatchard analysis (Fig. 2, inset), ALR was found to have high affinity receptors on Kupffer cells with a K_d of 1.25 ± 0.18 nM and a B_{max} of 0.26 ± 0.02 fmol/μg DNA. Similar ALR binding characteristic has been reported earlier (Wang et al., 2006). ALR, time- and concentration-dependently stimulated association of [^{35}S]GTPγS with G-protein in Kupffer cell membranes (Fig. 3A,B). The association was maximal at 1 h and half-maximal at ALR concentration of 5 ± 2 nM.

Effect of ALR on NO and cytokine synthesis

Since NO plays an important role in liver regeneration (Hortelano et al., 2007), and ALR administration significantly increased iNOS expression in the liver and NPCs after partial hepatectomy, we determined the effect of ALR on NO production in Kupffer cells. ALR stimulated NO synthesis/release concentration-dependently, with half maximal activity at 4 ± 1 nM (Fig. 4A) and increased iNOS protein (Fig. 4B) and mRNA (Fig. 4C) expression.

TNF-α, IL-6, and TGF-β are major cytokines produced by Kupffer cells during liver regeneration (Michalopoulos and DeFrances, 1997; Michalopoulos, 2007). TNF-α and IL-6 support hepatic regeneration, while TGF-β inhibits hepatocyte replication. Kupffer cells also synthesize potent hepatocyte mitogens hepatocyte growth factor (HGF) and TGF-α. Since ALR does not stimulate DNA synthesis in rat hepatocytes (Gandhi et al., 1999; Thirunavukkarasu et al., 2008), we examined whether ALR might influence hepatic regeneration by affecting expression of these mediators in Kupffer cells. ALR stimulated synthesis of TNF-α and IL-6 concentration-dependently with half maximal activity at 1–1.5 nM and increased their mRNA expression (Fig. 5). ALR did not alter the expression of HGF, TGF-α, or TGF-β in Kupffer cells (not shown).

Polymyxin B (an inhibitor of bacterial lipopolysaccharide action) did not affect any of the ALR's effects (Fig. 6) suggesting that the rrALR preparations were not contaminated with lipopolysaccharide.

Effect of G-protein inhibition

Since ALR stimulated G-protein binding activity, whether inhibition of G-protein by pertussis toxin and cholera toxin (Gilman, 1984) modulates ALR's effects was ascertained.

The concentrations of the toxins used are shown to be effective in causing complete inhibition of the respective G-protein-coupled responses in several cell types including Kupffer cells (Gandhi et al., 1990, 1992). Cholera toxin inhibited ALR-stimulated association of [^{35}S]GTPγS and G-protein more strongly than pertussis toxin (Fig. 7A), and also abrogated ALR-stimulated NO, TNF-α, and IL-6 synthesis (Fig. 7B–D). Neither toxin affected the basal release of these mediators.

Effect of ALR on NFκB and MAP kinases

Many extracellular stimuli elicit a variety of cellular responses via activation of JNK-, ERK-, and p38-MAPK, and nuclear translocation of NFκB. To examine if these pathways are involved in ALR's effects, Kupffer cells were pretreated with specific inhibitors prior to stimulation with rrALR. While inhibition of JNK- and ERK kinases did not affect ALR's effects, that of p38 kinase blocked ALR-induced TNF-α and IL-6 synthesis but not NO synthesis (Fig. 8A,B). NFκB inhibitors MG132 and pyrrolidine dithiocarbamate blocked ALR-induced NO, TNF-α, and IL-6 synthesis (Fig. 8A,B). Consistent with these observations, ALR caused activation of p38-MAPK and nuclear translocation of NFκB in Kupffer cells (Fig. 8C). These results suggest the possibility that ALR stimulates distinct signaling pathways that may act in concert or independently to instigate cellular responses.

Effect of ALR on hepatocytes via Kupffer cells

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 absence and presence of ALR. Figure 9A shows that medium conditioned by Kupffer cells inhibited DNA synthesis in hepatocytes. However, when the medium was conditioned in the presence of increasing concentrations of ALR, there was progressive amelioration of the inhibition of hepatocyte DNA synthesis.

The robust TGF-α-stimulated DNA synthesis in hepatocytes was strongly inhibited by Kupffer cell-conditioned medium (Fig. 9B) indicating constitutive release of potent inhibitor(s). However, when the medium was conditioned in the presence of ALR, inhibition of TGF-α-induced stimulation of hepatocyte DNA synthesis was partially recovered (Fig. 9B). ALR by itself did not affect DNA synthesis in hepatocytes.

Discussion

This investigation demonstrates presence of cholera toxin-sensitive activation of G-protein-coupled high affinity ALR binding and ALR-stimulated synthesis of NO, TNF-α, and IL-6, but not of HGF, TGF-α, or TGF-β, in Kupffer cells. Pertussis toxin, which targets another class of G-proteins, caused modest inhibition of guanosine triphosphate (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 as yet unidentified ALR-stimulated processes.

NO plays a significant role in the hemodynamic and metabolic regulation in hepatic physiology and pathology. At high concentrations, NO is cytotoxic for hepatocytes by inhibiting protein synthesis (Curran et al., 1991), gluconeogenesis (Ou et al., 1996), and mitochondrial respiration (Szabo, 1998). Attenuation of LPS-induced mortality in iNOS-deficient mice (MacMicking et al., 1995) indicates that excessive NO production has detrimental consequences (Li et al., 1999; Li and Billiar, 1999). In contrast, at lower concentrations, NO plays important roles in several critical physiological processes (Li et al., 1999; Li and Billiar, 1999; Brüne, 2005; Ferran, 2006), is antiapoptotic (Kim et al., 1997; Li et al., 1999; Li and Billiar, 1999) and promotes liver regeneration (Hortelano et al.,

2007). Thus, the modest stimulation of NO synthesis by ALR in Kupffer cells and increased expression of iNOS protein in greater number of NPCs in ALR-treated partially hepatectomized liver suggest ALR's role in hepatoprotection and regeneration. It is important to note that all of the NPCs expressing iNOS (about 30/100 hepatocytes) cannot be Kupffer cells alone as there are about 15–20 Kupffer cells/100 hepatocytes. Thus, the effect of ALR on other NPC types should be considered in hepatic regeneration. In this regard, staining with ED2, a marker for Kupffer cells, did not show any increase in their number in ALR-treated rats than in vehicle-treated rats after partial hepatectomy.

ALR also stimulated synthesis of TNF-α and IL-6, two important signaling polypeptides in the overall hepatic metabolism and regeneration, in Kupffer cells. TNF-α causes transient activation of NFκB and STAT3, and several-fold increase in the mitogenic response of hepatocytes to HGF and TGF-α (Webber et al., 1998). TNF-α also stimulates hepatic DNA synthesis in vivo (Feingold et al., 1988; Beyer et al., 1990) and plays an important role as a priming agent by inducing early signaling pathways during hepatic regeneration (Fausto et al., 1995, 2006; Michalopoulos and DeFrances, 1997; Webber et al., 1998). However, whether TNF-α plays a direct role in hepatocyte replication has been questioned by the finding that administration of IL-6 prevents impaired hepatic regeneration in TNF receptor type I-deficient mice (Yamada et al., 1997). The importance of IL-6 in hepatic regeneration is further exemplified by suppressed hepatocyte DNA synthesis in partially hepatectomized IL-6 gene knockout mice (Wallenius et al., 2000). Consistent with a previous report (Scotte et al., 1997), TNF-α and IL-6 mRNA expression increased slightly at 2 h following partial hepatectomy, but their expressions were significantly greater in the ALR-treated group than in the vehicle-treated group. However, the expression of TNF-α was similar in vehicle- and ALR-treated groups at 24 h while that of IL-6 mRNA was greater in the latter group. These observations and ALR-induced TNF-α synthesis in Kupffer cells suggest that increased IL-6 expression in ALR-treated rats might be an effect of TNF-α. However, direct stimulatory effect of ALR on both TNF-α and IL-6 synthesis in Kupffer cells, as observed in the in vitro experiments (Fig. 5), cannot be ruled out as ALR caused an increase in mRNA expression both of TNF-α and IL-6 at the early time point after partial hepatectomy.

Hepatic regeneration is a complex process involving production and effects of several growth stimulators and inhibitors. Most of the evidence for the role of various mitogens/comitogens in hepatic regeneration is derived from 70% partial hepatectomy in which the regenerative response is very strong and from gene knockout or transgenic mice overexpressing these factors. Although HGF, TNF-α, epidermal growth factor, and TNF-α treatments were shown to increase hepatocyte DNA synthesis after 70% hepatectomy, continuous infusion of high concentrations of these substances was required to elicit the augmentation response (Webber et al., 1994, 1998).

Our data indicate that the effect of ALR on rat hepatocytes in vivo is indirect. Hepatic release of ALR shortly after partial hepatectomy with concomitant increase in serum ALR (Gandhi et al., 1999) suggests that the released ALR stimulates synthesis of promitogenic factors from NPCs. However, ALR-conditioned Kupffer cell medium did not stimulate DNA synthesis in hepatocytes per se. These results are in contrast to those of Wang et al. (2006) who reported stimulation of DNA synthesis in hepatocytes by ALR-conditioned Kupffer cell medium. On the other hand, results of our experiments (Fig. 9) indicate that ALR prevents release of inhibitor(s) of hepatocyte DNA synthesis by Kupffer cells. This effect cannot be attributed to TGF-β whose expression is not altered by ALR. Although our work has not identified the inhibitor(s) produced by Kupffer cells, the findings suggest that ALR promotes liver regeneration by preventing the release of inhibitory factors and by stimulating NO, TNF-α, and IL-6 synthesis in Kupffer cells. Experiments of this study were repeated at least three times with essentially similar results using highly pure Kupffer cell

preparations with stellate cells being the contaminating cell type at 1–5%. Thus, it is reasonable to suggest that the contribution by other cell types to the effects of ALR in these Kupffer cell preparations is negligible.

The evidence for the role of Kupffer cells in liver regeneration is conflicting. Chlodronate-induced deletion of Kupffer cells was shown to delay hepatic regeneration demonstrating their importance in the regenerative response (Meijer et al., 2000; Abshagen et al., 2007). In contrast, GdCI3-induced blockade of Kupffer cell function was reported to stimulate regeneration (Rai et al., 1996; Boulton et al., 1998). It is possible that the relative levels and the time course of the production of the mediators that regulate hepatic regeneration are differentially affected by chlodronate and GdCI3 treatments thus causing conflicting outcomes. Our data suggest that Kupffer cells are required for unabated liver regeneration. However, ALR's effects on hepatocytes via other NPCs cannot be ruled out as the number of NPCs expressing iNOS in ALR-treated group exceeds the number of iNOS-expressing Kupffer cells in the liver. In this regard, we observed the presence of ALR binding, similar to that in Kupffer cells in hepatic stellate cells (unpublished observation). It should be noted that although activated stellate cells promote liver regeneration of the fibrotic liver after partial hepatectomy, the role of quiescent stellate cells (present in the normal liver) is unclear. The primary reason for this is the unavailability of a reliable method to remove stellate cells from the normal liver or block their functions.

In summary, the present results provide first evidence for coupling of the Kupffer cell ALR receptor to cholera toxin-sensitive G-protein, activation of which causes synthesis of mediators that are known to positively influence hepatic regeneration. Given the important role of Kupffer cells in regulation of the overall hepatic metabolism via secretory molecules, ALR's effects observed in this study could be significantly important in liver pathophysiology.

Acknowledgments

This work was supported by a VA Merit award and a grant from the NIH (DK 54411) to C.R.G. We thank Ms. Holly Grunebach, Ms. Kristin Anselmi, and Mr. Chao Huang for excellent technical assistance.

Contract grant sponsor NIH; Contract grant number. DK 54411.

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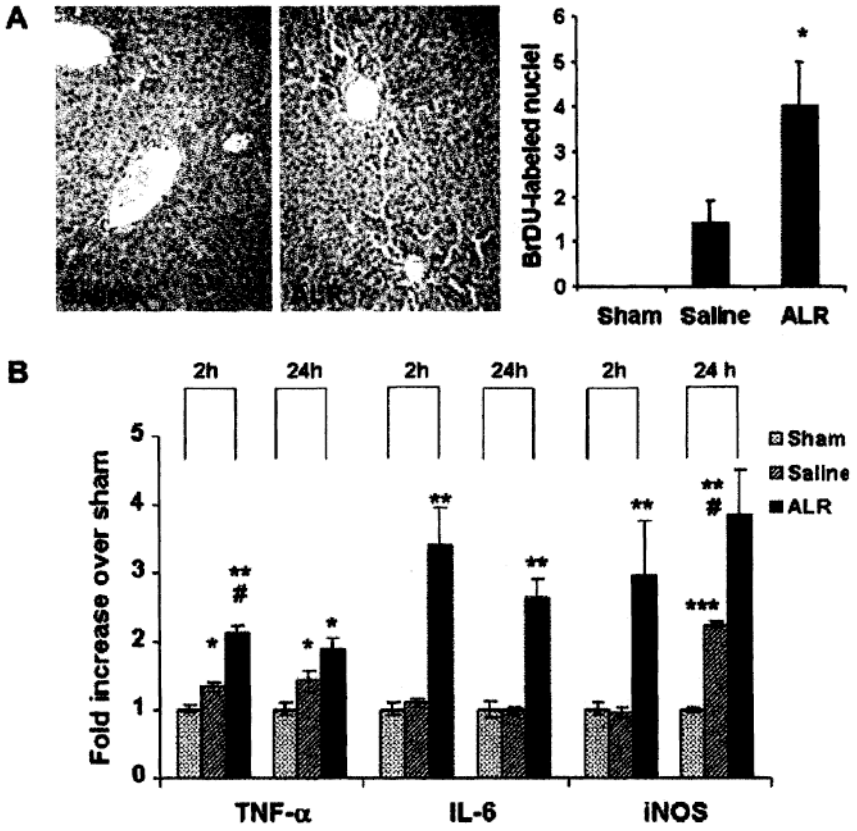
Abbreviations

ALR	augmenter of liver regeneration
BSA	bovine serum albumin
ERVI	essential for respiration and vegetative growth
FBS	fetal bovine serum
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HBSS	Hank's balanced salt solution
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
NO	nitric oxide
PBS	phosphate-buffered saline
PMSF	phenylmethylsulfonylfluoride
rrALR	recombinant rat ALR
TNF	tumor necrosis factor



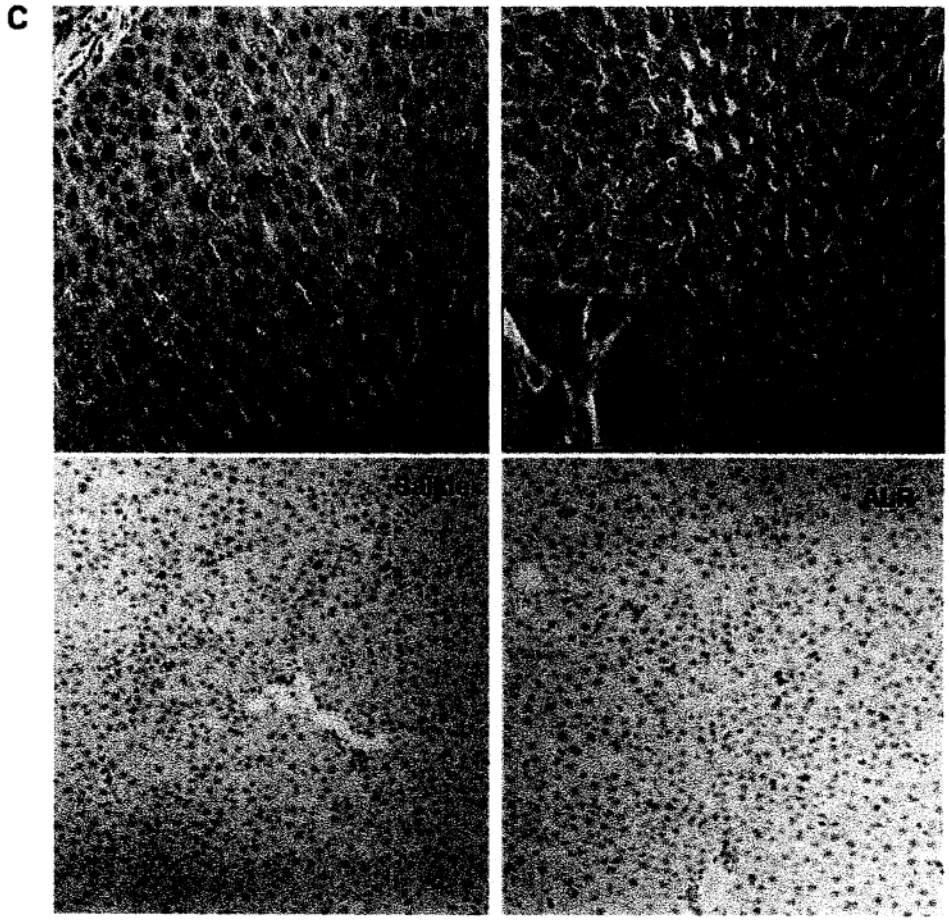


Fig. 1.

Effect of ALR on (A) hepatic regeneration, (B) mRNA expression of cytokines and iNOS, and (C) expression of iNOS protein after 40% hepatectomy. A: Photomicrographs show BrDU-labeled cells in saline- or ALR-treated rats, while bar graph shows BrDU-labeled cells/power field. **P*<0.05 versus saline. B: RNA was extracted from frozen livers and expression levels of various mRNAs were determined via real-time PCR. Bar graph shows mRNA expression relative to that of sham control. **P* < 0.05 versus sham; ***P* < 0.001 versus sham; ****P* < 0.01 versus sham; **P* < 0.05 versus saline. C: The formalin-fixed liver sections were immunostained with anti-iNOS (top parts) or anti-ED2 antibody (bottom parts). Staining for iNOS in much greater number sinusoidal cells (arrows) with higher intensity in ALR-treatment group can be seen as compared to the vehicle (saline)-treated group (magnification 20×); inset shows iNOS staining at higher magnification (100×). The number of ED2-positive cells is similar in the two groups (magnification 10×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

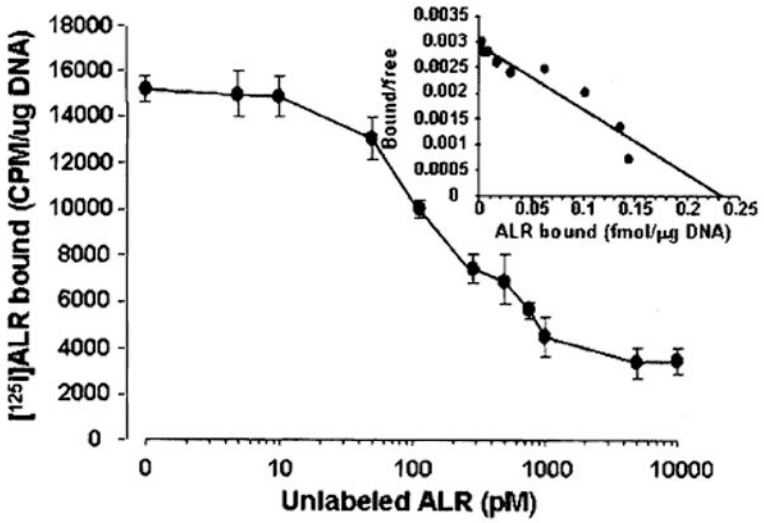


Fig. 2.

Binding of ALR to Kupffer cells. The [^{125}I]ALR competition binding assay was performed as described in the Materials and Methods Section. The incubation buffer contained 10pM [^{125}I]ALR and indicated concentrations of unlabeled rrALR. Values are means of triplicate determinations from a representative experiment repeated twice. Inset shows Scatchard plot of the [^{125}I]ALR saturation binding data.

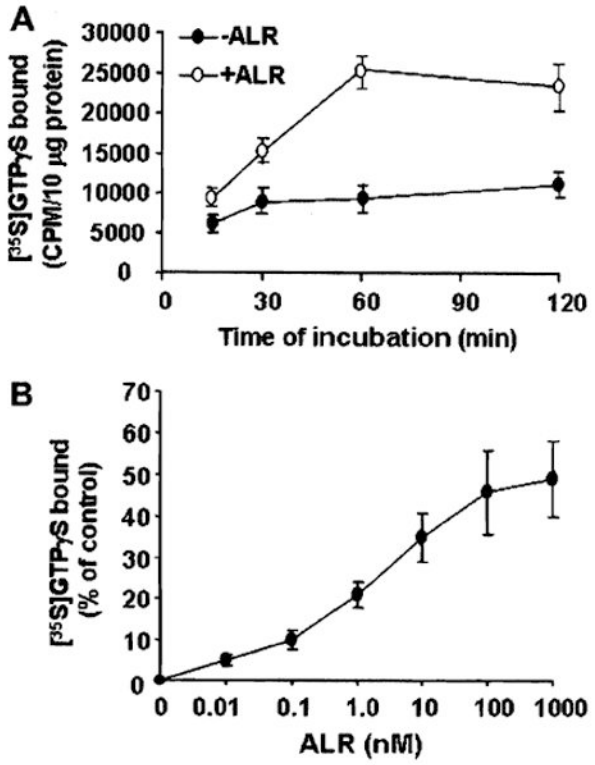


Fig. 3.

ALR-stimulated binding of GTP to Kupffer cell membranes. Membranes were incubated with the GTP-binding assay mixture containing (A) 45 nM ALR for indicated time points or (B) indicated concentrations of ALR for I h. The reaction was stimulated by addition of 10 μg membrane proteins. Details are described in the Materials and Methods Section.

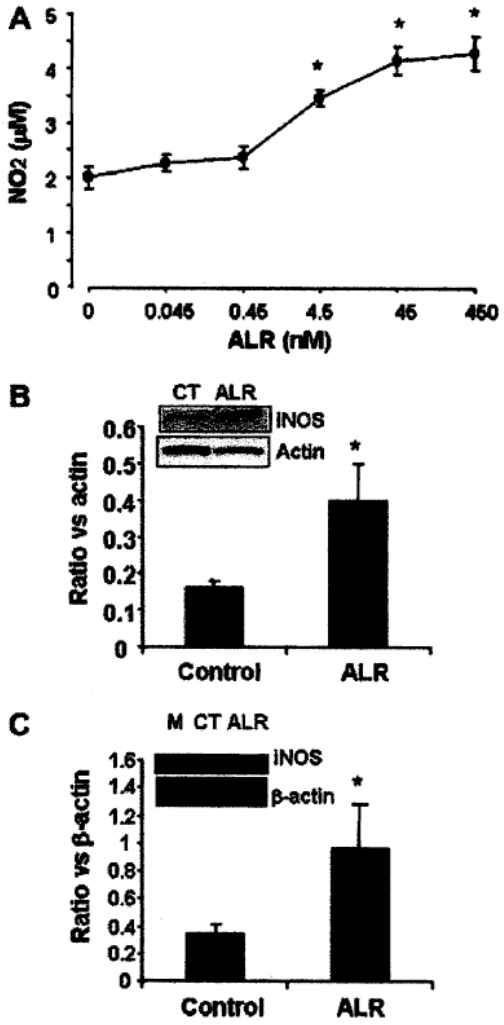


Fig. 4.

Effect of ALR on NO synthesis and iNOS expression. Kupffer cells were incubated with indicated concentrations of ALR (A) or 45 nM ALR (B,C) for 24 h. A: NO_2 concentration in the culture medium is shown. **P*< 0.005 versus "0" nM ALR. B,C: Protein lysates and RNA extracts were prepared from the cells for Western analysis and RT-PCR, respectively. Representative gels show protein expression of iNOS and actin (B) and mRNA expression of iNOS and β-actin (C). Bar graphs show relative expression of iNOS mRNA or protein versus that of actin. **P*<0.05 versus control (CT).

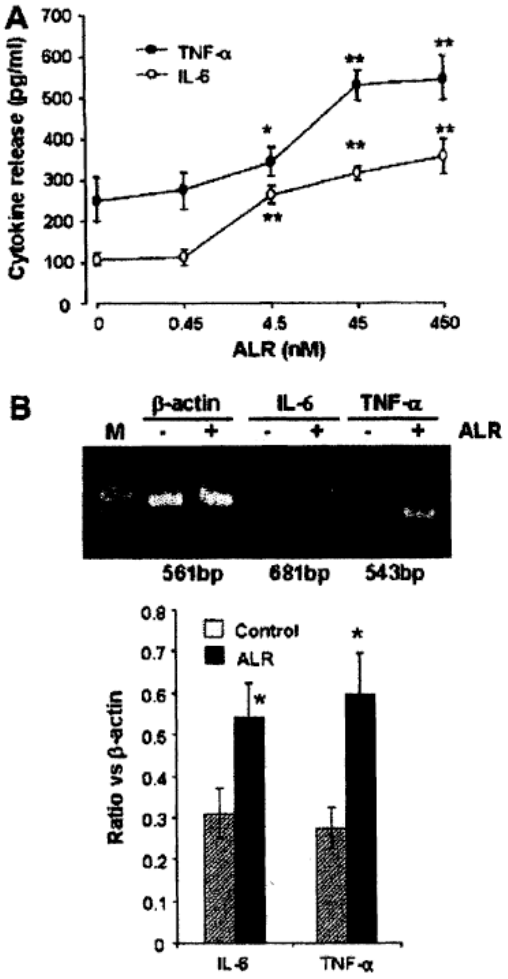


Fig. 5.

Effect of ALR on mRNA expression and release of cytokines in Kupffer cells. A: Kupffer cells were stimulated with indicated concentrations of ALR for 24 h, and TNF-α and IL-6 concentrations in the culture medium were determined. **P*<0.05 versus control; ***P*<0.01 versus control. B: After 24 h stimulation with 45 nM ALR, cellular RNA was extracted to determine TNF-α and IL-6 mRNA expression. The bar graph shows relative expression of IL-6 or TNF-α versus β-actin expression. **P*<0.05 versus control.

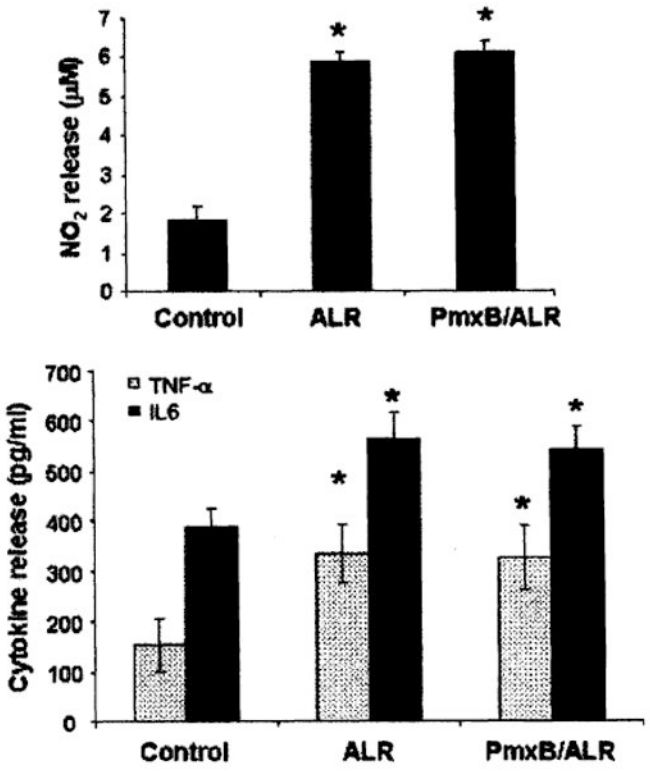


Fig. 6.

Effect of polymyxin B on ALR-induced synthesis of NO and cytokines in Kupffer cells. Kupffer cells were preincubated without or with 100 U/ml polymyxin B (PmxB) for 10 min. ALR (45 nM) was then added to the indicated wells and 24 h later, concentrations of NO_2, TNF-α, and IL-6 in the medium were measured. *P<0.05 versus control.

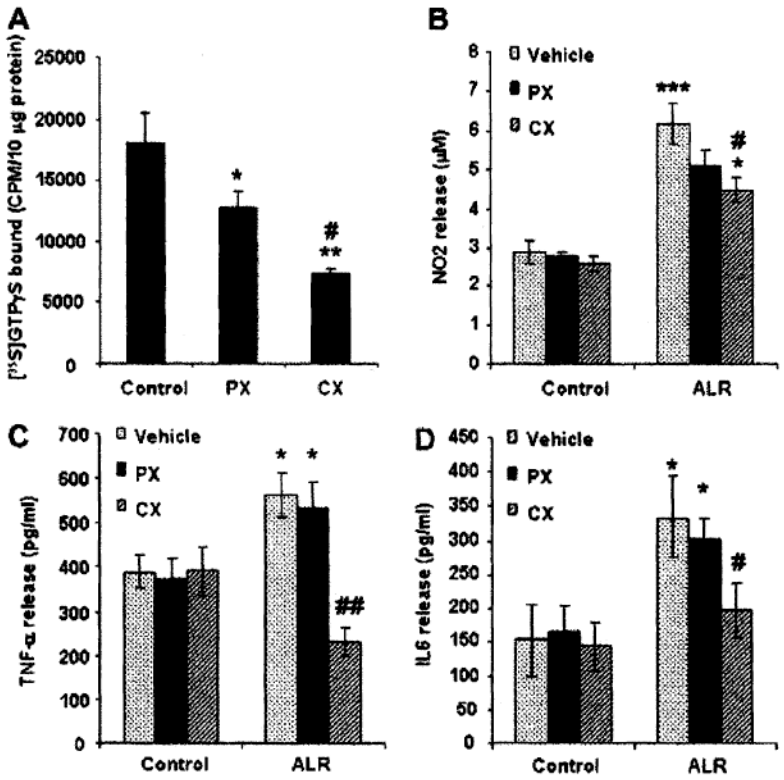


Fig. 7.

Effect of G-protein inhibition on ALR-induced synthesis of NO and cytokines in Kupffer cells. A: Kupffer cell membranes were incubated with the G-protein association mixture in the presence of 45 nM ALR without or with 50 ng/ml pertussis toxin (PX) or 2.5 μg/ml cholera toxin (CX) for 2 h, and membrane-associated radioactivity was determined. Specific [^{35}S]GTPγS association (differences between binding in the absence and presence of excess unlabeled GTPγS) is shown. B–D: Cells were preincubated for 15 min in the absence or presence of 50 ng/ml pertussis toxin or 2.5 μg/ml cholera toxin, then stimulated with 45 nM ALR. After 24 h. NO, TNF-α, and IL-6 synthesis were measured. **P*< 0.05 versus control; ***P*< 0.001 versus control; ****p*< 0.01 versus control; $^{\#}P$< 0.05 and $^{\#\#}P$< 0.001 versus ALR alone.

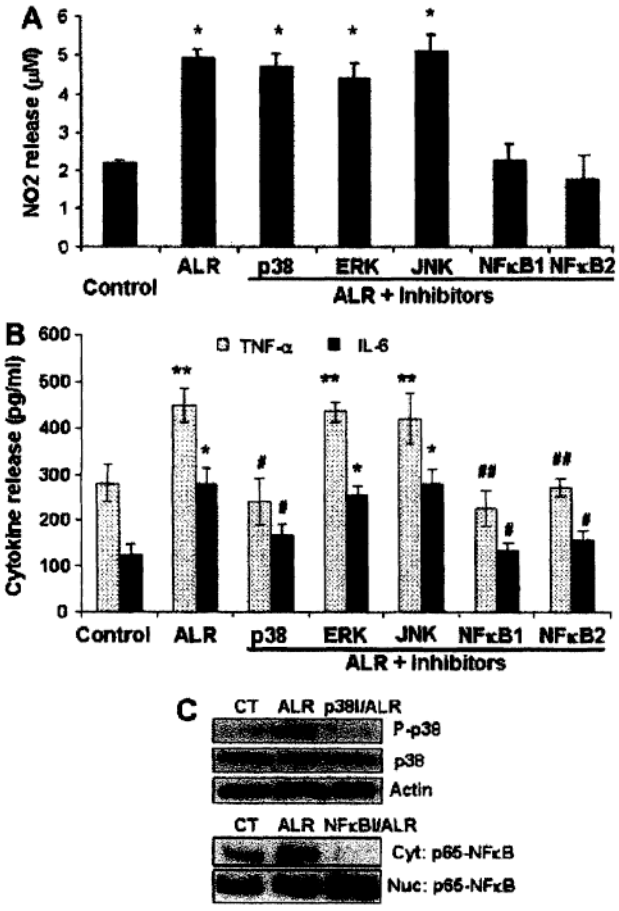


Fig. 8.

Involvement of NFκB and MAPK in ALR-induced NO, TNF-α, and IL-6 synthesis in Kupffer cells. Cells were preincubated with inhibitors of p38 kinase (SB283520), ERK1/2 kinase (PD98059), JNK kinase (SP600I25), or NFκB inhibitors (MGI32 or pyrrolidine dithiocarbamate: NFκB I and NFκB2, respectively) (all at 10 μM) for 30min before addition of 45 nM ALR. At 24 h, (A) NO or (B) TNF-α and IL-6 synthesis were determined. $^{*}P<0.05$ versus control; $^{**}P < 0.01$ versus control; $^{\#}P < 0.005$ versus ALR; $^{\#\#}P < 0.01$ versus ALR. C: Cells were incubated for 3 h with 45 nM ALR ± p38-MAPK inhibitor SB283520 or NFκB inhibitors MG132. Nuclear and cytosolic extracts or whole cell lysates were prepared for Western analysis. Cytosolic (Cyt) and nuclear (Nyc) expression of p65-NFκB, and total and phosphorylated p38 in the whole cell lysate are shown. Actin expression shows equal loading.

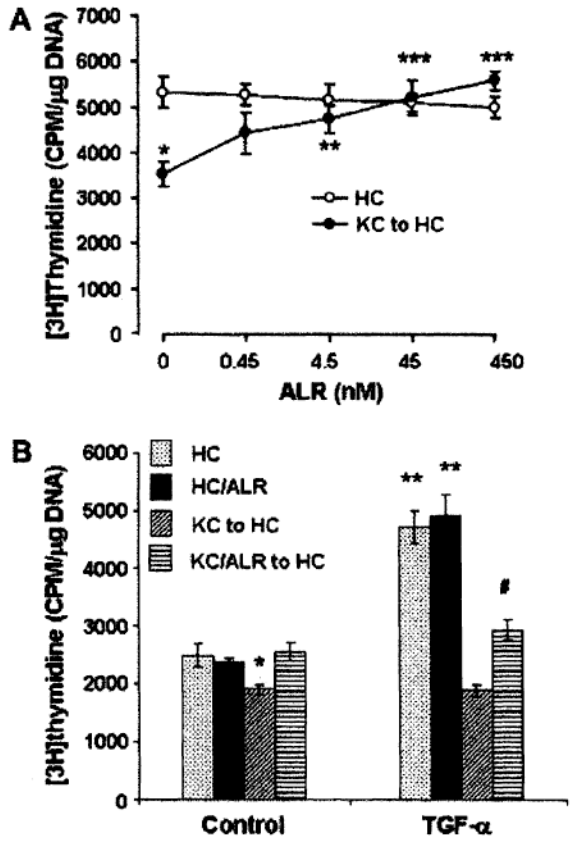


Fig. 9.

A: Effect of ALR-conditioned Kupffer cell medium on hepatocyte DNA synthesis. Kupffer cells were incubated with indicated concentrations of ALR for 24 h. The medium (KC to HC) was then transferred to the overnight culture of hepatocytes, and at 24 h DNA synthesis was measured via [^{3}H]thymidine incorporation assay. *P < 0.01 versus HC; **P < 0.05 versus "0" KC to HC; ***P< 0.01 versus "0" KC to HC. B: Effect of ALR-conditioned Kupffer cell medium on DNA synthesis in TGF-α-challenged hepatocytes. Hepatocytes were placed in unconditioned or Kupffer cell-conditioned medium in the absence or presence of 45 nM ALR. TGF-α (2 ng/ml) was added and at 24 h, DNA synthesis was determined. *P<0.05 versus HC or HC/ALR; **P<0.00l versus control; $^{\#}P$<0.05 versus KC to HC 4- TGF-α.