Role of RHOB in the antiproliferative effect of glucocorticoid receptor on macrophage RAW264.7 cells

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Abstract

Although glucocorticoid (GC) has been reported to inhibit macrophage killing activity and cytokine production in response to proinflammatory stimuli, the effect of GC on macrophage proliferation is controversial. In our previous study, we found that inhibition of glucocorticoid receptor (GR) expression in murine macrophage cell line RAW264.7 cells (RAW-GR(−) cells) by RNAi significantly promoted cell proliferation. In the present study, we provide the evidence that the expression of Rhob, a member of Rho GTTases with anti-cancer character, remarkably decreased in RAW-GR(−) and RAW264.7 cells transiently transfected with GR-RNAi vector. Overexpression or constitutive activation of Rhob in RAW-GR(−) and RAW264.7 cells by transfection with wild-type Rhob expression vector (Rhob-wt) or constitutively activated Rhob plasmid (Rhob-V14) resulted in decreased proliferation of the two cell lines. Oppositely, the proliferation of RAW264.7 cells was significantly increased when the expression of Rhob by RNA interference technique or the activity of Rhob by transfection with dominant negative Rhob mutant that is defective in nucleotide binding (Rhob-N19) was inhibited. In addition, enhanced activity of Akt, but not MAPK3/1 or MAPK14, was found in RAW-GR(−) cells. Blocking the pathway of phosphatidylinositol 3-kinase (PI3K)/Akt with the specific inhibitor LY294002 decreased the proliferation and elevated RHOB protein level, indicating that PI3K/Akt signal plays its role of proliferation modulation upstream of RHOB protein. In conclusion, these results demonstrate that Rhob plays an important role in the antiproliferative effect of GR on RAW264.7 cells by GR→Akt→Rhob signaling and Rhob negatively regulates the proliferation of RAW264.7 cells.

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Introduction

Glucocorticoid (GC) is a significant hormone in regulating diverse physiological processes such as immune and inflammatory responses, cell proliferation, differentiation, and apoptosis (Dondi et al. 2001, Kudawara et al. 2001, Distelhorst 2002, Yamamoto et al. 2002). The common use of GC in treating inflammatory and autoimmune diseases is mostly due to its function of immunosuppression, for instance, inhibition of growth and induced apoptosis in lymphocytes, and repression of cytokine production in macrophages (Distelhorst 2002, Frankfurt & Rosen 2004). Macrophages also play a key role in the immune and inflammatory process, mainly acting as antigen-processing/presenting cells and sources of inflammatory cytokines. Although GC has been reported to suppress macrophage killing activity and cytokine production in response to proinflammatory stimuli (Brummer et al. 2005), the effect of GC on macrophage proliferation is controversial (Llobers et al. 1998, Sakai et al. 1999, Pagniello et al. 2002).

It is well known that the action of GC was mediated by glucocorticoid receptor (GR) that belongs to nuclear receptor superfamily and is normally localized in the cytoplasm in an inactive state. On hormone binding, GR translocates to the nucleus where it binds to glucocorticoid response elements (GREs) on target genes, recruits coactivators, or corepressors to regulate the expression of target genes (Heitzer et al. 2007). But a few target genes have been known to mediate the antiproliferative effect of GC until now. Rhob is a member of Rho GTPase family and has a tumor-suppressive role, including inhibiting cell proliferation and inducing apoptosis in several human cancer cells, and inhibiting tumor growth in a nude mouse xenograft model (Prendergast 2001, Mazieres et al. 2004). Rhob expression level has recently been shown to dramatically decrease in several cancer cells and patient biopsies such as lung cancer, brain cancer, neck and head tumors as tumors become more aggressive, and ectopic expression of Rhob antagonizes oncogene-mediated transformation (Liu et al. 2001, Adnane et al. 2002, Forget et al. 2002, Jiang et al. 2004a,b, Mazieres et al. 2004, Huang & Prendergast 2006). Recently, we found that GC/GR increased Rhob expression in several tumor cells, such as human ovarian cancer cell lines HO-8910 and 3AO, as well as human hepatocellular carcinoma cell

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were generously provided by Dr Prendergast (2001). Although RHOB activation was required for mannose receptor-mediated phagocytosis of human alveolar macrophages (Zhang et al., 2005), the role of RHOB in the proliferation of macrophage was unclear until now. In our previous study, we found that Dex inhibited the growth of RAW264.7 cells, a murine macrophage cell line. Treatment of RAW264.7 cells with 10⁻⁷ M Dex for 4 days resulted in about 27% inhibition of cell proliferation. We also found unexpectedly that inhibition of GR expression in RAW264.7 by RNA interference significantly promoted cell proliferation. The cell number of RAW-GR(−) cells (stably transfected with GR) was almost twofold as that of the RAW-control cells (stably transfected with RNA interference control plasmid) after 4-day culture (Zhu et al., 2004). These results indicated that GC/GR negatively regulated the growth of RAW264.7 cell, and compared with GC, the expression of functional GR seemed to be more important in keeping cells a more differentiated and less proliferative phenotype. This prompted us to further clarify the molecular mechanism responsible for the anti-proliferative effect of GR on RAW264.7 cells. In this study, we investigated the expression of RHOB in RAW-GR(−) cells and its possible role in GC/GR-mediated proliferation inhibition of RAW264.7 cells.

Materials and Methods

Materials

Materials used in the present study included mouse monoclonal antibodies against β-actin and 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma–Aldrich Chemicals); rabbit polyclonal antibodies against GR and RHOB (Santa Cruz Biotechnology, Santa Cruz, CA, USA); goat anti-rabbit-AP and goat anti-mouse-AP conjugate (Bio-Rad Laboratories); RNA interfering expression vector pSilencer 2.1-U6 and negative control plasmid (Ambion, Austin, TX, USA); goat anti-β-actin and 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma–Aldrich Chemicals); rabbit polyclonal antibodies against GR and RHOB (Santa Cruz Biotechnology, Santa Cruz, CA, USA); goat anti-rabbit-AP and goat anti-mouse-AP conjugate (Bio-Rad Laboratories); RNA interfering expression vector pSilencer 2.1-U6 and negative control plasmid (Ambion, Austin, TX, USA); and BCA protein assay kit (Pierce, Pierce, IL, USA).

The wild-type RHOB expression vector (pcDNA3-RHOB; named RHOB-wt), constitutively active RHOB mutant containing a point mutation in the GTPase domain (G14V; named RHOB-V14), and dominant negative RHOB mutant that is defective in nucleotide binding (T19N; named RHOB-N19) were generously provided by Dr Prendergast (2001).

Cell lines and cell culture

Murine macrophage cell line RAW264.7 was obtained from American Type Culture Collection (Rockville, MD, USA). RAW-GR(−) and RAW-control cells were established by stable transfection of RAW264.7 cells with GR-siRNA expression vector and siRNA control vector respectively (Zhu et al., 2004). All the cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum at 37 °C in a humidified incubator of 5% CO₂.

Quantitative real-time PCR

Total RNA was extracted with the TRIZol reagent and 2 µg total RNA was used to synthesize first-strand cDNA by reverse transcription system (Promega), in accordance with the manufacturer's instructions.

Primers for amplification of RHOB gene were sense: 5’-CTG GCC CGC ATG CA-3’ and antisense: 5’-AGG CAG TCT GGT GTG CTC C-3’ (186 bp), and those for β-actin were sense: 5’-CTG TAT GCC TCT GTG CTG AC-3’ and antisense: 5’-TGA TGT CAC GCA CGA TTT CC-3’ (214 bp). SYBR Green from OPEN company was used as the fluorescence reagent. cDNA template (100 and 50 ng for the amplification of RHOB and β-actin respectively) was added to the mixture of 2 µl 10× buffer, 1-6 µl of 25 mM MgCl₂, 0.5 µl of 20 mM dNTP, 0.3 µl of 20 mM primers, 1.5 U Taq enzyme, and 1.0 µl of 20× SYBR Green to a final volume of 20 µl. Amplification of RHOB and β-actin was performed in different tubes, with the same process: denaturation at 94 °C for 15 s, annealing at 57 °C for 20 s, elongation at 72 °C for 15 s for 36 cycles, and a final extension for 10 min at 72 °C.

The mRNA ratio of RHOB and β-actin was calculated as follows: we first drew the standard curves of RHOB and β-actin by measuring the threshold cycles (CT) of standards of different concentrations, which had strong positive relationship with the start copy number (i.e. cDNA amounts) of a gene in linear amplification period. Then the cDNA amounts of RHOB and β-actin of each testing sample were calculated according to their standard curves, and cDNA amount of RHOB was normalized by the corresponding β-actin.

Western blot analysis

Whole cell extract was prepared with lysis buffer (Stamatakis et al., 2002): 10 mM Tris (pH 7.5), 0–1 mM EDTA, 0–1 mM EGTA, 0–5% SDS, 0–1 mM mercaptoethanol, 2 µg/ml of each of the protease inhibitors leupeptin, aprotinin, and pepstatin). The lysate was briefly sonicated on ice, and spun at 14 000 r.p.m./min for 10 min to remove the insoluble material. Protein concentrations of the supernatant were measured with BCA protein assay kit. Twenty to fifty micrograms of extracts were resolved on 10% SDS–PAGE gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, which were then blocked with 5% nonfat milk and probed overnight with antibodies against RHOB (1:200), GR (1:1000), total and phosphorylated MAPK3/1, MAPK14, phosphorylated Akt (1:500), or β-actin (1:10 000). Then the membranes were washed, exposed to alkaline phosphatase (AP)-conjugated secondary antibodies for 2 h, and finally detected by NBT/BCIP staining. The quantitativeness of protein expression was made by Tanon Bio-image software (Shanghai, China).
RNA interference constructs for Rhob and transfection

Using online siRNA sequence selector tool (BD Clontech), siRNA templates were designed to match nonconserved 19 nucleotide sequences within the mouse Rhob mRNA. The sequences of the oligonucleotides used to create Rhob-RAi were: 1) sense: 5'-GAT CCC GCG TAT GAC TCT GAG TTT CAA GAG TAG TCA TAG GCT TTT TGG GAA A-3' and antisense: 3'-GGA TAC TGA TGG AGC TCA AAG TCT TCT GTA GCT CCA TCA GAA CCC A AC TTT TCG A-5', and 2) sense: 5'-GAT CCC GCG TAT GAC TCT GAG TTT CAA GAG AAT TCT GGG ATC CGT AGC GCT TTT TGG GAA A-3' and antisense: 3'-GCC GCG ATG CCT AGG GTC TTA AAG RTC TCT TAA GAC CCT AGG CAT CGC GAA AAA ACC TTT TCG A-5'. Negative control plasmid was supplied from Ambion; it expressed a hairpin siRNA with limited homology to any known sequences in the human, mouse, and rat genomes. The oligonucleotides were synthesized by Sangon Technology Inc. (Shanghai, China) and were annealed/ cloned into pSilencer 2.1-U6 vectors. The clones were confirmed by DNA sequencing. Expression of siRNA for Rhob is under control of the U6 promoter.

pSilencer 2.1-U6-Rhob or pSilencer 2.1-U6 control plasmid was introduced into RAW264.7 cells with LipofectAMINE Plus reagent (Invitrogen). Briefly, the cells were transiently transfected with 3 μg DNA in each well of a 6-well plate, according to the manufacturer’s instructions.

Transient transfection of the cells

Cells were seeded onto 24-well plates (4×10⁴ cells/well) or 6-well plates (2×10⁵ cells/well) for 24 h and then transiently transfected with 1 (for 24-well plate) or 3 μg (for 6-well plate) of the blank vector (pcDNA3), Rhob-wt, Rhob-V14, Rhob-RNai, or Rhob-N19 plasmid using LipofectAMINE Plus reagent (Invitrogen), according to the manufacturer’s instructions.

Cell proliferation assay

Twenty-four hours after transfection with various Rhob plasmid as indicated above, cells were rinsed out and seeded in duplicate at 3×10⁴ cells per well onto 24-well plate for MTT assay or 2×10⁵ cells per well onto 6-well plate for cytometry. After incubation for 2 days, the cell number in each well was collected and counted with a hemacytometer or examined by MTT assay. Two hundred microliters of RPMI 1640 medium containing 5 mg/ml MTT (Sigma–Aldrich Chemicals) solution were added to each well. The cells were incubated for another 4 h at 37 °C, followed by solubilization with 200 μl DMSO solutions. Then the O.D. value was measured using a Bio-Rad (model 550) microplate reader at 550 nm with the DMSO as blank.

The linear response range of RAW264.7 cells between the results of MTT assay and the number of cells was explored, and it was found that there was a linear correlation when the cells were seeded at the densities from 1×10⁴ to 4×10⁵ cells per well onto a 24-well plate and cultured in the medium for 4 days (data not shown). Therefore, in our experiments, 3×10⁵ cells per well in a 24-well plate were selected, cultured in the medium for 4 days, and then cell proliferation was determined by MTT assay.

Statistical analysis

Data are expressed as X ± S.D. Statistical significance was determined by Student’s t-test with P<0.05 considered as statistically significant.

Results

Inhibition of GR expression in RAW264.7 cells by stable or transient transfection with GR RNA interference vector decreased Rhob expression of the cells

Considering that Rhob showed a proliferation inhibitory effect on several tumor cells, and the proliferation of RAW-GR(-) cells (stably transfected with GR-RAi vector) increased after inhibition of GR expression, we examined the expression of Rhob at mRNA and protein levels in RAW-GR(-) cells by quantitative real-time PCR (Fig. 1A) and western blot analysis (Fig. 1B) respectively. As we expected, the mRNA and protein levels of Rhob in RAW-GR(-) cells were reduced to about 22% and 28% of RAW-control cells respectively.

RAW-GR(-) cells were originated from a single clone. Several single clones of RAW-GR(-) cells were established and down-regulation of Rhob expression was also found in other clones (data not shown). To validate down-regulation of Rhob expression induced by a decreased level of GR, RAW264.7 cells were transiently transfected with GR-RAi vector and the protein level of Rhob was decreased in RAW264.7 cells (Fig. 1C), which indicated that down-regulation of Rhob expression in RAW264.7 cells stably or transiently transfected with GR-RAi vector was indeed a result from the knock down of GR.

Decreased expression of Rhob is involved in the increased proliferation of RAW-GR(-) cell

As increased proliferation was in concurrence with significantly decreased expression of Rhob in RAW-GR(-) cells, we hypothesized that decreased expression of Rhob might be related to the growth acceleration of RAW-GR(-) cells. To evaluate this possibility, we transfected RAW-GR(-) cells with Rhob-wt (Rhob wild-type construct) Rhob-V14 (a constitutively active Rhob plasmid) or blank vector to detect the effect of Rhob on the cell proliferation by MTT assay and cytometry. As shown in Fig. 2A, the protein level of Rhob increased about twofold in RAW-GR(-) cells transfected with Rhob-wt plasmid. The increase in expression and activation
of Rhob in RAW-GR(−) cells resulted in about 27 and 34% decrease in cell proliferation respectively (Fig. 2B). Similar results were obtained by cytometry (Fig. 2C). These data indicated that decreased expression of Rhob is involved in the increased proliferation of RAW-GR(−) cell.

**GC/GR induces the expression of Rhob at mRNA and protein levels in RAW264.7 cells**

Recently, we found that GC/GR increased Rhob expression in human ovarian cancer cell lines HO-8910 and 3AO, as well as human hepatocellular carcinoma cell SMMC-7721, and the proliferation inhibition of GC/GR on HO-8910 cells is partly due to the increased expression of Rhob (Chen et al. 2006). To determine the effect of GC on the expression of Rhob in RAW264.7 cells, we investigated the mRNA and protein levels of Rhob in the cells treated with 10⁻⁷ M Dex for different times by quantitative real-time PCR, and western blot respectively. Figure 3A shows that Dex induced the mRNA expression of Rhob in a time-dependent manner, with the maximal effect at 2 h after treatment with 10⁻⁷ M Dex. A significant increase in RHOB protein expression was also observed following incubation of cells with 10⁻⁷ M Dex for 4 h. Furthermore, RU486, an agonist of GR, could partially attenuate the up-regulation of Rhob by Dex, indicating that activation of GR is required for Rhob induction (Fig. 3B).

**Rhob negatively regulates the proliferation of parental RAW264.7 cells**

To examine whether Rhob has a direct inhibition effect on the proliferation of parental RAW264.7 cells, we used RNAi (RNA interference) to knock down endogenous Rhob expression in this cell line. The results of western blot showed that both the two Rhob-RNAi plasmids obviously reduced the expression of Rhob in RAW264.7 cells by about 73% [Rhob-RNAi(1)] and 88% [Rhob-RNAi(2)], when compared with that of control siRNA plasmid (Fig. 4).

Figure 1

Inhibition of GR expression in RAW264.7 cells by stable or transient transfection with GR RNA interference vector decreased Rhob expression of the cells. (A) Rhob mRNA level in RAW-GR(−) and RAW-control cells (stably transfected with GR-RNAi vector and RNAi control vector respectively) was detected by quantitative real-time PCR (X ± S.D., n = 3). **P < 0.01 versus RAW-control. (B) The protein level of RHOB in the two cells was assayed by western blot analysis. Similar results were obtained from three independent experiments. Using Tanon software (Shanghai China), the blots of Rhob in these two cells were quantified and normalized against β-actin. The normalized numbers from three independent experiments were compared. The values are expressed as the percentage of the level of RAW-control, as shown in the bar graph of B (X ± S.D., n = 3). **P < 0.01 versus RAW-control. (C) The protein level of RHOB in RAW264.7 cells transiently transfected with GR-RNAi vector or RNAi control vector was assayed by western blot analysis. Similar results were obtained from three independent experiments.
vector control cells. Similar results were obtained by MTT assay (Fig. 5B). These results further demonstrated that Rhob signaling negatively regulated the proliferation of RAW264.7 cells.

Akt phosphorylation, but not MAPK3/1 and MAPK14, is involved in the accelerating the growth of RAW-GR(−) cells

In recent years, evidence has emerged that GR plays important roles in regulating cell proliferation by modulating the activity of Akt (Leis et al. 2004, Chrysis et al. 2005, Nuutinen et al. 2006), MAPK3/1 and MAPK14 (Miller et al. 2005, Qin et al. 2005), so we examined the activated status of these kinases in RAW-GR(−) and RAW264.7 cells by western blot. We failed to find a significant difference in phosphorylated MAPK3/1 or MAPK14 level between RAW-GR(−) and RAW-control cells (data not shown), but found about a 79% increase in phosphorylated Akt in RAW-GR(−) cells compared with that of RAW-control cells (Fig. 6A). To validate the effect of Akt on the increased proliferation of RAW-GR(−) cells, we further examined the effect of LY294002, a specific inhibitor of phosphatidylinositol 3-kinase (PI3K)/Akt pathway, on cell growth by
MTT assay. As shown in Fig. 6B, treatment of cells with 25 μM LY294002 resulted in a more obvious growth repression in RAW-GR(−) cells than that in RAW-control cells, though the effect could be seen in both the two cells. These results indicated that enhanced activity of Akt, but not MAPK3/1 and MAPK14, was also associated with the increased proliferation of RAW-GR(−) cells.

Blocking PI3K/Akt pathway in RAW-GR(−) and RAW264.7 cells by LY294002 and inhibiting Akt activity in RAW264.7 cells by Dex-increased RhoB expression of the cells

Given that both PI3K/Akt and RhoB are involved in the growth of RAW-GR(−) cells, the relevance between the two signaling pathways was then investigated. RAW-GR(−) cells and its parental RAW264.7 cells were treated with 0–50 μM LY294002 for 12 h, and the expression of RhoB mRNA was then detected by real-time PCR. As shown in Fig. 7A, the expression of RhoB mRNA in RAW-GR(−) and RAW264.7 cells increased gradually with the dosage of LY294002, and the mRNA level of RhoB in RAW-GR(−) cells is about 2.3- and 3.2-fold of the control at 25 and 50 μM LY294002 respectively. The expression of RHOB protein was also further examined. The cells were treated with or without 50 μM LY294002, and the protein level was analyzed by western blot. Figure 7B shows that RhoB protein in RAW-GR(−) and RAW264.7 cells increased remarkably when compared with that of control when 50 μM LY294002 was added. These results demonstrated that PI3K/Akt signal was in the upstream of RhoB in these two cells and down-regulation of RhoB in RAW-GR(−) cells was resulted from the activation of PI3K/Akt pathway.

As shown in Fig. 3, we demonstrated that Dex increased the expression of RhoB in RAW264.7 cells at mRNA and protein levels. Does Dex also affect PI3K/Akt activity in RAW264.7 cells. Was the role of Dex on RhoB in the cells associated with Akt pathway? Therefore, we detected the effect of Dex on the protein level of p-Akt and RhoB in...
RAW264.7 cells at the same time. A significant decrease in p-Akt protein expression was observed, following incubation of RAW264.7 cells with 10\(^{-7}\) M Dex for 4 h accompanied by an increase in Rhob and RU486, which could partially attenuate the down-regulation of p-Akt by Dex (Fig. 7C), indicating that activation of GR is required for p-Akt reduction. These results demonstrated that Dex inhibited the activity of Akt in RAW264.7 cells and the decrease in Akt activity might be resulted in the increase in Rhob expression of the cells.

**Discussion**

In our previous work, we found that the inhibition of GR expression in murine macrophage RAW-GR(−) cells by RNA interference resulted in significant enhancement of cell proliferation (Zhu et al. 2004). These results indicated that GR plays an important antiproliferative role in these macrophage cells. Although it is well known that GR mediates GC action by regulating the expression of target genes (Dondi et al. 2001, Zhu et al. 2004, Bladh et al. 2005), but a few target genes have been known to mediate the antiproliferative effect of GC until now.

Our other previous work found that Rhob could be induced by GC/GR and it at least partially mediated the antiproliferative effect of GC/GR in ovarian cancer HO-8910 cell lines (Chen et al. 2006). In this study, we found that GC also induced the expression of Rhob at both mRNA and protein levels in RAW264.7 cells and activation of GR is required for Rhob induction. The levels of Rhob at both mRNA and protein markedly decreased in RAW-GR(−) and RAW264.7 cells transiently transfected with GR RNA interference vector. Ectopic expression or constructive activation of Rhob by transfection with Rhob-wt or Rhob-V14 obviously inhibited the proliferation of RAW-GR(−) cell. Furthermore,
inhibition of the expression or activity of Rhob by transfection with Rhob–RNAi or Rhob–N19 resulted in a significant increase in cell proliferation of parental RAW264.7 cells, while increase in the expression or activity of Rhob by transfection with Rhob–wt or Rhob–V14 inhibited the proliferation. These results demonstrated that Rhob negatively regulated the proliferation of RAW264.7 cells and down-regulation of Rhob expression was associated with increased proliferation in RAW-GR(−) cell. It needs to be mentioned that Rhob expression and the proliferation of RAW-GR(−) cells showed no significant difference between culture in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum and 10% charcoal-stripped serum (deprived of GC). The data indicated that the presence or absence of GC had no effect on Rhob expression when knock down of GR in RAW-GR(−) cells, while GC promoted the expression of Rhob remarkably in RAW264.7 cells through GR mediation. Therefore, we speculated that Rhob expression might be mainly regulated by GR in an agonist-independent way.

How GR up-regulates the expression of Rhob remains unknown. It is well known that GR up-regulates gene transcription usually through direct binding to GRE in the promoter region of target genes, where it serves to recruit other transcription factors usually through direct binding to GRE in the promoter region of target genes, where it serves to recruit other transcription factors. Bladh et al. (2004) demonstrated that the activity of a luciferase reporter gene containing a 1.9 kbp (−1756/+111) fragment of the promoter sequence of human Rhob gene was not induced by GC/GR in ovarian cancer HO-8910 cells, indicating that there was no functional GRE in this promoter region (−1765/+111; Chen et al. 2006). We also analyzed a 5 kbp fragment of the promoter sequence upstream of the transcriptional start site of the Rhob gene, and found no consensus GRE (Chen et al. 2006). In recent years, evidence has emerged that besides its role in cell cycle regulation, Rhob also plays an important role in the antiproliferative effect of GR on RAW264.7 cells by GR→Akt→Rhob pathway and Rhob negatively regulated the proliferation of RAW264.7 cells.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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