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HMG-CoA reductase regulates CCL17-induced colon cancer cell migration via geranylgeranylation and RhoA activation

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Abstract

Background: Simvastatin is widely used to lower cholesterol levels in patients with cardiovascular diseases, although accumulating evidence suggests that statins, such as simvastatin, also exert numerous anti-tumoral effects. **Aim:** The aim of this study was to examine the effect of simvastatin on colon cancer cell migration. **Methods:** Migration assays were performed to evaluate CCL17-induced colon cancer cell (HT-29) chemotaxis. In vitro tumor growth and apoptosis were assessed using a proliferation assay and annexin V assay, respectively. Active RhoA protein levels in CCL17-stimulated colon cancer cells were quantified using a G-LISA assay. **Results:** We found that simvastatin dose-dependently decreased CCL17-induced colon cancer cell migration. Simvastatin had no effect on colon cancer cell proliferation or apoptosis. Inhibition of Beta chemokine receptor 4, CCR4, reduced CCL17-evoked activation of RhoA in colon cancer cells. Moreover, administration of mevalonate reversed the inhibitory effect of simvastatin on CCL17-induced colon cancer cell migration. Interestingly, co-incubation with geranylgeranyl pyrophosphate (GGPP) antagonized the inhibitory impact of simvastatin on colon cancer cell migration triggered by CCL17. Moreover, we observed that simvastatin decreased CCL17-induced activation of RhoA in colon cancer cells. Administration of mevalonate and GGPP reversed the inhibitory effect of simvastatin on CCL17-provoked RhoA activation in colon cancer cells. **Conclusions:** Taken together, our findings show for the first time that HMG-CoA reductase regulates CCL17-induced colon cancer cell migration via inhibition of geranylgeranylation and RhoA activation. Thus, statins, such as simvastatin, might be effective tools to antagonize CCL17-dependent migration and metastasis of colon cancer cells.

Keywords: Chemokines, Colon, Metastasis, Migration, Simvastatin

Introduction

The incidence of colorectal cancer (CRC) is increasing and CRC is today the second leading cause of cancer-related death in Europe [1]. The main cause of death in patients with CRC is distant spread and organ metastasis [2]. The mechanisms behind cancer cell spread are not fully understood but convincing data indicate that increased expression of adhesion molecules and capacity to migrate in response to chemotactic stimuli are necessary for tumor cell metastasis [3]. Recent findings implicate that chemokines regulate multiple aspects of cancer cell biology, including proliferation, survival, angiogenesis, and migration [4]. CCR4 is a key receptor for regulating chemokine-dependent immune homeostasis and is selectively expressed on regulatory T cells and Th2 cells [5,6]. One study showed that CCR4 is involved in cancer cell evasion by stimulating recruitment of regulatory T cells in the tumor microenvironment [7]. CCR4 expression has been detected on several types of leukemic cells [6], gastric [8] and breast [9] tumor cells as well as on colon cancer cells [10]. Indeed, we recently reported that the CCR4 ligand CCL17 (TARC) is a potent stimulator of colon cancer cell migration [10]. At present, there is a need to develop ways to interfere with CCR4-CCL17 axis in colon cancer cell migration.

Although statins are mainly used to regulate cholesterol synthesis in patients with increased risk of cardiovascular complications [11], the literature suggests that statins, such as simvastatin, also exert anti-tumor effects, such as induction of cell cycle arrest [12] and apoptosis [13]. Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which is the rate-limiting enzyme in the formation of mevalonate [14]. Mevalonate is a precursor of farnesyl pyrophosphate and geranylgeranyl pyrophosphate (GGPP), which are critical components in protein isoprenylation of small regulatory G proteins of the Ras-homologous (Rho) family

[15]. Proteins of Rho family are required for a broad range of cellular functions, including membrane trafficking, cytoskeletal organization, growth, apoptosis, and differentiation [16]. Interestingly, a recent study showed that CCL17-induced colon cancer migration is dependent on Rho-kinase signaling [10]. However, the potential effect of simvastatin in CCL17-induced colon cancer cell migration remains elusive.

Based on the considerations above, we hypothesized that HMG-CoA reductase might regulate CCL17-induced and CCR4-depedent migration of colon cancer cells. Moreover, we wanted to investigate the signaling effects of inhibiting HMG-CoA reductase in colon cancer cells.

Materials and methods

Cells and reagents

The human epithelial colon adenocarcinoma cell line, HT-29, was obtained from American Type Culture Collection (HTB-38, ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, Stockholm, Sweden), supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin at 37ºC and 5% CO_2. Annexin V was from BD Bioscience Pharmingen (San Jose, CA, USA). Calcein AM, simvastatin, and mevalonate were obtained from Sigma-Aldrich. Recombinant human CCL17/TARC was purchased from Peprotech (Rocky Hill, NJ, USA). GGPP and the human CCR4 antagonist; 2-[1,4'-Bipiperidin-1'-yl-N-cycloheptyl-6,7-dimethoxy-4-quinazolinamine dihydrochloride] were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Simvastatin was activated prior to use by dissolving in absolute ethanol and adding 0.1 N NaOH and then incubated at 50ºC for 2h. The pH was adjusted to 7.0 by adding HCl and the volume brought up to 1 ml by sterile water. This gives stock solution of 10 mM, which was stored at -20ºC until use.

Chemotaxis assay

Chemotactic response of HT-29 cells was evaluated by using 24-well cell migration chambers with 8 µm pore size inserts (Corning Coster, Corning, NY, USA). The colon cancer cells were serum starved overnight and resuspended in serum free DMEM with 0.5% BSA. 8×10^5 cells/ml were pre-incubated with different concentrations of simvastatin (25 and 50 µM) for 24h. DMEM with 10% FBS with or without 100 ng/ml of CCL17 was added in the lower chambers and incubated for 24h at 37ºC (5% CO_2). Non-migrated cells were removed by cotton swabs from the upper surface of the

insert and cells in the lower surface of the insert membrane were fixed in ice-cold 100% methanol and stained with 0.5% crystal violet. In separate experiments, cells were treated the same way as in migration assay except that cells were also co-incubated with mevalonate (100 μM) and GGPP (10 μM). All migrated cells were counted microscopically using 100 × high power fields in at least 5 different fields. Migration index was then calculated as the ratio of the number of migrated cells divided by the number of cells in the control wells and expressed as percentage of migrated cells per field.

Proliferation assay

Cell proliferation was evaluated in quadruplicates after staining HT-29 cells with calcein AM. Briefly, cells were seeded in 96 wells culture plate at 3×10^5 cells/well in media with or without simvastatin (25 and 50 μM) for 24h at 37ºC (5% CO_2). To assess proliferation, 2 μM of Calcein AM was added per well and incubated for 1 h in dark at 37ºC (5% CO_2) and washed twice by ice cold PBS. Fluorescence was measured by use of a fluorometer (Tecan's Infinite® M200, Mannedorf, Switzerland) at excitation of 485 nm and emission of 530 nm and data expressed as mean fluorescence intensity.

Apoptosis assay

HT-29 cell apoptosis was evaluated by annexin V staining using flow cytometry. 1×10^5 cells/ml were incubated either with or without simvastatin (25 and 50 μM) for 24h. To detect apoptosis, cells were stained by annexin V and propidium iodide according to manufacturer's recommendations. Cells were analysed in duplicate for three independent experiments. The number of early apoptotic cells were counted and expressed as the percentage of annexin V positive and PI Negative cells.

RhoA activation assay

RhoA-GTP activity was measured using the G-LISA RhoA activation assay Biochem kit (Cytoskeleton Inc., Denver, CO, USA) according to manufacturer's instructions. Briefly, cells were grown to 60% confluence and then serum starved overnight. On the following day, cells were stimulated by 100 ng/ml of CCL17 for 10 min. Cells were washed twice by ice cold PBS and lysed in 0.3 ml lysis buffer of the kit on ice for 10 min. Then cells were homogenized using a 20-gauge needle for 20 strokes on ice and centrifuged at 14000 xg for 5 min at 4 ºC. Supernatants were collected, snap frozen in liquid nitrogen and stored at -80ºC until used. 30 µL of the supernatants were used for protein concentration determination using Precision Red Advanced Protein Assay supplied with the kit. For quantitative detection of active RhoA, 1 mg/ml of protein was used and absorbance was read at 490 nm using a microplate ELISA reader.

Statistical Analysis

All statistical analyses were performed using SigmaPlot® 10 software. For multiple comparisons Kruskal-Wallis One Way Analysis of variance on ranks followed by the Dunnett's post hoc test was used. *P*-value < 0.05 was considered significant.

Results

Simvastatin inhibits CCL17-induced colon cancer cell migration

To evaluate the effect of simvastatin on migration, HT-29 cells were treated with different concentrations of simvastatin (25 and 50 μM) for 24h in the presence or absence of CCL17. It was found that challenge with CCL17 caused a marked increase in HT-29 cell migration and co-incubation with simvastatin dose-dependently decreased CCL17-evoked colon cancer cell migration (Fig. 1).

Simvastatin has no effect on proliferation and apoptosis

Next, we examined the effect of simvastatin (25 and 50 μM) on colon cancer cell proliferation and apoptosis. It was observed that these doses of simvastatin had no effect on HT-29 cell proliferation and apoptosis (Fig. 2A and B).

CCL17-CCR4 axis activates RhoA

As shown previously [10], we observed that CCL17 significantly increased RhoA activity in colon cancer cells. Notably, it was found that co-incubation of HT-29 colon cancer cells with CCR4 receptor antagonist markedly decreased CCL17-induced RhoA activation in the colon cancer cells (Fig. 3A).

Role of HMG-CoA reductase and geranygeranylation in RhoA activation

As expected, CCL17 stimulation increased RhoA activation in HT-29 colon cancer cells. Interestingly, we found that simvastatin significantly attenuated CCL17-evoked activation of RhoA in the colon cancer cells. Co-administration of simvastatin-treated colon cancer cells with mevalonate abolished the simvastatin-induced inhibition of RhoA activity in HT-29 cells (Fig. 3B). In addition, we detected that co-incubation with

GGPP completely reversed the inhibitory impact of simvastatin on CCL17-induced RhoA activation in the colon cancer cells (Fig. 3B).

Role of HMG-CoA reductase and geranygeranylation in cell migration

We next asked whether the inhibitory effect of simvastatin on colon cancer cells may be due to inhibition of HMG-CoA reductase. For this purpose, we co-incubated simvastatin-treated HT-29 cells with mevalonate. We found that mevalonate significantly reversed the inhibitory effect of simvastatin on CCL17-induced colon cancer cell migration (Fig. 4). Moreover, it was observed that co-incubation of simvastatin-treated HT-29 cells with GGPP abolished the simvastatin-evoked reduction of colon cancer cell migration induced by CCL17 (Fig. 4).

Discussion

This study demonstrates that simvastatin is a potent inhibitor of CCL17-induced colon cancer cell migration. Moreover, mevalonate and GGPP not only reversed simvastatin-induced inhibition of HT-29 cell migration but also reversed the inhibitory effect of simvastatin on RhoA activation in colon cancer cells. Our novel findings indicate that simvastatin inhibits colon cancer cell migration via blocking of geranylgeranylation and RhoA activation. Based on our data, we suggest that targeting HMG-CoA reductase, geranylgeranylation and RhoA signaling might be useful ways to antagonize the spread of colon cancer cells.

Statins are mainly used to lower cholesterol values in patients with cardiovascular diseases. Convincing data have shown that statins exert pleiotropic effects besides their well-known potency to decrease levels of cholesterol, including anti-tumoral effects [17]. For example, previous studies have shown that statins, such as simvastatin, are powerful inhibitors of colon cancer cell proliferation [18]. Others have reported that simvastatin induces cancer cell apoptosis [19] and can inhibit tumor-associated angiogenesis [20]. Tumor cell migration is a critical step in the metastasis process of malignant cells [16]. In the present study, we demonstrate for the first time that simvastatin can attenuate colon cancer cell migration. In fact, simvastatin inhibited HT-29 cell migration in response to CCL17, which interact with CCR4, a chemokine receptor recently identified to be expressed on colon cancer cells [10]. In addition, our results showed that simvastatin decreased CCL17-induced HT-29 cell migration without any concomitant effects on cell proliferation of apoptosis, indicating a direct effect of simvastatin on colon cancer cell migration, which might be of beneficial value. Although this is the first report to show that simvastatin inhibits colon cancer cell migration in response to chemokine signaling, other investigations

have found that simvastatin can inhibit migration of breast [21], hepatic [22], renal [17] and prostate [23] cancer cells, supporting the concept that simvastatin is an effective inhibitor of tumor cell migration.

Statins, such as simvastatin, inhibits HMG-CoA reductase, which is the rate-limiting enzyme in the formation of mevalonate [24]. Mevalonate is not only used in the synthesis of cholesterol but is also a precursor of isoprenoid metabolites, including GGPP [25]. Isoprenoids are needed for relocation of members of the Rho family of GTPases, including Rho, Rac, and Cdc42, to the cell membrane where they can activate downstream signaling mechanisms [26]. In this context, it is interesting to note that overexpression of RhoA has been reported to be associated worse prognosis of patients with CRC [27]. As shown previously [10], we could confirm herein that CCL17 stimulation increased RhoA activity in colon cancer cells. Moreover, we found that inhibition of the CCL17 receptor CCR4 markedly decreased CCL17-induced activation of RhoA, indicating for the first time a functional role of CCR4 in regulating RhoA activity in colon cancer cells. We next wanted to examine the role of HMG-CoA reductase and geranylgeranylation in CCL17-dependent activation of RhoA in colon cancer cells. It was found that addition of mevalonate and GGPP both completely reversed simvastatin-induced inhibition of RhoA activity, suggesting that HMG-CoA reductase and geranylgeranylation constitute critical components in the regulation of CCL17-induced RhoA activation in colon cancer cells. Numerous studies have shown that RhoA regulates downstream activity of Rho-kinase, which phosphorylates myosin light chain and, thereby regulate actinomyosin assembly [28,29]. Knowing that increased expression of Rho-kinase in colon carcinomas is associated with increased tumor cell dissemination [30] and that Rho-kinase controls colon cancer migration [31], we next investigated the role of

HMG-CoA reductase and geranylgeranylation in simvastatin-evoked inhibition of colon cancer cell migration. It was found that co-incubation with mevalonate and GGPP reversed the inhibitory effect of simvastatin, indicating that both HMG-CoA reductase and geranylgeranylation play an important role in the simvastatin-induced interference with CCL17-provoked colon cancer cell migration.

Taken together, our novel findings suggest that simvastatin is a powerful inhibitor of CCL17-dependent colon cancer cell migration. Moreover, simvastatin-induced antagonism of RhoA activity and colon cancer cell migration are mediated via inhibition HMGCoA reductase and geranylgeranylation. Thus, these results provide new evidence indicating that statins may be of beneficial value in order to antagonize colon cancer cell metastasis.

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Figure legends

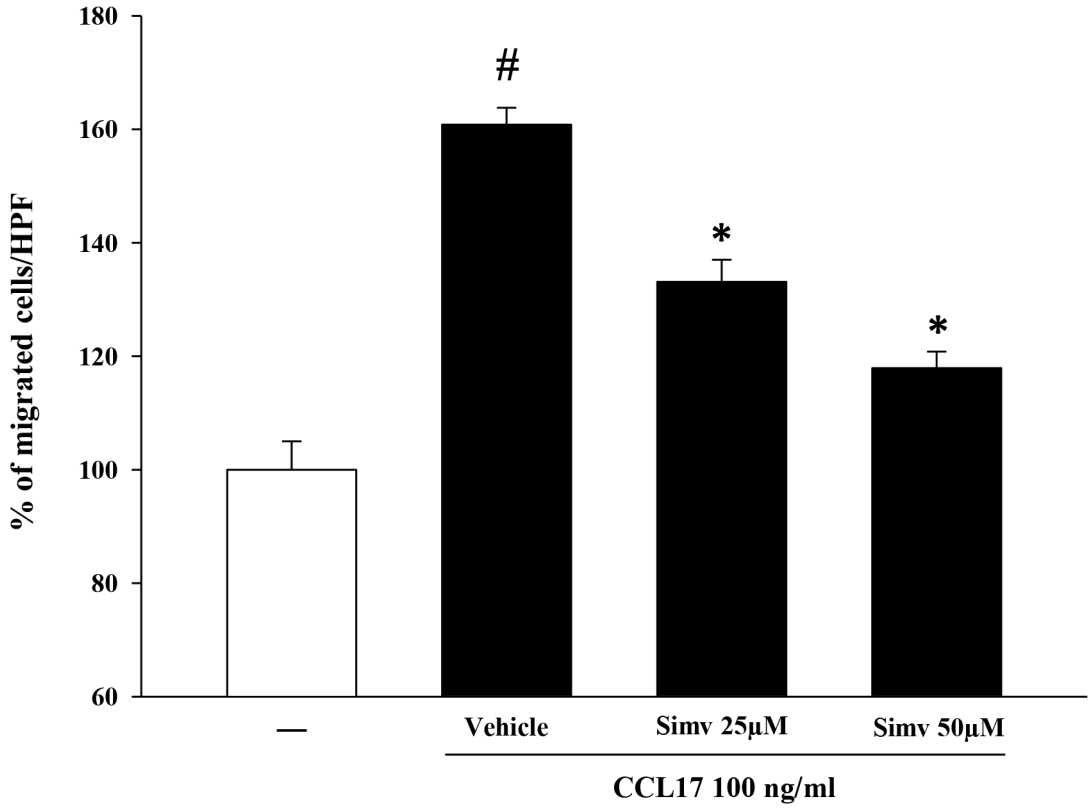
Fig. 1. Simvastatin inhibits CCL17-induced colon cancer cell migration. HT-29 colon cancer cells were pre-treated with simvastatin (25 and 50 μM) or vehicle for 24h and then stimulated with CCL17 (100 ng/ml) for 24h. Cells were counted microscopically using 10 High Power Fields in five different fields. Migration index was calculated as the ratio of the number of migrated cells on wells containing CCL17 divided by the number of cells in the control wells. Data represents mean ± SEM and $n = 4$. $^{\#}P < 0.05$ versus negative control and $^{*}P < 0.05$ versus vehicle + CCL17.

Fig. 2. No effect of simvastatin on colon cancer cell proliferation and apoptosis. (A) Proliferation of colon cancer cells was assessed using Calcein AM fluorescence assay. HT-29 colon cancer cells were seeded in 96-wells with or without simvastatin (25 and 50μM) for 24h at 37ºC (5% CO2). Fluorescence was measured at excitation 485 nm and emission of 530 nm. **(B)** Early colon cancer cell apoptosis was evaluated by Annexin V/FITC-PI assay as described in Materials and Methods. HT-29 colon cancer cells were incubated with or without simvastatin (25 and 50μM for 24h. The number of early apoptotic cells was counted and expressed as the percentage of annexin V positive and PI negative cells. Data represents mean ± SEM and n = 4.

Fig. 3. Effects of CCR4 antagonism and simvastatin treatment on RhoA activity. (A) CCR4 regulates CCL17-induced RhoA activation in colon cancer cells. HT-29 colon cancer cells were pretreated with a CCR4 antagonist (200 ng/ml) for 30 min and then stimulated with CCL17 (100 ng/ml) for 10 min. RhoA-GTP activation was quantified using G-LISA activation assay. Data represent mean ± SEM and $n = 4$. $^{\#}P < 0.05$ versus negative control and $^{*}P < 0.05$ versus vehicle + CCL17**. (B) Simvastatin inhibits CCL17-induced RhoA activity in colon cancer cells.** HT-29 colon cancer cells were pre-treated with simvastatin (50 μM) for 24h before stimulation with CCL17 (100 ng/ml). Cells were co-

incubated with mevalonate (100 μM) and GGPP (10 μM) as indicated. Active RhoA levels were quantified using G-LISA activation assay as described in materials and methods. Serum free media served as negative control. Data represent mean ± SEM and n = 4. #P < 0.05 versus negative control and *P < 0.05 versus vehicle + CCL17; **P < 0.05 versus CCL17 + simvastatin.

Fig. 4. Role of HMG-CoA reductase and geranylgeranylation in CCL17-induced colon cancer cell migration. HT-29 colon cancer cells were pre-treated with simvastatin (50 μM) or vehicle for 24h and then stimulated with CCL17 (100 ng/ml) for 24h. Cells were co-incubated with mevalonate (100 μM) and GGPP (10 μM) as indicated. Cells were counted microscopically using 10 High Power Fields in five different fields. Migration index was calculated as the ratio of the number of migrated cells on wells containing CCL17 divided by the number of cells in the control wells. Data represent mean ± SEM and $n = 4$. $^{\#}P < 0.05$ versus negative control and $^{*}P < 0.05$ versus vehicle + CCL17; **P < 0.05 versus CCL17 + simvastatin.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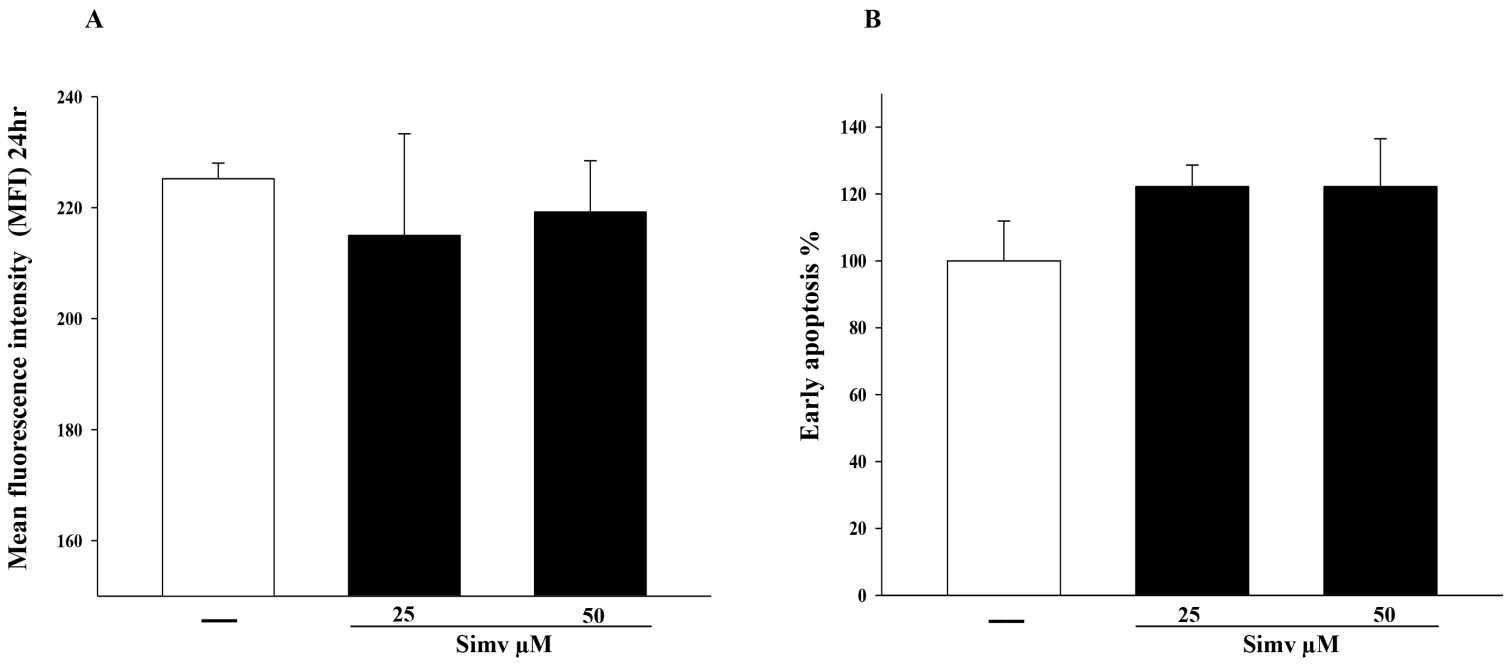
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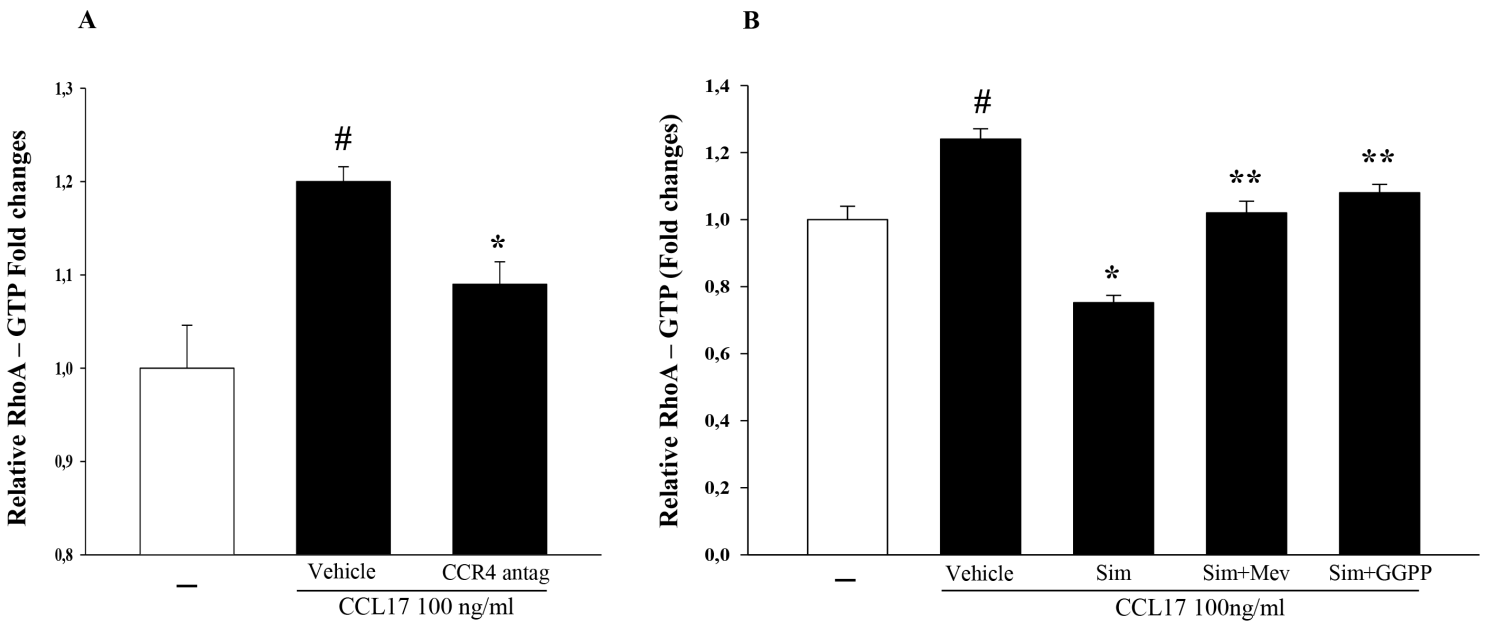
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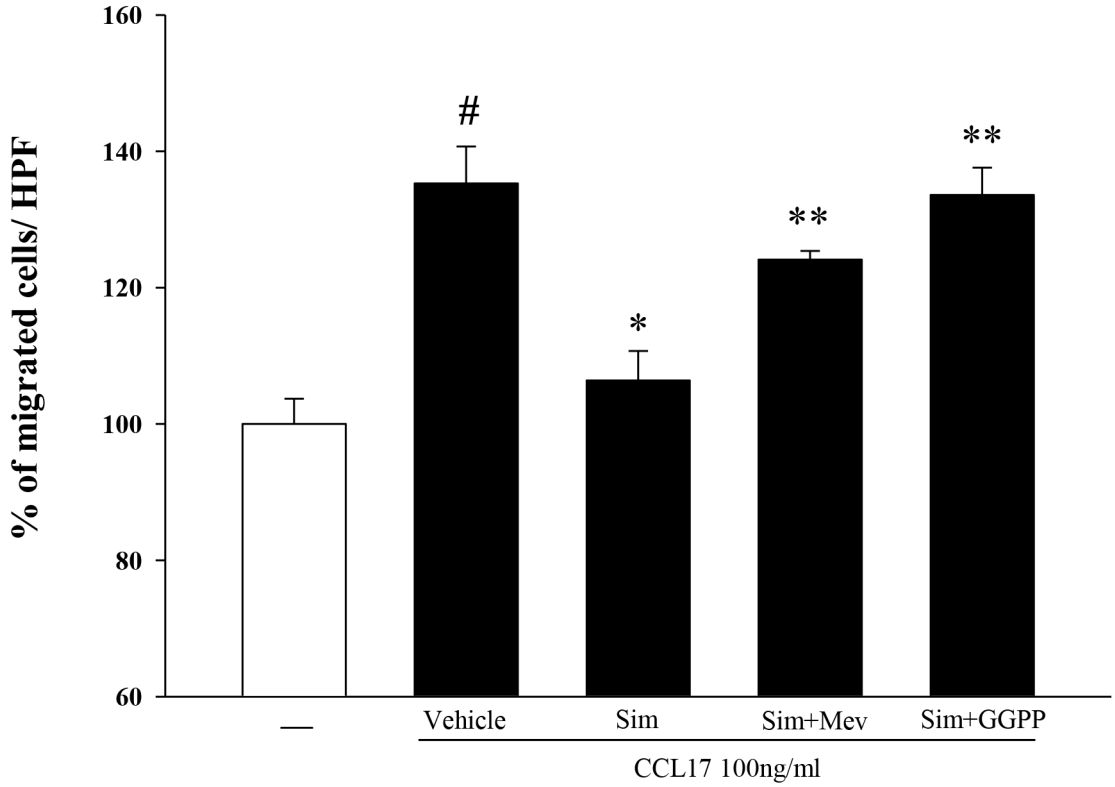
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