Regulation of S1P receptors and sphingosine kinases expression in acute pulmonary endothelial cell injury

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Background. Acute lung injury and acute respiratory distress syndrome (ALI /ARDS) is a severe clinical syndrome with mortality rate as high as 30-40%. There is no treatment yet to improve pulmonary endothelial barrier function in patients with severe pulmonary edema. Developing therapies to protect endothelial barrier integrity and stabilizing gas exchange is getting more and more attention. Sphingosine-1-phosphate (S1P) is able to enhance the resistance of endothelial cell barrier. S1P at physiological concentrations plays an important role in maintaining endothelial barrier function. Proliferation, regeneration and anti-inflammatory activity that mesenchymal stem cells (MSCs) exhibit make it possible to regulate the homeostatic control of S1P.

Methods. By building a pulmonary endothelial cell model of acute injury, we investigated the regulation of S1P receptors and sphingosine kinases expression by MSCs during the treatment of acute lung injury using RT-PCR, and investigated the HPAECs Micro-electronics impedance using Real Time Cellular Analysis.

Results. It was found that the down-regulation of TNF-α expression was more significant when MSC was used in combination with S1P. The combination effection mainly worked on S1PR2, S1PR3 and SphK2. The results show that when MSCs were used in combination with S1P, the selectivity of S1P receptors was increased and the homeostatic control of S1P concentration was improved through regulation of expression of S1P metabolic enzymes. **Discussions.** The study found that as a potential treatment, MSCs could work on multiple S1P related genes,
simultaneously. When it was used in combination with S1P, the expression regulation result of related genes was not simply the superposition of each other, but more significant outcome was obtained. This study establishes the experimental basis for, further exploring the efficacy of improving endothelial barrier function in acute lung injury, using MSCs in combination with S1P and their possible synergistic mechanism.

1   **Regulation of S1P receptors and sphingosine kinases expression in acute pulmonary**

2   **endothelial cell injury**

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18   **Abstract:**

19   **Background.** Acute lung injury and acute respiratory distress syndrome (ALI /ARDS) is a severe

20   clinical syndrome with mortality rate as high as 30-40%. There is no treatment yet to improve

21   pulmonary endothelial barrier function in patients with severe pulmonary edema. Developing

22   therapies to protect endothelial barrier integrity and stabilizing gas exchange is getting more and

23   more attention. Sphingosine-1-phosphate (S1P) is able to enhance the resistance of endothelial cell

24   barrier. S1P at physiological concentrations plays an important role in maintaining endothelial

25   barrier function. Proliferation, regeneration and anti-inflammatory activity that mesenchymal stem

26   cells (MSCs) exhibit make it possible to regulate the homeostatic control of S1P.

27   **Methods.** By building a pulmonary endothelial cell model of acute injury, we investigated the

28   regulation of S1P receptors and sphingosine kinases expression by MSCs during the treatment of

29   acute lung injury using RT-PCR, and investigated the HPAECs Micro-electronics impedance

30   using Real Time Cellular Analysis.

31 **Results.** It was found that the down-regulation of TNF-α expression was more significant when

32 MSC was used in combination with S1P. The combination effect mainly worked on S1PR2, S1PR3

33 and SphK2. The results show that when MSCs were used in combination with S1P, the selectivity

34 of S1P receptors was increased and the homeostatic control of S1P concentration was improved

35 through regulation of expression of S1P metabolic enzymes.

36 **Discussions.** The study found that as a potential treatment, MSCs could work on multiple S1P

37 related genes, simultaneously. When it was used in combination with S1P, the expression

38 regulation result of related genes was not simply the superposition of each other, but more

39 significant outcome was obtained. This study establishes the experimental basis for, further

40 exploring the efficacy of improving endothelial barrier function in acute lung injury, using MSCs

41 in   combination   with   S1P   and   their   possible   synergistic   mechanism.

Introduction

Acute lung injury and acute respiratory distress syndrome (ALI/ARDS) was first recognized as a clinical syndrome in the 1960s. It manifests as severe and acute respiratory failure with hypoxemia and lung infiltration and is often caused by pneumonia, sepsis and major trauma. The mortality rate of ALI/ARDS is as high as 30-40% (Rubenfeld et al. 2005). In the large number of drugs evaluated by clinical trials, none has proven to be effective or could be recommended as the standard treatment of ALI/ARDS(Cepkova & Matthay 2006). Supportive therapy is currently the major treatment, including protective ventilation and conservative medication (Brower et al. 2000; Wiedemann et al. 2006). Mechanical ventilation is a necessary and life-saving method, but it may delay the inflammatory response and ultimately results in pulmonary endothelial barrier dysfunction. Endothelial barrier dysfunction can lead to increased permeability, extravasation of fluid rich in proteins and pulmonary edema, which are all common symptoms of ALI/ARDS (Kumar et al. 2008). There is currently no treatment to improve pulmonary endothelial barrier function in severe pulmonary edema patients (Müller-Redetzky et al. 2014). Developing a therapy to protect endothelial barrier integrity and stabilize gas exchange is getting a lot of attention.

Sphingosine-1-phosphate (S1P) is a ubiquitous sphingomyelin and is an important regulator of vascular endothelial cell permeability and fluid balance *in vivo*. It is mainly present in plasma and tissues and is able to enhance the resistance of endothelial barrier. Endothelial barrier enhancement mediated by S1P is completed by activating the Gi and Racl signaling pathway through S1P receptor (Sun et al. 2012). Studies have demonstrated that S1P plays an important role in allergic reactions in the respiratory system. S1P promotes the adhesion of endothelial cells, which is the key in maintaining endothelial barrier and avoiding increased permeability leading to pulmonary edema (Lee et al. 1999). FTY720, structure analogue of S1P, was approved by FDA in the treatment of multiple sclerosis in 2010. FTY720 slightly differs from S1P in receptor binding activity, but they both have strong side effects at high doses (Natarajan et al. 2013). This indicates that in the treatment of lung disease, selectivity of S1P receptors and homeostatic control of S1P concentration is important.

69   Several studies have demonstrated that S1P, at physiological concentration, has an important

70   role in maintaining endothelial barrier function. For S1P and its structure analogues, their

71   limitations in therapeutic function, confirm the conception of sphingolipid homeostasis. The fate

72   of the cells is determined by the homeostasis of S1P concentration (Cuvillier et al. 1996). As an

73   endogenous bioactive molecule, S1P is an important regulator of vascular endothelial cell

74   permeability and fluid balance. Its anabolic and key molecules on signaling pathways are still

75   potential targets and focus of research in the treatment of ALI/ARDS.

76   Recent clinical studies have found that it is unlikely for any single drug to reverse the severe

77   pathological injury caused by acute lung injury. Therefore, in the therapy of ALI/ARDS, there has

78   been attention focused on cell therapy, especially mesenchymal stem cell therapy.

79   Mesenchymal stem cells (MSCs) are multi-functional cells with the ability of self-renewal.

80   They were first discovered in bone marrow and can differentiate into bone, cartilage, fat, muscle

81   and other tissues. At present, MSCs have demonstrated great efficacy in the treatment of cirrhosis

82   (Zheng et al. 2012), systemic lupus erythematosus(Wang et al. 2013) and other diseases.

83   Therapeutic effects have also been obtained in preclinical studies of Crohn's disease (Valcz et al.

84   2011), traumatic brain injury (Harting et al. 2009), sepsis (Nemeth et al. 2008), acute renal

85   failure(Bruno et al. 2009) and other diseases. In recent years, several studies have reported, using

86   a number of animal models of lung disease that allograft MSC therapy can reduce lung injury?

87   (Chang et al. 2009; Moodley et al. 2009; Ortiz et al. 2003)

88   Most recent studies involving MSCs in the treatment of ALI/ARDS are focused on the

89   evaluation of efficacy, with no further discussion of the immunomodulatory impact of MSCs on

90   pulmonary tissue cells, especially the endothelial cell barrier. The regulation of S1P metabolism

91   by MSCs is lacking in many systematic and comprehensive studies too. Homeostatic control of

92   S1P *in vivo* is a prerequisite to improving endothelial barrier and treating acute lung injury. The

93   anabolic process of S1P is regulated by many cytokines. The outcomes of many diseases when

94   MSCs were used demonstrate that it can secrete various growth and inflammatory factors, playing

95   a role in immune regulation. Besides, according to some studies, S1P is capable of promoting the

96   differentiation of MSCs *in vitro* (He et al. 2010). This suggests the possible synergistic mechanism

97   of MSCs and S1P in the treatment of ALI/ARDS. On the one hand, while S1P plays a role in

98   enhancing endothelial barrier function, MSCs modulates the host's immune response to injury

99   through its proliferation, regeneration and anti-inflammatory effects. On the other hand, with the

100   differentiation accelerating effect of S1P on MSCs, the MSCs have better immune regulation. The

101   effect of MSC on the expression of S1P receptors and metabolic enzymes, leads to better in vivo

102   homeostasis of S1P which in turn enhances the endothelial barrier.

103   Therefore in this study, by building an acute injury model of pulmonary endothelial cells

104   induced by LPS, the regulatory effect of MSCs on the expression of S1P receptors and

105   sphingosine kinase, in the treatment of acute lung injury was investigated. The efficacy of

106   improving endothelial barrier function in acute lung injury when MSCs were used in

107   combination with S1P and their possible synergistic mechanism are discussed. Thus it provides

108   theoretical and experimental basis for the treatment of acute lung injury.

109   **Materials and methods**

110   **Standards and reagents**

111   Sphingosine-1-Phosphate (S1P) purchased from Sigma-Aldrich Company was dissolved in

112   methanol and kept at -20°C. Lipopolysaccharide (LPS) E.coli O55:B5 purchased from Sigma-

113   Aldrich Company was dissolved in saline and kept at -20°C.

114   **Antibody**

115   Anti-EDG-1 (S1PR1), Anti-EDG-5 (S1PR2) and Anti-EDG-3 (S1PR3) were purchased

116   from Santa Cruz Company. Anti-β-actin was purchased from Sigma-Aldrich Company.

117   **Cell culture**

118   Newborn umbilical cord was taken under sterile conditions (The Affiliated Hospital of Military

119   Medical Science Scientific Research Ethics Committee Approval Report, № ky-2015-3-17)and

120   remnant blood in umbilical vein and artery was rinsed in D-HANKS solution containing 10^5U/L

121   penicillin and 100 mg/L streptomycin. Collagenase IV solution was added to digest the cells, at

122   37℃ for 2 hours. Single cell suspension of MSCs was obtained following filtration. After the

123   cells were counted, the suspension was inoculated into 10 ml MSC medium (Thermo Fisher,

124   USA) in a 100 mm petri dish with a cell density of 2.5 to $4\times10^4/cm^2$. After primary culture at

125   37℃ and 5% CO_2 in an incubator for 7 to 9 days, first passage was carried out according to

126   overall growth condition and local density. Incubation was continued until the density exceeds

127   80%, repeated the above operations. Subculture generations at P3 to P5 were used for subsequent

128   experiments. Human pulmonary artery endothelial cells (HPAECs) purchased from ScienCell

129   Company were cultured in endothelial cell medium (ScienCell, USA) at 37℃ in a 5% CO_2 in

130   incubator. P5 to P8 cells were used for subsequent experiments.

Acute Injury of cells

132   HPAEC cells between P5 to P8 were inoculated into a 16-well E-Plate (ACEA Biosciences,

133   USA) at a concentration of 2×10^4 per well in 100 µl, then cultured in an incubator at 37℃ and 5%

134   CO_2 for 12 hours following which LPS was added at varying concentrations of 0, 0.5, 1, 2 and 5

135   µM. The 16-well E-Plates were placed within the Real Time Cellular Analysis (RTCA) System

136   (ACEA Biosciences, USA) and cultured in an incubator at 37℃ and 5% CO_2. Micro-electrical

137   impedance of HPAEC cells were detected in real-time to investigate the effects of acute injury

138   caused by LPS on HPAEC cells, at different concentrations.

139   HPAEC cells between P5 to P8 were inoculated into a 24-well cell culture plate (Corning,

140   USA) at a concentration of 1×10^5 per well in 600 µl, then cultured in incubator at 37℃ and 5%

141   CO_2 for 6 to 12 hours after LPS was added at a final concentration of 1 µM. Cells were collected

142   at different time points and the expression of TNF-α was detected to investigate the acute injury

143   effects of LPS on HPAEC cells.

Cell co-culture

145   HPAEC cells between P5 to P8 were inoculated into a 16-well E-Plate (ACEA Biosciences,

146   USA) in 100 µl at a concentration of 2×10^4 per well and then LPS at a concentration of 1 µM was

147   added. In the meantime, 60 µl of MSCs at different proportions (wherein the ratio of HPAEC and

148   MSC were1:1, 1:2, and 1:4, the control group is MSCs medium only) were inoculated into a 16-

149 well E-Plate Insert (ACEA Biosciences, USA),   Then Insert was placed into Receiver Plate

150 containing 100 μl of MSC medium. The 16-well E-Plates were placed within the RTCA System,

151 together with the Receiver Plates, were cultured in an incubator at 37℃ and 5% CO_2 for 12 hours.

152 Following that, medium in E-Plates was discarded and replaced with 100 μl of fresh endothelial

153 cell medium and the Insert was filled to 60 μl with MSC medium in the corresponding Receiver

154 Plates. Then Inserts was placed into E-Plates containing HPAECs and co-cultured within the

155 RTCA System at 37℃ and 5% CO_2 for 8-24 hours. Micro-electrical impedance of HPAEC cells

156 were detected in real-time to investigate the effect of MSCs on HPAEC cell injury.

157 HPAEC cells between P5 to P8 in 600 μl medium were inoculated into a 24-well cell culture

158 plate at a concentration of 1×10^5 per well following which LPS at a concentration of 1 μM was

159 added. Meanwhile, 100 μl of MSCs with different proportions (wherein the ratio of HPAEC and

160 MSC were 1:1, 1:2, and 1:4, the control group is MSCs medium only) were inoculated into the

161 upper compartment of a 24 well Transwell plate (0.4 μm Polyester Membrane, Corning, USA),

162 and 600 μl of MSC medium was added into the lower compartment. The 24-well cell culture plates

163 and Transwell plates were incubated at 37℃ and 5% CO_2 for 12 hours. Then the medium in the

164 cell culture plates was discarded and replaced with 600 μl of fresh endothelial cell medium, the

165 upper compartment of the Transwell plates was filled to 100 μl with MSC medium in the

166 corresponding lower compartments. After 8 to 24 hours of co-culture with the upper compartment

167 of Transwell plates placed into cell culture plates, HPAEC cells were collected. The effect of

168 MSCs on HPAEC cell injury was investigated by determining the expression change of TNF-α.

169 **Detection of Cell Micro-electronics impedance**

170 16-well E-Plate was placed on Xcelligence RTCA DP system (ACEA Biosciences, USA) and

171 incubated at 37℃ and 5% CO_2. Detection was determined according to the manufacturer's

172 instructions.

173 **Real-time quantitative PCR**

174 Total RNA was extracted from collected HPAEC cells using RNeasy®Mini Kit (Qiagen,

175 USA) according to manufacturer's instructions. With 1 μg of RNA as template, reverse

176 transcription reaction was performed using QuantScript RT Kit (TIANGEN, Beijing, China)

177 according to manufacturer's instructions. Utilizing Bio-Rad iQ™5 Multicolor Real-Time PCR

178 Detection System and with GAPDH as internal reference, fluorescence intensity was detected

179 using SuperReal PreMix SYBR Green (TIANGEN, Beijing, China) according to the

180 manufacturer's instructions. The primers used are shown in Table 1. Relative expression level of

181 mRNA was calculated by 2-ΔΔCt method.

182 **Western blot analysis**

183 The collected HPAEC cells were washed with PBS twice. Then 200μl lysis buffer was added,

184 which contains 20mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton

185 X-100, 2.5mM pyrophosphate sodium phosphate, 1μg/ml leupeptin, 1μg/ml aprotinin and protease

186 inhibitors (Roche, USA). Cell lysate was centrifuged at $10,000 \times g$, room temperature for 10 min

187 and the supernatant was collected. After protein concentration was measured using BCA method,

188 20 to 50μg of protein was separated by 12% SDS-PAGE, then transferred to a PVDF membrane

189 and sealed with TBST buffer containing 5% BSA. Specific antibodies were added and incubated

190 overnight. On the next day, after washing and secondary antibody incubation, chromogenic assay

191 was performed using ECL chemiluminescence kit (GE Healthcare, USA).

192 **Statistical analysis**

193 All experimental data are represented as mean ± standard deviation (mean ± SD). One-way

194 ANOVA (Dunnett test) was adopted in multiple group comparison. Data was analyzed with

195 software GraphPad Prism 6.0. $p<0.05$ indicates that the difference is statistically significant.

196 **Results**

197 **Acute injury can be caused 12 hours after exposure of HPAECs to 1μM LPS**

198 LPS is widely used in the preparation of acute lung injury model. However, there is no

199 standard procedure that has been developed. Literatures shows that acute injury induced by LPS

200 will cause significant endothelial cell barrier disorder and increase in permeability. In the

201 meantime, there is abnormal expression of a variety of growth and inflammatory factors.

202 Particularly, the expression of TNFα is significantly increased (Meduri et al. 2009). Here HPAECs

203 were stimulated using LPS at varying final concentrations of 0, 0.5, 1, 2 and 5μM for 12 hours,

204 respectively. The micro-electrical impedance changes of endothelial cells were detected by Real

205 Time Cellular Analysis (RTCA) to reflect the changes in endothelial barrier function (Fig. 1A).

206 Results show that compared to other concentrations, micro-electrical impedance dropped

207 significantly when 1μM LPS was added to HPAECs (Fig. 1B) .This indicates that 1μM LPS is

208 more effective in acute injury. Furthermore, HPAECs were stimulated by LPS at a concentration

209 of 1μM for different time to detect the change in TNF-α expression. Results demonstrated that the

210 expression of TNFα was significantly increased at 12 hours after stimulation (Fig. 1C). This

211 suggests that HPAECs stimulated by 1μM LPS for 12 hours can produce an acute cell injury model

212 for subsequent studies.

213

214 **Fig. 1** Real Time Cellular Analysis (RTCA) of micro-electrical impedance changes of HPAECs.

215 **A.** A schematic showing the experimental design. **B**. Micro-electrical impedance of HPAECs

216 detected in real-time after 12 hours of culture with different final concentrations of LPS. **C**.

217 Histogram showing the change in TNF-α expression in HPAECs, at different time points using

218 RT-PCR, in the presence of 1 μM LPS (*$p < 0.05$).

219

220 **The effect of MSCs on acute injury of HPAECs**

221 HPAECs were incubated in a 24-well plate with 1 μM of LPS and were divided into 4 groups,

222 based on the treatment timeline, control group, 8, 16 and 24 h treatment groups. Following this the

223 cells were non-contact co-cultured with MSCs. The cells were collected and the change in

224 expression of TNFα was examined by RT-PCR. Results show that the effective treatment window

225 of acute endothelial cell injury by MSCs is 8h (Fig. 2A).

226 In addition the treatment groups were divided into another 4 groups based on the cell ratio as,

227 control group (HPAECs only), groups with inoculation ratio of HPAECs and MSCs 1:1, 1:2 and

228 1:4. They were non-contact co-cultured with HPAECs following treatment with LPS. Cells of each

229 group were collected and the change in expression of TNFα was examined by RT-PCR. Results

230 show that larger ratio of MSCs is not necessarily better. Best treatment effect was achieved when

231   the ratio of HPAECs and MSCs was 1:2 (Fig. 2B). Results from label-free Real Time Cellular

232   Analysis (RTCA) also show that, compared to that of the control group, the micro-electrical

233   impedance of groups where HPAECs were co-cultured with MSCs were significantly higher and

234   reached their peak after 10 hours. Among them, micro-electrical impedance of the group with the

235   ratio of HPAECs to MSCs 1:2 was the highest. 15 hours later, micro-electrical impedance of the

236   control group was significantly higher than that of the other groups (Fig. 2C). The results were

237   consistent with the change in expression of TNF-α, indicating that the effect of MSCs on acute

238   injury of HPAECs was best at a ratio of 1:2 and the best treatment time is about 8 to 10 hours.

239

240   **Fig. 2**. **A.** The change in expression of TNFα at different time points when injured HPAECs
241   were co-cultured with MSCs. Histogram showing TNFα expression in HPAECs, using RT-PCR.
242   (*p<0.05, **p<0.01). **B.** The change in expression of TNFα when injured HPAECs were co-
243   cultured with different proportions of MSCs as examined at different time points by RT-PCR
244   (*p<0.05 and **p<0.01). **C.** The micro-electrical impedance of HPAECs exposed to LPS and co-
245   cultured with MSCs as examined using RTCA system.

246

247   **The effect of S1P acute HPAEC injury**

248   Sphingosine-1-phosphate (S1P) is a biologically active ubiquitous sphingomyelin. It is

249   mainly produced by platelets and also present in plasma and tissues. When its physiological

250   concentration is in the range of 0.2 to 1.1 μM, it can effectively enhance the endothelial barrier

251   function. In this study, a HPAEC acute injury model was established using 1μM LPS and treated

252   with S1P at final concentrations of 0, 0.5, 1, 2, and 5μM . The effect of LPS on HPAEC injury was

253   studied by RTCA experiment. Results show that, S1P within physiological concentrations can

254   effectively enhance the micro-electrical impedance of HPAECs (Fig. 3A). In addition HPAECs

255   were cultured in a 16-well E-Plate and 1μM LPS was added to induce injury. Meanwhile, MSCs

256   at the twice concentration was inoculated into a 16-well E-Plate Insert. After incubated for 12

257   hours, the LPS medium was discarded and S1P at concentrations of 0, 0.5, 1, 2, and 5μM were

258   added in the E-Plate and E-Plate Insert for co-culture , then the effect on HPAEC injury was studied

259   by RTCA. Results show that S1P within physiological concentration (0.5 and 1μM) can effectively

260   enhance the micro-electrical impedance of HPAECs and alleviate LPS induced injury to HPAECs

261   (Fig. 3B). Both sets of data show that the enhancing effect of 0.5μM S1P on micro-electrical

262   impedance was superior to that of 1μM S1P in the presence or absence of MSCs. In addition, using

263   a Transwell plate, HPAECs exposed to 1μM LPS for 12 hours were co-cultured with MSCs at a

264   ratio of 1:2 and 0.5μM S1P for 8 hours. HPAECs were collected and the change in expression of

265   TNF-α was determined. Results show that compared to MSCs alone, the down-regulation of TNF-

266   α expression was more significant when S1P was combined with MSCs (Fig. 3C).

267

268   **Fig. 3.** The combined effect of LPS and S1P on micro-electrical impedance of HPAECs. **A**. The

269   effect of S1P on HPAEC injury studied using RTCA. Results show that, S1P within

270   physiological concentration (0.5 and 1μM) can effectively enhance the micro-electrical

271   impedance of HPAECs. **B**. The combined effect of MSCs and S1P on micro-electrical

272   impedance of LPS injuried HPAECs as examined using the RTCA system. **C**. Change in

273   expression of TNFα in injured HPAECs when treated with S1P and MSCs using RT-PCR

274   (**$p<0.01$).

275   **Effects of S1P and MSCs combination therapy compared to MSCs alone**

276   In order to further investigate the possible synergistic mechanism of MSC and S1P in the

277   treatment of HPAEC injury, the change in S1P receptors and sphingosine kinase expression in

278   HPAECs treated with MSCs alone or the combination of MSCs with S1P, were determined using

279   RT-PCR. After 12 hours following exposure to LPS, the expression changes of S1P receptors

280   (S1PR1, S1PR2 and S1PR3) and sphingosine kinases (SphK1 and SphK2) in HPAECs were

281   studied using RT-PCR. Results demonstrate that the expression of target genes were dramatically

282   increased following LPS induced injury. The expression of S1PR1 and S1PR3 were increased

283   significantly, while that of S1P2 was not. There was no significant difference in the expression

284   levels of SphK1 and SphK2 following LPS induced injury (Fig. 4). These results indicate that the

285   S1P related genes could act as potential therapeutic targets. It also suggests that acute lung injury

286   is a complex pathophysiological process and the treatment directed to a single target may not

287   achieve the best efficacy. The combination of MSCs and S1P may become an effective therapy.

288

289 **Fig. 4.** S1P receptor and Sphingosine kinase expression following acute injury. **A**.
290 Representative figure showing expression change of S1P receptors 1, 2 and 3. **B**. Histogram
291 demonstrating the expression change of sphingosine kinases 1 and 2. (*$p<0.05$, **$p<0.01$).
292

293   Furthermore, after 12 hours of LPS exposure, the medium was changed. According to the co-
294 culture condition, HPAECs were divided into 3 groups, that is, control group, MSC therapy group
295 and combined therapy group (with 0.5μM S1P and MSCs at a ratio of 1:2 of HPAECs). After 8
296 hours of non-contact co-culture, HPAECs cells were collected and total RNA were extracted. The
297 changes in expression of S1P related genes were detected using RT-PCR. Results show that
298 compared with MSC alone, the down-regulation of TNF-α expression was significant when MSCs
299 were used in combination with S1P. This indicated that there is a possible synergistic mechanism,
300 between MSCs and S1P, in the treatment of acute injury (Fig. 5A). The regulation results of S1P
301 receptors show that when MSCs work on injured HPAECs, its regulation of S1P receptors were
302 different, affecting only S1PR2 and S1PR3. The expression of S1PR2 and S1PR3 were further
303 reduced when MSCs were used in combination with S1P. There was no significant difference
304 regulatory effect on S1PR1 irrespective of whether MSCs were used alone or in combination with
305 S1P (Fig. 5B).

306

307 **Fig. 5.** Effect of MSC and S1P combination therapy on TNF-α, S1P receptors and Sphingosine
308 kinases. **A.** Histogram demonstrating the expression change of TNF-α. **B**. Histogram
309 demonstrating the expression change of S1P receptors 1, 2 and 3. **C**. Histogram demonstrating
310 the expression change of sphingosine kinases 1 and 2 (** $p<0.01$).

311

312   Studies have shown that the intracellular level of S1P is tightly regulated by the balance
313 between synthesis and degradation. *In vivo*, sphingomyelin (SM) is catalyzed to produce ceramide,
314 which is further hydrolyzed to sphingosine. Sphingosine is able to produce S1P by phosphorylation
315 catalyzed by sphingosine kinase (SphK1 and SphK2). Our study results demonstrate that the
316 expression of sphingosine kinase (SphK1 and SphK2) differs in HPAECs following LPS injury.
317 When MSCs were used alone, it mainly worked on SphK1, the expression level of which was
318 significantly reduced. When MSCs were used in combination with S1P, the expression of SphK2

319 was also significantly reduced (Fig. 5C). Through analyzing the expressions of S1P related genes,

320 we found that when MSCs were combined with S1P, their effect was not simply the

321 superimposition of each other, but more significant. This suggests that there might be some

322 synergistic mechanism between MSCs and S1P in the treatment of acute injury.

323 **Impact of MSC and S1P on S1P receptor Expression in acute injury**

324 After 12 hours of HPAECs exposed to LPS were divided into 3 groups, based on co-culture

325 conditions as, control group, MSC therapy group and combined therapy group (0.5μM S1P and

326 MSCs at a ratio of 1:2 to HPAECs). After 8 hours of non-contact co-culture, HPAECs cells were

327 collected and total proteins were extracted. The changes in expression of S1P receptors were

328 detected through Western-Blot. The results were consistent with that from the RT-PCR. The results

329 of regulation of different S1P receptors were different when MSCs were added, where S1PR2 and

330 S1PR3 were the main targets. The effect on S1PR1 was not significant whether MSCs were used

331 alone or in combination with S1P (Fig. 6).

332

333 **Fig. 6.** S1P receptors expression in HPAECs treated with MSCs. Pictograph showing Western
334 blot analysis of S1P receptor expression HPAECs exposed to LPS that were treated with MSCs
335 alone or in combination with S1P.

336

337 **Discussion**

338 Sphingosine-1-phosphate (S1P) is an agonist mainly present in plasma and tissues. It is an

339 important regulator of vascular endothelial cell permeability and fluid balance *in vivo* and is able

340 to enhance endothelial barrier. Its homeostasis *in vivo* is tightly regulated by the balance between

341 synthesis and degradation. *In vivo*, sphingomyelin (SM) is catalyzed by Sphingomyelinase to

342 produce ceramide (Cer), which produces sphingosine in hydrolysis catalyzed by Neuraminidase.

343 Phosphorylation of sphingosine produces S1P catalyzed by sphingosine kinase (SphK1 and

344 SphK2). Physiological concentrations of S1P plays an important role in maintaining endothelial

345 barrier function. It has been demonstrated that the activity of SphK1 and the dynamic concentration

346 balance of SphK1/S1P axis, are necessary for the modulation of inflammatory signals and are

347 related to the regulation of innate, adaptive and other immune cells. Studies have also shown that

348   abnormal changes in SphK1 or S1P lead to many inflammatory and autoimmune diseases,

349   including asthma, rheumatoid arthritis, sepsis, inflammatory bowel disease and so on(Spiegel &

350   Milstien 2011). Our study found that in LPS induced acute injury of HPAECs, the expression of

351   SphKs was increased dramatically. When MSCs were used alone, only the expression of SphK1

352   was reduced, and there was no significant impact on the expression of SphK2. When MSCs were

353   used in combination with S1P, the expression of SphK1 and SphK2 were reduced simultaneously.

354   This shows that MSCs play a role in the treatment of acute lung injury by regulating the expression

355   and activity of SphKs. It also suggests that a possible synergistic mechanism may exist, between

356   MSCs and S1P, in the treatment of acute HPAEC injury.

357   Studies on S1P show that endothelial barrier enhancement mediated by S1P is completed by

358   activating the Gi and Racl signaling pathway through the S1P receptor(Yu et al. 2012). There are

359   five different types of S1P receptors (S1PR1-5) and their distribution and function are different.

360   Those in endothelial cells are mainly S1PR1-3. Wherein the S1P receptor 1 (S1PR1) has a more

361   important biological role in enhancing endothelial barrier, the S1PR2 suppresses the endothelial

362   barrier function (Blaho & Hla 2014). Studies have shown that when the selective S1PR1

363   competitive antagonist is used in experimental mice, pulmonary endothelial cells integrity is

364   compromised (Rosen et al. 2007; Sanna et al. 2006). Besides, it has also been found that

365   stimulation of nitrification and release of S1PR3 in HPAECs helps to suppress the endothelial

366   barrier function (Sun et al. 2012). These demonstrates that S1P receptors can be the therapeutic

367   targets of ALI/ARDS to improve endothelial cell barrier. In this study We found that when MSCs

368   works on acute injured HPAECs, the regulation of individual S1P receptors were different, which

369   was mainly acted on S1PR2 and S1PR3. The expression of S1PR2 and S1PR3 were further

370   reduced when MSCs were used in combination with S1P. The regulatory effect on S1PR1 was not

371   significant irrespective of whether MSCs were used alone or in combination with S1P. This

372   indicates that MSCs may play a role in enhancing endothelial barrier function by reducing the

373   expression of S1PR2 and S1PR3 and enhancing specificity of S1P receptors.

374   Clinical studies have shown that any single target and drug are not likely to reverse the severe

375   pathological injury caused by ALI/ARDS and achieve great therapeutic effect quickly. Innovative

376   multi-target synergistic treatment mechanism may be an effective method to solve this problem.

377   In essence, stem cell therapy does not work on a single target or as a single drug in therapy, but on

378   multiple targets based on biological distress signals from the diseased tissues and as multiple

379   therapies. Our study found that when MSCs worked on HPEACs with acute injury, the expressions

380   of S1P receptors and sphingosine kinase were regulated. This indicates that MSCs are able to affect

381   the expression of multiple S1P related genes, improve endothelial barrier and cure acute lung

382   injury. Meanwhile, as an endogenous bioactive molecule, S1P is an important regulator of vascular

383   endothelial cell permeability and fluid balance. In the treatment of acute lung injury, it plays an

384   important role in improving endothelial barrier by regulating the expression of key enzymes in

385   anabolic processes and specific receptors on signaling pathways. Our study results demonstrate

386   that both MSCs and S1P can alleviate acute lung injury. But the combined use of MSCs and S1P

387   shows significant efficacy in the regulation of S1P related gene expression, suggesting that some

388   synergism between MSCs and S1P may exist in the treatment of acute injury. Further studies are

389   need to determine whether MSCs when used in combination with S1P, further improve the

390   endothelial barrier and if a possible synergistic mechanism exists and maybe to provide a novel

391   therapeutic strategy in the treatment of acute lung injury.

392

393   **Conclusions**

394   In this study we built a pulmonary endothelial cell model of acute injury by LPS, and

395   investigated the regulation of S1P receptors and sphingosine kinases expression by MSCs

396   combined with S1P. The study results demonstrated that both MSCs and S1P can alleviate acute

397   lung injury. When MSCs were used alone, only the expression of SphK1 was reduced, and there

398   was no significant impact on the expression of SphK2. When MSCs were used in combination

399   with S1P, the expression of SphK1 and SphK2 were reduced simultaneously. We also found that

400   when MSCs works on acute injured HPAECs, the regulation of individual S1P receptors were

401   different, which was mainly acted on S1PR2 and S1PR3. The expression of S1PR2 and S1PR3

402 were further reduced when MSCs were used in combination with S1P. These results show that

403 MSCs play a role in the treatment of acute lung injury by regulating the expression and activity of

404 S1P related genes, when MSC combined with S1P there is a possible synergistic mechanism exists.

405

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Real Time Cellular Analysis (RTCA) of micro-electrical impedance changes of HPAECs.

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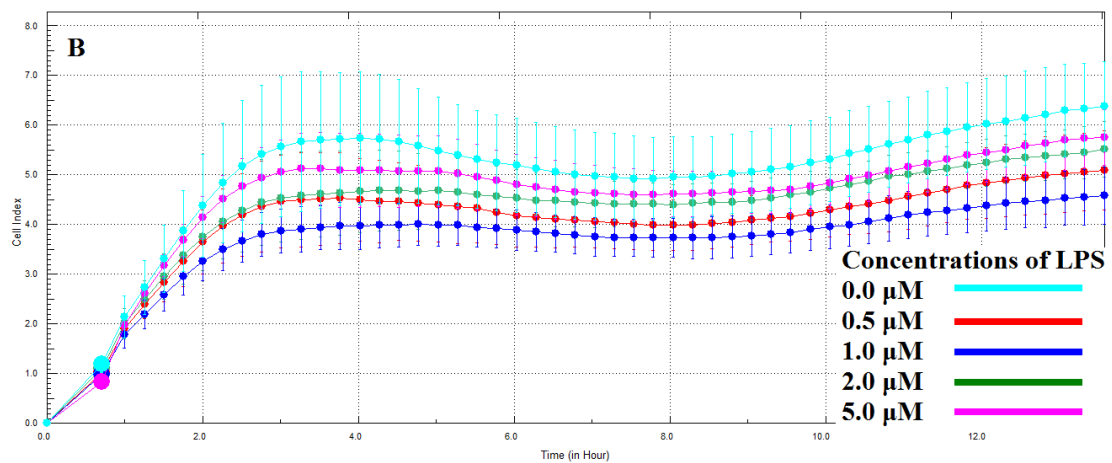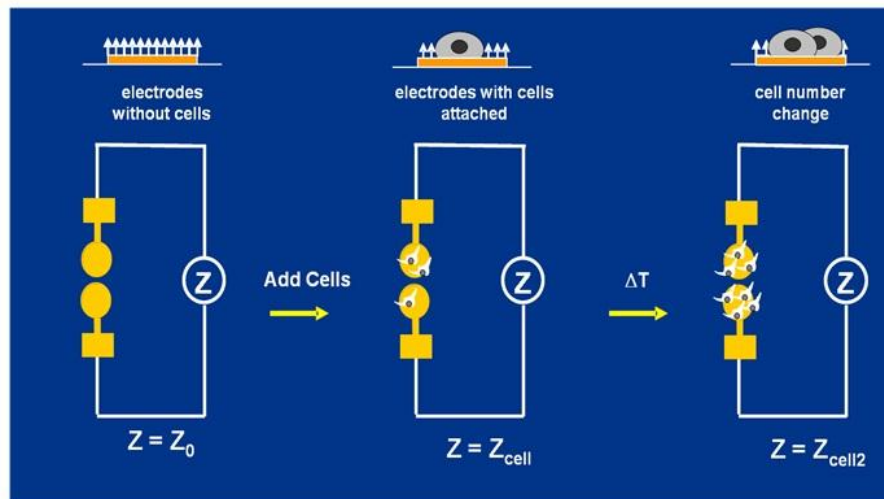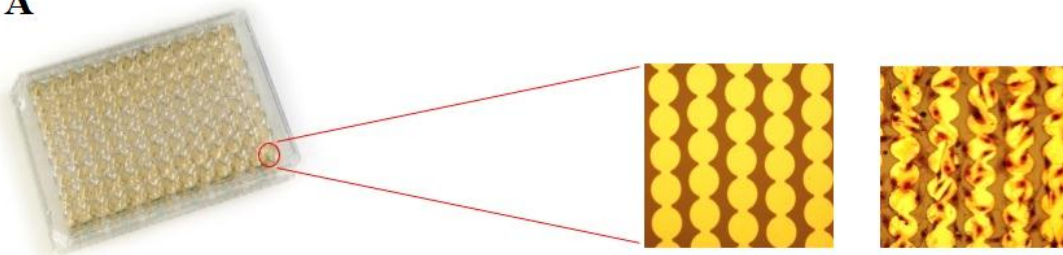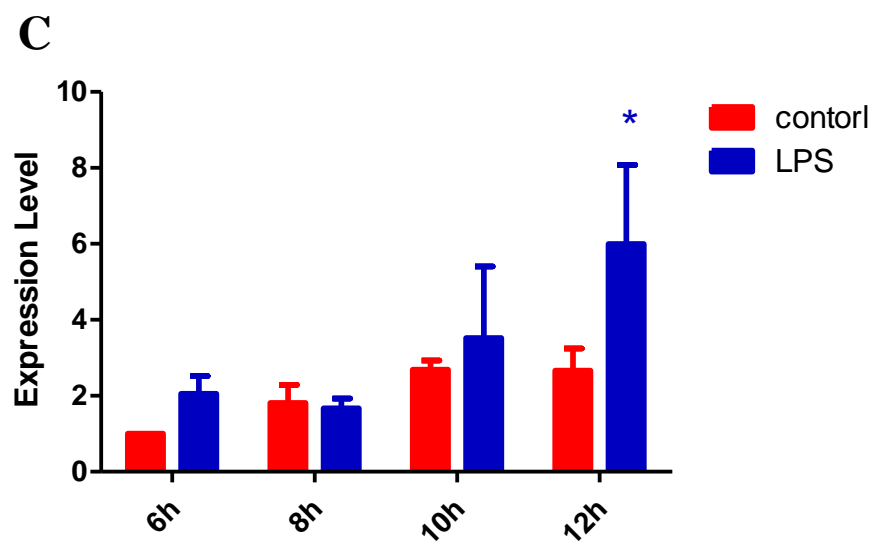
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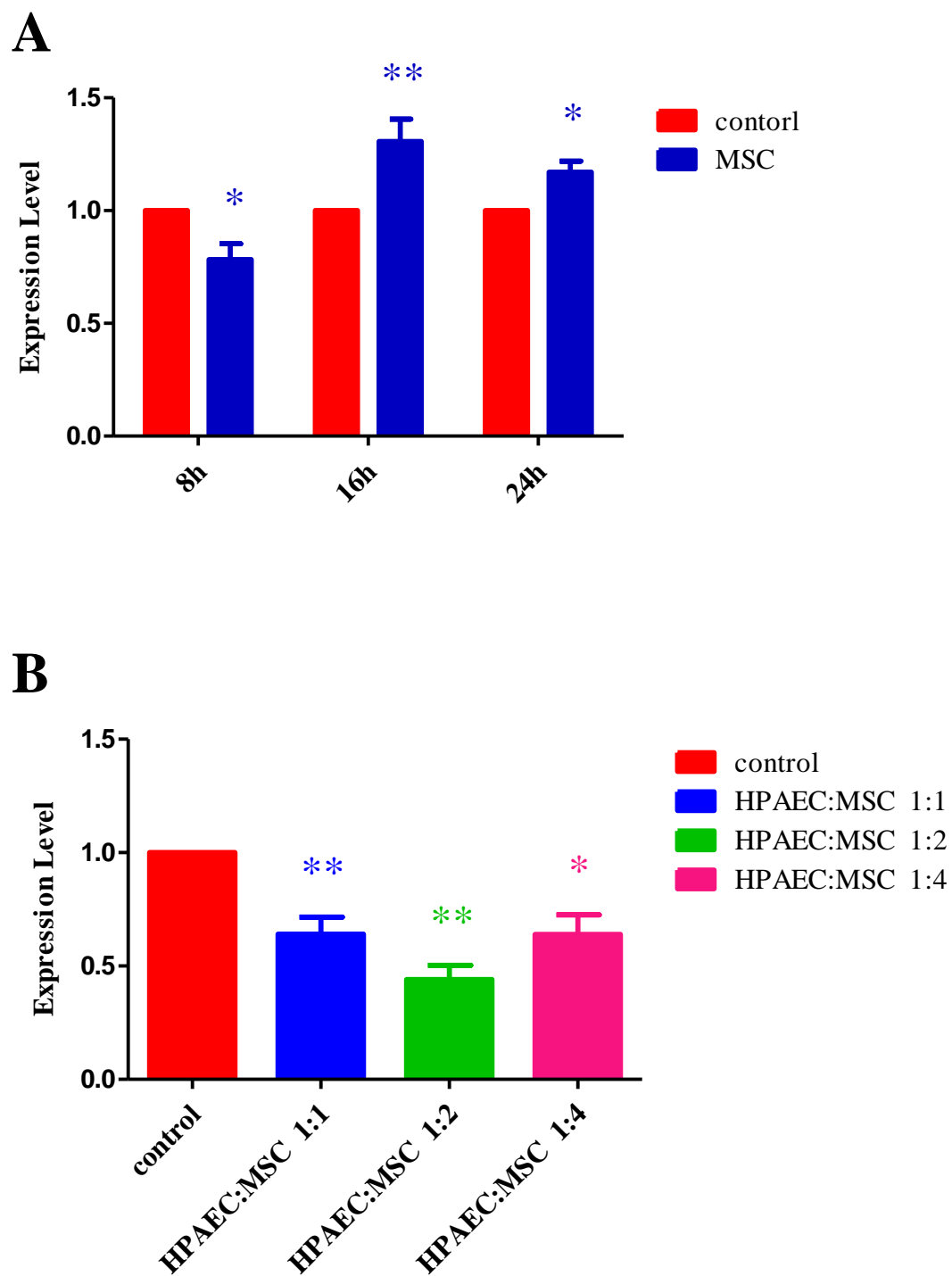
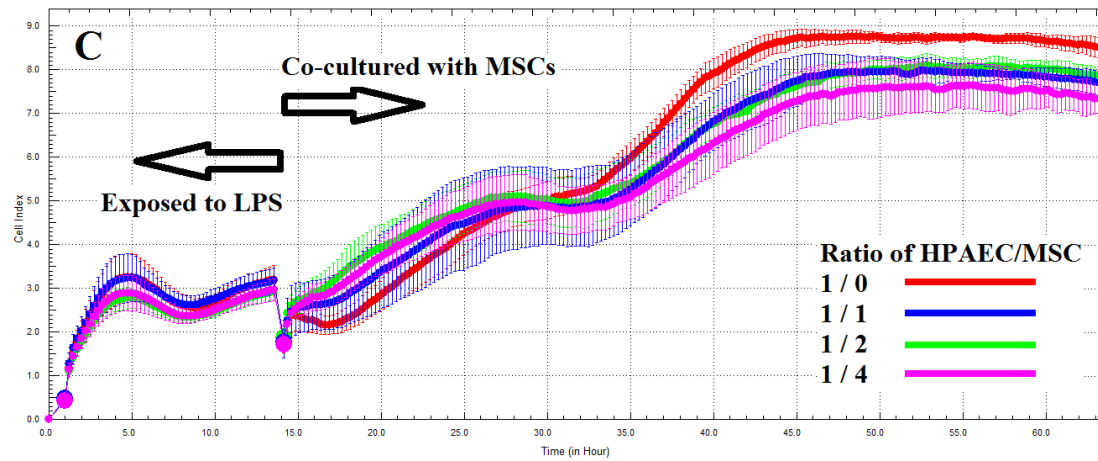
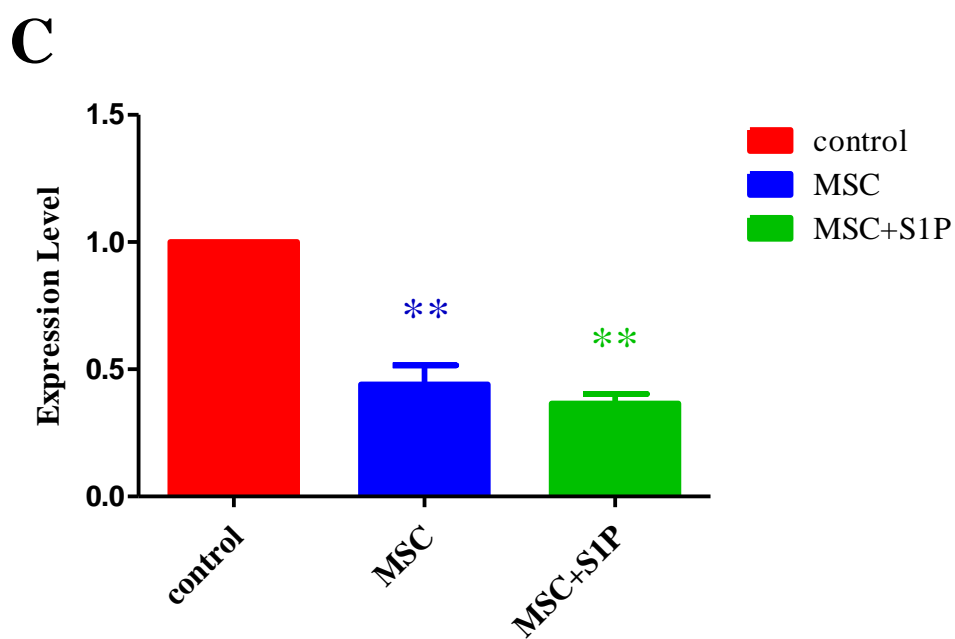
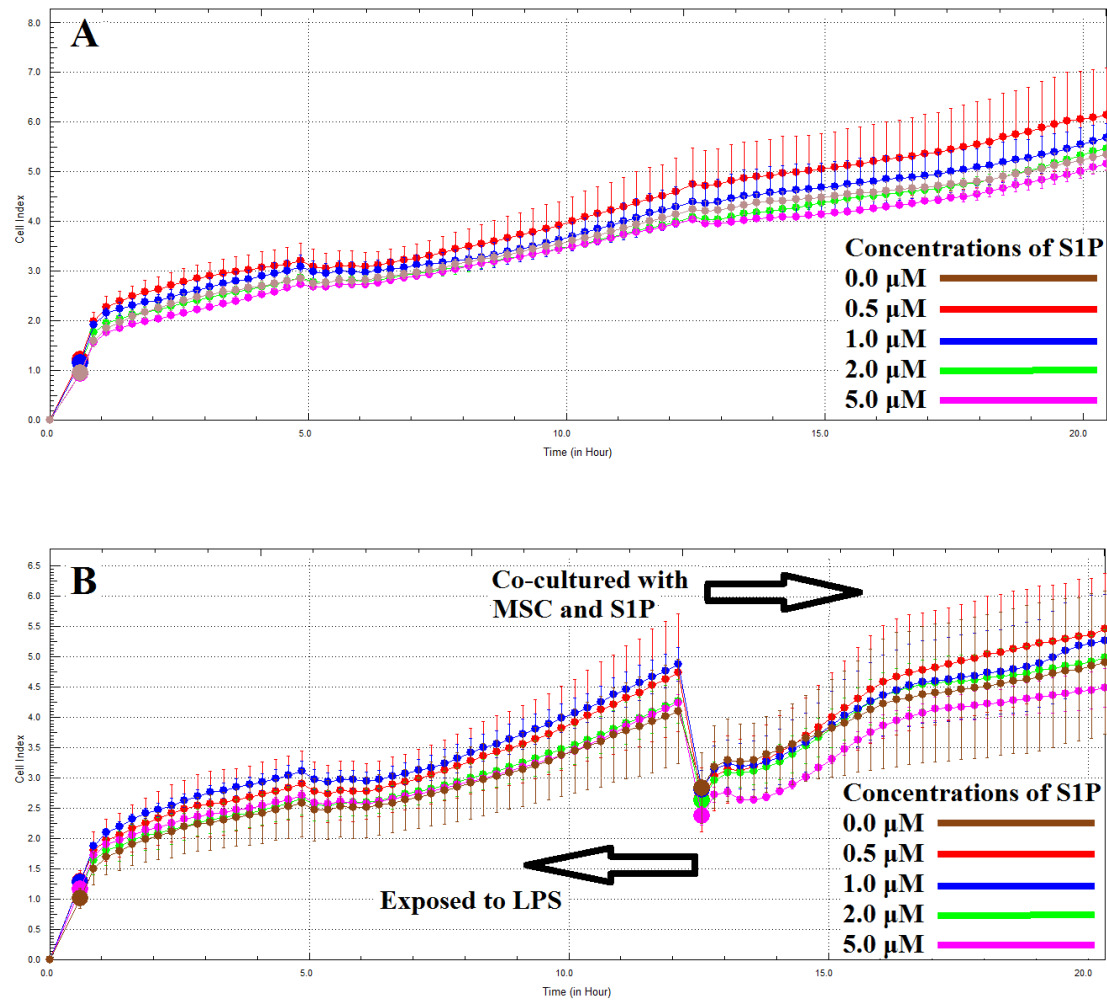
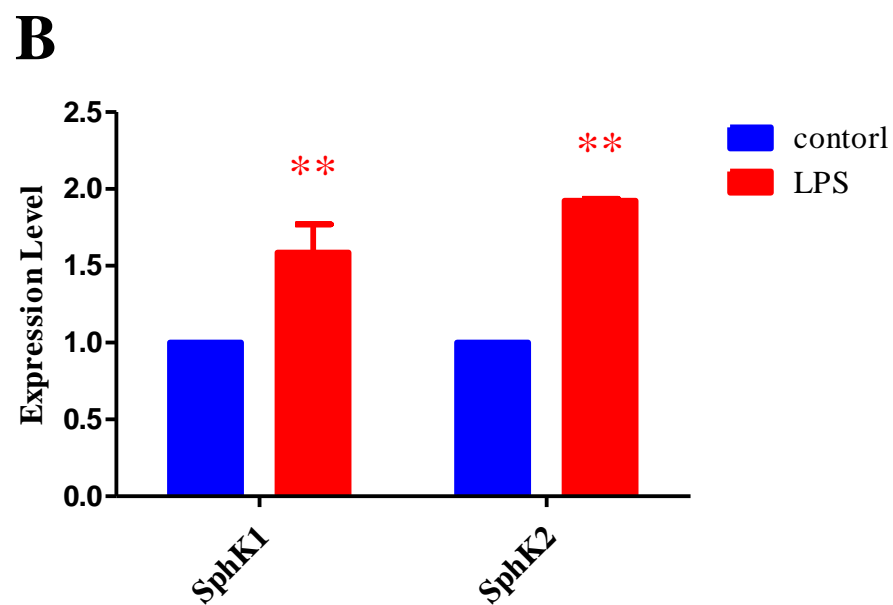
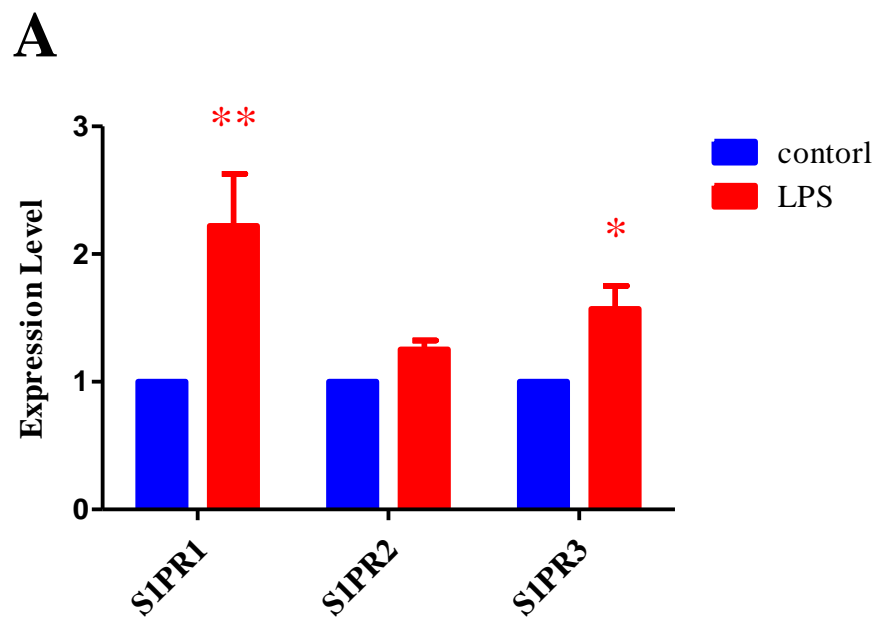

A

B

The combined effect of LPS and S1P on micro-electrical impedance of HPAECs.

S1P receptor and Shpingosine kinase expression following acute injury.

Effect of MSC and S1P combination therapy on TNF- a , S1P receptors and Sphigosine kinases.

A

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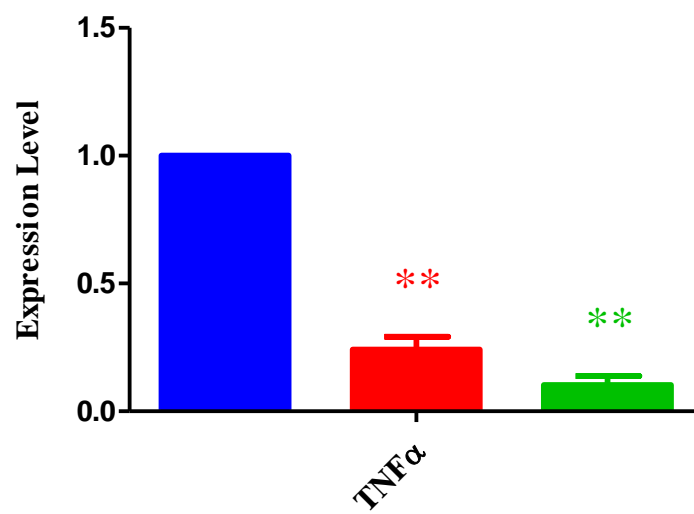
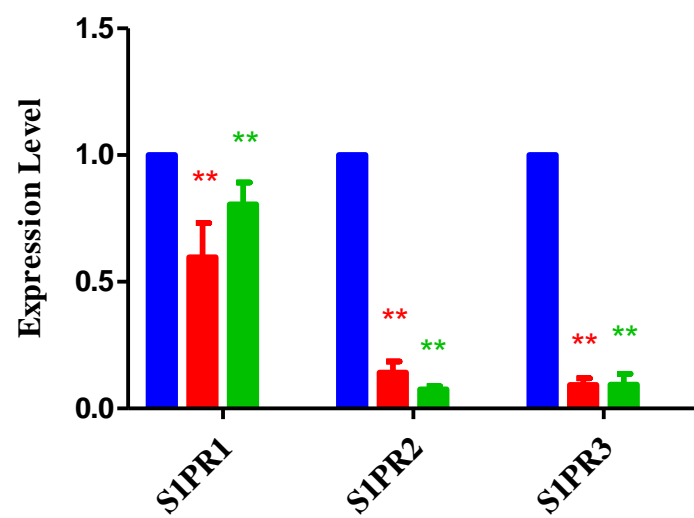
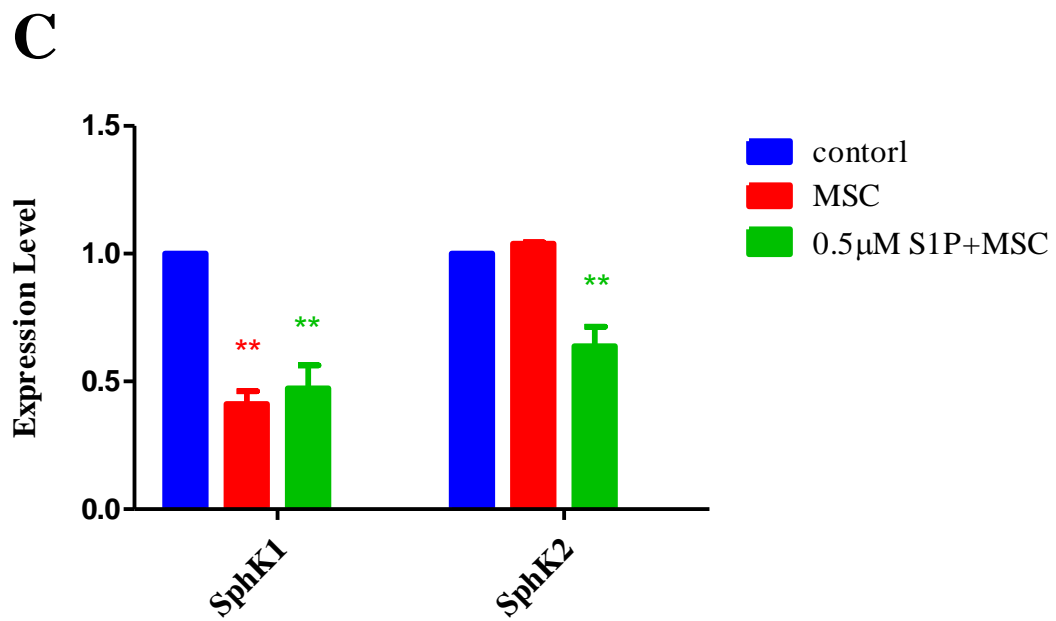
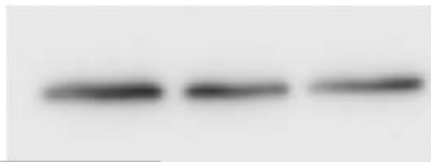

S1P receptor s expression in HPAECs treated with MSCs.

Primer sequences

引物	序列
S1PR1-F	5'-GCACCAACCCCATCATTTAC-3'
S1PR1-R	5'-TTGTCCCCTTCGTCTTTCTG-3'
S1PR2-F	5'-CAAGTTCCACTCGGCAATGT-3'
S1PR2-R	5'-CAGGAGGCTGAAGACAGAGG-3'
S1PR3-F	5'-TCAGGGAGGGCAGTATGTTC-3'
S1PR2-R	5'-GAGTAGAGGGGCAGGATGGT-3'
SphK1-F	5'-TCTGGGCACCTTCCTGCGTC-3'
SphK1-R	5'-CTCACTGCCCAGGTGCGAGTG-3'
SphK2-F	5'-TGCTGGAAGGTGGGCGTC-3'
SphK2-R	5'-AATAGACTCCGCCCTCAGCC-3'
TNFα-F	5'-TGATCCCTGACATCTGGAATCTG-3'
TNFα-R	5'-GCCAAGGTCCACTTGTGTC-3'
GAPDH	5'-GAAGGTGAAGGTCGGAGTC-3'
GAPDH	5'-GAAGATGGTGATGGGATTTC-3'