

The melanocortin 1 receptor (MC1R) inhibits the inflammatory response in Raw 264.7 cells and atopic dermatitis (AD) mouse model

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Abstract The alpha melanocyte stimulating hormone receptor (MC1R) is one of five G-protein coupled receptors belonging to the melanocortin subfamily, *MC1R* gene has been known to play a major role in regulating of fur color in mammals, and α -MSH and ACTH are endogenous nonselective agonists for MC1R. However, we found that MC1R was highly expressed in Raw 264.7 cells which were important inflammatory cells involved in the initiation of inflammatory responses. In addition, Cyclic AMP is not only a key molecule in the MC1R signal transduction pathway, but dampen innate immune-mediated responses. These intriguing biological results triggered the further conformation studies; it suggested that MC1R was very likely to be an important role in immunoregulation. In this study, we were to investigate the immunosuppressive effects of MC1R on inflammation in lipopolysaccharide (LPS) stimulated Raw 264.7 cells and LPS induced vivo 2-chloro-1,3,5-trinitrobenzene (TNCB)-induced atopic dermatitis (AD) model. The effects of the MC1R antagonist

psoralen on pro-inflammatory cytokines and signaling pathways were analyzed by enzyme-linked immunosorbent assay, western blot, real-time fluorescence quantitative PCR and Histological analysis. Our results show a consistent and marked effect of high concentrations of MC1R antagonist psoralen increased the level of MC1R mRNA in Raw 264.7 cells by cumulative feedback regulation through preferential binding of MC1R. Moreover, as evidenced by inhibiting the LPS-induced TNF- α , IL-6 and enhancing the expression level of cyclic AMP protein in vitro. In vivo study it was also observed that psoralen promoted on histopathologic changes in the skin tissue of TNCB-induced AD mice. Taken together, our results suggest that MC1R decrease the inflammation in vitro and vivo, and might be a therapeutic signaling pathway to against inflammatory diseases.

Keywords MC1R · Cytokines · Inflammation action · Psoralen · Atopic dermatitis

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Introduction

Atopic dermatitis (AD) is characterized by severe pruritic, chronic, relapsing inflammatory skin disease with a wide spectrum of clinical manifestations [1]. Serious AD can lead to skin allergy syndrome, immunological abnormalities, skin susceptible to certain cutaneous bacterial, fungal and viral infections [2, 3]. Recently, a small number of studies have shown that alveolar macrophage have a therapeutic effect on AD [4, 5]. The primary function of macrophages is phagocytosis and presentation functions by secreting various pro-inflammatory mediators such as tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6) [6, 7]. This different set of macrophage functions is decided

by what type of receptor is activated on the macrophage and the cytokine milieu in which the macrophage exists [8].

The melanocortin system has attracted much attention in recent years as a result of its involvement in a large number of important physiological functions, such as regulating of fur color, modulation of aggressive/defensive behavior and mediation of pain. The melanocortin receptor (MCR) sub-type family is a member of the GPCR superfamily and each of them has a different biological profile regarding the relative potency of the endogenous and synthetic melanocortin peptides. α -MSH and ACTH are endogenous non-selective agonists for MC1R, MC3R, MC4R and MC5R [9]. Melanocortin-1 receptor (MC1R) is composed of a single polypeptide featuring seven α -helical transmembrane domains (TMs), an extracellular N-terminus, three extracellular loops, three intracellular loops and an intracellular C-terminus. The major ligand of MC1R is α -melanocyte stimulating hormone (α -MSH) [10], and this is the primary signaling pathway of melanin pigmentation in mammals [11]. Liganded MC1R when occupied by α -MSH, activates adenosine cyclase, induces cyclic AMP production, and promotes eumelanin synthesis by activating the expression of tyrosinase. In addition, α -MSH when bound by MC1R activates the proliferation of melanocytes [12], so this signaling pathway plays an important role in pigmentation.

To date, The gene encoding MC1R have been described locating on the 3'-telomeric end of chromosome 16 in a locus that has a high density of genes, and MC1R acts between UVR stressed keratinocytes located in the stratum spinosum and the production and subsequent transfer of protective melanin from melanocytes [13]. MC1R have classically been considered to have a role in the skin pigmentation [14] and proliferation, melanogenesis and cell-cycle control [15]. Comparatively little attention has been given to the protection of MC1R on inflammation. Nevertheless, it has been reported that MC1R antagonist ACTH plays an important physiological role in stimulating fetal immune response which will be important for the study of cancer recently [16]. To further investigate whether MC1R could be a new therapy pathway that inhibits the inflammatory response, we analyzed the effects of the MC1R traditional antagonist psoralen on pro-inflammatory cytokines and signaling pathways in LPS-stimulated RAW 264.7 cells at different time. Psoralen, effective ingredient of a traditional Chinese medicine, has been isolated from *Psoralea linn*, Malay tea Scurfpea Fruit, *Psoralea tenuiflora* and Fruit of Malay tea Scurfpea, and its molecular formula is $C_{11}H_6O_3$. Psoralen was beneficial as a home remedy of vitiligo, the best therapeutic concentration of psoralen is 2.5–7.5 $\mu\text{g/mL}$, and the onset time in the body is 17 h. The intriguing biological result was that psoralen could obviously inhibit the α -MSH which was an agonist of MC1R to 82.23 % [17].

LPS stimulation activates mitogen-activated protein kinases (MAPKs) [18]. Increasing amount of evidence has demonstrated that LPS can trigger the most potent microbial initiators of inflammatory responses by activating numerous inflammatory cells [19, 20]. Macrophages are important inflammatory cells involved in the initiation of inflammatory responses, and play critical roles in the development of inflammation by secreting various pro-inflammatory mediators including tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6), TNF- α , IL-6 contribute to the pathogenesis of many inflammatory diseases [21–23].

In this study, we induced AD-like skin lesions in BALB/c mice by repeatedly applying TNCB and analysed the histological changes in clinical skin. Putative anti-inflammatory mechanism of MC1R on LPS-stimulated RAW 264.7 macrophages was elucidated by analyzing the level of MC1R mRNA and cyclic AMP protein, and total serum expressions levels of TNF- α and IL-6. In addition, in vivo anti-inflammatory effects of MC1R were further investigated in a mouse model of TNCB-induced AD.

Materials and methods

Chemicals and reagents

Psoralen was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Jilin, China). Dimethyl sulfoxide (DMSO), LPS (*Escherichia coli* 055:B5), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and TNCB were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), penicillin and streptomycin were obtained from Invitrogen-Gibco (Grand Island, NY). Mouse TNF- α , IL-6 enzyme-linked immunosorbent assay (ELISA) kits was purchased from Biolegend (CA, USA). Mouse monoclonal cyclic AMP antibody HRP-conjugated goat-mouse antibodies were provided by GE Healthcare (Bio-world Technology, USA). All other chemicals were of reagent grade.

Animals

Two-week-old BALB/c mice were purchased from the Center of Experimental Animals of Baiqiu Medical College of Jilin University (Jilin, China). The mice were housed in microisolator cages and received food and water ad libitum. The laboratory temperature was 24 ± 1 °C, and a humidity of 50 ± 10 %, under a 12/12 h light/dark cycle. Mice were housed for 2–3 days to adapt the environment before experimentation. All animal experiments were

performed in accordance with the guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Cell culture and treatment

The RAW 264.7 mouse macrophage cell line was purchased from the China Cell Line Bank (Beijing, China) and was maintained in DMEM supplemented with 10 % heat-inactivated fetal bovine serum, 3 mM Glutamine, antibiotics (100 U/mL penicillin and 100 U/mL streptomycin) at 37 °C under a humidified atmosphere of 5 % CO₂. Cells at passages 10–20 were used for the experiments. In all experiments, macrophages were incubated in the presence or absence of various concentrations of psoralen, which were always added 1 h prior to LPS (1 µg/mL) treatment.

Cell viability assay

Cell viability was evaluated by MTT assay. Briefly, 150 µL cells were plated at a density of 4×10^5 cells/mL in 96-well plates in a 37 °C, 5 % CO₂ incubator for 1 h. Then the cells were treated with 50 µL of psoralen of different concentrations (0–10 µg/mL) for 1 h. The total volume of each well was 200 µL, and the concentrations of psoralen were four times diluted finally. After 17 h of psoralen stimulation, 20 µL MTT (5 mg/mL) was added to each well, and the cells were further incubated for an additional 4 h. The supernatant was removed and the formation of formazan was resolved with 150 µL/well of DMSO. The optical density was measured at 570 nm on a microplate reader (TECAN, Austria).

Analysis of expression level of MC1R mRNA by QRT-PCR

Synthesis of QRT-PCR primer of MC1R

The sequence information of MC1R and glyeraldehyde-3-phosphate dehydrogenase was obtained from GenBank (GAPDH, XM-001725661.1). Primers were designed using primer 5.0 and synthesized by AuGCT Biotechnology Co (Table 1).

RNA extraction and real-time quantitative reverse transcription-PCR

Psoralen solubilized with DMSO was diluted with DMEM prior to treatment. RAW 264.7 cells were plated onto 6-well plates (10^6 cells/well), and incubated in the presence of either LPS alone 1 µg/mL and psoralen alone 2.5 µg/mL, or LPS plus psoralen 2.5, 5 and 7.5 µg/mL for 19 h.

Table 1 Sequence of primers of the target and house-keeping genes

Gene	Sequences of primer (5'–3')	Temp. (°C)
MC1R	F: 5'-GGACGATGCCTGCACTTGGCTCCC-3'	60
	R: 5'-TGCCACCCTCACCAGGAGCACTGC-3'	
GAPDH	F: 5'-TGTTGCCATCAATGACCCCTT-3'	60
	R: 5'-CTCCACGACGTACTCAGCG-3'	

Total RNA was extracted from differentiated RAW264.7 using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. For real-time quantitative reverse transcription (RT)-PCR, total RNA was reversely transcribed using an ImProm-II RT system (Promega) according to the manufacturer's instructions. For detection and quantification, a MyiQ real-time PCR detection system (Bio-Rad) was used. PCRs were performed using a SYBR Premix Ex Taq II (Takara, Seoul, Republic of Korea). PCRs were carried out in a final volume of 20 µL using 0.5 mM of each primer, cDNA, and 10 µL of the supplied enzyme mixture containing the DNA double-strand-specific SYBR Green I dye for detection of PCR products. PCRs were performed with a 3 min preincubation at 95 °C followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C. PCR products were verified by melting curve analysis, agarose gel electrophoresis, and DNA sequencing. The standard curve was exported from The MyiQ real-time PCR detection system (Bio-Rad). The difference in co-efficiencies was less than 0.1, indicating similar amplification efficiencies of the two cDNAs. The relative amount of mRNA to Gapdh RNA was described using the equation $2^{-\Delta CT}$ where $\Delta CT = (CT \text{ mRNA} - CT \text{ Gapdh})$ [24].

Western blotting analysis

To obtain total proteins, murine macrophage RAW 264.7 cells were plated onto 6-well plates and incubated for 24 h, then pretreated with psoralen incubating in the presence of LPS 1 µg/mL/psoralen 2.5 µg/mL, or LPS plus psoralen 2.5, 5 and 7.5 µg/mL for 1 h. After LPS (1 µg/ml) stimulation for 30 min, cells were collected and washed twice with cold PBS. The cells were lysed in lysis buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 5 mM EDTA (pH 8.0), 0.6 % NP-40, 1 mM Na₃VO₄, 20 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 2 mM p-nitrophenyl phosphate, and 1:25 Complete Mini Protease Inhibitor cocktail (Boehringer, Mannheim, Germany) and maintained on ice for 30 min. The cell lysates were washed via dilution and centrifugal concentration. Total protein of RAW 264.7 cells was extracted by Thermo Scientific M-PER Mammalian Protein Extraction Reagent. The

protein concentrations were determined using a BCATM protein assay kit. Aliquots of the lysates were separated on 10 % SDS–polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (BIO-RAD) with a glycine transfer buffer [192 mM glycine, 25 mM Tris–HCl (pH 8.8), 20 % methanol (v/v)]. After blocking the nonspecific site with blocking solution (5 % (wt/v) nonfat dry milk), the membrane was incubated overnight with specific primary antibody at 4 °C. The membrane was then incubated for an additional 60 min with a peroxidase-conjugated secondary antibody at room temperature. The immuno active proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detection kit [25].

Measurement of pro-inflammatory cytokines (TNF- α and IL-6) production

Psoralen solubilized with DMSO was diluted with DMEM prior to treatment. RAW 264.7 cells were plated onto 24-well plates (10^5 cells/well), dividing into six groups: control, LPS, psoralen and LPS + psoralen (2.5, 5 and 7.5 $\mu\text{g/mL}$), and incubated for 17, 18, 19 and 20 h. Cell-free supernatants were subsequently employed in pro-inflammatory cytokine assays using a mouse enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems), following protocol of the manufacturer (BioLegend, Inc, Camino Santa Fe, Suite E, San Diego, CA, USA).

Histological analysis

Mice were randomly classified into five groups: Control, TNCB 20 μL (3 $\mu\text{g/mL}$) and TNCB 20 μL (3 $\mu\text{g/mL}$) + psoralen 10 μL (2.5, 5 and 7.5 $\mu\text{g/mL}$). TNCB was daubed on the back of ears, neck of the BALB/c mice for 7 days, after that, and psoralen played a part in vivo for the next 7 days by intraperitoneal injection. Mice were sacrificed on day 14 after the first challenge; skin of ears and neck was removed for histological examination. Tissue samples were fixed with 10 % buffered formalin (pH 7.2), embedded in paraffin, sectioned at 4 μm . After hematoxylin and eosin (H&E) staining, pathological changes of tissue samples were observed under a light microscope.

Statistical analysis

Data are presented as mean $\pm$ SD. Comparison between groups was made with one-way analysis of variance (ANOVA; Dunnett's *t* test) and Student's *t* test. *P*-values of 0.05 or less were considered statistically significant.

Results

Effects of psoralen on cell viability

The potential cytotoxicity of psoralen was evaluated by MTT assay after incubating cells for 19 h in the absence or presence of LPS. The result showed that cell viabilities were not affected by psoralen at the concentrations used (2.5, 5, 7.5 $\mu\text{g/mL}$) (Fig. 1) Thus, the effects of psoralen on RAW cells did not attribute to cytotoxic effects.

Analysis of expression level of MC1R mRNA by QRT-PCR

Standard curves of MC1R and the housekeeping gene (GAPDH) were constructed. The slopes of standard curve of MC1R and GAPDH were 3.357 and 3.213 respectively and R^2 were 0.997 and 0.998 respectively. The slope difference value <0.1 so amplification efficiencies were identical for two genes. The data could be analyzed using $\Delta\Delta\text{Ct}$ method [24]. The melting curve of MC1R and GAPDH are shown in (Fig. 2a). The two genes appeared with only one peak at 86 °C (Fig. 2b) respectively, no nonspecific production or primer-dimer complication; all fluorescence resulted from specific amplification (Fig. 2c).

Real-time RT-PCR results of MC1R mRNA in Raw 264.7 cells with different group are shown in Fig. 3. In pre-treatment group of psoralen (2.50 $\mu\text{g/mL}$), the mRNA expression of MC1R was higher than control group by $\Delta\Delta\text{Ct}$ (>3.21 -fold, $P < 0.01$). In pre-treatment group of LPS (1 $\mu\text{g/mL}$), the mRNA expression of MC1R was higher than control group by $\Delta\Delta\text{Ct}$ (>2.59 -fold, $P < 0.01$). In pre-treatment group of psoralen (5 $\mu\text{g/mL}$), the mRNA expression of MC1R was higher than control group by

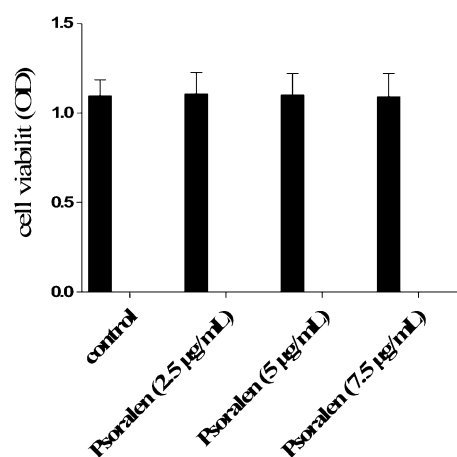


Fig. 1 Effects of psoralen on the viability of RAW 264.7 cells. Cells were cultured with psoralen (0–7.5 $\mu\text{g/mL}$) for 24 h. Cell viability was assessed by MTT reduction assays. Data are presented as mean $\pm$ SEM of three independent experiments

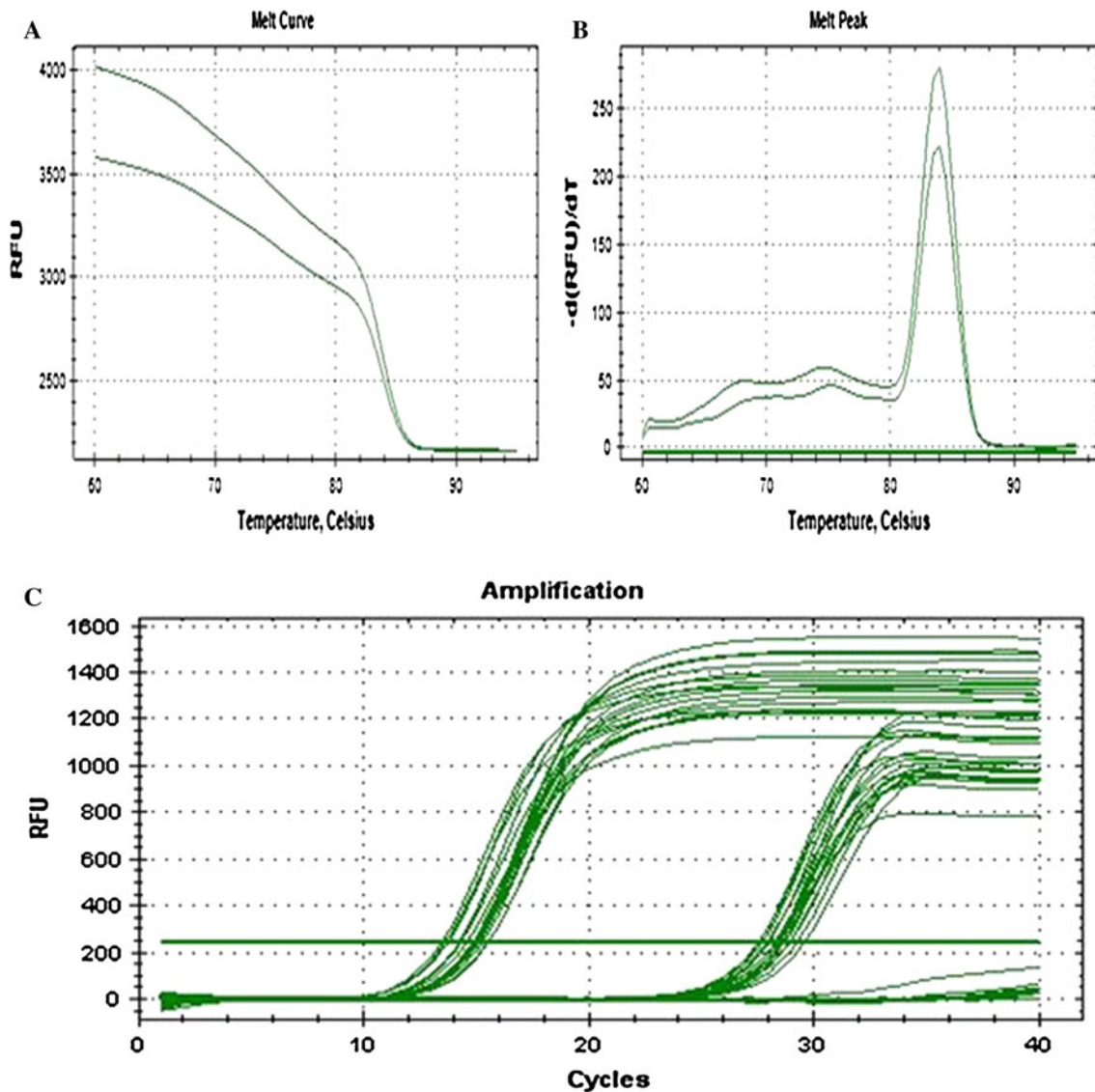


Fig. 2 Dissociation curve of MC1R and GAPDH. **a** The melt peak of MC1R and GAPDH in control group. **b** The melt curve of MC1R and GAPDH in control group. **c** The amplification of MC1R and GAPDH

$\Delta\Delta C_t$ (>4.11 -fold, $P < 0.01$). In pre-treatment group of psoralen ($7.50 \mu\text{g/mL}$), the mRNA expression of MC1R was higher than control group by $\Delta\Delta C_t$ (>6.64 -fold, $P < 0.01$), in addition, higher than the group of psoralen ($2.5 \mu\text{g/mL}$) by $\Delta\Delta C_t$ (>2.06 , $P > 0.01$) (Fig. 3).

Western blot analysis of cyclic AMP protein expression

Cyclic AMP is not only a key molecule in the MC1R signal transduction pathway, but dampens innate immune-mediated responses. To further prove the inhibition of inflammatory response by MC1R, the LPS-induced phosphorylation of cyclic AMP was examined by Western blot in RAW 264.7 cells pretreated with psoralen before LPS stimulation. As shown in (Fig. 4), following the increasing

concentrations, the phosphorylation of cyclic AMP was increased significantly.

Effects of psoralen on the production of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 cells

TNF- α , IL-6 may result in clinical syndrome of AD, including skin allergy syndrome, immunological abnormalities [1]. The levels of TNF- α , IL-6 in the culture supernatants were measured using ELISA kits. Treatment of RAW 264.7 cells with LPS alone resulted in significant increase in cytokine production compared to those of LPS groups and control groups (Figs. 5, 6). The levels of TNF- α and IL-6 in the supernatants of LPS stimulated cells

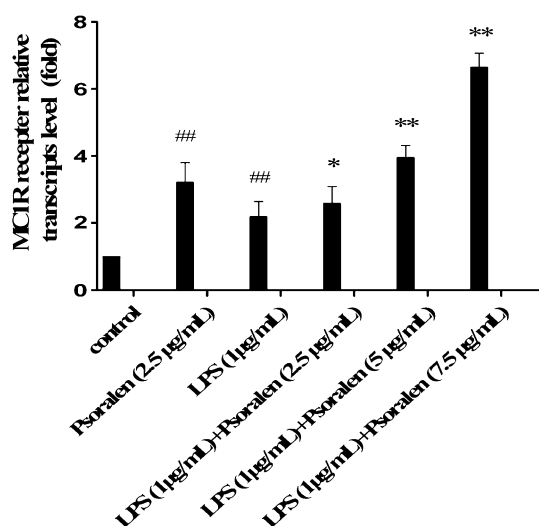


Fig. 3 RAW 264.7 cells were incubated with various concentrations of psoralen. After 19 h, the cells were harvested, and various groups including Control, LPS, psoralen and LPS + psoralen (2.5, 5 and 7.5 µg/mL) mRNA levels were analyzed with real-time quantitative RT-PCR using Gapdh as a reference. Values represent the means $\pm$ SD of triplicate samples. # P < 0.05; ## P < 0.01 indicates significant differences from the unstimulated control groups. * P < 0.05, ** P < 0.01 versus LPS. Each experiment was performed independently at least three times

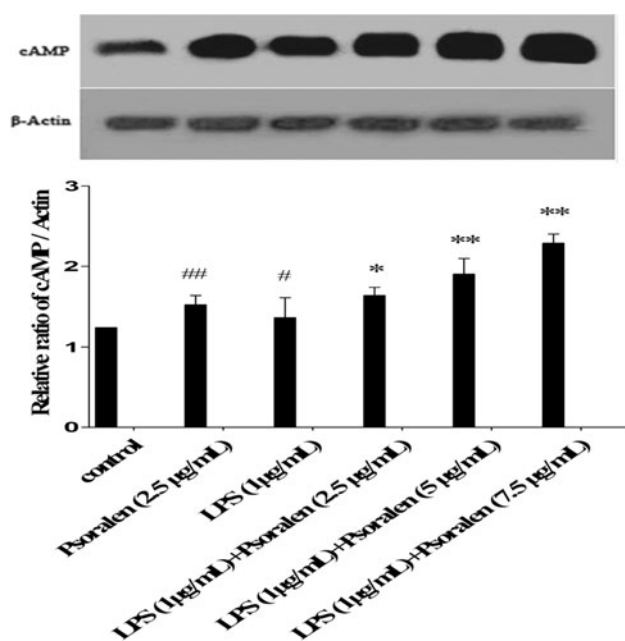


Fig. 4 Effect of psoralen on LPS-induced phosphorylation of cyclic AMP. The cells were stimulated with or without LPS (1 mg/L) for 30 min. Protein samples were analyzed by Western blot with phospho-specific antibodies. The data are representative of three independent experiments and expressed as mean $\pm$ SEM of three independent experiments. * P < 0.05; ** P < 0.01 indicates significant differences from the LPS groups, # P < 0.05 vs control group, ## P < 0.01 vs control group

pretreated with 2.5, 5, 7.5 µg/mL psoralen at 20 h were gradually reduced compared to LPS (P < 0.05) group in a dose-dependent manner. The level of TNF- α and IL-6 secreted into the culture medium of RAW 264.7 cells increased steadily after LPS and psoralen addition, and peaked at 19 h has determined by TNF- α and IL-6 ELISA (Figs. 5, 6).

Effects of psoralen on histopathological changes in the skin tissue of TNCB-induced AD mice

Mice in TNCB group proved AD-like skin lesions, such as depilation, erythema, hemorrhage, excoriation, scaling and dryness (Fig. 7b). In histopathological assessment of skin sections of mice in control group, intact structure and clear subcutaneous microvascular were observed (Fig. 8a). TNCB application produced marked epidermal thickening and angiectasis (Fig. 8b1, b2). As we expected, the group of psoralen 7.5 µg/mL promoted the progression of AD-like skin symptoms in clinical performance more than the group of psoralen 2.5 µg/mL and the group of psoralen 5 µg/mL (Fig. 7c, d, e). Furthermore, the severe symptoms of mast cell infiltration, hemorrhage and degranulation in the dermis were result in the group of psoralen 7.5 µg/mL (Fig. 8e1, e2).

Discussion

This study was to evaluate whether MC1R exerts anti-inflammatory effects in LPS-stimulated RAW 264.7 cells and analysis of expression level of MC1R mRNA and cyclic AMP protein with different concentration of MC1R antagonist psoralen to encourage them in vitro. As evidence, in vivo study it was also observed that psoralen promoted on histopathologic changes in the skin tissue of TNCB-induced AD mice. The collective results of this investigation demonstrate that MC1R antagonist psoralen increased the level of MC1R mRNA and the cyclic AMP protein by cumulative feedback regulation, leading to decrease of the LPS-induced TNF- α and IL-6 production in Raw 264.7. Beyond that, the psoralen further promoted the progression of AD-like skin symptoms in clinical performance in vivo.

Various pro-inflammatory mediators which are secreted by inflammatory cells play a central role in inflammation through stimulating of angiogenesis and cell migration. TNF- α , mainly produced by monocyte/macrophages, can elicit the inflammatory cascade and cause damage to the vascular endothelial cells. TNF- α can also induce alveolar epithelial cells to produce other cellular factors and chemotactic, such as IL-6 [26]. IL-6 is one of the most common inflammatory cytokines [27]. Circulating levels of

Fig. 5 RAW 264.7 cells were stimulated with psoralen (2.5, 5, 7.5 $\mu\text{g/mL}$), and LPS (1 mg/mL) for 17, 18, 19 and 20 h. The supernatants were collected, TNF- α concentrations were determined by ELISA. Values represent the means $\text{GS} \pm \text{D}$ of triplicate samples. $\#P < 0.05$; $\#\#P < 0.01$ indicates significant differences from the unstimulated control groups at the same time. $*P < 0.05$, $**P < 0.01$ versus LPS. Each experiment was performed independently at least three times

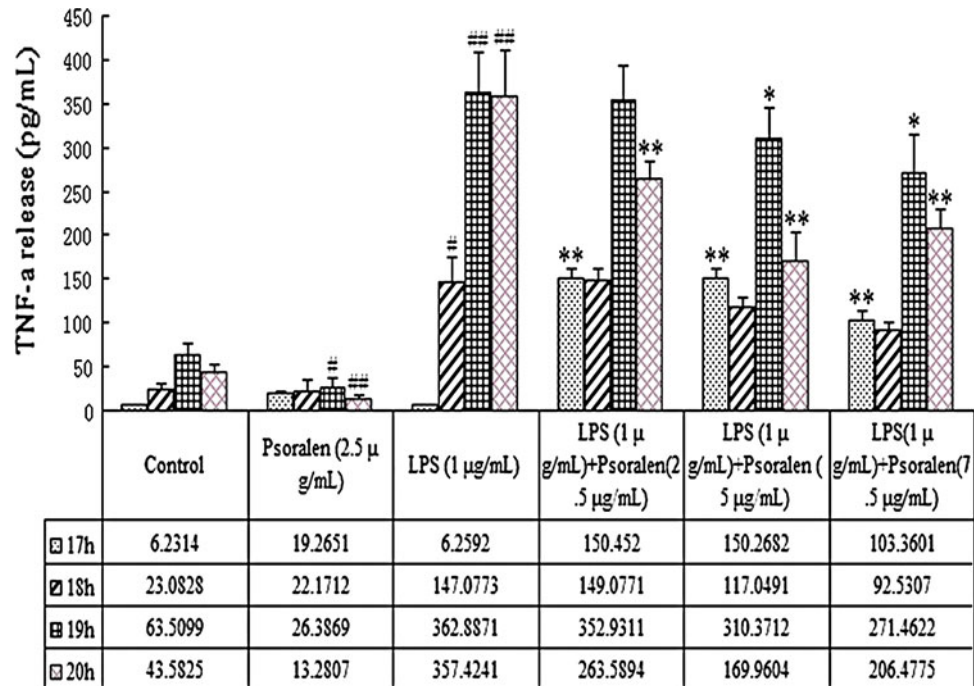
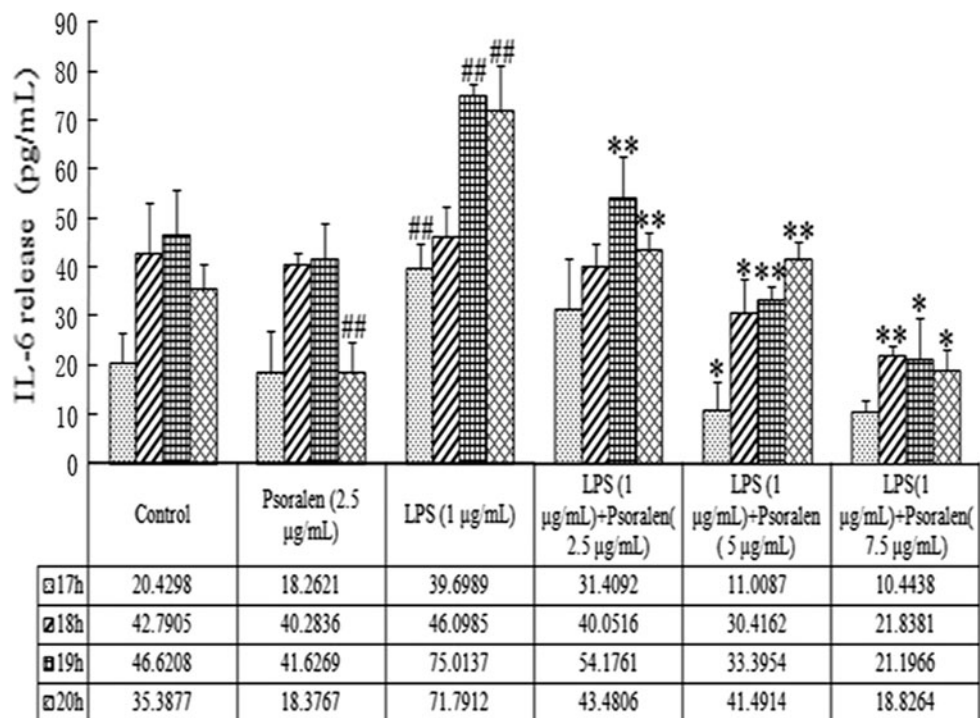


Fig. 6 RAW 264.7 cells were stimulated with psoralen (2.5, 5, 7.5 $\mu\text{g/mL}$), and LPS (1 mg/mL) for 17, 18, 19 and 20 h. The supernatants were collected; IL-6 concentrations were determined by ELISA. Values represent the means $\text{GS} \pm \text{D}$ of triplicate samples. $\#P < 0.05$; $\#\#P < 0.01$ indicates significant differences from the unstimulated control groups at the same time. $*P < 0.05$, $**P < 0.01$ versus LPS. Each experiment was performed independently at least three times



IL-6 have been shown to be excellent predictors of the severity of AD of different etiologies, such as sepsis and acute pancreatitis [28, 29]. Our results indicated that psoralen inhibited TNF- α , IL-6 productions ($P < 0.01$) in LPS-stimulated RAW 264.7 cells in a dose-dependent manner (Figs. 5, 6).

MC1R is a G-protein-coupled receptor located in the surface of melanocytes, encoded by the MC1R gene in the

extension locus [30]. It plays an important role in regulating black eumelanin and red pheomelanin synthesis in mammals [31]. MC1R has been studied in many species, and several loss of function or receptor-activating polymorphisms have been described [32]. The binding of α -MSH to MC1R receptors triggers multiple signal transduction cascades, primarily dependent on elevated intracellular cyclic AMP which dampen innate immune-mediated responses

Fig. 7 Photographs of the clinical features of BALB/c mice in **a** the naive control group **b** TNCB 20 μ L (3 μ g/mL) control. **c** TNCB 20 μ L (3 μ g/mL) + psoralen 10 μ L (2.5 μ g/mL). **d** TNCB 20 μ L (3 μ g/mL) + psoralen 10 μ L (5 μ g/mL). **e** TNCB 20 μ L (3 μ g/mL) + psoralen 10 μ L (7.5 μ g/mL). ($n = 3$ BALB/c mice per group)

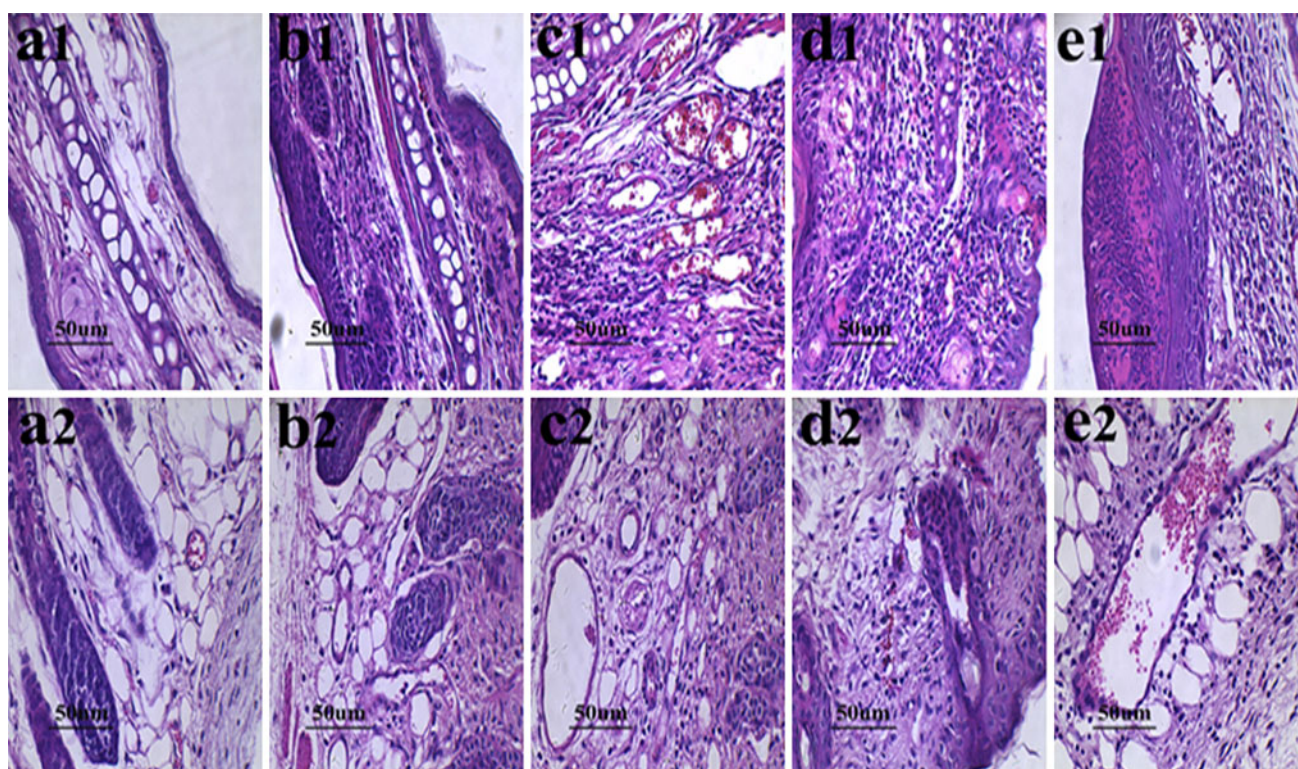
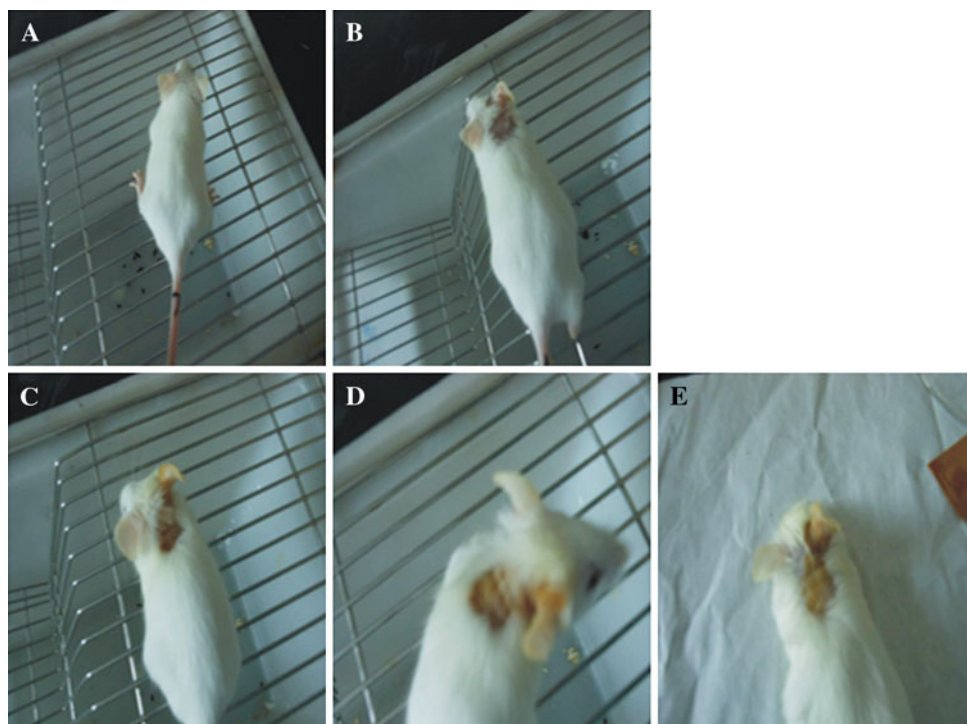


Fig. 8 Histological changes in the skin ($\times 200$). Skin ($n = 3$) sections (4 μ m) were obtained from **a1** the control group of ears. **a2** the control group of ears. **b1** TNCB 20 μ L (3 μ g/mL) control group of neck. **b2** TNCB 20 μ L (3 μ g/mL) control group of ears. **c1** TNCB 20 μ L (3 μ g/mL) + psoralen 10 μ L (2.5 μ g/mL) group of neck. **c2** TNCB 20 μ L (3 μ g/mL) + psoralen 10 μ L (2.5 μ g/mL)

group of ears. **d1** TNCB 20 μ L (3 μ g/mL) + psoralen 10 μ L (5 μ g/mL) group of neck. **d2** TNCB 20 μ L (3 μ g/mL) + psoralen 10 μ L (5 μ g/mL) group of ears. **e1** TNCB 20 μ L (3 μ g/mL) + psoralen 10 μ L (7.5 μ g/mL) group of neck. **e2** TNCB 20 μ L (3 μ g/mL) + psoralen 10 μ L (7.5 μ g/mL) group of ears

[33] resulting in the activation of a large number of genes [34], which together regulate several aspects of melanocyte biology such as pro-liferation, melanogenesis [35, 36] and cell-cycle control [37–39]. To further characterize the nature of the inhibitory effect of psoralen on cytokine production, we examined the level of MC1R mRNA and the effect of psoralen on the activation of cyclic AMP. As we expected, the level of MC1R mRNA and cyclic AMP protein was increased significantly with stimulating of LPS (Figs. 3, 4). Furthermore, following the increasing concentrations of psoralen, the level of MC1R mRNA and cyclic AMP protein was gradually increased in Raw 264.7 cells ($P < 0.01$). This finding makes it very likely that the competitive binding of psoralen to MC1R receptors triggers multiple signal transduction cascades, increasing intracellular cyclic AMP which could cause up-regulation of TNF- α and IL-6 secretion. By cumulative feedback regulation, the level of MC1R mRNA in Raw 264.7 cells increased.

Another finding in this study was that we induced AD-like skin lesions in BALB/c mice by repeatedly applying TNCB, after that, psoralen played a part in vivo for the next by intraperitoneal injection. We examined effects of psoralen on histopathological changes in the skin tissue of TNCB-induced AD mice. The same result was that psoralen promoted the progression of AD-like skin symptoms in the clinical performance, such as depilation, erythema, hemorrhage, excoriation, scaling and dryness. It can be speculated that the activity of MC1R signalling pathways to against the inflammatory response was induced by psoralen.

In conclusion, the data from in vitro assay and murine model of AD demonstrated that the MC1R antagonist psoralen increased the level of MC1R mRNA and the cyclic AMP protein in Raw 264.7 cells by cumulative feedback regulation. Meanwhile, it markedly promoted the LPS-induced TNF- α , IL-6 in vitro. Furthermore, in vivo study it was also observed that psoralen promoted on histopathologic changes in the skin tissue of TNCB-induced AD mice. Our current study provides MC1R is an effective target point of inhibiting the inflammatory response, further studies are warranted to investigate MC1R. However, the detailed mechanism of anti-inflammatory activities of MC1R is unclear, further studies are warranted to investigate the clinical usefulness of MC1R.

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References

1. Leung DY, Boguniewicz M, Howell MD et al (2004) New insights into atopic dermatitis. *J Clin Invest* 113:651–657
2. Morar N, Willis-Owen SA, Moffatt MF, Cookson WO (2006) The genetics of atopic dermatitis. *J Allergy Clin Immunol* 118: 24–34 quiz 35–36
3. Lubbe J (2003) Secondary infections in patients with atopic dermatitis. *Am J Clin Dermatol* 4:641–654
4. Nelson MP, Christmann BS, Werner JL (2011) IL-33 and M2a alveolar macrophages promote lung defense against the atypical fungal pathogen *Pneumocystis murina*. *J Immunol* 186:2372–2381
5. Liao CP, Lasbury SH, Durant Y et al (2009) *Pneumocystis* mediates over expression of anti zyme inhibitor resulting in increased polyamine levels and apoptosis in alveolar macrophages. *J Biol Chem* 284:8174–8184
6. Mosser DM, Edwards JP (2008) Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 8:958–969
7. Satoh H, Firestein GS, Billings PB, Harris JP, Keithley EM (2003) Proinflammatory cytokine expression in the endolymphatic sac during inner ear inflammation. *J Assoc Res Otolaryngol* 4:139–147
8. Taylor PR, Martine z-Pomares L, Stacey M, Lin HH, Brown GD, Gordon S (2005) Macrophage receptors and immune recognition. *Annu Rev Immunol* 23:901–944
9. Yingkui Y, Victor J, Victor JHruby, Chen Min (2009) Novel binding motif of ACTH analogues at the melanocortin receptors. *Biochemistry* 48(41):9775–9784
10. Kathleen G, Mountjoy L, Robbins S (1992) The cloning of a family of genes that encode the melanocortin receptor. *J Sci* 257:1248–1251
11. Schaffer JV, Bolognia JL (2001) The melanocortin-1 receptor: red hair and beyond. *J Arch Dermatol* 137:1447–1485
12. Liesel M, Fitz G, Jayne LF et al (2006) Effect of melantan, [Nle4.D-Phe7]-a-MSH, on melanin synthesis in humans with MC1R variant alleles. *J Pept* 27:388–394
13. D'Orazio JA, Nobuhisa T, Cui R et al (2006) Topical drug rescue strategy and skin protection based on the role of Mc1r in UV-induced tanning. *Nature* 443:340–344
14. Schaffler A, Scholmerich J, Buechler C (2006) The role of 'adipotropins' and the clinical importance of a potential hypothalamic–pituitary–adipose axis. *Nat Clin Pract Endocrinol Metab* 2:374–383
15. Steingrimsson E, Copeland NG, Jenkins NA (2004) Melanocytes and the microphthalmia transcription factor network. *Annu Rev Genet* 38:365–411
16. Wessells H, Gralnek D, Dorr R, Hruby VJ, Hadley ME, Levine N (2000) Effect of an alpha-melanocyte stimulating hormone analog on penile erection and sexual desire in men with organic erectile dysfunction. *Urology* 56:641–646
17. Jialing Y, Zhixue L (2008) High-Throughput Screening of crude antagonists of MC1R. *J shang hai university (natural science)* 14:216–220
18. Ho YS, Lai CS, Liu HI, Ho SY, Tai C, Pan MH et al (2007) Dihydrolipoic acid inhibits skin tumor promotion through anti-inflammation and anti-oxidation. *Biochem Pharmacol* 73:1786–1795
19. Tumurkhuu G, Koide N, Dagvadorj J et al (2008) The mechanism of development of acute lung injury in lethal endotoxic shock using alpha-galactosylceramide sensitization. *Clin Exp Immunol* 152:182–191
20. Mirzapourazova T, Kolosova IA, Moreno L et al (2007) Suppression of endotoxin-induced inflammation by taxol. *Eur Respir J* 30:429–435
21. Satoh H, Firestein GS, Billings PB, Harris JP, Keithley EM (2003) Proinflammatory cytokine expression in the endolymphatic sac during inner ear inflammation. *J Assoc Res Otolaryngol* 4:139–147
22. Korhonen R, Lahti A, Kankaanranta H, Moilanen E (2005) Nitric oxide production and signaling in inflammation. *Curr Drug Targets Inflamm Allergy* 4:471–479

23. Libby P (2000) Coronary artery injury and the biology of atherosclerosis: inflammation, thrombosis, and stabilization. *Am J Cardiol* 86:3J–8J discussion 8 J–9 J
24. Joshua SY, Ann R, Feng C et al (2006) Static analysis of realtime PCR data. *J BMC Bioinformatics* 7(85):1471–2105
25. Huo Meixia, Chen Na, Chi Gefu et al (2012) Traditional medicine alpinetin inhibits the inflammatory response in Raw 264.7 cells and mouse models. *Int Immunopharmacol* 12:241–248
26. Giebelen IA, van Westerloo DJ, LaRosa GJ, de Vos AF, van der Poll T (2007) Local stimulation of alpha 7 cholinergic receptors inhibits LPS-induced TNF-alpha release in the mouse lung. *Shock* 28:700–703
27. Hodge DR, Hurt EM, Farrar WL (2005) The role of IL-6 and STAT3 in inflammation and cancer. *Eur J Cancer* 41:2502–2512
28. Leser HG, Gross V, Scheibenbogen C, Heinisch A, Salm R, Lausen M et al (1991) Elevation of serum interleukin-6 concentration precedes acute-phase response and reflects severity in acute pancreatitis. *Gastroenterology* 101:782–785
29. Remick DG, Bolgos GR, Siddiqui J, Shin J, Nemzek JA (2002) Six at six: interleukin-6 measured 6 h after the initiation of sepsis predicts mortality over 3 days. *Shock* 17:463–467
30. Marklund L, Moller MJ, Sandberg L, Andersson L (1996) A missense mutation in the gene for melanocyte-stimulating hormone receptor (MC1R) is associated with the chesnut coat color in horses. *Mamm Genome* 7:895–899
31. Kijas JMH, Wales R, Tornsten A, Chardon P, Moller M, Andersson L (1998) Melanocortin receptor 1 (MC1R) mutations and coat color in pigs. *Genetics* 150:1177–1185
32. Garcí'a-Borro' n JC, Sa' nchez-Laorden BL, Jime' nez-Cervantes C (2005) Melanocortin-1 receptor structure and functional regulation. *Pigment Cell Res* 18:393–410
33. Mountjoy KG, Robbins LS, Mortrud MT, Cone RD (1992) The cloning of a family of genes that encode the melanocortin receptors. *Science* 257:1248–1251
34. April CS, Barsh GS (2007) Distinct pigmentary and melanocortin 1 receptor-dependent components of cutaneous defense against ultraviolet radiation. *Plos Genet* 3(1):e9
35. Steingrimsson E, Copeland NG, Jenkins NA (2004) Melanocytes and the microphthalmia transcription factor network. *Annu Rev Genet* 38:365–411
36. Cone RD (2005) Anatomy and regulation of the central melanocortin system. *Nat Neurosci* 8:571–578
37. Chhajlani V (1996) Distribution of cDNA for melanocortin receptor subtypes in human tissues. *Biochem Mol Biol Int* 38:73–80
38. Ni XP, Butler AA, Cone RD, Humphreys MH (2006) Central receptors mediating the cardiovascular actions of melanocyte stimulating hormones. *J Hypertens* 24:2239–2246
39. Cone RD, Lu D, Koppula S et al (1996) The melanocortin receptors: agonists, antagonists, and the hormonal control of pigmentation. *Recent Prog Horm Res* 51:287–317