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LRP1 receptor-mediated immunosuppression of α-MMC on monocytes

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ABSTRACT

Alpha-MMC is a type I ribosome-inactivating protein purified from bitter gourd that has strong anti-tumour and antiviral activity. Alpha-MMC also has immunosuppressive effects, but the mechanism of these immunosuppressive effects remains unclear. It is reported that the binding of α-MMC to its specific cell membrane LRP1 receptor is key to its biological effects. In this study, we investigated the effect of α-MMC on cytotoxicity and cytokine release regulation in three immune cells, human monocyte THP-1 cells, B-lymphocyte WIL2 cells and T-lymphocyte H9 cells, and explored the correlation between this effect and LRP1 receptor distribution on these three cell types. We demonstrate that α-MMC has a significant effect of apoptosis induction and cytokine release in THP-1 cells but has no effect on WIL2-S and H9 cells. Specifically, at a non-cytotoxic dose (80 μg/ml), α-MMC regulates THP-1 cells by inhibiting IL-1β, IL-2, IL-8, IL-9, IL-12, MIP-1α/β, MCP-1 and TNF-α expression and enhancing IL-1ra and RANTES expression, resulting in the inhibition of cellular immune function. Subsequent experiments showed that the cytokine expression regulated by α-MMC can be blocked by silencing the LRP1 receptor of α-MMC. Further research indicated that phosphorylation of 9 signalling proteins of the MAPK pathway was significantly regulated by α-MMC and was blocked by LRP1 silencing. We conclude that the regulation of cytokine expression induced by α-MMC in monocyte THP-1 cells is mediated by the LRP1 receptor, likely via the MAPK signalling pathway. Our results suggest that the inhibition effect on monocytes/macrophages mediates the immunosuppressive function of α-MMC. Due to the selective cytotoxicity and cytokine release regulation of α-MMC in monocytes/macrophages, α-MMC may be used for killing Tumour-Associated Macrophages (M2 subtypes) or inhibiting their cytokine release in the tumour microenvironment.

1. Introduction

Alpha-momorcharin (α-MMC), which is extracted and purified from the seeds of *Momordica charantia* L., exhibits multiple pharmacological properties, such as inhibition of tumour growth, anti-viral activity, and suppression of the immune response [1,2]. This protein is a type I ribosome-inactivating protein (RIP), and there are several subtypes of *Momordica* RIPs, including α-momorcharin, β-momorcharin, δ-momorcharin, γ-momorcharin, and the 30 kDa *Momordica* anti-HIV protein MAP30 [3,4]. Alpha-MMC has strong antitumour activity, as demonstrated for breast cancer, melanoma, and other cancers, which has made this protein a highly promising drug candidate for cancer therapy [1,5].

Alpha-MMC can inactivate the ribosome and induce apoptosis via its glycosidase activity and can regulate cell function via signal transduction [6]. Studies have found that α-MMC exerts these effects via the LRP1 (low-density lipoprotein receptor-related protein 1) receptor. LRP1 is a receptor that is shared by > 40 ligands, including α2-macroglobulin, ApoE, proteases, and protease-inhibitor complexes [7]. LRP1 receptors are widely distributed on tumour cells, such as

Abbreviations: α-MMC, α-momorcharin; RIPs, ribosome inactivating proteins; TCS, trichosanthin; LRP1, low density lipoprotein receptor-related protein 1; MAPK, mitogen-activated protein kinase; MFI, mean fluorescence intensity; STAT3, signal transducer and activator of transcription 3; TAMs, Tumour-Associated Macrophages

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choriocarcinoma, breast cancer, melanoma, and glioma cells, and are also widely distributed on normal cells, such as hepatocytes, monocyte/macrophages, central neurons and blood-brain barrier cells [8–10]. LRP1 is an endocytic receptor that has signal transduction activity [7,11,12]. Experiments have shown that α-MMC can specifically bind to the LRP1 receptor of the liver L02 cell line, leading to cytotoxicity via LRP1-mediated endocytosis, ribosome inactivation and LRP1-mediated JNK signalling [6]. Thus, the binding of the LRP1 receptor is an important part of the biological effects of α-MMC.

There is significant variability in the distribution of LRP1 receptors on immune cells [13]. The distribution density of LRP1 on the surface of monocytes is 12,618 ± 3766/cell, while that of lymphocytes is only 41 ± 44/cell. The high abundance of LRP1 receptors in monocytes/macrophages suggests an association with the biological activity of α-MMC, in that α-MMC may bind to the LRP1 receptor and be transported into monocytes/macrophages to induce apoptosis, or to stimulate signal transduction to regulate its cellular immune activity. It has been found that α-MMC can significantly inhibit macrophage activity, such as cytostatic and phagocytic activity, in vivo [14]. A single injection of a non-toxic dose of α-MMC in mice resulted in significant suppression of the delayed-type hypersensitivity response as well as humoural antibody formation towards sheep red blood cells, and a thioglycolate-induced migration macrophage migration was inhibited in vivo [14]. Is the immunosuppressive effect of α-MMC on immune cells initially mediated through its effects on monocytes/macrophages via LRP1 receptors?

Because monocytes/macrophages are important antigen-presenting cells and secrete a variety of immunopotentiating cytokines and chemokines that can promote immune cell activation, proliferation, differentiation and the production of immune effector molecules [15,16], the apoptosis or inhibition of cellular immunity induced by α-MMC on monocyte/macrophages is bound to cause inhibition of immune activity in the body. Therefore, in this study, we investigated the effect of α-MMC on cytotoxicity and cytokine release regulation in three immune cell populations, human monocyte THP-1 cells, B-lymphocyte WIL2 cells and T-lymphocyte H9 cells, and explored the mechanism of immunosuppression of α-MMC by analysing the correlation between this effect and the distribution density of LRP1 receptors on these three cells. In addition, we attempted to elucidate the LRP1 receptor-mediated signal transduction pathway.

Studying the regulation of α-MMC towards monocytes can help reveal the immunosuppressive mechanism of α-MMC and re-validate the functional mechanism of the LRP1 receptor-mediated pathway, laying the foundation for studying the mechanisms of other α-MMC-associated biological activities. Moreover, as monocytes/macrophages can be polarized to Tumour-Associated Macrophages (TAMs, M2 subtype) in the tumour microenvironment [17,18], which release various cytokines to promote tumour cell proliferation, tumour angiogenesis and tumour metastasis, the cytokine release regulation of α-MMC in M2 subtype macrophages may be an important mechanism of its anti-tumour effects.

2. Materials and methods

2.1. Preparation of α-MMC

Bitter melon seeds were obtained from the Institute of Agricultural Science and Technique of Sichuan Province, China. The bitter melon seeds were milled to a powder and extracted with 50 mM acetate buffer solution (pH 6.3). The crude extract was acidified by adding 250 mM HCl at 2 °C. After simple centrifugation, the supernatant was neutralized with 1 M sodium phosphate buffer (pH 7.0). Then, the proteins were precipitated with ammonium sulfate (AS). Further purification of α-MMC was accomplished using SP-Sepharose FF (GE, USA), Superdex 75 (GE, USA) and MacroCap™ SP columns (Piscataway, USA). Alpha-MMC was identified by SDS-PAGE and gel filtration HPLC. The molecular weights of the pure α-MMC were determined by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS), and N-terminal amino acid sequence analysis was performed by electrospray ionization quadrupole mass spectrometry (ESI-QUAD-MS). Finally, Sephadex G-100 column chromatography was used to remove endotoxins, and the endotoxin-free α-MMC solution was verified by the Limulus test.

2.2. Flow cytometry analysis of α-MMC-induced apoptosis in THP-1, WIL2-S and H9 cells

A human monocyte THP-1 cell line, a human B lymphoblast WIL2-S cell line, and a human T lymphocyte H9 cell line were purchased from the Typical Culture Preservation Committee Cell Bank of the Chinese Academy of Sciences. The cells were grown in complete RPMI-1640 medium (Gibco, USA) containing 10% heat-inactivated foetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Thermo, USA). The cells were maintained at 37 °C in humidified air containing 5% CO_2.

The Annexin V-FITC Apoptosis Kit (BioVision, USA) was used to detect α-MMC-induced apoptosis in the THP-1, Will2-S and H9 cell lines. Briefly, THP-1, Will2-S and H9 cells were seeded in 6-well plates at 2×10^5 cells/well, and then, α-MMC was added to each well at a final concentration of 20 μg/ml, 40 μg/ml, 80 μg/ml, 120 μg/ml or 160 μg/ml. After incubation for 24 h, the cells were collected and re-suspended in 500 μl of binding buffer with 5 μl of Annexin V-FITC and 10 μl of propidium iodide. Fifteen minutes later, the cells were subjected to flow cytometry analysis (BD FACS, USA).

2.3. Cytokine expression in cell lysates of THP-1, WIL2-S and H9 cells

THP-1, WIL2-S and H9 cells in the logarithmic growth phase were seeded on a 6-well plate at a density of 1×10^6 cells/well, and 2 ml of complete RPMI-1640 medium was added to each well. Each type of cell was treated with α-MMC (80 μg/ml) for 30 min, 2 h, 8 h, and 24 h. PBS was used as a negative control. Each group was repeated in triplicate. After centrifugation at 1000 rpm for 3 min, the cells were lysed, and the cell lysate samples were collected. All lysate protein concentrations were adjusted to 0.4 mg/ml, and the samples were stored at −80 °C.

Cell lysates from the THP-1, WIL2-S and H9 cell lines were analysed by a magnetic bead suspension array, using the Bio-Plex Pro™ Human Cytokine Grp I Panel (27-plex) (Bio-Rad Laboratories, Hercules, CA, USA, M500KCAF0Y) according to the manufacturer's instructions. The 27 human cytokines were IL-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, basic fibroblast growth factor (FGF basic), eotaxin, G-CSF, GM-CSF, IFN-γ, IFN-γ-induced protein 10 (IP-10), MCP-1, MIP-1α, MIP-1β, platelet-derived growth factor BB (PDGF-BB), RANTES, TNF-α and VEGF.

Briefly, the test procedure was as follows: consecutive four-fold serial dilutions of standards were prepared, and cell lysates were diluted to a concentration of 400 μg/ml. In each well of a 96-well plate, 25 μl of a magnetic bead suspension was added; the plate was washed; and 50 μl of the above samples, standards and controls were added. The plate was shaken at room temperature for 30 min. After three washes, each well of the plate was incubated with 25 μl of detection antibody and treated with PE-labelled streptavidin antibody. The plate was analysed on a Luminex 200 multiplex testing system (USA, gate setting: 8000–15,000).

2.4. Western blotting assays for the LRP1-receptor protein from THP-1, WIL2-S and H9 cells

THP-1, WIL2-S and H9 cells were washed with PBS in the logarithmic growth phase at a density of 1×10^6 cells/well. RIPA lysate buffer containing protease inhibitors (1:200) was added, and the cell lysate in each well was collected at 4 °C. The protein concentration of

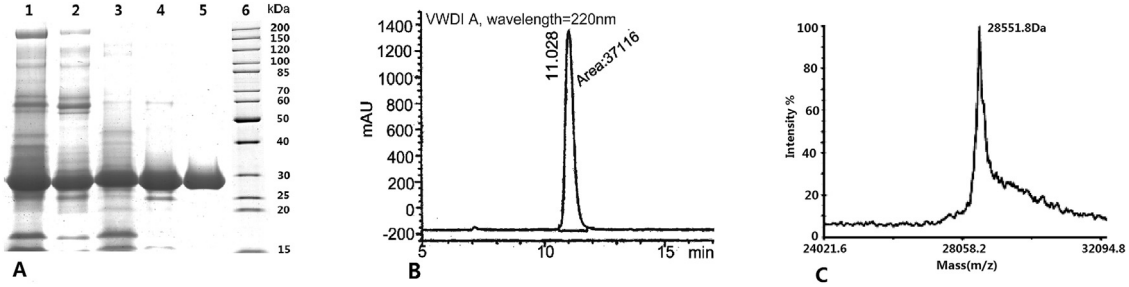

Fig. 1. Purification and identification of the α-MMC protein. A: lane 1, crude extract; lane 2, sample from AS precipitation; lane 3, eluate from SP-Sepharose FF chromatography; lane 4, eluate from gel filtration chromatography; lane 5, α-MMC purified by Macro-Cap-SP chromatography; lane 6: protein molecular mass marker. B: HPLC chromatogram of the α-MMC protein sample, analysed to verify > 97% purity. C: MALDI-TOF-MS analysis of α-MMC. The average MW of α-MMC was 28,551.6 Da.

each cell lysate sample was measured using the bicinchoninic acid (BCA) method, and Western blot assays were subsequently performed. The primary antibody was rabbit anti-human LRP1 (Abcam, USA); the secondary antibody was HRP-labelled goat anti-rabbit IgG (R&D, USA); and the substrate was luminol (ECL Millipore, USA). The cells were visualized using a chemiluminescence gel imager (Bio-Rad, USA), and a rabbit anti-human β-actin antibody (Novus, USA) was selected as the internal control antibody.

2.5. Detection of MAPK signalling proteins

A 9-plex MAPK phosphoprotein panel (Bio-Plex ProTM cell signalling assays, 171-304006M) was used to measure the phosphorylation levels of the 9 signalling proteins of the MAPK pathway. The 9 signalling proteins were activating transcription factor 2 (ATF-2) (Thr71), heat shock protein 27 (HSP27) (Ser78), extracellular regulated protein kinase (Erk1/2) (Thr202/Tyr204, Thr185/Tyr187), c-Jun N-terminal kinase (c-JNK) (Thr183/Tyr185), methionine enkephalin 1 (MEK1) (Ser217/Ser221), p38 mitogen-activated protein kinase (p38MAPK) (Thr180/Tyr182), signal transducer and activator of transcription 3 (Stat3) (Tyr705), protein 53 (p53) (Ser15), and 90 kDa ribosomal S6 kinase (p90RSK) (Ser380). The plates were shaken at 600–800 rpm overnight at 4 °C, and each well of the plates was treated with 25 μl of detection antibody and PE-labelled streptavidin antibody. Subsequently, the plates were analysed on a Luminex 200 multiplex testing system.

2.6. LRP1 knockdown in THP-1 cells

LRP1 (ID 4035) Trilencer-27 human siRNA (SR302723) and the SiTran1.0 transfection reagent (TT300001) (Origene, USA) were used to perform siRNA-mediated inhibition of LRP1 receptors at a concentration of 10 nM and a reaction time of 72 h [6,19]. There were three specific siRNAs targeting LRP1: siRNA A (5′-ACACCAAUAAGAA GCAGAUCAAUGT-3′), siRNA B (5′-AGAUUUGUCCACAGAGUAAGGC CCA-3′), and siRNA C (5′-GGCUGUGACUGACGAGGAACCGUTT-3′). The experimental method was as follows: THP-1 cells in 6-well plates at 8.0×10^5 cells/well were treated with 4 μl of 5 μM siRNA and 20 μl of Trans1.0 transfection reagent; 4 h later, the medium was replaced with RPMI-1640 containing 10% FBS and double antibodies. The cells were incubated for 72 h. The silencing effect of the LRP1-receptor siRNA was detected by Western blotting. A negative control without siRNA and a non-target control (NTC) were tested in parallel.

2.7. Effect of LRP1 silencing on cytokines and signalling proteins

THP-1 cells were seeded in 6-well plates at 8.0×10^5 cells/well, and the LRP1 siRNA treatment was performed as described above. Alpha-MMC was added at a final concentration of 80 μg/ml after 72 h of siRNA treatment. The cells were collected after α-MMC dosing and incubated for 30 min, 2 h, 8 h, and 24 h. Cell lysates were prepared and used to detect cytokines and phosphorylated protein levels in the MAPK signalling pathway, as described above.

2.8. Data analysis

The results are expressed as the mean ± standard deviation, and intergroup comparisons were performed with a one-way ANOVA (SPSS 19.0). The significance level was 0.05, and a *P*-value < 0.05 was considered significant.

3. Results

3.1. Verification of the α-MMC protein sample

The results of the purification and identification of α-MMC are shown in Fig. 1. The purified α-MMC protein was chromatographically and electrophoretically homogeneous, showing a single band on the SDS-PAGE gel (Fig. 1A) and a single eluting peak in the HPLC chromatogram (Fig. 1B). MALDI-TOF-MS analysis of α-MMC was performed, and the true MW of the protein was 28,551.6 Da (Fig. 1C). The N-terminal 5-amino-acid sequence N-Asp-Val-Ser-Phe-Arg was consistent with the sequences of α-MMC reported in the NCBI database. As shown in Online Resource 1 (http://www.ncbi.nlm.nih.gov/), the identified protein exhibited a representative mass spectrometric peak corresponding to YLLMHLFNYDGK, which was consistent with the sequence of α-MMC.

3.2. Dose-dependent α-MMC-induced apoptosis in THP-1

Flow cytometric analysis was used to detect α-MMC-induced apoptosis in THP-1, Will2-S and H9 cells at different dosages. The results (Fig. 2) showed that after 24 h of α-MMC treatment, the percent induction of apoptotic cells at doses of 0 μg/ml (PBS), 20 μg/ml, 40 μg/ml, 80 μg/ml, 120 μg/ml and 160 μg/ml was 2.5% ± 0.5%, 2.2% ± 0.6%, 3.0% ± 0.8%, 4.3% ± 1.1%, 10.4% ± 2.7% and 21.6% ± 4.5%, respectively, in THP-1 cells; 2.0% ± 0.4%, 3.2% ± 0.8%, 2.2% ± 0.5%, 3.7% ± 0.7%, 4.4% ± 1.2% and 10.8% ± 2.5%, respectively, in Will2-S cells; and 2.1% ± 0.3%, 2.2% ± 0.6%, 3.2% ± 0.8%, 3.7% ± 0.7%, 4.8% ± 1.2% and 12.6% ± 3.8%, respectively, in H9 cells. At a dose of 160 μg/ml, α-MMC treatment distinctly induced apoptosis in all the THP-1, Will2-S and H9 cells, while at a dose of 120 μg/ml, α-MMC treatment induced noticeable apoptosis in only THP-1 cells ($P < 0.05$). At doses of 80 μg/ml or less, α-MMC treatment could not induce significant apoptosis in these three cell lines within 24 h.

3.3. Regulatory effect of α-MMC on cytokine expression in immune cell lines

WIL2-S, H9, and THP-1 cells were cultured in 6-well plates and

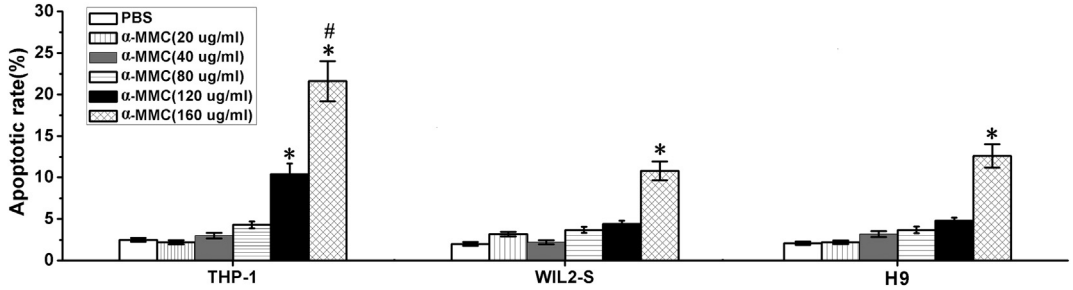

Fig. 2. Flow cytometric analysis of α-MMC-induced apoptosis in THP-1, Will2-S and H9 cells at different α-MMC dosages. Data are expressed as the means ± SDs (n = 3). *$P < 0.05$, significantly different from the negative control (PBS group) (one-way ANOVA). # $P < 0.05$, significantly different from that of Will2-S and H9 cells.

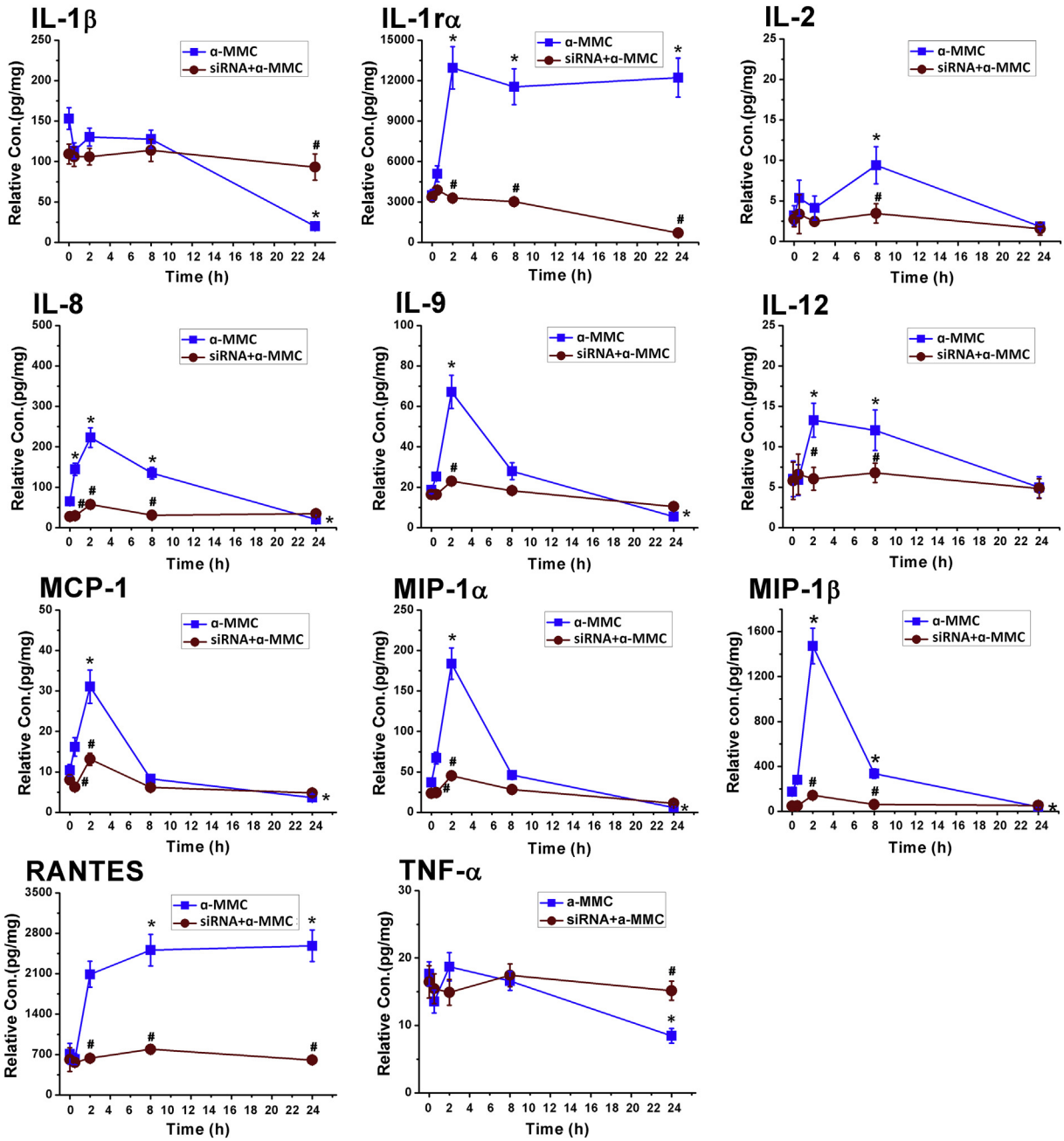

Fig. 3. Dynamic changes in 11 cytokines in THP-1 cells after treatment with α-MMC (80 μg/ml). Data are expressed as the means ± SDs (n = 3). *$P < 0.05$, significantly different from the pretreatment in the same treatment group (one-way ANOVA); #$P < 0.05$, significantly different from non-siRNA treatment group (one-way ANOVA).

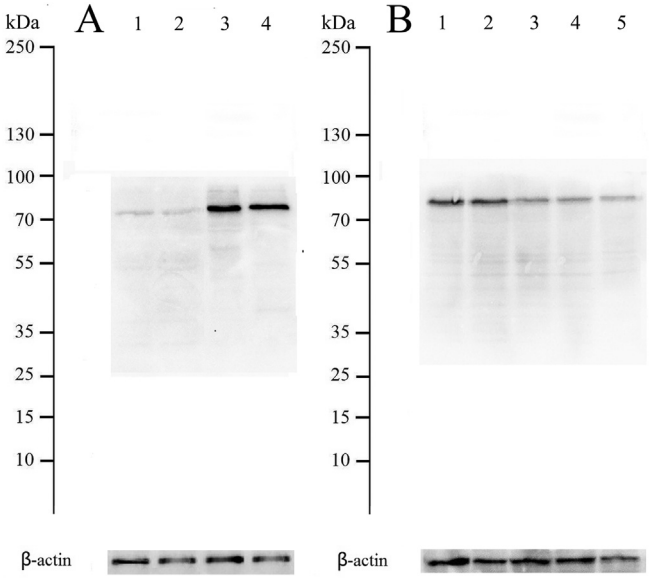

Fig. 4. Western blotting results for the LRP1 protein. A: LRP1 protein in different cell lines (non-siRNA treated) using the same total protein content (10 μg). Lanes 1 to 4 show the results for Will2-s, H9, THP-1 and L02 cells (human liver cells). B: Western blotting result for the effect of LRP1 silencing on the THP-1 cell line. Lane 1 is a negative control, and lanes 2, 3, 4, and 5 show the effects of NTC siRNA, siRNA A, siRNA B, and siRNA C, respectively.

collected after α-MMC treatment for 30 min, 2 h, 8 h, and 24 h. Twenty-seven cytokines were analysed in the cell lysates using the Bio-Plex Pro™ Human Cytokine Grp I Panel (27-plex) assay.

The α-MMC action time (h) is plotted on the abscissa, and the relative protein concentration (pg/mg) is plotted on the ordinate of the graph shown in Fig. 2. The relative protein concentration refers to the cytokine content (pg) in 1 mg of total protein in the cell lysate sample. The results showed that the cytokines were either not detected or were detected but did not exhibit significant changes in expression at any time point in WIL2-S and H9 cells. In THP-1 cells, α-MMC treatment regulated the expression levels of most of the tested cytokines. The primary effect of α-MMC treatment was a synthesis-promoting effect during the early phase (2–8 h) and a synthesis-inhibiting effect during the late phase (8–24 h) on IL-8, IL-9, MIP-1α, MIP-1β, and MCP-1. Alpha-MMC treatment resulted in persistent activation of the expression of IL-1ra and RANTES and persistent inhibition of the expression of IL-1β and TNF-α (Fig. 3).

3.4. Western blotting results of LRP1 expression in THP-1, WIL2-S and H9 cells

Western blotting showed that the LRP1 protein content was significantly higher in THP1 cells than in WIL2-S B-lymphocytes and H9 T-lymphocytes. The level was similar to the high-density distribution in the normal liver cell line L02 (Fig. 4A).

3.5. LRP1 silencing

Specific siRNAs targeting LRP1 was used to inhibit the expression of LRP1. Seventy-two hours after siRNA-mediated LRP1 silencing in THP-1 cells, Western blotting assays were performed to verify the inhibition of LRP1 expression. An anti-LRP1 light chain (85 kDa) antibody was used, and chemiluminescence detection indicated that siRNA C exhibited efficient silencing (Fig. 4B). Therefore, siRNA C was used to analyse the effect of inhibition of the LRP1 receptor.

3.6. LRP1 silencing inhibited the regulatory effect of α-MMC on cytokine synthesis

The expression levels of cytokines were significantly altered after LRP1 siRNA treatment. For example, the expression levels of IL-8, IL-9, MIP-1α, MIP-1β, and MCP-1 peaked at 2 h, but the peak value decreased significantly after LRP1 silencing (Fig. 3). For cytokines with persistently increased expression, such as IL-1ra and RANTES, LRP1 silencing also resulted in decreased changes in expression. The regulation of cytokine expression by α-MMC can be blocked by silencing of the α-MMC receptor LRP1, indicating that regulation of cytokine expression by α-MMC occurs via binding to this receptor.

3.7. Alpha-MMC inhibits the MAPK signalling pathway in an LRP1-dependent manner

Alpha-MMC treatment had a distinct effect on the phosphorylation levels of all 9 MAPK signalling proteins in THP-1 cells. Overall, phosphorylation (MFI values) increased during the first 8 h after α-MMC treatment, peaking at 8 h, and the MFI values of p-JNK, p-MEK1, p-p38MAPK and p-Stat3 also peaked at 0.5 h, indicating that these proteins were activated early by α-MMC. However, this activation stopped at 8 h; inhibition was observed from 8 h to 24 h; and the intensity of inhibition was far greater than that of the activation ($P < 0.05$) (Fig. 5).

During LRP1 silencing, the MFI values of these proteins decreased rapidly after α-MMC treatment and reached their lowest levels at 2 h, which was significantly different from the values at 0 h ($P < 0.05$). Then, the values gradually increased to the baseline level at 24 h. The kinetic profile of the LRP1 silencing group during the 2–24 h period was the opposite of that of the non-LRP1 silencing group (Fig. 5). The results suggest that LRP1 silencing blocked the regulatory effects of α-MMC on signalling proteins and had a strong inhibitory effect.

4. Discussion

Alpha-MMC is a type I RIP. In addition to anti-tumour and antiviral activity, α-MMC also has immunosuppressive effects [1,14,20]. It can inactivate the ribosome and induce apoptosis via its glycosidase activity and can regulate cell functions via signal transduction through the LRP1 receptor [6]. Because the distribution of LRP1 receptor is very different in monocytes/macrophages vs. lymphocyte membranes, the binding of α-MMC to the cell membrane LRP1 receptor is a key to its biological effects. In this study, we examined the apoptosis and cytokine expression induced by α-MMC in three immunocytes - WIL2-S, H9, and THP-1 cells - and attempted to elucidate the LRP1 receptor-mediated signal transduction pathway.

High-throughput liquid chip analysis was used to detect the cytokine expression profile and the phosphorylation levels of 9 MAPK signal transduction proteins [21], and LRP1 silencing was also used to verify the receptor-mediated effects. The liquid chip method offers higher sensitivity, greater capacity and better quantification than Western blots and ELISAs. The liquid chip method has been widely used to study protein expression and post-translational modifications of proteins, such as phosphorylation and glycosylation, and to screen for protein function and protein-protein interactions [22,23].

In the α-MMC-induced apoptosis experiment, α-MMC exhibited dose-dependent cytotoxicity towards WIL2-S, H9, and THP-1 cells, and the cytotoxicity towards THP-1 cells was significantly higher than that towards WIL2-S and H9 cells. For example, at a dose of 160 μg/ml, α-MMC treatment distinctly induced apoptosis in each of the THP-1, Will2-S and H9 cell populations, while at a dose of 120 μg/ml, α-MMC treatment induced noticeable apoptosis only in THP-1 cells ($P < 0.05$). When these three cell lines were treated with α-MMC at a final concentration of 80 μg/ml for 24 h, cytotoxicity was not observed. At this dose, among the 27 common cytokines tested, no significant increase or

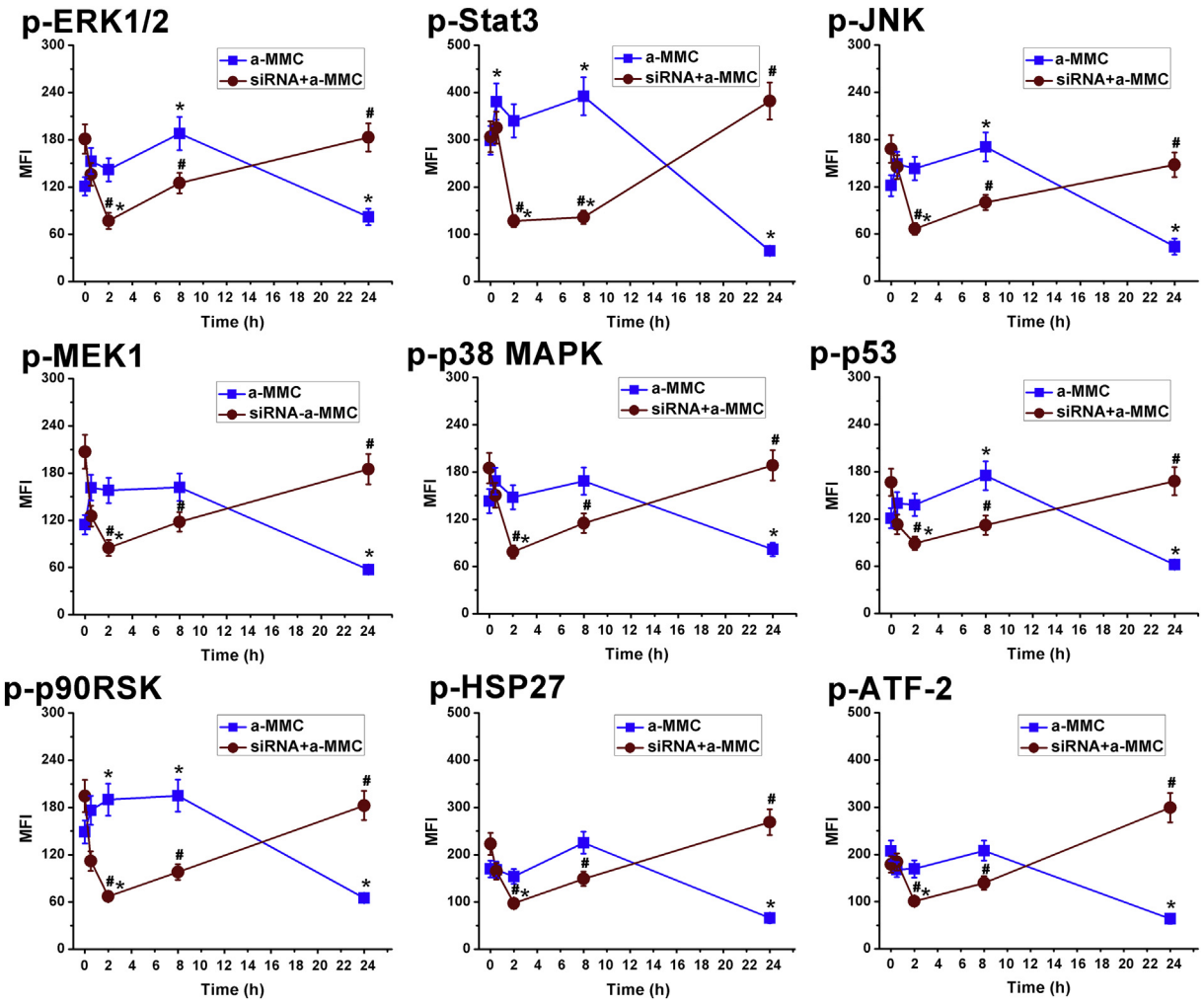

Fig. 5. Dynamic changes in phosphorylated MAPK proteins in THP-1 cells treated with α-MMC (80 μg/ml). Data are expressed as the means ± SDs (n = 3). *$P < 0.05$, significantly different from the untreated group (one-way ANOVA). #$P < 0.05$, significantly different from the non-siRNA treatment group (one-way ANOVA).

decrease in cytokine expression was observed when the WIL2-S cells and H9 cells were treated with α-MMC. However, α-MMC had a significant regulatory effect on cytokine expression in THP-1 cells. After 24 h of treatment with α-MMC, significant inhibition of IL-1β, IL-2, IL-8, IL-9, IL-12, MIP-1α/β, MCP-1 and TNF-α expression was induced. The data revealed that α-MMC had apoptosis effects on THP-1 cells and regulated cytokine synthesis in these cells but had no significant effect on WIL2-S and H9 cells. Our results may suggest that at non-cytotoxic doses, the immunosuppressive effect of α-MMC occurs primarily via regulating the release of cytokines from mononuclear macrophages.

It was also observed that α-MMC caused a significant increase in the synthesis of IL-1ra and RANTES in THP-1 cells. IL-1ra, an antagonist of IL-1, binds strongly to the IL-1 receptor and inhibits the immune activation function of IL-1. Therefore, elevated IL-1ra expression is also a factor that promotes immunosuppression. However, the increase in RANTES expression seems to be inconsistent with the inhibitory effect of α-MMC. RANTES is known to be a chemokine that favours T-cell activation, but we detected high levels of RANTES expression, which continued to increase over 24 h of experimental observation; this is an interesting new finding. However, because RANTES exhibits bidirectional promotion and inhibition in terms of its antitumour and anti-HIV infection activity [24–26], it remains unclear whether RANTES plays a positive or negative role in immune regulation.

The LRP1 expression assessed by WB assay was higher in human monocyte THP1 cells than in WIL2-S B-lymphocytes and H9 T-lymphocytes, demonstrating that a higher level of LRP1 protein content in the cell membrane leads to more apoptosis induced by α-MMC and an increased regulatory effect on cytokine expression. This indicates that the biological activity of α-MMC is closely associated with the presence of high levels of the LRP1 receptor. Furthermore, silencing of the LRP1 receptor by siRNA blocks the effect of α-MMC on cytokine expression in THP-1 cells, suggesting that α-MMC indeed exerted its effects by binding to this receptor. We have confirmed that the cytotoxicity mechanism of a-MMC in normal liver cells is through LRP1-mediated endocytosis and JNK activation [6]. When the LRP1 receptor was knocked down, the LRP1-mediated endocytosis of a-MMC was also blocked, and the apoptotic effect of a-MMC and the activation of JNK pathway were also inhibited.

In this study, our results initially confirmed that α-MMC regulates cytokine release through the MAPK signal transduction pathway. The results showed that phosphorylation of the 9 signalling proteins of the MAPK pathway was significantly regulated by α-MMC. After the LRP1 receptor was blocked by siRNA, the regulation of these signalling proteins by α-MMC was also blocked, indicating that signal transduction by α-MMC is mediated by the LRP1 receptor. In addition, the regulation of cytokine expression was also consistent with the MAPK signalling pathway of α-MMC because the “action-time curve” of cytokines and MAPK signalling proteins were consistent after α-MMC treatment. The curve exhibited an upward trend within 8 h after administration and peaked at 2–8 h; after 8 h, the curve exhibited a downward trend and

reached a nadir at 24 h. After LRP1 was silenced, both phosphorylation of the signalling proteins and cytokine expression were blocked.

LRP1 is an endocytic receptor with a signal transduction effect [7,11,12]. The LRP1 protein has two copies of the "NPxY" tetra-amino acid motif in the LRP tail, which can independently function as mediators of rapid endocytosis of the LDL receptor or as internalisation signals [27]. This receptor also undergoes regulated intramembrane proteolysis, and the released intracellular domain participates in transcriptional modulation [28]. The MAPK pathway is an important signalling pathway in eukaryotic cells and can transduce signals from the cell membrane to the cytoplasm and nucleus [29]. The MAPK signalling pathway plays an important role in cell proliferation, survival, differentiation, and apoptosis by affecting gene transcription and regulation in animal cells. There are three primary MAPK signalling pathways: the ERK, JNK/SAPK and p38MAPK pathways. In mammalian cells, the same stimulation can activate several MAPK pathways, which can cooperatively regulate biological activity [30–32]. In this study, we investigated 9 important MAPK signal transduction proteins in three pathways. For example, the terminal signal transduction proteins in the ERK pathway are important transcription factors in the nucleus that can regulate the transcription of target genes to alter the expression or activity of specific proteins, such as cytokines [33,34], as was observed in this study. STAT3 has been reported to regulate the expression of cytokines. The promoter region of the VEGF gene contains a STAT3 binding site, and constitutive activation of STAT3 can induce the expression of VEGF. In addition, STAT3 activation can upregulate the expression of IL-10 and TGF-β and downregulate the expression of IL-2 [35–39]. Thus, our experiments suggest that the inhibitory effect of α-MMC on cytokines was initiated by binding to LRP1 on the cell membrane, followed by inhibition of the MAPK signalling pathway. The hypothesised LRP1-MAPK signalling pathway and the elements with a central role in cytokine expression need to be further elucidated.

In summary, the experimental results prove that (1) due to differences in the distribution density of LRP1 receptors in the WIL2-S, H9, and THP-1 cell lines, the apoptosis and the regulation of cytokine expression by α-MMC occurred mainly in monocyte THP-1 cells; (2) the cytokine regulation manifested mainly as an inhibition of cell function, which may lead to inhibition of the immune response; and (3) cytokine regulation by α-MMC in monocyte THP-1 cells is mediated by the LRP1 receptor, possibly via the MAPK signalling pathway.

Cytotoxicity and immunomodulatory effects on monocytes/macrophages may be an important pathway and a leading factor for α-MMC immunosuppression. Simultaneously, this study demonstrates again the LRP1 receptor-mediated mechanism of the biological activity of α-MMC and that the distribution of the LRP1 receptor in histiocytes determines their sensitivity to the pharmacological or cytotoxic effects of α-MMC. Due to the high distribution of the LRP1 receptor in monocytes/macrophages [13,40], α-MMC is likely to exert an inhibitory effect on the release of cytokines from polarized M2 macrophages in the tumour microenvironment [17,18], and it is highly likely to kill M2 macrophages to inhibit tumour growth and metastasis. This will likely be a very exciting research direction in the future.

Conflicts of interest statement

All authors reviewed and agreed with the contents of this manuscript and report no conflicts of interest.

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References

[1] M. Puri, I. Kaur, R.K. Kanwar, R.C. Gupta, A. Chauhan, J.R. Kanwar, Ribosome inactivating proteins (RIPs) from *Momordica charantia* for antiviral therapy, Curr. Mol. Med. 9 (2009) 1080–1094.
[2] T.B. Ng, J.H. Wong, H. Wang, Recent progress in research on ribosome inactivating proteins, Curr. Protein Pept. Sci. 11 (2010) 37–53.
[3] W.P. Fong, Y.T. Poon, T.M. Wong, J.W. Mock, T.B. Ng, R.N. Wong, et al., A highly efficient procedure for purifying the ribosome-inactivating proteins alpha- and beta-momorcharins from *Momordica charantia* seeds, N-terminal sequence comparison and establishment of their *N*-glycosidase activity, Life Sci. 59 (1996) 901–909.
[4] E. Basch, S. Gabardi, C. Ulbricht, Bitter melon (*Momordica charantia*): a review of efficacy and safety, Am. J. Health Syst. Pharm. 60 (2003) 356–359.
[5] D. Cao, Y. Sun, L. Wang, Q. He, J. Zheng, F. Deng, et al., Alpha-momorcharin (alpha-MMC) exerts effective anti-human breast tumor activities but has a narrow therapeutic window in vivo, Fitoterapia 100 (2015) 139–149.
[6] L. Wang, F. Shen, M. Zhang, Q. He, H. Zhao, X. Yu, et al., Cytotoxicity mechanism of α-MMC in normal liver cells through LRP1 mediates endocytosis and JNK activation, Toxicology 357-358 (2016) 33–43.
[7] P. Boucher, J. Herz, Signaling through LRP1: protection from atherosclerosis and beyond, Biochem. Pharmacol. 81 (2011) 1–5.
[8] Yonghe Li, Nick Wood, Peter G. Parsons, David Yellowleesb, Peter K. Donnelly, Expression of alpha2-macroglobulin receptor/low density lipoprotein receptor-related protein on surfaces of tumour cells: a study using flow cytometry, Cancer Lett. 111 (1997) 199–205.
[9] Yonghe Li, Nick Wood, Philip Grimsley, David Yellowlees, Peter K. Donnellya, In vitro invasiveness of human breast cancer cells is promoted by low density lipoprotein receptor-related protein, Invasion Metastasis 99 (1998) 240–251.
[10] S.K. Moestrup, J. Gliemann, G. Pallesen, Distribution of the alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues, Cell Tissue Res. 269 (1992) 375–382.
[11] R.G. Fred, S.K. Boddeti, M. Lundberg, N. Welsh, Imatinib mesylate stimulates low-density lipoprotein receptor-related protein 1-mediated ERK phosphorylation in insulin-producing cells, Clin. Sci. (Lond.) 128 (2015) 17–28.
[12] X. Pi, C.E. Schmitt, L. Xie, A.L. Portbury, Y. Wu, P. Lockyer, et al., LRP1-dependent endocytic mechanism governs the signaling output of the bmp system in endothelial cells and in angiogenesis, Circ. Res. 111 (2012) 564–574.
[13] W. Maier, M. Bednorz, S. Meister, A. Roebroek, S. Weggen, U. Schmitt, et al., LRP1 is critical for the surface distribution and internalization of the NR2B NMDA receptor subtype, Mol. Neurodegener. 8 (2013) 25.
[14] S.O. Leung, H.W. Yeung, K.N. Leung, The immunosuppressive activities of two abortifacient proteins isolated from the seeds of bitter melon (*Momordica charantia*) [J], Immunopharmacology 13 (1987) 159–171.
[15] M. Haniffa, V. Bigley, M. Collin, Human mononuclear phagocyte system reunited, Semin. Cell Dev. Biol. 41 (2015) 59–69.
[16] D.A. Hume, The mononuclear phagocyte system, Curr. Opin. Immunol. 18 (2006) 49–53.
[17] J.W. Pollard, Tumour-educated macrophages promote tumour progression and metastasis, Nat. Rev. Cancer 4 (2004) 71–78.
[18] F.O. Martinez, L. Helming, S. Gordon, Alternative activation of macrophages: an immunologic functional perspective, Annu. Rev. Immunol. 27 (2009) 451–483.
[19] A. Sen, T.J. Nelson, D.L. Alkon, ApoE4 and Abeta oligomers reduce BDNF expression via HDAC nuclear translocation, J. Neurosci. 35 (2015) 7538–7551.
[20] Y. Meng, B. Liu, N. Lei, J. Zheng, Q. He, D. Li, et al., Alpha-momorcharin possessing high immunogenicity, immunotoxicity and hepatotoxicity in SD rats, J. Ethnopharmacol. 139 (2012) 590–598.
[21] Z. Liu, X. Yuan, Y. Luo, Y. He, Y. Jiang, Z.K. Chen, et al., Evaluating the effects of immunosuppressants on human immunity using cytokine profiles of whole blood, Cytokine 45 (2009) 141–147.
[22] Y. Yang, J. Wang, H. Wen, H. Liu, Comparison of two suspension arrays for simultaneous detection of five biothreat bacterial in powder samples, J Biomed Biotechnol 2012 (2012) 831052.
[23] R. Guo, H. Mao, X. Hu, N. Zheng, D. Yan, J. He, et al., Slow reduction of IP-10 Levels predicts HBeAg seroconversion in chronic hepatitis B patients with 5 years of entecavir treatment, Sci. Rep. 6 (2016) 37015.
[24] E.P. Adler, C.A. Lemken, N.S. Katchen, R.A. Kurt, A dual role for tumor-derived chemokine RANTES (CCL5), Immunol. Lett. 90 (2003) 187–194.
[25] E. Azenshtein, G. Luboshits, S. Shina, E. Neumark, D. Shahbazian, M. Weil, et al., The CC chemokine RANTES in breast carcinoma progression: regulation of expression and potential mechanisms of promalignant activity, Cancer Res. 62 (2002) 1093–1102.
[26] D.H. McDermott, M.J. Beecroft, C.A. Kleeberger, F.M. Al-Sharif, W.E. Ollier, P.A. Zimmerman, et al., Chemokine RANTES promoter polymorphism affects risk of both HIV infection and disease progression in the Multicenter AIDS Cohort Study, AIDS 14 (2000) 2671–2678.
[27] W.J. Chen, J.L. Goldstein, M.S. Brown, NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor, J. Biol. Chem. 265 (1990) 3116–3123.
[28] J. Herz, D.K. Strickland, LRP: a multifunctional scavenger and signaling receptor, J. Clin. Invest. 108 (2001) 779–784.
[29] Y. Sun, W.Z. Liu, T. Liu, X. Feng, N. Yang, H.F. Zhou, Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis, J.

Recept. Signal Transduct. Res. 35 (2015) 600–604.
[30] K. Kondoh, E. Nishida, Regulation of MAP kinases by MAP kinase phosphatases, Biochim. Biophys. Acta 1773 (2007) 1227–1237.
[31] W. Cui, Z. Zhang, P. Zhang, J. Qu, C. Zheng, X. Mo, et al., Nrf2 attenuates inflammatory response in COPD/emphysema: crosstalk with Wnt3a/β-catenin and AMPK pathways, J. Cell. Mol. Med. 22 (2018) 3514–3525.
[32] W. Zhou, X. Mo, W. Cui, Z. Zhang, D. Li, L. Li, et al., Nrf2 inhibits epithelial-mesenchymal transition by suppressing snail expression during pulmonary fibrosis, Sci. Rep. 6 (2016) 38646.
[33] A. Farooq, M.M. Zhou, Structure and regulation of MAPK phosphatases, Cell. Signal. 16 (2004) 769–779.
[34] C. Tárrega, C. Nunes-Xavier, R. Cejudo-Marín, J. Martín-Pérez, R. Pulido, Studying the regulation of MAP Kinase by MAP Kinase phosphatases in vitro and in cell systems, Methods Mol. Biol. 661 (2010) 305–321.
[35] N.I. Arbouzova, M.P. Zeidler, JAK/STAT signalling in Drosophila: insights into conserved regulatory and cellular functions, Development 133 (2006) 2605–2616.
[36] M. Saydmohammed, D. Joseph, V. Syed, Curcumin suppresses constitutive activation of STAT-3 by up-regulating protein inhibitor of activated STAT-3 (PIAS-3) in ovarian and endometrial cancer cells, J. Cell. Biochem. 110 (2010) 447–456.
[37] C. Wu, Q. Guan, Y. Wang, Z. Zhao, G.W. Zhou, SHP-1 suppresses cancer cell growth by promoting degradation of JAK kinases, J. Cell. Biochem. 90 (2003) 1026–1037.
[38] Z. Zhang, J. Qu, C. Zheng, P. Zhang, W. Zhou, W. Cui, et al., Nrf2 antioxidant pathway suppresses numb-mediated epithelial-mesenchymal transition during pulmonary fibrosis, Cell Death Dis. 9 (2018) 83.
[39] L.C. Li, D.L. Li, L. Xu, X.T. Mo, W.H. Cui, P. Zhao, et al., High-mobility group box 1 mediates epithelial-to-mesenchymal transition in pulmonary fibrosis involving transforming growth factor-β1/Smad2/3 signaling, J. Pharmacol. Exp. Ther. 354 (2015) 302–309.
[40] A.P. Lillis, L.B. Van Duyn, J.E. Murphy-Ullrich, D.K. Strickland, LDL receptor-related protein 1: unique tissue-specific functions revealed by selective gene knockout studies, Physiol. Rev. 88 (2008) 887–918.