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A surface antigen of *Orientia tsutsugamushi* activates human monocyte-derived dendritic cells via nuclear factor-κB & p38 mitogen-activated protein kinase pathways

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***Background & objectives*: Scrub typhus is a chigger-borne disease caused by *Orientia tsutsugamushi*. The immunological reactions to *O. tsutsugamushi* infection are not completely understood. In this study, we investigated the response of dendritic cells (DCs) to a major 56-kDa scrub typhus antigen Sta56.**

***Methods*: Monocyte-derived human DCs were incubated with different concentrations of recombinant Sta56 and analyzed for maturation based on phagocytic capacity, the ability to induce T-cell proliferation, expression of surface markers, cytokine secretion and activation of toll-like receptor (TLR)-dependent signalling pathways.**

***Results*: Treatment of DCs with Sta56 induced cell surface expression of CD80, CD83, CD86 and MHC Class II increased the production of interleukin-12 (IL-12) p40, IL-12 p70 and IL-10 and decreased DC phagocytic capacity. Furthermore, Sta56 increased the ability of DCs to activate T-cell proliferation and interferon-γ secretion. TLR4-specific antibodies neutralized Sta56-elicited effects on DC maturation, suggesting direct interaction between Sta56 and TLR4. Moreover, Sta56 activated nuclear factor (NF)-κB and p38 mitogen-activated protein kinase (MAPK) signalling as evidenced by decrease in Sta56-induced cytokine production and surface marker expression by specific inhibitors helenalin and SB203580, respectively, and increase in IκBα and p38 phosphorylation and NF-κB-DNA binding.**

***Interpretation & conclusions*: Our results showed that the surface antigen of *O. tsutsugamushi* activated DCs through interaction with TLR4 and activation of MAPK and NF-κB signalling, suggesting Sta56 as a potential candidate molecule for the development of vaccine against scrub typhus.**

Key words Antigen - dendritic cell - MHC Class II - NF-κB - *Orientia tsutsugamushi* - scrub typhus - Sta56

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Scrub typhus is an acute vector-borne disease caused by *Orientia tsutsugamushi*, an obligate intracellular Gram-negative bacterium of the Rickettsiaceae family. The disease is characterized by fever, rash and eschar and is endemic in China and the Asia-Pacific region. During the past several years, the incidence of scrub typhus has increased in several countries[1], including Taiwan where the annual number of confirmed cases increased from 39 in 1990 to 462 in 2005[2].

The major antigens of *O. tsutsugamushi* are proteins with molecular masses of 70, 58, 56, 47 and 22 kD. Of these proteins, both 56 kD and 47 kD are the major surface antigens of *O. tsutsugamushi* (scrub typhus antigen, Sta). The 56 kD protein (Sta56) is expressed on the outer membrane at a high concentration[3] and is recognized in almost all serum samples from patients of scrub typhus[4]. It has been demonstrated that mice immunized with Sta56 generated neutralizing antibodies and showed an increased resistance to infection caused by homologous strains of *O. tsutsugamushi*[5]. The 47 kD protein (Sta47) is found in the outer membrane of *O. tsutsugamushi* and contains both scrub typhus group reactive and strain-specific B-cell epitopes[6]. It has a potentially important role in the development of subunit vaccines against scrub typhus[7].

Dendritic cells (DCs) are important in the initiation of innate and adaptive immunity against pathogens[8]. Immature DCs reside in non-lymphoid tissues where they can capture and process antigens. Fully mature DCs show a high surface expression of major histocompatibility complex (MHC) Class II and co-stimulatory molecules (CD80 and CD86), although with decreased capacity to internalize antigens[9].

The induction of DC maturation is critical for the induction of Ag-specific T-lymphocyte responses. Endocytosis of foreign antigens may cause signalling through toll-like receptors (TLRs)-inducing activation of DCs and switching towards a DC1 or DC2 phenotype and initiating the production of Th1- or Th2-driving cytokines, respectively[10]. Interleukin-12 (IL-12) p40 production is an important marker for DC maturation and can be used to select Th1-inducing adjuvants. IL-10 that inhibits inflammatory and cell-mediated immune responses[11], has potential for the treatment of inflammatory and autoimmune disorders.

DC maturation or activation is a coordinated, regulated process that includes upregulation of MHC and co-stimulatory molecule expression and enhancement of adenomatous polyposis coli function. Nuclear factor (NF)-κB activation regulates DC maturation and blocking NF-κB prevents differentiation of DCs[12]. Previous reports have described mitogen-activated protein kinase (MAPK) activation in the process of human DC maturation[13]. There are at least three distinct MAPK signalling pathways in mammals, including the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK) and the p38MAPK pathways[14]. *O. tsutsugamushi* seems to be capable of replicating in DCs and the binding and uptake of bacterium particles by these cells may cause functional changes. The patients with *O. tsutsugamushi* infection have large amounts of bacterium proteins, and especially the major surface antigens. It is not clear whether the cytokine-inducing capacity of these bacterium proteins is dependent on interactions with specific cellular receptors or a direct effect on signal transduction.

Sta56 is the major antigen of *O. tsutsugamushi*, and it has been shown to be an important antigen in studying the relationship between rickettsial infections and the host. Though infectious microbial pathogens have established numerous strategies that disrupt DC functions to survive[15], there is limited knowledge of the role played by DCs in *O. tsutsugamushi* infections and the molecular mechanism of Sta56 in the activation and maturation of human DCs. Therefore, in the present study, we investigated the effect of Sta56 on human monocyte derived (MD)-DCs.

Material & Methods

This study was carried out in the department of Medical Research, National Taiwan University Hospital in Taipei City, Taiwan, from 2013 to 2015.

Expression of recombinant Sta56: *Escherichia coli* M15 was used as the host strain for the pET-32a expression vector (Qiagen GmbH, Hilden, Germany) carrying the Sta56-encoding gene[16]. Recombinant bacteria were grown in Luria-Bertani (LB) medium supplemented with ampicillin (50 μg/ml) and kanamycin (50 μg/ml) at 37°C with vigorous shaking overnight, and 1 ml of culture was used to inoculate 100 ml of fresh antibiotic-containing LB. Bacteria were grown at 37°C with shaking until optical density of 0.6 at 600 nm, and Sta56 expression was induced with 0.5 mM isopropyl-β-1-D-thiogalactopyranoside (Sigma, USA) for 4 h at 37°C. Bacteria were harvested by centrifugation at 2000 ×g for 10 min and analyzed for protein expression by sodium dodecyl sulphate-polyacrylamide

gel electrophoresis (SDS-PAGE) (5% stacking gel and 10% separation gel) followed by immunoblotting according to standard methods[17].

Purification of recombinant Sta56: Bacteria precipitated from 100 ml culture were resuspended in 3 ml of 10 mM Tris-HCl, *p*H 7.0, containing 1 mM EDTA, sonicated using an Ultrasonic Liquid Processor (VCX750) with a standard tapered microtip (Sonics and Materials, Inc., Newtown, CT, USA) at 150 W for 30 min using a six second cooling/six second sonication cycle and centrifuged. The recombinant Sta56 tagged with six consecutive histidine residues was purified from the supernatant by affinity chromatography on Ni-NTA resin (Qiagen GmbH, Hilden, Germany) under native conditions according to the manufacturer's protocol, and stored at −80°C before use for DC stimulation.

Endotoxin content determination: Endotoxin content was measured using the chromogenic *Limulus* amebocyte lysate (LAL) endpoint assay (Associates of Cape Cod, Falmouth, MA, USA) according to the manufacturer's instructions. Protein samples and the LAL standard were prepared in pyrogen-free vials. To construct a standard curve, LAL was two-fold serially diluted from 1 to 0.06 endotoxin units/ml in a 96-well plate. Samples and standards were incubated at 37°C for 20 min, and the absorption at 405 nm was measured using a microplate reader.

Purification and maturation of human monocyte-derived dendritic cells (DCs): DCs were obtained from human peripheral blood mononuclear cells (PBMCs) as described previously[18] and used at day 6 of culture. MD-DCs (1×10^6 cells/ml) were treated with IL-4 (1000 U/ml) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (750 U/ml) in the presence or absence of Sta56 (0.1, 0.2 and 1 μg/ml) for 48 h; lipopolysaccharide (LPS) stimulation was used as positive control. Cells and culture supernatants were analyzed by flow cytometry and ELISA, respectively.

Analysis of dendritic cell phagocytic capacity: MD-DCs were washed twice, resuspended in 1 ml RPMI 1640 with 10 per cent foetal calf serum (FCS) (HyClone, USA), rested on ice for 30 min and incubated with fluorescein isothiocyanate (FITC)-labelled dextran (0.2 μg/ml; Invitrogen, Carlsbad, CA, USA) at 4°C or 37°C for 1 h. Cells were washed thrice with cold buffer and analyzed using a FACSort cell analyzer (BD Bioscience, San Jose, CA, USA).

Autologous mixed leucocyte reaction (MLR): Autologous naïve $CD4^+$ T-cells were purified from PBMCs using magnetic beads (Miltenyi Biotech, USA), plated at 1×10^6 cells per well and incubated for five days in the presence of 1×10^5 stimulated MD-DCs. Then, tritiated thymidine (1 mCi/well; New England Nuclear, Boston, MA, USA) was added for 16 h and its incorporation was determined using a liquid scintillation counter.

Neutralization and pharmacological inhibition experiments: DCs were pre-incubated for 1 h with 20 μg/ml of TLR-1, TLR-2, TLR-3 and TLR-4 antibodies (eBiosciences, USA) and then with Sta56 (1 μg/ml) for 20 h, and culture supernatants were analyzed for cytokine secretion. Alternatively, immature DCs were pre-treated with helenalin, SB203580, PD98059 or SP600125 (Calbiochem, Schwalbach am Taunus, Germany) dissolved in dimethyl sulphoxide (DMSO) (Sigma-Aldrich Co., St. Louis, MO, USA) or 0.1 per cent (v/v) DMSO (used as negative control) for 60 min before stimulation and analyzed for cytokine production and expression of co-stimulatory molecules.

Cytokine detection: Cytokine production was measured in supernatants (100 μl/well) of DCs or activated T-cells cultured in 96-well plates using commercial ELISA kits (R&D systems, USA). DC supernatants were analyzed for IL-10, IL-12 p70 and IL-12 p40 levels after two days of culture with Sta56, and T-cell supernatants were analyzed for interferon (IFN)-γ levels after three days of co-culture with DCs.

Flow cytometry analysis of surface markers: DCs were harvested, washed with cold buffer [phosphate-buffered saline (PBS) containing 2% FCS and 0.1% sodium azide] and stained with FITC- or PE-labelled anti-mouse CD86, CD80, MHC Class I, MHC Class II antibodies or relevant isotype control antibodies (BD Pharmingen, USA) in cold buffer according to the manufacturer's instructions and evaluated by flow cytometry (BD Bioscience) using CellQuest software (BD Bioscience). At least 1×10^4 cells (excluding dead cells) were analyzed in each sample.

Western blotting: Protein expression was examined by Western blotting using anti-phospho-p38, anti-phospho-p42/44-P, anti-phospho-p46/54-P and anti-p38 polyclonal antibodies (Cell Signaling Technology Inc., USA) and anti-phospho- IκBα polyclonal and

anti-α-tubulin monoclonal antibodies (Santa Cruz Biotechnology, USA) as previously described[19].

Nuclear fraction preparation and NF-κB p50/p65 transcription assays: Nuclear fractions were obtained from DCs incubated with Sta56 for 2 h using a commercial nuclear extraction kit (Cayman Chemical, Ann Arbor, MI, USA) as previously described[20] and stored at −70°C until analysis performed within three days after sample collection.

NF-κB p50 and p65 binding to DNA was assessed using the NF-κB (human p50/p65 combo) transcription factor assay (Cayman Chemical). The binding of p50 and p65 to a DNA fragment (5′-GGGACTTTCC-3′) immobilized in 96-wells plates was analyzed separately using specific ELISA assays at 450 nm[20].

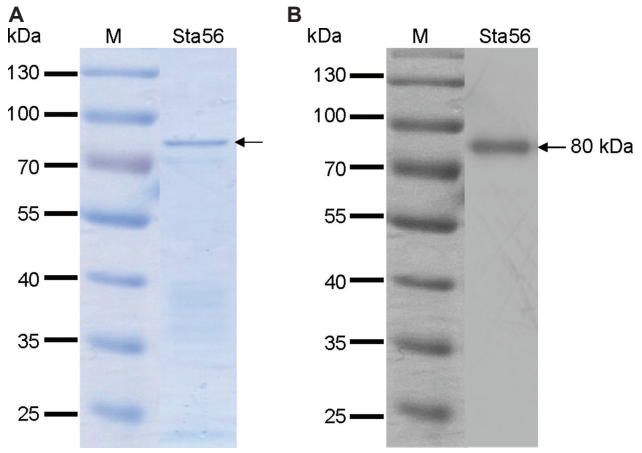

Fig. 1. Expression and purification of recombinant Sat56 protein in *Escherichia coli*. (**A**) The protein was separated by SDS-PAGE and stained with Coomassie blue. (**B**) Sta56 was separated by SDS-PAGE and detected with antibodies by immunoblotting. Lane M, protein molecular weight marker.

Results

Recombinant Sta56 expression in Escherichia coli: SDS-PAGE analysis showed that a protein with an apparent size of approximately 80 kDa corresponding to that of Sta56 was expressed in *E. coli* transformed with pET-32a/56 (Fig. 1A). Immunoblotting analysis indicated that the protein was recognized by serum samples from *O. tsutsugamushi* Karp-immunized mice and from homologous antigen-immunized mice as well as by Sta56 anti-serum (Fig. 1B), confirming that the recombinant protein was Sta56. The LAL assay indicated that the recombinant Sta56 initially contained 10-30 endotoxin units per 50 μg of protein, and the final preparation contained <0.1 endotoxin units per 20 μg of protein.

Sta56-induced interleukin-12 (IL-12) p40, IL-12 p70 and IL-10 secretion by MD-DCs: To determine whether Sta56 affected cytokine production in human DCs, cytokine levels were compared in the supernatants of DCs cultured with different Sta56 concentrations (0.1, 0.2 and 1 μg/ml). The treatment of DCs with Sta56 for 48 h induced the production of IL-12 p40, IL-12 p70 and IL-10 in a dose-dependent manner, and the effect of 0.2 and 1 μg/ml Sta56 was significant (P<0.05) (Fig. 2).

Sta56-induced maturation and decreased endocytic ability in MD-DCs: The surface expression of CD40, CD54, CD80, CD86, CD83 and MHC Class II

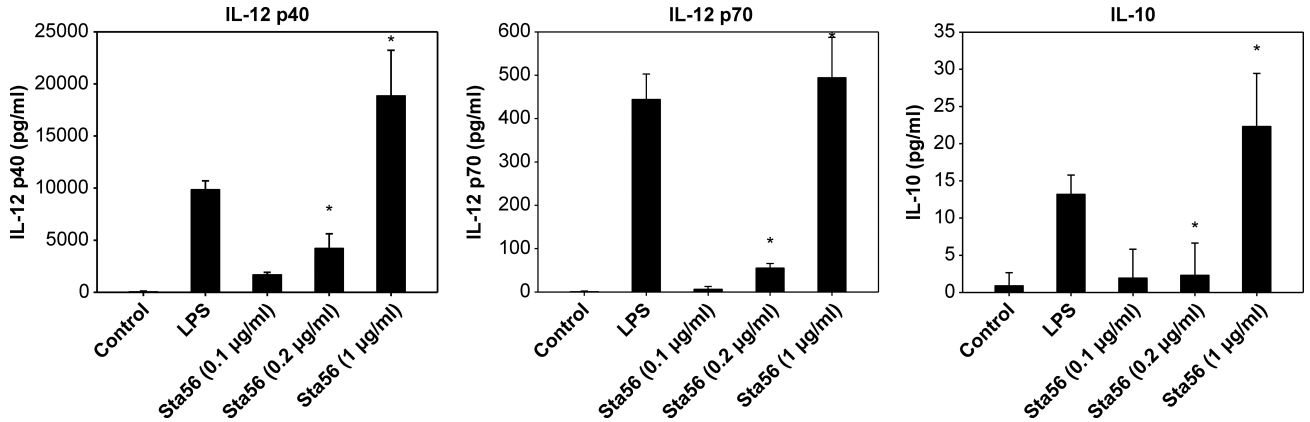

Fig. 2. Sta56 induced interleukin-12 p40, IL-12 p70 and IL-10 production in human dendritic cells *in vitro*. Human dendritic cells were cultured in the presence or absence of Sta56 0.1, 0.2 and 1 μg/ml for 48 h. The supernatants were the collected for cytokine analysis including such as IL-12 p40, IL-12 p70 and IL-10. Each data point represents the mean ± SE of three determinations. *P<0.05 unstimulated vs stimulated dendritic cells.

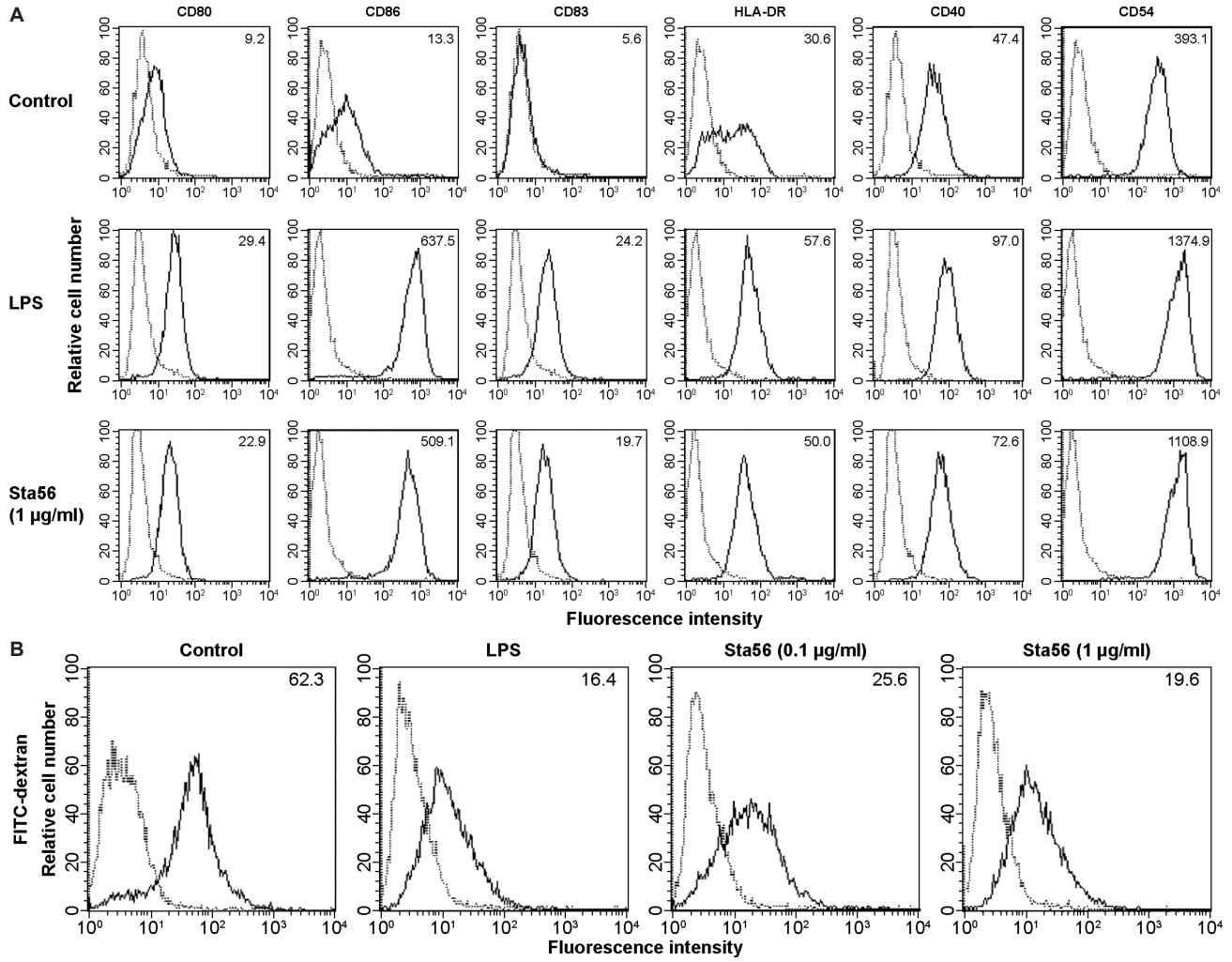

Fig. 3. Effect of Sta56 on (**A**) the expression of human dendritic cells. Human dendritic cells were treated in the presence or absence of Sta56 (1 µg/ml) or lipopolysaccharide (LPS, 100 ng/ml) for 48 h. The cells were harvested on day 7 of culture, stained with different mAbs and analyzed using flow cytometry (dotted line, isotype control; solid line, specific mAbs). (**B**) Phagocytic capacity of monocyte derived-dendritic cells. Immature monocyte derived-dendritic cells (1×10^6 cells/ml/well) were incubated for 24 h with or without Sta56 (0.1 or 1 µg/ml). The dendritic cells stimulated with lipopolysaccharide (100 ng/ml) served as the positive maturation controls. The cells were incubated with fluorescein isothiocyanate-dextran for one hour at 4°C (dotted lines) or 37°C (solid lines). The values shown in the flow cytometry profiles are the mean fluorescence intensity indexes indices. These results are representative of three independent experiments with similar results. CD, cluster of differentiation; HLA, human leukocyte antigen DR.

molecules characteristic for mature DCs was increased in LPS as well as in Sta56-treated human DCs (Fig. 3A). Furthermore, LPS and Sta56 reduced FITC-dextran uptake by MD-DCs compared to the untreated control MD-DCs (Fig. 3B), indicating that DC phagocytic activity was decreased. Overall, these data suggested that Sta56-induced maturation of human DCs.

Sta56 increased the ability of MD-DCs to activate T-cells: As Sta56 upregulated cell surface markers and increased IL-12 and IL-10 production involved in T-cell stimulation, we examined whether these effects were sufficient to promote activation of naïve $CD4^+$ T-cells. MD-DCs treated with Sta56 were incubated with autologous naïve $CD4^+$ T-cells, and the results indicated that Sta56 induced the ability of DCs to activate T-cells as evidenced by increased T-cell proliferation (Fig. 4A) and IFN-g secretion to culture supernatant (Fig. 4B).

Sta56 induced the production of IL-12 p40, IL-12 p70 and IL-10 in MD-DCs via TLR4: To examine the involvement of TLRs in Sta56 effects on the maturation of human MD-DCs, cells were incubated with anti-TLR1, -TLR2, -TLR3 and -TLR4 antibodies before Sta56 treatment and analyzed for cytokine production. The secretion of IL-12 p40, IL-12 p70 and IL-10 was significantly

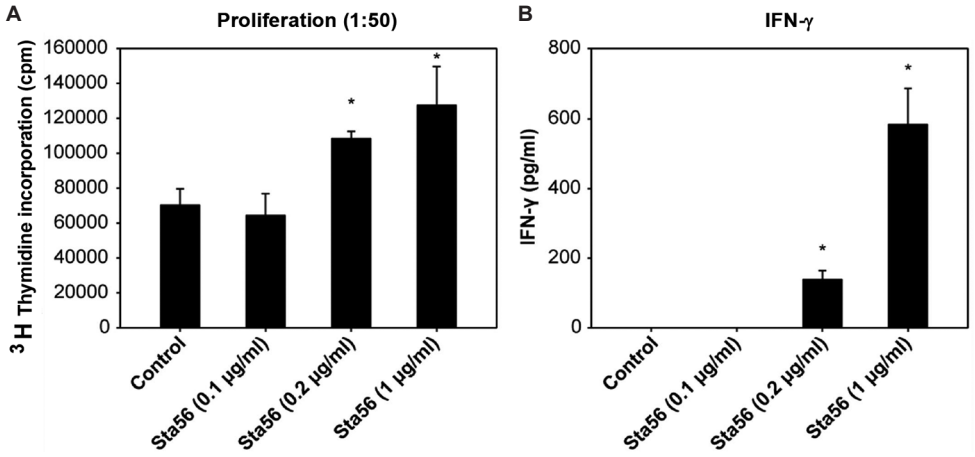

Fig. 4. Sta56 enhanced T-cell responses. (**A**) Immature dendritic cells were stimulated with Sta56 (0.1 µg, 0.2 µg, and 1 µg/ml) for 48 h. Autologous T-cell proliferation was measured after five days of co-culture with the dendritic cells. Data are presented as means ± SEM of three independent experiments. The supernatants were analyzed for (**B**) interferon-γ, interleukin-10 and interleukin-5 produced by the activated T-cells after two days of culture. $^*P<0.05$ compared to control. CPM, counts per minute.

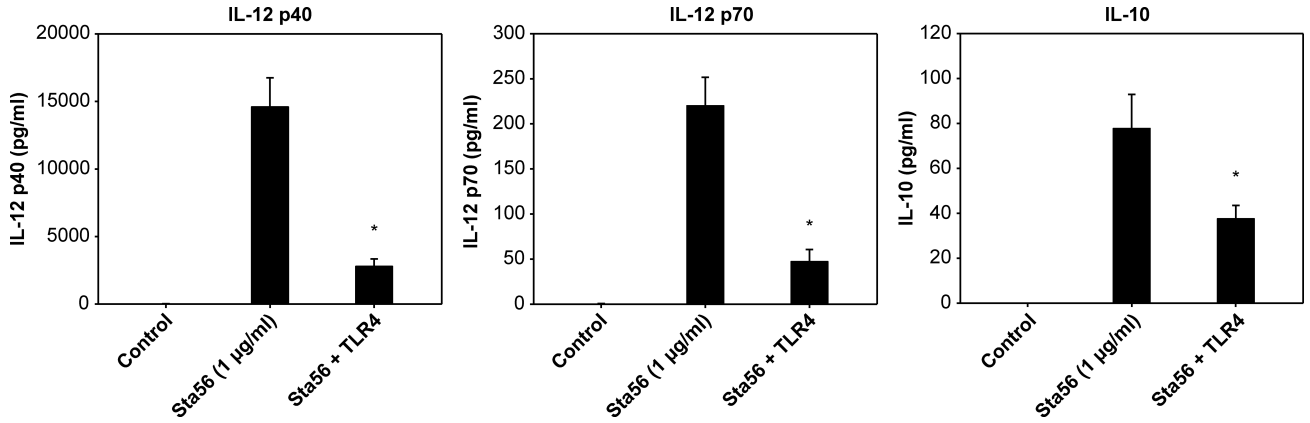

Fig. 5. Neutralization with anti-toll-like receptor-4 mAbs inhibits the synthesis of interleukin (IL)-12 p40, IL-12 p70 and IL-10. Human dendritic cells were pre-incubated with 20 µg/ml anti-toll-like receptor (TLR)-1, TLR-2, TLR-3 and TLR-4 mAbs separately for one hour. The dendritic cells were challenged with Sta56 (1 µg/ml) for 20 h. The cell culture supernatants were collected for IL-12 p40, IL-12 p70 and IL-10 analysis. Data are presented as mean ± standard error of three independent experiments. $^*P<0.05$ between the dendritic cells treated with and without Abs.

decreased by anti-TLR4 antibodies (Fig. 5), suggesting that Sta56-induced cytokine secretion by DCs through interaction with TLR4.

Inhibition of NF-κB and p38 mitogen-activated protein kinase (MAPK) blocked Sta56-induced maturation and cytokine production in MD-DCs: Immature human DCs were pre-treated with specific inhibitors of NF-κB and MAPKs p38, ERK and JNK before stimulation with Sta56 and analyzed for cytokine production. The results indicated that the increase in the production of IL-12 p70, IL-12 p40 and IL-10 caused by Sta56 was reversed by helenalin and SB203580, inhibitors of NF-κB and p38, respectively, whereas JNK inhibitor II SP600125 reduced only IL-12 p70 levels (Fig. 6A).

To further examine the involvement of NF-κB, p38 MAPK, ERK and JNK in the Sta56-induced activation of human DCs, we analyzed the expression of co-stimulatory and antigen-presenting surface molecules. NF-κB inhibitor helenalin significantly suppressed Sta56-induced upregulation of CD40, CD80, CD86, CD83 and HLA-DR, an MHC Class II cell surface receptor, whereas p38 inhibitor SB203580 suppressed that of CD80, CD86, CD83 and HLA-DR, but ERK and JNK inhibitors had no effect (Fig. 6B). These results indicated that the switch of human monocyte-derived DCs from the immature to mature phenotype induced by Sta56 was regulated by NF-κB- and p38 MAPK-dependent signalling, indicating an important role of these pathways in DC response to *O. tsutsugamushi* infection.

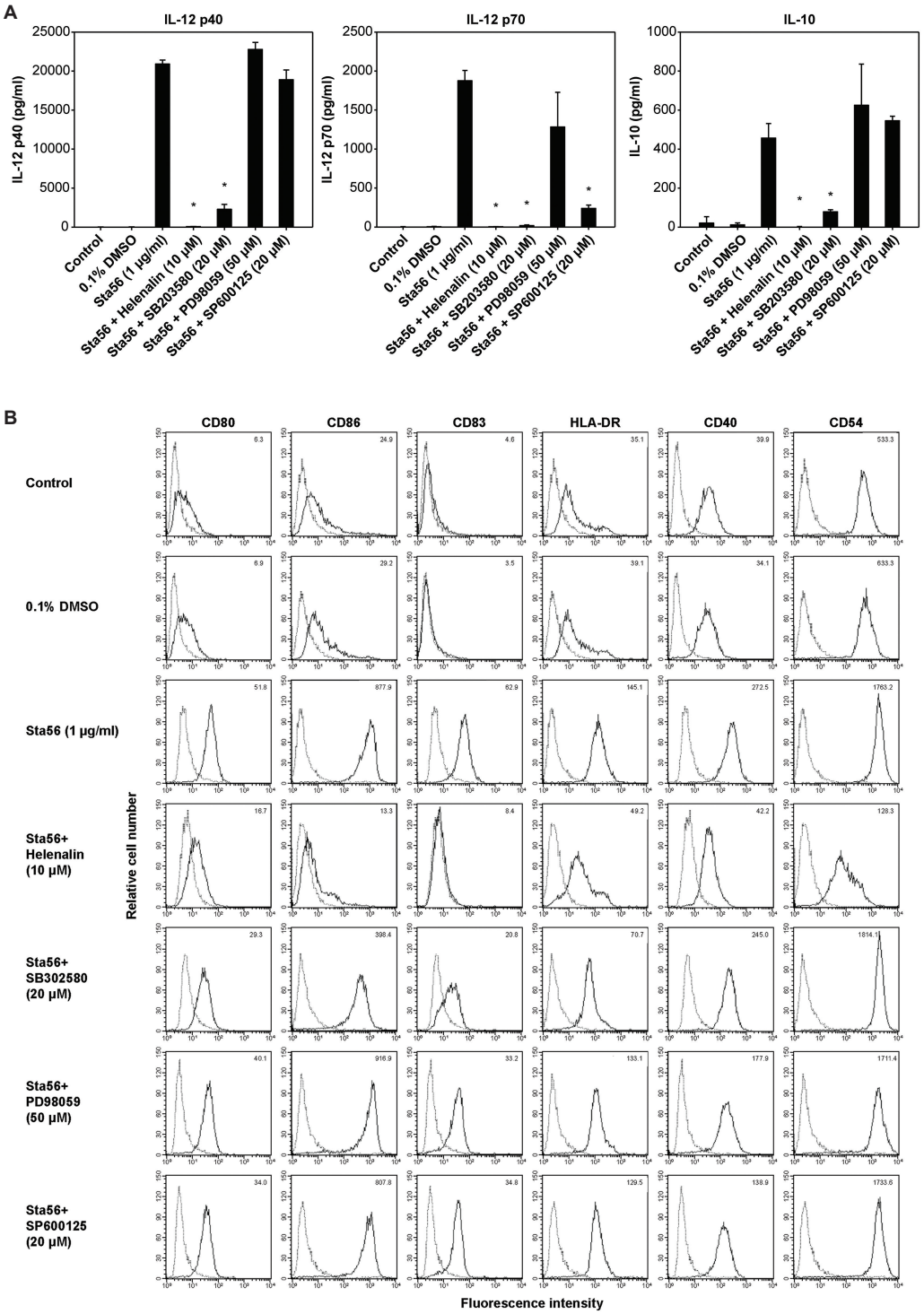

Fig. 6. The effect of NF-κB, p38 MAPK, extracellular signal-regulated kinase 1/2 and c-Jun N-terminal kinase inhibition on cytokine release and expression of surface markers in Sta56-treated dendritic cells. Cells were pre-treated with 0.1 per cent dimethyl sulphoxide (DMSO), 10 μM helenalin, 20 μM SB203580, 50 μM PD98059 or 20 μM of SP600125 for 1 h and incubated with 1 μg/ml Sta56 for 24 h. (**A**) IL-12 p40, IL-12 p70 and IL-10 levels in cell supernatants analyzed by ELISA. The data are presented as the mean ± standard error of three independent experiments. $^{*}P<0.05$ versus inhibitor untreated Sta56-induced dendritic cells. (**B**) The Sta56-induced upregulation of CD40, CD80, CD86, CD83 and HLA-DR in human monocyte-derived DC. Day 6 immature DC was pre-treated with as above. The cell surface expression of CD40, CD80, CD86, CD83 and HLA-DR was measured using the flow cytometry (dotted line, isotype control; solid line, specific mAbs). The values shown in the flow cytometry profiles are the mean fluorescence intensity indexes. These results are representative of three independent experiments with similar results.

Sta56-induced phosphorylation of IκBα and mitogen-activated protein kinases (MAPKs) in human dendritic cells: In the inactive form, NF-κB is associated with inhibitory proteins, including IκBα, and is sequestered in the cytoplasm; activation of NF-κB involves the phosphorylation and degradation of IκBα, leading to NF-κB nuclear translocation. To further examine Sta56-induced NF-κB activation, we analyzed IκBα phosphorylation in the DC cytoplasm by Western blotting. The results indicated that 45-min treatment with Sta56 induced the phosphorylation of IκBα in DCs (Fig. 7A), further confirming that Sta56 activated the NF-κB pathway. Moreover, Sta56 induced the phosphorylation of p38 MAPK, p42/44 ERK and p46/54 JNK (Fig. 7B).

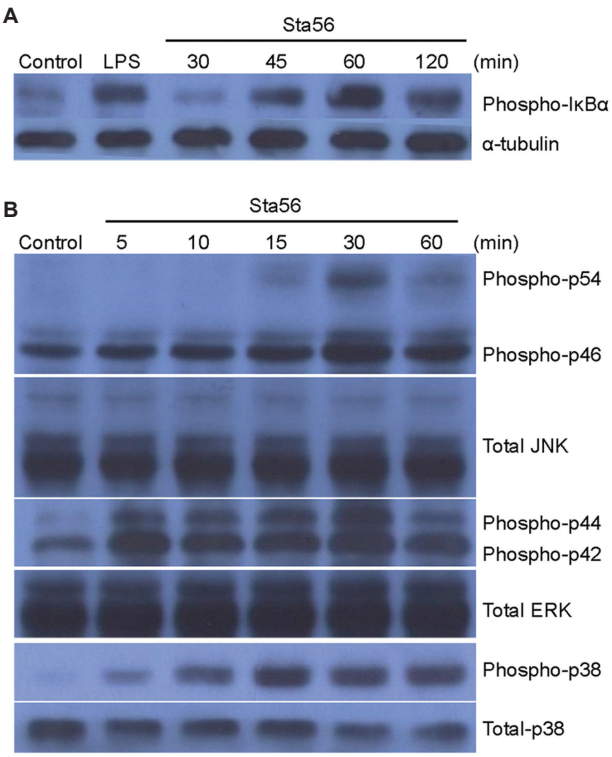

Fig. 7. Human monocyte derived-dendritic cells were treated with Sta56 (1 µg/ml) for the indicated time periods. Cytosolic fractions were prepared and analyzed for (**A**) the phosphorylation level of IκBα by Western blotting. The lower panel shows the blot probed for a-tubulin to demonstrate equal loading of the samples. (**B**) The phosphorylation level of mitogen-activated protein kinase by Western blotting. The lower panel shows the blot probed for anti-total p38 polyclonal antibody to demonstrate equal loading of the samples. This experiment was repeated three times with similar results.

NF-κB-DNA binding: To assess NF-κB transcriptional activity, we analyzed DNA binding of NF-κB p50/p65 from nuclear extracts of Sta56-stimulated DCs. Sta56 treatment significantly induced the binding of NF-κB p50 and p65 subunits to DNA compared to control (Fig. 8), which was consistent with the increase in IκBα phosphorylation indicative of NF-κB nuclear translocation.

Discussion

The process of DC maturation is a crucial step in the initiation of adaptive immune responses[8]. In our study Sta56 induced the phenotypic switch from immature to mature DCs, characterized by increased cell surface expression of MHC Class II, CD83, co-stimulatory molecules CD40, CD45, CD80, and CD86 and upregulation of IL-12 p40, IL-12 p70, and IL-10 secretion. Our results show that an *O. tsutsugamushi*-derived protein can elicit immune reactions *in vitro* similar to those observed *in vivo* in *O. tsutsugamushi* -infected patients[21]. The production of IL-12 by DCs is an early immune reaction[22] that serves as a link between the innate and adaptive immune systems. In the current study, Sta56 increased the production of IL-12 and IL-10 by human DCs and activation of naïve $CD4^+$ T-cells and decreased DC

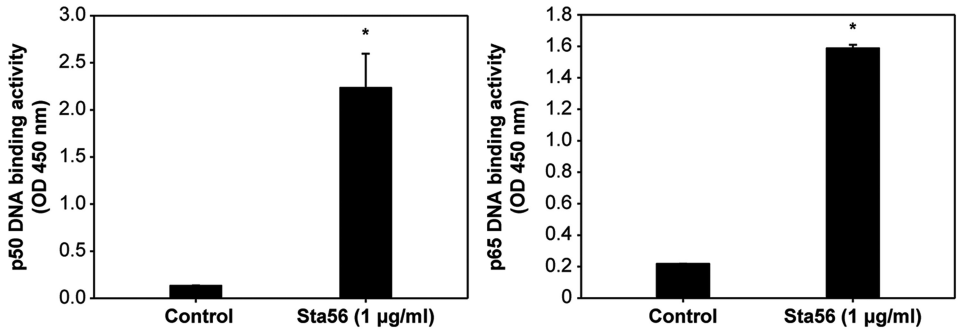

Fig. 8. ELISA of nuclear p50 and p65 nuclear factor-κB subunits of the dendritic cells treated with Sta56 for two hours. Each data point represents the mean ± standard error of three determinations. $^*P<0.05$ compared to control.

phagocytic activity as evidenced by the reduction in the uptake of FITC-dextran internalized by immature human MD-DCs through macropinocytosis through the mannose receptor[23]. We hypothesize that the role played by Sta56-stimulated DCs may be the initiation of a bystander effect. Sta56-host interactions may be more complicated in an *in vivo* system, as to whether the immune response of MD-DCs to Sta56 can exert immune modulation in the mice model, which has not been assessed in the present study. However, it is important to characterize the relationship to provide insights in solving the immunogenicity effect of Sta56.

Several putative binding factors have been described for Sta56; however, the mechanism of *O. tsutsugamushi* entry into DCs and the role of the Sta56 antigen in the process remain unknown. Similar to other intracellular bacteria or parasites, after internalization, rickettsiae upregulate phenotypic activation markers in DCs[24]. Both LPS- and rickettsia-stimulated DCs produced IL-2 with a similar kinetics, suggesting that the signal may be triggered through the same TLR, possibly TLR4. This notion is supported by the finding that *Rickettsia africae* can induce the activation of TLR4 on endothelial cells[25].

TLRs are pattern recognition receptors that detect microbial pathogens and trigger innate immune responses, thus providing host antimicrobial defence[26]. TLR signalling in DCs stimulates antigen presentation on MHC proteins for T-cell recognition, upregulates the expression of co-stimulatory molecules important for T-cell clonal expansion and induces the secretion of immunomodulatory cytokines which direct T-cell differentiation into effectors. Here, we showed that Sta56-activated DCs through interaction with TLR4, which resulted in DC maturation and upregulation of IL-12 and IL-10 secretion, indicating that *O. tsutsugamushi* induced DC-mediated immune responses through its Sta56 surface antigen.

NF-κB signalling is a key pathway involved in transcriptional regulation of immune responses, and many genes encoding pro-inflammatory cytokines have NF-κB-responsive elements in their promoters[19]. It is also known that p38 MAPK signalling is involved in NF-κB p65 transactivation[27]. Therefore, we investigated whether NF-κB and MAPK pathways played a role in human DC maturation induced by Sta56 interaction with TLR4. Pharmacological inhibitors of NF-κB and p38 significantly downregulated the secretion of IL-12 p40, IL-12 p70 and IL-10, whereas the JNK inhibitor downregulated the secretion of IL-12 p70 in Sta56-treated human MD-DCs. These results were consistent with the presence of NF-κB binding sites in the promoters of IL-12-encoding genes[28]. However, since there is no such site in the IL-10 promoter[29], the inhibition of its expression by helenalin may be attributed to other mechanisms. In addition, NF-κB and p38 inhibition reversed the Sta56-induced upregulation of MHC Class II, CD80, CD86 and CD83, whereas inhibition of other MAPKs, ERK1/2 and JNK had no effect. These data, together with the induction of IκBα and p38 phosphorylation, suggested that Sta56 promoted DC maturation through activation of NF-κB and MAPK p38. Our results are in agreement with the central role of NF-κB p50/p65 and MAPK pathways in immune cell functions, including DC maturation.

In conclusion, this study demonstrated that Sta56 could effectively and rapidly induce DC maturation through TLR4 and NF-κB and p38 signalling, suggesting Sta56 as a potential component of the treatment regimen to regulate host immune responses to *O. tsutsugamushi* infection. Our *in vitro* observations indicating the importance of NF-κB and MAPK pathways in DC maturation induced by the *O. tsutsugamushi* antigen need to be verified *in vivo* using experimental animals receiving Sta56-induced DCs to examine whether such transfer would confer immune activation and resistance to scrub typhus.

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***Conflicts of interest*:** None.

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