Ribonuclease T2 from *Aspergillus fumigatus* promotes T helper type 2 responses through M2 polarization of macrophages

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Abstract. Allergic bronchopulmonary aspergillosis (ABPA) is an allergic immunological response to *Aspergillus fumigatus* (*Af*) exposure, which induces a strong T helper 2 (Th2) response via mechanisms that have yet to be elucidated. The aim of the present study was to investigate the hypothesis that T2 ribonuclease from *Af* (*Af* RNASET2) induces M2-type macrophage polarization to produce a T helper 2 (Th2) immune response. Recombinant *Af* RNASET2 (r*Af* RNASET2) was expressed and purified in a prokaryotic pET system and BALB/c mice were immunized with r*Af* RNASET2 for *in vivo* analyses. Expression levels of M2 polarization factors were evaluated in RAW264.7 macrophages treated with r*Af* RNASET2 *in vitro* using flow cytometry, reverse transcription-quantitative PCR, and western blot analysis. The results predicted that the mature *Af* RNASET2 protein (382 amino acids; GenBank no. MN593022) contained two conserved amino acid sequence (CAS) domains, termed CAS-1 and CAS-2, which are also characteristic of the RNASET2 family proteins. The protein expression levels of the Th2-related cytokines interleukin (IL)-4, IL-10, and IL-13 were upregulated in mice immunized with r*Af* RNASET2. RAW264.7 macrophages treated with r*Af* RNASET2 showed increased mRNA expression levels of M2 factors [*arginase 1*, *Il-10*, and *Il-13*]; however, there was no difference in cells treated with r*Af* RNASET2 that had been inactivated with a ribonuclease inhibitor (RNasin). The protein expression levels of IL-10 in macrophage culture supernatant were also increased following stimulation with r*Af* RNASET2. In addition, r*Af* RNASET2 upregulated the expression of phosphorylated mitogen activated protein kinases (MAPKs) in RAW264.7 cells, whereas MAPK inhibitors attenuated r*Af* RNASET2-induced IL-10 expression in RAW264.7 cells. In conclusion, the present study reveals that high r*Af* RNASET2 activity is required for r*Af* RNASET2-induced M2 polarization of macrophages and suggests an important immune regulatory role for *Af* RNASET2 in ABPA pathogenesis.

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Abbreviations: ABPA, allergic bronchopulmonary aspergillosis; *Af, Aspergillus fumigatus*; ANOVA, one-way analysis of variance; ARG1, arginase-1; CAS, conserved amino acid sequences; COX2, cyclooxygenase 2; *Cs, Clonorchis sinensis*; ERK, extracellular signal-regulated kinase; Ig, immunoglobulin; IL, interleukin; iNOS, induced nitric oxide synthase; IPTG, isopropyl-β-D-thiogalactopyranoside; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinases; NCBI, National Center for Biotechnology Information; RNASET2, T2 ribonuclease; RNasin, ribonuclease inhibitor; *Sj, Schistosoma japonicum; Sm, Schistosoma mansoni*; Th1, T helper 1; Th2, T helper 2

Key words: ribonuclease T2, TH2, macrophages M2 polarization, *Aspergillus fumigatus*

Introduction

Patients who have abnormal pulmonary immune responses, such as those with atopic asthma or cystic fibrosis (CF), are prone to fungal colonization of the respiratory tract (1). The ubiquitous environmental mold *Aspergillus fumigatus* (*Af*) is the most common cause (2). A clinical study in America found that ~80% of children with CF also have IgG antibodies against Asp f 1, an immunodominant *Aspergillus* peptide antigen (3). *Af* antigen exposure following persistent fungal colonization of the lungs produces allergic bronchopulmonary aspergillosis (ABPA). There is a high prevalence (28%) of *Aspergillus* hypersensitivity and ABPA in patients with bronchial asthma, worldwide from a meta-analysis of observational studies between 1965 and 2008 (4). The pathogenesis of ABPA is not well understood; however, it is known that patients with ABPA have immunoglobulin (Ig)E, IgA, and IgG anti-*Af* serum antibodies (5).

The pulmonary immune response in patients with ABPA includes a higher than normal T helper 2 (Th2) response, in addition to elevated levels of IgE targeting the colonizing fungus (6). In human bronchial epithelium, *Af* exposure-triggered promotion of Th2 response is associated with inhibition

of interferon-β signaling through the JAK-STAT1 signaling pathway, which shifts epithelial responses from type Th1 to type Th2 (7,8), as well as, activation of protease-activated receptor-2 and tyrosine-protein phosphate nonreceptor type 11, which reduces CXCL10 expression, further favoring induction of a Th2 response (9). In addition, *Af* has been reported to promote Th2 responses through thymic stromal lymphopoietin production by human corneal epithelial cells (10). Sera from patients with ABPA show increased IgE reactivity to Asp f 2 and *Af* crude extract; and it has been hypothesized that the *Af* antigens, Asp f 1 or Asp f 2, may underlie upregulation of Th2 (11). However, it has been reported that an ABPA-associated Th2 response can be triggered in the absence of specific *Aspergillus* antigens (12). Thus, the mechanisms by which *Af* induces Th2 responses remain unknown. In particular, it is unclear whether the immunomodulatory effects of *Af* antigens are associated with the development of ABPA.

Th2 immune responses can be produced by differentiation of macrophages toward an M2 type (13). Induction of pro-inflammatory responses in human macrophages with *Af* has been shown to result in upregulation of tumor necrosis factor-α and interleukin (IL)-6 (14). In addition, *Af* produces a metabolite, gliotoxin, which downregulates vitamin D receptor expression on macrophages and airway epithelial cells, which has been shown to lead to increased production of the Th2 cytokines IL-5 and IL-13 (15). Notably, the T2 ribonuclease (RNASET2) protein was found to be a major inducer of Th2 polarization. ω-1, a glycosylated RNASET2 protein, which is secreted by *Schistosoma mansoni* (*Sm*) eggs and is abundant in soluble egg antigen extract, has been shown to condition dendritic cells to prime Th2 responses (16,17). The glycosylation and ribonuclease activity of *Sm* ω-1 are both essential to the conditioning of dendritic cells for Th2 polarization (17). In addition, *Schistosoma japonicum* (*Sj*) CP1412, which is a ribonuclease T2 family member, has been shown to promote M2-type macrophage differentiation (18) and recombinant *Sj* CP1412 has been reported to increase expression of CD206, arginase 1 (ARG1), and IL-10 in mouse macrophages (18).

The aim of the present study was to investigate the hypothesis that *Af*-induced Th2 responses, which are observed in ABPA, may involve RNASET2-mediated M2 polarization. The recombinant *Af* RNASET2 (r*Af* RNASET2) was expressed and purified in a bacterial pET system. Th2 cytokine expression was evaluated *in vivo* in mice immunized with r*Af* RNASET2. M2-type macrophage differentiation was examined in RAW264.7 macrophages incubated with r*Af* RNASET2 to further investigate whether *Af* RNASET2 may be an important immune regulatory factor in ABPA.

Materials and methods

Expression system components and reagents. The following reagents were purchased for recombinant protein expression: *Escherichia coli* (*E. coli*) BL21 (DE3) plysS cells (Merck KGaA), pMD 19-T vectors (Takara Biotechnology Co., Ltd.), pET-His vectors (Wuhan Miaoling Bioscience & Technology Co., Ltd.), and primer STAR HS DNA polymerase (Takara Biotechnology Co., Ltd.). *Af* RNASET2 cDNAs were synthesized by Nanjing GenScript Biotech Corp. Lysozyme (Sangon Biotech Co., Ltd.) and blot membranes (nitrocellulose and polyvinylidene fluoride; Merck KGaA) were used for pre-purification cell lysis and electrophoresis analysis, respectively.

Mouse model. A total of 18 female BALB/c mice (6 weeks of age; 20-22 g) were purchased from Guangdong Medical Laboratory Animal Center, and housed in a specific pathogen free facility with six mice per cage under a stable temperature (24±1˚C) and humidity (55±10%). Mice were kept in open polypropylene cages with clean chip bedding under a 12-h light/dark cycle with free access to a standard rodent diet. The animals were acclimatized to the laboratory for at least 1 week prior to the start of the experiments. The health status of experimental mice was monitored twice daily and humane endpoints were used to determine if mice met the criteria for euthanasia prior to the study end point, namely weight loss exceeding 10-15% of the body weight, lethargy, inability to stand, or anorexia. All surgical procedures were performed under 1.5-2% isoflurane anesthesia. At the end of the experiments, animals were humanely sacrificed using CO_2 asphyxiation. The flow rate of CO_2 displaced ~50% of the chamber volume per minute for euthanasia. The gas flow was maintained for at least 1 min following clinical death, and death was evaluated monitoring the lack of movement or visible breathing for 5 min. Both the breeder of the animals and the assessor of the results are blinded. All studies involving mice were performed according to protocols approved by the Animal Ethical and Welfare Committee of the School of Medicine of Shenzhen University. All efforts were made to minimize discomfort and suffering of the animals.

Cell culture. The RAW264.7 murine macrophages were purchased from Guangzhou Cellcook Biotechnology Co., Ltd., and cultured in complete DMEM with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), at 37˚C in a humidified incubator with 5% CO_2.

Homology analysis of RNASET2 genes. The analysis of the amino acid sequence alignment was performed to investigate the sequence conservation of *Af* RNASET2 gene in the ribonuclease T2 family. GenBank (https://www.ncbi.nlm.nih.gov/genbank) was used to download the amino acid sequences of *Af* RNASET2 [GenBank ID, XP754496.2; based on the *Af AF293* genome (Genome ID, 18)], *Sj* CP1412 (GenBank ID, AY570741.2), *Sm* ω-1 (GenBank ID, DQ013207.1), and *Clonorchis sinensis* (*Cs*) RNASET2 (GenBank ID, GAA50115.1) were imported into DNAMAN software (version 8.0; Lynnon Biosoft) for alignment. The sequences were saved in FASTA format for multiple *alignment analysis*. The homologous *alignment was calculated* according to the ratio of the number of conserved amino acids to total number of amino acids.

Recombinant protein expression and purification. The *Af* RNASET2 cDNA was synthesized following codon optimization and submitted to the National Center for Biotechnology Information (NCBI) database. The *Af* RNASET2 gene was amplified using PCR with Primer STAR HS DNA polymerase

and the aforementioned cDNA template. The following PCR primers were used: Forward, 5'-AGATGGATCCATGAA ATTCAACATAACTATCGC-3' and reverse 5'-AGCGAT AAGCTTATGTACATGTTAATTCTTTTTCA-3'; and the following thermocycling conditions: Initial denaturation at 94˚C for 30 sec, and 30 cycles of 55˚C for 30 sec, and 72˚C for 50 sec. The PCR product, confirmed by Sanger DNA sequencing (GenScript Biotech Corporation), was subcloned into a pET-His vector with *BamH*I and *Hind*III restriction enzymes. The recombinant expression vector pET-His-*Af* RNASET2 was confirmed by double enzyme digestion with *Bam*HI/*Hind*III. Following double enzyme digestion with *Bam*HI/*Hind*III for 2 h at 37˚C, the products were analyzed using a 1% agarose gel.

The recombinant plasmid pET-His-*Af* RNASET2 was then transformed into *E. coli* BL21 (DE3) plysS cells. The transformed *E. coli* were grown overnight in Luria-Bertani medium containing 100 mg/l ampicillin at 37˚C, and induction was performed using isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. Following cultivation and an additional 3 h at 37˚C, *E. coli* cells were harvested by centrifugation at 9,600 x g for 5 min at 4˚C. Following mixing with protein extraction buffer (20 mM Tris HCl, 150 mM NaCl and 1 mg/ml lysozyme), harvested cells were sonicated (50 kHz; 3 sec bursts, 5 sec inter-burst interval for a total of 20 min) in an ice bath, and then centrifuged at 9,600 x g for 20 min at 4˚C. Subsequently, 10% SDS-PAGE was performed to analyze the status of recombinant protein expression. The recombinant protein in the soluble fraction was purified with a Ni-NTA column (cat. no. 17040303) and gel filter (Hi Load Superdex 16/600; cat. no. 28-9893-33) (both from GE Healthcare). Specifically, r*Af* RNASET2 was expressed in the inclusion bodies, which were dissolved in 6 M urea with 25 mM β-mercaptoethanol. Denatured proteins were refolded and diluted 10-fold with 0.5 M L-arginine (pH 8.0) (19). Arginine buffer was added to the denatured protein solution slowly (flow rate of 0.5 ml/min) and incubated overnight at 4˚C. For renaturation, r*Af* RNASET2 was dialyzed with PBS at 4˚C for 24 h via a dialysis membrane (Sangon Biotech Co., Ltd.) (19). Following renaturation, protein concentrations were determined using the Bradford method (Sangon Biotech Co., Ltd). The r*Af* RNASET2 protein endotoxin was removed using an endotoxin removal kit following the manufacturer's instructions and quantitation was performed using an endotoxin assay kit, which revealed that the levels were reduced to <0.25 endotoxin units/ml (both Genscript Biotech Corporation).

Enzymatic activity analysis. Yeast RNA (Thermo Fisher Scientific, Inc.) was dissolved in reaction buffer (50 mmol/l Tris HCl, 50 mmol/l NaCl, pH 7.0) to prepare a stock solution (1 mg/ml). The r*Af* RNASET2 (1 μg) aliquots were mixed thoroughly with 50 μg yeast RNA at 37˚C for 20, 40, 60, 80, and 100 min. In addition, a 30-min pretreatment with an RNase inhibitor (RNasin; Thermo Fisher Scientific, Inc.) served as a positive control for confirmation of digestion by RNASET2. Ribonuclease activity was observed using the analysis of enzymolysis products in 1% agarose electrophoresis gels, which was stained with Gelview dye (BioTeke Corporation). Bands were visualized on a UV transilluminator (Analytik Jena US LLC).

In vitro analysis of macrophage stimulation. RAW264.7 macrophages ($1x10^6$) were incubated with the indicated concentrations (20 or 40 μg/ml) of rAf RNASET2 (pretreated with RNasin) in PBS at 37˚C under 5% CO_2. After 24 h of co-culture, the cells and supernatant were collected and centrifuged at 300-400 x g for 5 min at 4˚C. The cells were collected and incubated with the labeled antibodies (FITC-CD16/32 (cat. no. 11-0161-81; 1:500; BioLegend, Inc.) or FITC-CD206 (cat. no. MA5-16870; 1:500; Invitrogen; Thermo Fisher Scientific, Inc.) for M2 or M1 macrophage detection, respectively, and APC-F4/80 (cat. no. 17-4801-80; 1:500; BioLegend, Inc.) in PBS containing 1% bovine serum albumin (Amresco LLC) for flow cytometry. All incubations were performed at room temperature for 30 min in the dark and subsequently, the cells were washed with PBS. Macrophage surface markers were detected using a Cytoflex flow cytometer and analyzed using the CytExpert software (version 2.0) (both Beckman Coulter, Inc.). IL-10 levels were measured in the culture supernatant of RAW264.7 cells stimulated by incubation with rAf RNASET2 (20 μg/ml) alone or in the presence of MAPK signal inhibitors (U0126, 20 μM; SP600125, 20 μM and SB203580, 1 μM) for 24 h. The cells were centrifuged at 300-400 x g for 5 min at 4˚C and subsequently analyzed using ELISA kits according to the manufacturer's instructions (cat. no. HZ-IL-10-Mu; Shanghai and Shanghai Zhen Biotech Co., Ltd.).

RNA isolation and reverse transcription quantitative PCR (RT-qPCR). RAW264.7 macrophages were co-cultured with r*Af* RNASET2 (20 μg/ml), inactive r*Af* RNASET2 (pretreated with RNasin (2 U/μl)), and PBS and LPS controls at 37˚C with 5% CO_2 for 24 h, and with r*Af* RNASET2 (20 μg/ml) alone or in the presence of MAPK signal inhibitors (U0126, 20 μM; SP600125, 20 μM and SB203580, 1 μM) for 24 h, under normal cell culture conditions. RNA was extracted after the cells were centrifuged at 300-400 x g for 5 min at 4˚C, using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was subsequently reverse transcribed into cDNA using a TIANScript II RT kit (Tiangen Biotech, Co., Ltd.) according to the manufacturer's protocol: 42˚C for 50 min, followed by 95˚C for 5 min then cooled on the ice at 4˚C for 10 min. RT-qPCR analysis was performed with SYBRGreen (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the primers listed in Table I.

For the qPCR, 5 μl 2X SYBRGreen PCR buffer, 0.2 μl forward and reverse primers (both 10 μmol), and 5 ng template were used to a total volume of 10 μl with sterile water. The thermocycling conditions used were as follows: Initial denaturation 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The real-time PCR was performed on a qTOWER 2.2 system (Analytik Jena US LLC). All qPCRs were performed in triplicate with *18S* rRNA gene as a reference housekeeping gene. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (20).

Western blot analysis. RAW264.7 macrophages were co-cultured with r*Af* RNASET2 (20 μg/ml), inactive r*Af* RNASET2 (pretreated with RNasin (2 U/μl), and PBS and LPS controls at 37˚C in a humidified incubator with 5% CO_2 for 24 h. The cells were centrifuged at 300-400 x g for 5 min at 4˚C, and subsequently lysed in RIPA buffer (Beyotime

Table I. Sequences of the primers used in for reverse transcription-quantitative PCR.

Gene	Primer sequences (5'→3')	Accession number
18S rRNA	Forward: CGAGGGGTTCGGGATTTGTG Reverse: AAAGCCAACCCGAGCGTC	M35283.1
Arg-1	Forward: TGCGCCACATGAAAACCATC Reverse: TTGGGAGGAGAAGGCGTTTG	NM_007482.3
iNos	Forward: GGTGAAGGGACTGAGCTGTTA Reverse: TGAAGAGAAACTTCCAGGGGC	NM_007482.3
IL-10	Forward: AAGGGTTACTTGGGTTGCCA Reverse: CCTGGGGCATCACTTCTACC	NM_010548.2
IL-13	Forward: CCTGGCTCTTGCTTGCCTT Reverse: GGTCTTGTGTGATGTTGCTCA	NM_008355.3
TGF-β	Forward: GATCACCACAACCCACACCT Reverse: AGGTTCGTGGACCCATTTCC	NM_009368.3

Insitute of Biotechnology) with a protease-inhibitor cocktail (MedChemExpress LLC). The protein concentration was determined using the Bradford assay (Sangon Biotech Co., Ltd.) and the protein samples were subsequently diluted to 1 mg/ml, then separated (20 μg/lane) using 10 or 12% SDS-PAGE, following which the proteins were transferred onto PVDF membranes. The membranes were blocked overnight at 4˚C with 5% skimmed milk in TBS +0.05% Tween-20. The following primary antibodies were used: Phosphorylated (p)-ERK1/2 (cat. no. 4370; Cell Signaling Technology Inc.), ERK1/2 (cat. no. 46951; Cell Signaling Technology Inc.), p-p38 (cat. no. 4511; Cell Signaling Technology Inc.), p-38 (cat. no. 8690; Cell Signaling Technology Inc.) p-JNK (cat. no. 4668; Cell Signaling Technology Inc.), JNK (cat. no. 9252; Cell Signaling Technology Inc.), IL-10 (cat. no. ab33471; Abcam), induced nitric oxide synthase (iNOS; cat. no. ab213987; Abcam), ARG1 (cat. no. ab124917; Abcam), and cyclooxygenase 2 (COX2; ab179800; Abcam) (all 1:1,000) were used. The primary antibody for the loading control, GAPDH (cat. no. sc-365062; Cell Signaling Technology, Inc.) was used at a dilution of 1:2,000. After washing with PBS, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (cat. nos. sc-2004 and sc-2005) (both 1:2,000 and Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Proteins were visualized using an enhanced chemiluminescence kit (Dalian Meilun Biology Technology Co., Ltd.) according to the manufacturer's protocol.

Effect of rAf RNASET2 on immune responses in mice. Purified r*Af* RNASET2 was mixed with Imject Aluminum adjuvant (Thermo Fisher Scientific, Inc.) to form a water-in-oil adjuvant antigen. Mice were randomly divided into immunized, adjuvant control, and PBS control groups (n=6 per group), and injected intraperitoneally with 100 μg r*Af* RNASET2 and 2 mg of aluminum adjuvant (*Af* RNASET2 + adjuvant), 2 mg of aluminum adjuvant alone (adjuvant), or with PBS only (control) once a week for 3 weeks. A total of 7 days following the third immunization injection, about 50 μl orbital venous blood samples were collected and the sera were isolated following centrifugation at 3,000 x g for 20 min at 4˚C. The levels of IL-4, IL-13, IL-10, and interferon-γ (INF-γ) in mouse sera were detected using cytokine ELISA kits (IL-4, cat. no. HZ-IL-4-Mu; IL-10, cat. no. HZ-IL-10-Mu; IL-13, cat. no. HZ-IL-13-Mu and INF-γ, cat. no. HZ-INF-γ-Mu) (all Shanghai and Shanghai Zhen Biotech Co., Ltd.) according to the manufacturer's instructions.

Statistical analysis. Quantitative data are presented as mean ± SDs. Analyses were performed using Prism 7 software (GraphPad Software, Inc.). Student's t-tests or one-way ANOVA followed by Dunnett's post hoc test were performed, to detect statistical significance between groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characteristics of Af RNASET2. The amino acid sequence of *Af* RNASET2 (GenBank XP_754496.2) was obtained based on the *Af AF293* genome (Genome ID 18) in the NCBI database. The *Af* RNASET2 gene encodes 401 amino acids with a 15-amino-acid N-terminal secretory signal; the mature RNASET2 protein is 382 amino acids long. As shown in Fig. 1, a homology comparison based on amino acid sequence alignment revealed that *Af* RNASET2 was 11.84, 13.20, and 12.17% homologous with *Sj* CP1412, *Sm* ω-1, and *Cs* RNASET2, respectively. Notably, *Af* RNASET2 was predicted to contain two conserved amino acid sequence (CAS) domains (^{83}WTIHGLWP^{89} and ^{140}WEHEWNKHG^{148}), namely CAS-1 and CAS-2, and a conserved pair of cysteine residues (cysteine^{100} and cysteine^{158}) characteristic of T2 RNase-glycosylation sites were also found.

Expression and RNase activity of rAf RNASET2. To obtain the *rAf* RNASET2 protein, cDNA encoding mature *Af* RNASET2 (glutamicacid 26-arginine 407 region), without its signaling peptide (methionine 1-phenylalanine 25 domain) was synthesized, submitted to the NCBI (GenBank MN593022), and subcloned to a pET-His prokaryotic expression vector for expression and purification. cDNA from *Af* RNASET2 were generated using PCR amplification and subcloned into

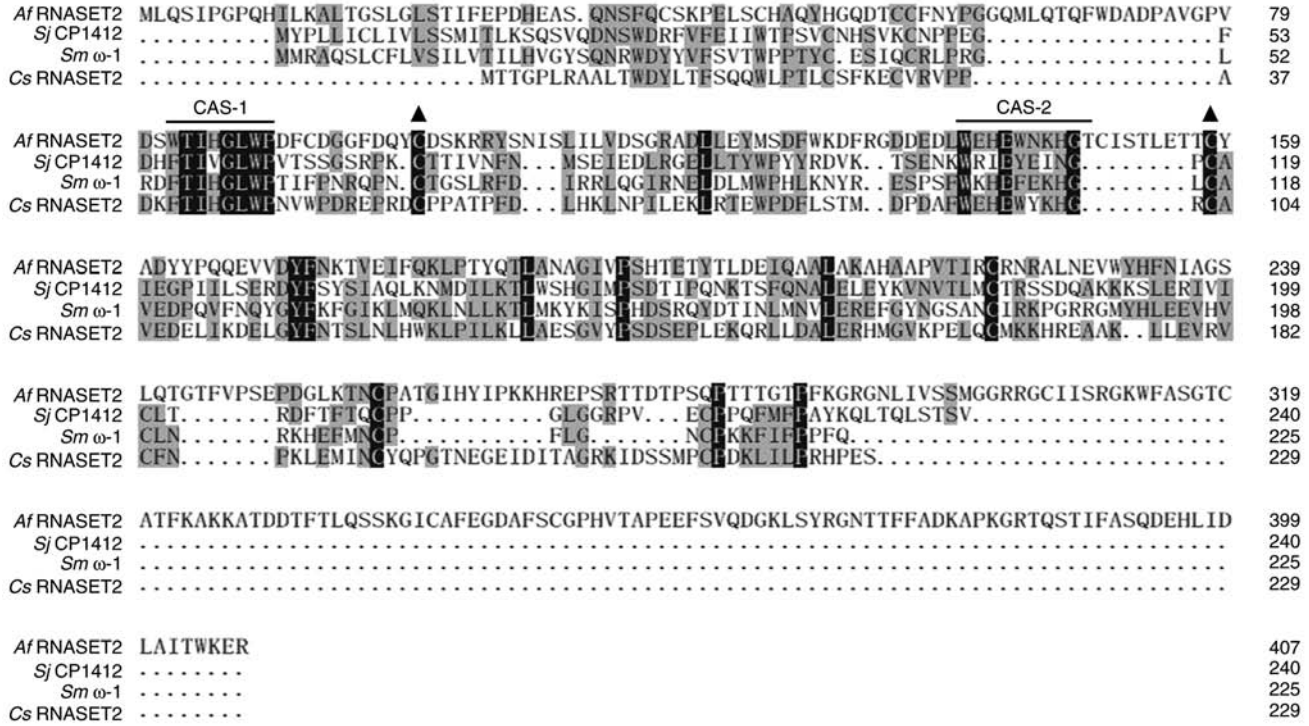

Figure 1. Amino acid sequence homology of *Af* RNASET2, *Sj* CP1412, *Sm* ω-1, and *Cs* RNASET2. Common amino acids are indicated by the grey shading. Black solid lines indicate conserved functional domains, CAS-1 and CAS-2. Solid black triangles indicate conserved cysteine residues. *Af, Aspergillus fumigatus; Sj*, *Schistosoma japonicum*; *Sm*, *Schistosoma mansoni*; *Cs*, *Clonorchis sinensis*; CAS, conserved amino acid sequences; RNASET2, T2 ribonuclease.

pET-His vectors using *Bam*HI/*Hind*III restriction enzymes. The band at 1,200 bp was consistent with the length of the *Af* RNASET2 gene (Fig. 2A). The pET-His-*Af* RNASET2 plasmid was confirmed using DNA sequencing. Subsequently, the pET-His-*Af* RNASET2 plasmid was transformed into *E. coli* BL21(DE3) plysS cells for expression and purification. SDS-PAGE revealed that r*Af* RNASET2 (~45 kDa) was expressed in the sediment following IPTG induction (Fig. 2B; Lane 3). A portion of the r*Af* RNASET2 protein sample was purified using Ni-NTA-resin and renatured into soluble proteins by gradient dialysis with urea (Fig. 2C). Purified soluble r*Af* RNASET2 lysed yeast RNA in a time-dependent manner and RNA digestion activity of r*Af* RNASET2 was effective with ribonuclease A (Fig. 2D) and could be inhibited with the ribonuclease inhibitor RNasin (Fig. 2E).

rAf RNASET2 promotes the Th2 response in vivo. The levels of the Th2 response-related cytokines IL-4 (P>0.01; Fig. 3A), IL-10 (P<0.01; Fig. 3B), and IL-13 (P<0.01; Fig. 3C) were significantly increased in r*Af* RNASET2-immunized mouse sera, collected 7 days following the third immunization compared with that in PBS control groups. In addition, there was no change in the expression of the Th1 response-related cytokine, INF-γ, between *Af* RNASET2+ adjuvant and the control group (P>0.05; Fig. 3D). This indicates that r*Af* RNASET2 enhanced Th2 responses *in vivo*.

rAf RNASET2 promotes M2 macrophage polarization. The flow cytometry results revealed that *in vitro* stimulation of RAW264.7 macrophages with purified r*Af* RNASET2 significantly increased the expression level of the M2 surface marker CD206 (Fig. 4B), Notably, CD206 expression was increased from 3.00 to 21.73% following treatment with 40 μg/ml r*Af* RNASET2 (P<0.01). However, there was no significant change in the level of the M1 surface marker CD16/32 (P<0.01; Fig. 4A).

The RT-qPCR results revealed that RAW264.7 cells treated with r*Af* RNASET2 for 24 h had significantly increased expression levels of M2-related genes, including *Arg1* (P<0.01; Fig. 5A), *Il-10* (P<0.01; Fig. 5C), and *Il-13* (P<0.01; Fig. 5D), without a significant change in the expression level of the M2 marker iNOS (P>0.05; Fig. 5B). Western blot analysis revealed marked increases in ARG1 and IL-10 protein levels, with no changes in iNOS and M1-related gene COX2 levels, in RAW264.7 cells treated with r*Af* RNASET2 (Fig. 5E). LPS upregulated the protein expression level of COX2 in RAW264.7 cells. An ELISA also revealed that the levels of IL-10 in the culture supernatant of RAW264.7 cells stimulated by r*Af* RNASET2 were also increased (Fig. 6B). These results indicate that r*Af* RNASET2 promoted M2 macrophage polarization in RAW264.7 cells.

Conversely, in a parallel experiment conducted with RNasin-inactivated r*Af* RNASET2, the aforementioned M2-like changes were not observed, as evidenced by no significant changes in the mRNA levels of ARG1, IL-10 and IL-13 (P>0.01 vs. control group; Fig. 5). These results indicated that high rAf RNASET2 enzyme activity was required for the M2 polarization/Th2 response of macrophages to r*Af* RNASET2.

rAf RNASET2 upregulated MAPK signaling in macrophages. Western blot analysis revealed that active, but not inactive, r*Af* RNASET2 upregulated the expression levels of p-ERK1/2, p-p38, and p-JNK in RAW264.7 cells (Fig. 6A). In addition, as IL-10 is important for Th2 responses (21), the

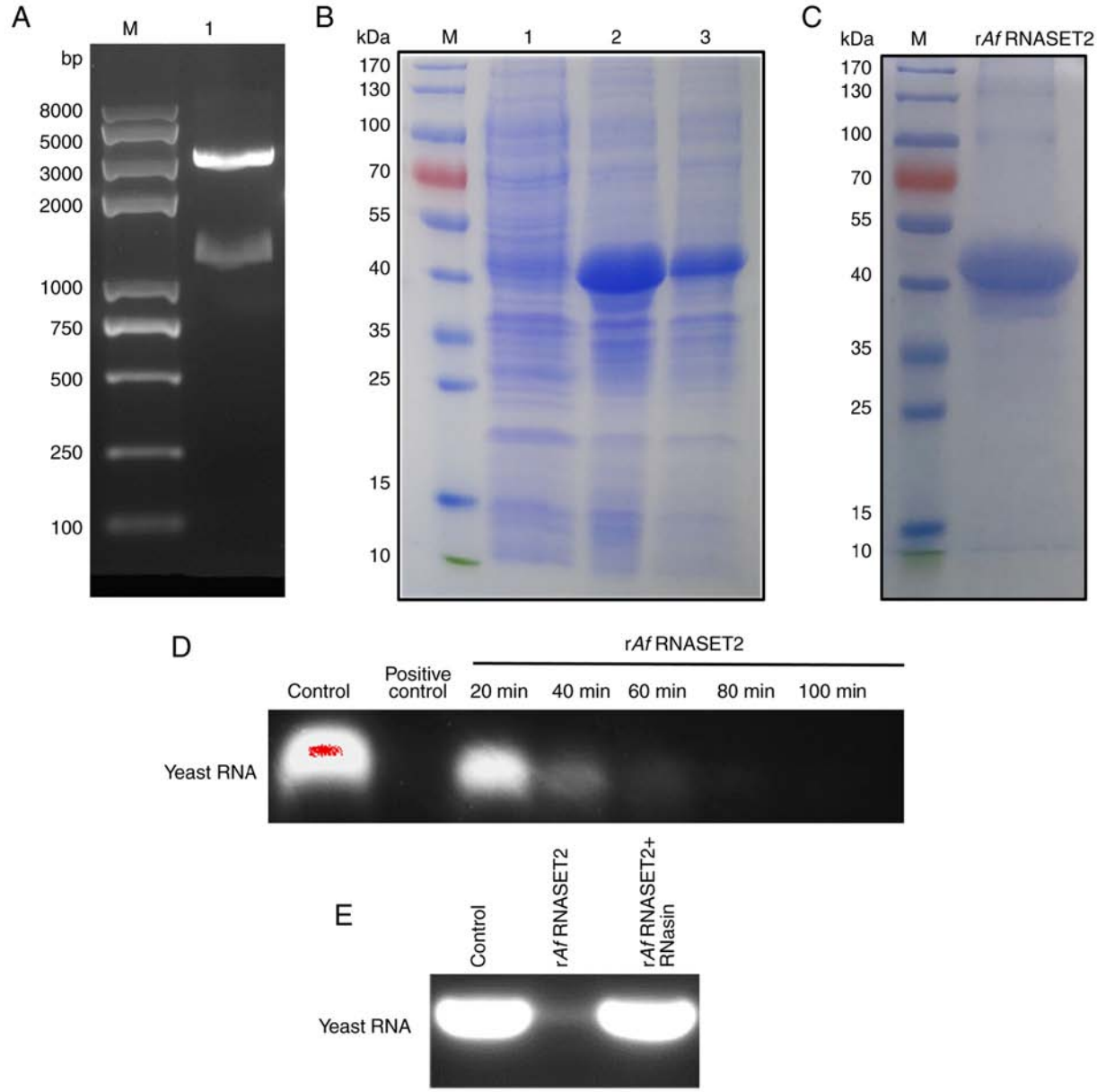

Figure 2. Expression, purification, and RNase activity of r*Af* RNASET2. (A) Analysis of the prokaryotic recombinant expression vector pET-His-*Af* RNASET2 (without signal peptides, Glutamic acid 26-Arginine 407) by double enzyme digestion. Lane M indicates the DNA marker. The 1,200 bp band indicates the *Af* RNASET2 cDNA insert. (B) SDS-PAGE analysis of pET-His-Af RNASET2 expression. Lane M, protein marker; lane 1, *E. coli* transformed with pET-His-Af RNASET2 prior to IPTG induction; lane 2, *E. coli* transformed with pET-His-*Af* RNASET2 following IPTG induction; lane 3, sediment from *E. coli* transformed with pET-His-*Af* RNASET2 plasmid following IPTG induction, and ultrasonication. (C) SDS-PAGE of renatured r*Af* RNASET2 protein following purification using Ni-NTA affinity chromatography. Lane M indicates the protein marker. (D) Analysis of RNase activity of r*Af* RNASET2 (1 μg) with yeast RNA (50 μg) at 37˚C at 20, 40, 60, 80 and 100 min). (E) Analysis of RNase activity of r*Af* RNASET2 with or without RNasin (RNAse inhibitor) pretreatment. RNase enzymolysis products were analyzed using 1% agarose gel electrophoresis. *E. coli, Escherichia coli*; r, recombinant; *Af, Aspergillus fumigatus*; RNASET2, T2 ribonuclease; IPTG, isopropyl-β-d-thiogalactopyranoside.

transcription and protein level of IL-10 was subsequently detected to reflect the effect of MAPK inhibitors on the rAf RNASET2-induced M2 macrophage polarization. RT-qPCR revealed that MAPK inhibitors (U0126, SP600125 and SB203580 for ERK, JNK and p38, respectively) significantly reduced the r*Af* RNASET2-induced increases in the protein expression levels of IL-10 in RAW264.7 cells ($P<0.01$; Fig. 6B). In addition, ELISA revealed that r*Af* RNASET2-induced IL-10 secretion in the cell supernatant was similarly decreased by MAPK inhibitors ($P<0.01$; Fig. 6C).

Discussion

In the present study, an *E. coli* expression system was used to produce r*Af* RNASET2 with robust ribonuclease activity. The amino acid sequence homology analysis revealed conservation of the T2 ribonuclease functional domains, CAS-1 and CAS-2. Glycosylation of r*Af* RNASET2 may have been unaffected by ribonuclease activity due to the lack of modification in a prokaryotic host. Similarly, recombinant *Sj* CP1412 protein (from pET bacterial transformation) mediated enzymolysis of yeast RNA, affirming its ribonuclease activity (18). Glycosylation mutants

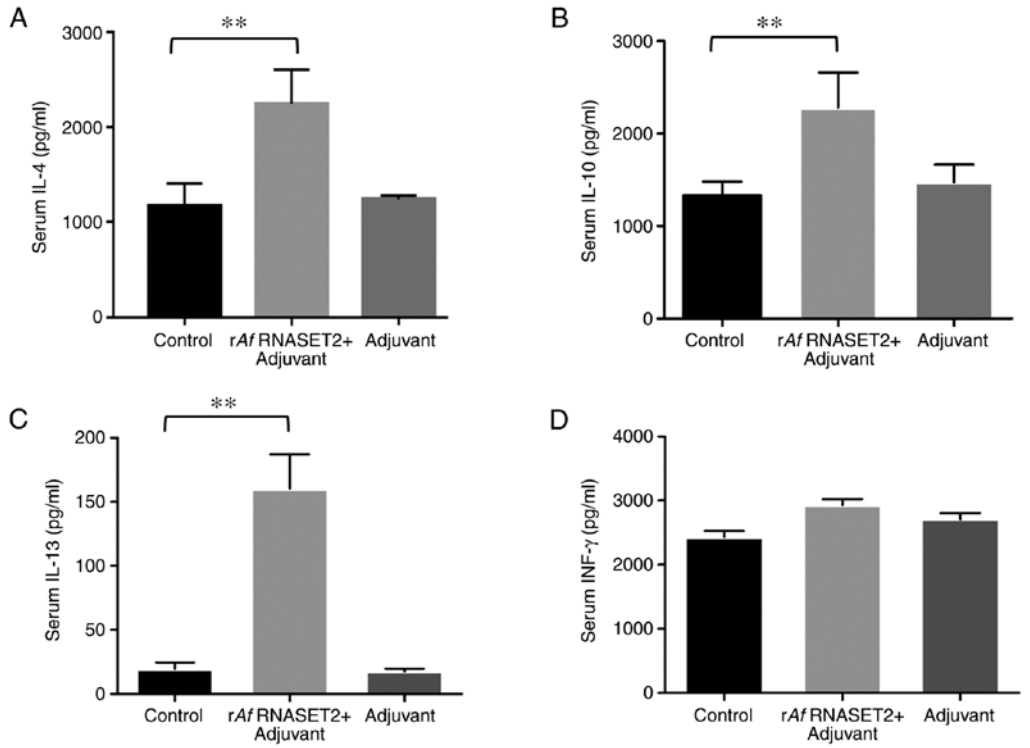

Figure 3. *In vivo* induction of immune responses by r*Af* RNASET2. Serum obtained from orbital vein blood (collected 7 days after the third immunization day) from BABL/c mice injected with rAf RNASET2 with aluminum adjuvant, aluminum adjuvant alone, or PBS only (n=6 per group) were analyzed using ELISA. Levels of (A) IL-4, (B) IL-10, (C) IL-13 and (D) INF-γ were increased in the rAf RNASET2 with aluminum adjuvant immunization group compared with that in the control groups. Data are presented as the mean ± SD from independent experiments. **P<0.01. r, recombinant; *Af, Aspergillus fumigatus*; RNASET2, T2 ribonuclease.

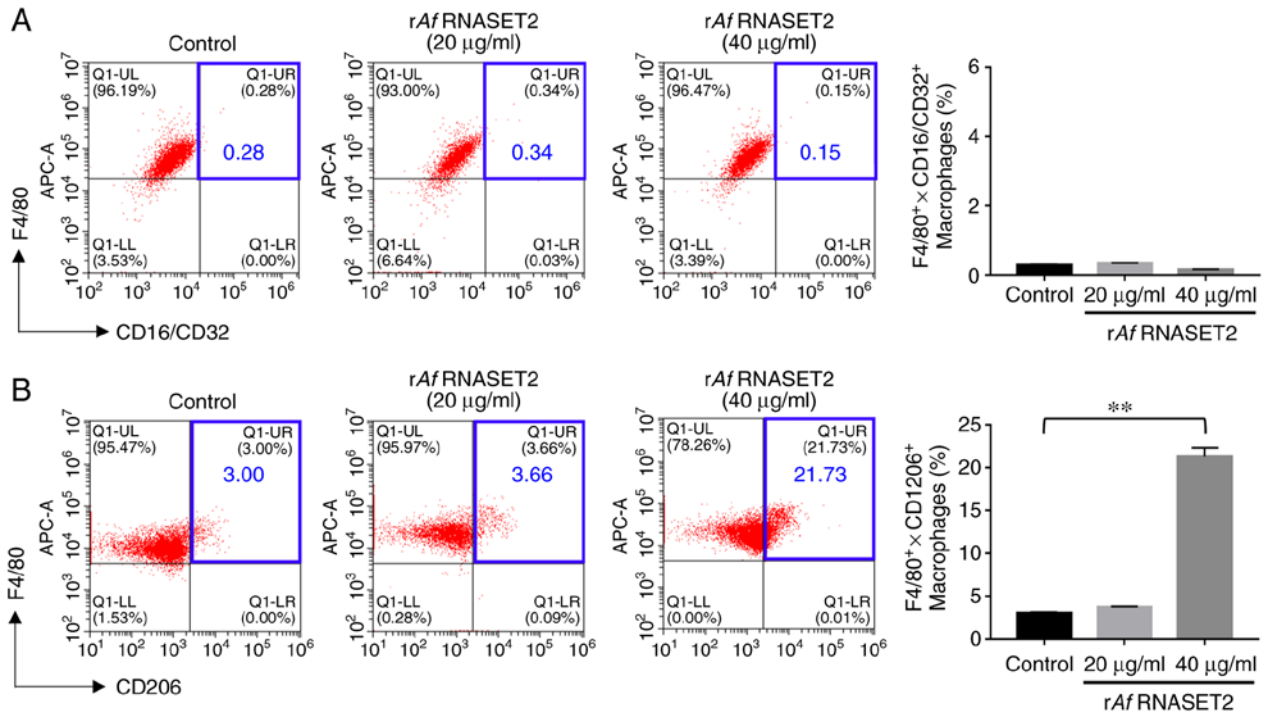

Figure 4. Macrophage polarization effect of r*Af* RNASET2. Flow cytometry of (A) CD16/32 and (B) CD206 expression levels on the surface of RAW264.7 cells stimulated with 20 or 40 μg/ml r*Af* RNASET2. Representative images from one of three independent experiments are shown. Data are presented as mean ± SD. **P<0.01. r, recombinant; *Af, Aspergillus fumigatus*; RNASET2, T2 ribonuclease.

of *Sm* ω-1, in which N-linked glycosylation sites (N71/176Q) were altered, did not have altered ribonuclease activity (16). These results suggest that glycosylation may be independent of RNASET2 protein activity and that prokaryotic expression methods are suitable for obtaining recombinant ribonucleases. Hence, although previously published data suggested that

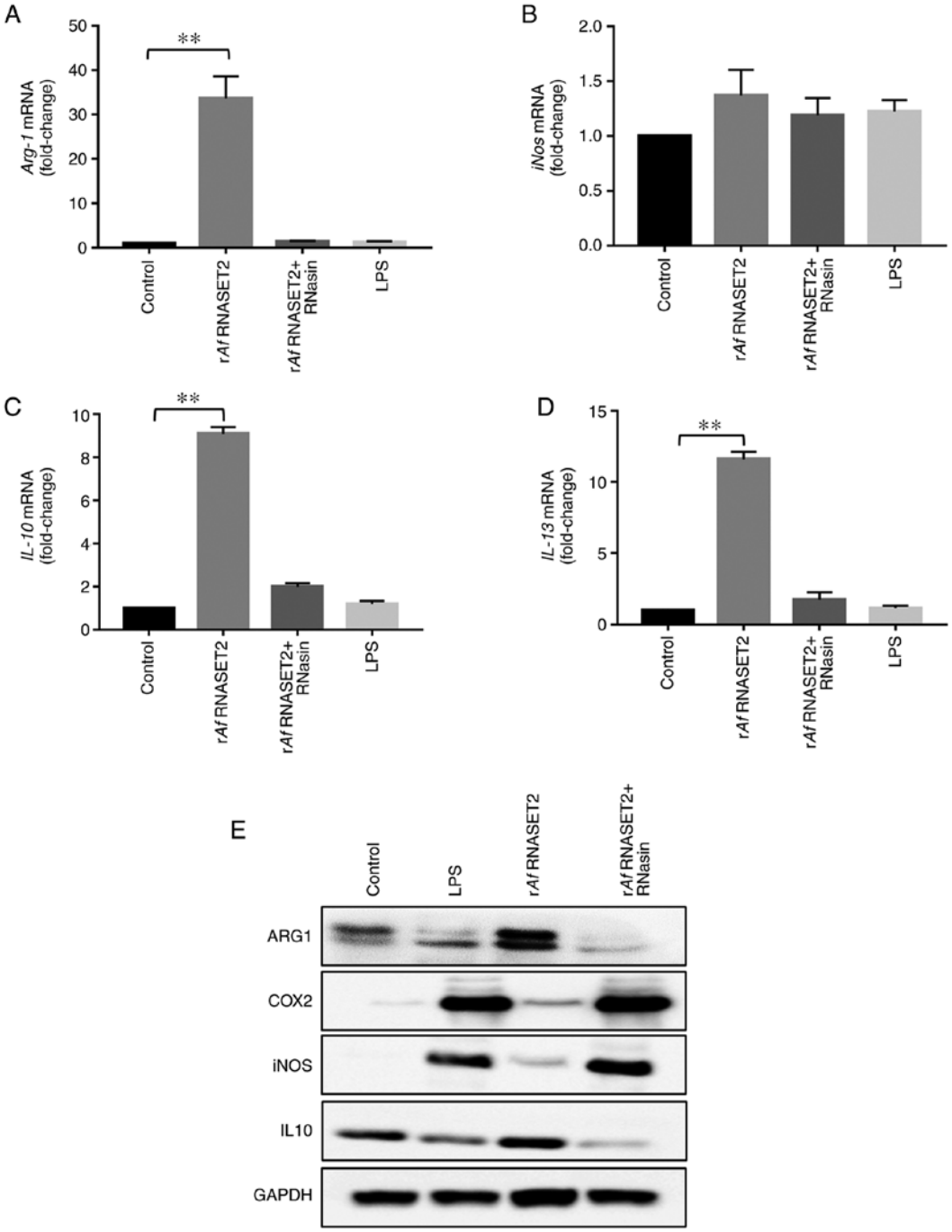

Figure 5. r*Af* RNASET2 increases expression of M2 polarization markers. Reverse transcription-quantitative PCR and western blot analysis were conducted with RAW264.7 macrophages co-cultured with r*Af* RNASET2 (20 μg/ml), inactive r*Af* RNASET2 (pretreated with RNasin), and PBS and LPS controls at 37˚C with 5% CO_2 for 24 h. mRNA expression levels of (A) *Arg-1*, (B) *iNos*, (C) *IL-10*, and (D) *IL-13*. Data are presented as mean ± SD from 3 independent experiments. **P<0.01. (E) Western blot analysis of ARG-1, COX2, iNOS, IL-10, and GAPDH in RAW264.7 macrophages following r*Af* RNASET2 (20 μg/ml) treatment for 24 h. r, recombinant; *Af, Aspergillus fumigatus*; RNASET2, T2 ribonuclease; LPS, lipopolysaccharide; iNOS, induced nitric oxide synthase; IL, interleukin; Arg-1, arginase-1.

glycosylation of *Sm* ω-1 may be necessary for conditioning dendritic cells for Th2 polarization (17), the findings in the present study indicate that glycosylation of *Af* RNASET2 was not associated with the observed immunoregulatory effect on M2 macrophage polarization.

Af antigen exposure induces ABPA in the majority of patients with atopic asthma or CF (22-24), as evidenced by a Th2 response and elevated IgE levels (25). *Af* proteases [i.e. Alp1 (26), Asp f 2 (27), Asp f 5 (28), and Asp f 13 (28)] have been shown to cause airway inflammation and remodeling in a murine inhalation model. The present study demonstrated that r*Af* RNASET2 immunization upregulated the expression of the Th2 cytokines, IL-4, IL-10, and IL-13, in sera, indicating a novel method of demonstrating whether there are specific *Af* molecules required to promote Th2 responses *in vivo*. The T2 ribonuclease proteins *Sj* CP1412 (18), *Sm* ω-1 (16), and *Cs* RNASET2 (29) can elicit Th2 immune responses *in vivo*. Moreover, the r*Af* RNASET2 produced in the present study

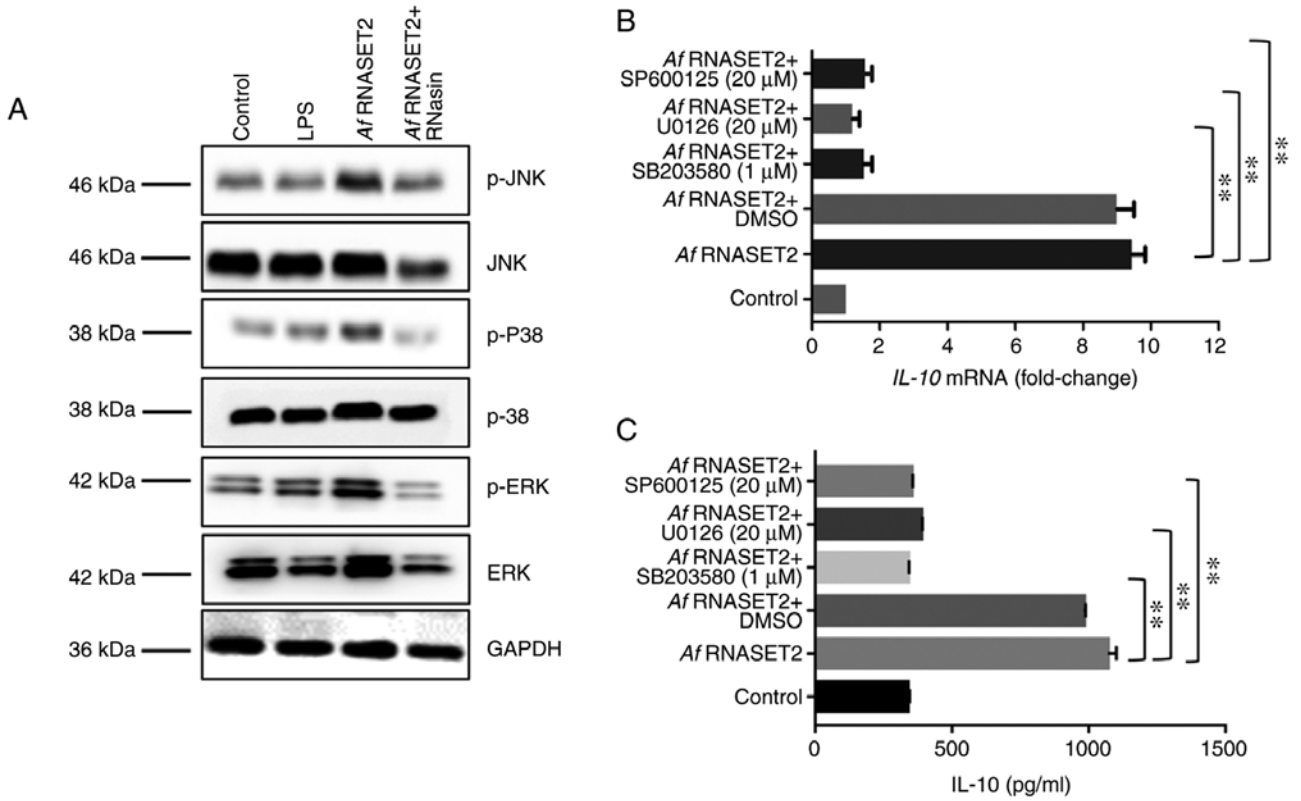

Figure 6. rAf RNASET2 upregulates proteins in the MAPK signaling pathway in macrophages. (A) Western blot analysis of JNK, p-JNK, p38, p-p38, ERK1/2, and p-ERK1/2 in RAW264.7 macrophages following treatment with r*Af* RNASET2 (20 μg/ml) or (24-h) RNasin-inactivated r*Af* RNASET2. Levels of IL-10 in RAW264.7 macrophages treated with r*Af* RNASET2 (20 μg/ml) alone or in the presence of MAPK signal inhibitors (U0126, SP600125 and SB203580 for ERK, JNK and p38, respectively) were determined using (B) reverse transcription-quantitative PCR and (C) ELISA. Data are represented as mean ± SD from 3 independent experiments. **P<0.01. r, recombinant; *Af, Aspergillus fumigatus*; RNASET2, T2 ribonuclease; p, phosphorylated; LPS, lipopolysaccharide.

elicited Th2 immune responses, supporting the hypothesis that *Af* RNASET2 is a Th2 response regulator in ABPA.

M2 macrophages can promote Th2 responses that could lead to ABPA by secreting IL-4, IL-10, or IL-13 cytokines or the chemokine CCL17 (30). Ke *et al* (18) revealed that *Sj* CP1412 induced M2 polarization of macrophages as evidenced by increased expression levels of CD206, ARG1, and IL-10. Notably, in the present study, stimulating macrophages with r*Af* RNASET2 also increased the protein expression levels of CD206, ARG1, and IL-10, with no changed in the M1 macrophage-related surface marker CD16/32, suggesting that r*Af* RNASET2 could promote M2 polarization in RAW264.7 macrophages. In addition, RNasin-inactivated r*Af* RNASET2 did not produce this M2 response, which is consistent with previous results with inactivated *Sm* ω-1 (16). These results suggest that a high level of r*Af* RNASET2 enzyme activity is required for M2 polarization and an associated Th2 response. Thus, suppression of *Af* RNASET2 activity may downregulate the immunoregulatory effect on M2 polarization of macrophages and prevent Th2 responses *in vivo*.

The effects of r*Af* RNASET2 on MAPK signaling were investigated as the MAPK signaling pathway has been shown to be involved in M2 polarization (31). The results from the present study reveal that r*Af* RNASET2 upregulated p-ERK1/2, p-p38, and p-JNK protein expression levels in RAW264.7 macrophages and that MAPK inhibition attenuated r*Af* RNASET2-induced IL-10 secretion detected in the supernatant of RAW264.7 cell cultures. These data indicate that MAPK signaling pathway is involved in the production of M2 macrophages following r*Af* RNASET2 induction.

The results from the present study support the hypothesis that *Af* RNASET2 may induce M2-type macrophage polarization, thereby leading to a Th2 response in ABPA development, and these effects were found to be dependent on ribonuclease activity. As ribonuclease proteins are relatively stable and resistant to degradation in the normal cell environment, the mechanism of the *Af* RNASET2-induced Th2 response effect provides important details regarding the predominant prevalence of *Af* promotion of ABPA development. Moreover, the current study supports the use of environmental intervention of ribonuclease inhibitors to suppress the effects of *Af* RNASET2-induced immune responses, as a prophylaxis against ABPA development. However, the efficacy of this method requires further investigation.

In conclusion, the results from the present study provide important evidence regarding the molecular pathogenesis of ABPA induced by *Af* exposure, which has not yet been elucidated, and supports the hypothesis that *Af* RNASET2 is an important immune regulatory factor in ABPA, primarily through augmentation of M2 macrophages polarization and thus induction of a Th2 immune response. These findings provide novel evidence pertinent to understanding the immune regulatory role of RNase T2 family proteins during *Af* exposure.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KJ and ZFZ conceived and designed the study. JJC, YSH, ZLC and YSL analyzed the data. JJC, YSH, XJZ and ZLC performed the studies. JJC and KJ contributed reagents, materials and analysis tools. JJC, ZFZ and KJ wrote the manuscript. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

All studies involving mice were performed according to protocols approved by the Animal Ethical and Welfare Committee of the School of Medicine of Shenzhen University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Donaldson SH and Boucher RC: Update on pathogenesis of cystic fibrosis lung disease. Curr Opin Pulm Med 9: 486-491, 2003.
2. Nayak AP, Croston TL, Lemons AR, Goldsmith WT, Marshall NB, Kashon ML, Germolec DR, Beezhold DH and Green BJ: *Aspergillus fumigatus*, viability drives allergic responses to inhaled conidia. Ann Allergy Asthma Immunol 121: 200-210.e2, 2018.
3. el-Dahr JM, Fink R, Selden R, Arruda LK, Platts-Mills TA and Heymann PW: Development of immune responses to Aspergillus at an early age in children with cystic fibrosis. Am J Respir Crit Care Med 150: 1513-1518, 1994.
4. Agarwal R, Aggarwal AN, Gupta D and Jindal SK: Aspergillus hypersensitivity and allergic bronchopulmonary aspergillosis in patients with bronchial asthma: Systematic review and meta-analysis. Int J Tuberc Lung Dis 13: 936-944, 2009.
5. Fricker-Hidalgo H, Coltey B, Llerena C, Renversez JC, Grillot R, Pin I, Pelloux H and Pinel C: Recombinant allergens combined with biological markers in the diagnosis of allergic bronchopulmonary aspergillosis in cystic fibrosis patients. Clin Vaccine Immunol 17: 1330-1336, 2010.
6. Nguyen NL, Pilewski JM, Celedón JC, Mandalapu S, Blanchard ML, DeRicco A, Hartigan E, Alcorn JF and Kolls JK: Vitamin D supplementation decreases *Aspergillus fumigatus* specific Th2 responses in CF patients with aspergillus sensitization: A phase one open-label study. Asthma Res Pract 1: 3, 2015.
7. Homma T, Bhushan B, Kato A, Norton J, Suh L, Sha Q, Gupta DS and Schleimer RP: *Aspergillus fumigatus* may promote Th2 activation by suppression of interferon signaling. J Allergy Clin Immunol 133: AB136, 2014.
8. Bhushan B, Homma T, Norton JE, Sha Q, Siebert J, Gupta DS, Schroeder JW Jr and Schleimer RP: Suppression of epithelial signal transducer and activator of transcription 1 activation by extracts of *Aspergillus fumigatus*. Am J Respir Cell Mol Biol 53: 87-95, 2015.
9. Homma T, Kato A, Bhushan B, Norton JE, Suh LA, Carter RG, Gupta DS and Schleimer RP: Role of *Aspergillus fumigatus* in triggering protease-activated receptor-2 in airway epithelial cells and skewing the cells toward a T-helper 2 bias. Am J Respir Cell Mol Biol 54: 60-70, 2016.
10. Wang L, Wang L and Wu X: *Aspergillus fumigatus* promotes T helper type 2 responses through thymic stromal lymphopoietin production by human corneal epithelial cells. Clin Exp Ophthalmol 44: 492-501, 2016
11. Knutsen AP, Hutcheson PS, Slavin RG and Kurup VP: IgE antibody to *Aspergillus fumigatus* recombinant allergens in cystic fibrosis patients with allergic bronchopulmonary aspergillosis. Allergy 59: 198-203, 2004.
12. Jolink H, De Boer R, Willems LN, van Dissel JT, Falkenburg JH and Heemskerk MH: T helper 2 response in allergic bronchopulmonary aspergillosis is not driven by specific Aspergillus antigens. Allergy 70: 1336-1339, 2015.
13. Furukawa S, Moriyama M, Miyake K, Nakashima H, Tanaka A, Maehara T, Iizuka-Koga M, Tsuboi H, Hayashida JN, Ishiguro N, *et al*: Interleukin-33 produced by M2 macrophages and other immune cells contributes to Th2 immune reaction of IgG4-related disease. Sci Rep 7: 42413, 2017.
14. Øya E, Becher R, Ekeren L, Afanou AKJ, Øvrevik J and Holme JA: Pro-inflammatory responses in human bronchial epithelial cells induced by spores and hyphal fragments of common damp indoor molds. Int J Environ Res Public Health 16: 1085, 2019.
15. Coughlan CA, Chotirmall SH, Renwick J, Hassan T, Low TB, Bergsson G, Eshwika A, Bennett K, Dunne K, Greene CM, *et al*: The effect of *Aspergillus fumigatus* infection on vitamin D receptor expression in cystic fibrosis. Am J Respir Crit Care Med 186: 999-1007, 2012.
16. Steinfelder S, Andersen JF, Cannons JL, Feng CG, Joshi M, Dwyer D, Caspar P, Schwartzberg PL, Sher A and Jankovic D: The major component in schistosome eggs responsible for conditioning dendritic cells for Th2 polarization is a T2 ribonuclease (omega-1). J Exp Med 206: 1681-1690, 2009.
17. Everts B, Hussaarts L, Driessen NN, Meevissen MH, Schramm G, van der Ham AJ, van der Hoeven B, Scholzen T, Burgdorf S, Mohrs M, *et al*: Schistosome-derived omega-1 drives Th2 polarization by suppressing protein synthesis following internalization by the mannose receptor. J Exp Med 209: 1753-1767, 2012.
18. Ke XD, Shen S, Song LJ, Yu CX, Kikuchi M, Hirayama K, Gao H, Wang J, Yin X, Yao Y, *et al*: Characterization of *Schistosoma japonicum* CP1412 protein as a novel member of the ribonuclease T2 molecule family with immune regulatory function. Parasit Vectors 10: 89, 2017.
19. Zhang Z, Cai Z, Hou Y, Hu J, He Y, Chen J and Ji K: Enhanced sensitivity of capture IgE-ELISA based on a recombinant Der f 1/2 fusion protein for the detection of IgE antibodies targeting house dust mite allergens. Mol Med Rep 19: 3497-3504, 2019.
20. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 5: 402-408, 2001.
21. Laouini D, Alenius H, Bryce P, Oettgen H, Tsitsikov E and Geha RS: IL-10 is critical for Th2 responses in a murine model of allergic dermatitis. J Clin Invest 112: 1058-1066, 2013.
22. Denning DW, O'Driscoll BR, Powell G, Chew F, Atherton GT, Vyas A, Miles J, Morris J and Niven RM: Randomized controlled trial of oral antifungal treatment for severe asthma with fungal sensitization: The fungal asthma sensitization trial (FAST) study. Am J Respir Crit Care Med 179: 11-18, 2009.
23. Chishimba L, Niven RM, Cooley J and Denning DW: Voriconazole and posaconazole improve asthma severity in allergic bronchopulmonary aspergillosis and severe asthma with fungal sensitization. J Asthma 49: 423-433, 2012.

24. Pasqualotto AC, Powell G, Niven R and Denning DW: The effects of antifungal therapy on severe asthma with fungal sensitization and allergic bronchopulmonary aspergillosis. Respirology 14: 1121-1127, 2009.
25. Murrison LB, Brandt EB, Myers JB and Hershey GKK: Environmental exposures and mechanisms in allergy and asthma development. J Clin Invest 129: 1504-1515, 2019.
26. Redes JL, Basu T, Ram-Mohan S, Ghosh CC, Chan EC, Sek AC, Zhao M, Krishnan R, Rosenberg HF and Druey KM: *Aspergillus fumigatus*-secreted alkaline protease 1 mediates airway hyperresponsiveness in severe asthma. Immunohorizons 3: 368-377, 2019.
27. Kurup VP, Xia JQ, Shen HD, Rickaby DA, Henderson JD Jr, Fink JN, Chou H, Kelly KJ and Dawson CA: Alkaline serine proteinase from *Aspergillus fumigatus* has synergistic effects on Asp-f-2-induced immune response in mice. Int Arch Allergy Immunol 129: 129-137, 2002.
28. Namvar S, Warn P, Farnell E, Bromley M, Fraczek M, Bowyer P and Herrick S: *Aspergillus fumigatus* proteases, Asp f 5 and Asp f 13, are essential for airway inflammation and remodelling in a murine inhalation model. Clin Exp Allergy 45: 982-993, 2015.
29. Xu Y, Lin J, Bian M, Chen W, Liang P, Wang X, Shang M, Qu H, Wu Z, Huang Y and Yu X: CsRNASET2 is an important component of Clonorchis sinensis responsible for eliciting Th2 immune response. Parasitol Res 114: 2371-2379, 2015.
30. Wang N, Liang H and Zen K: Molecular mechanisms that influence the macrophage m1-m2 polarization balance. Front Immunol 5: 614, 2014.
31. Neamatallah T: Mitogen-activated protein kinase pathway: A critical regulator in tumor-associated macrophage polarization. J Microsc Ultrastruct 7: 53-56, 2019.