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RESEARCH ARTICLE

Human pathogenic fungus *Trichophyton schoenleinii* activates the NLRP3 inflammasome

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ABSTRACT

The fungus *Trichophyton schoenleinii* (*T. schoenleinii*) is the causative agent of Trichophytosis and Tinea favosa of the scalp in certain regions of Eurasia and Africa. Human innate immune system plays an important role in combating with various pathogens including fungi. The inflammasome is one of the most critical arms of host innate immunity, which is a protein complex controlling maturation of IL-1β. To clarify whether *T. schoenleinii* is able to activate the inflammasome, we analyzed human monocytic cell line THP-1 for IL-1β production upon infection with *T. schoenleinii* strain isolated from Tinea favosa patients, and rapid IL-1β secretion from THP-1 cells was observed. Moreover, applying competitive inhibitors and gene specific silencing with shRNA, we found that *T. schoenleinii* induced IL-1β secretion, ASC pyroptosome formation as well as caspase-1 activation were all dependent on NLRP3. Cathepsin B activity, ROS production and K^+ efflux were required for the inflammasome activation by *T. schoenleinii*. Our data thus reveal that the NLRP3 inflammasome plays an important role in host defense against *T. schoenleinii*, and suggest that manipulating NLRP3 signaling can be a novel approach for control of diseases caused by *T. schoenleinii* infection.

KEYWORDS innate immunity, inflammasome, NLRP3, *Trichophyton schoenleinii*, infection

INTRODUCTION

Host innate immune response to microbial pathogens is usually mediated by pattern-recognition receptors (PRRs), which are mainly expressed by innate immune cells such as monocytes, macrophages and dendritic cells. This response is initiated from cellular recognition of conserved microbial structures, which are named as pathogen-associated molecular patterns (PAMPs). Till now, at least four families of PRRs have been identified, namely the Toll-like receptors (TLRs), C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs) and nucleotide-binding domain and leucine-rich-repeat containing proteins (NLRs) (Akira et al., 2006). A subset of NLRs, including NLRP3 and NLRP1, can respond to PAMPs as well as danger-associated molecular patterns (DAMPs) such as uric acid, and form a cellular complex called inflammasome which contains ASC and pro-caspase-1 (Twig et al., 2008; Franchi et al., 2009). Assembly of inflammasome leads to activation of caspase-1 and secretion of pro-inflammatory cytokines IL-1β and IL-18 (Martinon et al., 2002). Thus, the synthesis and production of IL-1β involves a two-step process: the first step is pro-IL-1β transcription and translation via NF-κB activation, the second step is pro-IL-1β cleavage by active caspase-1 largely due to inflammasome activation (Dinarello et al., 2012). IL-1β is a key pro-inflammatory cytokine for host defense, which helps oppose various invading microbes such as bacteria and fungi.

The fungus *Trichophyton schoenleinii* (*T. schoenleinii*) is an anthropophilic dermatophyte initially isolated from certain

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regions of Eurasia and Africa. Infection of this fungus is transmitted by contact between humans. It is the causative agent of Tinea favosa of the scalp, an infection which is characterized by the presence of yellowish, cup-like crusts or mats of hyphae occurring on the scalp. The cases of Tinea capitis caused by *T. schoenleinii* are increasing in South Xinjiang of China in recent years. Some epidemiological studies have been done to control this disease in the last few decades (Tai et al., 1992), but the Tinea favosa is not well controlled in South Xinjiang. Tinea favosa is the most serious clinical type of Tinea capitis, most of the patients can not take enough dosage of antifungal drugs, so the pathogen is transmitted person to person in the family or in primary schools.

A number of CLRs, TLRs and NLRs have been identified in responses to fungal pathogen infections, which involves signaling cascades resulting in the production of IL-1β (Gross et al., 2009; Hise et al., 2009; Hardison and Brown, 2012). IL-1β production via the NLRP3 inflammasome has been proven to be essential in host defense against pathogenic *Candida albicans* (*C. albicans*) and *Aspergillus fumigates* (*A. fumigatus*) infections (Gross et al., 2009; Hise et al., 2009; Said-Sadier et al., 2010). However, host immune responses to *T.schoenleinii* are not well characterized.

In the present study, we investigated the possibility of *T. schoenleinii* in activation of the inflammasome. We found that *T. schoenleinii* induced robust IL-1β production from human monocytes in an NLRP3 inflammasome dependent manner. Our study thus establishes an essential role for the NLRP3 inflammasome in host defense against *T. schoenleinii* infection, and suggests that positive manipulation of NLRP3 signaling may be a novel approach for control of favus caused by this fungus.

RESULTS

T. schoenleinii triggers IL-1β production from human monocytes

To determine whether *T. schoenleinii* induces IL-1β production from human monocytes, we monitored mature IL-1β level in human monocytic cell line THP-1 cells challenged with different doses of *T. schoenleinii*. IL-1β was strongly induced in a dose-dependent manner in this experiment (Fig. 1A). To figure out the time kinetics of IL-1β induction by *T. schoenleinii*, we went further and detected mature IL-1β level in THP-1 cells challenged with *T. schoenleinii* at different time points. It was observed that mature IL-1β level peaked at 48 h after *T. schoenleinii* challenge (Fig. 1B). Furthermore, to check whether *T. schoenleinii* induces ASC pyroptosome formation and caspase-1 activation, we treated THP-1 cells with *T. schoenleinii* (MOI = 1) and monitored the aggregation of ASC and autoactivation of caspase-1 using reported method (Fernandes-Alnemri and Alnemri, 2008). Indeed, we observed the formation of ASC pyroptosome (Fig. 1C) and mature caspase-1 secretion (Fig. 1D) in *T. schoenleinii* treated THP-1 cells and culture supernatants, respectively. Taken together, these data indicated that

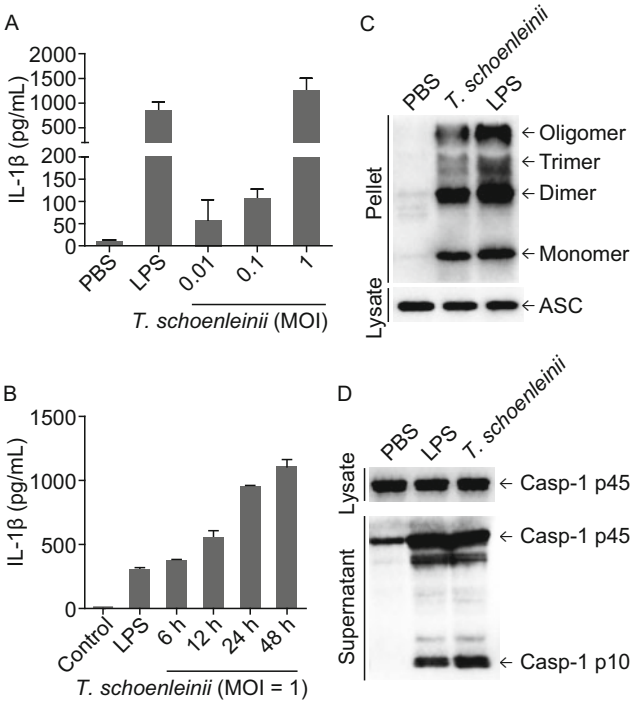

Figure 1. *Trichophyton schoenleinii* (*T. schoenleinii*) triggered IL-1β production and inflammasome activation in THP-1 cells. (A) 1 × 10^5 THP-1 cells were infected with *T. schoenleinii* with different doses (MOI), 24 h later the supernatants were harvested for IL-1β assay by ELISA. (B) 1 × 10^5 THP-1 cells were treated with *T. schoenleinii* (MOI = 1), supernatants were collected at different time points for IL-1β detection by ELISA. (C and D) 1 × 10^6 THP-1 cells were incubated with PBS, *T. schoenleinii* (MOI = 1) or LPS (100 ng/mL) for 6 h, the cells were harvested and treated using the method shown in Materials and Methods, the cell pellets and the supernatants were used for ASC pyroptosome and caspse-1 detection using Western blot, respectively. Data shown are mean ± SD from one out of three (A and B) or two (C and D) independent experiments.

T. schoenleinii triggered a strong IL-1β secretion as well as ASC pyroptosome formation and caspase-1 activation from human monocytes.

The secretion of IL-1β induced by *T. schoenleinii* is dependent on caspase-1

To confirm whether *T. schoenleinii* induced IL-1β secretion from THP-1 cells requires caspase-1-meadiated processing of pro-IL-1β, the cells were pretreated for 30 min with a specific caspase-1 inhibitor AC-YVAD-CHO (Allen et al., 2009) before subsequent infection with *T. schoenleinii*. Results from this experiment clearly demonstrated that *T. schoenleinii* induced IL-1β secretion was caspase-1 dependent, since the caspase-1 inhibitor decreased IL-1β production in a dose-dependent manner (Fig. 2A). As a control, the IL-8 secretion was not affected by this inhibitor as production of this cytokine is inflam-

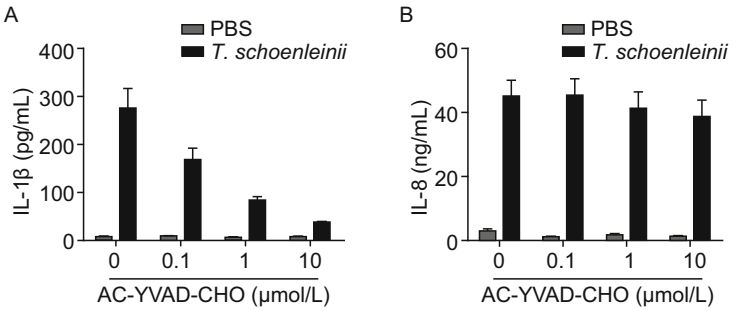

Figure 2. The secretion of IL-1β induced by *T. schoenleinii* infection was dependent on caspase-1. (A and B) 1 × 10^5 THP-1 were pretreated with caspase-1 inhibitor AC-YVAD-CHO and then challenged with *T. schoenleinii* (MOI = 1) for 6 h, the supernatants were harvested for IL-1β and IL-8 ELISA. Data shown are mean ± SD from one out of three independent experiments.

masome independent (Fig. 2B). Moreover, shRNA mediated silencing of caspase-1 also clearly decreased *T. schoenleinii* induced IL-1β secretion from THP-1 cells (Fig. 3A). From these data, we concluded that secretion of IL-1β induced by *T. schoenleinii* was mediated through caspase-1 activation.

T. schoenleinii induced IL-1β secretion is NLRP3 inflammasome dependent

Since the NLRP3 inflammasome has been shown to be responding to various fungal pathogen infections, we evaluated whether *T. schoenleinii* induced IL-1β secretion is NLRP3 dependent. We employed specific shRNA to silence the expression of ASC and NLRP3 in THP-1 cells. Results from these experiments clearly demonstrated that *T. schoenleinii* induced IL-1β secretion was NLRP3 inflammasome dependent, since the silencing of ASC and NLRP3 significantly decreased IL-1β (but not IL-8) secretion from THP-1 cells to the similar extent as caspase-1 knock-down cells (Fig. 3A and 3B). These results were further confirmed by immunoblotting assays detecting not only IL-1β release but also caspase-1 activation as well as ASC pyroptosome formation from *T. schoenleinii* infected cells, wherein silencing of caspase-1, ASC or NLRP3 strongly decreased IL-1β secretion as well as caspase-1 activation (Fig. 3C and 3D). Interestingly, the ASC oligomerization was attenuated only in NLRP3 and ASC silenced cells, while the knock-down of caspase-1 somehow promoted ASC oligomerization, speaking for the concept that caspase-1 activity is not required for the formation of ASC pyroptosome (Fig. 3C). Immunoblotting in Fig. 3D also showed successful silencing of the respective genes key for NLRP3 inflammasome assembly. Taken together, these results clearly demonstrated that *T. schoenleinii* induced IL-1β secretion through activation of the NLRP3 inflammasome in human monocytic cells.

To characterize whether other inflammasomes are involved in the production of IL-1β by THP-1 cells after treatment with *T. schoenleinii*, we treated THP-1 cells expressing shRNA specific to AIM2 or NLRP1. The silencing efficiency was well controlled (Fig. 3H). We observed that silencing of NLRP1 did not affect *T. schoenleinii* induced IL-1β secretion (Fig. 3E). In contrast, AIM2 knocking down significantly attenuated IL-1β production and slightly inhibited IL-8 production, indicating a possible role of AIM2 in *T. schoenleinii* triggered IL-1β production (Fig. 3E and 3F). Further experiments showed that AIM2 silencing did not affect caspase-1 activation and the formation of ASC pyroptosome, but decreased the expression of pro-IL-1β (Fig. 3G). Together, these data suggested that AIM2 might be involved in *T. schoenleinii* triggered inflammasome activation by regulating pro-IL-1β synthesis.

Lysosomal rupture, ROS production and K^+ efflux are involved in *T. schoenleinii* induced NLRP3 inflammasome activation

It's known that the activation of NLRP3 inflammasome by a variety of stimuli depends on lysosomal rupture, K^+ efflux and/or production of reactive oxygen species (ROS) (Ha et al., 2008; Tschopp and Schroder, 2010). To further explore the mechanism underlying IL-1β induction by *T. schoenleinii*, we first utilized CA-074 Me to inhibit activity of Cathepsin B, which is usually produced due to lysosomal rupture. In this experiment, we observed a significant attenuation of IL-1β induction by *T. schoenleinii*, and CA-074 Me almost completely blocked IL-1β release at the concentration of 100 μmol/L (Fig. 4A). We confirmed that this inhibition was not due to any toxic effect to the cells, since IL-8 secretion was not affected by this inhibitor (Fig. 4B).

Next, we pretreated THP-1 cells with ROS inhibitor diphenyliodonium (DPI), then challenged the cells with *T. schoenleinii* and measured the IL-1β secretion. As expected, DPI dramatically reduced *T. schoenleinii* induced IL-1β release (Fig. 4C). But in this case IL-8 secretion was also affected (Fig. 4D). Further experiments showed that DPI inhibited the expression of NLRP3 and pro-IL-1β proteins (Fig. 4G). Considering the minor difference in the sample loading, we attained the pixel value of the immunoreactive bands for NLRP3, pro-IL-1β and β-actin, then normalized the pixel value for NLRP3 and pro-IL-1β to β-actin. The results showed that DPI largely inhibited NLRP3 expression (Fig. 4H) and strongly inhibited pro-IL-1β (Fig. 4I). Quantitative real time PCR experiments

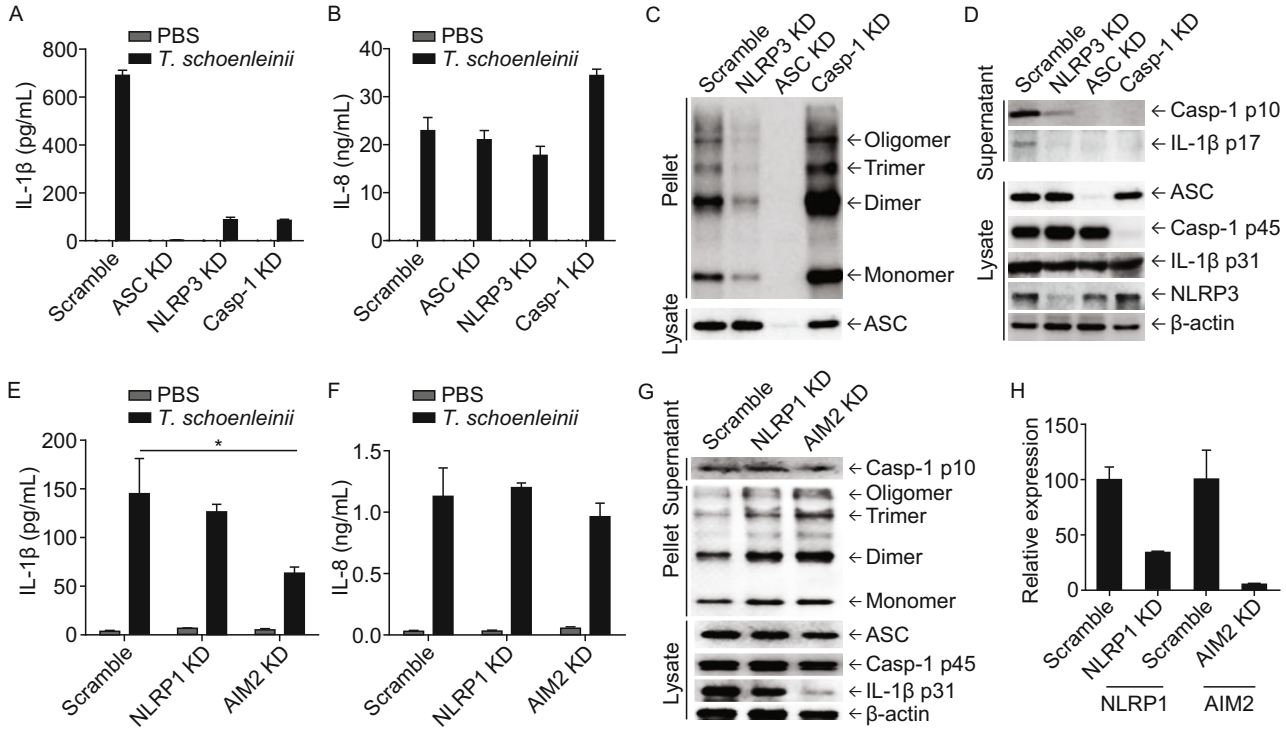

Figure 3. *T. schoenleinii* induced IL-1β production from THP-1 cells was NLRP3 inflammasome dependent. (A and B) 1 × 10^5 THP-1 Scramble, ASC KD, NLRP3 KD and Casp-1 KD cells were treated with *T. schoenleinii* (MOI = 1), 6 h later the supernatants were collected for IL-1β and IL-8 detection by ELISA. (C and D) 1 × 10^6 THP-1 Scramble, ASC KD, NLRP3 KD and Casp-1 KD cells were treated with *T. schoenleinii* (MOI = 1) for 6 h, the cells were treated using the method shown in Materials and Methods, the cell lysates were applied for Western blot to confirm the knockdown efficiency of inflammasome components. The cell pellets and the supernatants were used for ASC pyroptosome, caspse-1 and IL-1β detection. (E and F) 1 × 10^5 THP-1 Scramble, AIM2 KD and NLRP1 KD cells were treated using the same method as in A and B. The supernatants were collected to detect IL-1β and IL-8. (G) 1 × 10^6 THP-1 Scramble, AIM2 KD and NLRP1 KD cells were treated using the method same to C and D. The cells and supernatants were collected to detect caspase-1, ASC pyroptosome and inflammasome components. (H) The silencing efficiency of AIM2 KD and NLRP1 KD cells. Data shown are mean ± SD from one out of three (A, B, E and F) or two (C, D, G and H) independent experiments.

showed that mRNA transcriptions of NLRP3 and pro-IL-1β were both inhibited by DPI treatment (Fig. 4J and 4K). These data supported the recent finding that ROS inhibitors including DPI inhibit NF-κB activation instead of caspase-1 (Bauernfeind et al., 2011a).

We went further and blocked K^+ efflux by increasing the extracellular K^+ concentration. When THP-1 cells were pretreated with KCl, a significant reduction of IL-1β induction by *T. schoenleinii* was observed in a dose-dependent manner (Fig. 4E). As expected, this reduction was not due to KCl induced cell death since IL-8 production from the same cells was normal (Fig. 4F). Collectively, these data suggested that lysosomal rupture, ROS production and K^+ efflux all contribute to the activation of NLRP3 inflammasome in response to *T. schoenleinii* challenge.

Certain heat-sensitive component contributes to the IL-1β induction and caspase-1 activation by *T. schoenleinii*

To determine whether *T. schoenleinii* induced inflammasome activation is dependent on fungus growth, we treated *T. schoenleinii* at 90°C for 30 min. Successful heat inactivation was proved with further culture of heat treated *T. schoenleinii*, which did not grow up for new colonies (data not shown). We then used this heat-killed *T. schoenleinii* and live *T. schoenleinii* to challenge THP-1 cells followed with measurement of mature IL-1β and IL-8 levels. It was found that the production of both IL-1β and IL-8 was decreased compared with the normal *T. schoenleinii* infected THP-1 cells (Fig. 5A and 5B). We then measured the mRNA level of pro-IL-1β by q-RT-PCR, which was also reduced in the heat-killed *T. schoenleinii* treated THP-1 cells compared with control (Fig. 5C). For further characterization on protein level, we performed immunoblotting and observed decreased level of mature caspase-1 secretion and pro-IL-1β production in THP-1 cells after treatment with heat-killed *T. schoenleinii* (Fig. 5D). Thus, some heat-sensitive component from *T. schoenleinii* contributes to *T. schoenleinii*-induced IL-1β secretion, affecting both caspase-1 activation and NF-κB signaling responsible for pro-IL-1β synthesis.

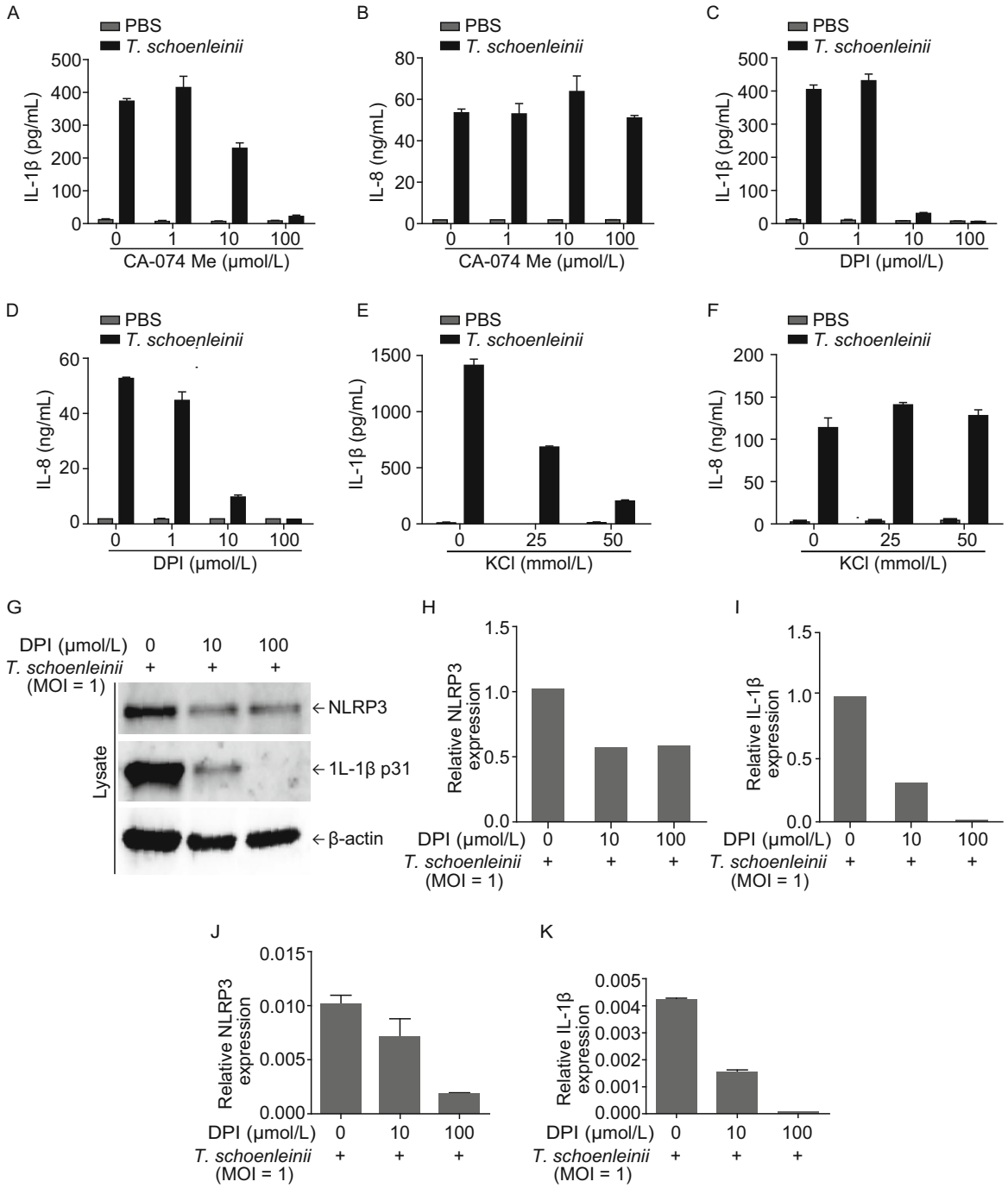

Figure 4. NLRP3 inflammasome activation in response to *T. schoenleinii* was dependent upon lysosomal rupture, ROS production and potassium efflux. 1 × 10^5 THP-1 cells were pretreated with CA-074 Me (A and B), DPI (C and D) or KCl (E and F), and then were challenged with *T. schoenleinii* (MOI = 1) for 6 h, the supernatants were harvested for IL-1β and IL-8 assay by ELISA. DPI pretreated THP-1 cells (5 × 10^5) were stimulated with *T. schoenleinii*, the cell lysates were harvested to detect NLRP3 and pro-IL-1β using Western blot (G) and qPCR (J and K). The pixel values of the immunoreactive bands for NLRP3, pro-IL-1β and β-actin were attained, then normalized the pixel value for NLRP3 (H) and pro-IL-1β (I) to β-actin. Data shown are mean ± SD from one out of three (A–F) or two (G–I) independent experiments.

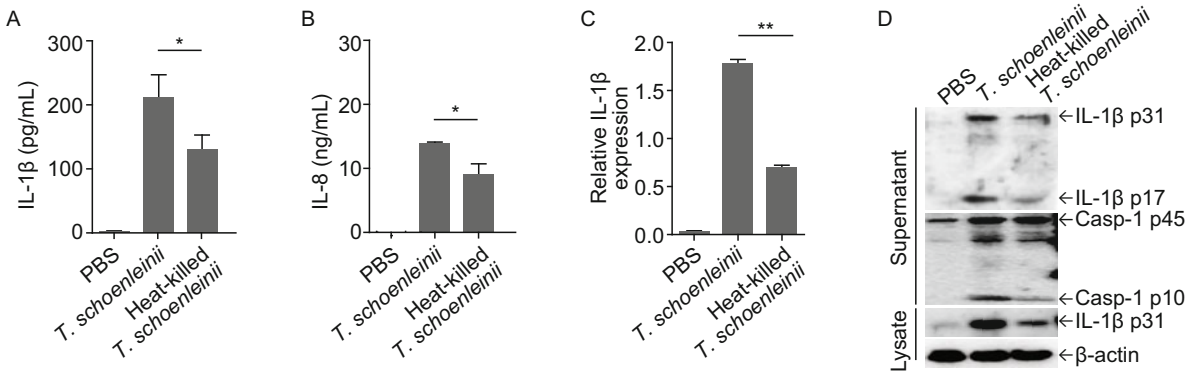

Figure 5. Certain heat-sensitive component from *T. schoenleinii* contributed to pro-IL-1β induction and caspase-1 activation. 1×10^5 THP-1 cells were challenged with *T. schoenleinii* or heat-killed *T. schoenleinii* (90°C, 30 min) for 6 h, the supernatants were harvested for IL-1β (A) and IL-8 (B) assay by ELISA, $^*P < 0.05$. (C) The expression of pro-IL-1β in *T. schoenleinii* challenged THP-1 cells was detected by real-time PCR, $^{**}P < 0.01$. 1×10^6 THP-1 cells were stimulated with *T. schoenleinii* or heat-killed *T. schoenleinii*, the cell lysates and supernatants were used for pro-IL-1β and caspase-1 detection (D), respectively. Data shown are mean ± SD from one out of three independent experiments.

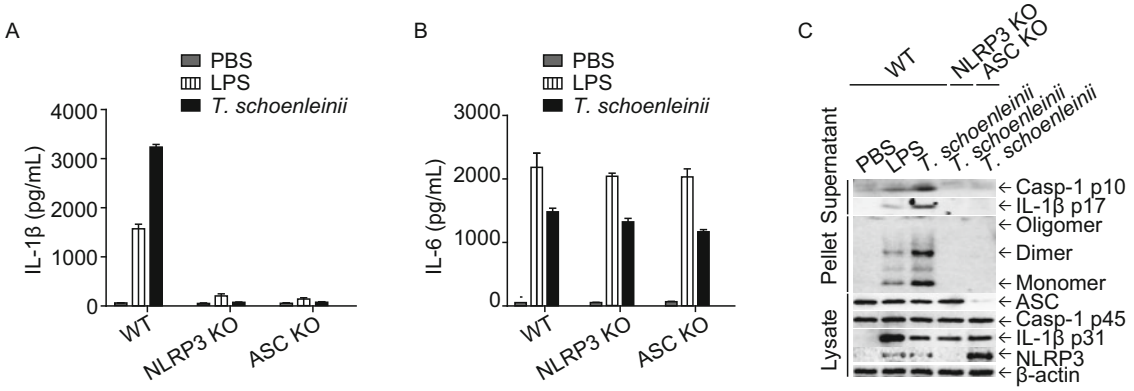

Figure 6. *T. schoenleinii* triggered IL-1β production from mouse BMDCs was dependent on NLRP3 inflammasome. 1×10^5 BMDCs (WT, NLRP3 KO and ASC KO) were treated with PBS, LPS (1 μg/mL) or *T. schoenleinii* (MOI = 1) for 6 h and the culture supernatants were harvested for IL-1β (A) and IL-6 (B) assay. (C) 1×10^6 BMDCs (WT, NLRP3 KO and ASC KO) were treated with *T. schoenleinii* (MOI = 1) for 6 h and the culture supernatants, cell pellets and cell lysates were harvested to detect IL-1β, caspase-1 and ASC pyroptosome by Western blot. Data shown are mean ± SD from one out of three (A and B) or two (C) independent experiments.

T. schoenleinii triggered IL-1β production in mouse BMDCs is dependent on NLRP3 inflammasome

To test whether *T. schoenleinii* is able to trigger inflammasome activation in mouse cells, we used PBS, LPS (1 μg/mL) or *T. schoenleinii* (MOI = 1) to treat mouse BMDCs for 6 h and the culture supernatants were harvested for IL-1β assay. Our results showed that *T. schoenleinii* treatment induced robust secretion of IL-1β in BMDCs (Fig. 6A). Moreover, ASC pyroptosome formation and caspase-1 activation (Fig. 6C) were also observed in *T. schoenleinii* treated BMDC pellets and culture supernatants, respectively.

We further checked whether *T. schoenleinii* induced inflammasome activation in mouse BMDCs was dependent on NLRP3 inflammasome activity. To this end, mouse BMDCs from WT, NLRP3 KO and ASC KO mice were treated with *T. schoenleinii* and the culture supernatants were collected for ELISA (IL-1β) and Western blot assay (IL-1β and caspase-1). From ELISA results, IL-1β release was strongly decreased in NLRP3 or ASC deficient cells (Fig. 6A), without affecting the release of IL-6 (Fig. 6B). In consistency with these data, Western blot results showed that the release of IL-1β, the activation of caspase-1 and the formation of ASC pyroptosome were all completely blocked in NLRP3 or ASC deficient cells (Fig. 6C). Taken together, *T. schoenleinii* induced IL-1β release and caspase-1 activation are also dependent on NLRP3 inflammasome in mouse BMDCs.

DISCUSSION

Opportunistic fungi, such as *C. albicans* and *A. funigatus*, are able to cause life-threatening infections in immunocompromised humans. Innate immune system plays a key role in the host defense against various classes of pathogens including these fungi *C. albicans* (Gross et al., 2009; Hise et al., 2009) and *A. funigatus* (Said-Sadier et al., 2010) were reported to be sensed by the NLRP3 inflammasome and lead to the synthesis, processing and release of IL-1β in mouse or human cells. In our present study, we analyzed the host immune responses to another opportunistic fungus, *T. schoenleinii*, and we found that *T. schoenleinii* induced caspase-1 activation and IL-1β production from human monocytes. Furthermore, we showed that the NLRP3 inflammasome was involved in IL-1β secretion, as there was a profound suppression of IL-1β release from NLRP3 and ASC silenced THP-1 cells.

The number of pathogens that activates the NLRP3 inflammasome is increasing, but the mechanisms by which NLRP3 senses its activators seem to converge on the intracellular perturbations such as lysosome rupture, ROS production and K^+ efflux (Schroder et al., 2010). Our data showed that Cathepsin B, ROS production and K^+ efflux were all required for the NLRP3 inflammasome activation by *T. schoenleinii*, since their inhibition resulted in a significant decrease of IL-1β secretion. A recent study (Bauernfeind et al., 2011b) showed that in mouse macrophages, the reason why ROS inhibitor such as DPI abolished IL-1β release was because of its inhibition of NLRP3 gene expression, our results confirmed this finding in human cells.

The fact that the NLRP3 inflammasome can be activated by a broad spectrum of stimuli indicated that they might not activate inflammasome directly (Juliana et al., 2010), some upstream molecules should be directly sensing the presence of microbes and NLRP3 might serve as an adaptor for inflammasome activation. Several PRRs, such as TLR2, TLR4, dectin-1 (Gersuk et al., 2006), dectin-2 and Mincle (Bugarcic et al., 2008; Yamasaki et al., 2008) have been observed to play a role in recognition and clearance of fungal infection (Hohl et al., 2005; Bretz et al., 2008; Werner et al., 2009) and triggering pro-inflammatory cytokine responses. The direct upstream receptors recognizing *T. schoenleinii* awaits further investigation.

From the fungi side, β-glucan is a key component activates host innate immune response indicated from early studies (Hohl et al., 2005; Kankkunen et al., 2010). Although we have not identified which components activated THP-1 cells for IL-1β production, our data suggested that certain heat sensitive component (likely a protein) of *T. schoenleinii* was responsible for the production of IL-1β triggered by this fungus. Moreover, since heat treatment also reduced the production of IL-8, pro-IL-1β synthesis and caspase-1 activation triggered by *T. schoenleinii*, the heat sensitive components might contribute to both pro-IL-1β synthesis via affecting NF-κB signaling and caspase-1 activity directly. Further study is needed to identify the components that engage NLRP3 dependent caspase-1 activation upon *T. schoenleinii* infection.

Interestingly, we observed increased level of ASC oligomerization in caspase-1 silenced cells. We speculate that this phenomenon was caused by several reasons. First of all, caspase-1 mediates the secretion of a number of unconventional proteins (Keller et al., 2008). It has been reported that inflammasome components, including ASC and NLRP3 could be detected in supernatant. It is possible that ASC oligomer may also be secreted through caspase-1 activation. Therefore, in the absence of a functional caspase-1, more oligomers retained in the cells. Secondly, the inflammatory cell death, pyroptosis, is dependent on caspase-1 (Fernandes-Alnemri et al., 2007). The lower level of caspase-1 mediated pyroptosis may allow caspase-1 knock-down THP-1 cells release less cellular contents including ASC oligomer, resulting in the relatively higher level of ASC oligomerization in these cells. Moreover, previous report showed caspase-1 independent role of ASC via activating caspase-8 (Pierini et al., 2012), it is possible that the interactions between ASC and caspase-8 or other caspases promote the oligomerization of ASC in caspase-1 knock-down THP-1 cells. These speculations can be tested with further work.

Another interesting point needs to note is the different capacity for *T. schoenleinii* and LPS in inducing IL-1β and IL-6 secretion in mouse BMDCs. *T. schoenleinii* induced a lower level of pro-IL-1β and IL-6 in BMDCs, but higher level of mature IL-1β than LPS (Fig. 6). LPS has a strong ability to prime the cells via potently activating NF-κB signaling, which promotes the synthesis of pro-IL-1β and NLRP3. This priming step works as signal 1 for IL-1β production. Besides the priming, a second activating signal (signal 2) is needed for IL-1β maturation in mouse cells. Our present findings indicated that *T. schoenleinii* was able to provide a stronger signal 2 than LPS in mouse BMDCs. Future study is needed to figure out the mechanism behind this different function for *T. schoenleinii* and LPS.

Taken together, our data suggested that the NLRP3 inflammasome is an important factor for control of *T. schoenleinii* infection, and manipulating NLRP3 signaling can be a novel approach for control of the disease caused by this fungus.

MATERIALS AND METHODS

Mice

C57BL/6 wild type (WT) mice were obtained from Shanghai Laboratory Animal Center (SLAC). ASC deficient mice were provided by Dr. Vishwa Dixit from Genentech and NLRP3 deficient mice were provided by Dr. Warren Strober from NIH. All animals were maintained at the SPF facility of SLAC. Animal care, use and experimental procedures complied with national guidelines and were approved by the Animal Care and Use Committee at Institut Pasteur of Shanghai.

Cell culture

THP-1 cells were maintained in RPMI 1640 media containing 10% FBS, 100 IU/mL penicillin, 1 mg/mL streptomycin, and 50 μmol/L mercaptoethanol. Mouse BMDCs were differentiated in the media mixture

consisting of 80% above mentioned RPMI 1640 media and 20% GM-CSF containing supernatant of Ag8653 cells. All the cells were cultured in a humidified 5% CO_2 incubator at 37°C.

T. schoenleinii culture and preparation

T. schoenleinii was inoculated on Potato Dextrose Agar Medium (PDA), and cultivated for 7 days at 27°C. The culture was triturated and washed with sterile PBS and counted using a hemocytometer prior to infection. To inactivate *T. schoenleinii*, we treated *T. schoenleinii* at 90°C for 30 min. Successful heat inactivation was proved with further culture of heat treated *T. schoenleinii*, which did not grow up for new colonies (data not shown).

Real-time PCR

Total RNA was extracted from the THP-1 cells using TRIzol reagent (Invitrogen). Reverse Transcription of mRNA and synthesis of cDNA was performed using TaqMan Reverse Transcription Reagents (Applied Biosystems). Real-time PCR was performed using the SYBR Green qPCR Master Mix (TOYOBO) on 7900HT fast real-time PCR system (Applied Biosystems). Relative quantification of genes was normalized against β-actin. The primers used were: β-actin, 5'-AGTGTGACGTG-GACATCCGCAAAG-(forward)3', 5'-ATCCACATCTGCTGGAAGGTG-GAC-(reverse)3'; pro-IL-1β, 5'-CACGATGCACCTGTACGATCA-(forward)3', 5'-GTTGCTCCATATCCTGTCCCT-(reverse)3'. NLRP3, 5'-AAGGGCCATGGACTATTTCC-(forward)3', 5'-GACTCCACCCGAT-GACAGTT-(reverse)3'; AIM2, 5'-TGGCAAAACGTCTTCAGGAGG-(forward)3', 5'-GATGCAGCAGGACTCATTTCA-(reverse)3'; NLRP1, 5'-ATTCCAGTTTGTGCGAATCCA-(forward)3', 5'-GTTCCTTGGG-GAGTATTTCCAG-(reverse)3'.

Quantification of cytokines by ELISA

Supernatants of THP-1 cells or BMDCs were harvested. Human IL-1β and IL-8 (BD Biosciences) and mouse IL-1β and IL-6 (eBiosciences) were analyzed by ELISA according to the manufacturer's instructions.

Generation of THP-1 cells expressing shRNA

shRNA vectors against human NLRP3, caspase-1, ASC, and their scramble vectors were gifts from Dr. Jurg Tschopp (Petrilli et al., 2007). AIM2 and NLRP1 shRNA vectors were constructed by cloning shRNA targeting AIM2 or NLRP1 into PLKO-puro vector. Lentiviruses expressing hairpins directed against these above genes were produced by transfecting shRNA vectors and the second-generation packaging plasmids pMD2-VSVG and pCMV-R8.91 (Naldini et al., 1996) into 293T cells. The virus particles were harvested 24 h later and infected THP-1 cells. After 48 h, 5 μg/mL of puromycin was added to the culture medium to select positive clones. The silencing efficiency was analyzed by real-time PCR and the most efficiently silenced cell subsets were used for further studies. Targeting sequences are: AIM2, GCCT-GAACAGAAACAGATG; NLRP1, GAGGGAAGAATCTGAGGAA.

Immunoblotting

THP-1 cells were pelleted by centrifugation at 3000 g for 3 min. The cell pellets were incubated in lysis buffer containing 50 mmol/L Tris (pH 7.5), 1% NP-40, 150 mmol/L NaCl, and protease inhibitor cocktail. After centrifugation at 12,000 g for 10 min at 4°C, the supernatants were harvested and mixed with SDS loading buffer. The protein-loading buffer mixture was then heated at 100°C for 10 min and separated on SDS-PAGE. The proteins were then transferred onto nitrocellulose membranes and were blocked with 5% fat free milk in 1× TBS containing 0.05% Tween-20 and then probed with primary antibodies as follows: polyclonal rabbit anti-human mature IL-1β and pro-IL-1β (sc-7884, Santa Cruz), monoclonal mouse anti-human NLRP3 (ALX-804-881, Enzo life sciences), polyclonal rabbit anti-human ASC (SC-22514-R, Santa Cruz), polyclonal rabbit anti-human caspase-1 (sc-515, Santa Cruz), monoclonal mouse anti-human β-actin (KM9001, Tianjin Sungene Biotech Co., Ltd). After incubation with appropriate HRP-conjugated secondary antibodies, the immunoreactive bands were visualized using ECL reagent (Amersham).

ASC pyroptosome detection

The procedure for ASC pyroptosome detection refers to previous report (Juliana et al., 2010). Briefly, THP-1 cells were seeded in 6-well plates (1×10^6 cells per well) and treated with PBS, LPS (100 ng/mL) or *T. schoenleinii* (MOI = 1) for 6 h. The cells were centrifuged at 3000 g for 3 min at 4°C. The cell pellets were resuspended in 0.2 mL of lysis buffer containing 50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% NP-40 and a protease inhibitor mixture, and lysed by sucking in and out of a 21-gauge needle for 10 times. The cell lysates were then centrifuged at 12,000 g for 10 min at 4°C. The resultant pellets were washed 3 times with PBS and resuspended in 500 μL of PBS. The pellets were then crosslinked with freshly prepared DSS (4 mmol/L) for 30 min at 37°C and were pelleted by centrifugation at 6000 g for 10 min. These crosslinked pellets were resuspended in 30 μL of SDS loading buffer for Western blot detection of ASC oligomerization.

In vitro T. schoenleinii challenge

1×10^5 THP-1 cells, scramble or NLRP3/ASC/caspase-1 silenced THP-1 cells were treated with *T. schoenleinii* (MOI = 1). The supernatants were harvested 6 h later for determination of IL-1β and IL-8 concentration by ELISA (BD Biosciences). In some cases, supernatants or cell extracts were collected for immunoblot analysis. 1×10^5 BMDCs (WT, NLRP3 KO and ASC KO) were treated with PBS, LPS (1 μg/mL) or *T. schoenleinii* (MOI = 1) for 6 h and the culture supernatants were harvested for IL-1β and IL-6 assay by ELISA (eBiosciences). 1×10^6 BMDCs (WT, NLRP3 KO and ASC KO) were treated with *T. schoenleinii* (MOI = 1) for 6 h and the culture supernatants, cell pellets and cell lysates were harvested to detect IL-1β, caspase-1 and ASC pyroptosome by Western blot.

Statistical analysis

Data was analyzed for statistical significance by two-tailed student's *t* test. A *P* value of ≤ 0.05 was considered as statistically significant.

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ABBREVIATIONS

ASC, apoptosis-associated speck-like protein containing a CARD; DAMPs, danger-associated molecular patterns; DPI, diphenylene iodonium; KCl, potassium chloride; IL-1β, interleukin-1 beta; NLRP3, NOD-like receptor family, pyrin domain-containing 3; PAMP, pathogen-associated molecular patterns; ROS, reactive oxygen species; shRNA, short hairpin RNA; *T. schoenleinii*, *Trichophyton schoenleinii*

COMPLIANCE WITH ETHICS GUIDELINES

Hua Li, Shuxian Wu, Liming Mao, Guowei Lei, Liping Zhang, Ailing Lu, Liguo An, Guiwen Yang, Paride Abliz, and Guangxun Meng declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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