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AUTHOR REPLY

Reply to Tantibhedhyangkul et al., 'Suspected Mycoplasma Contamination in the Study "Toll-Like Receptor 2 Recognizes *Orientia tsutsugamushi* and Increases Susceptibility to Murine Experimental Scrub Typhus"'

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Tantibhedhyangkul et al. speculate that the data presented in our recent study (1) may have been influenced by *Mycoplasma* sp. contaminating the Karp strain of *Orientia tsutsugamushi*.

They raise attention to a relevant point that was addressed by us before the Toll-like receptor 2 (TLR2) study was conducted. The *O. tsutsugamushi* Karp strain, which we obtained from the Australian Rickettsial Reference Laboratory, was propagated and tested for the presence of mycoplasma DNA in the preparatory phase of the study, using a *Mycoplasma* sp. 16S-specific PCR (2). This PCR yielded a positive result for mycoplasma, as demonstrated from a reference sample (Fig. 1A, sample ZK2009). Sequencing revealed the highest homology of the amplicon with *Mycoplasma orale*. As shown in Fig. 1B, the presence of *Orientia* DNA in the sample was verified by using an *Orientia*-specific PCR targeting the 56-kDa protein-encoding gene (3).

To purify the pathogenic *Orientia* strain contaminated with mycoplasma, we conducted a mouse passage, as was later suggested and discussed by Ogawa et al. (4). The strain was inoculated intraperitoneally (i.p.) into a female BALB/c mouse and reisolated from spleen homogenates taken during the acute infection phase. From then on, *Orientia* cell culture samples consistently tested negative by mycoplasma PCR, as shown by a representative sample from the end of the project phase (Fig. 1A, sample ZK2013). We thus exclude the possibility that our results were influenced by the presence of mycoplasma lipoproteins.

With regard to the fact that *Mycoplasma* spp. provide potent lipopeptide ligands for TLR2, e.g., macrophage-activating lipopeptide 2 (5), it is little surprising that Tantibhedhyangkul et al. found increased TLR2-dependent responses when using them to spike *Orientia* cultures. However, the authors neither experimentally addressed nor discussed other reasons for their failure to reproduce our findings. Specifically, they did not include heat-inactivated mycoplasma in their experiments to test their own hypothesis that "bacterial lysis and the subsequent release of more lipopeptides into the extracellular milieu" could induce a stronger inflammatory response in mouse dendritic cells. Additionally, we reported that the TLR2 ligand was, *inter alia*, proteinase K sensitive, unlike the synthetic lipopeptide Pam3Cys (1). Since digestion of lipoproteins with proteinase K preserves TLR2-ligating ability (6), this finding cannot be reconciled with mycoplasma lipoprotein contamination.

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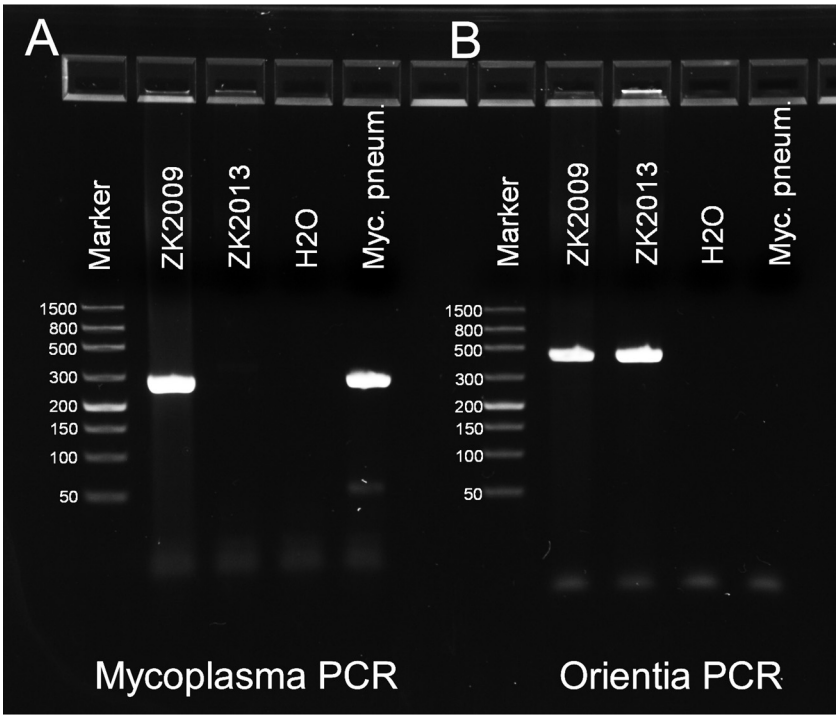

FIG 1 Decontamination of *O. tsutsugamushi* Karp from *Mycoplasma* sp. by mouse passage before the study of TLR2 involvement by Gharaibeh et al. *O. tsutsugamushi* Karp was propagated in L929 mouse fibroblasts (ZK2009). To decontaminate the strain from *Mycoplasma* sp., it was inoculated i.p. into a BALB/c mouse, reisolated from the spleen in L929 cell culture, and continuously propagated (ZK2013). DNA was prepared from reference samples ZK2009 and ZK2013 that had been stored at −20°C or *Mycoplasma pneumoniae* as a positive control (EZ1; Qiagen, Hilden, Germany) and eluted in 60 μl of AVE buffer (Qiagen). (A) A mycoplasma-specific PCR was run with the GPO-3/MGSO primer pair (MGSO, 5′-TGCACCATCTGTCACTCTGTTAACCTC-3′; GPO-3, 5′-GGGAGCAAACAGGATTAGATACCCT-3′) (2). The reaction mixture contained, in a total reaction volume of 25 μl, 5 μl of template, 400 nM GPO-3, 200 nM MGSO (both from MWG-Biotech, Ebersberg, Germany), 0.2 mM deoxynucleoside triphosphates, and 0.5 U of HotStarTaq DNA polymerase (Qiagen, Hilden, Germany). The cycling protocol was 94°C for 15 min; 35 cycles of 94°C for 20 s, 58°C for 30 s, and 72°C for 30 s; and 72°C for 7 min. (B) To detect *O. tsutsugamushi*-specific DNA, a PCR targeting a 409-bp fraction of the 56-kDa protein-encoding gene with the primer pair OtsuF/OtsuR was used (OtsuF, 5′-AATTGCTAGTGCAATGTCTG-3′; OtsuR, 5′-GGCATTATA GTAGGCTGAG-3′) (3). The reaction mixture contained, in a total reaction volume of 25 μl, 2 μl of template, 400 nM OtsuF, 400 nM OtsuR (both from TIB MOLBIOL, Berlin, Germany), 0.2 mM deoxynucleoside triphosphates, and 0.5 U of HotStarTaq DNA polymerase (Qiagen, Hilden, Germany). The cycling protocol was 94°C for 15 min; 40 cycles of 94°C for 20 s, 58°C for 30 s, and 72°C for 30 s; and 72°C for 7 min. Amplification products were visualized on a 2.2% agarose gel (Lonza, Basel, Switzerland). The values to the left of the marker lanes are molecular sizes in kilodaltons.

Moreover, Tantibhedhyangkul et al. claim that live *Orientia* bacteria generally induce higher levels of cytokines in monocytes and macrophages than inactivated organisms do (7, 8). This is in contrast not only to our data (1) but also to previous work that demonstrated increased production of tumor necrosis factor alpha (TNF-α) and interleukin-6 by murine macrophages that were stimulated with heat-killed *Orientia* organisms rather than infected with live *Orientia* organisms (9). A reason that could explain the authors' failure to observe downregulation of inflammatory responses by live *Orientia* bacteria may be the exclusive use of very high multiplicities of infection (MOIs) of $>$50 to 1,000/cell. A recent work by Tsai et al. showed that small infection doses (MOIs of $<$20) suppress TNF-α production, while larger doses (MOIs of $>$20 to 1,000) are strong inducers of TNF-α (10). Thus, the experimental conditions selected by Tantibhedhyangkul et al.—a restricted range of MOIs and no investigation of inactivated organisms—may have precluded the detection of TLR2-dependent recognition of *Orientia*. Instead, other recognition pathways may have become more relevant upon infection with high MOIs, associated with active suppression of all TLR2-mediated signals.

The authors were furthermore concerned about the timing of cytopathic effects. We do observe the onset of cytopathic effects in *Orientia*-infected L929 cell cultures between days 7 and 10 p.i. For the experiments presented in the paper, however, the cultures were harvested late in infection, at 14 days p.i. In light of this timing, it appears possible that TLR2 ligands are perhaps not stably expressed by *Orientia* but that their synthesis sets in late in infection. Similarly, it has been shown for *Staphylococcus aureus* lipoproteins that environmental factors such as an acidic pH or a postlogarithmic growth phase cause enrichment of diacyl rather than triacyl forms, which are known to have diverging TLR2 heterodimer specificities (11–13).

In summary, we demonstrate that the *O. tsutsugamushi* Karp strain used for the study by Gharaibeh et al. (1) was not contaminated by *Mycoplasma* sp. The limited investigation by Tantibhedhyangkul et al. leaves many questions unanswered and does not warrant concluding that TLR2 ligands are absent from *O. tsutsugamushi*. We would, however, like to emphasize that further research is required for a better understanding of the synthesis of TLR2 ligands by *O. tsutsugamushi* and their recognition.

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