Author’s response to reviews

Title: Caveolin-2 Associates with Intracellular Chlamydial Inclusions Independently of Caveolin-1

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PDF covering letter
Dear Dr. Borthwick,

We received our manuscript MS: 141174531535340, entitled “Caveolin-2 Associates with Intracellular Chlamydial Inclusions Independently of Caveolin-1” along with the listing of the reviewer comments. We appreciated the reviewer comments and have specifically addressed each of them as is indicated below, and in the text alterations. We hope that the reviewers will find that these changes have provided the clarification and requested additional information necessary to meet the criteria for publication in BMC Infectious Disease. The specific comments and our responses are presented below.

Major compulsory Revisions:

(1) Demonstration that caveolin-2 antibody is not cross-reacting with chlamydial EBs.

The reviewer expressed concern that many commercial antibodies cross-react with chlamydial EBs and therefore it is likely that the EBs might contain antigens that are being recognized by the caveolin-2 antibody. To rule out the possibility that the colocalization of caveolin-2 with the chlamydial inclusion is due to cross-reactivity of the antibody used, we performed the suggested western blot procedure using renografin gradient purified EBs and the caveolin-2 antibody. As the results demonstrate (figure 4), there is no reaction of the EBs with the caveolin-2 antibody. It is important to also note that caveolin-1 did not cross-react with EBs either (data not shown). We feel that the addition of this figure to the manuscript is adequate in addressing this reviewer’s major concern.

Minor Essential Revisions

(1) Reviewer “A” suggests that the volume of data in each figure is too much to effectively see the fluorescent staining pattern and that the data may be clearer if an enlarged image of only 1-2 cells were shown in each panel.
Please note that these figures are confocal images and will not give greater detail of staining pattern. These figures were taken at 600X magnification and when compared to pictures at 1000X, there is no greater clarity where staining pattern is concerned. We have previously shown the pattern of caveolin-1 colocalization with *Chlamydia* and is now mainly focusing on this colocalization with caveolin-2. The colocalization patterns for these two proteins with *Chlamydia* are indistinguishable, except for species specificity. The main point of the paper is that there is colocalization of the chlamydial inclusion with caveolin-2, and more importantly, that this colocalization takes place in the absence of caveolin-1.

(2) The reviewer requested that scale bars be added to each figure. We apologize for this oversight and have corrected this by adding scale bars to the figures. Each figure in a panel has been reduced to a similar degree from the original size to fit in the panel and was taken at the same magnification.

(3) The reviewer states that “It is not clear from the images that caveolin-2 staining is non-random and is in apposition to the pathogen cells.” It is important to note that the caveolin staining is not homogenous, nor does it cover the entire surface of the inclusion membrane. Rather, the caveolin protein appears as small packets in the *Chlamydia* inclusion membrane. These packets, when observed in the Z-section, appear to be at the same position coincident with the EBs/RBs in the inclusion. We therefore stated that the caveolin and the EBs “appear” to be coincident. We have no proof that there is a direct interaction of the EB/RB with the caveolin proteins and do not claim that such an interaction is taking place. We are merely stating what we have observed based on our confocal observations.

(4) Staining pattern of EB staining in figure #2. The reviewer suggests that the EB staining pattern in the Z-series does not seem to represent different focal planes of the cell and that this should be addressed in the discussion section. Please note that there are EBs in every plane of the Z-series, as would be expected when one takes sections through an inclusion membrane. Please see chlamydial developmental cycle in pictures at the *Chlamydiae* website maintained by Michael Ward (http://www.chlamydiae.com/docs/biology/biol_devcycle.htm). We believe that this staining pattern is characteristic of what should be seen. The outer rim of the inclusion is not expected to stain brightly with caveolin proteins since the caveolin proteins are not “inserted” in the inclusion membrane, nor are they distributed in a homogenous pattern in the membrane. Rather, caveolin proteins appear as tiny packets and are distributed over the surface of the inclusion. When viewing the compiled stack of Z-sections from the confocal micrographs, the overall intensity of the fluorescence results in what would appear to be a homogenous staining. However, under the microscope one can still see these small packets.

(5) Reviewer “B” pointed out the unintentional implication in the background section of the manuscript that the genus Chlamydia consists of a single species. This was
not our intension and we have amended this to include the other members of the genus, *C. muridarum* and *C. suis*, (Page 3, paragraph 2, lines 2 and 3).

(6) Reference #14 misspelt: The name of the author for reference 14, Thylefors, was spelt incorrectly as pointed out the reviewer. This spelling has been corrected.

**Discretionary revisions**

(1) Uninfected control cell stained with caveolin-2 antibody.

The reviewer thought it would be helpful to see an uninfected control of cells stained with the anti-caveolin-2 antibody. While one can see uninfected cells in the figures showing colocalization, we felt that it would be helpful to include such a control. We have therefore included a figure 3B depicting uninfected FRT cells stained with anti-caveolin-2. It is clear from this figure that the caveolin-2 is localized in a peri-nuclear position. This is presumed to be the Golgi, since it has been demonstrated that caveolin-2 localizes to the Golgi in the absence of caveolin-1 and does not migrate to the cell membrane. Importantly, none of these cells demonstrate membrane staining.

(3) Reviewer “B” inquired “….How, if at all, is clathrin and caveolins functionally related”. The caveolin proteins are structurally and functionally distinct from clathrin. Moreover, caveolae are likewise structurally and functionally distinct from clathrin-coated pits. Caveolae, and the lipid raft domains in which they are found, are briefly described in the manuscript. Also, we noted that an earlier publication from our laboratories demonstrates that some (but not all) chlamydial strains enter host cells via lipid rafts, rather than via caveolae, which are sometimes considered a special form of lipid raft.

(4) What role if any might the use of cycloheximide play? Have the experiments been repeated in the absence of cycloheximide? We have carried out these experiments in the absence of cycloheximide with no change in colocalization patterns or entry status. Thus, cycloheximide has no effect on caveolin/chlamydial colocalization. The cycloheximide was utilized only to enhance the growth of the *Chlamydia*.

Thank you for the opportunity to respond to the reviewers’ concerns.

Sincerely,

Wilmore Webley, Ph.D.