28 September, 2009

Dear Dr. Susan Banks-Schlegal

After careful consideration of the fact that the reviews of my R21 application # 1 R21 HL095948-01A1 entitled “Cross regulation of divergent host responses to viral and bacterial pathogens” do not reflect the contents of the application, I have decided to appeal the review. Clarifying some concerns raised in review of the first submission explicitly in the resubmission did not seem to have had any positive effect.

Basically there seem to be an inherent bias against the use of some of the best suited facile experimental model organisms from non-mammalian phyla for the stated goals. In addition, and probably due to this, the reviewers seem to have ignored the details already provided in the application. I base this conclusion on the fact that the referees made numerous criticisms (many technically flawed) based on the above stated misconceptions of the proposed model system. I outline some of these aspects below. Since all the arguments highlighted here are directly taken from the application where there are references cited, I have not used citations in this “Appeal” document.

In this application I proposed to study the cross regulation of two diverse arms of the immune response using well established invertebrate model systems and two classes of pathogens (RNA viruses and bacteria). The primary responses to these two classes of pathogens are through the two divergent arms of the innate immune response. One of them, involves the machinery of RNA silencing (to viral pathogens), and the other (to bacterial pathogens) through the extensively studied innate immune response to pathogen-associated molecular patterns that activates the production of antimicrobial effectors and other host strategies. Activation of the latter arm involves conceptually conserved signaling modules and pattern recognition receptors across different kingdoms.

1. Mammalian/medical relevance not clear

In the application, I proposed that the cross regulation between these arms of immunity is sparsely studied and hence poorly understood. One primary reason for this (that is addressed through the major advantages of the models of this study) is the increased complexity resulting from the interaction between the innate and the classically defined adaptive immune components in mammalian systems. In contrast to mammalian systems, the different model systems that I propose to use have complementary advantages and facilitate the study of the existence of the hypothesized cross regulation that likely hasn’t been recognized due to the above mentioned layers of complexity. Since innate immunity is among the first lines of defense even before adaptive immunity is activated the relevance still holds despite removing that layer of complexity. A number of lines of evidence are provided to substantiate the likelihood and relevance of this

proposed cross regulation. Prominent among them being the facts that (i) host microbe interactions co-evolve to negate each others advantages, (ii) the fact that there is continually increasing evidence of host and viral encoded miRNA affecting the outcome of viral pathogenesis, and (iii) there is direct evidence that where mammalian viral components interfere with both the interferon response (well established mammalian antiviral response) as well as RNA silencing. Though a prominent role for RNAi is still a matter of debate in mammalian systems, the RNA silencing machinery (that includes RNAi, miRNA mediated processes and some aspects of epigenetic regulation) include some shared and other structurally related components in the different kingdoms. Besides emphasizing this aspect in several sections of the application with appropriate examples and references, I also pictorially depict this rationale in Fig. 1 where I represented the commonality by using the term “silencesome” and included the different aspects mentioned above to further emphasize this. In the case of bacterial pathogens it has recently been demonstrated that they harbor effectors (delivered to the host through type III secretion system into the host) that interfere with RNA silencing machinery. Thus the proposal to test cross regulation between these two arms and the premonition that it has not yet been uncovered in mammals due to the complexities of the mammalian immune response is within the norms of scientific reasoning.

2. Rationale for use of multiple models and multi-pathogens not clear

It has been shown from numerous studies (including many leading contributions from Prof. Fred Ausubel’s laboratory that I am currently affiliated with) that the two invertebrate models have utility in highlighting different aspects of host-pathogen responses including innate immunity, and in many cases have direct utility in design and study of those aspects relevant to mammalian systems. In several instances, shared components of mammalian and agriculturally relevant pathogens have been unambiguously demonstrated. As to the question of rationale for using multiple pathogens (simultaneously or sequentially), I have explicitly stated that the use of viral and bacterial pathogens *per se* as opposed to known components or mutants when possible should highlight more aspects than the use of mutants in currently known dominant pathways. In accordance with the stated purpose of the RFA “Novel Approaches To Study Polymicrobial Diseases “ and of R21 applications, I propose exploration and development of model systems with unique advantages to the study of changes in outcome during multi-pathogen infections at the same site or at different sites with emphasis on cross regulation of the two divergent arms of immunity. The above mentioned studies address the question of relevance of these models in the study of diseases of mammalian systems. The previous studies that are still in progress deal with individual pathosystems, while this proposal is aiming to take it to the next higher level of complexity. Many aspects of biology have benefited enormously by the use of model systems, sometimes quite distant from mammalian biology (that the reviewer considers artificial). To cite an example from one of my personal contributions, I demonstrated during an earlier

postdoctoral experience by (artificially) expressing an avirulence gene product of a bacterial pathogen transgenically in plants that the site of action of such proteins is inside the (model) host plant cell even though the bacteria never invade the host. This paved way to the first published report of now what is considered a major demonstration of commonalities between plant and mammalian pathogens – the conservation of type III secretion systems.

Additionally, the understanding of the breadth of knowledge generated using these models and the thought process as to how to maximally exploit this understanding has led me to the different aspects of the intricate experimental design that I propose. In each case, it is clearly stated in the text as to what that advantage or knowledge is that can be exploited using that particular system and why that choice of system or design is specially suited is explicitly stated. This again points to the fact that a predetermined judgment against the use of these model systems for these kind of studies and that a lack of time and/or familiarity with these models played a major role in eliciting the negative responses from these reviewers. If the infection model involving *C.elegans* and TEV is a success then the unique advantage of having the ability to use same pathogen in two divergent hosts will be self-evident.

Questions from the referees such as: if a signal is found what relevance it would have to mammalian systems or and if a natural virus is found by deep sequencing of field isolates of *Caenorhabditis* sp. it may not be propagatable, further emphasize the flaws in the referees' reasoning. In the first case one wouldn't know until one finds a signal and tests if conceptually, structurally or modularly similar mammalian signals also exist. As to the second question, it is like questioning the deep sequencing of the gut microbiome or of a deep sea sampling of microbes – since most of the microbes cannot be cultured, what is the point? In fact, however, these deep sequencing projects have led to enormous interest and have shown great potential to develop novel and essential advances.

3. Lack of experimental detail, feasibility difficult to assess

In each case, the experimental design is depicted pictorially as to what is being tested and how. As to outcomes: (i) all of these are extensively studied models for single pathogen infections – thus the assays of infection are very well established, (ii) there is a section devoted to clearly how and what will be evaluated, even though many are well established protocols, (iii) in addition there are proposed new adaptations to these procedures that will be developed during the course of the proposal that would capitalize on newer developments in technologies. The overall goal to study the effect of pre- or concomitant infection of one pathogen on the other that uses these well established assays. In addition, even if the new model involving TEV - *C.elegans* is not successful, there are other backup viral infection systems from previous studies (incorporated into the experimental design) that will be modified to suit the goals

of this study. These alternate infection systems (proposed as backups) have to be modified because they are not in the right format to address the questions of this proposal – though they served to answer the questions those studies were addressing.

In every case I have stated what could pose difficulty and how it will be overcome or circumvented. Since it is an exploratory proposal testing the effect of one pathogen infection on the other under specific conditions, the exact phenotypic outcome cannot be predicted in advance in many cases.

Given the fact that I have published evidence for experience with all aspects of the experimental design, the feasibility of testing the effect of one pathogen on other should not be a question. A reviewer questioned if I have experience with viruses. I have worked with TEV, and other viruses (including TuMV) and their interactions with hosts including a high-throughput Arabidopsis mutant screen for two years in the laboratory of James Carrington (whose lab has carried out bulk of the published work on molecular aspects of TEV over the years). In addition I have established the system in my current location, which accounts to over four years of experience with that system. The relevant information is in the Biosketch and in the publication list. In the case of worm-bacteria interactions, I do not have publications yet, but I have been doing these assays on and off over the last two and half years at the Ausubel laboratory. Evidence to this effect can be inferred from preliminary experiments included in the application. People from various parts of the world come to this laboratory for a couple of weeks to a couple of months to learn worm pathogen assays and go back and establish the system in their own laboratories, evidence that this is not a daunting task.

Another example of the referees' failure to read the proposal carefully is their questioning whether TEV-GUS infects *C. elegans* -- a question that is being addressed by the proposal. The rate of whole or significant parts of the worm showing GUS activity (controls don't under these conditions) is 5-10%. 'Mutants not named in figure legends' is a factually incorrect statement. The only relevant mutant to be named in any of the figures is *rde-1* in Fig. 5 and it is mentioned. Adding specific mutant names in the other figures that depict appropriate mutant genotypes to be included in the experimental design do not serve any purpose at this stage of the proposal. The only study related to the proposal that was published between the two submissions of this application came to a conclusion preinfection with TuMV makes the host more susceptible to a bacterial challenge. I explicitly state that TuMV causes extensive phenotypes, though not apparent at that time point used in that study (an example can be seen in my published article that has both these viral infections) and that TEV is unlike TuMV. The reviewer again questioned that no visual symptom does not mean extensive physiological compromise. A lot of evidence including my extensive qualitative study clearly indicate infection of Arabidopsis with TEV does not have any such drastic effect (including subsequent growth and development or qualitative yield of progeny seeds). Such non-drastic changes in host physiology prevailing during

subsequent challenge is likely to be more relevant than otherwise, and makes this a very attractive model.

I would appreciate appropriate remedial action in this issue and getting my application funded.

Sincerely,

Suresh

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