
RECOMBINANT DNA ADVISORY COMMITTEE

Minutes of Meeting

December 4, 2015

**U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
National Institutes of Health**

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[Note: The latest Human Gene Transfer Protocol List can be found on the Office of Biotechnology Activities website at <http://osp.od.nih.gov/office-biotechnology-activities>.]

**U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
NATIONAL INSTITUTES OF HEALTH
RECOMBINANT DNA ADVISORY COMMITTEE
Minutes of Meeting¹**

December 4, 2015

The Recombinant DNA Advisory Committee (RAC) convened for its 144th meeting at 8:30 a.m. on December 4, 2015, at the National Institutes of Health (NIH), Building 35, Conference Room 620/630, Bethesda, Maryland. Dr. Hans-Peter Kiem (RAC Chair) presided. In accordance with Public Law 92-463, the meeting was open to the public from 8:30 a.m. until 4:20 p.m. on December 4, 2015. The following individuals were present, either in person or by teleconference, for all or part of the December 2015 RAC meeting.

Committee Members

Michael Atkins, Georgetown University School of Medicine
Paula Cannon, University of Southern California
Saswati Chatterjee, City of Hope National Medical Center
Mildred Cho, Stanford University School of Medicine
William Curry, Harvard Medical School
David DiGiusto, City of Hope National Medical Center
Kevin Donahue, University of Massachusetts Medical School
Rebecca Dresser, Washington University School of Medicine
Marie-Louise Hammarskjöld, University of Virginia School of Medicine
Angelica Hardison, Augusta University
Patrick Hearing, Stony Brook University
Howard Kaufman, Robert Wood Johnson Medical School, Rutgers, The State University of New Jersey
Hans-Peter Kiem (RAC Chair), University of Washington School of Medicine/Fred Hutchinson Cancer Research Center
Dean Lee, University of Texas MD Anderson Cancer Center
Douglas McCarty, Ohio State University College of Medicine
Joseph Pilewski, University of Pittsburgh
Richard Whitley, University of Alabama at Birmingham School of Medicine (*via teleconference*)
Dawn Wooley, Wright State University
Laurie Zoloth, Feinberg School of Medicine, Northwestern University

NIH Office of Biotechnology Activities (OBA)

Lyric Jorgenson, Office of the Director (OD), NIH

Nonvoting Agency Representatives

Lisa Buchanan, Office for Human Research Protection, NIH
Denise Gavin, U.S. Food and Drug Administration (FDA)
Carrie Wolinetz, Office of Science Policy, NIH

NIH/OD/OBA Staff Members

Linda Gargiulo
Morad Hassani
Robert Jambou
Maureen Montgomery

¹ The Recombinant DNA Advisory Committee is advisory to the NIH, and its recommendations should not be considered final or accepted. The Office of Biotechnology Activities should be consulted for NIH policy on specific issues.

Marina O'Reilly
Eugene Rosenthal
Aparna Singh

Attendees

There were 52 attendees at this 1-day RAC meeting.

Attachments

Attachment I contains a list of RAC members, nonvoting agency and liaison representatives, and attendees present for the bioethics discussions. Attachment II contains a list of public attendees. Attachment III contains a list of abbreviations and acronyms used in this document.

I. Call to Order and Opening Remarks

Dr. Kiem, the RAC Chair, called the meeting to order at 8:30 a.m. on December 4, 2015. Notice of this meeting under the *NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules (NIH Guidelines)* was published in the *Federal Register* on November 23, 2015 (80 FR 72978). Issues addressed by the RAC at this meeting included a report from the Gene Transfer Safety Assessment Board (GTSAB, a subcommittee of the RAC), public review and discussion of three gene transfer protocols, and public review and discussion of biosafety issues involving a proposal to transfer chloramphenicol resistance to several *Rickettsia* species and a request to lower containment level for in vitro subculture work involving an Ebola virus construct.

RAC members introduced themselves by name, affiliation, and research interests.

Dr. Jorgenson reminded RAC members of the rules of conduct that apply to them as Special Government Employees, read into the record the conflict-of-interest statement, and suggested that related questions be addressed to the OBA committee management officer.

II. Minutes of RAC Meeting, June 9, 2015

RAC Reviewers: Drs. Curry and Wooley

Dr. Wooley commented that the minutes reflected the Committee's discussion, with a few minor changes that were previously provided. No other comments or changes to the minutes were suggested by Dr. Curry or other RAC members.

A. Committee Motion 1

Dr. Kiem asked the RAC to approve the minutes of the June 9, 2015, RAC meeting. The RAC voted unanimously by voice to do so.

III. Minutes of RAC Meeting, June 10, 2015

RAC Reviewers: Drs. Atkins and Kaufman

The reviewers found the minutes to be accurate, with some previously made clarifications. No other comments or changes to the minutes were suggested by other RAC members.

A. Committee Motion 2

Dr. Kiem suggested that the RAC approve the minutes of the June 10, 2015, RAC meeting. The RAC voted unanimously by voice to do so.

IV. Minutes of RAC Meeting, September 9, 2015

RAC Reviewers: Drs. Donahue and Hearing

The reviewers found the minutes to be accurate. No other comments or changes to the minutes were suggested by other RAC members.

A. Committee Motion 3

Dr. Kiem suggested that the RAC approve the minutes of the September 9, 2015, RAC meeting. The RAC voted unanimously by voice to do so.

V. Review and Discussion of Human Gene Transfer Protocol #1510-1468: A Phase I, Multicenter, Open-Label, Single-Dose, Dose-Ranging Study to Assess the Safety and Tolerability of SB-318, a rAAV2/6-based Gene Therapy in Subjects with Mucopolysaccharidosis I (MPS I)

Presenters: Chester Whitley, M.D., Ph.D., University of Minnesota Medical School
Thomas Wechsler, Ph.D., Sangamo BioSciences, Inc.
R. Scott McIvor, Ph.D., University of Minnesota College of Biological Sciences

Sponsor: Sangamo BioSciences, Inc.

RAC Reviewers: Drs. Chatterjee, Hammarskjöld, and Ross

Drs. Cannon and Kiem were recused from consideration of this protocol due to conflicts of interest. As a result of Dr. Kiem's recusal, Dr. Chatterjee chaired this section of the December 2015 RAC meeting.

A. Protocol Summary

Mucopolysaccharidosis type I (MPS I), also known as Hurler or Hurler-Scheie syndrome, is a rare genetic lysosomal storage disorder caused by an autosomal recessive inherited deficiency of the enzyme, α -L-iduronidase (IDUA). The disease pathology is attributed to the loss of function of IDUA, which is required for the lysosomal catabolism of the complex polysaccharides heparan sulfate and dermatan sulfate. These polysaccharides, called glycosaminoglycans (GAGs), accumulate in the tissue of MPS I patients, resulting in characteristic storage lesions and diverse disease sequelae. Progressive accumulation of GAGs leads to widespread organ dysfunction.

Clinical severity depends upon the nature of the mutational changes and the degree of residual enzyme activity. Affected individuals may develop organomegaly, corneal clouding, joint stiffness and skeletal deformities (including abnormal spinal bones), hearing loss, and hernias. Patients with more severe forms of MPS I develop symptoms related to GAG storage in the central nervous system (CNS), which may include hydrocephalus, spinal cord compression, and cognitive impairment. Symptoms of developmental delay typically present before age 1 in these patients; halted growth and progressive mental decline are evident by ages 2 through 4. The life expectancy of patients with the severe form of MPS I is less than 10 years. Patients with attenuated forms of MPS I share most of these clinical manifestations but have no CNS involvement and do not suffer from mental retardation.

There currently is no cure for MPS I. Treatments exist for the systemic effects of MPS I, including enzyme replacement therapy (ERT) and hematopoietic stem cell transplant (HSCT), but these interventions have limited effect on the neurological symptoms associated with MPS I. HSCT can have a delayed effect on

the CNS, but survival rates for Hurler patients have been reported to vary between 50 percent and 85 percent, and engraftment failure is frequently reported. Intravenous (IV) ERT does not cross the blood–brain barrier and therefore has limited potential to treat CNS symptoms.

The proposed study will use a genetic editing technology to disrupt the endogenous albumin gene in hepatocytes cells using zinc finger nucleases (ZFNs), which causes a double-strand DNA break and subsequent repair by the natural DNA repair mechanisms of the cell to insert α -L-iduronidase complementary DNA (cDNA) by homologous recombination. The trial is a multicenter Phase I, open-label, dose-ranging study to assess the safety and tolerability of a single intravenous infusion of a gene therapy product, SB-318. SB-318 is composed of a set of three adeno-associated virus 6 (AAV6) vectors; two that encode the left and right halves of the ZFN and a third that encodes a promoter-less IDUA donor. Two vectors encode a nuclease that is designed to specifically “cut” the human albumin gene; a third vector contains an MPS I transgene, which is designed for insertion into the “cut” albumin gene. If successful, this approach could become a treatment for systemic MPS I. Up to nine research participants will be enrolled in the trial, with the possibility of expanding to 12 research participants. Two participants will be enrolled into each of three dose cohorts, and up to five research participants will be enrolled in the maximal tolerated dose (MTD) cohort. Enrollment within a cohort may be increased to four research participants if a Grade 3 adverse event (AE) related to the study drug occurs in one of the first two research participants. A safety monitoring committee (SMC) will meet 1 month after the last dose in each cohort, before dose escalation. Enrollment will be limited to adults with MPS I (Hurler, Hurler-Scheie, and Scheie variants) who are age 18 and older.

B. Written Reviews by RAC Members

Five RAC members voted for in-depth review and public discussion of this protocol. The trial was found to warrant public review both because it involves first-in-human use of this technology to insert the α -L-iduronidase gene into the genome for the treatment of MPS I and because of the risks of off-target effects given the novelty of the technology.

Three RAC members provided written reviews of this proposed Phase I trial. Dr. Chatterjee read Dr. Ross’s comments into the record during the meeting.

Dr. Chatterjee had the following specific comments and questions about the protocol:

- What is the frequency of insertions of the IDUA cDNA into intron 1 of albumin? What fraction of insertion events are inverted terminal repeat (ITR) mediated? Data presented on on-target analysis showed an insertion rate of 5.1 percent to 30.5 percent in human primary hepatocytes. How many of those events included ITRs?
- ITR-mediated non-homologous end joining (NHEJ)–based integration will result in a duplication of the homology arm regions. What is the effect of this sequence duplication on the stability of the insert? Does the presence of ITRs interfere with transcription?
- The protocol and Appendix M indicate a decline in the plasma IDUA levels after 2 to 3 weeks. If the IDUA cDNA is stably inserted into the albumin locus in the liver and there is no toxicity, what is the basis for this decline?
- Data provided on editing is measured only by the frequency of insertion or deletion mutations (indels). Could similar data be provided for insertions? The frequency of indels is being used as a surrogate for insertions. Are there data to support this?
- What are the reasons underlying the fairly large (0.5 log to 1 log) difference in levels of editing observed between animals?
- The major criterion is the plasma level of IDUA. Was it determined that all of the expression observed originates solely from the albumin locus? Are there any data demonstrating co-transcription of IDUA with albumin?
- Regarding the specificity of ZFN expression, do the locus control region (LCR) and promoter used show evidence of ectopic expression? In what tissues other than the liver is the albumin locus accessible for editing? Is editing observed in such locations?

- In the study querying candidate off-target site modification with SB-318 ZFNs via next-generation sequencing (NGS), was this a direct NGS without prior amplification or linear amplification-mediated (LAM) polymerase chain reaction (PCR)?
- In the unbiased on-target analysis where the insertion was queried genome-wide, how was interference from the large amounts of episomal vector genomes dealt with? Presumably at multiplicities of infection ranging from 1×10^5 to more than 1×10^6 , there would be many unintegrated AAV genomes that may reduce the sensitivity of the assay.
- Were any in vivo effects seen long term from off-target cutting at the Smchd1 locus?

Dr. Chatterjee noted the following regarding Appendix M:

- The Appendix states, “In addition to the MiSeq data, genotyping data and TaqMan analysis confirmed integration and active transcription of the hIDUA donor at the cynomolgus monkey albumin locus.” What is the level of observed integration and active transcription? What is the proportion of IDUA transcription from intron 1 relative to wild-type albumin?
- Table 4 summarizes key in vitro studies of SB-318 using hepatoma cells. The table appears to be missing, however, and needs to be provided.
- Why are multiplicities of infection ranging up to 1.2×10^6 vg/cell required for the observation of 29 percent indels? Does AAV6 not transduce HepG2 cells and hepatocytes efficiently?
- The Appendix states that sequences that mapped to an incorrect locus were removed from further analysis. There is no information as to whether these were off-target sites.

Dr. Hammarskjöld considered the protocol to be interesting and well presented for the assessment of the effects of SB-318. This is the first proposed trial for the novel gene editing technology in MPS I, but another protocol using the same ZFN to insert a different transgene into the same locus was recently reviewed by the RAC. Thus, some of the potential risks (e.g., potential off-target effects of the nucleases) are likely to remain the same, whereas potential side effects specific to the transgene may differ. If this strategy is found to be safe and effective, it could represent a significant step forward and be used for many chronic diseases, potentially allowing long-term benefits after a single administration.

Despite the apparent promise of this approach, there are still potential known and unknown risks, since there are no data from human genome editing studies. Although some promising data have been obtained in animal models, these have obvious limitations, partially because different nuclease constructs have to be used. Furthermore, AAV vectors are known to behave differently in different species.

In several places in the proposal, the investigators point out the limitations of the current ERT and the fact that it is not “curative.” Specifically, ERT does not show any neurocognitive benefits, since the enzyme does not cross the blood–brain barrier. Given this background, Dr. Hammarskjöld asked whether the investigators believe that SB-318 may provide neurocognitive benefits. If so, why would this be different from the current ERT?

Per the protocol, “Most MPS I patients (97 percent) receiving Aldurazyme develop IgG antibodies to alpha-L-iduronidase. However, this is a polymorphic variant of alpha-L-iduronidase that is produced in Chinese Hamster Ovary cells. Therefore, it is unknown if antibodies will develop against a protein with a glycosylation pattern derived from human hepatocytes.” Dr. Hammarskjöld raised several questions about the potential for patients to develop an immune response against IDUA, including the following:

- How will previous enzyme replacement therapy affect enrollment? It appears that research participants will be enrolled regardless of previous ERT and can remain on ERT after the gene therapy.
- If this is correct, will research participants with previous ERT exposure be tested for antibody (Ab) responses before enrollment? Will previous evidence of immune responses affect the ability to be included in this trial?
- The protocol does not appear to explicitly address potential immune responses to the IDUA transgene. Will this be monitored? If so, will both antibody and potential cellular immune responses directed against the transgene be analyzed?

- If research participants are already on ERT, how will the effect of the gene therapy on enzyme levels be assessed?

Similar to Dr. Chatterjee, Dr. Hammarskjöld had comments and questions regarding potential off-target effects of the investigational product. Per the protocol, an “unbiased integration site assay” yielded a candidate list of 49 candidate cleavage sites, with the top ranked locus being the intended target site in the intron 1 of the albumin gene. A follow-up indel analysis performed in hepatocytes revealed significant modification at the albumin locus (36.8 percent on-target) and 2.4 percent at one of the off-target sites (in exon 38 of the Smchd1 gene). Smchd1 has been linked to chromosome X-inactivation, DNA repair, facioscapulohumeral dystrophy, and tumor suppression.

The investigators note that most of these functions would not be likely to be significantly affected or cause problems in this protocol and that because “...SB-318 will exclusively target the liver in patients with a normal cancer risk profile, no increased cancer risk is expected”. The potential effects on tumor suppression remains a concern (in spite of the fact that mouse studies show only cancer in “pre-disposed mice), however. Cancer risk is affected by a number of factors throughout life, and the proposed research involves a potentially life-long genome modification that could be a factor in cancer development at any point in life. Further, it is not clear how much we can really extrapolate from the mouse studies. Given these issues, Dr. Hammarskjöld asked whether the investigators have a plan for long-term follow-up to monitor the potential development of hepatic cancer.

Regarding Appendix M, Dr. Hammarskjöld inquired about the following issues:

- Has the in vitro soft agar transformation assay to assess potential genotoxicity of ZFN activity been completed? If so, what were the results? Are any other experiments performed to assess the potential genotoxicity of SB-318?
- Per the Appendix, studies where cultured hepatocytes were co-transduced with the AAV2/6 ZFNs and hDUA donor, efficient expression and secretion of active hDUA protein was confirmed in both primary and transformed human hepatocytes. Was any NGS or other RNA analysis performed to look at the mRNA expressed from the modified loci?
- Since assessing hDUA activity and expression levels in plasma and liver in cynomolgus monkeys was not possible due to the high and variable levels of endogenous enzyme, were there any experiments done to look at mRNA expression from the inserted transgene? Dr. Hammarskjöld noted that because the 5’ end in this case originates in the albumin gene, this should be feasible. In addition, this would enable the investigators to determine that the mRNA had the expected sequence and how much of the mRNA originated from loci modified by NHEJ and homology-directed repair (HDR), respectively.

Dr. Hammarskjöld noted that some parts of the informed consent document (ICD) are well written and present material at the appropriate “lay language” level. However, the investigators should consider the following revisions and changes:

- The potential risks should be presented earlier in the document, preferably before the details of the protocol, so that the research participants are informed about the experimental nature and the known and unknown risks “up front.”
- Although the experimental nature of the protocol is explained, the document also calls the investigational product a “drug” and says that the goal of the study is to determine whether it “helps” with MPS I. This is potentially misleading, since SB-318 is not a drug and the goal is to determine whether the protocol is safe. The investigators should consider rephrasing this information to reflect these points.
- The statement that patients may be able to stop taking laronidase appears inappropriate for a Phase I protocol. Maybe this could be changed to say that “there is a possibility that you may be able to stop taking [it].”
- The potential benefits of longer follow-up because of possible long-term risks of off-target editing (although hopefully low) should be mentioned in the section describing the 39-week follow-up period.

- The statement “SB-318 may help your MPS I, [but] there is no guarantee that being in this study will help you” is inappropriate for the current study and should be changed to a statement such as “There is a possibility that SB-318 may help your MPS I.”
- The “Risks associated with immune responses” section should include information about potential immune responses to the transgene.
- The “Potential risk of cancer section talks about the potential risks of AAV vectors but does not mention the fact that this is a “genome editing” protocol with inherent potential off-target effects.

Dr. Ross’s comments and questions, as relayed by Dr. Chatterjee, were as follows:

- The protocol, answers to Appendix M, and consent forms are easy to read. The data and safety monitoring plan is ethically appropriate.
- The study should be referred to as a gene transfer protocol rather than a gene therapy protocol, given that the efficacy of SB-318 is not known.
- Inclusion criterion 4, “A history of reaction to IDUA infusion that the PI feels would contraindicate participation in the study,” appears to be an exclusion criterion.
- Research participants can be encouraged to comply, but if they drop out (and they have the right to do so), they cannot be forced to comply with safety evaluations. Furthermore, the investigators cannot require even a reduced follow-up testing schedule to “enforce” research participant compliance. No additional treatments are being offered after infusion of the test article, only testing. This needs to be made clear in the consent form. In addition, the consent document needs to explain that research participants can drop out of follow-up but not out of the experimental treatment, because it is a one-dose treatment and, once it is done, there is no undoing it.
- An off-target integration site is the *Smchd1* gene, which has been linked to chromosome X inactivation, tumor suppression, DNA damage repair, and facioscapulohumeral muscular dystrophy. These risks need to be added to the ICD.

Additional comments regarding the ICD noted by Dr. Ross included:

- The font type and size change and vary in different places of the document. The document should be revised so that the font is consistent throughout.
- The reason for the risk associated with ERT’s “inability to enter into the brain” should be explained (i.e., because ERT cannot treat neurological symptoms of MPS I). In addition, it may be better to word this statement as “inability of the *medicine* to enter into the brain”.
- References to the patients’ hemophilia appear to have been cut and paste from a different protocol and need to be removed.
- There is no mention of possible off-target integration (particularly of the *Smchd1* gene), which could harm health, in the risks section of the ICD. Even if the investigators are convinced that the risk of integration into the *Smchd1* gene is trivial, off-target integration presents a generic risk of gene transfer experiments and should be mentioned.
- The reason for and meaning of the statement that participants may be given “too much of the study product” are not clear and need to be clearly explained. In addition, any risks associated with this issue need to be specified (e.g., will extra product interfere with albumin?).
- The consent needs to clearly explain that although participants can stop being followed, once the infusion is done, they cannot undo the infusion/intervention. In addition, the investigators need to be careful with respect to statements that the study doctor or staff will tell research participants are any new information that might change participants’ decision about continuing in the study.
- The consent needs to specify whether the study sponsor will pay for the 24-hospital observation period and longer if necessary. If not, this needs to be stated along with whether participants will need to cover this cost.
- The statement, “You have the right to withdraw your sample from additional research studies providing samples are available by contacting the study doctor,” is unclear. The reference to providing samples should be deleted and replaced with language such as, “You have the right to withdraw your sample from additional research studies by contacting the study doctor.” The phrase “in writing” should probably also be added to read: “You have the right to withdraw your sample from additional research studies by contacting the study doctor in writing.”

- The reason for advising participants to talk to a genetics counselor before enrolling in this study is not clear and needs to be provided. In addition, the consent should state who would cover the cost of this counseling.

C. RAC Discussion

During the meeting, the following additional questions, concerns, or issues were raised by RAC members:

- The reviewers found the presentation to be clear and their concerns and questions to be well addressed. They went through their comments and the investigators' responses to their queries and suggestions. Dr. Chatterjee reviewed Dr. Ross's questions and the investigators' responses.
- Dr. Hammarskjöld noted the investigators' point that MPS I is only one of many diseases for which additional therapies are needed to provide affected individuals with a more or less normal life or at least improvement in their life quality. The current treatment, ERT, is limited. The proposed experimental intervention will require several infusions, but if successful, it could eventually replace ERT or possibly serve as an add-on to provide further benefit to the combination of BMT and ERT. Whether any neurological response will develop against IDUA remains to be determined, however. In addition, because most patients on ERT already have antibodies to this enzyme, it could be difficult to tease out the effect of the modified gene product. However, because the enzyme is being made intracellularly with the gene product (in contrast with ERT/transplantation), there is an opportunity for presentation on the cell surface and generation of cellular immune responses (e.g., cytotoxic T-cell response) that wouldn't be seen with the extracellularly delivered enzyme.
- Dr. Hammarskjöld also inquired about risk of liver cancer, given the potential for long-term injury to the liver, and the lifetime cancer risk, given the immune response combined with knocking out tumor suppression (e.g., via mutation of the *Smchd1* gene).
- The investigators were encouraged to evaluate mRNA expression of the fusion transcript (as done in the two NHP animals) to determine the structure of the product being made from the transgene.
- Dr. Chatterjee inquired about the proportion of the insertions that have ITRs versus those that do not and how this is measured. She also asked about the effect of duplication of intron sequences in the homology arms on stability of the insertion and on the presence of ITRs and the impact of ITRs in unspliced sequences. Continuing with this focus, Dr. Chatterjee questioned why, if the insertion is stable and there is no toxicity, in vivo studies in mice show a decline in the level of the transgene product.
- The percentage of indels increases over time (e.g., from about 25 percent at day 28 to about 50 percent at day 60), but the reason for this change is not clearly delineated. Dr. Chatterjee asked if this increase might be due to continuous transduction or perhaps because the ZFN activity proceeds slowly. She also inquired as to whether percent-indels is a valid surrogate for donor insertions, whether the relatively large level of variability in editing across species might be related to the leakiness of the LCR that controls ZFN expression, and how the threshold for detection of off-site background activity is set.
- Dr. Chatterjee asked about the accuracy of the oligonucleotide-based assay to reflect delivery via an AAV vector and whether the investigators have investigated the availability of the ZFNs is different when provided by an AAV vector versus mRNA transfection.
- Dr. Zoloth inquired about the age of the animals at sacrifice for the various studies conducted to date and the parameters used to assess efficacy and adverse outcomes in long-term studies. A better understanding of the effects of the gene editing over the course of the lifetime of the animal models is needed to inform plans for human use.
- The data indicate that in addition to neutralizing antibodies, antibodies to the enzyme are elicited, suggesting the potential for adverse effects of the antibodies. High antibody titers are included in the exclusion criteria, but it is not clear how titers will be determined. Dr. Lee noted that the antigen-antibody complexes are highly restrictive from the blood-brain barrier, which is the primary area for treatment. The complexes increase excretion of the investigational product and decrease its half-life. While antibodies to the virus are checked at multiple time points over the course of the trial, it does not appear that the antibodies to the enzyme are systematically

monitored again as part of the secondary enzyme correlate studies. The protocol should specify how antibody titers will be measured, and the consent document should include the potential risks associated with development of antibodies to the therapeutic transgene.

- Dr. Curry asked which markers were checked to evaluate off-target effects resulting from mutation of the *Smchd1* gene and whether these markers will be useful for the planned liver biopsies.

D. Investigator Response

1. Written Responses to RAC Reviews

The molecular assays to quantitatively measure the frequency of targeted integration at the albumin locus are still under development. Preliminary data from an assay that detects NHEJ-mediated targeted integration via the ITRs indicate that up to 4 percent of the albumin alleles have the IDUA cDNA inserted in vivo in non-human primates (NHPs). However, further assay development is necessary to confirm these numbers. Other less quantitative PCR-based assays indicate that the majority of integration events are mediated by NHEJ via the ITR sequences rather than HDR. The numbers mentioned in the Appendix M experiments (5.1 percent and 30.5 percent) reflect the percentage of albumin alleles that contain insertion and deletion mutations (percent indels), which are generated by error-prone DNA repair after ZFN cleavage. This experiment was designed to assess off-target activity (measured by percent indels) by transducing cells with ZFNs only, so no hIDUA cDNA donor template was provided to integrate at the albumin locus. The investigators noted that while it is technically difficult to follow an integrated donor over time, they have followed treated mice for several months and saw stable levels of IDUA expression. Using a similar approach to insert a human Factor IX (FIX) transgene into the albumin locus of treated mice, the team observed stable levels of human FIX protein in the plasma for more than 14 months (60 weeks). Therefore, ITR-mediated integration of the donor appears to be stable.

Clonal cell lines (derived from the human hepatoma cell line HepG2) have been generated to study transcription at the albumin locus after SB-318 treatment. These cell lines have SB-IDUA donors integrated at the albumin locus by either HDR or NHEJ (including ITRs). Consistent with similar data generated for the SB-FIX program (using a cDNA donor for FIX), there was no indication that the ITRs interfere with transcription and expression of the integrated transgene. Cell lines with the hIDUA donor integrated by NHEJ showed the same splice patterns when expressed from the albumin locus as those with the donor integrated by HDR. Two transcripts were observed, the albumin-IDUA fusion and the wild-type albumin transcript expressed from the unmodified allele. Thus, in these cells, both alleles (wild type, hIDUA donor integrated) are expressed, and the complete wild-type albumin mRNA and the predicted albumin-hIDUA fusion mRNA can be detected.

As described above, assay development for the quantification of cDNA donor integration is ongoing. Because ZFN cleavage is required to induce genome editing that results in the generation of indels or targeted integration at the targeted locus, the frequency of indels and targeted integration is expected to be directly linked. For other programs (e.g., SB-FIX), mouse studies using different AAV2/6 doses have demonstrated a clear correlation between the percent indels and the expression (and hence integration) levels of cDNA transgene donors. This has also been found in in vitro studies using human hepatocytes and low and high doses of SB-318 reagents. The percent indels has provided a good surrogate measurement for the frequency of targeted integration.

The decline in hIDUA levels in mice is likely due to a humoral immune response to the human IDUA protein. Human IDUA is known to be highly immunogenic in MPS I mice. Mouse liver hepatocytes analyzed at the end of one study (day 60) show high levels of albumin gene modification (up to 50 percent indels, compared with about 25 percent indels at day 28) that are consistent with previous studies. Furthermore, there was no evidence of liver toxicity (serum chemistry and histopathology evaluations), which suggests that the SB-318 ZFNs are well tolerated and do not induce a cytotoxic T-cell response that would target transgene-expressing hepatocytes. From these results, the reduction in plasma IDUA levels is most consistent with the induction of a humoral response against the human IDUA protein, as previously reported.

The underlying difference in levels of editing observed between animals is likely due to individual animal variability with respect to multiple factors, including dose administration, AAV vector uptake into hepatocytes, AAV vector unpackaging within the cell, ZFN expression and activity, and donor integration. A similar degree of animal-to-animal variability has been observed in previously published NHP studies, using systemic delivery of recombinant AAV vectors to the liver. Although animals were pre-screened for the presence of anti-AAV6 antibodies, some animals may have undetectably low levels of anti-AAV6 antibodies that could reduce hepatocyte exposure to the vector.

Human hDUA expression is anticipated to occur only as a result of integration at the albumin locus. Several key properties of the vectors help ensure the specificity of this reaction. First, the albumin ZFNs are under the control of a hepatocyte-specific promoter, which restricts ZFN expression and activity to the liver. Second, the hDUA donor lacks a promoter and can be expressed only after becoming inserted at the site of ZFN-driven cleavage at the albumin locus. Based on the team's past biodistribution studies in mice and NHPs, AAV serotype 6 vectors primarily transduce the liver in vivo.

In preclinical studies in MPS I mice, one of the control groups was treated with the hDUA donor only. If there was ectopic expression from the promoter-less hDUA cDNA, it would have been detected in that treatment group. Integration of the hDUA donor integrated at a genomic site other than albumin would most likely not result in productive expression and secretion of hDUA, because the transgene lacks the hDUA pro-peptide and would require both the signal and pro-peptide encoded by albumin exon 1. To study transcription at the albumin locus, the research team has generated clonal cell lines (derived from the human hepatoma cell line HepG2) using SB-318. These cell lines carry one albumin allele that is wild type (or has a small indel as evidence for ZFN activity) and one albumin allele with an hDUA donor integrated by either HDR or NHEJ (with ITRs). In these cells, both alleles are expressed and the complete wild-type albumin messenger RNA (mRNA) and the predicted albumin-hDUA fusion mRNA have been detected.

Experiments have been done to evaluate mRNA expression from the inserted hDUA donor. The quantitative hDUA donor integration assay is still in development, and the level of donor integration, therefore, was not determined in the cynomolgus monkey study. However, integration of the donor at the albumin locus was confirmed by using non-quantitative PCR assays. By using quantitative reverse transcription polymerase chain reaction (RT-PCR), the level of albumin-hDUA transcription was measured in relation to the housekeeping gene ribonuclease P protein subunit p30 (RPP30). This analysis confirmed robust expression of hDUA mRNA in the two NHP study animals with the highest levels of gene modification (percent indels) at the albumin locus. The albumin-hDUA fusion mRNA from these monkeys was not reverse transcribed and sequenced. However, previously described studies using HepG2 derived clonal cell lines showed the expected hDUA mRNA sequence in stably transduced cells. The proportion of hDUA transcription from intron 1 relative to wild-type albumin has not been assessed.

The ZFN constructs contain a hepatocyte-specific promoter, and ZFN expression and activity are expected to be restricted to the liver. The hDUA donor lacks a promoter and can be expressed only after being inserted into the ZFN-induced double-strand break site at the albumin locus. The 6-month GLP mouse pharmacology, biodistribution, and toxicology study was used to support the SB-FIX program, which utilizes the same albumin "safe harbor" approach and recombinant AAV2/6 vector system to express human FIX from liver. Several non-targeted tissues (e.g., spleen, heart, testes) in addition to the target tissue liver were analyzed for evidence of ZFN expression by NGS/MiSeq deep sequencing. At 6 months, high peak levels of gene modification (30 percent to 48 percent indels) were seen in liver, with no evidence for ZFN activity in other tissues. Thus, editing was not observed in any non-target tissues. A similar 6-month GLP mouse study is ongoing for the SB-318 program.

The wording in the NGS/MiSeq Method section describes the removal of raw sequences that do not map to the target genome (e.g., human genome version hg19 or 38), which are discarded as artifacts. This passage does not specifically refer to discarding off-target sites; rather, it refers to discarding random, artifactual DNA sequences from the analysis.

High MOIs are required to transduce cells in vitro due to unknown factors, and each cell type will have an empirically defined optimum MOI. AAV2/6 thus does not transduce HepG2 cells and hepatocytes at similar MOIs. The investigators have evaluated several in vitro human hepatocyte systems (including HepG2 cells) by transducing these cells with an AAV2/6 GFP or AAV2/6 ZFN/cDNA donor vectors. Although there are some differences between these cells, they all show dose-dependent behavior but require fairly high MOIs for efficient transduction (1×10^4 to 1×10^6 vg/cell). This makes HepG2 cells useful for proof-of-concept studies, off-target evaluation, and the generation of clonal cell lines carrying an integrated hIDUA donor at the albumin locus.

The sponsor clarified that the validation of off-target sites in step 2 of the oligo integration assay in human hepatocytes was by direct NGS without prior amplification or LAM PCR. LAM PCR was used only in step 1 for the identification (not the quantification) of the integration sites of the oligo. The first step of the genome-wide off-target search in K562 cells does not use AAV2/6 vectors. Rather, in this step, the SB-318 ZFNs are delivered as mRNA and co-transfected together with an oligonucleotide that integrates into the sites of double strand-break formation, which provides a molecular tag for identifying the sites of ZFN cleavage. The sites of oligo integration are then identified by LAM-PCR. In the second step, human hepatocytes are transduced with SB-318 ZFNs (AAV2/6) without a hIDUA donor. The ZFNs encoding AAV2/6 vectors carry no sequence homology to the on-target albumin locus or any off-target loci; no interference caused by these episomal vectors in the NGS analysis has been observed. Further details and a flow chart with graphics of this multi-step process were provided in the written response.

There were no long-term in vivo effects in mice or cynomolgus monkeys considered related to administration of AAV2/6 ZFN and/or hIDUA donor vectors. The long-term (3- to 6-month) endpoints evaluated included clinical observations, food consumption, body weights, clinical chemistry, hematology, coagulation, and gross and microscopic pathology. In vitro studies showed that NHP surrogate albumin ZFN components used in the cynomolgus monkey study have low off-target activity (0.1 percent) at the Smchd1 locus at high on-target activity (>25 percent) in hepatocytes. Liver tissue collected at 3 months following dosing in the NHP study showed no off-target activity at the Smchd1 locus.

No data to date suggest that SB-318 may provide neurocognitive benefits. However, organ systems (e.g., bone, pulmonary) may improve substantially with a constant level of IDUA in the circulation. Weekly infusions (6 hours/week) provide tissue-saturable levels of IDUA in the circulation. Thus, there is considerable potential improvement in height, mobility, and breathing functions that could be obtained with a long-term expression of IDUA from the liver. ERT is limited in many respects, requiring weekly infusions with a significant time commitment by the patient (e.g., 4 hours/week), constant risk of infusion reactions, and an intrinsic limitation in the maximal amount of drug that can be delivered (i.e., 0.58 mg drug/kg/week). In addition, at a minimum of \$100,000/year, the cost of lifetime treatment is prohibitive. One-time treatment with SB-318 offers significant improvements over existing ERT.

Regarding the potential development of antibodies to IDUA and eligibility criteria, the sponsor noted the following:

- Research participants with concurrent ERT therapy or bone marrow transplant (BMT) will be eligible to enroll. There is considerable room for improvement in joint, cardiac, and breathing function and mobility (walking) that can still occur, and for comparing the pharmacokinetics (PK) of weekly IV infusions of laronidase and the potential constant levels provided by gene expression in the liver.
- Since almost all research participants on ERT therapy have antibodies to IDUA and these antibodies do not seem to affect the clinical response, the investigators do not plan to exclude research participants with pre-existing antibodies to IDUA. In the very rare cases in which patients develop a neutralizing antibody against an ERT or other infusible protein (e.g., FIX), the widely accepted remedy is to provide constant or frequent infusions of the protein to induce tolerance. Thus, it is very likely that constant production of α -L-iduronidase from SB-318 will induce tolerance and/or minimize the induction of neutralizing antibodies.
- The standard FDA-approved (label) dose of laronidase (Aldurazyme) is 0.58 mg/kg, administered once-weekly as an intravenous infusion. According to the label, "50 of 55 patients (91 percent) treated with Aldurazyme were positive for antibodies to laronidase. The clinical significance of

antibodies to laronidase is not known, including the potential for product neutralization.” The team’s experience is consistent with this assessment. The development of neutralizing antibodies has been reported, but these are exceedingly rare. The standard of care for patients receiving laronidase is to monitor anti-laronidase antibodies. Physicians caring for these research participants will be encouraged to send antibody testing on a regular basis on research participants enrolled in this SB-318 clinical trial.

- A single laboratory designated by the manufacturer-distributor (BioMarin/Genzyme) does this testing. Use of this test is limited by the manufacturer-distributor, and independent laboratories may not be able to obtain the necessary laronidase reagent. Those developing new therapies of this type often develop such assays late in the FDA review process or in the post-marketing phase. The investigators are unaware of any method of assessing a cellular immune response in the few minute specimens of liver obtained in this study, but they are open to considering approaches suggested by the committee.
- The investigators plan to measure trough leukocyte α -L-iduronidase levels, as well as levels in liver biopsy, and compare levels after administration of study agent to baseline levels. In addition, the levels of urinary and liver GAG will be measured for comparison to baseline to evaluate for differences post IDUA gene transfer. In future long-term studies, more direct clinical responses will be measured (e.g., 6-minute walk, cardiac function, pulmonary function).

Long-term follow-up to monitor for potential development of hepatic cancer will include evaluation of the liver by liver function tests; liver biopsy at baseline, 6 months, and 1 year; and MRI of the liver and spleen at baseline 3 months, 6 months, and 1 year. Alpha-fetoprotein will be added to this evaluation and measured every 6 months for the next 3 years of the study, as will MRIs. After completion of this study, a separate long-term follow-up study will be designed with the PI, based on the safety and efficacy data obtained and submitted to the FDA.

The GLP soft agar transformation assay using human fibroblast cells transfected with ZFN mRNA has been completed, and the test results were negative for colony formation in soft agar. There are no other formal studies to assess the potential genotoxicity of SB- 318, but additional in vitro studies are available to characterize the levels of ZFN on- and off-target modification in SB-318 transduced cells.

References to Table 4 in Appendix M should have been deleted when the table was removed for confidentiality reasons.

Inclusion criterion 4, regarding history of reaction to IDUA infusion, is an exclusion criterion and will be changed accordingly.

The investigators and sponsor agreed with the recommendations regarding the ICD and will modify the proposed ICD accordingly.

2. Responses to RAC Discussion Questions

The investigators noted that short-term studies in mice have been conducted and that a longer-term study is underway. Most normal mice live about 2 years. In contrast, the typical untreated MPS 1 mouse survives about 1 year. Animals sacrificed at 1 year are considered “middle aged.” The longer-term studies will address efficacy and safety over an extended period. Some preliminary findings indicate that disease models live longer with treatment. Work in MPS 1 and also in hemophilia (as presented at the September 2015 RAC meeting) involves monitoring animals for more than a year to assess stability, transgene expression, and safety including identifying any toxicities or other effects that might be caused by the genome editing.

The consequences of providing a normal enzyme to an enzyme-deficient patient are of great interest. Dr. (Chester) Whitley summarized the history and experience gained with ERT since the first FDA approval in 1991. He noted that there is considerable potential for assessing the effects of antibodies to the enzyme, but that to date, few problems have been identified. Most patients receiving lysosomal ERT develop some level of antibody against that drug. For the vast majority of cases, the antibody against the therapeutic

enzyme is not a neutralizing antibody, and the Ab level remains low and constant. In other cases, such as with Pompe disease where much higher doses of the vector are administered, patients develop neutralizing antibodies, which makes the treatment useless. In one case, the team was able to tolerize the patient and allow him to receive an effective treatment by giving him small daily doses of enzyme. Participants on ERT will be eligible to enroll in the protocol. Once they start the gene transfer intervention, however, they will be off ERT if they have had a transplant, from which non-modified IDUA would have been produced. While it is not difficult to measure antibodies against the enzyme, the caveat is that there may be subtle differences between the enzyme administered as a licensed made drug and the enzyme expressed following administration of AAV. The clinical efficacy of the proposed agent will be assessed in part through measurement of urine GAGs and results of liver biopsies. If the patient has had low urine GAG levels (i.e., a therapeutic response) and then those levels go up, this indicates that the enzyme is not working. This would occur if the cell that is making the enzyme has been destroyed or if there is an antibody against the enzyme.

The FDA currently does not require long-term antibody testing. The researchers do monitor antibody titer levels, however, and use a commercial assay for this purpose. Interpretation of results can be challenging. Further assessment may be warranted if a patient has high titers, but the investigators are careful to not overstate the clinical meaning of such findings. Information about the assay used for antibody testing will be added to the protocol. The consent will include the risks of antibodies to the transgene and the enzyme. Dr. Whitley noted that the investigators will consult with Sangamo on developing a method for measuring antibody to the modified enzyme.

Plasma, WBC, and liver levels of the enzyme will be measured at baseline and then subsequently at various intervals over the course of the study. Because of the way modified enzyme presumably is being made and functions there is no transgene expression or labeling outside of the cell surface. As a result, a T-cell response is not expected. In other systems in which cDNA for IDUA is expressed, if the cells are destroyed, enzyme activity falls off. Liver biopsies in these cases similarly show an absence of a T-cell response once the cells are destroyed. Thus, even with liver biopsies, it would be difficult to detect expression of and an immune response to the modified enzyme. In contrast, immune response to the AAV vector would be easier to monitor.

Long-term follow-up will be done to monitor for cancer and other potential on- and off-target effects. Given a possible integration event, extended follow-up will be particularly important because all potential risks and outcomes are not known. Only small amounts of tissue will be needed for analysis of genomic DNA or mRNA isolations, but detection of such events (i.e., unexpected rearrangements or deletions) will depend on how much material can be obtained from the liver biopsies. Increased risk of liver damage and liver cancer is not expected given the small number of cells that will be transduced and the regenerative capacity of the liver. Very little liver damage (including fibrosis) has been seen in animal models given the construct; in addition, the agent has not been shown to be oncogenic. Dr. Whitley noted that a clinical feature of MPS 1 is some underlying fibrosis and that with ERT and BMT, the fibrosis tends to improve. For the planned study, the investigators anticipate that there would be either immediate tolerization and/or that the transduced cells will be destroyed, thereby reducing risks of injury to the liver.

Determining the proportion of insertions with and without ITRs is difficult to measure using PCR due to the repetitive nature of the integration events, which, in turn, has hampered efforts thus far in developing quantitative assays, including those using Taqman or digital droplet PCR. The finding of more end joining-mediated integrations is based primarily on radioactive PCR, which has a lower number of cycles but has good detection due to the radiolabeling; per this assay, the stronger bands are seen for NHEJ-mediated insertions. Better quantitative assays are needed, however, and other tests are being evaluated. Preliminary data from the SB-FIX program indicate that about 1–4 percent of the albumin alleles have FIX cDNA inserted in a monkey that had about 8-9 percent indels, suggesting a very high level of gene modification in that animal. The 1:10 ratio reflects ten times more indels versus donor integration in other assays under development. The ratio of HDR to NHEJ is much more difficult to estimate.

The end joining integration mechanism does not interfere with transcription, based on analysis of the clones and subclones, which have gone through many cell divisions with the donor component being

integrated. The insert is still present in its natural form in these subclones, from which the entire integrated end joining mediated integrated donor can be sequenced, suggesting that the insert is stable, not unstable. The same clone has been followed over time, and stable expression has been observed through 1 year in mouse models. Transcription of the locus has been good, despite integration. The investigators have not looked at different splicing patterns, but they have not found any other transcripts than the two cited earlier (i.e., the albumin-IDUA fusion and the wild-type albumin transcript expressed from the unmodified allele). Thus, based on the analyses done to date, there does not appear to be any mis-splicing at the target locus. The team has not done any isolation of unspliced mRNA, which involves different methodology.

Regarding the decline in the transgene product (in animals), Dr. McIvor noted that the data presented are from studies conducted in immunocompetent wild-type mice and that there most likely was an immune response prior to the decrease in the level of expression. Studies in immunosuppressed mice are still ongoing. The increase in the percent-indels probably reflects persistence of the AAV vector for a certain amount of time. After the ZFNs are expressed and the cut occurs, the target site can be repaired error-free or error-prone. It likely takes time to accumulate indels, and the persistence of ZFN expression may account for the reported increase in indels.

The team has not checked the IDUA cDNA number to assess stability or whether that sequence is being cut out of the genome. The only indication that the transduced cells are not lost is that the indels are stable or increase over time. Whether an immune response occurs as a result of antibodies to the modified enzyme or loss of the transgene is still in question.

In response to whether indels is a valid surrogate for donor insertion, Dr. Wechsler pointed out that in dose titration studies for other programs (e.g., the GLP tox study for Factor IX), there is a good, dose-dependent correlation between indel formation in the mice being tested and expression of FIX. The tight correlation is likely because the DNA repair mechanisms are fixed in all of these cells, and, as a result, there is no variability between the cells. The only difference is in the activity of ZFN. A fixed ratio between indel formation and donor integration is assumed, and sufficient data exist to support indel formations as good indicators of integration. Indel formation is being used as a surrogate at this point because of technical limitations with PCR. Once the technical issues with deep sequencing are resolved, it will be much easier to determine the integration rate directly and on a more quantitative basis.

Extensive biodistribution and tissue analysis of FIX in mice failed to produce evidence of indels, with detection levels up to only 0.02 percent. The investigators perform these analyses across studies and in multiple tissues and organs, with a focus on the liver and tissues closest to the liver, and fail to find any activity. Similar results were found in earlier animal studies, indicating that for these different programs (FIX and MPS 1), there is no evidence of leakiness of the LCR that controls ZFN expression.

The study team has compared the AAV integration assay with the oligo capture assay for both the FIX and MPS 1 programs and found that the AAV capture assay is much less efficient and less sensitive for detecting the integration sites than the oligo capture assay. The differences are due mostly to the nature of the ITRs and the few unique sequences in the flanking regions. Thus, it is much easier from a technical standpoint to design an oligo with all of the properties for the preparation of unique PCR primers for sequencing. The investigators use a two-step assay in which the oligo serves only for the identification of those cut sites and confirmation is then done in the relevant primary human hepatocyte system transduced with AAV2/6 (the clinical reagent).

Data suggest that the *Smchd1* gene is involved in the DNA damage response and that *Smchd1* is recruited to the site of DNA damage and that its depletion could alter DNA damage response signaling and cell survival. Potential off-target effects involving the *Smchd1* gene are detected using markers for apoptosis (cleaved Parp1) and DNA damage (Kap-1 phosphorylation). Monitoring for these markers has been done in NHP hepatocytes transduced with AAV2/6 virus. Using these markers in the proposed trial would be difficult, however, because double-strand breaks are repaired fairly quickly and the markers do not persist, liver biopsy would need to be done immediately after the AAV infusions, which is not practical,

especially for patients. In addition, the optimal timing for collection of these markers is not known and would need to be determined by checking levels at different post-infusion time points.

E. Public Comment

No comments from the public were offered.

F. Synopsis of RAC Discussion and RAC Observations and Recommendations

The following observations and recommendations were made during the RAC's in-depth review and public discussion:

- Your data shows that the AAV Inverted Terminal Repeat (ITR) sequences are inserted at the albumin locus. This is one of the first examples of transcription of the ITRs. Since the high GC content and palindromic nature of the ITRs may affect the stability of the transcripts, the effect of inclusion of ITRs in the transcript should be examined in both animal models and human participants.
- An assay should be developed to directly assess the level of AAV genome insertion at the albumin locus, rather than using the frequency of indels as a surrogate.
- As shown in in vitro studies, there is a low level of off-target editing with these constructs. A threshold for detection of potential off-target editing in human participants should be established and justified.
- Although there is a paucity of data relating to the formation of antibodies against and cellular immune responses to α -L-iduronidase that will be expressed from the insertion at the albumin locus, a plan should be developed to monitor for an immune response and to mitigate any untoward effects.
- In addition to indicating that there are potential risks of generating an immune response to the AAV construct that will be administered to deliver the transgene, the informed consent document should indicate that there is a potential risk of an immune response to the enzyme.

G. Committee Motion 4

Dr. Chatterjee summarized the RAC recommendations to be included in the letter to the investigators, expressing the comments and concerns of the RAC. Dr. Hammarskjöld requested a vote, and the RAC approved these summarized recommendations by a vote of 9 in favor, 0 opposed, 0 abstentions, and 2 recusals (Drs. Cannon and Kiem).

VI. Review and Discussion of Human Gene Transfer Protocol #1508-1455: The Effect of Vorinostat and AGS-004 on Persistent HIV-1 Infection (The VOR VAX Study)

Investigators/Presenters: Cynthia Gay, M.D., M.P.H., University of North Carolina at Chapel Hill (UNC-CH) School of Medicine
David Margolis, M.D., UNC-CH School of Medicine

RAC Reviewers: Dr. Hammarskjöld, Ms. Hardison, and Dr. Wooley

A. Protocol Summary

Although many drugs are now available for treatment of HIV/AIDS, none of these provide a cure and no vaccine is available. The main obstacle to a cure appears to be the reservoir of persistent latently infected cells. Testing of potential strategies to eliminate these reservoirs is therefore of great importance. Resting CD4-positive lymphocytes make up the largest and best characterized reservoir of HIV infection that persists despite antiretroviral therapy (ART). One of the latency mechanisms is the recruitment of histone deacetylases (HDACs) to the HIV long terminal repeat (LTR) promoter. The relevance of this mechanism has been validated in the resting CD4-positive T cells of HIV-infected individuals on ART.

The proposed trial will assess the combination of two different treatments given to HIV-infected individuals. The rationale for the study is based on the hypothesis that a combination of an immunotherapeutic agent, AGS-004; and an HDAC inhibitor (HDACi), vorinostat, will result in a depletion of persistent, latent HIV infection. Vorinostat is FDA approved for treatment of certain cancers but remains experimental for HIV. AGS-004 is an investigational agent in which autologous dendritic cells are modified by using four RNA-encoding antigens (Gag, Vpr, Rev, and Nef) from HIV replicating in the research participants prior to initiation of ART. The choice of HIV antigens for AGS-004 is based on data showing that these four elements are immunogenic and may contribute to viral load control. Previous Phase I and Phase II clinical studies with AGS-004 showed some effects on the rebounding of viral replication after ART interruption. An ongoing trial is assessing the effects of AGS-004 in HIV-infected individuals that are suppressed on ART. Although vorinostat and AGS-004 have been studied in individual trials, the proposed study is the first to combine the two agents.

The planned trial is a Phase I single-center pilot study in adult HIV-infected individuals on stable ART with durable viral suppression. Up to 48 individuals will be screened to obtain 12 eligible participants to be enrolled and followed for up to 96 weeks. Participants will take two doses of vorinostat (400 mg/dose), with two to four days separating the doses. AGS-004 will be administered as intradermal injections in the lymph node area; injections will be done every three weeks, for a total of four visits. A total of 1.2×10^{10} cells will be injected at each dosing visit. The investigators will evaluate the association of serial AGS-004 vaccinations and serial vorinostat dosing on the frequency of resting CD4-positive T-cell infection in all participants who demonstrate both an *ex vivo* response and an *in vivo* response to vorinostat and who complete all parts of the study through dosing with both agents (i.e., protocol steps 5 and 6). The team will also explore whether therapeutic immunization that enhances HIV-specific responses can reduce viral expression and the size of the latent viral reservoir in HIV-infected participants on ART. At the end of study (or termination visit), research participants will be entered into a registry for ongoing follow-up. Research participants will be contacted annually for five years to see whether they were diagnosed with any cancers or other events that might be linked to vorinostat.

B. Written Reviews by RAC Members

Nine RAC members voted for in-depth review and public discussion of this protocol. The trial was found to warrant public review because this is the first clinical trial to study the combination of vorinostat and AGS-004.

Three RAC members provided written reviews of this proposed Phase I pilot study.

The reviewers found the study to be well designed and the proposed intervention, the combination of two different agents, vorinostat and AGS-004, to be an interesting and novel approach to address persistent HIV infection.

Dr. Hammarskjöld noted that throughout the protocol, the investigators acknowledge the potential serious side effects of vorinostat. The consent is well written and informative and presents reasonable claims for a Phase I safety trial of this type. The protocol includes ongoing safety monitoring for these risks using several different tests and parameters and identifies several appropriate safety endpoints. In addition, potential research participants will be enrolled in the study only if their resting CD4 cells show a significant induction of resting cell-associated HIV RNA (rca-RNA) in response to vorinostat *ex vivo*. The research participants will continue their ART regimen throughout the study and will be carefully monitored for any increase in viral load (which may potentially reflect “release” of resistant virus from the latent pool, if the patients have been on different previous ART regimens). This approach is different from previous trials of AGS-004 that involved treatment interruptions.

Dr. Hammarskjöld asked whether there will be a time limit as to how long before the start of ART the sample to measure viral load will be obtained and whether sequence information from more than one time point will be considered in the preparation of AGS-004 if the research participants are known to have been infected for a long time before the initiation of ART. These factors might be relevant because of the possibility that the latent pool may represent an archive of viral sequences present throughout the

infection. If there was a long-standing infection prior to ART, there may have been a significant change in the viral antigens in response to immune responses over time.

Dr. Hammarskjöld also requested clarification regarding 'withdrawal' of research participants if their viral load is >150 copies/mL on two consecutive determinations while on active study follow-up. Specifically:

- Does this mean in the period after active dosing or any time during the study?
- Will an HIV sequence analysis be performed if this occurs?
- Could this signify release of "resistant" virus from the latent pool?

Dr. Hammarskjöld had the following additional comments involving the ICD:

- Language in the consent describing how AGS-004 is made ("...from your own dendritic cells [these are a type of white blood cell (WBC)] and your own HIV, in combination with...") is not phrased well and is not correct, since the cells are not made from HIV. The statement should be revised to language such as "...the investigational study treatment AGS-004 consists of your own dendritic cells (this is a type of white blood cell) that have been modified with RNA that contains genetic information from your own HIV virus. This is combined with..."
- The statement "AGS-004 is an investigational therapy that will be made from HIV virus in your blood before you start ART and from your own dendritic cells" is potentially misleading, since HIV virus is not used. Alternative language was suggested: "AGS-004 is an investigational agent that will be made by modifying your own dendritic cells using genetic information (in the form of RNA) from the HIV that was present in your blood before you started ART."

Dr. Wooley noted that the timing of administration of the investigational treatments with respect to each other may be important, as other drugs similar to vorinostat have been shown to decrease T-cell responses, which is not desirable for a vaccine strategy.

Dr. Wooley had the following additional comments and questions regarding the overall proposed strategy for this trial:

- Deep sequencing studies have revealed some insights into the latent HIV reservoir. Overall, seeding of the latent reservoir appears to begin very early, within days after HIV infection. Some recent evidence indicates that if ART is not initiated quickly (within three months of infection), then the latent reservoir becomes dominated by cytotoxic T-cell lymphocyte (CTL) escape mutants. Keeping in mind that the time of infection is often not known, do such findings indicate a limit to the utility of this study, and should the study be limited to those individuals placed on ART within the first three months of infection (when it can be pinpointed)?
- The proposed vaccination method targets HIV antigens thought to elicit relatively good CTL responses, and the proposal states, "Use of autologous viral antigens addresses the inherent problem of HIV extreme genetic diversity when using consensus or reference HIV protein sequences as immunogens." Even with the more modern HIV drug treatments, however, inpatient HIV diversity is still relatively large. Furthermore, a recent study provided evidence that episomal HIV DNA found in peripheral blood mononuclear cells (PBMCs) comes from a cellular or anatomical reservoir that is not detected by proviral sequencing of PBMCs. Thus, how can it be known whether the amplified RNA sequences to which the human research participants are being immunized are representative of the latent reservoir and not the result of selective polymerase chain reaction amplification?
- Numerous studies have shown early seeding of the brain reservoir for HIV and a unique evolution of sequences, resulting in compartmentalization. Furthermore, a recent study indicates that mutations in brain-derived HIVs may be less responsive to the HDACi class of drugs. Thus, what are the implications of the proposed strategy with regard to a potential lower efficacy of HDACi drug action in the brain, which is a known reservoir for HIV, and how will the vaccine work against HIV variants in this immunologically privileged site?

Dr. Wooley posed the following questions regarding research participant selection for the proposed trial:

- Is it correct to say in the that research participants must be on a three-drug cocktail—two nucleoside reverse transcriptase inhibitors (NRTIs) and either a non-nucleoside reverse transcriptase inhibitor (NNRTI) or at least one protease inhibitor?
- Would a chemokine receptor type 5 (CCR5) inhibitor be prohibited if it were combined with one of these acceptable ART regimens?
- All participants will be required to be on a stable regimen of ART for more than six months prior to study screening and throughout the entire study until study completion. However, there is some concern that research participants on older ART regimens or on ART for an extended period may have more chance to harbor drug-resistant strains or CTL escape mutations. Given these concerns, is there any exclusion for research participants who have been on older ART regimens, and is there any limit to the length of time that someone has been on ART?

Dr. Wooley had the following additional comments and questions regarding Appendix M:

- The investigators state that the questions regarding the description of the preparation, structure, and composition of the materials that will be given to the human research research participant or used to treat the research participant's cells is not applicable to the AGS-004 product. Dr. Wooley noted, however, that *in vitro* transcribed RNAs that are being used to treat the research participant's cells would seem to fall within the definition for synthetic nucleic acids per the *NIH Guidelines* ("...[those molecules] that are chemically or by other means synthesized or amplified, including those that are chemically or otherwise modified but can base pair with naturally occurring nucleic acid molecules.") Per these guidelines, the following questions should be answered: How are these RNAs purified? Have the researchers shown a single molecular species? Has the RNA been analyzed electrophoretically? Is the *in vitro* transcribed RNA that is transfected into the dendritic cells shown to be free of other contaminants?
- Additional information is needed regarding the commercial kits that will be used to synthesize and purify DNA and RNA, specifically, which commercial kits are used and whether there is any restriction on human use.
- The investigators provide some detail on the *in vitro* transcription of the RNA for CD40L but not for the HIV RNAs. Additional detail should be provided as to how the HIV RNA is amplified and synthesized from the archived pre-ART plasma sample. For example, how do the investigators go from a patient's HIV genome to the individual RNAs for Gag, Vpr, Rev, and Nef that are to be transfected into the DCs? Does the process include a cDNA step using reverse transcriptase, and if so, how is the cDNA priming done? In addition, are there any intermediate cloning steps? Dr. Wooley also inquired as to whether it would be possible to transfer DNA if the DNase step failed after *in vitro* transcription, and whether there any testing to verify the success of the DNase step.
- The investigators should to address the concerns cited in Lucera et al. (*J Virol* 88:10803-10812, 2014) that vorinostat could make uninfected CD4+ cells more susceptible to HIV infection and that use of the this drug, even in the presence of ART, may seed new viral reservoirs in the body.

Dr. Wooley agreed with the other reviewers' suggestions for the ICD and had the following additional comments:

- Regarding the statement, "We will make sure it is safe for you to receive the products," the investigators need to clarify if this is possible and, if so, how this will be done.
- The statement, "We are conducting this study to find out: If it is safe to give you," should read, "We are conducting this study to find out of it is safe for people."
- To better explain what a failure to show a significant response means to the patient, the following language was suggested: "Failure to show a significant response by step 3 indicates that the drug vorinostat did not significantly activate (flush out) your hidden HIVs. This is not a bad indication for your disease progression; it simply means that we will not be able to measure the necessary components in order for you to continue to the next step of the experiment." At the end of this section of the document, it would be helpful to reiterate these points with language such as, "Again, this is not a bad indication for your disease progression; it simply means that we will not be able to measure the necessary components in order for you to continue to the next step of the experiment."

- The investigators should consider removing the words “small” and “low” when describing the possible outcomes and risks associated with a detectable viral load since there are not enough data to quantify these risks. It would be better to say, “There is a chance that your HIV could become drug resistant during the time when your viral load becomes detectable. This chance is lowered by continuing to take your ART medications throughout the study as directed.”

Ms. Hardison’s comments focused on the ICD. She found the ICD overall to be satisfactory and the criteria for progression of the research participant from step 1 through step 8 to be well defined. The table that describes what is done at each study visit will contribute to the research participants understanding of how the study proceeds in such a way that they are well informed.

Ms. Hardison suggested reorganization of some information in the consent document and clarification of some consent language to improve research participant understanding, as follows:

- The description of the eight steps of the study under the section titled “What will happen if you take part in this study?” is very difficult to understand. Ms. Hardison suggested the following alternative language: “To be eligible to participate in this study, we need to locate a sample of your blood that was drawn and stored when there was measurable HIV virus in your blood. We will contact your healthcare providers for a sample.”
- The investigators should consider highlighting the following statements in the section titled “Study Drugs” as an added safeguard: “You should not crush or open the vorinostat capsule but should swallow the pill whole. The medicine inside the capsule can be dangerous if it gets in your eyes, mouth, or nose or on your skin.”
- Also in the Study Drugs section, the paragraph describing the risks associated with vorinostat seems to be out of place, as is the information in the section of the ICD titled “Risk Associated with AGS-004 Injections.” This information should be combined with the overall discussion of risks so that all risk disclosures are in one comprehensive section, which in turn might lead to better understanding of the risks and benefits for the patient.

C. RAC Discussion

During the meeting, the following additional questions, concerns, or issues were raised by RAC members:

- Dr. Hammar skjöld reviewed the 90-day requirement for collection of the pre-ART sample to measure viral load and issues related to a latent virus pool in participants with long-term HIV infection, including whether some “archived” virus sequences in the reservoir could have evaded treatment and detection. In addition, while most people now have ART very early in their infection, patients used to have to go below a certain CD4 count before starting treatment. She clarified that it would be unlikely to have resistant virus but that it is not possible to fully assess blips in HIV replication because the source (i.e., type of cell that can be induced to express HIV) is not known.
- Dr. Wooley shared Dr. Hammar skjöld’s concern that the virus that is being flushed out with vorinostat could be drug-resistant or immune escape mutants and that the HIVs being vaccinated against do not represent the latent reservoir. There still is a lot that is unknown about HIV reservoirs, including reservoirs in the brain that are seeded very early. This risk could be minimized by selecting individuals who have been placed on ART within three months of their infection or at least who have no history of treatment failure. Participants need to be on stable ART and have undetectable virus levels for greater than two years. However, someone who is on stable treatment and has been infected long-term may have been on five other regimens, but only the most recent regimen has worked; in such cases, the latent reservoir likely includes some drug-resistant mutants. The criterion that research participants must be able to have a salvage regimen suggests that there are some drugs that they have not yet tried or that might work. For those on newer regimens, the virus is suppressed from the start.
- It might be preferable to enroll this group of individuals first before proceeding to research participants who have followed other regimens. Given these scenarios, it might be preferable to apply other eligibility criteria to reduce this risk (e.g., individuals with no history of treatment failure, who have been on a regime that was successful from the beginning). Since this is the

first-in-human trial of this combination of drugs, the cohort could be expanded after testing is done in a more select patient population.

- Dr. Wooley asked how the plasma samples will be processed (e.g., using whole plasma without centrifugation or centrifuged pellets for isolating RNA). She also asked whether testing to assure that there is no cross-contamination is between research participants or for each research participant, and inquired about the manufacturing process for AGS-004 and how potential nucleic acid contamination is avoided. A control (e.g., a negative control without reverse transcriptase; a transcribed RNA treated with RNase) should be included in this process, and the protocol should describe the quality control (QC) steps for such a control.
- Per the description of the manufacturing process, the cDNA must be 70 percent of the expected size to proceed. It is not clear, however, if there is a QC step for the RNA to make sure that the bulk of what is transcribed is, for example, 70 percent of the expected size for that RNA. The final RNA should be checked on a gel to make sure that the size corresponds to what it should be.
- The ratio for the four HIV sequences is 1:1:1:0.25:1, where the 0.25 is for Nef. The reason for why there is less of the Nef gene compared to the other genes is not clear. Dr. Hammarskjöld suggested that a lower amount of Nef might be included because Nef affects antigen presentation; too much Nef, in turn, could impact the efficacy of the antigen presentation.
- Dr. Wooley requested further clarifications regarding research versus human grade materials in the kits used in several steps in the manufacturing process (e.g., to purify RNA) but not for preparation of the final injectable product.
- The efficiency of gene delivery based on the CD40 ligand and indirect testing via the IL-12 potency assay is estimated to be between 60 and 82 percent. The efficiency for the HIV genes is not specified, but it is not clear why expression of the HIV genes in the final product cannot be measured and if a cut-off for efficiency (e.g., at least 50 percent of cells expressing some HIV genes) can be identified to determine whether to proceed with the analysis or not. Since the immune response of the vaccine has been low, setting a threshold could help move the research forward. The investigators might want to consider doing flow cytometry on the dendritic cells for this purpose.
- The number of RNA copies per cell was not specified even though the gene ratios are known, and an estimated five percent of the RNA enters the cell.
- Additional details about the system used to measure the half-life of RNAs in DCs are needed.
- Further information was requested regarding use of Class 1 selective HDAC inhibitors, which one paper suggested do not affect uninfected CD4 cells.
- Dr. Wooley noted that no animal models appear to have been used to date to study the proposed intervention, even though good simian immunodeficiency virus (SIV) models exist. She asked whether this combination of agents should be tested in one of these monkey systems before conducting a clinical trial.
- The consent should not refer to the proposed intervention as a “treatment,” which is misleading, because treatment implies efficacy. The agents can be referred to as drugs, and the consent should explain what a lack of response means and that a lack of response is not necessarily bad.
- Dr. Zoloth considered the proposed consent suggestions to be reasonable. She asked whether an aim of the research is to coax out all of the latent HIV. Whether this is a goal needs to be explained more clearly in the consent.

D. Investigator Response

1. Written Responses to RAC Reviews

The investigators modified the suggested language regarding failure to respond as follows: “Failure to show a significant response to vorinostat by step 3 does not mean the treatment did not have any impact. The lack of a response will not affect your health. It simply means that we are not able to measure a change in order for you to continue to the next step.” The investigators agreed with the other recommendations regarding the ICD and have modified the proposed consent document accordingly.

There will be a time limit for the pre-ART sample collection. Per the inclusion criteria, frozen plasma samples must be collected within 90 days before starting ART, preferably within 30 days. Notably, the most proximal pre-ART sample with virus will be used as specified in the protocol to provide the best sample regarding sequences present and for use in the manufacture of AGS-004 for individual participants. Frozen plasma can be thawed and re-frozen only once prior to use for this purpose.

Confirmed viremia (>150 copies/mL on any two consecutive determinations) at any time during the study would result in the participant being discontinued from the protocol. HIV genotyping and resistance testing will be performed at the time of drawing the confirmatory. It is unlikely that participants in this study would have release of drug-resistant virus since the eligibility criteria (1) mandate that research participants have durable viral suppression on stable current ART regimen and (2) exclude individuals with an ART interruption >one month since starting ART from which pre-ART plasma sample was drawn.

The investigators explained why they do not believe that the proposed study should be limited to those starting ART during early HIV infection. They acknowledged that Deng *et al.* (*Nature* 15:381–387, 2015, cited by the RAC reviewer) found that in almost all participants who began ART in chronic infection, there was evidence of T-cell escape in replication-competent virus recovered from resting CD4-positive T cells. The investigators pointed out, however, that Deng and colleagues looked at a selected number of epitopes. In contrast, the T-cell response to HIV-1 infection involves multiple T-cell responses targeting different epitopes; that is, the T-cell response exhibits breadth. While Deng *et al.* found evidence of escape, there was no evidence that escape was ubiquitous across all epitopes. Indeed, all of the participants studied exhibited CD8-positive T-cell-mediated virus inhibition of autologous CD4-positive T cells infected with replication-competent virus. This means that some of the patient's circulating CD8-positive T cells must have been able to recognize epitopes in the replication-competent virus. Moreover, they showed that virus inhibition increased when CD8-positive T cells were stimulated with a standard set of HIV-1 peptides *in vitro*. Peptide stimulation induces *in vitro* expansion of CD8-positive T cells, increasing the relative frequency of HIV-1-specific T cells in the culture. Therefore, the increase in the patients' existing CD8-positive T cells increased virus inhibition. The therapeutic vaccine in the proposed study is designed to achieve the same result *in vivo*. The investigators commented that the findings from Deng *et al.* do not limit the planned study; rather, the results support the proposed trial.

Furthermore, the vast majority of HIV-infected individuals initiate ART with chronic infection. Any strategy to clear latently infected cells should address the resting cell reservoir that exists during chronic HIV infection. The planned study is a Phase I trial with a primary aim of determining the safety of the intervention and whether there is any virologic impact with combining a latency-reversing agent with an immune enhancing treatment. The investigators do not anticipate that this Phase I study will result in eradication of all latent HIV and note that additional studies that include those potentially used combination immunotherapeutic approaches will be needed. If no effect of the combined therapy is seen in this study, the investigators would conclude that vorinostat may not induce the expression of antigen in a sufficient number of cells and/or that immune responses induced were insufficient to clear those cells in the major patient population of relevance. This would significantly inform and direct future research.

At present, the nature/source of the amplified RNA sequences cannot be known. The purpose of this early phase protocol is to evaluate the impact of AGS-004 on latently infected cells in the presence of latency-reversing therapy (VOR) by determining whether the elicited responses result in a decrease in the frequency of resting cell infection. It cannot be overstated that measures of provirus and HIV DNA are problematic, given that they do not distinguish between replication competent and incompetent HIV DNA genomes; thus, whether the HIV DNA measured is in fact a reflection of relevant HIV antigen to be cleared is also uncertain. An important aspect of this research study is that participants are virally suppressed on their current ART regimen to be eligible and that they continue suppressive ART during the study.

The implication of the proposed intervention on the CNS compartment would be relevant if it can first be shown that this strategy is safe and reduces the frequency of latent infection. The investigators recognize that this point is arguable but note that the field is in its very early stages and that the planned study is the initial trial to combine a latency-reversing agent with an immune enhancing strategy. Furthermore, the

claim of Gray *et al.* (*Mol Psychiatry*, 2015, cited by the RAC reviewer) vis-à-vis the HDAC inhibitor effect in the CNS is based on the observation that promoters from CNS-derived isolates, when cloned and transfected on DNA expression vectors into immortalized cells, are upregulated less (i.e., fold induction compared to baseline) compared with an HXB2 consensus sequence. The investigators noted that this data set actually shows weak induction by vorinostat, but not less than the HXB2 sequence. In general, such studies of chromatin effects are not generally done using transfected DNA constructs, as native chromatin structures are not reliably formed and responsive, especially in immortalized cells.

If the proposed strategy is found to be effective, further studies would be needed to determine whether and, if so, to what extent other compartments such as the CNS, gut-associated lymph node tissue, and other lymphoid tissues are affected by the combined “kick and kill” approach. The investigators feel that it is prudent to assess strategy in general before proceeding to more invasive procedures to assess the impact on compartments.

Whether the vaccine will work against HIV variants in this immunologically privileged site (i.e., CNS compartment) remains unknown and, further, is not the aim of this Phase 1 study. HIV variants in the CNS are generally characterized by their ability to infect cells with lower surface levels of CD4, rather than a distinctive immune escape phenotype. However, the immune response in the CNS is unique, and will be a goal of future studies, should the proposed strategy (or any approach) show promise.

The investigators found the *in vivo* relevance of the observations in the experiments described in Lucera *et al.* (*J Virol* 2014) to be unclear, particularly in patients on stable ART. Lucera and colleagues demonstrate convincingly in *in vitro* experiments that vorinostat increases the efficiency of post-entry steps of viral replication, likely via inhibitory effects on HDAC 6 that may alter cytoskeletal functions. The levels of vorinostat needed to induce these effects appear to occur at or above 500 nM for at least 4 hours. Such exposures generally do not occur with clinical dosing at 400 mg. Furthermore, enhancement of these post-entry events in an *in vitro* infection is seen only in the absence of ART and is ablated by the addition of an RT inhibitor (efavirenz) or an integrase inhibitor (raltegravir), even if the antiviral drug is not added until more than 20 (efavirenz) or 30 (raltegravir) hours after infection.

The investigators confirmed that the inclusion criteria regarding ART stipulate that participants must be on combination ART per current treatment guidelines, as specified in the protocol (i.e., a three-drug cocktail, either two NRTIs plus an NNRTI or two NRTIs plus a protease inhibitor). Administration of a CCR5 inhibitor which is not a recommended first-line regimen, is a specific exclusion criterion in the protocol. A participant on an ART regimen that includes a CCR5 inhibitor (e.g., maraviroc) is likely to have received this drug as a part of therapy for multidrug-resistant HIV. There currently is little use of maraviroc in the team’s clinic population. The interaction of maraviroc with vorinostat is unstudied and difficult to predict. During the FDA review of the proposed trial, it was suggested that this drug be excluded. Exclusion of this drug is not expected to have any impact on recruitment.

The proposed trial will have no exclusion criteria based on past ART regimens. This issue is addressed by limiting enrollment to individuals who are on a stable ART regimen with durable viral suppression. This concern is also addressed through exclusion of persons with a history of treatment interruption of ART for more than 1 month and if it is not possible to construct a fully active alternative ART regimen based on previous resistance testing and/or treatment history. There are no exclusion criteria for maximal duration of ART administration and suppression.

The investigators acknowledged that, per the NIH definition for human gene transfer, AGS-004 is being classified as a gene therapy product. They provided the responses to the questions in Appendix M that they previously thought did not apply to this investigational agent.

The purity of the CD40L/HIV *in vitro* transcribed (IVT) RNA is measured at the intermediate steps for the individual IVT RNAs and after formulation using UV absorption at 260 nm and by denaturing gel electrophoresis. For the HIV cDNA, there is an in-process purity assessment for band with expected size; the purity needs to be greater than 70 percent. In addition, HIV cDNA is tested for identity as part of quality control. Each cDNA is sequenced, and the sequence of each antigen is analyzed to positively

identify the target antigen (Gag, Vpr, Rev, and Nef of HIV). A phylogenetic tree analysis is also performed for characterization; this test allows for analysis of amplified sequences to make sure that there was no cross contamination between research participants' sequences. The uncapped HIV RNA is tested using an ultrasensitive fluorescent nucleic acid stain for quantifying RNA down to 1 ng/mL in solution. Based on treatment with DNase and purification methods, the remaining species is intended to be IVT RNA without contaminating nucleotides and DNA. If these intermediates pass in-process analysis, the materials are formulated and retested for integrity, bioburden, and endotoxin. The response to the reviewers' questions provides additional details regarding the in-process tests and acceptance criteria for the capped IVT CD40L and HIV RNAs and the specific tests used to control the intermediates. The investigators note that additional in-house technical studies have demonstrated that potential residual process contaminants are minimal.

Argos is in the process of performing a risk assessment and qualifying all reagents including the kits used for HIV antigen production under a raw materials qualification project. The investigators explained that these kits are not used in the final steps of producing a direct injectable product; rather, they are used "for further manufacturing". Whenever possible (e.g., for the final kits for IVT of both RNAs), the reagents have been upgraded to higher-than-research grade reagents. A risk-based approach based on International Conference of Harmonization (ICH) guidelines is used to qualify and control reagents for manufacturing. The details of the HIV amplification process were provided in the response to the reviewers and in a PLOS paper cited in the response (Tcherepanova et al., 2008). In brief, during this process, the reverse transcription of the HIV genome is converted into individual cDNAs encoding Gag, Vpr, Rev, and Nef using gene-specific primers that are multiplexed to overcome the inherent issue with HIV genome variability. The identity of each cDNA product is determined via in-process QC testing to verify that only the target regions (and not any other sequences) were amplified. The cDNA is then directly transcribed into RNA without an intermediate cloning step.

Extensive details were provided in response to the questions in Appendix M regarding the efficiency of the delivery system, the percentage of the target cells contain the added recombinant or synthetic nucleic acid, the number of copies per cell, and the stability of the added recombinant or synthetic nucleic acid both in terms of its continued presence and its structural stability. The investigators stated that given that the HIV and CD40L RNA lacks structural elements that could facilitate replication, integration, or rearrangement, the structure of the added RNA in the AGS-004 cells will not deviate from the structure of the IVT RNA. The HIV and CD40L RNA are electroporated into the cytosol of the dendritic cells. The RNA is not integrated into the dendritic cell genome, is not rearranged, and is not capable of integration into the patient genome after administration of the AGS-004 product. HIV and CD40L RNA is added to the cytosol of cells at a ratio of 3:3:3:0.75:3 µg Gag:Vpr:Rev:Nef:CD40L electroporated per million DCs. Experiments conducted at Argos have established that less than 5 percent of that amount is introduced into the cytosol of the cells and that RNA degradation is well underway by the time that AGS-004 is administered to patients.

No models were used to assess in vivo or in vitro efficacy of the system using HIV RNA or in any models for HIV. Because AGS-004 is an autologous, research participant-specific cellular product, animal species are not appropriate models for predicting the safety or efficacy of this product when used in the humans from whom the product components were derived. Therefore, no animal testing has been conducted for AGS-004. The capacity of the AGS-004 manufacturing process to generate cells capable of independently inducing adaptive cellular immune responses has been assessed via an in vitro cell culture system that closely approximates the AGS-004 manufacturing process, albeit with healthy volunteer PBMCs rather than PBMCs from HIV patients. While this in vitro system cannot replicate the complexity of a human adaptive immune response in vivo, it is based on freshly derived donor cells (not cultured cell lines) and is perhaps the best available approximation to examine questions which cannot be assessed in vivo. The system was a good predictor of the type of response that was later found in patients in the immuno-monitoring studies; results indicated a statistically significant correlation between these memory T cell responses and overall survival among renal cell carcinoma research participants enrolled in another trial (NIH RAC protocol # 0712-888).

Regarding an estimate of the minimal level of gene transfer and/or expression necessary for the gene transfer protocol to be successful in humans, the investigators noted that HIV expression in the AGS-004 product is not measured, but that CD40L expression was measured (as percent CD40L protein positive cells) during product development using a potency assay for expression of IL-12. Data from a Phase IIB clinical trial indicate that multi-functional HIV-specific immune responses can be generated in chronically infected HIV research participants on ART and that these immune responses persist with no immune responses detected in research participants receiving placebo.

The investigators noted that because the AGS-004 mechanism of action does not require transfer into the cellular genome, the minimal required levels of gene transfer and expression have not been studied. Expression analyses done during the AGS-004 manufacturing process show that this process is capable of generating a dendritic cell therapy product that can induce an adaptive cellular immune response to protein encoded by the RNA payload electroporated into the cells. In the case of AGS-004, this RNA payload consists of amplified HIV RNA encoding at least three of the four HIV antigens present in the research participant's own disease (Gag, Vpr, Rev, and/or Nef). Using a similar manufacturing process for mature DCs electroporated with CD40L RNA and tumor RNA from research participants with advanced renal cell carcinoma in a Phase 2 clinical trial, Argos has shown a statistically significant correlation between anti-tumor memory T cell responses and overall survival among these patients.

The CD40L and HIV RNA includes only a single coding region (an open reading frame) and lacks the structural elements necessary for genetic integration. After electroporation of CD40L RNA into the cytosol of mature dendritic cells (MDCs, a cell type that does not normally express CD40L), expression of CD40L in these cells is driven wholly by the transcript rather than by any surrounding genetic material. During process development, electroporation efficiencies of 60–82 percent were observed in DCs with high fluorescent intensity, as measured by CD40L expression using intracellular staining and flow cytometry. Similar efficiency is expected to apply to the autologous HIV RNAs since the CD40L and HIV IVT RNAs are formulated together and co-electroporated. The indirect measure of electroporation efficiency is the IL-12 potency assay. Dendritic cells without expression of CD40L do not induce IL-12 and thus do not have the desired biological activity. CD40L expression is required for IL-12 expression, and there is a prerequisite for one of the three signals necessary to allow AGS-004 to induce an adaptive cellular immune response.

Due to the autologous nature of the product, the HIV RNA is present in the research participant because it is isolated from the research participant's blood. All RNA is introduced back to the patient within the exogenously matured dendritic cells; other cells are therefore not exposed to the HIV or CD40L RNA. For AGS-004, RNA is being introduced into the cytosol of the autologous matured dendritic cells by electroporation as a protein transcription template. The RNA is replication incompetent; thus, once it is digested by intracellular RNases, no more of the associated protein will be produced by the dendritic cell. According to the literature, electroporated RNA in DCs has a relatively short half-life with a pulse of protein following electroporation as the RNA is transcribed. In-house studies show detectable HIV RNAs at 22 hours post-electroporation for Gag; at four hours for Vpr and Nef, and at seven hours for Rev. CD40L protein expression peaks at four hours and decreases thereafter.

The investigators note that the risks of AGS-004 to the research participant are discussed in detail in the ICD and investigator brochure. The most common adverse events identified thus far are transient local reactions related to and at the site of intradermal administration; these reactions have been mild to moderate. Personnel involved in the manufacture of AGS-004 undergo training to reduce risks of handling the product, which is treated as an infectious substance during the entire process.

Because AGS-004 is an autologous product manufactured from the patient's own blood and viral agents already present within each patient, there are no additional risks beyond what would otherwise be encountered from handling an HIV-infected person's blood. The risk of exposure to other individuals would be from a needle stick to the healthcare professional or administration to the wrong research participant. As a precaution, only trained healthcare professionals are allowed to administer AGS-004, and a strict chain of identity is maintained throughout the manufacturing and administration process so that AGS-004 is only administered to the research participant for whom it was manufactured. There is no

significant possibility that the added RNA will spread from the human research participants to other persons or to the environment given the nature of AGS-004 and how it is produced. Appropriate cleaning and destruction of biohazardous waste are performed to reduce any potential risks.

The investigators note that there is no routine in-process testing to verify the success of the DNase step. However, data obtained during development indicate that the approximate levels of residual pARGCD40L plasmid DNA are well below the regulatory limit of 100 pg DNA per dose.

2. Responses to RAC Discussion Questions

The investigators confirmed that a pre-ART plasma collected within the 90 days before starting antiretroviral therapy must be available for research participants to enroll in the planned trial. Another inclusion criterion requires participants to have no more than one month off ART from the time they started treatment. Thus, to be eligible, research participants who would be enrolled in this study would have durable suppression without a time off therapy since starting treatment from which the pre-ART sample was collected. The investigators acknowledged the reviewers' concerns about latent virus sequences but also noted that setting parameters for the protocol is more of a question of the practical versus the ideal. The team is currently working to harvest RNA from the research participant's resting cell pool as a next-generation step using a GMP-compliant approach. Since the vaccine that has already been given to research participants induces an immune response and preliminary data suggest that the immune response to the vaccine has at least a transient clinical effect, the investigators hope that the proposed strategy, while not generating a "perfect" response might produce an observable effect.

The investigators noted that per the protocol, research participants must have a treatment history such that a salvage regimen can be constructed. They will consider adding an inclusion criterion to address the reviewers' additional concerns issues regarding prior treatment failure. The investigators do not consider the issue of the potential spread of drug-resistant virus to be well founded. They pointed out that patients often have 'blips' of virus and that evidence suggests that ongoing therapy prevents the spread of drug-resistant virus. Patients would have to have triple-drug-resistant virus in the reservoir to fail therapy, and the small amount of induction that can be measured is unlikely to overcome the standard (HA)ART that patients are taking.

The investigators did not agree with the suggestion to select research participants with a history of no treatment failure or who started ART soon after infection. They recognized the benefits of focusing first on research participants who started ART (particularly a newer regimen) very early in their infection and for whom HIV replication (and thus, the virus reservoir) is most likely very minimal. However, since the ability to reactivate the virus at this point is limited, by starting with low residual virus, it probably would not be possible to induce sufficient (measurable) resting/latent cell infection. Further, the generalizability of approaches such as the planned strategy would be very limited if they are developed and tested only for a highly select group of patients. To date, there has been no measurable viremia, even at the level of a single copy assay, in the approximately 30 patients given vorinostat. Most of these patients were treated for chronic infection, and many were treated in acute infection; some had a history of drug failure and resistance, but all had been suppressed for at least 2 years.

The investigators clarified that testing to check for any cross contamination is between research participants. As for vaccine preparation, including the steps for sample processing and RNA isolation, the investigators will be following the routine protocol that Argos has established and used to over the past 10-15 years. During the manufacturing process, HIV RNA is isolated directly from standard plasma samples; the samples are not ultracentrifuged. The plasma samples will be drawn prior to enrollment, usually from patients who have participated in other studies and had their samples banked. Drawing largely from this pool of patients is a limitation for the proposed trial because it would be very difficult to find other individuals who have a qualifying plasma sample in the appropriate time window.

The investigators will consider adding QC steps that use control(s) and RNase in the AGS-004 manufacturing process with their colleagues at Argos. In addition, they will go through the protocol and Appendix M to make sure the appropriate information regarding this process is added.

The lower amount of Nef is based on literature indicating an inhibitory effect of the gene. A T7 protocol will be used in the primers for the in vitro transcription. The investigators were not certain as to PCR efficiency of the primers used by Argos, but they will ask their colleagues for this information.

The Argos vaccine has been reviewed and approved to be used in several studies. The investigators will need to follow up on questions as to whether the reagents used in the manufacturing process leading up to the final product are qualified to use in research participants.

What constitutes an effective vaccine for HIV is not known. Electroporation of DCs induced immune responses in the vast majority of patients given the vaccine. However, which proteins and epitopes are most important, and at what levels, needs to be determined before a cut-off for efficiency of delivery of gene delivery can be set. The proposed trial is one the first studies to consider this question.

Dr. Margolis noted that the combination of agents to be studied in the proposed trial is difficult to test. Some testing has been done primate and murine models, but the results were not promising. One reason is that antiretroviral therapy in primates is not as optimized as that in humans. Additional animal studies are likely to be conducted in the future. The investigators recognize the importance of animal research but pointed out that they are trying to use the safest and most advanced techniques to determine if a signal is present, if antigen is being presented when vorinostat is given, and if an immunotherapy can clear some of those cells before moving forward.

Dr. Margolis noted that the inhibitory effect of HDAC6 described in the Lucera paper is a post-entry event involving upregulation following infection of a cell. In the presence of ongoing therapy, such an effect is negligible; as demonstrated, the addition of a single antiretroviral agent up to 20 hours after the cells were infected blocked this effect. Thus, this is not expected to be a concern for patients on therapy. Vorinostat is a Class 1 selective inhibitor that also inhibits HDAC6.

Dr. Gay explained that a goal of this research is to show whether some virus can be reactivated and to enhance HIV-specific immune responses to clear the reactivated cells, not to coax all of the latent HIV out of the reservoirs. This will be stated more clearly in the consent. At this point, the type and amount of those responses needed to achieve this goal are not known. Whether the proposed study generates positive or negative findings will be very informative for the field.

E. Public Comment

Public comments from Robert Reinhard and Lynda Dee were offered in support of this clinical trial. Dr. Jorgenson read into the record a letter from Dr. Reinhard. Their testimony is provided verbatim in Appendix A. Dr. Kiem thanked Dr. Reinhard and Ms. Dee for sharing their feedback and suggestions. It is always helpful for members of the RAC to hear from patients and family members to better understand the patients' perspective and needs. In response to comments from Ms. Dee, Dr. Kiem noted that the staff and the RAC are working on implementing regulations for gene transfer protocols and the criteria from the 2013 Institute of Medicine report regarding public hearings for the RAC.

The RAC members recognize and understand the competing concerns identified in the public comments and do not want to delay initiation of research studies. However, a centralized process, such as that provided through the RAC reviews, is important to ensure that safety is maximized and risks are minimized in the trials submitted for review. It was noted that most of the gene transfer protocols submitted to OBA do not need the additional level of review provided by the RAC and thus do not undergo public review. The current protocol was forwarded for public review to assure that several specific questions and issues, which were not initially addressed but were subsequently responded to per the written materials and meeting discussions, were fully considered and clarified.

F. Synopsis of RAC Discussion and RAC Observations and Recommendations

The following observations and recommendations were made during the RAC's in-depth review and public discussion:

- To help ensure the quality of the RNA being transferred into the autologous dendritic cells that will be subsequently transferred into the research participants, the following quality control measures should be considered, in the event they are not already being performed:
 - Verification of the DNase step conducted after *in vitro* transcription to confirm that no significant levels of contaminating DNA are being transferred. For example, a negative control containing no reverse transcriptase at the cDNA synthesis step could be included.
 - The final RNA product could be directly analyzed by ultraviolet absorbance and denaturing gel electrophoresis.
- The following sentence on page 4 of the informed consent document should be revised to eliminate the use of terms such as "treatment" and "impact" to avoid unrealistic expectations of clinical improvement by the research participants: "Failure to show a significant response does not mean the treatment did not have an impact, but that we are not able to measure all the parameters necessary to move you to the next step."
- Analyses of HIV gene expression in the dendritic cells might be of use for future correlation with clinical outcomes.

G. Committee Motion 5

Dr. Kiem summarized the RAC recommendations to be included in the letter to the investigators, expressing the comments and concerns of the RAC. Dr. Kiem requested a vote, and the RAC approved these summarized recommendations by a vote of 16 in favor, 1 opposed, 0 abstentions, and 0 recusals.

VII. Review and Discussion of Human Gene Transfer Protocol #1510-1469: A Phase I/II Study of Autologous T Lymphocytes with Antibody-Dependent Cellular Cytotoxicity in Subjects with Relapsed or Refractory CD20-Positive B-Cell Lymphoma

Investigators/Presenters: Seth Ettenberg, Ph.D., Unum Therapeutics
Michael Vasconcelles, M.D., Unum Therapeutics
Philip Cross, M.S., Phillip J. Cross & Associates
Limei Michelle Poon, M.D., National University Hospital, Singapore
(via teleconference)

Sponsor: Unum Therapeutics

RAC Reviewers: Drs. Kiem, Pilewski, and Zoloth

A. Protocol Summary

B-cell lymphoma is a cancer of the blood, where a specific type of white blood cell, B lymphocytes, grow and multiply uncontrollably in the blood and lymph nodes. Many people with B-cell lymphoma can be cured with combination treatments, usually including chemotherapy and a drug called rituximab. Rituximab, a monoclonal antibody used against CD20-positive malignancies, is designed specifically to attach to cancer cells to make it easier for the body's immune system and chemotherapy drugs to destroy. For some patients with B-cell lymphoma, the cancer is eliminated by treatment and does not return. For others, the cancer stops responding to treatment (refractory disease) or comes back after being successfully treated in the past (relapsed disease). Patients with refractory or relapsed disease have a grave prognosis, as no approved therapies have been shown to increase progression-free or

overall survival. For these patients, additional treatment strategies, including radiation therapy or stem cell transplant may be appropriate.

The proposed study is an experimental treatment for patients with B-cell lymphoma who are not cured after receiving available therapies. T cells taken from the patient (autologous T cells) will be modified to express antibody-coupled T-cell receptors (ACTR), making a change in the T cells that is designed to help rituximab therapy effectively kill the cancer cells. The trial is an open-label dose escalation Phase I/II study to investigate the safety and efficacy of a single dose of autologous T cells expressing ACTR for the treatment of relapsed or refractory CD20-positive B-cell lymphoma in combination with rituximab. Patients will first receive cytoreductive conditioning with fludarabine and cyclophosphamide before their ACTR T-cell product infusion. One day prior to the ACTR T-cell product infusion, patients will receive the anti-CD20 monoclonal antibody rituximab. During Phase I, up to 12 patients with CD20-positive B-cell lymphoma will be enrolled to evaluate the safety of ACTR T-cell product in a 3 x 3 dose escalation study. In Phase II, the MTD of the ACTR T-cell product from Phase I, or a lower dose selected by the Dose Escalation Committee (DEC), will be administered to all patients; this phase will enroll up to 33 subjects. The primary endpoint for Phase I is the incidence of dose-limiting toxicities (DLTs). The primary endpoint for Phase II is objective response rate. Subjects will be followed for 24 months after ACTR T-cell product infusion under this protocol for safety and additional endpoints. Research participants will then be followed for an additional 13 years under a separate long-term follow-up protocol, which will assess survival, general health, and potential long-term toxicity of the ACTR T-cell product. Enrollment in the proposed Phase I/II study will be limited to adults between ages 18 and 75.

B. Written Reviews by RAC Members

Nine RAC members voted for in-depth review and public discussion of this protocol. The trial was found to warrant public review because it is a first-in-human study to combine rituximab with autologous T cells that are genetically modified by retroviral transduction to express an ACTR for the experimental treatment of relapsed/refractory CD20-positive B-cell lymphoma.

Three RAC members provided written reviews of this proposed Phase I/II trial. Dr. Zoloth was not able to submit her written review before the meeting and presented her comments and questions during the meeting. All three reviewers expressed concern that significant portions of the protocol and Appendix M were redacted, which limited the reviewers' ability to evaluate the vector and other details and aspects of the proposed study.

Dr. Kiem had the following comments and questions regarding the protocol:

- A main concern is whether there could be nonspecific binding of the ACTR T cells in vivo and thus nonspecific toxicity. Triggering activation of chimeric antigen receptor (CAR) T cells nonspecifically could result in significant toxicity. It would be helpful to review the in vitro and in vivo data to document specificity. Because many parts of the protocol are redacted, it is unclear what was done in an in vivo setting to study off-target toxicity. While in vitro models are helpful, some in vivo data would be more reassuring.
- Given the above concerns, would it be reasonable to first test the ACTR T cells without rituximab?
- Please clarify the definitions for the DLTs.
- The initial target dose is redacted, preventing assessment of and comment on whether it is a "safe" dose. It is not clear why the starting dose is not shown. (The same comment was made for Appendix M.)

Dr. Kiem had the following additional comments and questions regarding Appendix M:

- The research product is a gamma-retroviral vector, which, for T-cell modifications, has been safe in the past. The backbone of the product is not shown, however, and most of the Preparation and Testing of Research Product section of the Appendix is redacted. As a result, it is not possible to review the safety of the construct.
- One experiment is presented with in vivo studies in NSG mice with Daudi cells. Four mice received rituximab and nothing else; five other mice received rituximab plus 1×10^7 ACTR T

cells. Tumor cells expanded in mice without the ACTR T cells. While this experiment shows efficacy of the approach, it does not provide information about toxicity, which is a critical part of a Phase I/II study.

- How can the investigators be sure that there will be no nonspecific binding of the patient's genetically modified T cells? Why would the modified T cells only bind to rituximab?, How can the investigators be sure that the modified T cells do not severely deplete any normal B cells in these patients?
- How did the investigators determine the initial dose in patients given that the toxicity is not known?
- Lymphodepleting chemotherapy will be given before dosing, which will improve engraftment and maintenance of gene-modified T cells and thus increase potential toxicities.
- The investigators state, "The two principal toxicology questions are (1) whether ACTR T cells interact with endogenous antibodies to kill healthy cells in a way that would be a toxicity concern and (2) whether there are toxicology-related off-target effects of rituximab-directed killing by ACTR cells." These are critical questions. The investigators argue that mouse studies are not predictive because of many differences, including the fact that rituximab does not bind mouse CD20. The investigators also argue that NHP studies would not be useful, but they refer to a study done in rhesus monkeys, not cynomolgus macaques, that concluded that NHP studies can be useful to study on-target/off-tumor toxicity. It remains unclear how the investigators can assess whether ACTR T cells interact with endogenous antibodies to kill healthy cells and cause a toxicity concern. This is an important point and needs to be addressed adequately.
- An ongoing Phase I pilot study (NCT02315118) uses ACTR T cells in combination with rituximab for patients with B-cell malignancies; this study was approved by the Singapore Health Sciences Authority (HSA) at the National University Hospital (Singapore) and Singapore General Hospital. Electroporation of mRNA is being used as a means of delivering ACTR to patient T cells, creating a transiently active form of the therapy. Dr. Kiem asked whether this is the same ACTR construct that will be used in the proposed protocol? What are the results of this pilot study to date? Why would the investigators in the current study not use mRNA electroporation?.

Dr. Pilewski had the following comments and questions:

- How did the investigators choose the starting and target numbers of infused ACTR T cells for the Phase I study?
- In Phase II of the trial, subjects may receive additional cycles of rituximab as long as ACTR T cells persist. How will persistence of ACTR T cells be defined?
- Additional information is needed to ensure that the members of the Dose Escalation and Data Safety Monitoring Committees are (or will be) independent from the sponsor and the study investigators.
- The inclusion criteria include patients with chemotherapy refractory disease, which is defined as "stable disease with duration of stable disease being <12 months, or progressive disease, after most recent chemotherapy-containing regimen OR disease progression or recurrence <12 months after prior autologous stem cell treatment (SCT)." As such, patients with stable disease are defined broadly, and this criterion seems to allow inclusion of a wide range of disease severity. Per this definition, it appears that the investigators could potentially enroll patients with recently completed salvage therapy whose disease may not progress.
- Given that this is a toxicity study, the inconsistency of IgG replacement therapies (e.g., "according to the guidelines of the study center") may influence the frequency of infectious complications. Consider a standardized approach to use across studies.
- Adverse events will be collected after day 28 by the investigator for 12 months or until disease progression, whichever occurs first. Given that the tables of study visits are redacted, it was not possible to assess the frequency and thoroughness of clinical and laboratory monitoring. How will adverse event monitoring be accomplished to ensure identification of complications and completeness for safety assessment?
- Given the risk of viral reactivation, will monitoring for reactivation of cytomegalovirus (CMV) be performed? If so, at what interval and duration after treatment?

Dr. Pilewski had the following additional comments and questions regarding Appendix M:

- The preclinical data are limited to in vitro and immunodeficient mouse studies using Daudi and Ramos cells as a model of B-cell lymphoma. The data shown in the paper by Kudo et al. (*Cancer Research*, 2014) provide strong evidence for cytotoxicity in vitro and in mice when ACTR T cells are given in conjunction with rituximab. Effector/target ratios are defined in vitro, but the data presented appear to inform choices of doses needed for an effect in humans. The investigators should address whether and how the preclinical studies inform the dose-escalation phase of the proposed study.
- The text repeatedly refers to reference 2 for studies of ACTR, but the correct citation is reference 16.
- The key safety issues are clearly summarized with respect to whether ACTR T cells interact with endogenous antibodies to exert antibody-mediated cytotoxicity to normal cells and whether there are off-target effects. The rationale for not pursuing murine or primate studies to assess safety of the proposed approach is discussed, and the investigators refer to an ongoing human Phase I safety study using a transiently active form of ACTR T cells/rituximab (NCT02315118). Dr. Pilewski commented that the safety results of these studies should be fully completed prior to final design and initiation of the proposed Phase I/II study.

The following comments and suggestions regarding the ICD were posed by Drs. Kiem and Pilewski:

- Chemotherapy is discussed in the consent document, but similar information is redacted in other parts of the protocol.
- The consent needs to explain that patients cannot simply “withdraw” once the study agent is delivered, since the T cells cannot be removed from the body. Subjects may withdraw from follow-up, but there is no real withdrawal from the intervention after cell infusion.
- The consent should not include a reference to potential for (direct) benefit with participation. Such a statement implies an expectation that the cancer will respond to the experimental intervention, which is not known for this Phase I study. The statement should be revised to clarify that there is no known direct benefit and that it is possible that the tumor will not respond.
- The ICD should explicitly state that the Phase I study is a safety study with little to no expectation of benefit and that the Phase II study will assess both safety and effectiveness.
- The Risk section does not explicitly describe the risk of autoimmunity that could occur if the ACTR T cells recognize endogenous antigens.
- In the Duration of Study Involvement section, the investigators indicate long follow-up times that are likely irrelevant to this patient population given the mortality. Death should be explicitly mentioned to acknowledge this outcome, both here and in the discussion of risks from the intervention.
- Follow-up is described as “specified visits” without any indication of the frequency and number. Details about follow-up visits and procedures (e.g., frequency, number, intensity) are important for patients to assess protocol burden and decide whether participation is appropriate for them.
- Consider replacing the term “ventilation” with “respirator” or “lung support machine.”
- Death should be mentioned as a possible outcome associated with the T-cell product and rituximab.
- Additional detail should be provided regarding risk of infection based on the risk with rituximab alone and in combination with other T-cell therapies.

C. RAC Discussion

Dr. Zoloth noted the importance of transparency for review of the protocol and thanked the investigators and sponsor for unredacting the previously blocked-out information (see below). She found the changes and responses to the reviewers’ questions and comments to have improved the original submission considerably. Additional comments and questions from Dr. Zoloth were presented during the meeting, as follows:

- The rationale of the proposed study is clear, and the logic is sound. However, the protocol and consent do not explicitly state or convey that B-cell lymphoma is a fatal disease. The status of patients who are eligible to participate in this trial, in turn, raises ethical issues, because the study

will be enrolling dying patients. To mitigate this concern, palliative care should be offered to or included for participants.

- The consent describes the participants' disease as "stable," but this term is usually used in reference to nonfatal conditions. The consent should include language such as, "You have a fatal disease...", "We are looking for the best dose of the study agent as a possible treatment for B-cell lymphoma...", and "You may have a lot of side effects..." Dr. Zoloth acknowledged that this language is stark. However, the information needs to be clearly presented to patients so that they understand the purpose of the study and risks of participation. The full language suggested is, "You have a fatal illness, and while we cannot be certain, we do not think you will live longer because of your participation in this study. There is nothing we can offer you now to cure you, but scientists are studying your disease and want to ask you to take drugs as an experiment that might someday be used to treat people with a disease like yours. This treatment is in its very early stages. Taking the drug at this stage does not mean it will help you. There is only a very slight chance of that happening. The experiment is to test the right dose in people and to see whether the drug might be safe or dangerous in people. The scientists are looking for side effects that would make the drug [or "procedure"] impossible to take. That means you are part of an experiment, and you may have very bad side effects from this procedure. Being part of a study like this will mean a lot of tests and doctor visits for you. If you participate, while you will not likely experience any physical benefit, you will have the satisfaction of being a courageous volunteer in the long process of studying how scientists can use these sorts of therapies to manage or cure diseases."
- When participants are exposed to the modified T cells expressing ACTR, they are also treated with rituximab, a powerful cytotoxic agent against B-cell malignancies. However, the protocol does not adequately describe the potential risks of rituximab, which are well known and can be severe (e.g., anaphylaxis, death). Other study components and procedures, such as bone marrow biopsy or aspiration and intensive follow-up, also carry risks and discomforts that are not trivial, particularly for this vulnerable patient population. These risks need to be fully addressed in the protocol and consent document.
- The investigators' response clarified references to the patients who "previously failed" more than two lines of treatment, since it was not clear whether that meant that the patients had failed because of side effects or because the prior treatment had not affected their cancer or the growth of their tumors.
- Dr. Zoloth agreed with the point made by the other reviewers that subjects may withdraw from follow-up but that no withdrawal from the intervention is possible after cell infusion.
- The goal of the proposed trial is to test both safety (toxicity) and efficacy. However, labeling the protocol as a Phase I/II study is problematic structurally, in that it sets up the pretext of an incentive to proceed (after a preconditioning regimen) to a "treatment that will either succeed or fail. Dr. Zoloth was particularly concerned about the language used, such as "if patients succeed," "if you don't fail," and "effective therapy," phrasing that is misleading and creates unrealistic expectations. As an alternative to the proposed design, the investigators should consider using an objective set of parameters for safety and initial endpoints from a Phase I trial to be used in a larger Phase II study after 24 months as an alternate approach. The subjects will be followed for an additional 13 years under a separate long-term follow-up protocol, providing a potentially broader range of endpoints.
- The participants need to be aware of how few Phase I clinical trials lead to actual benefit. The ICD refers to the planned intervention as "therapy" in several places, which could be interpreted as meaning that the intervention is effective or might be effective. The language on the risks and potential benefits of participation is not appropriate for a Phase I clinical trial and needs to be revised to better reflect the experimental nature and objectives of this first-in-human testing of the intervention. All references to therapy/treatment and benefit need to be removed (e.g., as in the sample language above).
- Because priming can produce severe effects, the investigators should consider revising the study design to wait 48 hours before giving the ACTR product.
- The DSMC needs to be fully independent—that is, outside the study and sponsor—to avoid any potential or perceived conflict of interest. Paying the members of the DSMC presents an actual

conflict of interest. The investigators should consider federal/public oversight to address this issue.

During the meeting, the following additional questions, concerns, or issues were raised by RAC members:

- The reviewers found the presentation to be clear and their concerns and questions to be well addressed. They went through their comments and the investigators' responses to their queries and suggestions.
- Dr. Pilewski requested additional details about the parameters that will be used as a threshold for T-cell persistence, particularly to define the population of patients who stay on rituximab even in the absence of T-cell persistence. In addition, he noted that the investigators are in the process of revising and refining the inclusion criteria, particularly regarding how patients with chemotherapy-refractory disease are defined for this study. Additional detail is needed on the assessments that will be done as part of the standardized follow-up period.
- Dr. Pilewski noted that the exclusion criteria and ongoing monitoring will be helpful in assessing safety and identifying off-target effects. He inquired about the status of the Singapore pilot study in forming these aspects of the proposed protocol.
- Dr. Kiem asked about the rationale for the starting dose for the Phase I arm of the protocol. He also asked about the conditioning regimen that will be used in the proposed trial.
- Dr. DiGusto noted that, to date, clinical testing of the T-cell construct has been within the context of a transient expression system, which is self-limiting. In the proposed trial, however, the expression system will be permanently placed in T cells. The responses between these systems are likely to differ, in that the planned study will have a persistent population in which a T-cell receptor signal is being induced. Details of a plan for how the investigators will control for any cross-linking via Fc receptors, which is particularly likely with infusion of IVIG (intravenous immunoglobulin), should be provided.
- Another question focused on the potential for antigen-antibody complexes to generate serious toxicity and whether steroids or life-long immunosuppression would be sufficient for such a response. For example, the presence of glomerulonephritis could cause significant reactivation of the modified T cells and, as a result, considerable injury. The investigators were asked whether this situation was a parameter that would be part of the safety monitoring for the proposed study.
- Dr. Hearing noted that antigen-antibody complexes could generate serious toxicity and questioned whether steroids will be sufficient to address a T-cell response.

D. Investigator Response

1. Written Responses to RAC Reviews

Dr. Vasconcelles clarified in both the written response to the reviewers' comments and during the meeting that information regarding dose estimation and data from the ongoing Phase I pilot clinical study in Singapore that were redacted in the original documents submitted for review have been unredacted and made available to the RAC. In addition, in an effort to allow for a thorough review of the viral construct, the list of viral vector elements for the proposed trial has also been provided. The investigators noted that the retroviral construct and packaging cell line to be used in the study were chosen because these elements have been used safely in previous clinical trials.

During Phase I of the planned United States-based dose escalation clinical study, three subjects will receive an initial target dose of 0.5×10^6 ACTR T-cell product/kg biweekly (bw) in combination with rituximab. If tolerated, the dose level will be escalated to the next dose of 1.5×10^6 ACTR T-cell product/kg bw in combination with rituximab for the next three subjects. If this dose is tolerated, the dose level will be escalated to the target dose of 5×10^6 ACTR T-cell product/kg bw in combination with rituximab.

The investigators have used two methods to guide dose selection for the planned study. First, they surveyed the current published clinical trials based on similar construct design and alternate construct designs to define the range of doses used. Second, they reviewed the experience in an ongoing Phase I

ACTR mRNA pilot study (Singapore pilot study) to estimate a safe starting dose. This study was approved by the Singapore Health Sciences Authority and is currently ongoing at the National University Hospital (Singapore) and Singapore General Hospital. The use of published literature is supported by the fact that the ACTR therapy uses the identical signaling domains as several CAR T constructs that have already proceeded into early clinical trials. A survey of the dose range used in these studies confirms doses between 1.5×10^5 and 1.6×10^7 CAR T-positive cells/kg bw, with a median dose of 7.4×10^6 . The ongoing Singapore pilot study supports this proposal and has dosed three patients with an intended starting dose of 0.5×10^6 and a second dose cohort of 0.5×10^7 ACTR T cells/kg bw.

Similar to the CAR T-cell field, persistence of ACTR T-cells will be measured via both quantitative PCR (qPCR) and flow cytometry. qPCR using ACTR transgene-specific primers will be performed on genomic DNA isolated from blood samples. Flow cytometry will be used to detect the percentage of CD16-positive and CD3-positive T cells in isolated PBMCs using a panel of markers to exclude other immune cell populations.

The investigators plan to obtain peripheral blood for these analyses at two time points at baseline (prior to ACTR T-cell administration), at least weekly through day 28, and before and after each subsequent dose of rituximab administration. The definition of what constitutes threshold(s) for ACTR T-cell persistence has not yet been established. As currently written, the protocol states that subjects may receive additional cycles of rituximab as long as ACTR T cells persist. The study team is considering modifying the protocol to remove this requirement and to allow subjects to receive rituximab treatment independent of ACTR T-cell persistence. Per this modification, rituximab treatment would be based on demonstration of clinical benefit (i.e., at least stable disease). The investigators will submit an amended version of the protocol to the RAC for review, as required, to provide for this change.

The DEC membership is planned to include the Phase I study investigators and the sponsor-designated medical monitor. Responsibilities and accountability of the DEC will be governed by a DEC charter. The DSMC membership is planned to be independent of the study sponsor and the clinical investigators and will include experts in the fields of oncology drug development, medical oncology, clinical immunology, rheumatology or internal medicine, and/or drug safety. Similar to the DEC, responsibilities and accountability of the DSMC will be governed by a DSMC charter, including its relationship to the DEC. The investigators state that accountability for dose escalation decisions during Phase I is best held by the clinical trial investigators and medical monitor most familiar with the specific clinical scenarios emerging among these first-in-human clinical trial subjects. As the clinical program with ACTR T-cells progresses, however, it will be important to expand participation for interpreting and managing the emerging safety profile of the investigational product, through the creation of a DSMC composed of subject matter experts with complementary expertise to other stakeholders. This approach is intended to support an environment that facilitates the most robust insights and recommendations to the Sponsor regarding safety and risk/benefit assessments in support of the clinical development programs that include ACTR T cells.

The investigators agree that stable disease following the subject's immediate prior therapy, even for less than 12 months (as currently proposed in the study eligibility criteria), may not appropriately define the intended patient population for the proposed trial. The study team is in the process of reviewing this eligibility criterion. The objective is to define patient eligibility criteria that appropriately restrict patient enrollment to those for whom no reasonable therapy is available and who have demonstrated clear evidence of progression of disease (or an equivalent clinical scenario) following their most recent prior treatment regimen. The refined eligibility criteria will be submitted to the RAC in a protocol amendment.

The investigators concur with the comment regarding the inconsistency of IgG replacement therapies and are working toward a standardized approach for intravenous immunoglobulin (IVIG) replacement. This change will be provided to the RAC in an amended version of the protocol, when available.

The investigators plan to modify the existing protocol to accommodate full adverse event reporting during the entire study period. Following study entry, the study period will continue for subjects until one of the following criteria are met: (1) documented disease progression, (2) 30 days beyond the last dose of rituximab, or (3) 365 days from study entry, whichever occurs first. During the study period, subjects will

have study visits at least weekly between day -6 (the first day of the conditioning regimen) and day 28, and approximately every 10 days if rituximab dosing is continued.

Following the completion of rituximab administration, subjects will be enrolled in a long-term follow-up study with the intent of follow-up for up to 15 years from the time of study entry into the Phase I/II protocol. The frequency and intensity of subject visits during long-term follow-up is intended to vary, depending on whether the subject enters long-term follow-up with an ongoing measurable response of at least stable disease attributable to the study intervention. Adverse event collection and reporting during the extended follow-up period is intended to meet expectations of existing regulatory guidance pertaining to the long-term follow-up of trial subjects following investigational products containing recombinant DNA. The long-term study will include continued follow-up of any unresolved adverse events arising in the intervention study, as well as additional SAEs and predefined medical events of interest anticipated to be attributable to the combination therapy.

The investigators acknowledge the potential risk of viral reactivation of CMV following the proposed treatment regimen in the anticipated study population. The investigators will provide the RAC the interval and duration of CMV assessments and treatment guidance with re-activation in an amended protocol.

Regarding the role of preclinical studies in informing the dose-escalation phase of the proposed study, the investigators explained that adoptively transferred T-cells do not expand in immunodeficient mouse models to the same extent seen in an autologous human setting, so it is not informative to extrapolate cell dose requirements or thresholds from mouse models to humans. In addition, *in vivo* efficacy similar to that reported in Kudo et al. (*Cancer Research*, 2014) has been observed, but with an approximately 10-fold lower dose of ACTR T-cells. Results of previous CAR T cell studies and the team's current experience with the Singapore pilot study have been used to select cell doses for the proposed trial. The planned starting dose of 0.5×10^6 ACTR T-cell product/kg bw is lower than the effective dose in most CART studies.

The investigators noted that the chemotherapy drugs and doses listed in the ICD should not be redacted in the protocol. The investigators will change language in the consent document to state that the primary endpoint for both parts of study will be safety but pointed out that tumor response will be assessed as a secondary endpoint. The ICD will be revised regarding the meaning of the term "withdrawal" within the context of this gene therapy trial, as recommended. The investigators agreed with the other recommendations and suggestions regarding the ICD and have modified or will revise the proposed consent document accordingly.

2. Responses to RAC Discussion Questions

Dr. Vasconcelles found the reviewers' comments germane to the study. He noted that the team had received Dr. Zoloth's review the day before the meeting and that the investigators had tried to address as many of the issues and questions that she and the other reviewers raised during the presentation as possible.

Dr. Vasconcelles agreed that the alternative to the proposed intervention will likely be palliative care alone, which, in addition to participation in the clinical study, will be critical to the patients' optimal care. Patients enrolled in the trial will have already received rituximab as a single agent. This pre-study requirement provides a reasonably high probability that subjects have tolerated the drug and that the side-effect profile for rituximab for a particular patient will be well understood. Preclinical data to date indicate that the ACTR T-cell infusion does not affect the adverse events associated with the patients' underlying disease. Thus, proceeding with the combination seems to be a reasonable decision. As the program progresses, the contribution of effects of the two agents together will be better understood.

The investigators acknowledged that providing payment to DSMC presents a conflict of interest. Dr. Vasconcelles noted that the sponsor of the Investigational New Drug (IND), Unum Therapeutics, will take accountability for the investigational application. However, the DSMC charter is clear that the members of the DSMC will be completely independent of the study and the IND sponsor and that they will

have unfettered access to all clinical trial data. The DSMC's recommendations will be presented independently from both the clinical investigators and the IND sponsor. This information will be clarified in the protocol.

The threshold for defining T-cell persistence in the clinical setting will be established by both PCR and flow cytometry and through the validation of those assays. This testing is underway.

Full safety assessments will be performed not only during the DLT assessment period (the first 28 days post-infusion) but also for any patient who continues with single-agent rituximab. In addition, as patients roll over to the long-term follow-up (LTFU), both ongoing AEs at the time of entry into the LTFU study and unresolved SAEs or SAEs that may be related to or expected from the ACTR T-cell intervention will be monitored. Medical events of interest will also be tracked.

Enrollment into the proposed trial probably will not start for several months. During this time, the pilot Singapore study will continue to inform the proposed protocol as details of the trial are finalized. The starting T-cell dose for the trial is based on data from the Singapore study and other available data. The investigators have taken a conservative approach to deciding on the dose levels for the planned trial. The initial dose will be 0.5×10^6 ; the dose level will then be escalated in two steps to 5×10^6 . Thus far, patients in the Singapore pilot study have been dosed at 10^6 (three subjects) and 10^7 (one subject). Dr. Poon noted that there have not been any unexpected SAEs in the Singapore study to date. Of the four subjects enrolled thus far, only the patient at the highest dose has had an SAE (i.e., neutropenia). No CRS has been observed. The safety monitoring plan for the proposed trial will include individual subject monitoring during immunoglobulin infusion, whether the infusion is done in the clinic or possibly in the hospital setting for the first few patients. If any severe adverse events were to occur, the team would rely on approaches and interventions used in other programs (e.g., steroids). Incorporation of a suicide gene into the construct is also being considered. Frequent monitoring during the study period will help identify and better understand the types of events that might occur with intermittent administration of an exogenous antibody (rituximab) and how that may affect persistence of ACTR. The safety monitoring/management plan will be delineated in the protocol, and staff at all sites will be educated as to how to address different scenarios. Dr. Vasconcelles noted that the study sites for the protocol are transplant centers and/or have had prior experience with other CAR T cell therapies and are very adept at responding to these types of situations. The best approach for addressing antibody-antigen toxicity is being assessed in the pilot study and will also be informed with full safety monitoring.

The conditioning regimen that will be used in the proposed trial is among the most commonly used regimen based on the literature. The regimen is somewhat different from that used in the pilot Singapore study, which allows for somewhat more flexibility in the regimens that can be used. For the proposed multi-institution trial, the team preferred a more standardized approach.

E. Public Comment

No comments from the public were offered.

F. Synopsis of RAC Discussion and RAC Observations and Recommendations

The following observations and recommendations were made during the RAC's in-depth review and public discussion:

- The investigators should consider splitting the protocol into two separate trials, a Phase I study and a Phase II study, instead of combining the two studies under one protocol.
- Provide a plan for assessing autoimmune complications and for managing autoimmunity, including autoimmune glomerulonephritis.
- To reduce conflict of interest, ensure that the DSMC is truly independent of the protocol and the sponsor and remove payment to DSMC members.
- The consent needs to stress that patients are enrolled in a Phase I/II study and that the aims of the study are to assess the safety of different doses of an experimental intervention. The consent

needs to convey that the experimental intervention is not a treatment and that there is no direct (medical) benefit from participating in the study.

- The consent should clarify that the patients' condition is fatal and delineate the risks of participation. Palliative care should be offered or provided as part of the protocol.

G. Committee Motion 6

Dr. Kiem summarized the RAC recommendations to be included in the letter to the investigators, expressing the comments and concerns of the RAC. Dr. Kiem requested a vote, and the RAC approved these summarized recommendations by a vote of 13 in favor, 0 opposed, 0 abstentions, and 0 recusals.

VIII. Discussion Regarding the Deliberate Transfer of Chloramphenicol Resistance to *Rickettsia felis*, *R. rickettsii*, and *R. typhi*

Moderators/Chairs: Drs. Whitley and Wooley

Presenter: Olaf Schneewind, M.D., Ph.D., University of Chicago

A. Presentation by Dr. Schneewind

Dr. Schneewind provided background information on the characteristics and prevalence of rickettsiae, how the organisms are transmitted, and how infections are treated. In addition, he described the request to conduct research involving deliberate transfer of chloramphenicol resistance to four different *Rickettsia* species and the generation of a shuttle vector using one of these species.

Rickettsiae are small gram-negative aerobic coccobacillary α -proteobacteria. They are obligate intracellular bacteria with a life cycle that involves both vertebrate and invertebrate hosts. Rickettsiae depend on hematophagous arthropods as vectors and primary reservoirs. Small mammals serve as amplifying hosts. In humans, rickettsiae invade endothelial cells and cause vasculitis.

Rickettsiae are classified into four groups:

- The spotted fever group (SFG), including *R. conorii* and *R. rickettsii*
- The typhus group (TG), including *R. prowazekii* and *R. typhi* (*R. mooseri*)
- The transitional group (TRG), including *R. felis* and *R. akari*
- The ancestral group (AG), including *R. belli*

Rickettsial species were originally discovered in Montana by Howard Taylor Ricketts. Because there are no available genetic systems to study pathogenic rickettsial species, very little is known about these organisms. The proposal is a request to gain knowledge about this species solely for research purposes.

The most common rickettsial disease in the Americas is Rocky Mountain spotted fever (RMSF), which is caused by *R. rickettsii* and transmitted by ticks. *R. rickettsii* and RMSF are confined to the United States. In contrast, murine typhus, which is caused by *R. typhi*, is found worldwide, with fleas as the vector. *R. felis* has worldwide distribution and causes a flea-borne spotted fever-type disease in some vertebrates. The main symptoms of rickettsial diseases are fever, headache, abdominal pain, vomiting, muscle pain, and rash. In contrast with diseases associated with other species, *R. felis* does not cause disease in humans in a manner that has been characterized for other species.

The availability of treatment of RMSF has led to a drop in the fatality rate associated with this disease, from 25 percent in the 1930s to less than 1 percent in 2007. Of the 6,388 cases of RMSF reported between 1981 and 1998, 213 patients died (3.3 percent). The death rates for these patients differed considerably based on the treatment they received. Approximately 1.5 percent (60/4,059) of the patients treated with tetracycline died during this period, while 7.6 percent (79/1,038) of patients given chloramphenicol died. Between 1999 and 2007, of the 7,738 reported cases of Rocky Mountain spotted fever, 1,845 (23.8 percent) were hospitalized and 40 (0.5 percent) died. The number of cases of murine

(endemic) typhus (*R. typhi*) has also dropped significantly worldwide, from more than 5,000 cases annually in the 1940s. In the 1980s, murine typhus was endemic in parts of California, Hawaii, and Texas, with 3 to 21 cases reported annually in California, 5 to 6 cases in Hawaii, and 9 to 72 cases in Texas. The case fatality rate of murine typhus with antimicrobial therapy is less than 1 percent; without antimicrobial therapy, it is approximately 4 percent. In 2002, a total of 47 cases of murine typhus were identified in Hawaii, owing to an outbreak, and in 2008, 33 confirmed cases appeared in Austin, Texas. Of the 33 patients in Texas, 23 (70 percent) were hospitalized, and none died. Most of these patients were treated with tetracycline. About 100 human infection cases of *R. felis* have been reported worldwide in the past two decades, but no known deaths are attributable to *R. felis*. Human infection is most likely due to close proximity to pets. Recently reported cases of *R. felis* infection have been diagnosed using serologic testing and PCR analysis.

Rickettsial organisms are susceptible to a broad range of antibiotics, with the exception of erythromycin. In the case of erythromycin, *R. rickettsii* has a moderate resistance level, whereas that of *R. felis* is considerably higher. Resistance to doxycycline, the most important antimicrobial in treatment of rickettsial diseases, has never been reported. The clinical effectiveness of antibiotic treatment for rickettsial infections has been studied in both randomized and non-randomized trials. Two studies (a double-blind randomized study and a retrospective study) suggest that ciprofloxacin (versus doxycycline) is associated with a significantly longer hospital stay and delayed defervescence in individuals with *R. typhi* infection. In one nonrandomized prospective study, data collected from 87 patients admitted to a hospital in Greece between 1993 and 1998 showed that the duration of fever was shorter for patients who received doxycycline versus ciprofloxacin or chloramphenicol. The combinations of chloramphenicol plus doxycycline or ciprofloxacin did not improve the clinical outcome of this disease over monotherapy with doxycycline. No deaths were reported for this cohort. In contrast, two independent cases (in Nepal and Cyprus) reported successful use of ciprofloxacin. The treatment of epidemic typhus, which is caused by *R. prowazekii* and is not part of the current proposal, has been attempted with both chloramphenicol and doxycycline, as well as with Bactrim (sulfamethoxazole/trimethoprim), trimethoprim, and sulfamethoxazole. Data reported in 1973 show that doxycycline is superior to both chloramphenicol and sulfamethoxazole/trimethoprim in reducing the defervescence period of patients with epidemic typhus.

The clinical effectiveness of a newer macrolide antimicrobial, clarithromycin, has been tested in Europe, where the spotted fever disease is caused by *R. conorii* and the complexes are referred to as Mediterranean spotted fever (MSF). In one randomized controlled study of 51 children with suspected MSF, clarithromycin was the superior therapeutic agent compared with chloramphenicol. Another study of the clinical effectiveness of antibiotic treatment for *R. conorii* found no significant differences among three drugs: chloramphenicol, clarithromycin, and azithromycin. A retrospective analysis of antibiotic treatment of 415 children hospitalized with *R. conorii* infection between 1997 and 2004 and an open-label randomized controlled study of 87 children with MSF found no significant differences in effectiveness between chloramphenicol and azithromycin. Rickettsial organisms are also very susceptible to another antimicrobial drug that is not widely used in the United States, josamycin, which has been shown to be as effective as doxycycline in treating MSF.

The Centers for Disease Control and Prevention (CDC) identify doxycycline as the drug of choice for treatment of all rickettsiosis in children and adults. The American Academy of Pediatrics Committee on Infectious Diseases revised its recommendations in 1997 and identified doxycycline as a drug of choice for treating presumed or confirmed RMSF in children of any age. Resistance to doxycycline and relapses in symptoms after the completion of the recommended course of treatment have not been documented. Potential side effects of doxycycline include gastrointestinal irritation and increased photosensitivity. A prospective study of children treated with doxycycline for RMSF demonstrated a minimal risk of tooth staining, which was the primary concern for not using doxycycline in pediatric populations. CDC recommends alternative therapies, including chloramphenicol, in cases of life-threatening allergies to doxycycline and in some pregnant patients for whom the clinical course of RMSF appears mild. The oral form of chloramphenicol is no longer available in the United States, but the intravenous form may be used. Side effects of chloramphenicol can be very serious and include aplastic anemia, leukemia, sudden death, and gray baby syndrome. Other alternative treatments include rifampin with erythromycin in combination or josamycin alone.

Following review of this background information on rickettsial species, diseases, and treatments, Dr. Schneewind shifted the presentation to genetic manipulation of rickettsiae, which is the focus of the proposed research, and then to the details of the request. Genetic manipulation of *Rickettsia* species has relied on antibiotics for selection; these have included rifampin, erythromycin, and chloramphenicol. Rifampin has been used in the past as an antimicrobial and as a genetic selection marker in various rickettsial species. In *R. prowazekii* and *R. rickettsii*, random transposon mutagenesis has been attempted with a transposon that carries the rifampin resistance determinant. Erythromycin has been used as a resistance determinant in *R. prowazekii*, while another antibiotic resistance marker, chloramphenicol acetyltransferase (CAT), a protein synthesis inhibitor, has been used in *R. prowazekii* and in two *Rickettsia* strains in the spotted fever group, *R. monacensis* and *R. montanensis* (aka *R. montana*).

Dr. Schneewind noted the drawbacks of using rifampin instead of CAT in the proposed genetic experiments. As a drug that interacts with RNA polymerase, rifampin is subject to well-documented spontaneous resistance mechanisms that occur at a background mutational frequency rate of about 10^{-7} to 10^{-8} . The transformation efficiency of *Rickettsia* spp., obtained by isolating and then electroporating the organism with purified DNA, ranges in the order of about 10^{-8} , slightly higher than or equivalent to the spontaneous mutagenesis frequency that would yield rifampin-resistant rickettsial determinants. This, in turn, is the pitfall and limitation of rifampin as a selectable marker in *Rickettsia*. In contrast, the spontaneous resistance frequency for chloramphenicol typically exceeds 10^{-10} to 10^{-11} and does not generate level of background noise of spontaneous rifampin resistance. This difference is particularly important because rickettsial species are propagated inside mammalian cells and must be placed under continuous selection to maintain the resistance trait and thus differentiate them from the sensitive organisms.

The research proposal submitted by Dr. Schneewind involves four organisms: *R. rickettsii*, the causative agent of RMSF (the Sheila Smith stain, a pathogenic isolate); *R. typhi*, a member of the typhus group (the Wilmington strain); *R. conorii*, a member of the spotted fever group, and *R. felis* (the LSU isolate), which infects humans but is nonpathogenic. Two experiments are planned, and the antibiotic resistance marker that is proposed for these experiments is CAT. The first involves a transposon mutagenesis with an EZ-Tn5-type transposon. This is an engineered transposon that involves a variant transposase from the Tn5 transposon of *E. coli* that forms a heteroduplex with a DNA; in this case, it is a mini-transposon carrying the CAT transposon itself. There is no known Tn5 transposase in the *Rickettsia* genome; the mini-transposon that is generated does not carry the genetic determinant for the transposase and, as such, is not mobilizable. The protein-DNA complex will be electroporated into the recipient rickettsiae, and the organisms will then be selected for insertional lesions carrying the mini-transposon. The second experiment involves the generation of shuttle vectors that can replicate in both *E. coli* and *R. felis*. Plasmid vectors are rarely found in rickettsial species, but *R. felis* is known to carry a determinant called pRF, which is thought to be a conjugative plasmid that has the capacity to transfer plasmids by a conjugative transfer mechanism. The newly constructed shuttle vectors will be produced by fusing the natural plasmid pRF from *R. felis* to the pET/pUC plasmid from *E. coli* with the CAT antibiotic marker. The shuttle vectors will then be used to clone genes of interest in *E. coli* for complementation studies in *R. felis* and potentially other *Rickettsia* following plasmid transformation.

The primary goals of the proposed research are to introduce chloramphenicol resistance into several *Rickettsia* strains to generate a mutant library in *R. rickettsii*, *R. conorii*, and/or *R. typhi*, using CAT as a selectable marker, and to generate shuttle vectors that can replicate both in *E. coli* and rickettsia, using *R. felis*. Antibiotic sensitivity will be evaluated. All mutants harboring CAT genes will be subjected to *in vitro* tissue culture assays to determine their susceptibility to chloramphenicol, josamycin, and doxycycline; MIC values will be compared to those of wild-type strains. Any mutant strains exhibiting increased resistance to doxycycline will be destroyed immediately. Mutants will be screened for their ability to invade and replicate within mammalian cells *in vitro*. Newly identified virulence factors will be carefully examined for their requirement to cause disease in animal models for rickettsial diseases.

The proposed experiments will be conducted and contained in the BSL3 facility at the Howard T. Ricketts Laboratory (HTRL) located within Argonne National Laboratory, about 22 miles outside of Chicago. Dr. Schneewind noted that the HTRL facilities are designed to meet or exceed requirements outlined by the CDC, NIH, and Occupational Safety and Health Administration (OSHA) specifications for biocontainment. The HTRL is also certified by CDC as part of the Select Agent Program. On an annual basis, all personnel will be required to undergo extensive training required by the University of Chicago and HTRL to participate in this research and on an annual basis. In addition, all individuals entering the BSL3 facility must be cleared by the Federal Select Agent Program. A comprehensive emergency response plan in case of occupational exposure is in place and was delineated at the RAC meeting. The recommended therapy for exposure is a prophylactic treatment of 100 mg doxycycline orally every 12 hours; alternative antibiotic therapies will be considered by the biosafety committee of the University of Chicago.

The primary goals of the proposed research are to introduce chloramphenicol resistance into four *Rickettsia* strains (*R. rickettsii*, *R. conorii*, *R. typhi*, and *R. felis*), to generate a mutant library in *R. rickettsii*, *R. conorii*, and/or *R. typhi* by using CAT as a selectable marker, and to generate shuttle vectors that can replicate both in *E. coli* and *R. felis* as well as other *Rickettsia*. Antibiotic sensitivity will be evaluated. All mutants harboring CAT genes will be subjected to *in vitro* tissue culture assay to determine susceptibility to chloramphenicol, josamycin, and doxycycline; minimum inhibitory concentration (MIC) values will be compared to those of wild-type strains. Any mutant strains exhibiting an increased level of resistance to doxycycline will be destroyed immediately. Mutants will be screened for their ability to invade and replicate within mammalian cells *in vitro*. Newly identified virulence factors will be carefully examined for their requirement to cause disease in animal models for rickettsial diseases.

B. Presentation by Dr. Wooley

Dr. Wooley reviewed the request and the role of the RAC in this process, provided a brief history of prior decisions for similar requests, and presented the issues raised by the RAC Biosafety Working Group (BSWG) for consideration during the current meeting. The presentation was put together by Drs. Wooley and Whitley and the NIH/OBA staff and was reviewed by the members of the BSWG who attended the conference call to discuss Dr. Schneewind's request.

The current request involves four rickettsia species, three of which are Risk Group 3 organisms (*R. rickettsii*, *R. conorii*, and *R. typhi*). The official Risk Group classification for *R. felis* is not clear. The proposed research has two main parts. The first is to introduce chloramphenicol as a selectable marker, using a transposon system that has been modified so that it cannot mobilize unlike normal transposons that usually insert, excise, and move around in the genome. The second part involves creating a shuttle vector. Generally shuttle vectors can propagate in more than one species; in this case, the investigators plan to propagate the shuttle vector in *E. coli* and *Rickettsia* by fusing a plasmid from *E. coli* and the pRF plasmid from *R. felis*, which presumably has the ability to conjugate. As shown in Dr. Schneewind's presentation, the bacterial cells contact each other using proteins and then transfer genetic information via this protein apparatus.

This request is considered a major action and is being brought to the RAC in accordance with the *NIH Guidelines*, Section III-A-1-a, which states, "The deliberate transfer of a drug resistance trait to microorganisms that are not known to acquire the trait naturally...if such acquisition could compromise the ability to control disease agents in humans, veterinary medicine, or agriculture, will be reviewed by the RAC." The RAC approved a prior request in 2007 that permitted the conduct of experiments to deliberately introduce a gene encoding chloramphenicol resistance into *R. conorii*. In another major action, also decided in 2007, the RAC disapproved a request to introduce chloramphenicol resistance into *R. typhi*. New requests to transfer chloramphenicol resistance into *R. conorii* have not been handled as major actions since these 2007 decisions. Rather, such requests have been handled administratively by NIH staff.

A number of stipulations were made in the previous RAC decision that allowed the transfer of chloramphenicol resistance into *R. conorii*, including the following:

- Only the laboratory making the request is permitted to carry out this experiment.
- The experiment must be conducted at BL-3 physical containment level with restricted access.
- Only well-trained personnel essential to the conduct of the experiment are permitted in the containment area. A standard training procedure should be in place for initial and ongoing training.
- The laboratory should be off-limits to anyone with a known allergy or sensitivity to doxycycline or any other tetracycline.
- A back-up power source must be in place to maintain the laboratory's security system, which is controlled by a computer.
- A unique identifier should be engineered into the genome of the drug-resistant strain to facilitate the identification of the laboratory-created strains.
- The health surveillance program for laboratory personnel should involve storage of a baseline blood sample, training of all personnel, and development of a detailed standard operation procedure (SOP) in the case of a laboratory exposure or infection.

The charge to the RAC at the current meeting was to make a preliminary assessment of the request based on input from the BSWG, which met via teleconference to review the request. A final determination by the RAC will be made at an upcoming meeting.

The BSWG identified the following questions and issues for consideration during the recent conference call:

- What is the natural resistance to chloramphenicol for these species of *Rickettsia*?

To the best of their knowledge, participants on the call were not aware of any reported naturally occurring chloramphenicol resistance in *Rickettsia*. Treatment failure data, which may indicate resistance, is crude and uncontrolled; there are reasons for treatment failure other than resistance, such as starting treatment too late, an immunocompromised host, and other host factors.

- What are the therapeutically available antibiotics for each *Rickettsia* species?

Regarding therapeutically available antibiotics for each *Rickettsia* species, the first-line treatment in the United States is doxycycline, a member of the tetracycline family. The second-line treatment is chloramphenicol, which has more side effects than doxycycline and is available in the US only in IV form. In some countries, chloramphenicol is the first-line treatment due to availability and cost. More information is needed on worldwide usage of chloramphenicol. Drugs showing promise in clinical trials and anecdotal reports include ciprofloxacin, a member of the quinolone family, and azithromycin, a member of the macrolide family.

- What are the public health considerations of introducing chloramphenicol resistance in each organism?

Chloramphenicol is the first-line treatment in some countries and in certain situations (e.g., allergy to doxycycline, pregnancy). Introducing chloramphenicol resistance into *Rickettsia*, however, would remove one of only two available treatment options that are known to be effective. Some researchers believe that public health risk is low, because the disease does not spread directly from human to human; however, if resistance were introduced into the reservoir population, it could still spread via vectors (ticks/fleas) and contaminate the environment.

- Is the current requirement of experimenting with *R. conorii* first still valid? If so, is this applicable to all or limited to *R. typhi*?

There are no published data indicating the the use CAT as a selectable marker has been successful in experiments with *R. conorii*. The BSWG did not recommend requiring proof-of-

principle first with *R. conorii* before proceeding with experiments in *R. typhi*. Given this recommendation, excluding any experiments involving *R. typhi*, further consideration about whether proof-of-principle in *R. conorii* should be demonstrated before proceeding with *R. rickettsii* and *R. felis* is warranted.

Several additional concerns were identified by the BSWG. Inserting a chloramphenicol resistance gene into a shuttle vector that has conjugation capability is above and beyond simply using chloramphenicol as a selectable marker. In this experiment, the chloramphenicol resistance gene would be placed on a molecular construct that can spread. The host range for *Rickettsia* is not well defined, and there is still a lot to learn. Some members thought that additional research to develop methods of selection other than through antibiotic use should be encouraged. In addition, there were concerns about the similarity of *R. prowazekii* (the causative agent of epidemic typhus) to *R. typhi* and the potential for explosive epidemics.

Additional questions raised by individual members of the BSWG included the following:

- Should the introduction of chloramphenicol resistance (Cm^R) into *R. typhi*, *R. rickettsii*, and *R. felis* be allowed to proceed?
- Given that human-to-human transfer is more likely through arthropod transmission, should work with Cm^R rickettsia in arthropods be allowed to proceed?
- Excluding any experiments involving *R. typhi*, would proof-of-principle in *R. conorii* be recommended prior to proceeding with *R. rickettsii* and *R. felis*?

Of the nine members present during the BSWG teleconference, most or all voted against allowing (or disagreed with) each of these additional three questions/recommendations.

The BSWG identified the following two draft recommendations for the RAC:

- The landscape for treatment of *Rickettsia* and drug resistance has not changed much since 2007.
- Because the transfer of Cm^R into *R. conorii* is not a major action, the NIH stipulations from the last major action in 2007 shall apply.

C. RAC Discussion

Dr. Zoloth requested clarification regarding the goal(s) of the proposal and whether one ultimate aim of the research is to address treatments for rickettsial diseases. Dr. Schneewind explained that the primary goals of this research are to generate insertional lesions in the chromosome of rickettsial species and to gain insights from studying the biological properties of the variants. He added that because many antimicrobials have been shown to be effective against these rickettsial species, this research is not designed to lead to improved therapeutics. However, this does not mean that the results of the research might not have translational value. Research that identifies key virulence vectors could lead to attenuation of those vectors and, in turn, to the design of subunit vaccines for the prevention of disease.

Dr. Donahue commented that the decision in 2007 was initially accompanied by very stringent requirements and was followed by the rather nebulous statement that since then, essentially all other requests have been under administrative review. He asked whether there is any information on how much deterioration, if any, has occurred in the criteria since 2007, and how much additional risk is involved should the current proposal proceed. If the RAC were to move in the direction of approval, it might be prudent to set up some contingencies, such as making the approval solely for the current request and/or stating that full review would be required if there is evidence that the initial criteria are not durable over time. Dr. Wooley noted that the prior criteria are durable and that the decision from 2007 set a precedent for that case and that all other requests thereafter were not considered major actions. Her understanding is that any new researcher who wants to introduce chloramphenicol resistance into the species has to follow all of the requirements from the 2007 decision. Dr. Jorgenson agreed with this interpretation and added that the OBA staff can review this history and clarify and confirm the requirements.

It was noted that some modifications were made to the process involved in review of these requests, in a subsection of Section III-B-2 of the *NIH Guidelines* entitled "Experiments that have been approved under Section III-A-1 as major actions under the *NIH Guidelines*." This section of the guidelines states that upon receipt and review of an application from the investigator, NIH may determine that a proposed experiment is equivalent to an experiment that has previously been approved by the NIH Director as a major action, including experiments approved prior to implementation of these changes. An experiment would only be considered equivalent if, as determined by NIH OBA, there were no substantive differences from previously approved experiments and no pertinent information had emerged since submission of the initial III-A-1-a experiment that would change the biosafety and public health considerations for the proposed experiments. If such a determination were made by NIH OBA, these experiments would not require review and approval under Section III-A. This clarification was designed to make it easier for investigators who plan to conduct an experiment that has been previously reviewed and approved by the NIH Director. If the researchers are doing the same experiments under the same conditions (e.g., containment, species), the work can proceed. If, however, these experiments are not deemed to be similar, then the investigators would need to come before the RAC to discuss a major action.

Dr. Lee requested clarification regarding use of prophylactic antibiotics. The presentation specified the antibiotics, doses, and frequency but not duration of treatment, so it is not clear whether exposures are treated as if they were in an infected patient or if there is an established shorter duration for prophylactic therapy. Dr. Schneewind explained that when individuals who work in containment are exposed to an agent, a protocol that involves consultation with occupational health physicians is followed. A hotline is set up for this purpose, and these physicians decide the plan of action on an individual case basis. In general, for exposure to *Rickettsia* species, it is likely that a full course of antimicrobial therapy (doxycycline) to prevent disease would be recommended. Dr. Dasch, an *ad hoc* consultant to the BSWG and researcher with the CDC, clarified that the recommendation in cases of laboratory exposure is to treat with doxycycline only if there are signs of actual illness, not immediately upon exposure (or possible exposure), given the mechanism of action of the drug, which is bacteriostatic, not bactericidal.

Dr. Lee inquired about the source and nature of the transposase in the system that is being used. He also asked whether the transposable element (i.e., with the transposase) that is transferred to *E. coli* becomes a serially transposable element. Dr. Schneewind noted that the origin of the EZ-Tn5 transposon is an *E. coli* transposon. The variants were isolated in Bill Reznikoff's lab at the University of Wisconsin, and a company, Epicentre, provides this transposase commercially as a protein that is free of the genetic determinants found in natural transposons. As such, the transposase protein can be mixed with defective mini-transposons that can then form a heteroduplex leading to insertion of a DNA sequence into the chromosome. However, the gene that gives rise to the transposase protein will not be introduced into *Rickettsia*. Given the mechanism of this system, there is no reason to assume that there would be any genetic transfer between organisms or that the modified *E. coli* with the inserted mini-transposon would mobilize that genetic element into *Rickettsia* in the wild.

Dr. DiGusto made several points to strike a balance between allowing the science to proceed and taking steps to prevent a disaster of any magnitude. Because effective treatment is available for rickettsial diseases, exposures would not be associated with untreatable disease. To reduce risk, the RAC should consider limiting batch size and require that the only applications allowed are those that can be addressed in other species. Otherwise, investigators will have to present a compelling justification to use a more infectious or dangerous species and meet minimal qualifications for those studies. These issues should be taken into account in considering whether to amend the existing recommendations.

Dr. Wooley pointed out that in some countries and in certain situations, chloramphenicol is the only option to treat rickettsial diseases. By potentially eliminating this useful antibiotic, the diseases may not be as treatable as in the United States, especially if these organisms were to get into the environment. This was the main concern and the driving force of the votes by the BSWG.

Dr. Chatterjee asked whether any other antibiotic resistance genes could be used instead of that for chloramphenicol which is used to treat humans with the infection. In response, Dr. Schneewind noted that antibiotic resistance alleles can be generated in microbes through spontaneous selection but that, as the

name or the procedure implies, this occurs at frequencies that are consistent with the mutational noise of chromosome replication. Such resistance can be obtained for macrolide protein synthesis inhibitors but not for chloramphenicol or tetracycline; thus, the limitation of these determinants is similar to that of rifampin. The major obstacle for rickettsial species is the very low level of transformation, which is why the selection of mutants is either impossible or very arduous and why genetic analysis of these organisms is limited. While there are other antibiotic resistance genes that could be isolated and then placed into various plasmid vectors, the rate of transformation whereby the DNA will be introduced into the recipient organism would not exceed that of spontaneous mutational frequency. In this scenario, most of the resistant organisms that would be isolated are not the desired mutants but, rather, are spontaneous resistance determinants.

Dr. Hackstadt, an *ad hoc* consultant to the BSWG and investigator at the Rocky Mountain Laboratory, noted the benefits of having other antibiotics that could be used for resistance selection.

Dr. Wooley asked whether any of the newer gene editing tools would be helpful in creating libraries of rickettsial mutants. Dr. Zoloth asked specifically whether the proposed work could be done using the CRISPR (clustered regularly interspaced short palindromic repeats)–associated protein 9 (Cas9) tool. Dr. Schneewind pointed out that these tools have the same limitations with respect to insertion of DNA and mutation frequency as described earlier. The advantage to using the mini-transposon system lacking the transposase is the ability to maximize transformation of target *Rickettsia* using electroporation.

Dr. Wooley commented that the Cas9 system might be more efficient than other tools at making mutations in the *Rickettsia* genome and then for selecting phenotypes. Dr. Schneewind noted that the Cas9 system is very elegant and works by using a protein, Cas9, a very specific RNA-guided DNA endonuclease enzyme associated with the CRISPR adaptive immunity system to generate mutations. However, because the same obstacles for the genetic transfer of material into *Rickettsia* occur with use of Cas9, it does not appear to be a remedy for this research. Dr. Cannon pointed out that it has not been established whether the CRISPR Cas9 will work in *Rickettsia* but that even if this had been demonstrated, Cas9 is not appropriate for screening, given how the system works (i.e., by making a guide RNA against a specific chromosomal target). In addition, although rifampin can be used for selection, it is problematic, because it has a background of spontaneous selection. An alternative might be to combine rifampin resistance with a molecular tag such as green fluorescent protein (GFP) to more easily select mutants with the inserted lesions versus those with background mutations. Dr. Schneewind explained that the problem with working with *Rickettsia* is that the organisms replicate inside target cells, so that sorting is not for the microbe but, rather, for the infected cells. The selection process therefore involves use of drugs that penetrate replicating, living mammalian cells and subsequently into the intracellular *Rickettsia*. Being able to enter the cell is already an obstacle for many antibiotics, and the number of cells that would be required to combine GFP screening with rifampin selection would not be feasible for the proposed research.

Dr. Schneewind has worked on this type of technology development for several different organisms, with the goal of creating large libraries of mutants and then screening the mutants for various phenotypes. These technologies have moved fields like that for *Staphylococcus aureus* research forward tremendously. The proposed research could do the same for rickettsial species. Through advances in the field, the obstacles cited can be addressed, as proposed, with a mini-transposon that does not carry the transposase itself, using the current delivery mechanism of choice, electroporation. Another option could involve generating mutations that have no selectable attributes and then analyzing each variant through whole genome sequencing to determine whether a mutational lesion is present. However, this technology is very costly and labor-intensive, does not provide a mechanism to ensure that there is only one insertion or mutation per chromosome, and is not a breakthrough in the technology that will move the field forward in as expeditious a manner as the approach described in the proposal.

Dr. Schneewind has identified what he believes is the best solution to achieve the objectives of this research and asked the RAC to assess whether the technology and plan presented is acceptable for the agents specified in the request.

Dr. Chatterjee revisited the concern about the impact of eliminating chloramphenicol as an effective and relatively inexpensive treatment for rickettsial diseases that is used more widely in other parts of the world. It was noted during the BSWG conference call that chloramphenicol is available as an over-the-counter drug in many countries and that people often purchase and use the drug in the event of a fever. Another participant pointed to the situation in Vietnam when chloramphenicol became less useful as an over-the-counter medication for treatment of typhoid fever. This resulted in a significant expansion of the number of cases of rickettsiosis. This experience suggests a suppression of background disease as a consequence of over-the-counter chloramphenicol treatment of fevers that have a rickettsial etiology and are not due to other organisms. Dr. Dasch noted that while *R. rickettsii*, the agent of RMSF, is a major concern in the United States, this species and RMSF extend through Central and South America to Argentina. Various states in Mexico, and urban areas in particular, are currently experiencing major breakouts of RMSF, demonstrating that these cases are not unique to North America. Dr. Schneewind clarified that in his presentation, he focused on U.S.-based epidemiologic data for *R. rickettsii* and RMSF and did not mean to imply that *R. rickettsii* occurs only in this country. In addition, as pointed out in Dr. Schneewind's presentation, the CDC recommends chloramphenicol as an alternative to doxycycline in cases of life-threatening allergies to doxycycline and in the first and second trimesters of pregnancy in patients for whom the clinical course of RMSF appears mild. Thus, there are clear situations in which chloramphenicol is used within the United States.

Given these concerns, Dr. Wooley reviewed excerpts from a letter (dated 5 November 2015) from Dr. Keiji Fukuda, World Health Organization (WHO) Special Representative of the Director-General for Antimicrobial Resistance, regarding chloramphenicol and the treatment of rickettsial infection and the proposed request to insert a gene for chloramphenicol resistance into several species of *Rickettsia*. In this letter, Dr. Fukuda notes that the WHO model list of essential medicines for rickettsial diseases does not include parenteral doxycycline but does include both oral and parenteral chloramphenicol. His letter concludes by saying that despite the known drawbacks related to chloramphenicol, treatment using parenteral chloramphenicol may be the sole option for severe rickettsial infections in many countries.

Dr. Dasch asked whether the investigators plan to sustain the isolate in the presence of chloramphenicol once the initial selection in the presence of chloramphenicol is successful and they confirm that they have a mutant. This is a concern in part because that natural chloramphenicol resistance has been generated by passage in the presence of the antibiotic. Dr. Schneewind replied that he has not made a rickettsial mutant that carries a chloramphenicol resistance determinant and therefore cannot answer this question. However, he has made mutations in many bacterial genomes, often with CAT as the selectable marker. Once the desired mutants have been isolated and the location of the DNA insertion identified, chloramphenicol selection is no longer needed.

Dr. Dasch shared others' concerns about the potential risks of some of the agents proposed for research under the current request. He was not convinced that the conjugation system for fusing the plasmids from *E. coli* and *R. felis* would be successful because the genes involved in plasmid transfer found in the *R. felis* pRF plasmid are not intact. As an alternative to the proposed *Rickettsia* species, consideration should be given to organisms that are less hazardous to work with and for which (unlike *R. rickettsii*) there are suitable animal models, such as *R. akari*. Use of such agents would allow some of the goals of this project, including assessing the safety of the modified organisms within the laboratory setting and demonstrating feasibility of the approaches, without the same public health concerns as those involved with the planned organisms. Dr. Hackstadt commented that while there is an argument to be made for using less virulent strains, real pathogens are needed to study pathogenesis and to identify the virulence determinants that make an agent pathogenic. Given the nature of the selected *Rickettsia* species—that is, strict obligate intracellular parasites that do not spread from person to person and that have very limited stability in the environment outside of host cells—the benefits of these new tools outweigh the risks.

To better inform this decision, it would be helpful to know what other work has been done since the 2007 decision and what knowledge has been gained from that research. For example, the description of a project at the University of Texas Medical Branch mentions the development of spontaneous resistance to single antibiotics such as chloramphenicol. Given these issues, the logic of creating resistance to this

particular antibiotic is not clear, and more detail regarding the justification for working with *R. rickettsii* and *R. typhi* was requested.

Dr. Schneewind said that he has proposed these organisms because they are disease-causing agents and he is interested in studying their biology. The proposed approach of inserting chloramphenicol resistance, which can be easily selected for, will expedite the work of creating the mutant library and learning more about the association between the mutation and phenotype. In contrast with chloramphenicol, the spontaneous mutation rate for other antibiotics is such that the investigators may not be able to identify or quickly select for the mutant organisms.

In response to a question about who will be supervising and performing the proposed experiments, Dr. Schneewind noted that Dr. Kim will be conducting the research under Dr. Schneewind's supervision. Dr. Kim is not a faculty member at the University of Chicago. Further, Dr. Schneewind will not be continuing the work of Dr. Martinez, who is no longer at the University of Chicago.

Dr. Jorgensen noted that the discussion about resistance to *Rickettsia* will continue. Since a determination regarding this issue is considered a major action, it requires input from the public. A notice for public comment will be posted in the *Federal Register*. A plan to reconvene via teleconference in January or February will be finalized pending polling of RAC members for availability.

D. Public comment

No comments from the public were offered.

IX. Containment Discussion for ΔVP30 Ebola

Moderator/Chair: Dr. Cannon

Presenters: Yoshihiro Kawaoka, DVM, Ph.D., University of Wisconsin
Peter Halfmann, Ph.D., University of Wisconsin

A. Presentation by Dr. Kawaoka

The Ebola virus is a filamentous organism whose genome is a single-stranded RNA that includes eight genes, seven of which code for structural proteins. The Ebola virus initiates infection by binding to cell surface receptors, which is followed by fusion of the viral envelope with cellular membranes and then entry of the virions into the cell. All replication cycles occur within the cytoplasm; thus, no nuclear phase is involved in this process.

Dr. Kawaoka's team has developed a method to generate infectious Ebola virus from cloned cDNA. The method requires that the full-length Ebola cDNA genome be inserted downstream of a T-7 promoter so that upon introduction of this construct into cells, together with a plasmid expressing T-7 polymerase and plasmids expressing the viral proteins NP, VP30, VP35, and L (RNA polymerase), which are essential for replication of RNA, the infectious Ebola is produced. This work was then extended to generate virus that lacks the VP30 gene, which is essential for virus replication. A neomycin-resistant gene is used in place of the VP30 gene, and when this system is introduced into cells expressing VP30 constitutively, the Delta VP30 Ebola virus is produced. Different versions of the Delta VP30 Ebola virus exist, depending on the insert used to replace the VP30 gene. Because VP30 is essential for virus replication, the Delta VP30 virus does not replicate in normal (wild-type) cells, but it does replicate in cells expressing VP30 protein.

VP30 is a transcription activator that is essential for Ebola virus replication. No known cellular protein can substitute for VP30. The Ebola ΔVP30 virus system is designed to prevent or minimize the possibility of generating replication-competent viruses. In the ΔVP30 system, the VP30 open reading frame is replaced with a reporter gene, and cells expressing VP30 protein do not have the non-coding region of this segment of the genome. Thus, the Ebola ΔVP30 system lacks a gene that is critical for replication. The

product has no overlapping sequences that could lead to homologous recombination. A plasmid encoding VP30 is not used to generate Ebola Δ VP30 viruses, rather the VP30 gene has been inserted, following retroviral transduction, into the cellular chromosome. There is no selective pressure for the generation of replication-competent viruses, because the VP30 protein is produced constitutively. Given these parameters, gene conversion and the possibility of reversion to a wild-type virus indicate that the generation of replication-competent viruses is highly unlikely.

The Ebola Δ VP30 virus system is approved under enhanced BSL3 (BSL3+) conditions. The enhancements include the use of scrubs (after removal of street clothes) and the following personal protective equipment:

- A water-resistant Tyvek® suit
- Shoe covers for feet
- Nitrile gloves
- Dedicated shoes with a pair of shoe covers
- A powered air purifying respirator (PAPR) or an N100 respirator with a hair cover and safety glasses

A shower is mandatory upon exit from the lab.

Steps taken to date to lower the Ebola Δ VP30 virus system from BSL4 to BSL3+ include the following:

- Generation of an Ebola Δ VP30-neo virus under BSL4 containment that possessed the neomycin resistance gene instead of the VP30 gene. Amplification of the defective Ebola Δ VP30-neo virus was performed by complementation of the VP30 function using retrovirally transduced Vero cells constitutively expressing the VP30 protein (VeroVP30 cells).
- Passaging the virus seven consecutive times in VeroVP30 cells at an MOI of 0.01 to favor many rounds of virus replication and consequently to also favor the possibility of recombination and rescue of wild-type Ebola virus.
- At each passage, infection of VeroVP30 cells with Ebola Δ VP30 virus resulted in cytopathic effects; however, no replicating virus was detected in wild-type Vero cells.
- Isolating viral RNA at passage 7 and sequencing a genomic fragment spanning the Ebola Δ VP30 neo gene region. Analysis of these products revealed that the sequences of 20 molecular clones were identical to the Ebola Δ VP30 cDNA construct, attesting to the stability of the viral genome.
- Performing three blind passages of the Ebola Δ VP30-neo virus harvested at passage 7 by infecting wild-type Vero cells at an MOI of 5 at each of three blind passes.
- Testing the culture supernatant for the presence of any replicating virus by incubating with wild-type Vero cells and then performing plaque assays and immunostaining with anti-Ebola VP40 antibody.
- This entire process was repeated three times starting from the generation of Ebola Δ VP30-neo virus from cDNA and supporting expression plasmids. No infectious virus was detected at any stage of viral replication.

Following these multiple steps, the team generated Ebola Δ VP30 viruses *de novo* under BSL3+ containment. Additional *in vitro* and *in vivo* experiments were performed, with approval from the Centers for Disease Control and Prevention (CDC), to evaluate the safety of defective Ebola Δ VP30 virus. Challenge studies using STAT-1 knockout mice (which are highly susceptible to wild-type Ebola virus infection), demonstrated no signs of illness or death with this animal model during the entire observation period (28 days) after inoculation with Ebola Δ VP30-neo virus, Vero VP30 cells, or a mixture of both. In contrast, mice given wild-type Ebola virus showed signs of disease including ruffled fur and weight loss within 3 days after inoculation, and all animals succumbed to infection by day 6.

No materials currently under BSL3+ containment will be transitioned to BSL2 containment. However, the team is planning to generate Ebola Δ VP30 viruses under BSL2 containment starting again *de novo* from defective genomic cDNA, supporting expression plasmids and VP30 complementing cells. For daily tissue culture experiments, the typical yield of Ebola Δ VP30 viruses is up to 10^7 focus-forming units

(FFU), and for vaccine experiments, up to 10^8 FFU of Ebola Δ VP30 viruses have been produced at one time. *In vitro* safety testing for the Ebola Δ VP30-GFP virus was performed once, and viruses of different passage histories were used for *in vitro* safety testing. Virus that had undergone between 1 and 10 passages was used to inoculate wild-type Vero cells. Ten days after inoculation, cells were immunostained using anti-Ebola VP40 antibody to detect replicating virus. No virus replication was detected.

Ebola Δ VP30-neo and Ebola Δ VP30-GFP viruses from passage 2 were used for *in vivo* safety testing using STAT-1 knockout mice, Balb/c mice, and guinea pigs, while passage 2 Ebola Δ VP30-GFP virus alone was tested in rhesus and cynomolgus non-human primates (NHP). No signs of viral replication were detected in the blood of NHPs by real-time PCR on the day of inoculation. Additional safety testing was done following three blind passages with passage 7 of Ebola Δ VP30-neo virus by infecting wild-type Vero cells. No infectious virus was identified in the culture supernatant following incubation with wild-type Vero cells and immunostaining with anti-Ebola VP40 antibody. This entire process was repeated three times starting from the genomic cDNA of Ebola Δ VP30-neo virus, supporting expression plasmids and VP30 complementing cells. For sensitivity testing, the total number of viral particles tested *in vitro* was 1.02×10^9 FFU, while total amount of virus tested *in vivo* was 4.9×10^8 FFU. Three blind passages were also performed. To date, no events indicating reversion of defective Ebola viruses to wild-type have been detected.

Examples of experiments to be conducted under BSL2 containment with the Ebola Δ VP30 system include evaluation of:

- Monoclonal antibodies
- Small molecule compounds/inhibitors/chemicals
- Small interfering RNAs/short hairpin RNAs (siRNAs/shRNAs)
- Overexpression of cellular cDNA
- Mutant viruses and structure-function analysis of viral proteins
- Sensitivities of various cell lines (human, bat, pig, etc.)

Before performing any BSL2 experiments, all personnel must undergo a Federal Bureau of Investigation background check and complete a select agent security risk assessment. Biosafety measures in place for BSL2 experiments using the Ebola Δ VP30 system include:

- A dedicated biosafety cabinet, CO2 incubator and reagents for Ebola Δ VP30 virus work
- Ebola Δ VP30 viruses will be temporally and spatially separated from other viruses
- Validation of the genetic stability of Ebola Δ VP30 viruses will be performed at each passage

Biosecurity measures implemented for experiments to be performed under BSL2 containment include the following:

- The research group is housed in a dedicated stand-alone structure, and no other groups are present in the building.
- The facility has undergone a thorough security risk assessment by the University of Wisconsin–Madison Police Department before approval.
- There is monitoring inside and outside the building, and agents are secured behind two physical barriers.
- Campus police patrol the area.
- Access and entry to the facility are limited to approved staff only.

B. Presentation by Dr. Cannon

Dr. Cannon reviewed Dr. Kawaoka's request to lower containment for work on the Ebola Δ VP30 virus system and the history of the generation and testing of this system under BSL4 and BSL3+ conditions, as delineated in the presentation by Drs. Kawaoka and Halfmann, and per discussions by the BSWG

regarding this request. Dr. Kawaoka is seeking approval from the NIH OBA to change the biosafety containment of replication-incompetent Ebola viruses lacking the VP30 gene (Ebola Δ VP30 viruses) from BSL3+ (enhanced) to BSL2 containment for *in vitro* (tissue culture) experiments based on safety results to date. The request is limited at this time to *in vitro* tissue culture experiments using the Ebola Δ VP30 system and is specific to Dr. Kawaoka's lab.

Drs. Kawaoka and Halfmann described the generation of defective Ebola viruses in a paper published in the Proceedings of the National Academy of Sciences (PNAS) in 2008. Based on safety data in this paper, Dr. Kawaoka requested in early 2008 that the CDC Division of Select Agents and Toxins (DSAT) determine whether or not the Ebola Δ VP30 virus was still subject to the select agent regulations, in accordance with 42 CFR Part 73. The CDC DSAT set the containment level for *in vitro* experiments using this virus at BSL3+ (reduced from BSL4) but did not exclude it from the select agent regulations. The lowered biosafety level required the following:

- The work should be restricted in time and space when working with other viruses
- *In vitro* experiments should be expanded to include *in vivo* experiments in a well-characterized NHP model under BSL4 containment (i.e., to provide more safety data)
- More experience with the Ebola Δ VP30 system to rule out the possibility of reversion to wild-type virus

In 2013, based on additional data from Dr. Kawaoka's group, the Ebola Δ VP30 virus system was determined to be excluded from the requirements for a select agent under 42 CFR Part 73, as noted in the following statement: "Ebola Δ VP30 replication incompetent virus (effective 01-02-2013): This virus lacks the gene encoding for the VP30 protein; therefore, biologically contained Ebola virus (Ebola Δ VP30 viruses) are replication incompetent and do not form infectious progeny in wild-type cells due to the lack of the VP30 gene. The genome of the Δ VP30 viruses is stable, infection of Vero cells and various animals with Ebola Δ VP30 virus particles did not indicate a single event of virus replication, [and] infection of animals with Ebola Δ VP30 virus particles did not cause disease in infected animals."

Dr. Cannon noted that the additional experimental data that led to the decision in 2013 included results presented by Drs. Kawaoka and Halfmann in support of the request to lower containment from BSL3+ to BSL2. With these data, safety testing for *in vitro*-based manipulations using tissue culture is complete.

The key questions raised by the BSWG in response to this request were:

- What is the nature of the components of the Δ VP30 system, specifically, the defective the defective virus and its complementing cell line?
- What is the production flow chart from transfection to defective virus?
- What is the projected scale of production?
- What safety testing has been done, and what are the findings of this testing?
- What are the sensitivity and power of assays to detect a "rescue" event?
- What experiments are planned under BSL2 containment?

Regarding the components of the Δ VP30 system, the BSWG requested additional information to better understand how this system was engineered to prevent or minimize the possibility of rescue or another event that could lead to a replication competent virus. In particular, members had questions about whether or not there were any overlapping sequences between the VP30 complementing component and the Δ VP30 genome that could lead to recombination. As noted in today's presentation, there are no overlapping sequences that could give rise to a rescue event by virtue of homologous recombination. The BSWG also discussed the possibility of gene conversion events, the possibility of reversion to a wild-type virus, the risk of rescue of a replication competent or wild-type Ebola virus following DNA transfection of producer cells with viral cDNAs and supporting plasmids during the initial stage of generation of the virus. As Dr. Kawaoka explained, the process for generating the Δ VP30 virus does not involve transfection of a VP30 expression plasmid and that the VP30 component is stably inserted into the chromosome of the cells used for transfection.

The second set of questions involved the production work flow, from the initial transfection through producing stocks of the defective virus. In their presentation, Drs. Kawaoka and Halfmann provided details of the step-by-step process of the transfection initially performed at BSL4, subsequently at BSL3+ and what will be done at BSL2. Information on how the various components obtained at each of these steps would transition to the lower containment level, the plan for transitioning the components, and the scale of production was also presented.

The BSWG also requested additional information and clarification regarding the type of safety and sensitivity testing that has been done, the power of these assays to detect replication competent virus, and the results of this testing. Drs. Kawaoka and Halfmann addressed these issues in detail in their presentation. They also pointed out that there has never been a case of reversion to wild type for defective Ebola viruses.

Additional questions focused on the specific experiments that will be conducted under BSL2 containment and the safety and security measures in place for personnel and the actual lab(s) and building(s) in which the experiments will be done. The investigators satisfactorily answered these questions. The research will be conducted in a stand-alone building, and the security measures in place are rigorous.

Dr. Cannon noted that based on the data and information from Drs. Kawaoka and Halfmann, the BSWG concluded that the Δ VP30 system is unlikely to produce a wild-type revertant of the Ebola virus and that a reduction to BSL2 with added biosafety measures should be considered.

The NIH is seeking input from the RAC to consider if containment can be lowered to BSL2 for experiments involving the Ebola Δ VP30 system (i.e., the defective VP30 genome and the complementing stable Vero-VP30 cell line). The NIH determination to lower containment shall apply only to Dr. Kawaoka at the University of Wisconsin and to the Ebola Δ VP30 virus system and shall include stipulations to ensure appropriate biosafety and biosecurity measures. This determination does not change the risk group classification for the Ebola virus.

C. RAC Discussion

Dr. Cannon requested further clarification about the process for experiments done under BSL2 containment, specifically, if the investigators go back to the initial transfection step when preparing a stock, if they passage an existing stock of transfected cells to produce a working stock, or if they use a combination of these approaches. In addition, she asked about the ongoing program to validate the new stocks against prior ones, including when validation is done and whether all or only some stocks are tested for this purpose. Dr. Kawaoka explained that for each Δ VP30 virus, the investigators use a DNA transfection step to generate the defective virus, and amplify the defective virus to make a large virus stock. Transfection is initially done using 293T cells stably expressing the VP30 protein because the transfection efficiency in these cells is very high. Once the Δ VP30 virus is generated, amplification in the VeroVP30 cells follows. This entire process will be performed under BSL2 containment, after which a portion of the viral stock is brought into the BSL3 laboratory to test the stock for the presence of infectious virus. Although the investigators have not detected any infectious virus to date and the testing of these stocks could be done under BSL2 containment, they prefer to perform the testing under BSL3-enhanced containment as an added safeguard to exclude any possibility of working with infectious virus in the BSL2 laboratory. In addition, an appropriate positive control can be added to the validation tests at BSL3+.

Dr. Cannon also asked if there is a maximum number of effective passages of the VeroVP30 cell line that the investigators would not go beyond. Dr. Kawaoka noted that when a large stock of virus is depleted, the new stock is made from seed stocks obtained from the initial transfection, not from the large (depleted) stock of transfected virus. The investigators have gone up to 10 serial passes without evidence for rescue of wild-type virus. They try to limit the number of passages, however, by producing and storing sufficiently large amounts of defective virus to avoid running out of material on a regular basis. Every passage and every stock is validated to confirm the absence of infectious virus.

Dr. Wooley noted that many of the questions raised during the BSWG conference call focused on production issues. She provided a schematic to help clarify the production process and noted one difference in the current process used to generate the Ebola Δ VP30 virus compared to that delineated in the 2008 PNAS paper. In the original paper, the first step of the process involves the simultaneous co-transfection of a T-7 promoter-driven full-length cDNA of Ebola virus lacking the gene for VP30, four helper plasmids that encode the essential proteins (including VP30) for replication and transcription of the Ebola genome and a plasmid expressing the T-7 RNA polymerase. All the genes necessary for the viral RNA to be functional need to be present in the cell at the same time as the cDNA. Dr. Kawaoka pointed out that the process described in the 2008 paper is no longer used and that the team now uses 293T cells that constitutively express the VP30 protein. The VP30 open reading frame is stably integrated into the chromosome of the engineered 293T cells. That is the only difference between what was presented in the 2008 paper and the current process.

Dr. Wooley identified two points in the process for generating the Ebola Δ VP30 virus that present an opportunity for recombination: The first is when all necessary genes for viral replication are present in the cell at one time in the form of DNA, although as Dr. Kawaoka pointed out, the VP30 gene is now stably integrated into the cellular chromosome. The second opportunity for recombination occurs when mRNA coding for the missing VP30 is present in the cytoplasm as it is being translated into protein. Dr. Cannon questioned this conclusion and noted that the only Ebola DNA in the Vero VP30 system is VP30 gene located in the cellular chromosome. All other Ebola genes and supporting proteins are brought in by the virus including its RNA genome that is copied only as RNA. In response, Dr. Wooley referenced publications showing evidence of recombination occurring in Ebola viruses isolated from great apes and other primates in the wild, as well as recombination occurring between human respiratory syncytial viruses following co-infection in a laboratory setting. Dr. Kawaoka pointed out that while mRNA for all of the Ebola virus genes is present in the Vero VP30 system, there is no selective pressure to force recombination because the VP30 protein is always present. Further, during transcription the virus genome is fully coated by the viral nuclear protein, for both the genomic and anti-genomic RNA single strands.

Dr. Wooley asked for further clarification on the transition of the Ebola Δ VP30 system from BSL3+ to BSL2 and where testing of the material would occur. Dr. Kawaoka explained that a very small quantity of the BSL2 seed stock is checked in the BSL3 setting to test for the absence of infectious virus. The rest of the seed stock stays in the BSL2 lab, and the final stock is made in the BSL2 lab. He clarified that the BSL2-produced seed stock that is verified in the BSL3 setting will be discarded and that no product transferred from BSL2 to BSL3 is removed from BSL3 containment.

Dr. Hearing inquired about the function of VP30 in the virus life cycle and whether it is possible to generate a mutant that could overcome the absence of VP30. Dr. Hearing also asked about the number/yield of infectious viruses that are generated with the cDNA system and whether recombination could occur if cells infected cells at a high MOI are repeatedly passaged, in contrast with multiple passages at a low MOI where recombination does not occur. Dr. Kawaoka noted that there is no viral genome transcription without VP30 and that it is unlikely that a mutation could overcome this deficiency given the critical role of VP30 in replication. As for yield per cell, with 10^6 cells, up to 10^7 plaque-forming infectious units are typically generated. Passage of the modified virus in cells at high MOI would produce defective particles that are not viable because they are deleted for many viral genes and would be more likely to mask recombination events should they occur. Dr. Kawaoka noted that the highest viral titers produced by his lab are in the range of 10^7 to 10^8 FFU. As for all viral stocks, each batch is retested for the presence of infectious virus.

Dr. McCarty requested additional information regarding the types of experiments that the investigators plan to do with this virus stock, specifically, if there are plans to infect any cells other than the VP30 Vero cells and, if so, whether this would create a condition in which there is selective pressure for recombination to occur. Dr. McCarty noted the examples cited by Dr. Wooley and asked whether positive selection for the recombinant and, in turn, generation of replication-competent virus could occur in a cell type other than the Vero VP30 cell. Dr. Wooley continued the question by asking what the possible outcomes could occur if the virus that is missing VP30 is introduced into naïve 293T cells or another cell

line without VP30. Dr. Kawaoka pointed out that there are no known genes that substitute for the VP30 gene or code for the VP30 protein, as tested to date in human and in NHP cells. No reversion or infectious virus has been seen in experiments involving multiple passages and with introduction of the passaged product into animals.

Dr. Donahue considered these experiments within the context of adenovirus, where replication deficiency and reversion is well described. He noted that in his lab, reversion happens about once every 5 years. Thus, while this reversion is rare, it is a well-accepted event that occurs at a frequency that is considerably less than what Dr. Kawaoka's team has tested. Applying real-world data from the adenovirus example, the potential for reversion of the Ebola Δ VP30 virus seems to be very small. However, because the denominator for the Δ VP30 experiments is low, the number of replicates, in turn, may be too low to give a high level of confidence in the conclusions presented for the Ebola Δ VP30 system. Dr. Kawaoka noted that the Ebola virus does not replicate to the titers that adenoviruses can, and he did not agree that the lower virus yield (i.e. denominator) is so low as to question the confidence in the estimated low reversion frequency for the Ebola Δ VP30 virus. Each virus is tested at its own specific titer, whether Ebola, adenovirus, or another organism, and as Dr. Cannon noted, the safety assays that have been done for the Ebola Δ VP30 virus are at the scale of the volumes and titers of the stocks that will be produced and are higher than the amount of virus that will be used in the proposed experiments.

Dr. Lee asked a follow-up question regarding the theoretical possibility of recombination with RNA given that there is no homology between VP30 and the new vector. Specifically, what is known about RNA recombination events? For example, are the events mediated by homologous sites, which presumably would require a complete section of the RNA and blunt-end ligation of three pieces of RNA to combine these segments together as needed? Dr. Wooley noted that she could not find evidence for this and commented that little is known about negative-strand RNA viruses such as Ebola. For other viruses, non-homologous recombination occurs and for retroviruses in particular, it occurs very easily. How often non-homologous recombination events occur for the Ebola virus is not known.

A safety strategy used with many viral vectors involves split genomes, so that the more molecules must recombine, the less chance for a successful recombination to occur exists. In the current scenario, the genome is missing one gene, and the sequences are present on a second molecule. Dr. Wooley asked whether the investigators have tried or at least considered making a viral vector system that is missing two genes (e.g., VP30 and L) because by adding just one other molecule, the risk of successful recombination can be dramatically reduced. Dr. Kawaoka commented that this approach would be the next step for the team to try.

Dr. Chatterjee requested further information to explain why viral infection was not seen in mice given different constructs of the Δ VP30 Ebola virus (e.g., the Ebola Δ VP30-neo virus) and the VP30 Vero cells. Dr. Kawaoka noted that the viruses produced from the VP30 Vero cells are still defective and would not replicate in the animal, as a result, no virus is detected. Even with introduction of the Vero VP30 cells, there was no detectable amplification of the Ebola Δ VP30 virus *in vivo*.

Dr. Donahue inquired about the security requirements for the virus now that it is no longer a select agent. As detailed in the presentation, the entire laboratory building (in which the investigators also work on the influenza virus) and is monitored 24 hours a day by the University of Wisconsin police. In addition, everyone with access to the lab is required to undergo an FBI background check.

Dr. Lee noted that from a regulatory standpoint, although safeguards are being built into the recommendations, the RAC (and other groups) should proceed with caution to assure that there is confidence in the research conclusions when setting or advising new guidelines. Dr. Cannon clarified that the issue at hand for the RAC is not a rule request. Rather, it is a request specifically for work being proposed for Dr. Kawaoka's lab only.

Dr. Kiem asked about the impact on this research if the request to progress to BSL2 is not approved at this time. The investigators noted that the basic research would be enhanced under BSL2 containment. For example, two-photon microscopic experiments would be possible, and vaccine production and testing

would be much easier to perform at BSL2. In response to a follow-up question regarding additional work, Dr. Kawaoka noted that the Ebola Δ VP30 system has demonstrated protection in NHPs when tested as a candidate vaccine. The full-length Ebola genome (cDNAs) is classified as BSL2, and it must be maintained in a separate room and with specific biosecurity measures in place. Dr. DiGiusto requested further clarification regarding the request under consideration given that the investigators already appear to be conducting experiments with the Ebola construct under BSL2 containment. In response, it was noted that the request is being made because nothing live can be taken out of BSL3 into BSL2. Dr. Wooley added that this was a major discussion point during the BSWG conference call, that is, even if defective is produced under BSL3 and shown to be free of replicating wild-type virus, it cannot be removed from higher containment and placed in lower containment. The solution is to produce the defective virus *de novo* under BSL2 containment.

Dr. Jorgenson noted that no formal vote by the RAC is required. The NIH OBA is discussing the pros and cons of this request, which will be taken into consideration in making a determination going forward.

D. Public Comment

No comments from the public were offered.

X. Gene Transfer Safety Assessment Board Report

RAC Reviewers: Drs. Atkins, Curry, Donahue, Kaufman, Kiem, Lee, Pilewski, and Whitley

A. GTSAB Report

Dr. Kiem opened the session by presenting the charge to the GTSAB:

- Review in closed session, as appropriate, safety information from gene transfer trials for the purpose of assessing toxicity and safety data across gene transfer trials,
- Identify significant trends or significant single events, and
- Report significant findings and aggregated trend data to the RAC and thereby disseminate it to the scientific and patient communities and to the general public.

The current GTSAB roster includes eight RAC members and one FDA representative (Dr. Gavin).

Dr. Kiem then presented the GTSAB report for the fourth quarter of 2015. Within the past 3 months, OBA received a total of 22 protocol submissions, 19 of which were not selected for public review at this RAC meeting. Of the 19 protocols not selected for public review, 13 were oncology protocols, 5 were monogenic disease protocols, and 1 was a salivary gland hypofunction protocol. Among these 19 protocols, 6 used lentiviruses, 5 used AAVs, 3 used plasmids, 2 used attenuated *Listeria monocytogenes* (*Lm*), 1 used RNA, 1 used adenoviruses, and 1 used herpes simplex virus. For the fourth quarter of 2015, the GTSAB reviewed initial and follow-up reports on 26 serious adverse events (SAEs) from 23 protocols. (Information about these trials was made available on the OBA website after this RAC meeting and in the future will be available in the NIH Genetic Modification Clinical Research Information System, also known as GeMCRIS.)

Of note, the GTSAB discussed an event involving listeriosis in a Phase II trial (*protocol 1082*) evaluating AVXS11-001, an attenuated *Lm* vaccine strain for the treatment of persistent or recurring squamous or non-squamous cell carcinoma of the cervix. A recent SAE suggests a possible delayed listeriosis and bacteremia more than 2 years after the last dosing of a subject with the attenuated *Lm* vaccine strain. The study is currently on hold, and the company is investigating the case.

ADXS11-001 is an XFL-7 strain carrying a modified *prfA* gene. *PrfA* is a regulator of virulence genes in *Lm*. Dr. Kiem noted that 19 protocols currently are using different attenuated *Lm* strains as vaccines for various target diseases (i.e., 18 target malignancies, one targets hepatitis C/liver disease). CRS-207, another commonly used *Lm* strain, carries Δ *inlB* Δ *actA* mutations.

Most of the other SAEs are from CAR and T-cell receptor (TCR) studies, as described below. Dr. Kiem noted that similar events, including signs and symptoms of cytokine release syndrome (CRS), were seen across different protocols:

- **CD19 CAR for B-cell lymphoma (protocol 940):** Two SAEs were reported in one protocol: (1) hypotension, seizures, mental status changes, and hallucinations and (2) cognitive dysfunction, somnolence, dysphagia, and muscle weakness.
- **HER2 CAR for advanced sarcoma (protocol 969):** Mild CRS was reported.
- **HLA-A2–restricted NY-ESO-1 for sarcoma (protocol 1071):** Severe CRS plus tremors, complicated by respiratory failure, pancytopenia, sepsis, and readmittance with prolonged cytopenia and possible CMV infection, were reported in this protocol.
- **Anti-EGFRvIII CAR for gliomas (protocol 1095):** Respiratory distress was observed shortly after T-cell infusion, resulting in death attributed to pulmonary edema and distributive shock.
- **CD19 CAR for acute lymphoblastic leukemia (ALL) (protocol 1279):** Fever progressing to severe CRS a few days later with nausea, tachycardia, shortness of breath, visual field defects, and rash were reported. CRS resolved with treatment with tocilizumab and corticosteroids. In this case, the research participant had conditioning chemotherapy followed by transplant.
- **Anti-BCMA (B-cell maturation antigen) CAR targeting multiple myeloma (protocol 1303):** SAEs were reported for two research participants. One research participant had severe CRS (fever, tachycardia, hypoxia, and hypotension) that resolved with tocilizumab, but the research participant developed prolonged neutropenia and thrombocytopenia. Another research participant developed severe CRS, with neurotoxicity (Grade 3 delirium) and elevated creatine phosphokinase (CPK), which was complicated by prolonged pancytopenia and transfusion-dependent thrombocytopenia. Neither research participant showed evidence of myeloma on their last bone marrow analyses.
- **CD19 CAR against refractory NHL (protocol 1339):** Severe CRS, dyspnea, encephalopathy complicated by pancytopenia and sepsis were reported for one research participant, who subsequently died following an intracranial hemorrhage.

For the fourth quarter of 2015, OBA received notification that 14 new protocols opened, one of which was publicly reviewed:

- *OBA Protocol #1405-1313: A Phase I Trial of the Safety and Immunogenicity of a Multiple Antigen Vaccine (STEMVAC) in HER2-Negative Advanced Stage Breast Cancer Patients*

Dr. Kiem reported that the FDA has approved talimogene laherparepvec (IMLYGIC) as the first oncolytic viral therapy in the United States in addition to being the first licensed gene therapy product in the United States. Talimogene laherparepvec is indicated for the local treatment of unresectable cutaneous, subcutaneous, and nodal lesions in patients with melanoma recurrence after initial surgery. Dr. Kiem noted that this approval is good news for the gene therapy field.

B. RAC Discussion

A RAC member inquired about the different genes in the 19 protocols using attenuated *Listeria* strains. It was noted that 7 of these 19 studies are using a product that has a prfA defect, while another seven studies are using CRS207, which has double mutations (actA and inlB). Three additional protocols are using combinations of mutated genes, one using a product with a triple mutation, including an actA mutation and a prfA mutation. To date, 2 years after initial treatment, there has been no evidence of immunosuppression in the research participant with delayed listeriosis (i.e., the case involving treatment with a vaccine strain carrying a modified prfA gene).

C. Public Comment

No public comments were offered.

XI. Closing Remarks and Adjournment

Dr. Kiem thanked the RAC members and the OBA staff and adjourned the December 2015 RAC meeting at 4:20 p.m. on December 4, 2015.

[Note: Actions approved by the RAC are considered recommendations to the NIH Director; therefore, they are not considered final until approved by the NIH Director.]

Lyric Jorgenson, Ph.D.
RAC Executive Secretary

I hereby acknowledge that, to the best of my knowledge, the foregoing Minutes and the following Attachments are accurate and complete.

This Minutes document will be considered formally by the RAC at a subsequent meeting; any corrections or notations will be incorporated into the Minutes after that meeting.

Date: _____

Hans-Peter Kiem, M.D., Ph.D.
Chair, Recombinant DNA Advisory Committee

Attachment I: Recombinant DNA Advisory Committee Roster

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Executive Secretary

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**Attachment II:
Public Attendees**

(This list includes only individuals who are not identified elsewhere in this document. One name on the public sign-in sheet is not legible, and at least one attendee who joined the meeting by phone is not listed or named on any of the sign-in sheets or elsewhere.)

Greg Dasch, CDC (via teleconference)
Lynda Dee, Collaboratory of AIDS Researchers for Eradication (CARE) Community Advisory Board
Steve Dumler, University of Maryland (via teleconference)
Amie Eisfeld, University of Wisconsin
Lawrence Fox, National Institute of Allergy and Infectious Diseases (NIAID), NIH
Sandra Bridges Gurgo, NIAID, NIH
Ted Hackstadt, Rocky Mountain Laboratory (via teleconference)
Tia Morton, NIAID, NIH
Betty Poon, NIAID, NIH
Kathy Wetzelman

Attachment III: Abbreviations and Acronyms

AAV	adeno-associated virus
Ab	antibody
ACTR	antibody-coupled T-cell receptors
AE	adverse event
AG	ancestral group
ALL	acute lymphoblastic leukemia
ART	antiretroviral therapy
BCMA	B-cell maturation antigen
BMT	bone marrow transplant
BSL	biosafety level
BSWG	Biosafety Working Group
CAR	chimeric antigen receptor
CARE	Collaboratory of AIDS Researchers for Eradication
Cas9	CRISPR-associated protein 9
CAT	chloramphenicol acetyltransferase
CCR5	chemokine receptor type 5
CDC	Centers for Disease Control and Prevention
cDNA	complementary DNA
Cm ^R	chloramphenicol resistance
CMV	cytomegalovirus
CNS	central nervous system
CPK	creatine phosphokinase
CRISPR	clustered regularly interspaced short palindromic repeats
CRS	cytokine release syndrome
CTL	cytotoxic T-cell lymphocyte
DSAT	CDC Division of Division of Select Agents and Toxins
DCs	dendritic cells
DEC	Dose Escalation Committee
DLT	dose-limiting toxicity
DSMC	Data and Safety Monitoring Committee
ERT	enzyme replacement therapy
FDA	U.S. Food and Drug Administration
FIX	Factor IX
FFU	focus forming unit
GAG	glycosaminoglycan
GeMCRIS	Genetic Modification Clinical Research Information System
GFP	green fluorescent protein
GTSAB	Gene Transfer Safety Assessment Board
HDAC	histone deacetylase
HDACi	HDAC inhibitor
HDR	homology-directed repair
hIDUA	human IDUA
HSA	Health Sciences Authority
HSCT	hematopoietic stem cell transplant
HTRL	Howard T. Ricketts Laboratory
ICD	informed consent document
ICH	International Conference of Harmonization
IDUA	α -L-iduronidase
IgG	immunoglobulin G
IND	Investigational New Drug
indel	insertion or deletion mutation
ITR	inverted terminal repeat
IV	intravenous
IVT	in vitro transcribed
IVIG	intravenous immunoglobulin

LAM	linear amplification-mediated
LCR	locus control region
<i>Lm</i>	<i>Listeria monocytogenes</i>
LRA	latency-reversing agent
LTR	long terminal repeat
MDCs	mature dendritic cells
MIC	minimum inhibitory concentration
MOI	multiplicity of infection
MPS I	mucopolysaccharidosis type I
mRNA	messenger RNA
MSF	Mediterranean spotted fever
MTD	maximum tolerated dose
NGS	next-generation sequencing
NHEJ	non-homologous end joining
NHP	non-human primate
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health
<i>NIH Guidelines</i>	<i>NIH Guidelines for Research Involving Recombinant DNA Molecules</i>
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitors
OBA	Office of Biotechnology Activities, NIH
OD	Office of the Director, NIH
OSHA	Occupational Safety and Health Administration
PAPR	powered air purifying respirator
PBMCs	peripheral blood mononuclear cells
PNAS	Proceedings of the National Academy of Science
PCR	polymerase chain reaction
PI	principal investigator
pg	picogram
PK	pharmacokinetics
QC	quality control
qPCR	quantitative polymerase chain reaction
RAC	Recombinant DNA Advisory Committee
rca-RNA	resting cell-associated HIV RNA
RMSF	Rocky Mountain spotted fever
RPP30	ribonuclease P protein subunit p30
RT	reverse transcriptase
RT-PCR	reverse transcription polymerase chain reaction
SAE	serious adverse event
SFG	spotted fever group
SIV	simian immunodeficiency virus
SMC	safety monitoring committee
TCR	T-cell receptor
TG	typhus group
TRG	transitional group
UNC-CH	University of North Carolina at Chapel Hill
WBC	white blood cell
WHO	World Health Organization
ZFN	zinc finger nuclease

Appendix A: Public Comments on Protocol #1508-1455

[This testimony is provided verbatim, as reported in the transcript of the December 2015 RAC meeting.]

Testimony of Robert Reinhard (via letter, read by Dr. Jorgenson)

Thank you for the opportunity to submit public comments for the RAC discussion of protocol number 1455. Dr. Margolis has pioneered the study of the HDAC as potential latency-reversing agents, or LRAs, especially with his initial demonstration using vorinostat, the compound to be studied in this protocol.

First, I note that some features of the proposed study design are highly desirable to mitigate risk for this stage of research, including the means to test the hypothesis using assays that preserve continuous participant use of the CAR-interrupted ART. Dr. Margolis' earlier demonstrations were critical to advances in the field for testing other LRAs. The protocol discusses two of them comparatively: Vermadepsom (ph) and Pataministat. Several other compounds and—from other classes have been proposed as elements of a combined approach, like this study, to pair an LRA with several candidate immunotherapeutics. In other words, menu of theoretical paired option grows and some other pairs are also in clinical trial progress.

My comments request the RAC evaluate how well vorinostat meets criteria toward its pairing with the AGS-004 in comparison to other possible LRA choices.

The protocol provides the relevant criteria: (1) potency of the agent to stimulate expression of proviral HIV in vivo, (2) avoiding concerns with increased susceptibility of uninfected cells to HIV infection, and (3) detrimental impairment of cTAL function needed to contain viremia.

Further evaluation of vorinostat to weigh in on all three counts would be helpful as follows: One, recent comparative study is not mentioned in the protocol of LRAs, and vorinostat in particular can be added to review. Vorinostat may be relatively weak compared to other candidate LRAs delivered either singly or in dual combination, according to the paper by Laird (ph) et al. And catch one modified figure slide, which, I believe, is up, illustrating their comparison. Another recent study found that response to vorinostat is very heterogeneous among participants and suggests that vorinostat treatment does not induce killing of transcriptionally activated latent cells in vivo in the majority of individuals. And thus, according to this model, the reductions in reservoir size were minimal or absent in most participants.

Another study not mentioned in the protocol demonstrated that vorinostat significantly increases the susceptibility of CD4-positive T cells to infection by HIV dose- and time-dependent manner. It is independent of receptor and co-receptor usage and the risk of receding the reservoir. Although the protocol discusses the study by Jones et al., it may mischaracterize the conclusions. The investigators observed that romidepsin, panobinostat, and SAHA all rapidly suppressed interferon production from virus-specific CD8 T cells. Critically, each of these HDACs also impaired the ability of HIV-specific CTL to illuminate infected CD4. This affect was exacerbated with repeated dosing. It is unclear if the protocols offer to measure the ability of circulating CTLs to inhibit bio recovery from infected resting CD4-positive T cells addresses this risk sufficiently.

These considerations may point to increased scrutiny of the design measures for the LRA for this very pertinent study. Perhaps also consider an added LRA arm from another class. Of course, that would require major redesign, and I am mindful that this proposal is a component of a well-funded stage investigation program for the Argos therapeutics immunotherapy.

I also comment on the protocols protections and informed consent for participants. In previous comments I made to the RAC on other protocols, I've asked these cure studies include a commitment to treat any research-related injuries without charge to the participant. This protocol does not appear to offer the commitment clearly and seems to refer participants to their usual care providers in some circumstances, Section 816.

I understand the RAC previously declined to adopt the principle I requested across the board in all cure studies. Please note, as a new model of clinical trial protections, that the NIH-sponsored HBTN HIV vaccine studies do now provide this commitment in all cases. I repeat my same request.

Thank you for your consideration. My comments can be summed up in the lyrics of a song sung by Pat Benatar: If you are going to do this kind of study, then hit me with your best shot.

Sincerely,
Robert Reinhard
Public Global Health Consultant

Testimony of Lynda Dee

Good morning. My name is Lynda Dee. I'm from AIDS Action Baltimore and the AIDS Treatment Activist Coalition. I'm also the co-chair of the CARE Collaboratory Community Advisory Board.

And let me just say initially that Dr. Margolis did not ask me to come down here. Hopefully, he will be glad when I'm done and not sorry, like he usually is when I start. But anyway, I want to thank you for your service. I have been on a number of these boards, and I know how thankless it is and how time consuming it is. I am sorry that my friend Rich Whitley is not here, who I haven't seen in many years. I wanted to say hello to him, but hello, Dr. Whitley, anyway.

[Dr. Whitley: I'm on the phone, Lynda, so don't worry.]

[Ms. Dee: No, I know. All right.]

I wanted to remind you of the Institute of Medicine report from 2013 that indicates that the public hearings for the RAC should only be called to order in the most extreme cases. I mean, the report gives three specific instances, and it is also—I think in 2014, the report received the imprimatur of Dr. Collins as far as sticking to that, because there are all kind of reviews, and this might be one layer that might not be necessary.

I don't think it was necessary. I mean, I thought some of the questions were interesting, but just for instance, let me get this one off my chest first. For the last 20, 25 years, people from the community have been arguing with researchers to include people that are chronically infected. And years ago, it was, "Oh, well, the drugs will work better in people that aren't so sick, and we want to be sure that we don't lose the chance for a drug because we are in the wrong patient population." And I just would say to you, I wonder how many people would be dead today if we had let that sort of strategy proceed. I think, aside from the latent measuring problems and issues that Dr. Margolis described, the HIV community wants people tested that are not only acutely infected—that would make these different strategies look better and look more promising. We want it done in everybody and at the same time whenever possible and whenever it is safe.

As far as the animal studies are concerned, these two drugs are relatively safe compared to some of the things that are in the pipeline. And I think that the animal studies are very expensive, and most people try and save them for drugs like panobinostat and other drugs that are much more toxic than vorinostat to see what happens to them so that we cannot have to put people through those sorts of things.

The other thing's about the contamination stuff. I mean, I would think that the FDA, having reviewed this, is in a perfect position to decide whether something has a risk of being contaminated, so I would just defer to them on that.

I think that real delays have real consequences. And just for instance, in this particular instance, because of the public hearing process, this—the RAC process took 4 months longer than the North Carolina UNC IRB, the NIH review process from the CSRC, and the—what was the other one?—the FDA process. So already, one of the milestones for this protocol—the first patient enrollment date has been missed. And

that was exactly 4 months ago, as long as it has taken to get this protocol reviewed by this group. So I would just say to you that these real consequences have real effects for real people aside from the investigators and to increase your workload.

The HIV community wants ethical protocols, but we want them to be expedited, especially in this arena. One of our concerns is, hello, before the money runs out. So we really need to get this work done. Investigators are under pressure from the funders to get this stuff done and get it out the door. And I would just, again, cite that Institute of Medicine report; it says that the RAC review process might be redundant—is arguably redundant, I believe it says, in light of FDA and IRB reviews.

So if this committee doesn't want to go by the way of the dinosaurs, I think you should save your public hearing for instances where it is absolutely necessary. I mean, I think a lot of these questions and answers could have been done without the public hearing process, and I think we need to just speed this up as much as possible, whenever possible, as long as patient safety is not going to be sacrificed.

So again, I also have two suggestions. I wonder if it wouldn't be possible to do a little bit differently than you do now as far as when a public hearing is voted on and whatever. And maybe a majority or even two-thirds of the group voting affirmatively for a public hearing might be a better way to go, under the circumstances. I also think that it would behoove you to have a very verbal and knowledgeable community person on your group, which I think most NIH boards do.

So again, I think that I again want to thank you for your work, and I hope you will pass this without further delay.

And that is it for me.