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# WORKSHOP ON ADJUVANTS AND ADJUVANTED PREVENTIVE AND THERAPEUTIC VACCINES FOR INFECTIOUS DISEASE INDICATIONS

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TUESDAY
DECEMBER 2, 2008

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The workshop convened at 8:40 a.m. at the Bethesda North Marriott Hotel & Conference Center, 5701 Marinelli Road, Rockville, Maryland, Jay Slater, M.D., Deputy Director, Center for Biologics Evaluation and Research, Moderator, presiding.

## Introduction and Welcome

ANTHONY S. FAUCI, M.D., Director, NIAID/NIH
JESSE L. GOODMAN, M.D., M.P.H., Director, CBER/FDA

Session 1: Background

NORMAN BAYLOR, Ph.D., Director, Office of Vaccines Research and Review (OVRR)/CBER/FDA
DAN ROTROSEN, M.D., Division of Allergy, Immunology, and Transplantation/NIAID/NIH

Session 2: Specific Adjuvants Overview

ELIZABETH SUTKOWSKI, Ph.D., Co-Chair, CBER/FDA
BALI PULENDRAN, Ph.D., Co-Chair, Emory Vaccine Center

FABIO RE, Ph.D., University of Tennessee Health Science Center
DEREK O'HAGAN, Ph.D., Novartis Vaccines and Diagnostics, Inc.
EUGENE MARASKOVSKY, Ph.D., CSL Limited
BRUCE BEUTLER, M.D., Scripps Research Institute

NATHALIE GARCON, Pharm.D., Ph.D., GlaxoSmithKline Biologics
GEERT VAN den BOSSCHE, D.V.M., Ph.D., Bill and Melinda Gates Foundation

Session 3: Preclinical Safety

ETHAN SHEVACH, M.D., NIAID/NIH, Co-Chair

MARION F. GRUBER, Ph.D., Co-Chair, OVRR/CBER/FDA
JAN WILLEM VAN der LAAN, Ph.D., National Institute for Public Health and the Environment, The Netherlands
CARL ALVING, M.D., Walter Reed Army Institute of Research

SARAH GOULD, Ph.D., Sanofi Pasteur
DEBORAH NOVICKI, Ph.D., Novartis
HANA GOLDING, Ph.D., Division of Viral Products (DVP)/CBER/FDA
WILLIAM WARREN, Ph.D., VaxDesign Corporation

TABLE OF CONTENTS

P-R-O-C-E-E-D-I-N-G-S

8:42 a.m.

DR. SLATER: Good morning and welcome to the FDA NIH Workshop on Adjuvants and Adjuvanted Preventive and Therapeutic Vaccines for Infectious Disease Indications.

Welcome to all of you. I'm Jay Slater, and I have a couple of introductory sort of housekeeping comments before we get on to the main part of the program.

First of all, you are probably all aware that there are a lot of you here. This session was well over-subscribed. We were originally planning for about 250 participants. We have over 400 people signed up. So my guess is that as the morning goes on, it will become more crowded.

The purpose of this is to warn you that tomorrow our setting will be a little bit more intimate. We were successful at getting two rooms on the first day when we realized how heavily subscribed we were going to be.



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But we were unable to get two rooms for the second day.

So tomorrow we will all be in basically half the space. Obviously the tables will not be here. So we'll be a little bit closer. You will get to know your neighbors and fellow participants a little bit better tomorrow. But everybody should be able to safely fit in.

Those of you who parked outside in the parking lot, please make sure at some point during the day to go to the registration desk and get parking vouchers in case you haven't already. I will talk a little bit later about your lunch options as well.

Speakers, if there are speakers out here who have not yet given in your talks, Mr. Sandoval, who is sitting right there at the AV desk in the corner, will help you with your talks and get you loaded on here during the next appropriate break.

You will also notice that we have



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a very full schedule today and tomorrow. It is very tightly packed. The ability to ask questions will be at the session chair's discretion.

The one thing I would warn you about is that with a schedule like this, we could easily be a half hour ahead of schedule or a half hour after schedule. So don't treat this like a train schedule that is going to be particularly precise. If you are a speaker, please make sure you are here well in advance of the time of your presentation.

There will be time at the roundtable discussions tomorrow, I hope, for more questions. The other thing to keep in mind is this meeting is being transcribed. Therefore, if you are going to ask any questions, please take advantage of the six or seven microphones that are out in the audience.

So it is my pleasure to introduce for you the first two speakers of the session

at this introduction. We're fortunate to have both Dr. Fauci and Dr. Goodman introducing our session at this time. Dr. Anthony Fauci, as you know, is the Director of the National Institute of Allergy and Infectious Diseases. Dr. Jesse Goodman is the Director of the Center for Biologics Evaluation and Research.

These are the two organizations that cosponsored this meeting. And, therefore, we're going to ask both of them to come up and give some introductory remarks.

Dr. Fauci?

DR. FAUCI: Thank you very much, Jay. It's a great pleasure to be here with you all this morning. And I'd like to welcome you on behalf of NIAID. And you will hear very shortly from Jesse from CBER also welcoming you.

I'm only going to take just a few minutes because I want to really get us on to the main guts of this meeting. But I want to make some introductory remarks on a few

slides, likely telling you things that you are extraordinarily well familiar with. But I think it is worth mentioning as we start on this.

If I were to be given a 20 second time slot, I would just come up here and say you have my absolute commitment that work on adjuvants and the importance of adjuvants in both adjuvanted preventive and therapeutic vaccines is extraordinarily key and of a high priority to NIAID.

Having said that, I'm only going to spend just a couple of minutes just going through some issues, again, that I know you are familiar with. The impact of vaccines in the United States and globally is profound.

If you look at this slide, many versions of which get circulated, you see that of all the things we do in countermeasures, be they drugs or preventive measures, whenever we go before the Congress or talk to anyone nationally or internationally, vaccines are



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always the issue that is brought up for the highest cost-benefit ratio for the individual on public health as shown on this slide with the baseline cases in the 20th century in the pre-vaccine annual cases of a variety of important diseases compared to both the cases as well as the percent decrease.

I don't think there is any intervention in medicine that is as good as this, something that people in this room are very well familiar with. Recently, this is not just something of ancient decades ago, but recently vaccines that have been developed have continued to change the course of lives of people, again both in this country and in the developing world.

And these are just three examples of relatively recently developed vaccines and their potential in children less than five years old with the pneumococcal conjugate, the Hib vaccine, and the rotavirus vaccines.

NIAID from a research component,

and again when you are dealing with vaccines, there are multifaceted aspects of it. Sometimes this confuses people. There are broad general surveillance and public health issues. There are fundamental basic research issues. There are developmental research and developmental product issues. And there are regulatory issues.

NIAID is one of an important part of a very heterogenous and multifaceted approach towards vaccines. I show you this website of ours, which essentially describes the research agendas for vaccine development.

And I'm not going to talk about that. And I'm not going to bring up the research questions. You'll hear a little bit more about that from Jesse and how that impacts on the regulatory area.

But for those of you who are not familiar with this -- and I would hope that you were -- this is a site that you can get virtually all of the information for what we

at NIAID are doing.

Now with regard to vaccinology, it is an old and very well respected discipline. But as, again, all of you are aware, science has proceeded at a very rapid pace, particularly over the last couple of decades.

So now what we are -- really it behooves us -- it is an imperative for us to do is to apply the new science as it evolves to this very old and respected discipline of vaccinology, utilizing the tools and the platforms that are discussed or listed on this slide from the explosion in the arena of genomics, the fact that we now have structure-based vaccine designs utilizing crystallography.

We have biotechnology with nanotechnology, systems biology, high throughput systems, bioinformatics, different delivery systems, particularly in the arena of viral vectors, which have caught on very, very hot over the last several years, production

technology, one example in influenza, the now rapid progression from cell culture-based -- from egg-based to cell culture-based manufactory.

And last on this slide is the issue of adjuvants, which is the subject of our discussions in the workshop here today. The NIH vaccine adjuvant programs range from very fundamental, basic research on innate immunity, looking for the molecular mechanisms of the actions of adjuvants, to the discovery and development of actual new adjuvants, new products, be they from computational models, high throughput discovery platforms, new technologies of optimizing of lead candidates, as well as the important area that we are very heavily involved in is the clinical studies of adjuvants.

The paradigm is changing, again, things that people in this room are extraordinarily well familiar with, the old perspective in classic vaccinology, that

antigen may be sufficient to induce a protective immune response, antigen alone, that is true in some cases.

But it is becoming very clear, particularly with the new challenges that we have with certain vaccines -- influenza, influenza in general, influenza for the elderly, HIV almost certainly, is that adjuvants, either endogenous or exogenous, contribute to the effectiveness of vaccines and that the addition of adjuvants or other immunomodulators will be necessary for optimum response in many settings.

So this is something that will happen. The question is how do we, from the research and developmental standpoint, make it happen quickly and safely.

Now adjuvants were big black boxes. Many of you -- most of you -- all of you, I would say, know decades ago. But with the delineation of the molecular aspects, receptors, and ligands, and signal

transduction mechanisms associated with the interplay of the innate and ultimately the adaptive immune system, I found it curious and interesting as I was going over the history of this, if you look at this paper from Medzhitov and Janeway, in 2000, which was just really relatively recently, in the New England Journal of Medicine, delineated what we knew, at that time, of the Toll-like receptors and other receptors on cells and the ligands that are used to trigger the innate immunity, and look at the studies that have gone on from 2000, which is right about the middle of this slide, up until the end of 2007, and that of the number of publications, that if you do a MEDLINE search on innate immunity, much of which relates to adjuvant potential, you can see the extraordinary growth in knowledge such that a recent paper, this one from Host Cell and Microbe just this year -- and this is a simplified version of that, is the important complexity of the host innate immune receptors

and how we might use that in our scientific delineation of where we may go with adjuvants.

The pipeline of new adjuvants is robust, as you see from this slide. There are a number of them that are in clinical trials or on their way, ranging from CpG to the Lipid A mimics, RNA-based peptide carbohydrate, small molecule activators of TLR signaling an early discovery. So have a very, very robust and fruitful, potentially fruitful -- and I know will be fruitful discovery chain in the arena of adjuvants.

The goals for the future adjuvants are familiar to all of you. We need earlier, more robust and durable immunity with fewer boosters and less antigens. I'm going to show you an example of that in just a moment -- one example of many -- broader coverage and enhanced cross protection, and adjuvants which are designed according to the immunization route, be it subcutaneous, intramuscular, mucosal, or what have you.

The example I was referring to was the issue that we faced a few years ago -- and this is The New England Journal paper from 2006 from John Treanor and his colleagues, when we were putting a full court press on trying to develop an H5N1 vaccine in the classical manner. It was successful, but it wasn't optimal, as all of you know.

The dose that was required was non-practical from a domestic or global standpoint and a relatively small percentage of individuals were actually induced to give an immune response that you would predict by standard guidelines to be protective.

There was another study that came out, this one from GSK using their proprietary adjuvant, and, in fact, with the same goal in mind, were able to use a much lower dose. Instead of 90 mics times two, it was 3.8 mics times two.

Not only did it encompass a greater percentage of individuals -- 75

percent or more -- but there was also induction of cross-reactive neutralizing antibody against a clade of H5N1 that was a bit drifted from the original immunizing clade, something extraordinarily important.

Now this is a mental exercise on this next slide. It's not something that has been done. But if you just take those data and put them together with the stockpiled H5N1 vaccine of close to 23 million 90 mic doses, and if, in fact, the adjuvant data proves to be practically applicable, you would see that literally we would have 542 million instead of 22 million doses, which is extraordinarily important when you are dealing with stockpiling not only for diseases like potentially pandemic flu but a variety of others.

Also important, it extends the supply, it increases the level of the immune response, importantly for something like pandemic flu whose strains drift regularly, as

we know, an increase of the breadth of the immune response with protection against drifted strain, and the option for a single-dose priming instead of multiple doses.

And finally, having said that just as a quick introduction, the challenges ahead of us from a pure disease standpoint are extraordinary. And these are just some selected examples of vaccine candidates that almost certainly would benefit greatly if we had a wider array of adjuvants, ranging from HIV through malaria, TB, and the neglected tropical diseases, I already mentioned pandemic influenza but also seasonal influenza in the elderly as well as those vaccines that require multiple doses such as varicella in children.

So in closing, I want to again welcome you and to again reinforce the commitment of NIAID from the research standpoint to work closely with you, the people who are involved in the research, in

the development, biotech, pharma, FDA, CDC, and all of our other partners in what I am sure is going to be one of the most important endeavors that we undertake.

Thank you.

(Applause.)

DR. GOODMAN: Well, thank you very much, Tony, and thank you for your support and vision in this field.

I'm very happy to be here. It is wonderful to see this turnout. I was thinking that I would like to see this meeting be an adjuvant for adjuvants. Okay. So we can really stimulate work in the field and stimulate progress.

This is one of the adjuvants you don't want to get on the cover. This is, I think, complete Freund's adjuvant. So it certainly shows you one extreme.

But let me just give also a few introductory remarks, again to thank colleagues both at NIAID and CBER and others

for sponsoring this and also these people here who I'm informed are the organizing committee. I'm sure there are others who have helped support this, and I thank them as well.

One of the interesting things that I think has happened in adjuvants is that there are almost two universes, and the crosstalk recently has started. And that is going to be very productive. One universe is the vaccinologists who see a problem in the immune response and basically take an empiric approach. Another are the immunologists who tend to focus on their pathway or molecule.

And I think one of the goals we should have, particularly our colleagues at NIH but also at CBER is to bring these disciplines together so we're really applying science to what we're trying to accomplish clinically. So that's another great reason for this co-sponsorship.

Tony has mentioned many of these things. What are the needs, potential needs

for and benefits of adjuvants. Well, they can affect the immune response in many ways and there are many examples of these different kinds of effects. And they all have meaning, particularly when we are dealing with emerging infectious disease threats, bioterrorism, et cetera.

One is to enhance the rapidity. And this could also potentially effect the number of doses needed and/or the height or intensity of the immune response. So it could occur earlier. There could be a higher level of antigen or cellular immunity.

This is very important for many antigens that are out there of poor immunogenicity. I often like to think that we've tackled a lot of the infectious diseases that, for which the host really makes a good protective immune response, and this is about the complex interplay of an antigen that may be poorly immunogenic and a host that may not respond well to that pathogen.

So we're left with these pathogens which either aren't terribly antigenic or with a host response that is defective. This could also enhance the breadth of the response. And you heard Tony mention this with cross protection against clades of pandemic flu. So this could protect against pathogen evolution.

The duration of the response is also important. We are discovering more and more issues where memory or priming is surprisingly not as good as we may have thought it was and where people are needing to be re-immunized at various points in life. And there is evidence that certain adjuvants can direct more immune resources towards memory cells.

And, of course, what has driven a lot of this has been the finding that H5N1 influenza was a very poor antigen and the exciting results that some of the novel adjuvants may really improve that situation and, as Tony showed, perhaps result in solving

problems of manufacturing capacity, which, if we think it is critical in this country, is even more critical globally, and also in dealing with the likelihood that when a pandemic strain emerges, it is not going to be exactly what we have been studying or predicted.

Okay. And then as I mentioned, there seem to be a bunch of pathogens in which either vaccines don't work very well or the host doesn't work very well. And I suspect those are just different sides of the same coin. And these are some of the things that were listed by Tony.

But, again, as we look at opportunities to prevent malaria, TB, or HIV, or in the whole arena of therapeutic vaccines where we are dealing with a host failure to mount an effective immune response against an invader such as a cancer cell, these adjuvants could be particularly important.

Now Tony listed some of the things

under study, and I won't go through this in detail. But just to say there are many approaches to adjuvants that range from the commonly used mineral salt such as alum to the more recently used oil-in-water emulsions and then a number of things in earlier stages of investigation.

Also worth pointing out, that as we understand more mechanistically and we understand the deficiencies in the immune response to certain pathogens, it appears possible and even beneficial to combine adjuvants that target different places in that diagram -- that increasingly complex diagram that Tony showed.

What are some of the overall selective mechanisms of action? I know you have some talks from people who have really delved deeply into these. I think the important overriding message is they are still often poorly understood.

As a non-immunologist who used to

work a lot on infectious diseases in the laboratory, I always found immunology very frustrating because I always felt like you could prove anything or nothing. But perhaps that is an unduly skeptical view of it. And as we get to a more molecular level, we'll do better.

But we often find -- the flip side of this is that evolution is wonderfully complex. And whether you look at the clotting system or the immune system, nothing is ever as simple as one receptor or molecular. And these are really complex control loops.

And so that the idea that an adjuvants works on just one thing would probably be a very naive idea. And they often work at multiple steps.

But some of the things that have been identified or interactions with antigen uptake through antigen-presenting cells, or prolongation of that uptake, similarly and related a traction of mononuclear cells,

dendritic cells, even neutrophils to process and present antigen, direct effects on cellular membranes, and, of course, increasingly interactions with these pattern response recognition molecules, including the whole family of TLRs.

And many of these will result in downstream and fairly nonspecific manifestations such as cytokine, chemokine release, and enhanced body and T cell responses. Another thing that others have pointed out that I think is worth considering is when -- while it is a blunt instrument, when you do unleash this whole cytokine response, you also tend to unleash a counter-regulatory response, which actually may be protective against some of the negative effects we worry about for adjuvants.

So the point is that all of these mechanisms can lead to immune and inflammatory responses. That is part of what is desirable. But it also leads to the increased reactions

and sometimes systemic effects that we see and worry about.

And we also -- very important to think about the complexity of the needs for adjuvants and the responses. They may differ with different antigens. There's no reason to think that all antigens would behave the same with one adjuvant or vice versa in different clinical settings, children versus adults, et cetera, or priming verses recall.

So at FDA, we are asked to make some difficult judgments ranging from clinical trial judgments to approval judgments to where to put resources in terms of trying to stimulate product development. And it comes down to, in this area, enhanced immunity versus inflammation, adverse events, and potentially autoimmunity.

And I like to remind people, particularly when many people here are maybe engaged mostly in laboratory investigation, that these products ultimately interface with

humans who are your children, our children, our country's children, the world's children.

And it is very important to understand that large numbers of people get vaccine products and we don't walk through a single day at CBER without recognizing that confidence in all of immunization, which, as Tony showed you, has been a remarkable public health advance, and in our very institutions, is dependent on whether we get this right. So we have a very serious scientific and regulatory responsibility.

So what are some of these potential concerns we want to keep in mind? As I mentioned, you could get an antigen-specific or nonspecific increase in potency of immune and inflammatory stimulation.

We typically see, for effective adjuvants, increased reactogenicity, an FDA term for feverishness, sore arm at the site, things we typically see with non-adjuvanted vaccines but often see more in the presence of

an adjuvant.

I want to point out it is very unclear whether these ever correlate with more severe adverse events. You know occasionally they do. But we have not found, to date -- but the flip side is it would be difficult to find, for example, that increased local reactogenicity or feverishness down the road increases the commonality of some of the more severe adverse events that we might be concerned about such as neurologic events.

There just aren't those data. I think to some degree that may reflect the weakness of our tools to look at it.

Issues have been raised about the potential role of autoimmunity. There is an interesting article just recently in JID from the folks at CDC Penn and other places about what seemed to be antigen-specific reactions to flu vaccine that may cross-react with the GM1 neural ganglioside and could potentially be related to the rare cases of GBS that have

occurred after flu vaccine. And also concerns have been raised about autoimmunity and immune disease in general.

One question that has been raised with children is for some potent agonists, are there plausible risks to a developing immune system. And I don't know of evidence that that would be true but I ask the scientists is this plausible and are there ways that we need to look at it. And I think that is one of the questions at this meeting.

And I'd like to end up by saying we see some reassuring observations to date. One is, as I said, even strong pattern recognition signaling is likely similar to natural infection. It's not -- you know you go through life and you get some pretty bad infections.

And you get a lot systemic reactions, for anybody who has had one of these bacterial infections, and on the other hand, a caveat if people are aware of the

recent study with monoclonal antibody that was an agonist to CD28, which would certainly be a costimulatory pathway, that unexpectedly and despite negative studies in primates, this monoclonal antibody stimulated near lethal effects through essentially T cell stimulation. And, again, people who know much more about this could probably comment on that.

The other good thing is there is no evidence to date of major problems with those compounds being most actively considered. But we always point out the absence of evidence is not evidence of absence. It just tells you a little.

There are very few of these studies with adequate numbers of controls with long-term follow up or with children.

So Norman will probably say more about this but we are here to assess the current knowledge base. And I think really to stimulate a research agenda. And I'd take

that one step further -- to stimulate research collaboration, to be sure we learn from basic science studies what can help us with patients and be sure we've applied basic science to patient studies much more often to learn from those as well.

We're going to review the clinical data, some of the clinical data, and I think an important area that I'll comment once more briefly on is, you know, the toxicology of vaccines, not to mention the toxicology of adjuvants has been a really neglected area.

And, you know, we've tended to only recently pay attention to this. And we've had just tools of conventional toxicology, which largely focus on drug effects on organs. And, of course, when you are talking about immunotoxicology, there are not a lot of good models.

I think there is a huge opportunity for the scientific community to develop better nonclinical or non-human models

and even human studies that could tell us about the safety of novel vaccines and adjuvants.

The good side is I really think this meeting and some of the investment -- and, again, I credit our colleagues in HHS and industry as well in trying to get better flu vaccines -- that all these things are going to bring us to a place where we are going to have successful development and evaluation of vaccines for some of these unmet challenges.

So just to finalize, a few overarching scientific questions that occurred in me, more as an infectious disease person but also as somebody who sees the beginning of your innovations, the question has been asked are there some cases where there is a reason we don't respond to certain antigens that actually may protect us. I'm not sure how important that is but we always need to keep that in mind.

Or is the organism designing how

it presents the antigen to simply evade our immune system? And if so, not only could we possibly design better adjuvants but can we better design antigens or present them in more antigenic manners and have an adjuvant effect in itself without a chemical adjuvant.

And certainly the use of particulate presentations may, in fact, be doing some of that. And alum may do some of that.

As we understand host protection -- and I think this is where the basic science is very important -- can we design adjuvants that work far more specifically? Or will they not work? I don't think we know the answer to that yet.

But, for example, if we are more distal in a pathway, can we get less undesirable information but let's say more turning on of T cells? That would be a question.

And then I mention can we get



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better approaches to vaccine toxicology in general. And I see there are a number of talks at the end of today but I've seen very little where genomics are applied where responses of human cells to certain antigens or substances are applied to look at can we recognize profiles that would be associated with both effectiveness and safety. And I think there is huge opportunity there to bring together the basic scientists with clinical and animal studies.

Very important to remember as we look at models, again, the incredible complexity and, again, just skimming the surface of some of this literature, all different mice with different TOR responses, which may or may not be relevant to humans, so the importance of looking at animal studies with more global knowledge than most of us have and with some skepticism.

This is just one study I recently found though that looked to be a very



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specific, more distal use of an adjuvant approach. And this was a study using a costimulatory ligand for CD137 as an adjuvant for cytotoxic T cell responses. And what you can see on the right, that highest line is the lysis of influenza-infected target cells when this is occurring in a background of this molecule for IBBL being constitutively expressed.

So this is just an example of a very specific molecular tweak on a very specific pathway. Now what I don't know is how many other pathways this then goes and influences. And an immunologist could probably teach me a lot about that.

So, again, I think you for your interest. I showed this slide in various places. But I'm hoping what we end up with is new, improved antigens and vaccines and solutions to our public health problems that are safe and that protect our people.

So thank you very much.

(Applause.)

DR. SLATER: Thank you, Dr. Goodman. Thank you, Dr. Fauci, for those introductory remarks.

Session 1 is focused on background to get us pointed in the right direction in terms of our discussions today. The two speakers in Session 1 will be first Dr. Norman Baylor who is the Director of the Office of Vaccines Research and Review.

And following him Dr. Daniel Totrosen, who is the Director of the Division of Allergy, Immunology, and Transplantation at NIAID.

Dr. Baylor?

DR. BAYLOR: Good morning.

What I want to try to do, in the brief time I'm speaking, is to sort of set the stage, give you a little background about the meeting and sort of where we are going. And also build upon a little bit of what Dr. Goodman and Dr. Fauci stated earlier.

Just as an introduction, when we think about vaccine development, what we want to do for an ideal vaccine is we want to provide the safest vaccine we can, we want to provide a vaccine that has a maximum efficacy, and we want a vaccine that requires the least amount of antigen and the number of doses, preferably one dose.

Now as it has been stated today, the interest in vaccine adjuvants and new delivery systems has significantly increased over the past decade. And a variety of new technology and advances in vaccine development present significant challenges to the national regulatory authorities such as the FDA. However, these products may present opportunities for advancing public health as well as have been presented by the previous speakers.

The FDA, as the national regulatory authority in the United States, we must be in a position to develop new

scientific and regulatory criteria to facilitate the development of these new vaccines, including vaccines with novel adjuvants. And we need to evaluate these vaccines for their safety and effectiveness.

As most of you know, adjuvants are not licensed separately from vaccines which they have formulated in the United States. And currently only aluminum-containing adjuvants are used in U.S.-licensed vaccine.

It is the individual vaccine-adjuvant combination in the United States that is licensed. And this necessitates a case-by-case evaluation of these compounds. But when you start evaluating on a case-by-case basis, this makes it very difficult in developing guidelines that would apply in all situations.

And so what we are trying to do is collect as much information as we can, evaluating the science to try to formulate guidelines that will apply across many situations.



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The other challenges that we see with adjuvants, of course, are the safety concerns which, as has been mentioned to some extent by Dr. Goodman. And so we must, as we do with all vaccines, adjuvanted or not, evaluate benefit versus risk.

One of the issues with the adjuvants is the lack of universality. Adjuvants are currently not considered active ingredients in prophylactic vaccines. So we license and we evaluate the adjuvanted vaccine, not as separate.

And also the immune responses that are obtained with one antigen adjuvant combination cannot always be -- and most of the time cannot be extrapolated to another antigen or even the same combination given by different routes.

Other challenges with evaluation adjuvants is the manufacturing, such as scale-up, consistency of manufacturing from lot to lot, evaluating potency and stability of the

combined product.

And also from a clinical perspective determining the clinical endpoints for assessing safety and efficacy. These are challenges that are presented to us as not only from a development point of view but also from the regulatory point of view.

So the objectives of the workshop over the next couple of days, we will look at mechanisms of action of adjuvants, try to identify the scientific gaps, and also look at approaches to nonclinical safety evaluation for adjuvanted vaccines, what criteria for selecting the appropriate route of administration, doses, schedule, are there animal models that can be used in evaluating these new adjuvants. And also alternate methods. And, of course, clinical experience with respect to safety.

There will be a couple of roundtables today. And just some of the questions that we'll try to get out to really



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tease out in the roundtables, if you think about this there is really your nonclinical and your clinical. Those are your big areas.

And so looking at the current approach to adjuvant toxicology testing is one of the topics we really want to try to get a handle on today. And find out what information do we know.

For example, is it sufficient to test only the highest human dose of the vaccine-adjuvant combination and adjuvant alone? Should the dose ranging studies be conducted on the adjuvant alone? Should other parameters such as cytokine levels or other biomarkers be assessed in evaluating these adjuvants? And are other aspects of current study designs, such as the route of administration or the regimen appropriate?

These are just some of the questions that will come up in the nonclinical discussion roundtable today. And there are a number of others that will come out. This is

sort of -- I hate to use the word free-for-all but it is a free-for-all because we're trying to collect as much information as we can and open up the discussion as we try to evaluate these adjuvants.

Less so, there will be a clinical issue. The clinical issues involved, this will not be as in depth but the things that we'd like to know are what type of clinical studies are needed to, for instance, detect age-specific differences in adjuvant responses going from a pediatric population to an elderly population? What type of long-term safety information needs to be provided? As well as dose ranging data on adjuvants as well as the antigens that they are stimulating.

And what kind of clinical studies can be designed that will incorporate safety information from the preclinical data? So can you build upon the preclinical data as you move into your human studies? Can you translate that data as you are looking and

trying to design clinical studies going into humans?

And, of course, there are a number of other clinical issues that are out there that we probably will not be able to address all of those today. This is, again, an evolving dialogue, trying to really get some understanding of how we're going to evaluate these products and also bring these products to licensure.

So just in summary, the development and evaluation of novel adjuvants present unique challenges. I mean that's obvious. The use of adjuvants in vaccines also can provide an opportunity to improve public health.

In many of the examples that Dr. Fauci showed in his presentation of antigen-sparing, increasing the amount of vaccine, access to vaccines -- I mean the adjuvants may have a huge impact on our ability to improve public health globally.

And then keeping in mind that nonclinical safety assessment as well as the clinical safety evaluation of adjuvant vaccines are critical and those two will be the focus of the panel discussions later on today.

And I believe that's it. Thank you.

(Applause.)

DR. ROTROSEN: Let me thank you again for all the participants today joining us.

I'm going to finish up the introductory session with a little more background on the NIAID perspective and our goals in cosponsoring the workshop today.

The background has been covered amply by all the previous speakers. But just very rapidly, there has been a tremendous growth in information on adjuvant activity. We know a lot now about distinct classes of adjuvants in innate and in receptors that is

fairly new information.

The complexity of the signaling pathways is clearly evident. And these insights provide the potential to further dissect and more important to direct immune responses.

There is growth, although not all that great yet, in the numbers and classes of adjuvanted vaccines entering clinical trials. And we should learn a lot from these examples.

And finally, these developments offer unprecedented opportunities but they will require new research and regulatory approaches.

And our goal at NIAID in cosponsoring this workshop, one of our major goals is to expand the dialogue that we already have ongoing with many of you more on an individual basis to a collective dialogue on how we can position our research portfolio to address these issues and facilitate further vaccine discovery and development.

I think it is worth taking just a couple of moments to kind of review some of the recent history. And Dr. Fauci and Dr. Goodman have mentioned the tremendous growth over the past two decades.

It was just about 20 years ago when Charlie Janeway published this monograph on the Cold Spring Harbor Symposia where he was musing about what he had termed the immunologists dirty little secret, the fact that in animal models immunologists knew that purified proteins rarely generated an immune response. And when one was demonstrated, it was usually weak.

What you needed was the addition of an adjuvant, and at that time, it was usually Freund's adjuvant, to generate robust immune responses. And what Charlie posited was that immune receptors will be discovered that would recognize generalized structural patterns in molecules found on microorganisms but not in mammalian cells.

And it was about ten years later that he and Ruslan Medzhitov demonstrated that was actually the case with the discovery of the Toll-like receptors in mammalian cells. And that triggered the explosion of growth and publications in this area that Dr. Fauci already mentioned.

So there are a number of new insights and emerging opportunities that are quite recent over the past six months or so in fact. We now know that alum signals via the NLRP3 inflammasome. And this insight is really a wonderful example of basic research answering questions that had been rather murky for decades.

And the fact that alum is now known to signal through a particular innate immune receptor and pathway provides a tremendous opportunity for growth in adjuvant engineering, the design of specific adjuvant combinations that signal through distinct but complementary pathways and the like.

We have new technologies published only in the last year or so that reveal that vaccine responses are far more robust than previously appreciated. For example, flu vaccine elicits unexpectedly high number of flu-specific B cells, roughly about six percent of circulating B cells if measured at an appropriate time after vaccination.

And similarly, smallpox vaccine elicits unexpectedly high number of CD8-positive T cells, almost 40 percent of circulating T cells. And these kind of tools for immune profiling coupled with systems biology approaches and transcriptional profiling may provide a variety of new opportunities for dissecting and directing the immune response.

Here's just one example published last summer from the group at Novartis looking at the transcriptional profiles and cytokine activity of mouse muscle cells and the inflammatory cells in those muscles triggered

either with MF59, CpG, or alum -- and you can see the Venn diagrams show a surprisingly distinct set of genes upregulated by each of these with some degree of overlap.

And then on the right Venn diagram a combination of MF59 and CpG versus MF59 alone or CpG alone. So the tools for immune profiling and transcriptional profiling are tremendous.

Another study that came out just this week from the Emory group and Institute of Systems Biology in Seattle took a slightly different approach looking at yellow fever vaccine and the correlates of immunogenicity after yellow fever vaccination. And I think Bali Pulendran will probably speak about that later.

So the potential utility of transcriptional immune profiling is obvious I think. We have great opportunities to identify correlates of vaccine safety and efficacy, to disassociate drivers of

protective immunity from toxicity and reactive genecity.

And to adjust and optimize antigen adjuvant content and formulation to achieve these goals. And explore and compare responses across species, in vitro versus in vivo.

And in special populations we have unique problems in vaccinating the very young and the very old. And perhaps this type of transcriptional immune profiling will help us identify approaches that would be more effective in these populations.

So to sum things up, I want to reaffirm the commitment that Dr. Fauci voiced earlier to supporting fundamental research at the interface between innate and adaptive immunity, in particular to enhance the understanding of the biochemistry and the biophysics and formulation issues and how they influence adjuvant activity.

It is a topic that NIAID has not

supported all that substantially. And industry has supported much more robustly. But there is an important role for academic scientists in this area as well.

We are committed to enlarging the pipeline of potential adjuvants and developing safer and more potent adjuvants. And finally to supporting a highly-trained cadre of investigators and providing them with the tools they need to pursue these cross-disciplinary approaches.

And with that I'll thank you for your participation today. And we'll begin the main session.

(Applause.)

DR. SLATER: Thank you all very much.

We're now going to begin Session 2. I'm going to ask the Session 2 co-chairs and speakers to come up to the lecterns. Session 2, which is our specific adjuvants overview, will be co-chaired by Dr. Elizabeth

Sutkowski and Dr. Bali Pulendran.

Dr. Pulendran is from the Emory Vaccine Center. Dr. Sutkowski is from CBER/FDA. And they will introduce the session.

DR. SUTKOWSKI: Good morning everyone. And thank you for coming to this NIH and FDA cosponsored public workshop on adjuvants and adjuvanted preventive and therapeutic vaccines for infectious disease indications.

I'd like to thank Drs. Jay Slater of CBER and Chuck Hackett of NIAID for asking me to co-chair the specific adjuvants overview session along with Dr. Bali Pulendran.

I'd like to open by quickly highlighting a just a few of the initiatives that have been undertaken in the past few years regarding vaccine adjuvants and adjuvanted vaccines. The first entry here is a reminder that exactly six years ago today, on December 2nd and 3rd of 2002, CBER co-

sponsored a two-day workshop together with the Society of Toxicology on the nonclinical safety evaluation of vaccines in general in which a couple of talks were on adjuvants or adjuvanted vaccines.

Then came the WHO guidelines on nonclinical evaluation of vaccines, which was published in 2003. And it contained a special consideration section that focused on adjuvants. And then in 2005, the EMEA published a guideline that was dedicated specifically to vaccines adjuvants which was quickly followed by a note on immunomodulators in 2006.

And now we have this two-day workshop on adjuvants alone and adjuvanted vaccines. So we've come a long way.

In the EMEA's guideline and explanatory note, adjuvants are called adjuvants if they are included in the formulation with the antigen but they are called immunomodulators if they are given

separately from the antigen, whether given at the same time or at a different time.

It should be noted, however, that although there is the distinction in their names, the principles of the EMEA guideline on adjuvants published in `05 apply to both adjuvants and immunomodulators.

In the next few slides, I'd like to go over just a couple of definitions and regulations. Our office, the Office of Vaccines Research and Review, or OVRR, regulates the preventive and therapeutic vaccines for infectious disease indications.

This is in contrast to therapeutic vaccines for other types of indications such as cancer vaccines. Those vaccines would be regulated by OCTGT, the Office of Cell, Tissue, and Gene Therapy within CBER.

And since they are targeted for a different patient population than most preventive vaccines are targeted for, they would likely result in a different risk versus



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benefit assessment.

As far as definitions of adjuvants go, we, in the Office of Vaccines in CBER, would define adjuvants as agents added to or used in conjunction with vaccine antigens to augment or potentiate and possibly target the specific immune response to an antigen.

It is also important to point out, as was already mentioned, that in the U.S. adjuvants alone are not currently licensed as such but rather each specific antigen plus adjuvant formulation is licensed as one adjuvanted vaccine.

With respect to vaccine regulatory requirements, the IND regulations are covered under Section 312 of the Code of Federal Regulations, or CFR. And these include the items that are required to be an investigational new drug application or IND.

For example, the chemistry manufacturing and control or CMC information and the pharmaceutical. tox. information,

which specifically should include data from in vivo or in vitro studies on the basis of which it can be concluded that the product is safe for use in humans.

The licensure-relevant regulations are covered in Section 610 of the CFR. And, for example, these include the requirements that were already mentioned by Dr. Baylor for lot release, potency, general safety, sterility, purity, and identity, et cetera.

Also in this Section 610 are the regulations that are specifically relevant to adjuvants. And these include those under Section 610.15 on constituent materials, which includes ingredients, preservatives, diluents, and adjuvants and states that like all other vaccine components, adjuvants shall meet generally accepted standards of purity and quality.

This means that for clinical studies a certificate of analysis for the adjuvant would need to be provided to the IND

and is often also provided to a cross reference master file for the adjuvant. This regulation also states that an adjuvant shall not be introduced into a product unless there is satisfactory evidence that it does not effect adversely the safety or potency of the product.

This will be the topic of the session that will follow this session that is going to occur soon. This following session will be later today after lunch. And also, of course, the clinical session would address this as well.

So as far as the product-relevant data that is required to be submitted in an IND, it should include sufficient information regarding the adjuvant and the adjuvanted vaccine formulation. This routinely includes info on the source of the products, how they are purified, the general QC testing, and product-specific QC testing conducted, as well as lot release and stability data, if

available.

For an adjuvanted vaccine, this testing would include an assessment of antigen and adjuvant content in the final formulation and of the particle size distribution for the adjuvant, for example, if appropriate, as well as an assessment of the integrity of the antigen adjuvant mixture upon storage.

It is also helpful when a sponsor provides functional information on the adjuvanted vaccine formulation to include the rationale for including the various components and the rationale for the particular dose of adjuvant if such data are available from pilot studies, for example.

Also sponsors are encouraged to demonstrate that the product causes an immune response in animals and to demonstrate immune response enhancement by the adjuvant.

So having said that, the goals of this session are to provide updates on how several different types of adjuvants are

thought to work. We've invited several speakers to provide information on how specific adjuvants activate both innate and adaptive immune systems and to discuss their lessons learned with respect to how well animal studies predict human responses and their experiences regarding formulation issues.

So without further delay, I'd like to just now invite the co-chair of this session, Dr. Bali Pulendran, to provide a few introductory remarks. He is a professor at Emory in the Emory Vaccine Center. And his area of expertise is the innate immune system.

DR. PULENDRAN: Thank you very much, Liz.

Good morning. I'd like to thank the organizers for inviting me to participate in this very interesting and exciting workshop. And basically I'd like to introduce the speakers for this Session 2.

And as Liz mentioned, the goal of

the session is to stimulate discussion about adjuvants, what is known and what we would like to know about the biology underpinning the mechanism of action of adjuvants and also what we would like to know about the mechanisms that might mediate the toxicity -- mediated by some of these adjuvants.

So just to sort of set the tone from a historic perspective, if you take stock of the major vaccines that have been made since Edward Jenner's smallpox vaccine in 1798 right through to the first recombinant vaccines to be licensed, say, for example, the Hepatitis B vaccine, what I find very interesting about this slide is that despite the success of many of these vaccines, we really do not understand the mechanisms by which they stimulate immune responses, okay.

Why? Because most of these vaccines have been made empirically. So the notion that these induce strong immune responses is really driven by empiricism and

by what we see.

So given recent advances in immunology and innate immunity that Dr. Fauci and Dr. Rotrosen and others have spoken about, the question is to what extent we can deconstruct some of these empirically-derived successful vaccines. And to what new insights can be gain from such deconstruction that might be useful in designing new and emerging vaccines, okay.

So one of the dilemmas that vaccinologists have is that if you look at the timeline and if you go from Jenner's smallpox vaccine right the way through to the first recombinant vaccine, even though the vaccine purity has progressively increased with time, we also see that there is an increasing requirement for exogenous adjuvants, okay.

Now all of us in this room know why this is the case in hindsight but this was not so obviously as recently as ten years ago. I don't think any one of us could have told

ourselves why is it that some of these highly successful vaccines are successful.

Well, we now know that innate immunity, the so-called science of adjuvants has really demystified this area of adjuvant research, which is a bit like a witch's brew but now with all the new insights about Toll-like receptors, C-type lectins, NOD-like receptors and so on that we're going to hear much more about from Drs. Bruce Beutler and Fabio Re and other this morning.

And the idea that cells of the innate immune system like dendritic cells macrophages play an absolutely key role in sensing vaccines and adjuvants and then translating this information into useful or productive immune responses.

So these insights are now beginning to guide the future, development of new adjuvants and vaccines. So just as a point of example, a few years ago in my lab we demonstrated that this highly successful

vaccine, the yellow fever vaccine, which is, in fact, a live virus was working because it was engaging multiple Toll-like receptors.

Toll-like receptor 9, 8, 7, and 2. And here was a vaccine that had been in use for the past 70 years or so given to 600 million people globally. And it was engaging good old Toll-like receptors.

So in a sense, one might make the argument that Toll-like receptor ligands, indeed a combination of TLR ligands has already been licensed for use to be given, okay. So deconstructing some of these vaccines has been very fruitful.

Another example that the innate system does not work simply through Toll-like receptors comes from the work of Dr. David Nemazee and Dr. Bruce Buetler who showed that, in fact, some of the adjuvants that are used in animals but also in humans, for example alum, does not engage TLRs or do not require TLRs for the induction of antibody responses.

And I think we're going to hear more about this from Bruce and from Fabio Re.

Now the other side of the coin has been something that we've neglected. So immunogenicity is one thing and we are beginning to apply innate immunity trying to figure out how to make immunogenicity better. But I think we have been relatively negligent about the other side of the coin, which is toxicity.

So some of the questions that I think we should focus on are number one, what are the mechanisms that mediate vaccine toxicity? Number two, are these mechanisms similar to those that mediate vaccine immunogenicity or are they quite distinct.

So, for example, last year in Science there was a paper that showed that MPLA, which is TRL4 ligand activates mostly the TRIF pathways signaling. And that this might account for the reduced toxicity of MPLA relative to some of the other TRL4 ligands.

Another question is to what extent can toxicity measurements in animal models be extrapolated to humans? And here, you know, what comes to mind is the fact that these innate immune receptors showed differential expression profiles in mice versus humans. And so, for example, TLR9 is expressed only on human PDCs whereas it has a much broader express profile in humans.

And so how does this impact the evaluation of toxicity profiles between these two species. This is the key, key area which I think Dr. Bob Coffman will address tomorrow in his discussion.

And then finally, to what extent do formulations and delivery systems impact on the toxicity of adjuvants in vaccines. So, for example, if you have nano particles or ISCOMS that target antigen presenting cells, does this mitigate the indiscriminate bystander activation of undesirable cells of the immune system, okay.

So this is an area that is under active research. And Eugene Maraskovky and Derek O'Hagan will address this issue.

So with that said, let's move on with the agenda. Here it is. We have eight presentation -- actually seven presentations. Firstly Fabio Rey will talk about the activation of the inflammasome by adjuvants.

This will be followed by two talks on liposomes, micro particles -- first one by Derek O'Hagan and the second one by Eugene Maraskovsky on ISCOMS. And then we'll have a coffee break and then Bruce Beutler will tell us about TLRs and how they regulate vaccine responses.

This will then be followed by Nathalie Garþon who will talk about adjuvant development from an industry perspective. And then Dr. Geert van den Bossche from the Gates Foundation will tell us how to use adjuvants, the perspective from the Gates Foundation.

And then finally I will give a few



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comments on the possible synergy between TLRs and CLRs and the applications of systems biology in predicting the immunogenicity of vaccines.

So with that, I think we can move on with the next speaker who is Fabio Re from the University of Tennessee.

Fabio.

DR. RE: Good morning and I would like to start by thanking the organizer, in particular Elizabeth and Bali for the invitation and the opportunity to show you some of our results still unpublished regarding deactivation of the NALP3 inflammasome by different adjuvants.

And as we heard before by Dr. Rotrosen, 20 years ago Charlie Janeway would famously declare adjuvant immunology's dirty little secret. And we heard that from that time that immunity has really bloomed in great part thanks to Janeway.

And we have learned how some of

the adjuvants work or start to understand how they work, in particular adjuvant-like microbial products which clearly stimulate pathway recognition receptor.

We -- the general assumption should be that these substances that act as adjuvants may mimic biological activities which are associated with live pathogens. And that is clearly the case with microbial products.

We know much less about a whole variety of other substances that works as adjuvant, in particular particulate adjuvant, we know very little about how this molecule works until recently.

So particulate adjuvant comprised a wide variety of substances, including solid carrier particle such as polystyrene microsphere, chitosan. Chitosan, I'll show a little bit about chitosan. Chitosan is, as you may probably know, a fragment of the exoskeleton of crabs, basically a

polysaccharide, alum, you are all familiar with, the immune stimulatory complex, which are lipid particles and saponin, which is QuilA, QS21, emulsion particles, such as the adjuvant MF59.

So the proposed mechanism of action for this class of adjuvants, particular adjuvants at least the most cited is the so-called antigen depot theory. So what is believed is that the antigen, by absorbing to the particle of adjuvant, would lead to an increased stability and concentration of the antigen at the injection site.

This would prolong the time of interaction between the antigen and antigen presenting cells. This would also enhance the antigen uptake, being a particle, through phagocytosis or endocytosis. And finally, also importantly, would ensure the delivery of antigen and adjuvant to the same antigen presenting cells.

Now these are clearly -- these

different effects are clearly responsible for the adjuvant property of these different substances, however, the antigen depot theory has been challenged by quite a few reports. What is being shown, for example, is that it is not really true that the antigen remain and the antigen concentrate and stability is increased at the injection site.

For example, it has been shown that the antigen elude pretty quickly from the adjuvant particle. Also it has been shown that you can still elicit an immune response even if you inject antigen and alum separately if you use enough antigen concentration.

So that suggests that other mechanisms may also be involved in the mechanism of action of these substances. So among the particular adjuvant, alum is clearly the most successful one. And, as we heard before, the only one that is really approved by FDA in the United States.

So these are alum. These are

crystals of aluminum hydroxide and aluminum phosphate. Alum promote bias responses with high IgG1 and IgE titers. And it probably the major limitation of these antigen, of these adjuvant, and these prevents its use in those situations where you would rather have elicitation of a Th1 type of immune response.

So what is the mechanism of action of alum? As I said, the most believed is that antigen depot theory with the caveat that I mentioned before. So suggesting that other mechanics may also account for the activity of alum.

Alum is being tested by many different laboratories and it clearly does not activate Toll-like receptor and does not induce dendritic cells maturation.

So among the other activity that has been ascribed to alum, which may well account for its adjuvant ability, is the ability of alum to fix complement. It has also been illustrated that alum injection

result in formation of a granuloma containing antibody-producing plasma cells. And in some cases, in some extreme cases, this may actually result in a sterile abscess. So it is clearly an inflammatory reaction at the site of injection caused by alum.

Alum has been demonstrated to induce -- injection of alum induce influx of neutrophils and interleukin-4 expressing eosinophils in the spleen and these cells are then being shown to be able to prime D cells.

What is interesting is that it has been demonstrated that in IL4 not compromised or otherwise IL4 nonresponsive animals, mice, alum induced only a Th2 response but also Th1 response, suggesting that IL4 has been already known down-regulate the Th1 response.

And finally something that has been known for some time is alum induces necrosis at the injection site. And in the second part of the talk, we will see a little bit about necrosis and how these may actually,

indeed, be mechanisms of action of alum.

So one question that was clearly important to address and that many labs try to address is does alum mimic features of pathogens. And so the way we -- that would mean does alum activate any pattern recognition receptor.

And so as you all know, innate immunity relies on the ability of cells to recognize microbial products and endogenous danger signals through classes family of pattern recognition receptors, which can be soluble, it can be expressed on the cell surface, can be expressed in the cytoplasm.

And these recognition events would trigger a signaling event which lead to production of a wide variety of inflammatory molecules and to the reprogramming of the antigen-presenting functions of the dendritic cells and other antigen-presenting cells, which eventually culminate in the antigen process and presentation and activation of

that immunity.

So among pattern recognition receptors, the mouse studies -- the first correct rising details are the Toll-like receptors, which are expressed on the cell surface or in the endosoma compartment of several different cell types.

And they recognize microbial products or endogenous danger signals and activate signally pathway, most notably the NF kappa B, the MAP kinase, and interferon reconstructor pathways which lead to a transcriptional response without regulation of a wide variety of proinflammatory mediators, including here -- and I put here also including the cytokine belonging to interleukin-1 family.

Now more recently, too, a family of pattern recognition receptors has come to prominence. One is the RIG-like helicases, which detect viral genomes in the cytoplasm of cells in contrast to Toll-like receptor which

really scanned the extracellular and endosomal compartment.

And the pathway activated by RIG-like helicases are largely similar and overlapping with those of TLR, including NF kappa B, MAP kinase, and interferon reconstructor, again leading to production of inflammatory mediation.

Another family of pathway recognition receptor is the NOD-like receptor or also known as nucleotide binding leucine-rich repeat containing receptor. These are expressing also in the cytosol of cells and they recognize, again, microbial product or danger signal.

And in contrast to Toll-like receptor and RIG-like helicases, the pathway they activate is not really the MAP kinase and NF kappa B but rather they, as far as we know right now, they use as an effector molecule Caspase-1, lead to activation of caspase-1, and activation of these proteases is a key

step for the secretions of the interleukin-1 family of cytokines. And this would be interleukin-1 beta, interleukin-18, and interleukin-33.

So this slide illustrates the architecture of these molecules that are over more than 20 NLRP member in humans.

They are correctly classified central domain, which is a nucleotide binding domain, a leucine-rich repeat domain, and an end terminal domain which is either -- which can be classified into a pyridine domain so the nomenclature for this family of molecules, which was quite confusing, is now called this NLRP if they contain a pyridine domain and the end terminals or NLRC if they contain a CARD domain. This is a caspase activational recruiting domain such as IPAF.

So the most studied NLRP family members are IPAF and NLRP3. And these are the ones that I will talk in more detail today. Also NOD1 and NOD2 have received a lot of

attention although it is still not clear how -- whether they are really part of an inflammasome.

So I didn't introduce the inflammasome yet so activation of caspase-1 mediated by these NLR molecules, of course in the context of a multi-protein complex which has been termed the inflammasome.

And these illustrate the composition of the inflammasome that is better studied right now is the NLRP3 inflammasome.

Again, this is also -- this molecule is also known as NALP3 or cryopyrin or CIAS1. There are quite a few names. So the way we think the inflammasome is activated is is illustrated here.

So it is believed that in the resting state, this molecule is inactive by probably an intramolecular interaction between the leucine-rich domain and that NALP domain and the nucleotide-binding domain.

In reference to recognition of



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ligand, and there is a big question mark here -- what are these ligand, and if we have time, we may go into that in detail, there is oligomerization of these molecules, which recruit an adaptor molecule called ASC, which then bridge and activate and recruit also caspase-1, which is the effector molecules.

This brings to activation of caspase-1, which then proteolytically cleave pro-interleukin-1-beta, pro-interleukin-18, and pro-interleukin-33 to give the mature form of of interleukin-1-beta, which is now being secreted.

So secretion by a synthesis of interleukin-1 family then requires at least two key steps, one is the induction of the messenger and the pro-immature protein, which is triggered by classical inflammatory stimuli, in particular Toll-like receptor, and then activation of these inflammasomes, which is regulated by these NLR molecules.

This slides summarize what we know

so far about these NLR molecules, at least the one that activates inflammasomes. So NLR1 and NAP1 activate inflammasome in reference to anthrax leukotoxin, IPAF, or as it is known now, NLRC4 mediate inflammasome activation in reference to a wide variety of intercellular bacteria or bacteria that possess type 3 or type 4 secretion systems.

And it has been proposed that the actual ligand might be the flagellin, which -- a immunometric which is injected through these types of secretion systems into the cytosol cells.

And finally now, three cryopyrin or NLRP3, and I will call these molecules NLRP3 from now on, which is activated and inflammasome activation in reference to a wide variety of product. You have intercellular, and otherwise bacterias which activate this pathway.

You have muramyl dipeptide. This is a breakdown product of the fetal ligand

which, by the way, has also been demonstrated to be an adjuvant. This is actually the active compound of Freund complete adjuvant which has been demonstrated to activate these pathways.

It is also interesting that MDP has also been shown to activate also NLRP1 as well as NOD2. So this molecule may act on different -- target different NLR molecules.

Recently it has been demonstrated that several particles such as asbestos fiber or silica particles activate this pathway, thus explaining the activities of these compounds -- the proinflammatory activity, which we've known for a long time about is compounds.

Extracellular ATP has also been demonstrated to activate this pathway. This would represent an endogenous danger signal. So a molecule that is released -- for example when cells die by necrosis -- and therefore, it is believed the immune system learned to

recognize these as a danger signal and, therefore, activate pathway that is protected.

And importantly, NALP3 inflammasome has been demonstrated to be activated by monosodium urate crystals. So these are the crystals that accumulate -- these are urate crystals which accumulate in the joints of people with gout disease. And it has been known for a long time this was a chronic inflammatory disease and nobody really knew how this works.

So the group in Lysine published these observations. And that is what really triggered our interest in the inflammasome and in the alum. So the question that we asked is well these are what is important, the urate has to be a crystal -- has to crystalize.

Soluble urate would not activate this pathway so it isn't -- and, in general, it is now clear that several different particles and crystals are able to activate this pathway. So what we ask ourselves is



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well, does alum, which is a crystal, activate this pathway. And that is clearly the case.

So what we do here, we stimulate human PBMCs with different combinations of alum and LPS and then measure production of natural IL-1 beta in the culture supernate. And as you can see here, we are using three different formulations of alum. This would be aluminum phosphate, aluminum hydroxide, and this is the alum inject stuff you buy from peers which may turn out to be not really aluminum.

So in any case, when you stimulate cells with this compound alone, you don't have any production of IL-1 beta. When you stimulate cells with a clean preparation of LPS at low concentration, you have an negligible amount of IL-1 beta release.

However when you add LPS together with the different alum, you have a robust production of IL-1 beta. So this response is blocked by an inhibitor of caspase-1, showing

specificity for the inflammasome.

You can achieve the same effect if you proliferate the cells with a different Toll-like receptor agonist. This would be a synthetic lipopeptide that activates TLR2 and, again, in the presence of alum you have production of IL-1 beta.

So the activation of the inflammasome by alum is blocked by cytochalasin B, suggesting that phagocytosis of the alum particle is required for activation of this pathway in contrast, for example, to the response -- the activation of the pathway by ATP which is now sensitive to cytochalasin B and also occurs with a different kinetics.

You may look at the other cytokine belonging to interleukin-1 family, IL-18, and you will find again that the cytokine, the mature form, is produced in cells stimulated with LPS plus alum.

So this slide illustrates from a

biochemical point of view the activation of the inflammasome. So caspase-1 is also produced in an inactive, immature form, which is then self cleaved into an active p20 and p10 sub unit so the presence of the p20 caspase-1 sub unit in culture is a measure of activation of caspase-1.

And, again, you see that caspase-1 -- the mature caspase-1 is present only in cells stimulated with LPS plus alum. This is blocked by the caspase-1 inhibitors. And interestingly, you see that alum alone, without any LPS, is also able to activate caspase-1.

And down here, you have also a demonstration of the cleavage and maturation of IL-1 beta. So again when you look in the cell lysis of cells stimulated with LPS in the presence of -- or with or without alum, you will see a similar amount of pro-IL-1 beta. This is the immature form of the proteins.

But then when you look in the

culture supernate and you will find the mature form only in cells illustrated with alum and LPS. Again, caspase-1 inhibitor blocked the processing. And importantly, alum alone does not induce any IL-1 beta production.

So the big question -- so we published those results and then the big question was which NLR molecule mediated a response inflammasome activation by alum. And so to make a long story short, it took us a long time to get the mice.

So I pair up with -- I team up with Jenny Ting who had the mice and send us some bone marrow of mice deficient in the ASK molecule. This is the adaptor which links NLR to caspase-1 and then mice deficient in NALP3, NALP3 cryopyrin or NLRC4, which is IPAF, and asked -- we made dendritic cells out of these cells and stimulate them, measuring IL-1 beta.

And as you can see, dendritic cells derived from wild-type mice or IPAF,

NLRC4-deficient mice, are still able to produce mature IL-1 beta in response to LPS hydrogel and our cells deficient in ASK and NALP3 are unable to produce IL-1 beta.

These are just the specificity of controls so these are cells infected with listeria monocytogenes, which is known to activate NALP3. And, indeed, you lose the response in NALP3 and NLRP3 knockout cells and, in contrast, salmonella, which mediate activation of the inflammasomes through IPAF is still working in NALP3 knockout cells.

You look at other cytokines, such as IL-6, which do not depend on the inflammasome, and you will see that they express an equal amount between what are now NALP3 knockout cells.

ASK cells consistently have lower amount of these and other cytokines, which probably suggests that ASK may be involved in other pathways other than inflammasome activation

This slide, again, simply is a demonstration of activation of the inflammasome at the biochemical level and we can skip through this one.

So what we asked is how common is activation of this pathway by adjuvants which look at other adjuvants, in particular chitosan, as I mentioned -- these are the fragments of the exoskeleton of crabs -- and QuilA -- this is attracted by tree bark.

And as you can see, you have a combination of LPS plus chitosan and quillaja results in production of IL-1 beta. This is blocked by the inhibitor caspase-1, similarly for IL-18. And then when you look in the mice, again you see that this response, secretion of IL-1 beta or IL-18, in reference to LPS chitosan or LPS Quil, again it is lost in NALP3 knockout mice.

So here we have at least three adjuvants and a fourth one if you consider IMDp, which are known as -- now have been

demonstrated to activate a NALP3 inflammasome. And what is interesting to note is that this cytokine, IL-1 beta, IL-18, and IL-33 have all been associated with the Th2-type of response.

IL-1 beta has been demonstrated to be an adjuvant which suggests that they may play an important role for -- in the action of alum, which is also a Th2-type of immune stimulator.

And then finally we asked the big question -- is alum still an adjuvant in IL-3 knockout mice? So what we did here we vaccinated mice with a commercial vaccine. This is a pediatric diphtheria, tetanus toxoid which is adjuvanted by alum or with a homemade vaccine which is avomine absorbed to aluminum hydroxide.

And as you can see, then we measure total IgE or antigen-specific IgG1 production. As you can see, this response is reduced in the NALP3 knockout mice. However, it is not completely abrogated, suggesting to

us that other mechanics are also responsible.

So at the same time, we published our observation, the group of Richa Shlavel also reported similar results in this paper. What they see, in contrast to our result, is a complete lack of response in the NALP3 knockout mice.

More recently, two other groups -- Shaw Group and Gabrielle Nuniz in Michigan they also reported activation of the the NALP3 inflammasome by alum in contrast to our result. So the -- so the Shaw Group saw a difference in the IgE -- different than we saw but not much in IgG1. More interestingly, the saw an actual increase in the production of IgG2c, which is a Th1-associated type of hemoglobin, suggesting that if you lack NALP3 inflammasome activation and the four you lack, IL-1 beta, IL-18, IL-33 production, you may skew the response to Th1.

And finally, the group of Gabrielle Nuniz didn't observe an appreciable

difference in vivo for the vaccination in mice.

So that tells you that there are clearly other mechanisms that are responsible for the alum adjuvant effect. And that clearly one measure or point that comes out of this is that we need really to standardize experiment and vaccination protocols.

I'm running kind of slow so I probably should stop, I guess. So let go to the conclusion and just to leave you with some open questions, so one thing that is important to us, at least to my lab, to understand is what is the role of the interleukin-1 family in the alum adjuvant effect. As I mentioned, all these cytokines are known to be adjuvant and associated to Th2 type of response, which is the same, which is activated by alum.

And also what is the role of necrosis and release of endogenous danger signals indicating an adjuvant effect.

I'll just briefly mention -- I

didn't have time to show you -- it has been known that alum activate some form of necrosis, at least in vivo, and necrosis is associated with release of these endogenous danger signals.

And we have evidence that -- I couldn't show you -- that necrosis activate inflammasomes and also that it has been known for some time to be an adjuvant necrosis to be immunogenic. So I think this is another important area to explore.

So let me thank Hanfen Li, who follows most of the studies. And Jenny Ting that initially provided us with the knockout bone marrow. And finally Vishva Dixit, which now has given us -- has provided us the mice. And NIH for and R01 R21 grant.

So I'll take some questions.

(Applause.)

DR. MALONE: Can you take a question?

DR. PULENDRAN: Yes.

DR. MALONE: Robert Malone speaking as an empirical vaccinologist. Can you comment on the role of these pathways in VLP activity? And can you comment on potential toxicology associated with inflammasome activation?

DR. RE: Well so -- yes, I'll start with the last one. So the toxicology implication, I find it interesting that -- so there are a few syndromes -- out inflammatory syndromes which are due to a mutation in NALP3. And so patient with this disease have mutation in NALP3 which lead to conservative activation of NALP3, which results in their symptoms, which are recurrent fevers and skin rashes and atralgia.

So these mutations result in a conservative active NALP3 pathway. These patients don't have -- so far, there isn't any evidence that they have any other disease like out inflammatory or, you know, there isn't much evidence on that so I don't know if that



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may answer your question.

So you may envision using substances that activate inflammasomes without that many side effects. This is just a very naive -- so I'm not sure I -- what was the first one?

DR. MALONE: (Speaking from unmiked location.)

PARTICIPANT: If you could please use the microphone.

DR. MALONE: Viral-like particles. So part of your thesis is that alum is activating -- and other crystalline formulations are activating your inflammasome activity. And that's contributing to the potency of the formulation, right?

So virus-like particles clearly appear to have enhanced potency relative to non-particular formulations. And so I'm wondering whether you can comment on whether VLPs enhanced potency or apparent enhanced potency may be a consequence of an activation



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of inflammasome activity.

DR. RE: VLP meaning bacterially lipoproteins?

DR. MALONE: Virus-like particles. Okay. We're in very different fields, I guess.

DR. RE: Yes.

DR. MALONE: Remember I'm an empiric vaccinologist so we use the terminology VLP to refer to virus-like particles.

DR. RE: Oh, VLP, okay.

Yes, it is, you know, it is clearly -- you know the consensus is that this inflammasome, at least the NALP3 inflammasome, is activated by particle. These are crystals.

One of the things that have been -- not all particles activate these inflammasomes. For example, some microsphere, polystyrene microsphere, in our hands do not really activate inflammasomes.

So one of the ideas that has been



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proposed is that there is this frustrated microphages. So a microphage that tries to phagocytose a particle, like crystals or asbestos fiber, which has not really been prepared to, in nature during evolution, to phagocytose.

And this may lead -- I didn't have time to go into detail -- to destabilization -- may lead to destabilization of the lysosome. And that may lead to release and leakage of lysosome proteases in the cytokine. That what might be what activated the inflammasome.

So I don't know if viral -- viralized particle could activate inflammasomes.

DR. PULENDRAN: Thank you, Fabio.

We're running behind time so if you can just take a couple of -- two quick questions. I think you and then you. Thank you.

DR. REED: As far as the mechanism of alum in producing the responses you talked



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about -- this is Steve Reed from Infectious Disease Research Institute -- you showed that alum alone was inert in terms of inducing IL-1 for example. But alum plus LPS had enhanced over LPS alone.

How is this relevant to a vaccine? What happens if you put alum on a real vaccine target? Did you see anything like that? What is the conclusion? Alum is actually responsible for this enhancement rather than just changing the form of LPS?

DR. RE: Well, alum -- so it's -- as I show you, you don't require LPS. Actually I didn't show you. You can, for example, pre-activate the cells with TNF. This will lead to production of IL-1 beta and alum would activate, again, the inflammasome. Is that what you were asking?

So it's really -- and, again, so the other things that one might ask is what happened in vivo when we inject alum but there is no LPS. So one of the things we are



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thinking is that, for example, IL-18 is already present and conceivably able to express in many cell types.

So alum alone could be sufficient. You don't need in that -- for IL-18 -- and there is evidence -- and it may be the case also for IL-33 -- you don't need the classical proinflammatory priming of cells to build up the pro-IL-1 beta or IL-18 because many cells express conservatively pro-IL-18.

So in that case, alum may induce or release just on itself IL-18 and IL-18 has been demonstrated to then trigger transcriptional activation -- transcription of Il-1 and IL-33. So that could be a mechanism in vivo.

DR. REED: Thank you.

DR. PULENDRAN: One last question.

PARTICIPANT: So given the restriction of the inflammasome, what is the role that it plays in the secondary versus primary responses?

DR. RE: You know, I can very little here.

PARTICIPANT: Have you look at secondary responses before for the inflammasome?

DR. RE: Secondary response meaning --

PARTICIPANT: Memory.

DR. RE: Oh, no. Yes, no, sorry. No, we didn't. Not yet.

DR. PULENDRAN: Okay. Thank you, Fabio.

We'd like to move on to the next speaker, who is Derek O'Hagan from Novartis who is going to be talking about first generation adjuvants, the use of liposomes and microparticles.

Derek?

DR. O'HAGAN: So, good morning. And I'd just like to start by thanking the organizers for the opportunity to be here.

So I'm going to talk about first



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generation vaccine adjuvants and I'll define better what I mean by first generation in a moment.

I wanted to highlight that I'm going to be talking predominantly in the context of vaccines for infectious diseases against to protect against -- not therapeutic vaccines. So the risk-benefits evaluation is somewhat different.

I was going to start with a slide that kind of highlighted why we include adjuvants in vaccines but I'm already starting to realize that probably every speaker has their own version. So this may be somewhat repetitive.

But in essence, you know, we include adjuvants for practical, pragmatic reasons. You know some of them are very important in relation to pandemic influenza, dose bearing, higher titers, responses more rapidly. And the breadth of response is really important.

So adjuvants are here because vaccine is increasingly purified, soluble recombinant proteins, poly immunogenic, we need them.

So this is my attempt, after many years looking at it, to find some kind of classification that we can understand vaccine adjuvants. I've tried it many times in the past. They define easy definitions, there are no two ways about it.

And in relation to generation one, what I'm really talking about are the kinds of particulate carriers, dispersions, particulates. These have been around for quite some time.

Aluminum, we've talked about, but clearly the most well established, licensed in Europe, licensed in the U.S.

Other approaches came along somewhat not long after. Freund first brought forward water and oil emulsions. And interestingly, it was another famous

vaccinologist, Jonas Salk, who really looked at water and oil emulsions with flu vaccines in the 1950s and made some pretty key observations that you enhance the response and you allow a significant dose reduction.

So water and oil emulsions were very effective but their tolerability profile was not great and not appropriate for prophylactic vaccines. So oil-in-water emulsions were developed. Subsequently I'll talk a lot about those since they are the most prominent really for new generation adjuvants.

Calcium phosphate was on the market in Europe for quite some time, then kind of replaced by aluminum. Liposomes are licensed in Europe to be used with influenza vaccines. And tyrosine is used for allergy vaccines.

More recent developments include microparticles and nanoparticles, which I'll talk about at the end of the talk if I have time. So many of these technologies have been

around for quite some time.

And generation two adjuvants, in essence they mostly represent the first generation with something added. So something added are the kind of things we've started talking about today already -- TLR agonists, NLR agonists. The most advanced is AS04. And Nathalie Garþon will talk about that, I believe.

But I just wanted to add, you know, a couple of dates here. The concept of generation two adjuvants has been with us for quite some time.

People were adding TLR agonists in the `60s and the `70s without understanding what they were, what they did. We just knew they were immune potentiators.

So they have been around for quite some time. But obviously it is only relatively recently with AS04 have they started to gain acceptance and approval.

And then there are some newer



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concepts like ISCOMS and IC31. ISCOMS will be spoken about later.

So here I wanted to try and say what are we looking for, what are we trying to achieve with vaccine adjuvants. And kind of on the right-hand side is what we perceive as ideal. What do we really want?

Certainly we want something that is safe and not associated with any long-term effects. But also we need it to be well tolerated. So short-term reactogenecity is a key issue if you are going to have a successful adjuvant.

Other important factors -- it needs to be simple, easy to scale up, the manufacturing needs to be reproducible, and it needs to be easily characterized and perhaps these will be discussed a lot more about characterization.

Ideally, it should be made from abundant, inexpensive components, things that are readily available and not hugely

expensive. These components should be biodegradable and biocompatible.

Ideally the adjuvants should be compatible with many different antigens if you are going to develop the adjuvants, if you can use it broadly, obviously that is beneficial. And in relation to generation two, if you can use it as a platform to deliver other adjuvants, then that is pretty important, too.

So this is an adjuvant I've been involved with for quite some time. I called it a successful adjuvant. I mean successful because it is included in licensed products. I think generally speaking we have many adjuvants. We're not short of adjuvants. We're short of adjuvants that have achieved success in terms of product licensure.

So MF59 is an oil-in-water emulsion. From a pharmaceutical perspective -- I'm a formulation scientist -- it is relatively simple. It is a low content of oil -- I'll say more about the oil in a moment --



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squalene. It is biodegradable. It has two nonionic surfactants, which have been broadly used in a range of alternative products.

It has a low viscosity so it is easy to inject. It is easy to add to other components, to add to other antigens. And its size is important. It is 160 nanometers prepared by microfluidization.

So MF59 is a squalene oil-in-water adjuvant. Arguably, it is most well established and there are others coming behind. You'll probably hear about AS03 from GSK and also AF03 from Sanofi. So there are other squalene-based adjuvants coming forward.

So the major component of MF59 is squalene. Chemically it is very simple, C30H50. Structurally, it is rather more complex. It is over here. That is what the structure looks like.

But this is a normal metabolite of all of us. So it is produced by humans. It is a precursor to cholesterol and steroid



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hormones. And you see as simplified biosynthetic pathway here where you end up with the steroid hormones and cholesterol.

So it is synthesized in the liver and skin. It is secreted in significant quantities by sebaceous glands. It is used in a broad range for other purposes. So it is biodegradable, biocompatible, a normal component of all of us. So that is a fundamentally important characteristic we believe.

So I didn't want to go into too much of the preclinical data. This adjuvant has been around since the mid-`90s. And there is quite a lot of experience accumulated in the preclinical setting.

Certainly in the mouse setting, significant dose reduction, several hundredfold. Probably the most important data we generated a long time ago was that it restores the immune response of old mice. Old mice, like old people, respond badly to flu

vaccines.

You give them the adjuvants. And their responses back up to what you see in the young mice. And just out of interest, we summarize a lot of this experience about the mouse model, using the mouse model, the limitations of the mouse model, but how you can use them optimally in a publication at the end of last year.

More recently we've shown improved heterologous and homologous challenge in ferrets. And we're looking at the pig model as a large animal model of flu vaccines.

But to get into a little bit of data, in Novartis, we are bringing forward a new generation flu vaccine based on flu cell culture. So we had the opportunity to ask again, is MF59 as good as it gets? Or are there other adjuvants that can be equally potent or even more potent?

So we did a competitive evaluation of the ones we had easy access to. So see

these are some of the adjuvants you saw earlier, CpG oligonucleotides, calcium phosphate, PLG microparticles. This is just the three strains included in seasonal influenza vaccine.

And it is pretty striking and clear that emulsions are very effect adjuvants for flu vaccines. And this, in essence, this is rediscovering what was discovered by Salk in the 1950s but using an adjuvant that is much better tolerated and, we believe, is very safe.

Kind of a -- this is an interesting aside looking in the pandemic setting but still in the mouse model, this is actually a collaboration with Kanta Subbarao with NIH.

And it was asking the same question. Is MF59 as good as it gets? Or can we make it better? Can we have a more effective vaccine. And this is kind of using MF59 as generation one and adding something to

it.

So in this situation, this is looking at T cell responses to MF59. The one in the middle, the vaccine alone, and on the right, MF59 plus CpG oligonucleotides. And this is the kind of color scheme at the top. If it bluish, it is a Th1 response. If it is reddish, yellowish, it is Th2.

So in Balb/c mice, a mouse predisposed to Th2 responses, MF59 gives a potent T cell response dominated by Th2 cytokines. If you add CpG, the magnitude of the response is not increased but the quality changes significantly. Now it becomes a much more Th1 response.

And the question is does that make for a better vaccine or not? And Kanta went ahead and did some challenge studies. So this is one of the studies she did. And this is looking at 50 LD50 challenge dose, a pretty significant challenge dose.

The observation was PBS or vaccine



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alone, all the mice died over ten days. MF59 or MF59 CpG all the mice survived. So clearly one adjuvant helps. In this setting, the second doesn't.

But actually I must say she also did a challenge dose of 50,000 LD50, a huge challenge dose. And in that setting, the CpG combination actually offered improved protection. So that was interesting.

Just to clarify something that is kind of sometimes misrepresented, MF59 gives a Th1 response in flu-exposed mice. So this is the same Balb/c mice.

In this situation, you are looking here at naive mice. The Balb/c mice are inherently predisposed to a Th2 response. So the MF59 gives a Th2 response. If you previously infect the mice, then you use MF59, it is a completely Th1 response.

So in essence, MF59 is more like T80. Whatever is predisposed in the situation, the MF59 enhances. Humans are not

Th1 or Th2. We are kind of mixed.

This is a complicated slide but it is a very simple message. And I think it is an important one. This is another study to look at a range of generation one adjuvants, different particulate carriers.

So we're looking across the bottom, microparticles, tyrosine, calcium phosphate, MF59, aluminum, we are testing these different alternative adjuvants against a number of traditional vaccines and new generation.

Tetanus toxoid, diphtheria toxoid, a protein polysaccharide conjugate against MenC, Hepatitis B surface antigen, and a recombinant antigen, Neisseria meningitidis serotype B -- on the left-hand side, ELISA titer, the right-hand side, a functional titer if you could do it. And you generate at two-dose levels.

And kind of clear picture emerges. The MF59, as the architype oil-in-water



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emulsion, tends to be very potent. And tends to be the winner amongst all these particulate carriers. It is very striking for the recombinant antigens here and here.

Probably alum works best with these traditional bacterial toxoids. And that is how alum was originally introduced, as an adjuvant for diphtheria and tetanus.

So we've had MF59 for quite some time. When we first developed it back in the `90s in Taiwan there was a lot of work done on its mechanism of action. And we thought we had a reasonable understanding. It looked mostly to be a delivery system promoting antigen uptake, that kind of thing.

But more obviously over a decade, the techniques, the technologies improved significantly. So relatively recently we've gone back, applied a bunch of new techniques and asked the question again. How does it work?

We've looked in human cells and



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we've look in mouse in vivo because they are the easy things you can do. And then we've done the mouse in vitro cells trying to link the two fine connections between mouse and human and make sure what you are seeing is consistent.

And this is a slide that was actually shown already. This is looking at the gene expression profile in the mouse muscle. And it was kind of interesting. Numerically, MF59 is the most active in terms of activation of transcription. But when you focus on the immune response genes, it is surprisingly more active than CpG, for example.

So MF59 activates 891 genes. CpG, less alum, and then there is some overlap. And here you see the time profile. And you see the red for MF59. And the combination MF59 CpG, in essence some things were down-regulated, which surprised us a little.

But in essence, surprisingly, MF59



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was the most potent activator and it induced transcription of chemokines, cytokines. It was activating innate immunity. It's not just a delivery system. It is doing a lot more at the injection site.

So to summarize a significant amount of work on the mechanism of action, which is still ongoing, in the human work, we identified three target cells, microphages, granulocytes, and monocytes. We saw that MF59 rapidly recruits cells into the injection site.

We saw that MF59 induces the release of chemoattractants and activate innate immunity. And, you know, relevant and interesting to some of the other discussions today, MF59 does not activate any TLR. And as far as we can see so far, it does not appear to activate inflammasomes.

So it certainly generates a local immunostimulator environment and the work is continuing. And we kind of published this

work and we tried to put a picture together of what we think it is doing. And this was published in JI earlier this year.

And this is what we think is going on in terms of the immune stimulator environment in the muscle, the release of chemokines, the recruitment of lots of cells, the activation of those cells, and then moving off to the lymph nodes to promote the immune response.

So I'll finish up now with where I think adjuvants may be going in the future. Maybe I'm thinking about generation three here. So we are looking at discovery of new adjuvants.

And because we are a large company that does drug discovery in addition to vaccine-related work, we have the capability to utilize the mechanisms of high throughput screening drug discovery to look for new generation adjuvants.

And this is a schematic

representation of a TLR-based screen to look for what we are calling small molecule immune potentiators, abbreviated to SMIPs. So you look for compounds that activate through TLRs, activate immune cells. And then you formulate and deliver these compounds to make more effective vaccine adjuvants.

And so, you know, what are the advantages of SMIPs? Why are we focusing on these small molecules? And, you know, these are some of the advantages.

Certainly there are simple synthetic pathways. We know how to make drugs very inexpensively. They have well-defined chemical structure. And there is a lot of history of manipulating the structure to modulate the response that you get.

Certainly there are 100 years of successful development so people know how to develop drugs for a variety of purposes. We see no reason why we can't develop them for use in vaccines.



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And certainly there is an established safety profile. How much of what is traditionally done for these compounds is relevant to a vaccine setting, obviously we need to discuss with the regulators. But we believe we know the kind of work that is necessary.

Certainly these are easily degraded and excreted, biodegradable. And we know that the delivery systems are well established, the delivery systems to control the related release and delivery of these drugs.

And one delivery system that we are particularly interested in is something -- it is a biodegradable microparticle. So it is a polymer called PLG, which is an abbreviation of polylactide-co-glycolide.

It is biodegradable and safe. It has already been included in 11 licensed products. So the particles degrade and leave no tissue residue -- completely biodegradable.

Because of the size of the particles, antigens stuck on the surface a targeted to immune cells. And the absorption of the antigen retains the integrity and the structural features of the antigen as it does with aluminum.

But the important feature is that microparticles can co-deliver an antigen and an immune potentiator. So microparticles were developed for the controlled release of small molecular weight drugs.

So we can utilize that technology, we believe, to deliver these SMIPs. And the idea is to encapsulate the SMIPs, to limit their systemic distribution to improve their safety profile, keep them at the site, keep them focused on the immune cells that you want to activate. Don't allow them to circulate away from the site.

And this is the basic concept, again put into a picture, traditional vaccines, like a whole bacteria, a couple of



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microns or so in diameter, a lot of immune potentiators contained inside, antigens generally on the surface.

So the idea is you make a synthetic microparticle of this completely degradable polymer. It is about the same size for uptake into antigen presenting cells. You absorb the antigen on the surface.

You put in the small molecule immune potentiators so they are delivered to the immune cells that take up the particulates. And you don't allow them to distribute any further than the injection site.

So that's the basic idea. Maybe this is generation three.

And I don't think I'm going to be brave enough to actually discuss this but I think there are certainly many regulatory challenges in relation to development of new adjuvants. These will be talked about in greater detail as we go through the day.

I guess I just wanted to highlight the basic researchers, like myself, need to be aware of these challenges as we go through our programs. And we need to design the programs appropriately to meet the needs.

And thanks for your attention. If there is any time, I'll deal with questions. Or we can do it tomorrow.

(Applause.)

DR. PULENDRAN: Thank you, Derek.

We have time for a couple of questions.

DR. SEAN SULLIVAN: Sean Sullivan, Vical.

Derek, I had a question about your expression profile studies. You said something interesting in that if you look at -- if you have mice that are infected and you give them MF59 versus if you give them MF59 with the antigen alone, in your PNAS paper you were characterizing expression profiling. There's really no antigen present.

And I was wondering -- and I know you have, you know, there are a variety of antigens you can look at but do you see a change in the response when you look at expression profiling in the presence of an antigen? And also if the animal has been exposed to a pathogen?

DR. O'HAGAN: Yes, it is a very good question.

You know, of course the gene expression profiling work, in looking at adjuvants, is kind of novel and new. So we start off with the most simple situation where you have only the adjuvant.

When you put the antigen, it becomes more complicated. And it depends on the antigen. So it is a more cloudy picture. And I would expect that if you have a pre-exposed infected animal, it would be much more complicated still.

We may get to that level of evaluation. We've started with the relatively



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simple studies.

DR. SEAN SULLIVAN: Could you also comment in your random screening on what you use to screen for, like what are the cell types and what kind of markers you look for, especially relevant to what you had for a comparison between human and mouse?

DR. O'HAGAN: Yes, I mean we look at human cells and we look at a variety of human cells. It is not a single target. And we look at TLR transfectants. And we look at native cells. So there is a variety of cell types we look at for confirmation of the hits with any one screen.

DR. SEAN SULLIVAN: Thanks.

DR. PULENDRAN: Okay.

So thank you very much, Derek.

The next speaker is Eugene Maraskovsky from CSL in Melbourne, Australia, who is going to be talking about ISCOMS. Eugene?

DR. MARASKOVSKY: Thank you. I'd

like to thank the organizers for inviting me to present at the workshop.

And today I wanted to give everyone an overview of saponins and ISCOMS and, in particular, ISCOMATRIX adjuvant. So basically I'll introduce what saponins are. I'll summarize what saponin-based adjuvants there are out there and what are currently in clinical development. And in particular then focus on our understanding of our particular saponin-based adjuvant, that is ISCOMATRIX adjuvant.

Now saponins are actually high molecular weight glycosides that are consisting of sugar moieties linked to a triterpene. Now there is a distinction that I need to make between ISCOMS and ISCOMATRIX to basically clarify that they are not interchangeable terms.

ISCOMS are actually a complex of saponin, cholesterol, and phospholipid where the antigen has been purposely incorporated



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into the cage-like structure during the formulation. So the full components are formulated together. And the antigen is associated.

ISCOMATRIX adjuvant is actually the cage-like structure made out of the saponin, cholesterol, and phospholipid. And you can make an ISCOMATRIX by then adding the antigen to that cage-like structure. So that is quite a different componentry.

Now the structure of quillaja saponin is essentially this triterpenoid moiety component here with a fatty acid and there's also three areas of carbohydrate or sugar moieties attached to that.

It's actually derived from the quillaja saponaria tree, which is an indigenous tree to Chili and Peru. And crude quillaja has actually been used in many industrial processes from agriculture, cosmetics to the foaming agents in our beers and soft drinks, so we actually ingest



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saponins during our lifetime, too also extraction purposes in mining. And clearly what I'll focus on is the vaccine use of saponins.

The other important point to point out is that saponins have actually been used in the context of vaccines for over 80 years. So -- and they've actually been going through an evolutionary process of further defining what is immunogenic in the saponin and what is actually the reactogenic component.

But I think it is important to note that we have quite a long history of experience of the use of saponins in the vaccine adjuvant setting.

And it has been in the more recent terms where we have made some revolutionary steps in minimizing the reactogenic potential within the saponin fractions and focusing on what is really the immunogenic potential of the saponins and how to actually formulate these in a safe and robust way.

Now there are several types of companies and commercial versions of saponins that are being used. They vary from the QS21, which is a highly defined saponin, to what we use in the ISCOPREP, which is actually saponin that's formulated with the lipids, cholesterol, and phospholipid. And various other sort of formulations.

Now the issues with saponin are that the naked saponin or saponin alone actually has quite haemolytic activity, particularly at the injection site, which results in reactogenicity.

And also it is quite susceptible to alkaline breakdown. And one of the solutions for actually overcoming some of these issues was to complex it with cholesterol and other lipids. And also to optimize the fractions that are selected, particularly to move towards fractions that maintain the immunogenicity and minimize the reactogenicity.

ISCOMS, which is the four components associated together -- that is the antigen with the saponin and the lipids -- also have issues in terms of they are quite complicated to produce and manufacture in sort of a robust process.

And so the solution that CSL has used is to actually devise the ISCOMATRIX adjuvant, which is the cage-like structure that you can then formulate with your antigen independently, and inject into patients.

Now in terms of the saponin-based adjuvants that are in advanced clinical development, these not only include the CSL ISCOMATRIX adjuvant but also other saponin-based adjuvants such as the AS series that are being developed by GSK as well as the more naked QS21.

The one thing to point out here is what we understand of saponins from our studies is they don't actually act through the TLR or Toll-like receptors at all. And in

order to activate that pathway, you'll need to actually add some of these TLR agonists. But ISCOMATRIX adjuvant is quite a potent adjuvant but doesn't actually act through the TLR pathway.

What we do know, though, is that we get quite a balanced immune response that is generated in mice and monkeys and also in humans in measuring antigen-specific vaccine responses. We can actually detect both Th1 and Th2 cytokines in mice and humans.

We definitely see a broad and Th1/Th2-type profile when it comes to antibody isotypes where we've looked and also we see quite a robust responses, both CD4 and CD8 responses against multiple epitopes to the protein antigen that is used in the vaccine, which gives this quite an advantage in terms of what type of immune response you want to actually gear towards.

So just to summarize quillaja saponins, in particular, they have a long

history as immunomodulators in vaccines. Purified fractions are required for human use.

Although in vet vaccines you can away with the more crude fractions, you need to complex them with lipids such as cholesterol and phospholipid. And ISCOMATRIX adjuvant has no addition immunomodulators that are added to it in the way that we are using it in the clinic.

And sort of the program of activity that CSL is pursuing is really setting where we have vaccines that are not actually showing sufficient immune conversion in patients, whether they are hyperresponsive, such as settings in the elderly, chronic infectious disease in cancer where patients may be immunosuppressed, and our need to try and focus on the therapeutic vaccine setting has basically made us want to understand what the mechanisms by which vaccines induce CDI T cell responses are at.

And most of our work is really

focused on the CTL side of the equation although we have quite a good understanding of antibody responses as well with this adjuvant.

In terms of mechanisms of action in vivo, what we do understand is that the ISCOMATRIX adjuvant is really a sort of dual-focused adjuvant. It has both antigen delivery and immunomodulatory capacities. And it seems to integrate these two very nicely.

It's not a depot adjuvant in the sense of depot antigen at the injection site but we believe that -- and I'll show you today -- prolonged antigen exposure in vivo at the antigen presenting cell level is where we are getting some of this benefits of the adjuvant in terms of its delivery capacity as well as the cytokines that are responsible for the immunomodulatory effects. And that's summarized here.

ISCOMATRIX targets and activates APCs in vivo -- and I'll show you that data -- enhances the mechanism of cross-presentation -

- this is by which antigen, exogenous antigen gets into the antigen presentation pathway into dendritic cells in a noncanonical way.

It escapes into the cytocell, which allows it to be processed in the Class I MHC pathway, which is critical for the generation of cytotoxic T lymphocytes.

Most other exogenous antigens would normally be processed in Class II MHC, which is great for CD4 T cell responses and antibody responses. ISCOMATRIX has this unique ability to also target the Class I pathway.

We get prolonged presentation in the drained lymph node and, as I mentioned earlier, the immunomodulatory potential relates to recruitment and activation of innate immune cells as well as cytokine and chemokine induction.

And I'll show you the data for that right now. If we inject mice with ISCOMATRIX alone and have a look in either the

draining node or the spleen, we find that most of the activity of T cell generation after the prime is essentially in the lymph node.

And when we actually look at what is happening in the lymph node, what we find is that very shortly after injection, we get a large influx of dendritic cells into the draining lymphocyte. This is transient so it's rapid and transient in the setting of RDC influx.

What we also find, which is quite unexpected, is that we get a very rapid presentation of peptide on Class I MHC molecules on the surface of those dendritic cells actually within about four hours after injection.

And what we are finding is that ISCOMATRIX actually is directly trafficking to the draining node as opposed to remaining at the injection site, loading dendritic cells in the node. They are initiating the presentation process very rapidly and

generating these T cells.

The other thing to point out is that we get about a hundredfold more presentation going on in the draining node than if we used antigen alone. And the other advantage is that we have this prolonged presentation over a period of three days, which is continuously stimulating the T cell immune response.

So we asked the question what dendritic cells are the ones that are actually either recruited into the node and are the resident dendritic cells in the node also responsible for the T cell expansion that is going on?

And the first thing we found was that ISCOMATRIX activates these dendritic cells in the node. It activates both the CD8 positive lymph node resident dendritic cells. It also activates the plasmacytoid dendritic cells that are in the blood.

It causes transient but rapid

induction of cytokines that can be detected in the lymph -- and this is using sheep cannulation studies -- and this is quite important to point out, they are quite rapid and high production of cytokines but they are transient and reversible.

And these also result in a rapid and transient recruitment of many types of innate immuneffectors into the node. Within 24 to 48 hours, we have NK cells, neutrophils, macrophages, NK T cells directly trafficking into the node that is downstream of the injection site.

If we look at the contralateral node, there is no influx of those cells in the contralateral non-injected node.

When we actually harvested the dendritic cells out of those draining nodes and asked can they present peptide from the vaccine that was being carried with the ISCOMATRIX adjuvant, what we find is that in the early time points, predominantly the CD8

resident dendritic cells are doing all the presentation of the vaccine, which is evidence that the vaccine is getting to the node directly.

We have a second wave of presentation that occurs at about 24 hours onward. And these are actually the migratory dendritic cells from the injection site finally getting to the node and starting to present themselves.

And we find that this is probably responsible for the prolonged presentation that we are seeing within the node following ISCOMATRIX vaccine injection. At the later time points, interestingly, most of the cross-presentation is occurring by the migratory dendritic cells.

In terms of the TLR pathway and with ISCOMATRIX as a TLR agonist, we've done various types of experiments. We've looked at NF-kappa B activation as one of the surrogate readouts for TLR downstream effects and find

that ISCOMATRIX adjuvant does not activate the TLR pathway at the level of NF-kappa B.

We've also looked at knockout T cells and knockout APCs and even knockout mice from the various TLRs, and again we find no evidence for a TLR mechanism. We have found, however, that there is a MyD88-dependent pathway that ISCOMATRIX adjuvant employs.

And what I can say is that MyD88 is not only a TLR downstream signaling moiety but also shared between the IL-18 and IL-1 and IL-33 pathway.

And we found in particular that IL-18 signaling is important for the way that ISCOMATRIX activates the immune response in that IL-18 receptor knockout mice, RL-18 knockout mice are showing defective T cell responses following ISCOMATRIX vaccination.

So ISCOMATRIX adjuvant targets and conditions multiple dendritic cell populations in vivo, enables DCs to cross-present to CDI T cells, does not activate TLRs, but does

require IL-18 for induction or CTL responses via MyD88, and conditions the draining lymph node environment for both Th1 and Th2 responses.

In terms of how ISCOMATRIX gets into the dendritic cells, we've looked at this by confocal microscopy. If you look at tagged antigen alone, fed to human dendritic cells, what you find is that within the first ten minutes, most of the antigen is actually within endosomal compartments that we actually can define using various late and early endosomal markers.

If you look at ISCOMATRIX, within ten minutes a lot of the antigen is actually in the cytosol. So we have cytosolic escape or translocation into the cytosol of the antigen, which is a prerequisite for getting into the Class I pathway for stimulation and presentation to CD8 T cells. And we think this is quite an important mechanism by which we get this robust CTL response in vivo.

The other aspect is that dendritic cells are particularly sensitive to translocating antigens into the cytosol. If we look at monocytes and macrophages -- monocytes being the precursor of this type of dendritic cell. They actually can't translocate very efficiently and ISCOMATRIX vaccine into the cytosol as compared to the monoDCs.

And macrophages, similarly seem to capture the antigen to these endosomal compartments and very little is translocated into the cytosol.

So there is something very particular about dendritic cells and their ability to translocate.

Now the final sort of points that I want to make is in terms of pulse chase experiments in human DCs, if we look at pulsing human dendritic cells with peptide, washing them, putting them back in culture, and then sampling those cells periodically to

see how much peptide is on the surface that a T cell can see and make interferon gamma, we find that peptide pulsed dendritic cells rapidly lose peptide over time so that by 48 hours, those dendritic cells have hardly any peptide recognized on the cell surface as it has been replaced by other competing peptides.

If you pulse and chase dendritic cells with protein, it is very poorly cross-presented onto Class I MHC so very little is detected on the surface of those dendritic cells.

If you use an ISCOMATRIX-formulated antigen, you find that this is rapidly translocated and expressed on the surface of Class I peptides as detected by T cells and you have this very prolonged presentation over a 72-hour period.

And it is at this time point where you see the big differential and the advantage of an ISCOMATRIX adjuvant. And this is where I'm talking about the intercellular depot of

the saponin-based adjuvant as compared to it being an injection site depot adjuvant. This is very similar to the sort of findings we found in the mouse studies, too, by the way.

Now the other things that we found out about ISCOMATRIX is that it not only translocates antigen into the cytosol but it can actually generate epitopes in a proteasome independent fashion as opposed to using the more canonical proteasome-dependent mechanism.

And these rules seem to also vary depending on that antigen and the epitope within the same antigen. So there is a very complex array of rules which we are analyzing at the moment in terms of how isotopes are expressed on MHC Class I.

But the bottom line is that what we get is a very broad capacity for epitope generation that dendritic cells can express as a result of an ISCOMATRIX-formulated vaccine.

So the final summary really is ISCOMATRIX targets and conditions multiple

dendritic cell populations in vivo. It actually enables multiple dendritic cells to cross-present. And this actually results in prolonged Class I presentation, which can either be proteasome dependent or independent, and generates tumor-relevant T cell effectors of broad specificity in humans.

The sorts of things we're currently doing now is extending some of these mechanisms of action questions. We're also looking at cytokine profiling that can be detected in vivo, either by ISCOMATRIX alone or ISCOMATRIX vaccines, and looking at some of the more sort of systems biology approach and network biology to understand what is happening in the draining lymph node because that's really the side where most of the vaccine ends up in our system.

So the four take-home points, I think, for today's talk is that ISCOMATRIX adjuvant is an immunomodulator and an antigen delivery vehicle. And these are both



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integrated sort of properties of this adjuvant. They recruit and activate immune cells not using the TLR pathway.

They accelerate and provide prolonged presentation. And that these integrated mechanisms result in the production of broad specificity, both antibody and T cell responses both in mice and man.

And I'll leave it at that. Thank you.

(Applause.)

DR. SUTKOWSKI: Since we're running overtime, perhaps we can -- unless there are any burning questions for clarification, leave the question -- Jan Willem? Maybe just one question. Then maybe we'll have to shorten our break.

DR. van der LAAN: Just a very short, very short one.

Your colleague from Isconova last year presented the idea that you can give your ISCOMATRIX in your left arm and your antigen

in your right arm. So then it is more an immunomodulator. What is it? Is it an immunomodulator or an adjuvant?

DR. MARASKOVSKY: We actually haven't done those experiments ourselves so I can't really comment on their data. But what I can say is that from the experiments that we've actually been looking at, we have both an immunomodulatory and an antigen delivery component there.

We don't see the immunomodulatory effects in the contralateral nodes in terms of recruitment of cells at least with our adjuvant system. So most of the activity we tend to find, at least in the priming phase, is all happening in the draining node.

Upon boosting the vaccine, you do see now activity going on in the spleen. So a lot of the boosting of the immune response will result in antibody and T cell responses detectable in the spleen. So you do end up with a systemic effect after boost.

But I can't really comment on their data in terms of, you know, delivering antigen in one side and the adjuvant in another.

DR. SUTKOWSKI: Thank you.

Dr. Slater, do you think we need to shorten the break or -- no? Okay. Okay.

If the speakers for the next --

(Applause.)

DR. SUTKOWSKI: Thank you everybody.

(Whereupon, the foregoing matter went off the record at 11:15 a.m. and went back on the record at 11:35 a.m.)

DR. SUTKOWSKI: Okay. So now we're ready to finish up this session on the various specific adjuvant overviews. And the first speaker is Dr. Bruce Beutler. He is coming to us from Scripps Research Institute. And he will talk about his many years of experience with Toll-like receptors.

DR. BEUTLER: Well, thank you very much. It is a great pleasure to be here. And it has been a very interesting meeting for me so far.

I was given a very long title sometime during the lead up to this meeting. So I parrot it here. But what I have to say will be relatively simple. And I'll concentrate on the biochemical mechanisms of TLR adjuvanticity.

But while I was sitting listening to the first talks, I had a number of thoughts of my own. And so the first slide is based on those.

It is a big question in immunology just what the switch is that activates an adaptive immune response. And as was pointed out, we've known about adjuvants for close to a hundred years beginning with alum, then there was Freund's complete adjuvant and Freund's incomplete. And there were many serious attempts to understand just what the

relevant molecules were that would ignite an adaptive response over all that time.

By 1989 when adjuvants were effectively renamed the immunologists' dirty little secret, this, in itself, didn't really advance our understanding of how they work.

If you think about it for a moment, just substituting a synonym like that or making a catchy phrase, it's nice and it helped to focus attention on the field but in itself, it wasn't really an advance nor was the use of the term pathogen associated molecular patterns to lump molecules like LPS double-stranded RNA and also DNA that were already very well known to have endogenous adjuvant effects.

On the other hand, finding discreet receptors for these molecules was an important advance. It did enhance our understanding and it continues to do so. And understanding the signaling pathways that lead to adjuvant effects is also important.



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But at this point, what I think all of you know, is that there is a lot of redundancy in this field. There are many ways to activate an adaptive response. And maybe that is the central message that I have to give you.

The TLRs are extremely important in this regard. And their discovery was part of the broader question of how innate immune sensing operates. How we know when we have an infection.

And the story of TLRs, from my own perspective, began with the story of lipopolysaccharide, which again was more than a hundred years in the making. LPS was identified as something that was inherently toxic about gram negative bacteria.

And by the early 1980s, it was clear that it worked by interacting with macrophages. And in some of my own early work, I found that it would induce the production of cytokines that had LPS mimetic

effects, TNF being the key one among these but certainly not the only one.

Victor Jongeneel and his colleagues showed by 1990 that the TNF response was entirely dependent on NF-kappa B. And if you mutated more than two of the NF-kappa B binding motifs in the TNF promoter, you didn't get TNF production.

TNF and other cytokines, of course, work in a very complicated way. They interact with receptors present on many cells throughout the body.

And where this meeting is concerned, the most important point to make is that since 1955, since the work of Condie and Good, it was known that LPS was endowed with adjuvant activity. If co-administered with a protein antigen, it would greatly augment the antibody response that could be measured.

If we went forward a few decades from then, we would say it was not the macrophage but the dendritic cell that was of

key importance there. And people pointed to the up-regulation of costimulatory antigens and also the Class I and Class II MHC antigens themselves as being key events in driving an adaptive immune response.

But for LPS, the mystery remained. What is the LPS receptor? That is where it all must start.

We had good information from the 1960s that there must be just one LPS receptor, one solitary pathway for LPS responses because it had been shown that there were mice of the C3H/HeJ strain, for example, also C57 black/10ScCr where a single mutation that had been mapped to chromosome 4 could be ablate all responses to LPS. And they said that probably there was an LPS receptor and only one such receptor.

Where adjuvant effects went, it was shown by Skidmore and Weigle in 1975 that these animals derive no adjuvant response from LPS. So the adjuvant effect, like all effects

of LPS, was mediated by this receptor.

We positionally cloned this receptor over a period of about five years and discovered that it was a mutation in Toll-like receptor 4, which to that time had been described as something similar to the Drosophila receptor Toll, something that was known to activate NF-kappa B but it had no known ligand nor did any of the other Toll-like receptors.

And so the picture that emerged was one in which Toll-like receptor 4 was the membrane-spanning component of the LPS receptor. It was assisted in recognizing LPS by CD14 and later, as it turned out, by a small molecule called MD2, which we now know really directly engaged the lipid A moiety of LPS.

Also very exciting at the time was the fact that this was one member of a family of paralogues that we now know have 13 representatives at least in mammals, 12 in the



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mouse and ten in humans.

And as we suggested, each of them has a specificity for different molecules of microbial origin. And this is quite a minimalistic view of how they work.

In my lab over a period of years, we've taken a genetic approach to deciphering how Toll-like receptors signal. And by screening about 30,000 mice with randomly induced mutations, we began to put together a fairly comprehensive picture of the biochemistry of TLR signaling.

We know first of all that the LPS receptor, TLR4, is predominantly on the surface of cells and signals there. We now that it activates two pathways by interacting with a pair of adapters called MyD88 Mal on the one hand or Trif and TRAM on the other hand. And where the MyD88 signaling pathway goes, it activates NF-kappa B and drives the production of hundreds of cytokines.

The key thing to remember about



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the Trif TRAM pathway is that this is the only way the LPS receptor is able to drive the production of Type 1 interferons. And it does so by interacting with a kinase called TBK1, then with IRF-3, a transcription factor that activates interferon beta and one thereby gets interferon production.

By combining different mutations that we created, we are able to ablate parts of the pathway piecemeal. And we know that by deleting two of the adaptor proteins, MyD88 and Trif, we arrive at a situation where the Toll-like receptors can't signal at all anymore.

And under those circumstances, mice are severely immunocompromised. It is quite rare that they survive to weaning age, although they sometimes do. And with great effort, one can maintain a stock of double-deficient mutants.

But the important thing to note, which I will return to, is they retain very

robust adaptive immune responses to all adjuvants except those that worked directly through the Toll-like receptors, purified ligands like LPS or CpG DNA or poly IC and the like.

The adjuvant effect of LPS we know now is mediated chiefly through Trif. And we know that applies also to double-stranded RNA or poly IC. Remember the adjuvant effect of LPS has been known since 1955 and in 1975, it was shown to depend upon the LPS locus in that it was absent in C3H/HeJ mice. So we've known for a long time it must depend on TLR4.

And we decided to look at adjuvant effects by monitoring the up-regulation of costimulatory proteins, including CD80, CD86, and CD40 on antigen-presenting cells in response to LPS. And we used our mutant mice to see whether the MyD88-dependent pathway or the Trif-dependent pathway was of key importance.

We looked both at LPS and at

double-stranded RNA, or really poly IC, in order to make our judgments about this. And you can see quite clearly just from the top panel here that in the wild-type, you get up-regulation of all three of these molecules on bone marrow-derived macrophages, for example, or on dendritic cells I think are shown here if you use LPS.

If you take a Trif mutant, one with a point mutation that we called Lps2, you have no up-regulation. If you take a MyD88 knockout, then you have fairly robust up-regulation.

The situation is more complicated for double-stranded RNA. There neither mutation will independently ablate the up-regulatory process. And we know today, this is because there are redundant pathways for sensing poly IC, especially MDA5, a cytoplasmic sensor of the RIG-I-like helicase family, will do the job.

So not only a mutation in Trif but



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also a mutation in TLR3 fails to completely abrogate the up-regulation of costimulatory molecules, again because there is this redundant pathway embodied by MDA5.

Because the endpoint of the Trif pathway, at least in large part, is the production of Type 1 interferons, at least that is the unique endpoint, we wondered whether the interferons were what was really causing the up-regulation. And prior to this point, it had been assumed and written quite widely that this was an NF-kappa B-dependent response.

But it turned out not to be. It turned out to be an interferon-dependent response. And it was specifically IRF3 dependent if you are talking about the TLR signaling pathways.

In Panel A, you can see that if we take wild-type mice and we stimulate with LPS, we get up-regulation of costimulatory molecules. If we use the Trif mutant mice, we

don't get such up-regulation. And up-regulation isn't restored by any of a panel of cytokines that we applied, including IL-18, IL-15, IL-12, IL-1 TNF. But it is restored if we co-administer either Type 1 or, to some extent, Type 2 interferon.

If you look down then at Panel C, you can see that the Type 1 interferons, by themselves, do a pretty good job of inducing the up-regulation of costimulatory molecules. And finally in Panel D, we antagonize the Type 1 interferons with antibodies and we get a significant decrement of the response.

But antagonism with antibodies isn't always 100 percent effective. So, of course, we looked at interferon Type 1 receptor knockout mice.

And just to be quick, if you look at the bottom panel of the slide here, you see that in mice that are IfnR mutants, you get no up-regulation of CD80 or 86 or CD40 in response to either LPS or double-stranded RNA.



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This is an absolute requirement for the costimulatory response.

Now in the meanwhile, as you may remember, some years ago in early years after the turn of the century, there was a lot of excitement about TLRs being the pathway for activation of adaptive immunity. And you will recall there were a lot of papers published saying that TLRs were required for an adaptive immune response.

It was written that Toll-like receptors control activation of adaptive immune responses by APCs, that they play an essential role in the induction of innate and adaptive immune response, that they are responsible for the induction of DC maturation, which is responsible and necessary for the initiation of adaptive immune responses. And also the generation of T-dependent antigen-specific antibody responses requires activation of TLRs in B cells.

These statements led many to

assume that this was a concomidant of adaptive immune activation, that this was it, this was the pathway. And we had reason to be skeptical. And we, ourselves, began to look at it very closely.

By we, I should mention that this was the work of my lab together with David Nemazee, who is the senior author on these studies. We noted first that by no means were MyD88/Trif double-deficient mice agammaglobulinemic. They could clearly make adaptive responses to some antigens if we simply looked at their serum immunoglobulin levels.

But what we did notice was that there was skewing so that in the double mutant mice, for example, there was exaggerated representation of IgE and there was diminished representation of IgG3. And so we thought perhaps there was a problem in these mice with class switching in the ambient microbial environment.

We then began to immunize the mice and look directly at their immune responses. And in one instance we used alum as the adjuvant. Alum was used just to test the thesis that anything would require TLRs to activate an adaptive response.

And as you can see, it certainly did not. We got an IgM response, an IgG1, IgG2c, IgG2b, IgG3, IgE response. And where there were significant differences, it was the double deficient mice that actually were hyperresponsive.

Of course we could be criticized and we could face the objection that of course alum doesn't require TLRs to generate an adjuvant effect because it is not microbially derived. But to our surprise and, I think, to the surprise of many, complete Freund's adjuvant also doesn't require TLRs.

It works perfectly well in mice that can't signal the TLRs. And so you see again you've got good responses of all the

different subtypes. And you find that where is a significant difference between the knockout, the double knockout, and the wild-type, it is usually in favor of the double knockout, which is hyperresponsive.

So neither alum nor CFA depend upon TLR signaling for adjuvant effect. We asked since that time what about a real microbe? What really is TLR dependent when it comes to adaptive immune responses?

We began to test this question, to look at the question using mouse cytomegalovirus, which provokes a very strongly Th1-biased IgG2c antibody response. It is known to trigger signaling via TLR3 and actually it should read TLR9, not 7, but no other TLRs.

We know that signaling by TLRs 3 and 9 but no other TLRs is essential to survival during the first week following infection with ten to the fifth PFU of the virus. So it is part of the innate response.



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And on the other hand, if lower doses of the virus are given, the mouse can mount an adaptive response quickly enough to survive infection. And even in mice that lack those signaling pathways, we can evaluate the adaptive response overall.

We looked then at mice that were wild-type or that lacked TLR9 of the CpG1 mutant induced by ENU or have a mutation called 3D which abrogates signaling via TLRs 3, 7, and 9 or mice that lacked all TLR signaling.

Now as you can see, you've got perfectly adequate adaptive immune responses looking out to 90 days. We wanted to repeat this experiment, of course, and so we did.

And we did it again all over with fresh mice. This time we looked at just MyD88/Trif double deficient mice or wild-type. And again you get adequate responses. Notice that the wild-type responds much better than the -- rather the wild-type responds not as



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well as the double deficient mouse.

Our interpretation of this is that probably the double deficient mouse gets a higher burden of virus and so it makes a more robust antibody response in time.

We then looked at the question of memory B cells were compromised in any way if there was a lack of TLR signaling because this, too, had been claimed. And so we took B cells from either wild-type or double deficient animals and here I simply show you that these animals, which had been inoculated with the virus themselves mounted a very good response in terms of IgG production against MCMV. Those were the donors of B cells.

And we transplanted the B cells into a T deficient environment. And then we challenged the mice with irradiated virus to produce an anamnestic response.

If the mice were naive and had never been immunized, if we used that kind of a donor, we got no response. If we used



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immunized cells -- cells from an immunized donor but didn't boost them, we got no response.

If we took wild-type B cells from an immunized mouse and boosted, we got a robust antibody response. And if we took B cells from double deficient mice and boosted them, we got a robust response although perhaps a little bit less than what was observed in the wild-type. In no way could we say that the B cell response was really dependent on TLR signaling.

Of course we repeated this experiment as well. And I just show you the repetition. Exactly the same thing was done except in this case I'm not showing you the controls where we didn't immunize or where we used naive cells. Again, you see that the wild-type B cells respond to immunization and so do the double knockouts.

So our conclusions are that TLR signaling certainly does augment an adaptive

response as could be deduced from experiments that were performed more than 50 years ago.

But TLR signaling is not required at any level, whether it is antigen presentation or helper T cell function or B cell activation for an adaptive immune response to classical adjuvants nor to MCMV nor to any microbe as far as we know at this time.

TLR signaling does influence class switching in the ambient microbial environment. And there is a modest decrease in B cell memory responses to an authentic viral pathogen if primary immunization is performed in mice that lack both MyD88 and Trif. But TRL signaling is not required for B cell memory per se.

So we might guess that there are redundant pathways for adjuvant effects. And the question is how can we look for these pathways? We've always favored a genetic approach, particularly when we don't

understand a system very well.

And we've begun to look at this question using recombinant Semliki Forest virus. And we used this system as an immunization protocol because it was shown by Gunilla Karlsson Hedestam and Asa Hidmark, her graduate student, to be a type of adaptive response that is completely TLR independent.

So we wanted to begin with a system where we knew there wouldn't be interferons from TLRs and try to understand exactly how the adjuvant effect might work.

We know that in this system if you take an antigen and you immunize it in recombinant Semliki Forest virus vector, then there is a strong response to immunization with boost. We can use vectors that have variable expression of the encoded antigen and one can then run a genetic screen in both directions. One can test for both high and low responders to two different antigens at the same time in the same mouse.



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And as was shown already by Asa Hidmark paradoxically in view of what I told you about the interferon dependency of TLR adjuvant effects, a weak antigen gives a much stronger immune response in the absence of Type 1 interferon signaling.

Now here is the beginnings of our screen. We've gone through six or 700 mice by this time. And this is work that is funded by the Gates Foundation.

But you see we run it in both directions. We can take a weak antigen, which is OVA, or a strongly expressed antigen, which is Beta-gal and on the one hand we look for mutants where there are exceptions and you have an exaggerated response to the OVA -- and here we have three candidates which we are evaluating now -- or we can look for mutants like perhaps this one where you have a diminished response to the strong immunogen. And in this way we hope to ferret out non-redundant components of these signaling

pathways.

To show you the effect of an interferon mutation -- I don't know how well you can read from there -- but this is the primary response to immunization with the OVA vector, the weak antigen, weakly expressed antigen.

This is the boost response. And in this column you see the effect when we immunized mice that are deficient in the Type 1 interferon receptor. There we do get a response. We don't get a response in wild-type mice. And in this case, we are looking at black6 mice given varying doses of the viral vector. This is a secondary response looking strictly at antigen-specific IgG.

So I want just to conclude at that point. This is my group as it stands now. I didn't tell really what most of them do. But for the most part, we do take a forward genetic approach. We make no judgment about how adjuvants really might work.

But our goal is to dissect them by making random mutations that impair their function or augment it.

And I want also to thank Kasper Hoebe who is now at the University of Cincinnati, Gunilla Karlsson Hedestam at Karolinska Institutet and David Nemazee at Scripps whose work I mentioned during the course of my talk.

I'll take any questions you might have. Thanks.

(Applause.)

DR. SUTKOWSKI: Thank you for very provocative data there.

We have time for a couple of questions since the speaker stayed on time.

DR. PULENDRAN: Bruce, thank you that really elegant presentation.

Maybe I could ask you a question. All the data for the immune responses you showed us concerned humeral responses. Have you looked at T cell responses in response to

various viruses and TLR knockouts?

And I ask this because some of our own work shows that the yellow fever vaccine responds very poorly in terms of T cell responses in MyD88 knockout mice suggesting that at least for that vaccine that you do need MyD88 signaling to get a T cell response.

DR. BEUTLER: It's a particular interest of Kasper Hoebe to look at responses of CD8 cells. And that's where we've done the most work.

We find that a CD8 response is elictable, let's say, by TLR signaling. But not very strongly. And what drives a CD8 response much more is the induction of programmed cell death by several different means.

One can cause death my NK cell killing, by UV or gamma irradiation, by fas ligation, all of these things will drive a strong response to any antigen that is carried by the cell that is undergoing death.

We know that a CD8 response is also strongly elicited by recombinant Semliki Forest virus. To what extent that's death dependent we don't really know as yet.

Oh, I'll mention those are totally TLR independent, by the way, the death pathways.

DR. SUTKOWSKI: Okay. So now our next speaker is Dr. Nathalie Garþon. Nathalie was Vice President and head of research and R&D in North America in GSK. And since September of this year, she's heading now the Adjuvant Center for Vaccines at Overseas Adjuvant Activity from Research to Life Cycle Management.

DR. GARþON: Good morning. Thanks for the introduction, Elizabeth.

So we're going to switch gears a little bit here. The presentation and the topic, as I understood it also, was to bring lessons learned that we have learned in the development of adjuvants. And in the 20

minutes that I have to present, I will try to put you through some of the experience we have had and the lesson we took from that.

So I think we have discussed that several times this morning but again I will tell you where need new adjuvants or adjuvant systems.

One of the lessons is that you need to know your component and your adjuvant system;

That they need to be designed to elicit a tailored immunity that you are looking for;

That the formulations need to consider the physical/chemical properties of your component;

That the formulation can impact on the immunogenicity of the vaccine even if you use the same immunomodulator;

And also that one name for one molecule can refer to different molecules when you look in the literature.

And I will conclude.

So as it was already presented several times, clearly infectious diseases require new strategy for the development of efficacious vaccine. And that can be linked both to the target population but also the pathogen you are targeting.

And if we consider the targeted population, clearly there is a need to induce a long-term persistence of the protection. There is a need for vaccines that are adapted to fully-responsive population and that can be elderly in particular. And there is, in some cases, clearly a need for antigen sparing.

That can be linked also to the target pathogen. There are complex pathogens that can evade or subvert the immune defenses. There are pathogens that require complex multi-stage immune response. There are antigens that are potentially weak and we had this morning a presentation actually where clearly by going from live vaccine to purified



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recombinant, we did lose part of the ability of the antigen to induce an immune response.

There are pathogens that exist with multiple strains, serotype, or genotype. And there is a need for that to induce a cross-protection. And there are pathogens that may not give us time to develop a vaccine and pandemic flu is a clear example.

So there are clearly needs for new approaches in adjuvant and adjuvant system is one of them. So what is the GSK approach for adjuvant system? Basically that was touched upon already also this morning is that classical vaccines are made of antigen and what you could refer to as classical adjuvant, which are aluminum salt, emulsion, and liposomes.

And adjuvant systems basically are based on the combination of one of those and/or an immunomodulator, which can be MPL or QS21, CpG and alpha-tocopherol.

I won't talk about CpG today and I

will talk about the three others. And clearly the goal of doing that is to tailor the immune response to achieve an enhanced protection.

What is obvious also is that if you don't need an adjuvant system, you don't use an adjuvant system. So a new component adjuvant system -- so MPL is registered so MPL, as defined by Corixa and produced by Corixa, which is now part of GSK, is a pure TLR4 agonist.

It is derived from the lipopolysaccharide from Salmonella minnesota. This is a detoxified form. And MPL can return the adjuvant activity with a much reduced toxicity.

So what does it do? So as I was telling you, it is clearly a TLR4 agonist. MPL acts on monocytes, mDC, but not the plasma situate CD8 T CELL. And this is per the TLR4 expression on cells.

What is important is that one of the adjuvant systems we are using is called

AS04. It is a combination of aluminum and MPL. If you look at the ability of AS04 to activate dendritic cells -- and this is a box I just added -- there is no difference in the production of TNF-alpha whether the MPL is absorbed or not on aluminum.

So that formulation maintains the ability of MPL to activate DCs. And that translates in vivo in mice when we use the HPV VLP antigen also to an increase in the antibody production for both VLPs as compared to the aluminum dioxide alone.

So QS21 enhances CTL induction. There was a presentation earlier on ISCOMS and ISCOMATRIX. QS21 is a purified fraction from Quillaja saponaria so you have a certain number of fractions in Quillaja. QS21 is one of them. And it is part of the triterpene saponin family.

So what it does, QS21 enhances the CTL induction and as observed in animal models. And here you see an example with OVA

where whether you use PBS over OVA MPL, there is no really detectible CD8 response. Only do you see such a response when you use QS21.

So you can go one step further and depending on the type of immune response you are looking for, you can combine different immunomodulators. And this is a case here of a combination of MPL and QS21. And by combining them, they act synergistically on the innate and adaptive immune responses.

So you combine both of those molecules and what you see is that it does impact on the innate immune responses is the lower left box. And looking at interferon gamma production by APCs, the production you induce with the combination of MPL and QS is more than MPL and QS separately.

And this has an impact also on the immunity as you see that looking at the antibody response which is induced. There is a clear increase in that antibody response when you combine both MPL and QS versus each

of them separately.

What is important also to note is -- and that's what I've circled -- those molecules have different physical/chemical properties. And this is important because not only do you need to ensure that you induce the type of immune response you are looking for but you also need to ensure that your formulation can be done through a process that could be done at large scale and used for final product.

So MPL is a hydrophobic molecule and tends to aggregate for clumps so you need to have a process that allows you to have particulate that are serofilterable and QS21 is a nonspecific molecule with clearly defined properties.

So alpha-tocopherol directly impacts the immune response in the elderly. So this is also a hydrophobic molecule.

And it has been recently published that tocopherol helped to reverse the excess

acidity effect in T cell response. And we have also seen that when in oil-in-water emulsion, alpha-tocopherol results in the increased of production of cytokines. And this translated to an increase of antibody response.

Tocopherol has been used in a lot of vaccines, veterinary vaccines since many years, in particular in poultry. So it's not such a new immunomodulator.

So adjuvant systems are designed to elicit immune response. We use, as an example, the RTS,S malaria candidate antigen. RTS,S is a particulate antigen which is based on mixed particles that are made of S antigen from hepatitis B and a part of the circumsporozoite surface protein. And this is referred to as RTS,S.

If you look at the left panel, which is the first experiment we did in monkeys and we looked at antibody response in immunity, we tested three different adjuvants



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and adjuvant systems. The LMNPL, which is known as AS04, the MPL QS21, which is known as the AS01 family, and the emulsion MPL QS, which is known as the S02 family.

What came as a surprise to us was the results we had with MPL QS because from the mouse data, we didn't expect to have such a low response and in particular when comparing to LMNPL.

Actually it turned out that in that formulation, QS21 was not stable. We were at a pH that was inducing degradation of QS21. And hence we didn't have any adjuvant effect.

The oil-in-water emulsion that we used in that first experiment turned out to be unstable after six months. So though we had great results pre-clinically, clearly there was an issue of production of the formulation.

So we went back and reworked the emulsion and we tested different types of emulsion that actually were all based on the

same principle of density and particulate size. So we defined the particulate size that we were looking for and a density. But they were varying in their composition.

And what you can see here is that if you look at oil-in-water 2 and oil-in-water 3, both of those are emulsion, oil-in-water emulsion, however they don't give the same type of immune response in the monkeys, whether it is antibodies to DTH.

When you start an immunomodulator to those systems, you see that you impact both on the DTH and the antibody response. And the highest impact is seen when you combine the three together.

So here what you see that not all oil-in-water emulsion are equal, that you can have different types of immune response, and that when you do add an immunomodulator, depending on the one you add had how you add it, you do induce a different type of immune profile.

This is also true when you look at challenge models so that is the ferret challenge model for flu where we used ferrets that were first infected with the virus and then vaccinated once with trivalent flu vaccine. And they were challenged 49 days after the immunization.

And what you follow here is temperature. So the ferret has the ability to give you the set time of clinical symptom that you have in humans in a raise in temperature. It is a marker if you don't have any raise of temperature of the efficacy of your vaccine.

And what we saw is that using two different split trivalent activitated vaccine, whether you used one type of oil-in-water emulsion or none, there was no difference. There was no protection that was seen.

However, if you use those two split trivalent activated vaccine, we saw that by adding the oil-in-water emulsion that did contain alpha-tocopherol, we saw a complete

protection in those ferrets.

That was also correlated with a decrease in the virus shedding and in the ferret we can't look at cell-mediated immunity but there was a clear difference in the antibody level when comparing both the group that protected and the one that didn't.

So, again, not all emulsions are equal. So when you do the formulation of your adjuvant system or your adjuvant, you need to consider the physical/chemical properties of the component.

As it was pointed out earlier for ISCOMS, QS21 has the ability to degrade in an alkaline pH. And by doing so, QS21 becomes what has been described as DS1 or it is known also as QS21h.

And what you see in the right panel is that indeed QS21 in water remains stable even at 16 hours at 37 degrees. This is the lower line. However when you put QS21 at a pH 9.0, QS21 transforms in 16 hours in 94

percent of QS21h. So you lose almost all of your immunomodulator.

And this does have an impact on the immunomodulator property of your molecule and that has been published around 2000 when it was seen that QS21 was capable of inducing CD8 immune response whereas the degraded QS21h, which is the black square at the bottom, didn't have this ability anymore.

And actually you do see the same impact on the humoral immune response as well. So losing the the acylated chain that is on the QS21 abrogates its activity.

One other property of QS21 is that it is an amphiphilic molecule. And again it was pointed out earlier that QS21 has lytic activity. This is what you can see here if you take red blood cells in water, this is the first group. There you see your red blood cells.

When you put them in PBS it settled. And when you saw that in QS21, you



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have amylase that appears in your sample. And that amylase is proportional to the amount of QS21 you introduce.

Doing it in vitro on red blood cells actually is a marker of what happens when you inject QS21 intramuscularly. Lysis of cells is not restricted to red blood cells. It is a phenomenon you can see also in muscles and can lead to necrosis at the injection site.

So how can a formulation help you? Well, you can reformulate QS21 in such a way that putting it at pH 9.0 for 16 hours at 38 degrees, you do not have any degradation any more. This is the lower line of the table.

Your QS21 remains as QS21 and does not perish into QS21h. And also you can formulate your QS21 in such a way that the necrosis that you can see in the picture here at the bottom, which is induced in the muscle of a rabbit when you inject 50 microgram of QS21, disappears when you reformulate QS21

that abrogates the lytic activity.

So formulation can impact the immunogenicity of the vaccine. Again, this is using the malaria antigen as an example. And here the two adjuvant systems that were used both contained the same immunomodulator and pure QS21. What is different is the formulation which is used.

And what you can see on the left side is that in mice, looking at antibody -- this is the upper panel -- they do have the same type of antibody profile induction profile. However, when you look at CD4 T cell response, you can clearly see that -- the AS01 is the next to the last if that can help you -- you clearly see there is a difference in the induction of CD4 double positive CD4 T cells to an interferon-gamma.

When you look at what happens in humans when you compare both of those formulations, you do see that there is a difference -- in that case a significant

difference in the antibody titers that were induced with AS01 versus AS02. And you do also see a trend for a difference in the CD4 T cell positive induction. And here clearly we saw the same ranking from mice to humans, going through monkeys.

So one then can refer to different molecules. So different LPS of different agonist activities, gram-negative LPS, rTLR4 agonist, gram-positive bacteria, rTLR2. And also depending on which MPL you are looking at -- and here I'll take the MPL what is called the MPLR and is sometimes referred in the literature as a GMP form of MPL versus mpl8, which is referred in the literature as non-GMP material.

If you look at the MS profile of those molecules, you clearly see they are very similar but they are different and, in particular, both MPLR produced from Salmonella minnesota.

However, mpl8 does show a peak at

the profile which it doesn't exist in MPLR. And this is most likely due to the difference in process where the first one only has an acidic hydrolases in the production process whereas the MPLR includes both acidic and basic hydrolases. And this is important because actually each do present different cytokine activation patterns on human monocyte cells.

And actually what we have seen is MPLR is a poor inducer of Trif pathway on human monocytes, which is different from what was discussed earlier that showed that MPL was a Trif-inducer. So one has to be careful and specific on what he's using when he is testing molecules.

So in conclusion, adjuvants and adjuvant systems, clearly the knowledge of the molecular action guides the vaccine development on the what and the how. The formulation can impact the physical/chemical property of the adjuvant or the adjuvant

system.

And it is possible through formulation to reduce or abrogate the core reactogenicity. And clearly formulation of semi-immunomodulator can lead to increased immunogenicity.

And finally, adjuvant systems are designed to elicit immunogenicity. And not all adjuvant/adjuvant systems induce the same immune response and they need to be selected and justified appropriately.

And I can't name all the people that have been involved that work since we have ten years. But we certainly thank all of them whether they are within GSK Bio or external collaborators.

Thank you.

(Applause.)

DR. SUTKOWSKI: Does anybody have any questions for Dr. Garþon or lessons learned?

(No response.)

DR. SUTKOWSKI: Okay. Thank you.

Okay, our next speaker, Dr. Geert Van den Bossche comes to us from the Bill and Melinda Gates Foundation where he is the Senior Program Assistant for Global Health Discovery. And he will be talking to us about additional lessons learned.

DR. VAN DEN BOSSCHE: Hello everybody.

I thank the organizers for inviting me and I congratulate them on this initiative.

It's just amazing if I look at this audience, such an interest and attention paid finally to adjuvants. I would say wow. I mean this really seems the field is moving.

And we are really happy about it. So I will come back to the mission of the Gates Foundation later on in this talk.

So obviously since I joined the Gates Foundation, I consider myself as a knockout scientist. And my hands-on gene got

severely deleted. I hope a couple of other genes felt up-regulated but it probably changed my phenotype. So whatever.

So the agenda -- I skipped a number of slides for the introduction. I was just -- you know since we saw that biophysical aspects are going to be maybe on the priority list of adjuvants and better understanding their interaction, the interaction of adjuvants with membranes and so on, I'm just going to limit this to one single slide.

And then move straight on to adjuvant safety. What are the challenges? What are the issues? And what can we do about this just to end up with a number of practical recommendation?

So obviously I'm not going to show you any hard data. What I want to do is just to share with you some insights that are based on my background in adjuvants. And you will see the statements that I'm going to make are backed up by a number of references from



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literature that I appended at the end of the presentation.

So this is really the one slide that I always start out with where you see -- I call it the discrepancy we are currently observing between the world of the two Ps -- the publications and the products.

On the one hand side, we have adjuvant discovery where I think we have been doing a fabulous job over the last ten, fifteen years. There has been tremendous progress in, you know, for example, innate immune biology, discovery of new adjuvants, discovery of new receptors.

We have established discovery tools to better analyze immune signaling cascades, transcription, activation of transcription factors, and also to analyze the expression of inflammatory cytokines.

And frankly this has lead to a huge amount of information and we don't always know what to do with all this information.

It's -- one is getting the impression sometimes -- getting a little bit lost in the whole thing. But it is very obvious that this has been very, very useful and contributed to a better understanding of how adjuvants work and of innate immunity in general.

So when it then comes to adjuvanted vaccines, I would say to vaccine development, well, the approach has been quite different. It has been largely characterized by empiricism so far.

And we have had some difficulties to translate this discovery into really product development, adjuvant development. And at the end of there, adjuvanted vaccines. That is what we are looking for.

So this is basically due to the fact that, of course, we have -- and we acknowledge this, of course -- that we have to formulate these compounds. And we have to put them into delivery vehicles.

We have to process these



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compounds. And by doing so, by formulating them, we sometimes change the physical properties as we just heard. And then we sometimes encapsulate this stuff and we absorb it on particles or we present this at multimeric particles and whatever.

So in the meantime, we maybe have forgotten that we are generating, by doing this, a number of physical interactions that not only we don't always understand but that we don't usually characterize enough. And that we do not always control.

And this may have lead to a number of issues. I think that the lack of rationale, sound rationale to why -- how do we formulate these things and also a lack of a more multidisciplinary approach to the understanding of what is the relationship between the physical properties and the biological behavior has led to a number of issues like, for example, reproducibility.

How much aid the stability -- and

I'm not talking about only chemical stability, also physical stability? And one of the challenging things -- and this is a question I would like to address in this presentation -- to what extent could these be responsible, for example, for the lack of the association between adjuvant potency and toxicity? I think this is one of the key targets of use of adjuvants.

So what are the challenges to adjuvant safety? Well, we have already seen this before. An adjuvant shall not be introduced into a product unless there is satisfactory evidence that it does not adversely effect the safety or the potency of the product.

We all know, of course, that vaccines are going to induce some side effects. We have some local side effects. We have some, you know, systemic effects often due to some cytokines circulating around.

But what we really want to avoid



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is that the use of adjuvants would enhance local reactogenicity or even worse, would also enhance systemic reactions.

So what we want to avoid is severe reactogenicity. And we are especially, I think, scared of a kind of generalized, unspecific stimulation of innate immune cells breaking tolerance, for example, things that may lead to immune pathology.

So how can we avoid this? Well just first of all a couple of statements that I -- citations from literature. And we may have a number of questions around these statements.

But, you know, at least I think they clearly illustrate that, indeed, vaccine safety and tolerability are critical regulatory issues. And probably one of the greatest barriers to the approval of new adjuvants. And the fact is we have only a few adjuvants that are approved right now -- only alum in the U.S. and a couple of others in



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Europe.

So do we have to live with this? No pain, no gain. So this was, you know, the kind of spirit I tried to raise my kids in saying, you know, if you want to achieve something, it is first going to hurt you. But they are saying, you know, this is obsolete.

I'm kind of old fashioned and they may be right because if it hurts too much, people don't want to have vaccines anymore. And that's not good either. It would be bad for the perception and the acceptability of the vaccines.

So we know where this dogma is coming from. And it seems like in the past we thought we have to make a kind of trade off. If it is for a very important disease, you know, it can hurt a little bit more.

So the question really is can we disassociate this? Do we need to continue to live with this dogma? And what can we do about this?

So first of all, very simple, I would say let's have a look in the causes of adjuvant-related safety issues. On the left-hand side, we see local reactogenecity.

And what are the reasons for local reactogenecity or the cause? Well, it is either going to be some local -- I don't know whether this works -- it doesn't seem to work -- some local irritation, some local tissue insult that could be caused by a number of compounds. And that is going to generate some local inflammatory reaction.

Or it could be by a local activation of the -- I don't know, it would be useful if it -- I don't know, I can't see it -- well, at the bottom on the left-hand side, it can also be provoked, of course, by the local activation of the innate immune system -- so to say the danger signals.

And if these influences become spread, become disseminated into -- over the body, then we get some systemic toxicity



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which, of course -- oh, sorry -- systemic toxicity which could be, for example, some of these compounds that pose some unspecific inflammatory reaction start gaining the systemic circulation and disseminate in different organs, we could get possibly some organ toxicity.

You know, this is not something not something that we are often observing. We know this material can, to some extent, be degraded, excreted by urine, et cetera.

So I think what we are more concerned about is when these danger signals start spreading to the systemic circulation and then, you know, send the immune system in a kind overdrive where we then get this generalized stimulation of innate immune cells. And that is definitely something we want to avoid.

So how do we achieve a potent adjuvant effect while reducing its likelihood of causing local reactogenicity? Well, it is



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pretty simple in fact. First of all, we'll use adjuvants with low or reduced intrinsic toxicity -- and I'm going to come back to this point and give you some more explanation.

And then secondly, and maybe even more importantly, restrict -- we have heard this already before today -- restrict the delivery to the site where you expect them to exercise their effect and certainly not into the systemic circulation.

So the message, obviously, is increase really the retention of the adjuvant at the injection site and avoid release from the injection site. This will also allow you to lower the dose, of course, of the adjuvant. And on top of this it is favorable for safety.

So first of all, yes, we'll use adjuvant with low and reduced intrinsic toxicity. I'm not going to explain all this in detail but, you know, we know that adjuvants can, indeed, be detoxified genetically or chemically. And I've listed a



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couple of examples there.

Some of the adjuvants, especially detergents, for example, can be physically quenched so that they are less toxic. And there are even natural mechanisms of detoxification which, for example, enzymatic degradation, this is a way, for example, some of these polyelectrolytes or polyionic adjuvants work.

They can cross-link structures on the surface of membranes and, therefore, induce signaling. So when they get degraded, this signaling will finally be weakened and stopped.

So the other way to reduce toxicity is to restrict adjuvant delivery to tissue- resident dendritic cells at the injection site, basically, I guess, dealing with parental vaccination.

I think we all agree that dendritic cells are the cells that we want to target. These are the guys that are going to



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traffic to the lymph nodes, that are going to present the antigen, that are going to be responsible for immune signaling, et cetera.

So if we think about adjuvants, for example, that enhance antigen presentation, we often call them antigen carriers, and we see that first of all -- well, if you look at this part of the cartoon, I think the message I want to convey there is that it is very important and we know that particulate formulations, for example, are particularly well suited for delivery and targeting to dendritic cells.

And so in order for dendritic cells to make optimal use of the antigen and to prime CD4 T cells, for example, and to induce the differentiation of CD8 and CLs, et cetera, well, to make optimal use of the antigen, the antigen should ideally be presented in MHC Class II or MHC Class I presentation.

And I'm not going to go into the

detail of the immunology here but the way the antigens are presented and the way they are processed by the dendritic cells very much depend on the mechanisms of internalization of the antigen, of the mechanisms of entry.

And there is -- so, as I said, I appended a number of literature -- references from literature on this -- it has now be fairly clearly proven, I would say, that, for example, lipid clathrin-mediated endocytosis is very favorable to the presentation of the antigen into MHC Class I presentation whereas receptor-mediated uptake by the dendritic cells through phagocytosis, for example, would rather favor the presentation of MHC Class II.

Now what is interesting is that these mechanisms of internalization very much depend upon the physical properties of the antigen. And so you see some sizes that I have, I would say, copies from what is cited in the literature, but it is, of course, much more complex that that.

It is not just about the size. It is also about surface charge, about potential, et cetera. Globally speaking, it is about the surface properties of these particles.

We should not forget that particles, in order to get internalized, they are going to interact with the lipid bilayers and the interfacial properties between the particles and the surface membrane is going to be very important.

These are some of the things we didn't pay enough attention to, I guess, in the past. We may want to not forget that, for example, all these things like antigen presentation, endocytosis, phagocytosis, like signaling, for example, also the key junction between the APC and the T cell, which is the key link between innate and adaptive immunities, all are about signaling membranes.

So if we manage to present small -- - the antigen using these antigen carriers, for example, as small particles in multimeric

colloids, it is going to favor these mechanisms of internalization, particularly colloids are very appropriate to induce lipid raft-mediated endocytosis whereas small particles, monodispersed particles are favorable to be taken up through phagocytosis.

So this is going to ensure maximal antigen presentation and very efficient antigen presentation. If you don't do this, for example, and we leave the antigens just like free monomeric compounds, then we will find out that they will simply diffuse in the systemic circulation. There is no antigen uptake whatsoever.

And we may think, well, it is not efficient so we will increase the dose. But then, in some cases, we may even end up with organ toxicity, which is, for example, if you are using cationic peptides or things like that, those things may be toxic if they start to circulate, broadly circulating.

So on the other hand, if we now



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use carriers that really transform these particles into large, large aggregates, for example, micro sized particles and big droplets, for example, then we are probably -- because this is not the ideal size -- this is not the ideal confirmational shape and confirmation for the antigens to be taken up by the dendritic cells -- we are probably rather causing local inflammation than any kind of beneficial antigen presentation.

And to gain, because this is not very efficient, we may want to increase the dose and even make the situation worse.

So the very same effect applies to immune potentiators. So the adjuvants, the real adjuvants that have the immune signaling effect -- and, we know, of course, their target cells are also the dendritic cells -- again small particles and multimeric colloids have been shown to really enhance -- and I'm dealing here especially with TLR receptors to enhance TLR signaling.

We know, for example, that transmembrane TLR signaling is associated with phenomena of lipid membrane dynamics, of lipid rafts, for example. This has been nicely documented by many scientists.

So well these multimeric colloids, for example, are going to favor lipid raft-mediated endocytosis. This is also, by the way, the mechanism by which we make the signaling transient because really these receptors get then endocytosed into the cell. And that makes the signaling transient.

So we also know that -- and this is really based on some empirical findings -- that if we want to make agonists for TLR C7, 8, 9, that interact with endosomal receptors, if we want to make them more active, we need to formulate them.

And people have found out that turning those guys into particles, for example, that then get taken up by endosomes and phagosomes is going to make their effect



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much more sufficient.

So it seems that also for the immune potentiator itself, it is going to be favorable to present them as small particles or multimeric colloids. It is going to allow you to reduce the dose and to improve on the ratio between biological activity and toxicity.

So, again, if you don't do that and you end up with small molecules like SMIPs, and there is nothing to say against these molecules, the only thing is, you know, you need really to formulate them. We know this.

If you leave them alone, they are going to diffuse in systemic circulation. There is no local adjuvant uptake. And this may result into poor biological activity. And then what do we do?

Well, we increase the dose and it is even worse. It might -- that's where the questions come up about immune pathology or we

go into to send the whole immune system in overdrive because these things start to circulate.

And, again, the same if we formulate adjuvants in a way that we end up with large particles. And there are some examples, for example, of lipid A aggregates, and I will show you some of those from literature, then again these particles will not be taken up efficiently by the dendritic cells, will not end up into efficient interaction with transmembrane or endosomal TLR receptors. And finally may be causing more local inflammation than anything else.

So ideally adjuvants should come in particulates and/or colloid suspensions. So that also means that inappropriate adjuvant formulation or control thereof may lead to diminished adjuvant potency. But also it is a major -- it could be a major cause of adjuvant reactogenecity, toxicity, and lack of consistency.

And we know -- I'm not citing here, I'm not mentioning the companies -- but all these are different techniques that companies are now using to make their adjuvants more particulate, to give them more complexity, to give them a more confirmational structure.

And it is all about association with particles, with delivery vehicles, absorbing on particles, integrating, encapsulating into particles and so on.

And I just wanted to show you one example on the physical importance, for example of a lamilar versus an inverted micellar lipid A, which -- for example, this adjuvant comes naturally in two different shapes. You see the lipid particles here and over here. And you should see the lamilar form here.

Well, this is called a lipid A polymorphism. And there is a kind of equilibrium between both. Well, people have

found out about 20 years ago -- and the tests they were using at that time were not as sophisticated as the immunological panels of tests that we have right now -- but they really found out that, for example, if you present lipid A -- and as I said, it is an equilibrium between the lamilar form, which is here on top of this slide, and the inverted micellar form.

And you can shift this equilibrium. It is depending on the environmental conditions. I'm not going to go into the detail but anyway they clearly found out that this is the biological form which is active, which is causing signaling, which is having the biological activity.

If you manage, by the way you treat this in the formulation, to shift the equilibrium to the inverted micellar form, you will end up with higher biological activity whereas the opposite is true if this fraction is going to more important. So I think this

clearly illustrates the importance of physical constraints on the biological activity. And safety is, of course, part of biological activity.

So just to give a couple of practical maybe implications of this, and to wrap up very clearly safety is the major concern of regulatory authorities, and we have seen some of these regulations summarized already. I just kind of wanted to focus on the three last bullets, which are about characterization, stability, and critical process parameters.

I would like to insist that we think of these bullets as not only being applicable to chemical and biological characterization, stability and critical parameters to be in control of, but also the physical -- the physical. So the characterizations need to include physical aspects. The stability needs to look after physical stability. And critical parameters



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need to be also applicable for physical parameters.

So in order to try to convince you even more of this, well, this is mayonnaise, right, this is the mayonnaise made by my wife. It's the perfect dressing. It smells good. It tastes good. It is just perfect.

When I'm doing the same thing, I'm using exactly the same ingredients, the same vinegar, the same mustard, I'm using eggs from the same hen. But obviously not knowing exactly what are the critical parameters, I don't do the mixer right. And you can see it is just a mess.

And the biological activity seems to be different, right? So -- and the only thing which is different is the physics of the whole thing.

So what is important, I guess, is that we control adjuvant delivery and ensure consistency. Well to do this, we usually tend to prefer using small-sized colloid

particulate suspensions. I would say it is better, probably, to stay away from soluble molecules, monomeric detergents, things like this, and certainly large, irreversible aggregates that do not dissolve, it's probably not going to contribute to biological activity either.

Characterize your adjuvant's dirty little secret versus well characterized product. I think to some extent the dirty little secret comes from all kinds of physical interactions that we don't well characterize. And we do have the tools right now. I cannot go into the detail of this but we do have the tools today to well characterize interaction and to well characterize also biophysical features of adjuvant formulations.

I just wanted to mention that also delivery can be an important aspect for safety. It is because the environment is different, depending on the route of administration, you may change also physical

constraints, physical parameters, and, hence, biological activity.

If you think, for example, of intradermal delivery, well it's going to be usually pretty safe. I mean we have already kind of topical administrations so you are likely to avoid systemic effects.

And also with the way we do this intradermal administration, for example, and also due to the physiology of the skin, it is less likely that you are going to induce local reactor.

There are some disadvantages, of course. Less local reactor means that you cannot rely on inflammation as a kind of initiator of adjuvanticity, which you can, for example, in a muscle. If you induce some local inflammation already, well, we know that inflammation can trigger adjuvanticity.

So other routes of delivery -- I'm not going, for the sake of time, to go into the detail but, for example, intrapulmonary,

I think we all agree that this one is pretty likely to favor systemic distribution.

Now is that what you want to do with adjuvants? I don't think so. So this one would be pretty tricky, you know, talking about adjuvanted vaccines of course.

As always, use common sense. I don't think it is very useful if you have a very complex adjuvant mixture, which is partly characterized, and then you envisage to administer this in a prophylactic context to, for example, young children. I think this makes sense.

Also avoid the delivery, as I was just saying, of adjuvants through administration routes that enhances systemic uptake. I would advise against this. And I think this is something we should be very cautious about.

So keep it simple is also very important. Avoid cocktails. Avoid chemical association between adjuvant and antigen.

Avoid interactions between antigen and adjuvant.

I know this is quite revolutionary but it is true -- and I'm not think especially about regulatory constraints there -- but it is true that if you generate all these interactions knowing that physical interactions and the outcome thereof may have an impact on the biological activity, it will be important to characterize this.

It will be important to control these things. And the less interactions you are generating, the easier you are going to make your job.

So avoid adjuvant that are potentially immunogenic. I think this is a no-brainer. And keep it TLR dependent. Well, I think TLRs or the TLR agonists, we have a lot of them already, and we have some tools to characterize them, we have these knockout systems. We can over-express the genes. We have kind of reporter gene systems that we can



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use.

They are fairly well characterized. And I think one of the advantages, as well, is that for the TLR agonists, we have kind of integrated action with the immune system. It's not only about stimulating innate immunity. They do have impact on adaptive immunity and even on the regulatory networks.

So optimize formulation and delivery as to be able to reduce the dose is obviously key. So keep it simple, which doesn't mean that we need to say with alum for the rest of our lives, right.

I'm not going, you know, to open this box of Pandora, but I think we all agree that, you know, we feel talking about diseases that require cellular-mediated immunity and things like that, alum will not be sufficient.

So just two words -- preclinical safety assessment. Preclinical safety assessment is obviously important because it

can give us some warning signs on safety profiles of adjuvants.

And I just wanted to highlight that -- and we have seen this already in the other sessions this morning -- that it is not about the effect in isolated human cells. Also the peripheral cells and the tissue can significantly contribute to induction of innate immunity.

And, therefore, it is interesting that today we have kind of systems that integrate several different immune-competent cells and also the inflammatory compounds. And those systems may be interesting to use for assessing and better understanding some mechanisms of innate immunity and adjuvants in general.

With regard to the animal model, I mean we could discuss for hours and hours. I'm just thinking that if you want to study really the delivery and the distribution of adjuvants and adjuvanted vaccines, well the

mice may not be the ideal model.

And I'm just thinking of this like, you know, squeezing an elephant in a Mini Cooper. And then you would ask the elephant to only sit in the driver's seat, for example, right.

So if you look at the mice and the volumes we are giving to the mice in relation to what we are doing in human clinical trials, it is very likely that because the routes of administration -- intranasal, for example, in the mice versus humans, it is not comparable.

Putting a large volume in the mice or a small animal, it may have an impact on the distribution, on the retention of the adjuvant and, therefore, not be a good model in terms of studying distribution and local retention.

So large animals are particularly useful for testing different delivery systems. I don't want to say that we should, you know, use only large animals but in terms of



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distributing the local effect, then the distribution may be very useful.

Should we do pharmacokinetics? Well, regulatory agencies have already been thinking about this. If there is an indication that an adjuvant might be distributed over the body and/or accumulate in well-defined tissues, pharmacokinetic studies should be considered.

Well this may be a way, you know, of finding out whether some of these adjuvants are distributing into the systemic circulation, something we would like to avoid.

It is not usually performed with vaccines because there is no relationship between plasmic concentration of antigen and immunogenicity but there might be some kind of relationship between systemic side effects and circulating adjuvants.

So conclusions in -- well just in a nutshell, I think it is important that you have a kind of good rationale for all the

different ingredients that you are using.

It is particularly important to really focus the effect on the key immune cells. And that can be done by using formulations -- formulations that are well conceived, that are well thought of, and that are also well characterized.

And I think this is going to help us to make products that are -- because these are the requirements of a product to be consistent, to be maybe more safe, and to make optimal use of the antigen and the adjuvant.

And it is my personal belief that this is not going to be possible to get the guys, you know, science, and technology to get these people around the table as well. This is going to be very, very important because it is going to trigger the upstream mechanisms of immune signaling.

We are mainly focusing always on downstream signaling. This is going to condition the interaction of antigen and

adjuvant with the target cells.

And obviously there is some more discussion needed on the animal model.

So adjuvant dose, the less the better. I mean, you know, also I would say in terms of the number of adjuvants. Well, the fewer adjuvants you are using, I think the more easy -- the easier it is going to be to develop them into true products that we can use in adjuvanted vaccines.

And well the more it is targeted, of course, the less the likelihood that you are going to run into toxic effects.

So we have apparently a mission here. There is a call for more interest, for more investment, for more resources in to adjuvant development, to make it possible to move some of these candidates forward into clinical development.

There is a call also to funding agencies. Well, we at the Gates Foundation, we are taking this very, very serious.



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We have in our portfolio, for example, diseases like HIV, malaria, tuberculosis, and we have a firm commitment to the development of vaccines. And we cannot have this commitment -- make this commitment without being also firmly committed to the development of adjuvants.

So that is basically what are our goals. We want to foster efforts that help us to move candidates forward into clinical development and to also make them available for developing countries.

So with these stats, I thank you. And, well, if there are any questions, I will be happy to take them. Thank you.

(Applause.)

DR. SUTKOWSKI: Okay. Just one quick question.

DR. MALONE: The Foundation has been an advocate for alternative vaccine delivery technology including jet injection, for example. My understanding -- when you

think about it, vaccines are really combination products. We have an administration device and the formulation.

Is there any evidence for any of these alternative delivery technologies altering the properties of adjuvant-formulated vaccines? Does that make sense?

DR. VAN DEN BOSSCHE: Yes, well I think we have been moving forward some of these efforts quite rapidly. And I think we are in the process of reviewing and better understanding what is going on because if you add on top of this -- so, for example, alternative routes of delivery, there is an additional component that you add on top of this which is, for example, the device, which is different from the needle.

So this is going to add to the complexity. And as I was just saying, the route of delivery, it may impact. So we need to take these things into consideration. We really need -- we don't have all the answers.

We're looking for the answers.

But we are sure that if we are going to better understand what is the impact of all this, that we are going, you know, to make better choices and that we are going to move forward these alternative technologies in a way that is going to be very useful for what are our goals in the end, which is to deliver safe vaccines to developing countries that are very efficient and that are widely available at low cost, right.

DR. SUTKOWSKI: Thank you.

Okay, our last and final speaker, Dr. Pulendran, he's -- if you listen to all this advice and you get the physical/chemical characteristics right and you get back to the biology here, Dr. Pulendran will be talking to us about a slightly different title than what is in the agenda. He'll be talking on his most recently published work on systems biology and vaccine development.

DR. PULENDRAN: Thank you very

much, Liz.

I realize that we are overdue for lunch so I'll be -- I'll try and be brief and not hold you back too much from lunch.

So the focus of my presentation is some work that we're doing to apply systems biology to try and understand how vaccines work and how adjuvants work.

And we've heard this -- this particular slide the message from this slide should be very obvious to all of us -- I mean a lot of speakers have spoken about this today and that is that the quality of the adaptive immune response is absolutely key in determining protection against different microbes or viruses.

So, for example, Th1, Th2, Th17, Treg cells are all very important in conferring protection against different microbes or pathogens. And, in fact, Tregs are also useful in controlling the immune system altogether.

So from a vaccinologist's perspective, a central question is what are the adjuvants and what are the mechanisms by which you could stimulate these different types of immune responses that could be optimally effective against different microbes?

And, again, this is now obvious to all of us that the dendritic cell, the antigen-presenting cell is absolutely key in this process.

In fact, there are many types of dendritic cells. And these seem to be programmed differently. They express different markers on their surface. They make different cytokines. And then they can lodge different types of immune responses.

And so the question is how can different adjuvants target these specific types of dendritic cells to program the immune response in a given direction? And then as we've heard from Bruce and others, the Toll-

like receptors and other pathogen recognition receptors are key in recognizing vaccines and adjuvants and then in programming the adaptive immune response.

There are many different TLRs, some expressed on the surface, some inside the -- in the intracellular compartments in dendritic cells. And so the question is how can we exploit them in vaccine design?

So with this very broad kind of perspective, really the questions that we'd like to focus on are as follows:

Firstly, can we apply this new knowledge in innate immunity to understand the mechanism of action of some of our most empiric, highly successful vaccines? And then if we can get insights from this kind of approach, in what way can these insights guide the development of new vaccines against emerging infections and pandemics?

So this is Sir Edward Jenner more than 200 years ago doing his most historic

experiment in human immunology of actually vaccinating this child with cox pox pus and then showing that this kid was then immune to further infection with smallpox.

And then as we've seen before, since that time, many, many vaccines have been developed, many highly successful vaccines. And so as I mentioned earlier, one of the paradoxes is that we really don't understand the mechanisms by which they stimulate immune response.

And so some work in our lab has focused for the last three or four years on understanding how exactly the yellow fever vaccine works. Why the yellow fever vaccine? Well, it just happens to be a very successful vaccine.

It is one of the most effective vaccines ever made. It is a live virus. One injection of the vaccine give you a very broad spectrum of immune responses, Th1, Th2, cytotoxic T cells, neutralizing antibody. And

remarkably, one injection of this vaccine gives you neutralizing antibody that can last for up to 30 years, okay.

So here we have a model, a model vaccine that we've given to 600 million people globally but we don't really understand how it works. So the simple question is can we deconstruct this vaccine immunologically such that then you could design adjuvants that do exactly the same thing, or facets of these kinds of immune responses that this vaccine does.

So this really summarizes all that we have been talking about this morning and that we believe that this black box called the innate immune system is absolutely key in this regard.

So what are some of the pathways and the receptors within this black box that control these different types of immune responses?

So as I mentioned to you earlier,



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we began a few years ago by demonstrating that this vaccine was triggering multiple Toll-like receptors and that this seemed to be relevant because depending on which TLR was engaged, you seemed to get either a Th1 or a Th2 bias, suggesting that one reason -- it's not a reason but one of the manifestations of activating multiple TLRs is to give you a balanced Th1/Th2 response.

And since then we've gone on and we've also understood that this vaccine is engaging or activating this pathway within the plasmacytoid dendritic cells, the so-called mammalian target of rapamycin, which seems to control the production of Type 1 interferons by plasmacytoid dendritic cells.

So this is an example of some new biological insight that one can get by trying to deconstruct a really successful vaccine, okay, that we wouldn't necessarily have done this experiment if not for the fact that we were looking at how this yellow fever vaccine

is working, okay.

But one major focus of our lab is this. And that is to apply this new science of systems biology to trying to understand if we can predict the immunogenicity of vaccines and, indeed, to predict the toxicity of vaccines, okay, in a completely unbiased kind of way.

So put simply, the question is are there innate signatures that are induced by vaccination in humans with which you can actually predict the subsequent immune responses or the toxicity of vaccines or adjuvants?

So a couple of years ago, we began to address this question with a small clinical trial in humans who were vaccinated with this yellow fever vaccine. Blood samples were removed at these different time points, including very early time points like Day 1, Day 3, Day 7. And then later on at Day 120, 180, 160.



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And we made various measurements. We measured the antigen-specific CD8 T cell responses and the neutralizing antibody titers. And then we also made measurements of so-called innate responses in the blood.

And so the question was by looking here early on, could we predict what is going on later on, okay? And why would we want to do this? Well, because when we design new vaccines of questionable efficacy, we would like to think that this kind of strategy could be informative in evaluating the potential success or efficacy of emerging new vaccines is the first reason.

Second reason is that the same kind of approach, we would like to think, could be useful in predicting potential toxicities that might be stimulated by vaccination or adjuvants.

And thirdly, because we think that this kind of unbiased global analysis would be useful in providing new biological insights

about the mechanism of action of vaccines and adjuvants, okay.

So the approach is shown here, that we measure cytokines in the blood using Luminex platform. We look at the activation of different types of dendritic cells and monocytes in the blood.

And then we do a microarray analysis of the gene expression profiles in the blood. And this was done in collaboration with Alan Aderem at the Institute for Systems Biology in Seattle.

So first when we look at the immune response -- so this is -- we have a tetramer that can stain for the yellow fever-specific CD8 T cells and you can clearly see that this is the population that comes up two weeks after vaccination.

And you can characterize the phenotype of those T cells using a number of different markers. And this was done in Rafi Ahmed's lab at the Emory Vaccine Center.

And what is apparent is that if you gate on those tetramer-positive T cells shown here as red dots and you overlay this on a profile that shows HLA-DR versus CD8 on gated CD3-positive T cells, in fact there is a remarkable correlation between the coexpression of CD8 HLA-DR and tetramer positivity. And this is shown nicely in this linear graph here.

And so, as I mentioned, you could phenotype these cells and it turns out that these tetramer-positive T cells at Day 15 are mostly effector cells.

They express high levels of CD27, higher levels of CD28. They are dull for BCL2. They are dividing because they are Ki67 positive. And they are CCR5 high and CCR7 dull, suggesting that they are highly activated effector cells.

And so if you look at the variation in the magnitude of the CD8 T cell responses in these 15 individuals, the first

surprise was that, in fact, there is a striking variation. So, for example, these vaccinees here seem to have a relatively poor response compared to these vaccinees, okay.

So we were initially worried because we thought that perhaps this was a technical flaw in terms of the vaccine not being administered successfully in the clinic.

But, in fact, when we looked at the neutralizing antibody titers in these individuals, for example, 1910 and 1920, who were relatively poor at CD8 T cells responses do just fine with the neutralizing antibody titers suggesting that no, these people, in all likelihood, did receive the vaccine.

So for us the question was could we predict this. For example, we see this variation in T cell responses or variation in the antibody responses. By looking early after vaccination, could be identify correlates that would predict how strong a T or B cell responses a particular vaccinee



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might have.

So, as I mentioned, we did a comprehensive analysis of cytokines in the blood. And the short answer is that despite the up-regulation of these two molecules, IP-10 and IL-1 alpha, in the majority of the vaccinees, really there was nothing that correlated with the magnitude of the T cell responses or the B cell responses.

And then we also looked at the activation of these various dendritic cell monocyte subsets and, again, you know, we see up-regulation of CD80/86 and so on but nothing that seemed to segregate people who had a high CD8 versus a low CD8 or a high antibody versus a low antibody.

This suggested to us that the so-called traditional correlates that we might have been programmed into looking for were not sufficient for this exercise. That we needed a much more unbiased kind of approach to identify potentially new correlates, okay.

So the approach we took was a microarray analysis of total peripheral blood mononuclear cells using the Affymetrix GeneChip platform. And the first question was whether we could identify genes that were reproducibly up- or down-regulated in the majority of the vaccinees who got this vaccine, okay.

And in the interest of time, I'm not going to belabor the statistical approach but we can talk about that later on if you'd like to.

So the message that came across quite strongly was that yes, there were a subset of genes, about 65 genes, that were reproducibly expressed in the majority of the people who got the vaccine.

nd so this is shown here in this heat map. And if you focus on Trial 1, which is the first trial with 15 individuals -- and so here we have the kinetics, Day 0, 1, 3, 7, and 21. And unfortunately you can't read the



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names of these genes here but I can point out some of the important ones.

So, for example, you have oligoadenylate synthetase 1, synthetase 2, synthetase 3, synthetase L, RIG-I, protein kinase R, MDA5, TLR7, Lgp2, all the usual suspects that you might normally associate with a viral infection. And remember this a live attenuated virus, okay.

So this was reassuring because this kind of approach had not been done with vaccines. And so this was -- and going into this, we didn't have any idea that we would see anything that any signature could be detectable in the blood because we are giving this vaccine subQ. But this was actually reassuring, that told us that perhaps this kind of approach does have merit, okay.

So the question was to what extent is this simply an artifact? And can we validate the signature using other approaches?

So the one thing that we did do



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was to use a realtime PCR approach to systematically evaluate the expression of all of those 65 genes that we had seen. And, again, we see a very nice correlation between the expression based on the microarray data and the realtime PCR data.

And the second thing was a couple of years after the initial trial was over, we set up a completely independent trial with funding from Sanofi Pasteur to vaccinate an independent cohort to vaccinees with this vaccine, again did the same kind of approach. And we see a remarkable concordance between the signature that we had seen with Trial 1 and Trial 2.

And also of interest in the signature are things like IRF7, STAT1, again transcription factors that mediate the Type 1 interferon response, okay.

So that was all very well. So you can actually now some bioinformatics modeling and you can put in those list of 65 genes.



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And, you know, what comes out is a signature which is pretty much a textbook signature of Type 1 interferon induction in response to viruses.

And so this was a nice confirmation that, indeed, this kind of approach is viable in terms of picking up genomic signatures in response to vaccination.

Now previously, as I mentioned, we had shown that this vaccine is triggering multiple Toll-like receptors but the expression of RIG-I, MDA5 raised the question as to whether it might also be engaging these additional non-Toll-like receptors.

So we tested this using cell lines which over expressed our RIG-1 or MDA5 with a reported gene. And you can see that, indeed, that yellow fever vaccination could induce NF-kappa B activation in response to engagement of either one of these receptors expressed on the cell line.

So this was again some new insight

that we had acquired through this kind of unbiased sort of approach.

So now some of you might be asking well are these changes in the gene expression that you see in vivo really bonafide induction of genes within a certain cell type or does it simply reflect changes in the cellular composition of the peripheral blood mononuclear cells -- migration, exit of cells, and so on, okay?

Well, this is a very difficult question to answer. But what we did was to do a poor man's experiment to address this. So we simply took PBMCs from a healthy individual, a couple of healthy individuals in vitro, previously unvaccinated with the yellow fever vaccine, and then just dumped this yellow fever vaccine in vitro. And then did a microarray at a couple of different time points.

And what we see is that the signatures that are induced in vitro are

remarkably similar to what we had seen in vivo in response to yellow fever vaccination suggesting that much of the changes that we are seeing are most likely due to de novo expression of genes rather than any alterations in the cellular composition, okay.

But if you recall, the original purpose of doing this experiment was to see if there were signatures that would predict the CD8 response or the antibody responses.

And so as much as we had hoped that the Type 1 interferon signature would be one such signatures, it apparently is not because we did not see any correlation between people who had a high level of expression of these Type 1 interferon genes and the magnitude of the T or B cell responses.

So we resorted to a second approach to select for genes that would correlate with the antibody response or the CD8 T cell response. Again, we can talk about this but I'm going to skip this in the



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interest of time.

And if you did that, from your chip, which has about 25,000 genes, you can come up with the signature of about 200 genes with which you can nicely segregate the vaccinees into two groups -- the yellow group and the red group, okay.

So you segregate them based on your signature that you have selected, okay. And what you see is that the folks here in the yellow group have a CD8 T cell response that is less than three percent whereas the folks in the red group have a CD8 T cell response that is greater than three percent, okay.

So this was encouraging, showing that this kind of approach can be useful in delineating a subset of genes that seem to correlate with the magnitude of the CD8 response.

But really the real test of such a signature is to ask to what extent it can actually predict, not simply correlate, but

predict immune responses in a completely independent trial. And that's what we did.

And working together with some bioinformatics folks at Georgia Tech who had developed a model called the DAMIP classification model -- Discriminant Analysis via Mixed Integer Programming.

What it is is that it is an algorithm that can sift through tons of data and recognize patterns in this data, okay. So, for example, it can look at the 200 genes and then it can begin to classify them in vaccinees, okay.

So using this model, we were able to come up with a set of about ten genes or so and a number of predictive rules. So we had obtained the signature using Trial 1. And then we were using a second trial of independent vaccinees to see whether we could predict the magnitude of the CD8 responses there.

And what we see is that with just



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a couple of genes here, for example the solute carrier family member 2 or 6 and then this gene, eukaryotic initiation factor alpha kinase 4, you can predict in Trial No. 2, with up to 80 percent accuracy, the magnitude of the CD8 response.

And you can generate a number of predictive rules of this type. For example, Rule 1, Rule 2, Rule 3, and so on. And you can do the opposite. You can actually derive the signature from Trial 2 and then use it to predict in Trial 1.

What is very interesting to us is that many genes, for example this gene, eukaryotic initiation factor 2 alpha kinase is multiply represented in very many of these signatures, okay.

So this now begs the question of what some of these genes might be doing. And if, indeed, they are so important in controlling the CD8 T cell response. And so this is exactly what we are doing now.

So basically it turns out that that gene, EIF2AK4, which is multiply represented in these signatures is also called GCN2 and it plays an absolutely key role in the so-called integrated stress response.

Now as many of you know, the integrated stress response is launched in response to various cellular stresses. For example, recognition of viral infection through protein kinase R or oxidative stress through HRI, or stresses in the endoplasmic reticulum perhaps due to a viral infection, through PERK, and also in response to proteasome inhibition.

The combination of all these stress response pathways is the phosphorylation of this molecule called eIF2 alpha, okay. And it turns out that this molecule plays an absolutely key role in the translational shutdown, global translational shutdown in cells, okay.

And one thing that happens when

that -- when the translation is shut down is that you have formation of what is called the stress granules, okay. So now this raises the question of whether this yellow fever vaccine might be trigger the stress response pathway. And if so, whether this has a link to the adaptive immune response, notably the CD8 T cell response.

So we have begun to do some biology here. So, for example, you can see that yes, indeed, that in PMCs, or in the cell line, the exposure to yellow fever vaccine does result in the phosphorylation of EIF2 alpha.

Secondly, consistent with this, you do find the formation of these stress granules. These are dense aggregates of proteins and RNA that appear within the cell when it is under stress. And the function of this stress granule is thought to be to protect untranslated message RNA from degradation, okay.



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So what influence does this pathway, does the induction of this and the formation of stress granules have on the CD8 T cell response mechanistically? This is an area that we are actually investigating using mice that are deficient in these various stress response genes.

But our early indications are that GCN2 activation is key in the induction of CD8 T cell responses to yellow fever vaccination.

Now I've talked a lot about CD8 T cell responses but we should remember that this vaccine works mostly -- or is thought to work mostly through neutralizing antibody responses. Can we predict neutralizing antibody responses?

And, again, the answer is yes. So we can actually do the same kind of approach. We can come up with these two clusters that, in fact, distinguish vaccinees based on the antibody titers and then we can do the DAMIP model.

And what is very interesting is that this gene, tumor necrosis factor receptor superfamily 17, here is present in every single DAMIP signature, either going from Trial 1 to Trial 2 or vice versa.

What is that gene? So it turns out that this gene is also called the B cell maturation protein, BCM, BCMA, CD269. And it is a receptor for BAFF BLyS, which we know to be absolutely key in the induction of antibody responses.

So here is another example of a gene, that we've come up a through completely unbiased sort of approach, that seems to be one of the best predictors for the neutralizing antibody responses 90 days after the initial vaccination, okay.

So, again, this provides one an opportunity to delve into the biology of this and to ask well, how is the yellow fever vaccine inducing this gene? Is it through a TLR dependent, TLR independent pathway and so

on and so forth.

So to summarize what I think we are seeing is that this vaccine, one of the best vaccines in the world, is engaging multiple pathways. It is engaging TLRs on multiple subsets of DCs and which downstream of that is the emptor-dependent regulation of interferons.

It is also engaging non-TLRs, for example RIG-I MDA5. In addition, some of these other genes like protein kinase R, oligoadenylate synthetase 1, 2, 3, and L, which are involved in innate immunity. And TRIM5 alpha and its complement component IqB, BAFF, BLyS -- I didn't have time to talk about some of these.

But most intriguingly, it seems to be activating many genes involved in the so-called integrated stress response. And I mentioned EFI2ASK4. I mentioned phosphorylation of eIF2 alpha and stress granules. But there are also these other



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genes.

So the question is in what way do any of these modules link to the ultimate immune response and then ultimately to protection?

So this is exactly what we are beginning to understand now using various animal models and knockout models. But the long-term goal of this kind of approach we would like to think is that this might be beneficial in predicting the efficacy or the immunogenicity of new and emerging vaccines of questionable efficacy.

But also, in the context of this workshop, in perhaps predicting the potential adverse reactions that might develop from vaccinations.

So thank you for your attention. And I'd be happy to take your questions. Thank you.

(Applause.)

PARTICIPANT: Bali, I really

enjoyed your and Troy's paper. I've got two quick questions. One is that in that first figure you can kind of separate out CD8 responses and antibody responses.

And when I was in Atlanta, I got 17D and I can tell you that from about Day 9 to Day 16 or 17, it was a really bad week. Do those adverse events correlate with broadly CD8s or antibody?

DR. PULENDRAN: Are you talking about adverse events meaning the adverse events that sometimes develop in response to this yellow fever vaccination?

PARTICIPANT: No, just overwhelming rough flu-like symptoms.

DR. PULENDRAN: Okay, okay, that's interesting. We haven't -- you know that is a good question -- we haven't done this analysis directly to see whether there is any correlation between the type of response and these mild adverse events.

What I can tell you is that there



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was an individual last year who developed very serious adverse events. He almost died unfortunately but then fortunately he survived.

And it turns out that in this individual -- and we had expected that perhaps the reason why this person almost died was because his immune system was compromised and there was very weak adaptive immune responses. But on the contrary, we saw the opposite.

In fact, there seemed to be an exacerbated CD8 response and tremendous neutralizing antibody titers that persist for a very long time. But interestingly this Type 1 interferon canonical signature was absent in that individual, basically completely diminished. So, yes.

DR. SUTKOWSKI: Okay. I guess it is time for lunch then.

Dr. Slater, do you have some announcements?

(No response.)

DR. SUTKOWSKI: Okay, I guess we'll plan to be back at two-thirty please. Thank you.

(Whereupon, the foregoing matter went off the record at 1:23 .m. to be reconvened in the afternoon.)

A-F-T-E-R-N-O-O-N  S-E-S-S-I-O-N

2:32 p.m.

DR. SHEVACH: Okay, I'd like to welcome you all to Session 3, which is entitled Preclinical Safety. And our first speaker will be Marion Gruber from the FDA, giving us a brief overview of current nonclinical testing requirements for adjuvant and adjuvanted vaccines.

DR. GRUBER: Yes, good afternoon. Yes, welcome to the session. In this preclinical Session No. 2 we are going to be discussing current and perhaps novel approaches to preclinical safety assessments of adjuvanted vaccines and adjuvants.

And whereby we don't really want to restrict the discussions to animal models but we also want to look at potential alternative technologies to really at least to support or supplement safety studies in animal models.

So the focus, at least how I would

see it, is really discussing the current nonclinical testing requirements for adjuvants and adjuvanted vaccines and we are going to give you the perspective from the U.S. FDA. And then we will hear from our European counterparts on their thinking about recommendations for preclinical safety assessments.

And at the same time, I think what we all should -- or what we would want to do is to perhaps challenges some of these approaches and see are they still relevant when we look at preclinical safety assessments of adjuvants or adjuvanted vaccines.

And in this regard, we have formulated a number of questions, nonclinical issues that should be discussed and they will be subject for tomorrow's Roundtable No. 1.

Dr. Baylor had showed you some of these questions this morning. And as I'll give the overview here on nonclinical testing requirements for adjuvanted vaccines, I'll

point out some of the issues that we want to discuss and hopefully improve upon.

So I think the goal should not necessarily be how can we do more but how can we improve the current methodologies that are available to us so that we can better inform clinical development.

This was pointed out this morning but I'd just like to remind everybody that the majority of vaccines, and it doesn't matter if they are adjuvanted or not, they are given to healthy subjects, including healthy children and that does place significant emphasis on their safety.

And it is especially critical in a time where, at least in developed countries where the immediate benefit of a vaccine in terms of preventing infectious disease, may not be immediately obvious because of relative absence of the disease in developed countries. And, therefore, the risk-benefit is looked at on the individual level and the perception of

risk by getting a vaccine may outweigh the perception of the benefit.

So it is crucial that vaccines really undergo a rigorous pre-licensure, preclinical, and clinical safety assessment. And thus increased focus has been given to nonclinical safety assessments, including toxicity studies in animal models, to support proceeding to clinical studies.

Now safety is always primary but safety is relative. It is not absolute. So in determining whether a vaccine product is safe, one has to look at the indicated target population, the nature of the product, the indication, and the circumstances under which the vaccine will be used.

And even if you read through the definition of safety in the Code of Federal Regulation, it will tell you that safety is -- or the definition of safety here is relative.

I don't really want to go over this here in detail. We have heard this

morning about the potential safety concerns as they relate to adjuvants and adjuvanted vaccines.

And because of safety concerns, the law in the Code of Federal Regulations under the IND regulations requires that adequate information about the pharmacological and toxicology studies that have been conducted or that should be conducted for the vaccine or adjuvanted vaccine need to be available.

And on the basis of these studies, the sponsor has to conclude that it is reasonably safe to conduct a proposed clinical investigation.

However, the law provides us with flexibility here in that it also states the kind, duration, and scope of animal and other tests that are required will vary with the duration and the nature of the proposed clinical investigation.

Dr. Sutkowski stated this morning

that adjuvants are considered constituent materials under 610.15. And, again, to remind you, the law also states that an adjuvant shall not be introduced into a product unless there is satisfactory evidence that it does not effect adversely the safety or potency of the product.

Now what are the goals in nonclinical safety evaluations? Well, first of all, nonclinical safety evaluations should help to support entry into clinical trials where human safety, of course, is ultimately evaluated.

So at least given the limitations of currently available animal models, and hopefully we will be discussing this this afternoon a little bit, one has to be mindful of the fact that certain toxicities, that is rare toxicities or perhaps toxicities that only occur in certain human subpopulations, may only be addressable in humans.

But given the limitations of

animal safety evaluations, such testing may help to determine a safe dose to be evaluated clinically and to also identify any potential or unknown toxicities or toxicities on certain target organs. So if we do toxicity evaluations in animal models, we are really looking for unexpected effects.

The current guideline that the FDA is referring to in terms of their guidance and recommendations for preclinical safety assessments of vaccines, including adjuvanted vaccines, is the WHO guidance that has been published in 2003.

And it is really a document that tries to harmonize the recommendation and requirements for nonclinical safety evaluations for vaccines across the regulatory agencies. And as such, it is recognized by the U.S. FDA as well as by the EMEA.

The toxicology studies for adjuvanted and adjuvanted vaccines that are typically conducted are local tolerance

studies and repeat dose toxicity studies whereby the term repeat dose toxicity study is really loosely used here because it doesn't really follow the testing paradigms as you may know it for the typical chemical drug entities. And I'll get to the design issues in a couple of minutes.

There is another form of toxicity assessment that is frequently required in these developmental toxicity studies, in particular if a product is indicated for a target population that would include females of childbearing potential. But I'm not going to be discussing that at this point.

Just a few words, toxicity studies need to be conducted in compliance with good laboratory practice regulations. Those are specified in 21 CFR 58. And the test article or the vaccine lot that is used in animal studies should be from a lot or from lots that are manufactured with the same production process as those lots intended for clinical



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use. That is the ideal situation.

Now in terms of the animal model, we have a couple of questions framed for discussion in tomorrow morning's roundtable, and that is really what is a relevant animal model and how do we choose animal models for these types of studies.

In general, what we have been recommending to date is that one species is sufficient. So we would not require toxicity evaluation in two different animal models in general. And we are recommending that a species is chosen in which antigen is immunogenic and in which the odd adjuvant augments the immune response.

Now we have to perhaps discuss this a little bit further in tomorrow's roundtable discussion to see what are really the limitations here. Do we have to redefine the relevant animal model, in particular since we heard this morning, you know, about the species specificity of the immune response,

the different distribution of Toll-like receptors, et cetera. So that is certainly an issue that we should be reevaluating.

If the species is sensitive to a pathogen, so that is if the animal model would allow challenge, that would be ideal. But that is not a current requirement of one animal model to be chosen for a tox study.

And, of course, there are a couple of other issues. There needs to be a sufficient number of animals per sex for groups and the number, of course, it is not really set in stone. And it depends somewhat on the animal model also that is chosen for the tox study.

Toxicology studies are usually conducted as combination safety activity studies whereby toxicological endpoints and immunogenicity endpoints are both evaluated in one study and we are recommending that to really preserve the use of animals.

Of course, it is important to

include relevant controls such as the saline placebo. For adjuvanted vaccines there is a recommendation to really also include an adjuvant-only study. And, of course, it is important to evaluate the vaccine-adjuvant combination that you want to study in clinical trials.

There is also an option to submit a document that we call a master file for the adjuvant only, which would typically include chemistry, manufacturing and control information of the adjuvant, and toxicology assessments that may have been performed on the adjuvant only.

A few words regarding the study design, our currently recommended study design. We have been recommending to administer at least one full human dose to the animal model. And that should not be scaled for body weight or surface area wherever that is feasible.

Of course, it is recognized that

sometimes, especially if you have a small animal model like a mouse, it may not be feasible to administer the full human dose. And in that case, there is a scaling usually or typically based on body weight.

Now this recommendation really comes from the discussions that we had on how to best do toxicological studies for vaccine antigens. And we were saying at that time -- and that was in 2002 -- since the dose -- the immunogenicity of the vaccine in the human should really sort of drive the dose, we thought it was sufficient to really do one dose only.

So there is no requirement for dose ranging study. But that, of course, is another issue that we are going to be discussing tomorrow in the roundtable for in terms of evaluating toxicity of adjuvants should there be a recommendation to perform dose ranging studies.

Since vaccines are administered

clinically as episodic dosing, episodic dosing and not daily dosing is also recommended for these preclinical safety studies whereby sufficient time between vaccinations should be allowed so that the host immune response can be developed.

And we typically recommend that at least one additional vaccination should be done in the animal model relative to the clinical trial so if the vaccine is given as three doses in the clinical trial, then four doses should be evaluated in the animal model. And we refer to this as the so-called end plus one rule.

We usually recommend for the same route of administration to be used as is planned for the clinical trial. And if there is an intention to use the vaccine-adjuvant combination with a delivery device, then that should be evaluated preclinically as well if this is possible. Sometimes, of course, the animal model chosen prohibits use of a certain

delivery device that is proposed to be used clinically.

And I already mentioned the importance of including appropriate control groups. Placebo as well as recovery groups, so that is usually one study of animals that is allowed to recover. That means it is followed up somewhat longer to really evaluate if the rare adverse events or adverse effects are noted are they reversible.

I already spoke to the number -- to the sample sizes here.

And also -- and I mentioned that at the beginning of the talk -- if we do these tox studies, we seldom are really after a certain adverse effect. What we want to look at is really unexpected effects.

Are there potential toxicities that we can be made aware of by doing these safety studies in the animal models? And, therefore, it is important to really include a broad spectrum of measures and parameters to

be evaluated such as in-life procedures that include daily clinical observations, weekly body weights, feed consumption, as well as physical examinations of the animals.

There should be an assessment of local reactogenicity and, of course, clinical chemistry, hematology, and immunological assessments after initial vaccination as well as scheduled necropsies.

Terminal procedures are conducted typically one to three days after final immunization and then, of course, after a number of weeks in the recovery group.

And there is an assessment, a histopathological assessment of the injection site and necropsy and histopathology on select tissues. The select tissues are usually the pivotal organs, those that may be primarily effected by vaccine administration. And also a histopathology on the immune organs.

When vaccines are adjuvanted, we have been recommending that the full tissue



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list be evaluated. And when I say full tissue list, I am referring to the tissue list that is included in the WHO guideline on nonclinical safety assessments for vaccines.

So that, in a nutshell, was the current overview on approaches to nonclinical safety assessment of vaccines and adjuvanted vaccines. And as you can see, there are probably multiple issues that we are going to be discussing.

I'm not going to really put up all these questions. We're going to do this tomorrow at the beginning of the roundtable discussion. But I think what we really should focus on is really, again, what does the current approach look like, how can we improve upon this so that we are perhaps going to be in the position that preclinical safety information can inform clinical development.

And I'll stop here. And if there are no pressing questions, I think we can introduce the next speaker.



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DR. SHEVACH: Well, we can take a couple of questions if there are any.

(No response.)

DR. SHEVACH: Nope? Okay. We'll go on to the European perspective, Dr. van der Laan.

DR. VAN DER LAAN: Thanks for invitation organizers, FDA and the NIH, for being here to speak for this unexpected large audience on adjuvants. I'm from the National Institute of Public Health in the Netherlands. But also representing the EMEA and the Vaccine Working Party.

I will first start just with a remark. In the EU, we have a guideline on adjuvants but my personal opinion is that guidelines are only guidelines and not the law.

So if you want to apply guidelines, they are meant to help you and not to block development. So please think before you apply.

When writing the first guidance on vaccines in 1997, the adjuvants has only this small paragraph. Adjuvants were included in other aspects and other exepients and more was not written. At that time, adjuvants were not very strong.

But later on there was a much stronger discussion. And for me it was very helpful to think about adjuvants as to make a differentiation between the type of signals they give.

You can differentiate along several other criteria but for me it was most helpful that you can take an adjuvant, what is really the purpose of the adjuvant and the mechanism of action? And Virgil Strands from the veterinarian company in the Netherlands has worked on it.

And, okay, we now know that most of the adjuvants are engineered to target the antigen-presenting cells, the key players in the innate immunity. And that is what we are

working on.

But in the EU, we have just looked at the alum as the traditional adjuvant and as it is so long on the market, we indicate that for alum, of course, I think you would still think about is it relevant to use it but we have no further strong requirements for that.

But for all other new adjuvants, the same applies, in fact, for the adjuvants as well as for the vaccines as a whole. Ideally, enhanced protection against the disease, that should be the final purpose of the adjuvant and you should test that also in this way.

In use by infectious agents, that is the ideal situation. But we have to admit that the fact that there a lot of human diseases specific for human and there are no animal disease models available, in that case, surrogate markers, for instance adequate responses in the immune system, might be used.

But, in fact, they have to be



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validated in a sense, at least evaluated as what are the right surrogate markers and antibodies are not primarily, for each and every disease, the right surrogate markers.

So the actual situation is although there is a guideline now written a few years ago, the scientific research on adjuvants is still based on trial and error. And those words have been on the screen already several times this morning.

And the research is not directed to requirements for marketing authorization. They are in a lot of cases carried out by small specialized companies or university laboratories. And if they were successful, they will be taken over by the bigger companies to sell out their dream.

From a pharmacological point of view, we feel, as the European authorities, that there is a lack of knowledge of mechanism of action. There is a lack of dose response relationships. A lot of studies are done with

only one, maybe two dosages, a lack of combination studies with different endpoints.

And most is the focus on immunological effects and there is hardly any idea about cardiovascular or CNS effects. You can imagine that if you have a vaccine leading to the release of cytokines, that there might also be cardiovascular effects, the safety pharmacology.

There are a lot of difficulties. There are no clear systematic data on high dosages of adjuvants. For some products, there might be historical data present. But sometimes fine distributed over the literature and difficult to find.

There are combination products fro different types of adjuvants with different types of quality of course. A very important point, as Marion Gruber already mentioned, the children is an important population. But are we -- do we know what is the effect of an adjuvant in very young children where their

immune system not very mature. And should we then test this in juvenile animals and at which time of juvenile animal?

So what to do with specific human diseases if there is no animal disease model, should we go for anther disease, a similar disease? We have seen that for HPV that you can use a type of disease that is specific for dogs or other and use that as a type of animal model.

And under specificity regarding the antigen, an adjuvant is combined with an antigen but are there coexisting antigens at the same time in humans? And, of course, also in men. And is there any interference with that? Or do we know what adjuvants are doing at the same time in humans for all other antigens that are present?

So the guideline on adjuvanted vaccines was presented in July 2005. And I will give a very short overview. It is just focusing on the proof of concept.

There are a lot of different mechanisms of action. And we have seen a lot of them this morning already. So there are a lot of possibilities.

One of the aspects of the use of adjuvants is that they are combined with subunit vaccines to get a sufficient approach. And just in our experience, that might also be the rationale for such an adjuvant.

We have done a lot of work on influenza that last few years because of the threat of a pandemic. And if you look at influenza immunization, there is a type of gradients. You can see that there is a maximal protection by a full infection experience. It is, of course, not complete but at least it gives a maximal protection.

There is broad cross-protection with live attenuated vaccines. However, most of the seasonal vaccines are just using whole-virion vaccines or just subunit vaccines and maybe the rationale for the addition of an

adjuvant might be to combine it with low immunogenic vaccines to gather more robust protection but also a broader cross-protection if we are now thinking about just the development of the pandemic vaccine.

And we are now discussing how broad is the protection against the mutual mutation shifts. Then also the use of adjuvants might be relevant in that respect.

The increased immunological response should be shown in a relevant animal model. Are the cells of the innate immune system really triggered? And to what extent are humoral and cellular immune responses activated? And is that relevant for the protection?

Of course, data from combinations of the adjuvants other antigens can be used as supportive evidence but nothing is the same. The adjuvant or antigens are different from each other as we have also seen this morning the difference between influenza and smallpox.

Public literature can be used as supportive information for the proof of concept.

So this gives the first aspect what I have told thus far was on just the proof of concept ideas. Of course, safety is an important aspect but it should be seen in relation to the efficacy.

If we look at the safety of the adjuvants and you can see -- you have to put it in the framework of how broad is the effect of an adjuvant, that's why we have the emphasis in Europe to also to test the adjuvant alone whereas the methodology should follow the pattern of use of the vaccine.

There might be a differentiation between the stimulation of the non-specific resistance to infections, the innate immunity, and increasing the immune response to the specific antigens.

The intended action is to induce long-lasting changes in the immune system by



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influencing the sensitivity to the defined antigen but as was said already, but to emphasize it here, what about the increased or decreased sensitivity to the unknown or unintended antigens?

Also with respect to the adjuvant alone, you should test it in two species unless you can justify that it is only sensitive in one. And preferably also in a non-rodent as we -- and maybe I can emphasize this as this morning we discussed mice lie. Mice can lie indeed.

Some adjuvants might exert a high level of species specificity and I think we also would -- what has been said about the specificity of the Toll-like receptors, we have to take into account that some animal species might be less responsive.

And ideally the selected species should be the same in which the proof of concept has been studied to see the differences. Of course, it might be difficult

if there is not that much experience.

But even for ferrets, if you have the handbooks of animal toxicology, you see large chapters on ferrets to be tested in toxicology not only for influenza vaccines but also for other purposes.

Toxicity endpoints, local tolerance is the first one but also hypersensitivity and anaphylaxis. Pyrogenicity is, in my view, a type of adverse effect or toxicological effect and not belonging to quality.

And under systemic toxicity, we require a full histopathology of primary and secondary immune organs and maybe also other organs if it is a new product. Of course you can limit yourself and the risk you take if you have just focusing on local applications.

Just a short word about reproductive testing, there is an FDA guideline but just -- and because of the facts that in the ICH S6 for biopharmaceuticals, we

are discussing about the relevance of reproductive toxicity studies with antibodies.

We have found that the placental transfer of antibodies is very low during organogenesis. And so focusing on the antibodies as an important endpoint of exposure during the whole part of the pregnancy is for me now questionable.

And I think it might be more important to think about the placental effects of the transfer of cytokines or cytokines and interferons. That might be difficult to do that in rodents. I think that that needs some further discussion.

We can be very short about genotoxicity and carcinogenicity. For the toxicity for the combination of adjuvant and antigen, we focus on local tolerance. And I would support the idea of the repeat of what has been said already about the repeated dose toxicity studies.

It is focusing on the immune

response as a type of phenomenon as we should think about the rationale of the why using that specific adjuvant in combination with that specific antigen. And I have to say that at least in the scientific advice procedure in Europe, there is a lot of improving concepts are now being developed.

What is on the market in Europe? We have accepted MPL as MPL/alum, ratio 1:10 AS04, and Fendrix, a hepatitis B vaccine, and Cervarix, as the HPV vaccine, and also MF59 in Focetria pandemic influenza vaccine. This is just a short listing of what was in the EPAR, the European Public Assessment Report, on the website of the EMEA.

For Fendrix, there is a specific remark that the MPL is completely absorbed at the element. And it is a reflection of the knowledge at that time that that was an important aspect. I'm not sure that we would all think that we know today that we should emphasize that too much today.

The immunogenicity was not done in relevant animals. Hepatitis B vaccine, there is no -- those animals are not representative of the immunogenicity. There are also reproductive toxicity study and the rabbit study that repeats the dose. You can question the relevance of those studies but at least they are in the dossier.

There is a safety pharmacology study but no species is mentioned in the report. And there are some toxicity studies.

Just with Cervarix, the same adjuvant system, there is an extension of the dossier with immunogenicity data in rhesus monkeys. Now the safety pharmacology is spelled out in rats and dogs for MPL alone.

There is some pharmacokinetics for MPL but that's not related to the activity of the MPL itself. There are also reproduction toxicity studies in rats for over the whole spectrum.

For the MF59, we have only one

product, the Focetria. It is a pandemic influenza vaccine with special regulations for the guidance on pandemic influenza vaccines. And so that specific concentrations indicate that limited evidence for the support of the safety and the efficacy of this adjuvant is accepted because of the threat of the pandemic.

There are some proof of concept data in ferrets but there was no control without an adjuvant so it is not fully clear from that data whether MF59 really stimulates the immune response.

And all other aspects on MF59 are shown only in mice. There is a safety pharmacology study in dogs for the only local tolerance. You see very limited supportive datasets but it was accepted because of the character of the vaccine.

So in conclusion, the EMEA guideline on adjuvants is reflecting the state of the art for the moment. We can discuss



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whether we would have more and new data. As the EU is opened to receive new applications, we see the discussions in the scientific advice procedure and we have something a bit more than only alum in our licensing procedures.

Thank you.

(Applause.)

DR. SHEVACH: Any pressing question? Thank you. Oh, there's one.

DR. FRIEDE: Martin Friede, World Health Organization.

So, Jan Willem, I was a little bit surprised to see the statement on MF59 being accepted because of its application in pandemic vaccine. And it was accepted because of that.

Because in Europe, especially in Italy, MF59 was accepted in a national licensing procedure prior to the EMEA. So there is 20, 30 million cases of human administration of MF59. So how does the EMEA



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then position the approval process for MF59 separate from what this historical data we have of a licensed vaccine within Europe?

DR. VAN DER LAAN: Yes. Maybe that's a reflection of the fact that I am Dutch.

(Laughter.)

DR. VAN DER LAAN: And I will explain to you that the product that you refer to is the seasonal vaccine, Fluad, with also MF59. That's indeed on the market in Europe. But not in all countries.

We have in the European Union, and that's not wholly Europe but at least the main part, 27 countries. And as far as I know -- but maybe someone can correct me -- the Fluad is on the market of 15 of the 27. And it is not accepted in all.

So that's why the European position is only because of the guideline on the vaccines and not -- there's no, at the moment, no full acceptance of that vaccine.



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DR. SHEVACH: Okay. Thank you. We'll move on.

Dr. Alving, from Walter Reed, Use and Limitations of Animal Models.

DR. ALVING: Well, thank you very much.

If we accept that the vaccines are initiated, that the principle that initiates the immune response in vaccines is the antigen, then the adjuvant's function to amplify the immune response or to channel the immune response in a particular direction, for example Th1 or Th2 -- and we haven't mentioned about a lot about other cites such as mucosal sites yet -- increase the duration of the immune response, and actually help to overcome tolerance when necessary. For example, for cancer antigens this might be important.

So the question that I'm going to address is can the functions and the safety parameters of adjuvants for humans be predicted either qualitatively or

quantitatively by utilizing animal models.

Now I want to make a caveat here. This is a pretty complicated topic. There are lots of different kinds of adjuvants and I'm going to focus mainly on adjuvants where certainly one of the major mechanisms is thought to be a depot effect, either a depot of the antigen together with the adjuvants or a depot of the adjuvant alone.

So with that caveat in mind, the answer to this question is, in some cases yes, and in some cases not. And I'm going to give two examples in the next three slides that illustrate both of these, the first being with respect to safety. And the second being with respect to efficacy.

So I want to start first with the safety. Now the first -- as we have been mentioning here, one of the most commonly used adjuvants at the present time is Lipid A. Well, Lipid A is the pyrogenic factor that is present at the -- is the anchor site of the



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bacterial level polysaccharide.

And there was a series of wonderful studies done by Sheldon Greisman at the University of Maryland in the 1960s. And what he showed was absolutely astonishing in my view. It's very, very interesting.

If you look here, these are the doses. This is the endotoxin, the lipopolysaccharide given to rabbits. And exactly the same lipopolysaccharide given to humans. And these are increasing doses.

So that at the lowest dose -- and this highest dose up here is a tenth of a nanogram, there is no response in either one of them. But when you go to the higher level, which I believe here is one nanogram to 1.4 nanograms, suddenly in each case here you get an immune -- sorry, a pyrogenic response.

This is an increase in temperature. And the -- actually what he shows in the study is he shows the subjective response that the human actually had at the

same time. And so they were looking at this in some detail. Now this is based on milligrams per kilogram, injecting intravenously into rabbits.

You get a slightly different response if you look at the total LPS that was actually injected into the rabbits. So that in this case, you do get some pyrogenicity when you look at the total, not on the milligram per kilogram basis in this. And so there are some discrepancies. But even in general, it looks like a pretty good representation.

So in this case, this is an excellent example where the adjuvant that we're talking about, and we'll get into some of the pyrogenicity studies of the monophosphoryl Lipid A actually, as I go along here, too, a little further. And this is actually for the total dose.

Now we have -- there are ways to influence this and actually we've done a

number of studies, Phase I trials in humans, where we have actually done pyrogenicity where we put the Lipid A into a carrier, the liposome carrier, and this is where we are looking at the -- this is the total Lipid A injected on a microgram per kilogram basis into the rabbits.

And so this is the free Lipid A right here. And as you can see, it is highly pyrogenic even at this dose here which is 0.022 micrograms per kilogram.

However, when you put the material into liposomes, and this is simply different amounts of Lipid A that were incorporated into the liposomes with an increasing dose, you get a pyrogenic response at a much higher dose.

In fact, when you look at the difference, for example, between what looks to be approximately the initial place where you get a pyrogenic response here, it is a 55-fold difference in the pyrogenicity.

If, in contrast, you go to the

chemical test for lipopolysaccharide endotoxin, the Limulus amebocyte lysate assay, this is a much more sensitive test. And this is a chemical test. This is obviously in an intact animal. This is in a tube.

And in this particular instance, when you put the endotoxin -- increasing amount of endotoxin, the Lipid A into the liposomes, despite the fact that there is endotoxin there, it is not detected as being any higher than the liposome lacking the Lipid A.

So that the Limulus assay doesn't necessarily prove the absence of Lipid A if it is there. So you can actually get liposomes that are Limulus negative and liposomes that are Limulus positive. So there is a hundred-thousand-fold difference there.

So the second example that I want to give you is really quite dramatic. It was the first circumsporozoite protein antigen that was developed by GlaxoSmithKline in



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collaboration under a cooperative research and development agreement with the Walter Reed Army Institute of Research. It was initially tested in mice and rabbits and found to be efficacious but it failed in humans.

So the question that arises then -- - at the final question at the bottom, what are some of the variables that influence the predictability of adjuvants in humans versus animals?

Well, one of the obvious things that could predict this is the differences in sizes between the different animals. This is actually from this paper by Freireich, et. al, in 1966, where they are comparing the body weight of these different species of animals and the surface area.

And this is the -- the km factor is the body weight over the surface area. And if you look at this, the human -- the adult human is 1,000 times heavier -- or 3,000 times heavier than a mouse. However, in contrast,

the surface area of the human is only 242 times greater than the mouse.

Now if you take the idea that you are looking at the internal organs, than maybe these great difference occur in the internal organs. After all, the volume, if you look at the internal peritoneum as a sphere, the gut volume goes up as the cube of the diameter, while the surface area goes up as the square of the diameter.

So the -- now let's look at the monkey. The monkey is -- the humans are 20 times heavier than a monkey and yet the surface area is only 6.7 times heavier than the monkey. So it is clear that this conceivably could be an effect. Here's the body weight over the surface area compared to the mouse versus the monkey compared to the human.

So the monkey is three times more than the human -- well, the mouse -- or the human is three times more than the monkey

compared to more than 12 times higher in the mouse.

While these may be factors, what could be the implications of this? This is a study -- I apologize that this was actually published in a very difficult to obtain thing if anybody would like a reprint.

But it was where we actually did -- and I think you'll see in a moment why we did this -- published it here -- the depot effects of liposomes absorbed to alum -- these are liposomes, I believe, containing monophosphoryl Lipid A. Yes, they were.

And what we did is we put phospholipids that were fluorescent -- there were two different kinds of fluorescent -- rhodamine and fluorescein dye attached to phosphatidylethanolamine.

These liposomes were then absorbed to aluminum hydroxide and the injection site was in the gastrocnemius muscle of the left rear limp of the mouse. Is the gastrocnemius



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mouse 1/3,000th the gastrocnemius muscle of the humans? I mean here you are injecting 50 microliters into a mouse. Are you going to inject 3,000 times that into a human? I don't think so.

But what we found was really quite extraordinary. Over a period of six days, there was apparently no change at all in the amount of material that was present at the injection site. In fact, when we looked at the -- we took a variety of mice, we let the pathologists look at the fluorescence blinded and grade the degree of fluorescence and so forth.

The first thing -- and if you look here at the intramuscular injection, the first was at 24 hours there was a little bit that appeared in the spleen but there was no detectable amount in the spleen at all after that. In contrast, in the lymph nodes it was continuously appearing through the whole time in the lymph nodes.

So the conclusion there is that there could be an idea that there may be -- the differences in the sizes of the animals may have -- there may be adjuvant effects that occur in the entire animal that would be similar that would be coming from a mouse and a human.

But the effect on the whole animal -- that is the rate of release may be similar. But it getting from the site of injection in a mouse, let's say, to the spleen of the mouse might be a lot different than getting to the arm of a human into the spleen of a human in terms of the distances and so forth that the material has to traverse.

Now I just want to now switch topics to another type of adjuvant, incomplete Freund's adjuvant. It is a water-in-oil emulsion in a light paraffinic mineral oil of low viscosity called Drakeoil that is stabilized by an emulsifier consisting of Arlacel A, which is isomannide monooleate.

Since water-in-oil emulsions require relatively low energy input for emulsification, the shearing forces obtained by pushing oil through a small orifice by connecting opposing syringes is usually sufficient. And the antigen is generally included in the water phase of the emulsion.

Incomplete Freund's adjuvant is one of the most potent adjuvants ever devised. The question is why is it not routinely used in humans.

Well, in 1964 to 1965, 900,000 people in U.K. received an influenza vaccine adjuvanted with incomplete Freund's. Forty persons developed local modules. And of these, nine developed a cyst that required local surgical aspiration or incision. The cysts were viewed as a toxic reaction similar to cysts sometimes seen in mice.

A tetanus toxoid vaccine in New Guinea and cholera vaccine in the Philippines containing IFA reportedly had higher levels of

local reactions. IFA induces tumors. This was the killer actually in the United States. It induces tumors in male Swiss mice. However it is very infrequent in female Swiss mice. And it does not induce tumors in Balb/c or C57 black mice. This was the death knoll for incomplete Freund's adjuvant.

IFA is known as a potent agent for induction of autoimmune arthritis in mice, a condition also known as adjuvant arthritis. It's all sounding bad. It is feared that IFA may cause cysts, cancer, or autoimmune arthritis, or other diseases in humans.

How does all this stand up to scrutiny? Well, the first thing that was done was to actually take a mouse and inject the mouse with incomplete Freund's adjuvant. Then let it go. I believe it went for 270 days actually.

And then the same incomplete Freund's was put in a bottle and put on the shelf in the laboratory. And then the mouse

was sacrificed at the end. Here is the dorsum of the mouse. And as you can see, you get what appears to be a separation here that looks very similar to the separation that you see in the bottle. And this is just a walled off cyst that occurs here.

And so this is actually something that -- the separation that you see where you get clear oil that occurred in the cyst, these were not infectious cysts. They were chemical cysts that occurred. It could simply be drained off it need to be. But anyway, it was problem.

Jonas Salk looked at the long-term safety of incomplete Freund's adjuvant. He was one of the greatest advocates for incomplete Freund's, particularly for the initial polio vaccine. He was forced to not use the polio vaccine with incomplete Freund's adjuvant and as a result of that, instead of having a single injection polio vaccine, he had to go with multiple injections to get the

same immune response.

So in the initial study, Salk, in collaboration with the Army, immunized 18,000 recruits with influenza vaccine emulsified in IFA at Fort Dix. Cyst-like reactions observed in some but were eliminated by purifying the Arlacel A.

Subsequent to this, there were no cysts that were observed. It appeared to be a degradation product of the Arlacel A that was responsible for these cysts.

Because these were military individuals, they had a long-term follow up in the military medical system, a nine to ten year follow up of the Salk cohort.

Cyst-like reactions required hospitalization treatment in 0.1 to 0.6 percent and outpatient treatment in 1.2 to 4.1 percent. Otherwise, no significant unexplained problems, no effect of vaccine on the incidence of mortality.

There was then a 16- to 18-year

follow up. There were no adverse correlations with diseases or death.

And then finally, this is another difficult one -- I have an original copy of this journal, Vaccine Research. It is now out of publication. It doesn't exist anymore.

But this is a wonderful study that was done by Abe Benenson actually in which he did a greater than 35 year follow up on these same individuals. There were no adverse correlations with 74 different diagnostic categories, including arthritis and autoimmune disease.

Decreased mortality was observed in five disease categories, significantly reduced mortality. A p of 0.01 was observed with respect to cancers of the digestive tract.

So that it appears that in the mouse, for example, the adjuvant arthritis and the cancer and so forth may, in fact, be diseases of mice. This is the thing that you

really have to worry about the possibility that there may be diseases of mice that will not occur in humans. But in any case, this is an example of a long-term study that was done.

Now I want to switch to another adjuvant and this is the MF59. This is with the Herpes simplex vaccine in which gD2 and gB2 were used in combination with MF59 as we've hear earlier.

This was one in which there was published a series of studies in which the adjuvant activity of MF59 was compared with the adjuvant activity of alum. And so the ratio of MF59 to alum are the numbers that are shown on this table with all of these different kinds of antigens here.

And as you can see, it goes all over the place -- five, 122, two, 42, and so forth. But if you look at the Herpes simplex material, the guinea pig, which was the primary model for looking at the Herpes simplex efficacy in the animal models, it

looked as if it had the best activity in the guinea pig right here.

But actually they should have looked at the baboon up here, which is a sevenfold lower activity because when they actually went to the human trial in the Phase III trial, there was no significant -- actually, it was very interesting. This appears to be a female vaccine. And there was a tendency towards efficacy in the females but it was not significant and there was no efficacy in the males at all.

In contrast, when what is now known as the AS04, the MPL absorbed to alum was utilized, then there was distinct efficacy in the females. So it did appear that the animal studies had not given exactly the type of thing.

Really I want you to just remember all of those differences in the adjuvant compared to the alum. And we'll get into that in this study right here. This is a study

with a malaria antigen, MSA-2. And this is where actually five different adjuvants were tested: Freund's complete/incomplete, Alhydrogel, SAF-1, which is an oil-in-water emulsion with this thionyl muramyl dipeptide in there, Montanide ISA 720, which is a water-in-oil material that looks pretty much -- that uses mannide monooleate -- it is pretty much similar to incomplete Freund's adjuvant, and liposomes containing Lipid A that we supplied.

And if you look at the -- this is the same thing. This is the adjuvant activity in sheep, rabbits, and mice of these five different types of things. These are two different experiments right here.

And you can see there are huge differences. For example, this 33 versus 155, 60 versus 466. If you look down here in the liposomes, it is a tenfold difference in the rabbit and the mouse.

It didn't seem to be that there was any rational way to figure out exactly

what was happening in these different animal species.

And actually what Glaxo did, again in collaboration with the Walter Reed Army Institute of Research and the Naval Medical Research Institute, now known as the Center, and Glaxo through a cooperative research and development agreement, there were studies done in Phase I studies with three different types of adjuvants in humans.

Now the first adjuvant is the AS04 that worked so well in the Herpes simplex vaccine. And that actually gave a slightly less response right here.

The AS03, which is simply the emulsion containing RTS,S in an oil-and-water emulsion looked pretty good. Actually it looked very good. These were very good results in terms of antibody levels.

And then finally the AS02, which was also shown there.

These individuals were then

challenged with malaria to determine the protection. And all three of these gave some protection. With the AS04, there was one individual protected. In the AS02 -- what is it -- the AS03, there were three protected. But in the -- so it is six out of seven were protected with the AS02.

So it looked as if the human results were giving the correct answer and this actually was a comparative adjuvant trial in humans.

So the summary of this is is that WRAIR and GSK initiated a rigorous comparative preclinical safety and immunogenicity evaluation of six GSK proprietary formulations of RTS,S in rhesus monkeys.

And during preclinical studies in mice, a synergistic effect was observed between QS21 and MPL. Nathalie Garþon has talked about these studies actually earlier today. And the combination of oil-in-water emulsion, MPL, and QS21 was selected based on

the results from the monkey studies.

And she actually showed this same slide. The purple numbers right here, this is the dramatic number right here which showed the effect that the AS02 gave that turned out to be the most protective one of all.

What I would emphasize is that -- oh, here is another example where we used liposomes containing Lipid A together, again, in collaboration with GSK at Walter Reed. And this is in monkeys where we are increasing the -- each one is an individual monkey, increasing the amount of Lipid A MPL that was in the liposomes and we got quite dramatic immune results here.

Based on that, we went to a human study of this last formulation that I just showed you. And this is where we actually -- each bar represents a single individual human. And this is the antibody levels. This is micrograms of IgG per milliliter that were observed -- very high levels here but it was

at a very high level of MPL that was done there. This is a dose ranging study.

And this is compared, for example, with the original work that was done with alum where the very poor results that were obtained. And that was the subject of the Washington Post article that I showed you at the beginning.

Actually it is my belief that an adjuvant researcher should actually receive the treatment that he actually gives to his children and to other people. And this has actually been a tradition in the Army.

Rip Ballou, over there, he received the same thing. I believe you got challenged and you got malaria though, did you, Rip. Yes, sorry.

(Laughter.)

DR. ALVING: But anyway, this is a very interesting thing here is the side effects. Now we had had monophosphoryl Lipid A here. We did the original pyrogenicity



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study on this in rabbits. And it was negative. It was nonpyrogenic in the rabbits. But it was positive in the Limulus studies assay. It was strongly positive in the Limulus assay. But negative in the rabbits.

And what we did is the vaccine was essentially nonpyrogenic and nontoxic even though at the highest dose, which is shown right here, which is Group 5, the volunteers received 2.2 milligrams of monophosphoryl Lipid A.

This actually was about 12 times higher than the previously established maximal safe dose of MPL when given intravenously. I'd just like to point out that most vaccines are not given intravenously. And this was, therefore, felt to be safe and potent. Despite this, my wife has expressed some reservations about how safe it was in view of my subsequent characteristics after receiving this vaccine.

(Laughter.)

DR. ALVING: So actually what I want to emphasize is that the animal studies may not give you the proper results. And you may have to resort to doing Phase I trials.

This is perhaps a little more expensive but what you should be looking at when you do sequential Phase I trials with different adjuvants is you should be looking for knockout results. Nobody wants to go around doing statistics actually. You want to do eyeball statistics.

And if you can't see it, it's just not worth looking at. And so you can see through the years, it looked as if it there was -- this is actually the last trial that was shown here -- this is a paper by Dan Gordon who was, actually, the first person ever protected by a synthetic malaria vaccine as part of this program.

However, this is not foolproof because -- okay, so based on this, the approximately theoretical minimum protective



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value -- some people may quibble with this -- but it is somewhere in this range around here where some people would get protected against the malaria.

But this is not a foolproof thing because it was subsequently shown by Steve Hoffman in a subsequent study using the Detox formulation that when he repeated this with a second Phase I trial that the results were not quite as dramatic.

So the conclusions from what I've discussed here is that the rabbit pyrogenicity assay is a reasonable predictor of endotoxin pyrogenicity in humans.

The Limulus amebocyte lysate assay may be less useful in this respect simply because it is stunningly sensitive. Even the liposomes in the example I showed you, even the liposomes lacking Lipid A gave some reactivity.

Pyrogenicity, in turn, is also a reasonable predictor of many types of

mediators such as IL-1, IL-6, TNF, et cetera, induced by endotoxin. Certain toxic effects induced by adjuvants in mice, such as adjuvant arthritis, autoimmune disease, and adjuvant-induced cancer have not necessarily been observed with the same adjuvants in humans.

The weights and surface areas of different animals might influence the predictability of safety and efficacy of certain types of adjuvants in humans. Perhaps because of this, nonhuman primates may be better than rodents, including mice, guinea pigs, and rabbits for predicting efficacies of adjuvants that rely mainly on depot effects.

Animal models are useful for investigating mechanisms of certain types of adjuvants but do not always predict safety or efficacy in humans. Comparisons for down selection for human vaccines of different types of adjuvants, especially those that rely mainly on depot mechanisms, are probably best evaluated in nonhuman primates or, better yet,

in sequential Phase I trials in humans.

And finally, the effects of adjuvants or adjuvant combinations that rely on different mechanisms such as depot effects, recruitment of antigen-presenting cells, TLRs, secretion of cytokines, or combinations of these cannot necessarily be reliably predicted by any given animal model when looked at prior to actually doing the experiments.

Thank you.

(Applause.)

DR. SHEVACH: Thank you.

We have time for one very quick question.

DR. GARÞON: Yes, I have a question on what you were saying about MF59 and the guinea pig model for Herpes and the no correlation of what was seen in guinea pigs versus efficacy in the clinical trial.

DR. ALVING: Yes.

DR. GARÞON: What is the question? The animal model or the readout that was used

as a correlate?

DR. ALVING: The readout, are you meaning the ratio over the alum?

DR. GARþON: Yes.

DR. ALVING: Well, this is -- the ratio of the adjuvant effect over the alum has been widely used. But I was just pointing that out. It may be the readout. You are right. It could be the readout. There could be other ways of looking at that.

DR. GARþON: But there was an antibody response, right?

DR. ALVING: There was what?

DR. GARþON: There was the antibody response you used as a ratio?

DR. ALVING: That was the antigen response, exactly. Yes.

All right. Thank you.

DR. GRUBER: Okay. It is a pleasure to announce the next speaker before the coffee break. And this is Dr. Ethan Shevach, my co-chair from the NIAID, who will

speak about long-term effects of adjuvants, consequences on the adaptive immune response. Thank you.

DR. SHEVACH: Well, thanks for having me. Thank the organizers for inviting me.

I have to also mention I'm sort of the odd man out here in that I'm the only one really interested in suppressing the immune response when all the rest of you are interested in enhancing the immune response. But perhaps something can be learned from someone who is interested in immune suppression rather than immune activation.

My only tie to adjuvants is a historical one. I've been a member of the Laboratory of Immunology of NIAID for 40 years. The Laboratory of Immunolgy at NIAID was founded by Jules Freund some 51 years ago for those of you that remember.

I'm going to talk about one of the key cells in the immune response that plays a



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role in absolutely everything. It hasn't really been mentioned here. It is the regulatory T cell.

This is the way I view the immune response. It is always imbalanced with our attempts to activate the immune response. This is why autoimmune disease or allergy or immunopathology or graft transplantation -- I don't have immunization with vaccines up here but it is in this column. And everything is balanced by regulatory T cells.

And it is nice when there is an even keel. But, of course, any time you are going to immunize an animal or man, one has to overcome the effects of these regulatory T cells.

Too much regulatory T cell action can lead to chronic infection. The overabundance of regulatory T cells is a feature of cancer.

What are regulatory T cells? So someone showed a slide this morning with a

number of papers referring to TLR receptors. I think the number of papers over the last ten years referring to regulatory T cells -- maybe I'm exaggerating a little bit -- is probably a log higher from 1997 to the present time.

Almost every single T cell subset has been endowed with regulatory function. For the sake of time, I'm not going to go through all these subsets. I don't think all these other ones are terribly important but one subset is definitely important.

And these are the so-called thymic derived Foxp3 positive regulatory T cells. Some probably come from the thymus. Some may be generated in the periphery. And we will address this concept.

This is a new idea. We used to think that all the regulatory T cells that expressed Foxp3 arose in the thymus and came out in the periphery. More recent studies have suggested that regulatory T cells can also be generated in the periphery, in the

gut, in lymphoid tissues, perhaps in response to certain kinds of immunizations.

Overall, in both mouse and man, and everything is the same in this field, about ten percent -- eight to 12 percent of CD4 positive T cells express the transcription factor Foxp3 -- we'll come back to that in a minute -- which appears to be the real lineage marker of this subset of cells.

We used to call these CD4 positive, CD25 positive T cells. That's how they were originally discovered by Shimon Sakaguchi. That's what we called them for many years.

But the real marker -- not necessarily the most perfect marker -- is the expression of Foxp3. And this population of cells controls the immune response to everything.

If there are any nonbelievers in the audience that these cells are important, I'm going to show you one simple experiment,



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a really adaptive experiment that Fiona Powrie and Bob Coffman did a long time ago. If one takes the CD4 positive T cells that don't express Foxp3 and inject them into a RAG knockout mouse, almost 100 percent of the mice develop severe inflammatory bowel disease as can be seen from this section of the colon.

If one injects regulatory T cells along with the Foxp3 negative cells into this immunodeficient mouse, the regulatory T cells completely protect against IBD. So regulatory T cells are real. They are a definite population. You can't argue about them. They exist.

So most of these cells were said to come from the thymus until about five years ago when a number of groups published that one could induce Foxp3 expression by merely culturing conventional CD4 positive T cells in the presence of TGF beta and and a TCR stimulus.

And indeed this works every time.

If we take a population of cells that expressed zero to no Foxp3 positive cells, culture them in the presence of TGF beta, a TCR stimulus, and a high concentration of Interleukin 2 -- and Interleukin 2 is absolutely required in this -- we can generate a population where you can see now 90 percent of the cells express Foxp3.

And these cells have all the properties, including the in vivo-suppressive effects of the thymic-derived regulatory T cells. TGF beta is an absolute here.

The three percent you see here is real. If we put in anti-TGF beta to the culture, we knock it down to zero. And there is a little bit of TGF beta course presence in the fetal calf serum we use for culture. So this is in vitro.

Does it occur in vivo? Can Foxp3 negative cells be converted to Foxp3 positive? And there are a couple of different ways of showing this. I'm going to show you a drastic



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example but it is the proof of the concept.

Several laboratories have generated Foxp3 as a transcription factor so it can't be used as a cell surface marker obviously for regulatory T cells. But GFP, the fluorescent dye has been knocked into the Foxp3 locus. So now that all Foxp3 positive cells are green, which is very convenient -- and you can see about ten percent of these CD4 cells are green.

We're not interested in those cells. We're interested in the cells that don't express Foxp3. So we sort the GFP negative cells. Again, we inject them into a RAG knockout mouse that has not T cells or B cells. And we just leave the mouse on the shelf for two to eight weeks.

And you can see gradually the development of Foxp3 positive cells both green and stained with antibody as well over a period of eight weeks. So by eight weeks, roughly 25 percent of the CD4 cells in this

particular mouse express Foxp3.

We can sort these cells, get them out of the mouse, and show they have most of the suppressive properties of the thymic-derived cells. So this occurs in the periphery. And this is a rather important idea.

And it raises the issue of what is stimulating these cells. Is it TGF beta in vivo? That's not really been proven.

Obviously if they can occur and develop spontaneously in this mouse, presumably they can develop normally in your peripheral tissues.

And I bring this up because we have to ask the question where do T regulatory cells come from in the adult man? Thymic output stops at puberty. Perhaps all the regulatory T cells hang around, divide very slowly.

Alternatively, regulatory T cells are constantly being generated under

conditions that we don't understand in our peripheral lymphoid tissues. And that in the 80 year old, which has exactly the same number of Foxp3 positive cell as a 20 year old, most of these cells may be derived in the periphery.

And that brings us to a sort of scary question. It used to be thought that the TCR repertoire of the Foxp3 positive cells, most of those generated in the thymus, is biased toward the recognition of self antigens. That's proven by sort of esoteric molecular approaches that I'm not going to show you.

Other studies suggested that pathogen-derived antigens could also be recognized by T regulatory cells. So there was nothing special about the repertoire really of T regulatory cells. Perhaps a self bias but a very diverse repertoire that could recognize anything.

And then you people have to ask

the question could a vaccine adjuvant preferentially expand a population of antigen-specific regulatory cells resulting in vaccine failure? That's sort of a caveat to understanding how vaccines can work.

And a few of the notions I heard this morning were sort of low dose, very little danger. That is really the ideal kind of vaccine that you would want in certain respects. Those are the kinds of conditions that people propose would induce regulatory T cells rather than boost up an immune response.

Of course my colleagues in the autoimmunity field would be rushing to patent this vaccine if it was specific for an auto antigen. So it really depends on how you look at everything what conclusions you draw.

They would love to have a vaccine to a tissue-specific antigen, to an auto antigen that would boost up a population of regulatory T cells and would function as a vaccine for autoimmunity.

So this is my sort of bad Chinese restaurant menu slide where you have one from Column A and one from Column B. How do regulatory T cells work? I've been in this field for about 13 years.

And first of all, it used to be considered that the CD4 positive regulatory T cells would act on CD4 responders and CD8 responders. That was a nice concept. It's still possible.

But over the years, papers -- reasonably good papers I have to say -- have appeared that regulatory T cells can act directly, I have to say, on every other possible cell type in the immune response. Most recently mast cells, if you look at the current issue of Immunity and also a paper published that I am a co-author on in the Journal of Immunology over the past year.

So regulatory -- how can they do it? How can one cell type suppress all these other cell types? Well, that's where you get

to Column B.

Column B are the proposed mechanisms by which these regulatory T cells function, some sort of easy to understand. They could secrete suppressive cytokines, IL-10, or TGF beta. The latest fad cytokine they might secrete is IL-35 -- really the publications of only a couple of laboratories.

It said they can do all kinds of other things. They express the antigen CLTA-4 on their cell surface. Perhaps that interacts with dendritic cells, with CD80/86 on dendritic cells, and induces the production of IDO or by some other means inactivates dendritic cell function.

I won't go through all of these. None of these have been -- these are largely the products of single laboratories or one or two laboratories, certain membrane molecules may be involved in their suppressive function.

My own view is we really don't know how they work. I wouldn't say they use

one mechanism. Perhaps they pick a mechanism depending on the nature of the environment they are in.

If they have to suppress inflammatory bowel disease where there is lots of inflammation, they probably have to make IL-10 most of the time. But not all the time. Is that all they do? We don't know.

So I think these are important research questions for the future. How exactly do regulatory cells work? And if you don't know how they work, it is hard to understand how you could manipulate their functions, which is really the goal.

If you want to stop regulatory T cell functions or inhibit it, how do you do it if you don't exactly know their mechanism of action or even their target cell?

So adjuvants, I was forced to think about adjuvants, having been invited to give this talk, and there are several papers suggesting that adjuvants, primarily TLR

ligands, might function by acting directly on regulatory T cells.

And regulatory T cells in both mouse and man probably express most of the TLRs, although that hasn't been done I'd say with the greatest care, but there is enough data to suggest that regulatory T cells, along with every other cell type including CD4 effector cells, express TLRs.

So adjuvants, and I'm going to use TLRs as my model here, might conceivably act directly on regulatory T cells to do something to them or, alternatively, act on another cell type, the antigen-presenting cells -- and this makes more sense -- that adjuvants, say CpG acting on an antigen-presenting cell would induce the antigen-presenting cell to make certain cytokines -- the examples I'll give are IL-6 and TNF alpha -- that would then act on effector T cells and somehow render the effector T cells resistant to the suppressive effects of the regulatory T cells. And that

is another way adjuvants can influence regulatory T cell function. So direct and indirect effects can be manifest.

I hate to show a list of papers that have been published in a field but this is a perfect example that really reflects our ignorance. So don't look at any of these things except the enhances, reverses, enhances, reverses, reverses, enhances, reverses, okay.

(Laughter.)

DR. SHEVACH: I'm not convinced by any of these papers, frankly.

The first paper claimed that LPS, for example, would enhance mouse T regulatory cell survival and suppressor function. That's definitely true. I tried extremely hard in my own laboratory to repeat that. It's not going to get published, of course, because I couldn't repeat it. It doesn't work.

So this is a mouse Treg study. Toll-2 Pam3CS K reverses mouse Treg function.



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Somebody in my laboratory has been trying to repeat those two papers from two different groups. We can't repeat one piece of data in them.

So a word of caution about believing anything on this slide. And the only thing I'll mention is the famous Pasare and Medzhitov paper that everybody loves to quote.

But this basically was what was shown on the previous slide -- that CpGs stimulated APCs to make IL-6 and probably something else that functions as growth factors for T effector cells.

And under those conditions, the T regulatory cells fail to manifest their suppressive effects. The regulatory cells were driven to expand by IL-6 and something else that was never defined in that original paper. So that is an indirect effect. And that's quite understandable.

But direct effects of TLR agonists

on regulatory cell function, none of these papers are very convincing. But it is something, of course, to worry about because you are all about to administer TLR agonists as adjuvants.

I'm going to finish up and talk about what happens when you don't have regulatory T cells because I was given the task of sort of defining the real dirty secret of adjuvants, which is could they cause autoimmunity, which is what some of you are worried about.

So if you lack regulatory T cells, humans that lack regulatory T cells develop something called the IPEX syndrome, which stands for immune dysregulation, polyendocrinopathy, enteropathy, X-linked.

And they developed essentially every single type of autoimmune disease. Many of the boys born with this disease develop diabetes in utero. It is the most severe autoimmune disease known to mankind.

They also develop IBD, they have tremendous lymphadenopathy, hepatosplenomegaly, most of them die at age two to three unless they get a bone marrow transplant. But even bone marrow transplantation is very, very hard to manage in this particular disease.

Fortunately very rare and I also should point out that the mothers of these children are completely normal even though they have had half of their X chromosomes inactivated, those cells that have the normal Foxp3 allele function normally in a trans-fashion and suppress their potentially auto-reactive cells.

And mouse and man are exactly the same in this field. There is something called a scurfy mouse. The scurfy mouse has a mutation in Foxp3 so it is a two base pair insertion in Foxp3 resulting in deletion of a big domain of the transcription factor.

And they develop a disease that

looks just like the human disease, exactly like the human disease lymphadenopathy, hepatosplenomegaly. They have an exfoliative dermatitis that I'll show you. And they also die.

And unfortunately if you are a male mouse, a male scurfy mouse that has no regulatory T cells, you die at about three weeks of age of flagrant, exuberant inflammation and autoimmunity.

So this is just to show you that regulatory T cells can really function. And in this particular experiment, we used regulatory T cells from a normal black 6 mouse that we induced in culture as I showed you on that early slide.

We took non-T regulatory cells and induced them to express Foxp3 and this is one of their in vivo functions. They can actually rescue the scurfy mouse. So this is a wild-type mouse. This is a scurfy mouse. And it is pretty evident when you give the scurfy

mouse these induced T regulatory cells on Day 1 of life and this is looking at about Day 30 of life, you've rescued this mouse from this runting syndrome.

And it is not just injecting any old population of T cells into this mouse. It's not just putting lymphocytes in because if you put into the scurfy mouse lymphocytes that have been expanded up in the presence of anti-TGF beta so there are no Foxp3 positive cells in your prep, the mouse is still runted.

And this is what a scurfy mouse tail looks like. There is a unique infiltrate into the skin of the tail. This is ear skin. It's what we usually look at the histopathology.

They have lymphocytic infiltrates everywhere: lung, liver, heart, pancreas. I'm just showing you a little small example of this. If we put these induced T regulatory cells in, the tail is completely normal, the ear is normal, and the lung, liver, or what

have you, are completely normal for as long as we keep the experiment going which, in this case, is about 30 days. But this is only one single shot of these induced T regulatory cells.

Why am I showing you this particular model? Why is it interesting? Because this is the ultimate test of an animal that has the propensity to develop autoimmunity. This animal develops autoimmunity to every single organ that it has.

So it is sort of like a person who has the ultimate -- you know you are all worried that a vaccine adjuvant might induce autoimmunity in someone of the right genetic background that has a propensity to develop say Type 1 diabetes. These animals besides having a cellular infiltrate also have high titers of autoantibody. In this case it is autoantibody to skin.

So I'm going to show you what -- I



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was sort of given the challenge to discuss -- and I'm just going to do it on this slide rather briefly -- how would you test whether and adjuvant could induce autoimmunity? And I gather that is a major issue in this field.

And I began to think about that problem and it wasn't so easy for me to come up with something that would really address the issue and give an answer.

And I'm a mouse organ-specific immunologist, so to speak. I'm interested in all kinds of organ-specific autoimmune diseases in the mouse. But, for the most part, their induction requires the use of Freund's adjuvant.

And there are very, very few spontaneous models of organ-specific autoimmune disease. But probably the best example is the NOD mouse which develops a disease like diabetes. And perhaps you could test functions of the -- the ability of a -- the potential ability of an adjuvant to induce

autoimmunity or autoimmune diabetes but that's only one disease. If that's not what the adjuvant really works on, you wouldn't see anything in other diseases.

Most of the mouse models are systemic autoimmune diseases. The NZB mouse, the MRL mouse, the GLD mouse are very slow to develop, mostly due to -- they obviously have helper T cell dysfunctions but most organ damage is primarily due to immune complexes.

And most of them have been characterized in molecular defects, for example, in the Fas or Fas ligand deficient, is characterized and that really isn't a characteristic of any human autoimmune disease for the most part. There are patients that do have mutations in Fas and Fas ligand.

The scurfy mouse, I think, is sort of the most interesting one. It develops immune pathology everywhere so it has the potential to develop autoimmune disease in every single target organ.

It looks just like the IPEX disease in man. It is the same disease. So I began to think about how I would test an adjuvant.

And I haven't done this experiment. I'm only showing you half the experiment which is sort of the control that I have done. And I'm not showing you how I would test an adjuvant but I will predict one result.

So you can't do this experiment in a scurfy mouse. The scurfy mouse is going to develop unbelievable autoimmune disease unless it gets a transfusion of regulatory cells. But what we've done is to take cells from a Day 13 -- ten to 13 day old- or even a week old-scurfy mouse.

So these are cells that have already been activated, in part, have the potential to recognize autoantigens because they have never been exposed to regulatory cells. And we transmit them to a RAG knockout

mouse.

And what we see is a recapitulation eventually of the disease in scurfy mouse. And I'm going to show you that data. And we do these experiments -- we can do these experiments in seven days. We can take these scurfy cells, transmit them to the RAG knockout and begin to see manifestations of autoimmunity by seven days of life.

Let me show you the experiment and let me show you what I would have done if I had worked for TeGenero. Thank God I didn't.

So this is taking scurfy cells and injecting them into a RAG knockout mouse and we do this either with purified CD4 cells or actually Eva Heuter, who did these experiments, did this with total scurfy cells. She just took everything from the spleen of a scurfy mouse and injected it into a RAG knockout mouse.

And this is histology scores at about 14 days after transfer. Skin, lung,

liver, you begin to see infiltrates. You begin to see lymphadenopathy. You begin to see increases in T cell numbers in every organ you look at.

And this is the control study where we -- this is why she did the experiment. She wanted to show that Foxp3 positive regulatory cells, in this case ones we induced in vitro, would prevent this disease in a co-transfer model -- something that we could control.

We can control the number and type of cells we inject. We can control the number of regulatory cells that we inject. And, indeed, when she injected these induced T regulatory cells, if you look at the middle bars, the triangles, she really completely suppressed the development of autoimmunity.

The other -- if we inject non-T regulatory cells, they seem to potentiate the disease.

So here is a model where we have

some effector cells that have been injected into the mouse. And the mouse will -- the recipient mouse will eventually develop organ-specific autoimmunity everywhere.

So one might ask the question what would happen if at this time of cell transfer of the effector population alone, one administered an adjuvant? Would it enhance the ability or increase the frequency and the intensity of the autoimmune response? Could you use this kind of model -- and I'm only showing this as a kind of model that I thought about.

And I can tell you one experiment which hasn't been done. But I can predict the result. So if one took the agonistic anti-CD28 antibody that was used by the TeGenero Company in humans -- and I think they actually had the mouse-equivalent antibody that had the same affinity for mouse CD28 as the human antibody had for human CD28, if one injected that antibody at the same time one transferred

these cells, this mouse would have been dead seven days after transfer of the effector cells because it would have really activated these effector cells and brought this score up to six and death rather rapidly.

So this is a kind of model where one could test. And that would be an experiment.

Even in the presence of Foxp3 positive cells where regulation is taking place, one could ask the question would an adjuvant be so strong that it would overcome, either by acting on the regulatory cells or by hyperactivating the effector cells, overcome the suppressive effects of the regulatory cells. And one would begin to see autoimmune disease under those conditions.

And it doesn't matter because conceivably you could only see autoimmune in the pancreas in this kind of model and you'd be protected against everything else. But that would still tell you that the adjuvant or

the agent you were using would have an effect in enhancing the immune response to autoantigens.

So let me just finish up very briefly. Multiple types of regulatory cells, but the most important one, the Foxp3 positive ones -- and these could be generated both in the thymus and in the periphery. In the periphery conceivably they could be generated in response to antigens, to exogenously administered antigens including vaccines.

The cancer people are very worried about this concept because they think that the regulatory cells really have a predisposition to see autoantigens. But I think this holds for every kind of antigen.

Unfortunately, what we'd really like would be an animal that is normal that doesn't have regulatory T cells. That would be really nice to have. You could immunize it. You could see what role regulatory T cells played in response to all kinds of

antigens.

It is impossible to make such an animal. If one completely depletes regulatory T cells from mice, they rapidly develop autoimmunity and are dead within 18 days. They look like a scurfy mouse. And this has been done using a Foxp3 controlling the diphtheria toxin receptor and giving the animal the diphtheria toxin to deplete the Foxp3 positive cells. So you can't have that kind of situation.

It would be really nice to know how these cells worked because if you really knew the molecular mechanism they worked, one might use drugs or antibodies to transiently inactivate them and then give the animal a vaccine.

There have been attempts to do this with a drug. It is a combination of IL2 and diphtheria toxin marketed by Ligand Pharmaceuticals. I haven't been impressed that it really depletes regulatory T cells

very effectively. And, of course, would only deplete those that express IL2 receptors.

Lastly, T regulatory cells do express TLRs. Perhaps TLR ligands can influence regulatory T cell function in certain ways. I'm unimpressed by what has been published in the literature as being real.

And one thing I think all of you have to consider, to conclude, is that any kind of test of an adjuvant effect really has to include some form of evaluation of the role of the adjuvant on regulatory T cell function, be that a direct effect of the adjuvant or an indirect effect of the adjuvant on another cell type with something happening to regulatory T cells.

Thank you.

(Applause.)

DR. GRUBER: Yes, thank you very much for this very interesting presentation.

I think we have time for one --

for two questions.

DR. SHEVACH: He, I know. You can ask the question.

DR. MALONE: Okay, as an empirical vaccine developer, it seems to me that inbred mouse strains with their own unique immunogenetics are going to be relatively poorly predictive of outbred human responses.

And it seems to me that the only way that we are really going to be able to assess this is if we get a little more sophisticated in how we assess our safety signals in our human clinical trials rather than having a preset, prescreened tox assay that we have to employ in order to get into the clinic.

DR. SHEVACH: Ideally you are right. But let's say you had an agent that really made that mouse I put a few effector cells into much worse, would you use it?

DR. MALONE: But is that going to be predictive of outbred humans? I think

we're going to have a small -- I think we're going to have a signal. I think there is a reasonable chance that we're going to have a signal in, you know, is it one in 10,000?

DR. SHEVACH: Well, I gave a mouse that could develop anything. That's why I picked it. Not, you know -- we can talk about it.

DR. MALONE: I'm just concerned about at this stage of development of Treg biology employing a tox screen threshold in our assessment of a vaccine or adjuvant candidate prior to entry into the clinic. I mean --

DR. SHEVACH: No, you are right. My talk is only -- it was only meant to make you think. I succeeded.

DR. MALONE: And I love Treg biology. Thank you.

DR. GRUBER: So one more question.

DR. SHEVACH: Bob, you can ask me later.

DR. SAHNER: Hi, David Sahner, NES Medical Consulting.

This actually plays off of the prior question quite nicely I think. Basically what you would propose with this experiment would be evaluating for exacerbation of an underlying autoimmune model.

Key to interpreting the data would be some sort of sense of what the threshold for significance might be potentially. And we already know from humans the mothers of the IPEX neonates that they can tolerate some relative deficient of Foxp3 cells compared with the rest of the population.

So knowing that having merely a subset of or a smaller quantity of what is typically found in the circulation of Foxp3 positive cells in these mothers, knowing that this smaller quantity of cells is up to the task of preventing these horrific autoimmune complications in humans, I think it becomes

very important to be sure in an animal model, if one is put forth, that we have a clear sense of the threshold.

And, of course, I have to agree with the first questioner. I think it is going to come from clinical data -- that the insights will come --

DR. SHEVACH: Well, let me give you one example that is different from the mothers. In one of the papers using the Toll-2 agonist Pam3CS, Eddie Lu's laboratory claimed -- and I can't reproduce this myself I must say -- that it decreased the level globally of Foxp3 positive -- of Foxp3 in all regulatory T cells by about 50 percent.

And there is a paper from Blovell's laboratory where he had a mutation that they discovered really by accident where the regulatory T cells expressed, on a per cell basis, all of them were 50 percent of the normal level. And that mouse developed a Th2-mediated autoimmune disease.



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So a little hard to predict but good question.

DR. GRUBER: Okay. I think we'll take an extreme quick coffee break. And we will reconvene at 4:20.

(Whereupon, the foregoing matter went off the record at 4:14 p.m. and went back on the record at 4:28 p.m.)

DR. GRUBER: So I'm very pleased that I can announce the next two speakers for this session that will give us an industry perspective on the design and challenges of conducting preclinical toxicological studies to support safety of adjuvanted vaccines to allow proceeding to clinical studies in humans.

And the first speaker is Dr. Sarah Gould. She's the head of Nonclinical Safety at Sanofi Pasteur.

DR. GOULD: Thank you, Marion. And thank you for inviting me today.

So we've seen some very interesting talks. And I'm a toxicologist and have been for a number of years.

And I'm not sure how many toxicologists are here in the meeting. Now there's one hand that's gone up. So there are some. Not many. So maybe as a toxicologist we have a slightly different perspective.

A lot of it is about risk assessment. The models that we use may not be 100 percent predictive. Can you hear me now? Sorry about that. But they are models and the aim is to be looking for signals and to be supporting to move forward into the clinic.

So the outline of my presentation today, I'm going to -- some of my presentation has already been picked up on already -- I'm going to look a little bit at the background and some considerations.

I'm going to give two case histories in brief. And then I'm going to leave you with a what-about because I'm going



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to stimulate, I hope, some thoughts. I'm going to ask some questions. And I don't necessarily have all the answers. And that's, hopefully, one of the reasons for this meeting here is, and particularly tomorrow, that we can hopefully sit down and go through some of those questions.

So as you've already seen clearly presented this morning, there are mainly two guidelines when we are looking at the safety evaluation of adjuvants. There is the EMEA guideline, which was specifically written to give advice on going into humans with adjuvants. And there is the WHO guideline, which does discuss moving forward with adjuvants.

In 2007, there was a DIA meeting in which EMEA, FDA, and industry got together. And there were many questions raised. There was a clear message given by the FDA and the EMEA. It's case by case. And I think that is correct.



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But, of course, that does bring its problems. And I think there is a clear industrial need to consider this further -- this subject further. And to evolve.

So these are some of our tools as a toxicologist that we sort of use. So there's the general toxicity studies. This is assessing systemic toxicity. We also include local tolerance in this. We try to include it within the same studies. We also have to consider the ethical use of animals.

There's reproductive and developmental toxicity studies. There's safety pharmacology studies, genetic toxicology, juvenile studies. There's also other specific toxicity studies that we may conduct, immunotoxicity, investigational studies, mechanistic toxicity studies. I haven't mentioned them all.

And there's also -- I can mention the paragenecity studies and hypersensitivity and anaphylaxis studies, which I'm going to

come back to in our what-about.

So when we're considering a toxicology assessment, the first question is what are we testing? What is the adjuvant? Now there can be a lots of different types of adjuvants which you have seen this morning in the presentations, giving you some ideas of the types of adjuvants out there.

So how do we define it? Do we define it as a new chemical entity? As an excipient? As a biological? So you really need to try and know what you are testing.

Then we need to consider are we assessing this adjuvant alone? And there's this master file concept. And we also must assess the adjuvant with the antigen.

Again, we've already seen this morning the discussion about when you use adjuvants. It is with a vaccine. You can administer this, vaccines and adjuvants, via various routes. Today I would say the most commonly used route is intramuscular and

subcutaneous. But obviously other routes are being developed: ID, IV, patch, nasal, oral, et cetera. Again, the list goes on. I haven't mentioned everything.

And the dosing schedule -- the dosing schedule of vaccines is usually short term. I put usually there because I'm not sure how we define flu vaccines which are given yearly or something like some of the immunotherapies for cancer or HIV.

So moving forward a little bit more to discuss about the toxicological assessment, looking more about the designs, and asking some questions. What studies do we do?

Well, our main interest is in the systemic toxicity and local tolerance for which we usually conduct either single or repeat general toxicity studies. And here we have to start asking some questions. What species? How many? What's the study design going to be like? What dosing schedule? How

many doses levels? And I'm going to take you through some of these points.

When we consider the number of species, let's just have a look at some of the guidelines. So for a new chemical entity, if we're looking at general toxicity, assessing systemic toxicity, we require two species, a rodent and a non-rodent. Now as you've seen, animals are not always predictive so we use a rodent and a non-rodent to try and help that.

For a biological, we tend to try and use a one relevant species. If we can't find a relevant species, then you might want to consider two.

So what do we want to use for an adjuvant? One species or two? Again, it's case by case.

In the EMEA guidelines, it should be tested in two species unclear otherwise justified. And ideally at least one species selected should be the same as that used in the proof of concept studies.

So if an adjuvant has no species specificity, say like an oil emulsion, perhaps you want to consider using two species. If an adjuvant exerts a high level of species specificity, e.g., like some of the cytokines, then perhaps one relevant species is enough.

Now looking at the dosing schedule, when you are assessing the adjuvant alone -- now how are we going to consider this? Are we going to consider it as a vaccine or a new chemical entity?

Well, the EMEA supports that a vaccine-like administration. The WHO is less specific. It refers to the ICH and excipient guidelines.

So, for an example, if we have a look at the sort of typical doses that we might administer, a clinical schedule, and then add one, it is the N+1, which was talked about earlier about Marion, giving one dose maybe every two to three weeks, looking at when we do necropsy, Day 2, and then maybe



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give a non-dose period, maybe 14 days after the last period.

Use the same route as the clinic, for example, intramuscular. And what dose level? The human dose? By volume? By mg/kg?

So how many dose levels? And what about the MTD? That's the maximum tolerated dose. So, again, when you are looking at new chemical entities, then the guidelines suggest that you are looking for a dose relationship should be established and you should reach maximum tolerated dose. You really need to understand what this small molecule might do if you are looking at a small molecule.

The adjuvant guidelines for the WHO is not specific detail. The EMEA suggests that a dose relation should be established, range of doses may be relatively low, reflect the clinical dose, and maximum tolerated dose is not needed.

And how many doses? If we're setting the adjuvant alone and developing a



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master file, this adjuvant may actually be used in more than one vaccine. So if it's being used in more than one vaccine, there may be more than one clinical schedule. So one vaccine may be one dose, another vaccine could be three.

And when you are supporting the clinic and you don't know what will the clinic do. Is the dose in the Phase I trial fixed? And then I think maybe tomorrow some of this will be discussed, the companies and projects may have a very different approach.

So there are some possibilities. Just administer one dose, a HEMO clinical dose. You have to make the assumption, therefore, that this is going to be a no-effect dose level. Or you administer more than one dose and, if so, what information are we looking for?

The MTD is not needed. Maybe it is going to help us understand the dose response, that is if we're seeing any



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toxicity. Maybe it's going to be used to investigate possible toxicity, in fact like push the dose up a little bit or to make sure we do get a no-effect dose level.

Again, about the doses, are they based on volume or mc/kg? And are you going to use this data for dose setting in the clinic? Or is the clinic going to assign the dose before you go into the clinic?

Genotoxicity, do we assess genotoxicity? Again, it may depend on the classification of your adjuvant. So for NCEs and excipients versus a biological, it might be different. For an oligonucleotide, we might was to consider DNA integration.

So for the EMEA, it states if it is considered a nonchemical entity, that we follow the ICH2a guidelines. I just put a little comment here that genotoxicity tests, as mentioned in the ICH2a guidelines, are conducted at the highest maximum tolerated dose.



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So hopefully you can see that's opposite to what we were trying to achieve with our adjuvants where we are saying we don't have to achieve a maximum tolerated dose.

These guidelines are currently being changed so this actually might change. But it is just something that is of interest at the moment, specifically for a toxicologist as to how we answer the question.

So now I'm going to take you through two case studies. And the first one -- I've named them Adjuvant X and Y for IP reasons.

This adjuvant is currently in Phase I clinical trials and it is an emulsion. And the idea here was to create a master file. So for the toxicology assessment to move forward into Phase I, we conducted a general and genetic toxicity study with the adjuvant alone.

We conducted a repeat dose

toxicity study and we used two species, a rodent and a non-rodent. So here we used the rat, which is a preferred rodent species -- the mice are okay but you can't get as many samples out of them so we prefer the rat -- and the rabbit as a non-rodent species.

Again, how you select the species can depend on a number of factors. And I don't think I'm going to go into that here because of I haven't got enough time.

We tested two groups, an adjuvant alone group and a saline control. And here we looked -- we did more than the human dose. We did three dose levels because we decided we were just going to push the dose up. We weren't aiming for maximum tolerated dose but we were going to just have a look to see what potential effects there may be partly due to because of some of the safety concerns that adjuvants have.

But we fixed our volume at -- the human volume or which is the normal human



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volume that we tend to use is not .5 ml or for the rat, for which you can't inject the human dose, we gave two doses of 250 microliters.

Now as we said, at this time we are creating a master file here so the clinical regime is unknown. So we have to sort of guess what might be the worst case scenario here.

So we looked at -- if you look across at the schedule of vaccines, on the whole, five to six is the maximum you'll ever see in our experience although you may have different experiences, so we decided that we'd dose five times and every three weeks.

The study design which, again, I'm not going to go into detail but it's based on the WHO, EMEA, and ICH standard guidelines so we were looking at various endpoints such as clinical signs, local reactogenicity, body weight, food consumption, clinical pathology, ophthalmology, histopathology, and organ weights. And we included a recovery period of

14 days after the last dose.

Because this was an emulsion and not a biological, we decided that we would just test the potential genotoxicity. So here we followed the standard ICH2a guidelines and we tested in an in vitro Ames and mouse lymphoma assay. And an in vivo mice/mouse micronucleus assay.

And as I said earlier, for this assay we have to really dose to the maximum tolerated dose. So, in fact, we did do a preliminary study. It's just a single dose that is given here. But we did a dose ranging finding single study to assess the maximum tolerated dose and then we did the pivotal study.

And, again, without going into major details, this is standard ICH design.

Now as we said, before you can move forward into the clinic if you are testing -- you must test your adjuvant with the vaccine. This was a prophylactic vaccine.



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And now the clinical plan is known.

So for this we supported this with a general toxicity study. And we supported in the rabbit as we believe this to be the most appropriate species. With the antigen that we were developing, we got an immune response in the rabbit, for us is a good model.

So we dosed the rabbit with the human dose, just the human dose, not .5 ml. We went in via the clinical route, intramuscularly, and we followed the clinical regime, N+1 -- dosing once every three weeks. And the study design was as I've discussed previous.

So that was what we did for Adjuvant X. And as I said, it's in the clinic.

So moving on to Adjuvant Y, which is a biological, and this is currently in the Phase I -- now actually I have to say that we didn't do the toxicological assessment so I can't give you some of the rationale behind

what was done.

But the idea here is to point out that there are some slight differences that you will see. So for the general toxicity study, they chose to do an acute study and a repeat dose toxicity study. And here they chose the mouse.

For the route, they chose two routes to assess, subcutaneous and intramuscular.

Now the acute study, they dosed at 2,000-fold the human therapeutic dose. And for the repeat dose toxicity study, they chose three dose levels at five-, 15-, and 45-fold of the human therapeutic dose. They included a Trif buffer control and they dosed five doses weekly.

They also conducted a local tolerance study. So some people do conduct these studies separately. And for this, they chose the rabbit. The mice isn't so good for assessing local tolerance. And, as you've

heard, the volumes in the mouse, you can't inject the same volume. So in this case, the rabbit is a better model for assessing local tolerance.

Again, they chose the routes subcutaneous and intramuscular and they gave a single dose of 10-fold the therapeutic dose.

They also conducted a genetic toxicity test, an in vitro, just an in vitro Ames, and they did a range of doses from three to 3,160 micrograms.

And as I said, this is currently now in the clinic.

So now just two last slides -- the what-abouts. So there are a lot of things that I haven't covered. There are still a lot of questions.

In these studies are we really investigating the appropriate endpoints? We've heard some of the appropriate endpoints in terms of potential autoimmunity. How are we going to test for that?



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What biomarkers are we testing? Well, we aren't really adding any additional biomarkers to what is classically used at the moment.

Safety pharmacology, I haven't discussed.

Developmental and reproductive toxicity studies, at the moment, we've only supported the Phase I clinical trial. We're slowly moving forward and these questions are coming up in which now we need to turn and consider the same questions, number of species, doses, schedule, et cetera.

I haven't covered toxicokinetics and there are questions there. And what about biodistribution, particularly for biologicals such as the oligonucleotides?

Pyrogenicity tests and the PAS and ASA tests which are mentioned in the EMEA guidelines, so these aren't always routinely conducted.

And I think with pyrogenicity

tests, we have to be careful as to when we measure the temperature and telemetry is probably really the best way. So I've certainly see studies where they've measured for pyrogenicity and it has come up negative and actually it is possibly because of the study design. And, again, species is important.

The PAS and the ASA tests, well, I'm not sure this is being used at the moment or that it is thought of as particularly predictive.

I haven't discussed antigen/adjuvant ratios. I haven't discussed about combination adjuvants, whether we can just considered it as one entity or we test them as two separate entities. And what about concomitant vaccines where you are giving more and lots of different adjuvants, et cetera?

What about the pediatric population? And I know this has already been mentioned by Jan Willem? And species can



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effect margins. So a rabbit is approximately the same weight as a baby. So what happens to our margin when we're dosing using the rabbit as our model? And what about juvenile toxicity?

And I think that's me. So thank you. Any questions?

(Applause.)

DR. GRUBER: Thank you very much, Sarah.

We have time for a couple of questions.

DR. TIM SULLIVAN: Could you remark please on -- you mentioned the biologic, the Adjuvant Y species activities and choice of the toxicology, you said it wasn't yours -- you didn't develop that.

DR. GOULD: Yes.

DR. TIM SULLIVAN: But does that come into play? Or can you make some general remarks on how that would come into play if it had been your project to run?

DR. GOULD: I think it is a good question actually and it depends on the product as to what you'd be looking for in terms of your species being relevant.

And in terms of relevant species, I think I have some general questions there that makes it very difficult to answer, particularly with adjuvants.

So it might depend on the mechanism of action. And sometimes, you know, you just have to say there isn't a relevant species. And then there's questions.

DR. TIM SULLIVAN: And not to make too big of a deal out of it but I'm Tim Sullivan from Idera and our product line is oligonucleotides. And I don't know how they are regulated in Europe but in the United States, they are not considered biologics. So that would be within CDER.

DR. GOULD: I didn't hear the question. Sorry.

DR. TIM SULLIVAN: It wasn't a

question so much as a comment.

DR. GOULD: Oh, all right, okay.

DR. TIM SULLIVAN: You had listed oligonucleotide as a biologics. And I would consider that still a chemical rather than a biologic.

DR. GOULD: You're saying it is not a biologic? I can't hear you.

DR. TIM SULLIVAN: Yes, not a biologic. It's just the CDER.

DR. GOULD: Okay.

DR. GRUBER: I think we need to clarify this a little bit. I had a little bit of trouble understanding but, you know, oligonucleotides or DNA oligonucleotides or DNA vaccines, in the U.S. FDA are actually regulated as biological products. But perhaps you can clarify. I may have misunderstood.

DR. TIM SULLIVAN: Well, as a vaccine certainly as an adjuvant.

DR. GRUBER: Well, you know, the point is -- I mean -- so you're talking about



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things like CpG motifs and -- well, you know, the point is -- and that's what we tried to get at this morning is, you know, the way you define an adjuvant.

It's really not an active ingredient. And as such, you know, we wouldn't really call it, in that case, biological product. It's like we go by the regulatory definition as being, you know, in the U.S. FDA, we call it constituent materials. The EMEA, I think, defines it as excipients.

So the term biological product, I think, is really reserved for what we call the active ingredient, that is the vaccine antigen, for instance.

DR. TIM SULLIVAN: Yes. You've got a good point. And I'll step back and say from the point of view of doing like a master file or something on the oligonucleotide by itself, apart from the vaccine application which obviously clearly would be a biologic.

DR. GRUBER: Yes. Liz, can you comment on this in terms of the master file issue? I mean I think we --

DR. SUTKOWSKI: It would all depend why you are completing a master file and what you plan to do with it, right?

I mean we certainly would have -- we would consider master files for oligonucleotides for use as adjuvants and we would consider -- we would expect to see the same kind of information come in as we would, you know, for other biologic components.

It's the same CMC-type information. It would just depend on what it is used for, I think. But I think his concept if probably very different from what we are talking about if it is something in CDER, possibly as a therapeutic, it might. I just don't know what else to say, you know.

DR. GRUBER: Okay. If there are no more questions, I think I'll go ahead and introduce the next speaker. And I'll do this

-- I'll remain seated by doing so.

The next speaker is Dr. Deborah Novicki from Novartis. And she is going to be presenting some of the company's experience in performing toxicological studies. And she is raising, I think, also some very interesting questions.

Thank you very much for being here.

DR. NOVICKI: Thank you for inviting me.

Let's see. Here I am. Thank you.

Okay, so we've heard a lot of different points of view today. Just -- I'm going to focus on earlier in this session and I'm going to try to not be redundant because it is almost unavoidable that there is some continuing theme that is running through these talks.

We heard about the U.S. and EMEA approaches. We heard about some limitations of animal models, some potential long-term

effects of adjuvant treatment, some of the programs that have been done by Sanofi for two of their experimental adjuvants. And I'm going to focus on MF59 adjuvant, which Derek O'Hagan spoke to this morning and described some of the research and mechanism of action and proof of concept-type of work. I'm going to focus on the toxicology program for this adjuvant.

Just to put the picture back up there to remind you, it is an oil-in-water emulsion and the squalene oil component is a natural compound and is highly metabolizable.

The current status of the file is a master file submitted in the U.S. We had a little discussion earlier today about Fluad being approved in several European countries but not all. It is -- my latest is 23 countries so I'm not sure. It changes, you know, month by month.

This vaccine is licensed for greater than 65 years old and younger age

groups are being studied.

Focetria was mentioned as a pandemic vaccine that is approved in the EU. And Derek showed some of the data that we have with nonclinical studies with a variety of different antigens and a variety of different species.

And tomorrow in the clinical section, there will be two presentations by Drs. Della Cioppa and Rappuoli about clinical aspects of MF59. So that will be something that will be a nice bridge from what you are hearing today on the nonclinical parts.

I'm not going to reach each of these WHO and EMEA points. I kind of put it up here to remind myself about the point that I wanted to make. Sarah did a very nice job kind of pointing out what the expectations are as well as each of the individual speakers presenting the U.S. and the EMEA perspective.

So from the pharmacodynamic standpoint, I think there was a lot of

discussion about that this morning. What does the vaccine do? What does the adjuvant do? What are the target cells? What is the species specificity? All of these thing play into the design of the pharmacodynamic and proof of concept studies.

So the studies that we've done are primarily mechanism of action studies in mice and studies with mouse and human cells to kind of bridge from mouse to human. And then a wide variety of immunogenicity and challenge studies that are actually oriented toward supporting the various vaccines that have been examined or are being developed with the MF59 adjuvant as a platform.

We touched on safety pharmacology studies. We did not do classic safety pharmacology study which would normally be something like continuous monitoring immediately post dose for a specified period of time, instrumented animals.

What we did -- and these studies

were done quite a while ago -- they were dog tolerability studies basically, not classic toxicology. And animals were treated with a saline control or MF59 adjuvant.

They received three injections that were spread apart approximately by two weeks. And at the end of the study period, one week post last dose, the animals were assayed for cardiovascular and for neurological function. So this was a week after the last dose.

So not your classical safety pharmacology but at least following three full doses to dogs. There were no effect on cardiovascular parameters. And toxicologically or tolerability-wise the vaccine was well tolerated in these same animals.

Pharmacokinetics, I think that we've had a brief discussion this morning that we do not do classical pharmacokinetics with either antigens or with adjuvants. But there

certainly was the point raised of being interested in understanding where is your adjuvant going. Is it local? Does it go systemic? And sometimes you find out that it is going systemic because you actually find systemic effects that you may not have been prepared for.

With MF59 -- and these are quite old studies, 1995, 1999 -- it was mostly focused on seeing how long did the MF59 persist at the injection site and where did it go. So there were radiolabeled studies done in rabbits and radiolabeled or fluorescently-labeled studies done in mice.

And basically MF59 clears fairly rapidly from the injection sites but these studies did not include a full assessment of all the different tissues and the different specific activities in these tissues. These were more mechanistically oriented.

Local tolerance has been discussed repeatedly. It is something that we do in

every study. We look at the injection sites from every study and we build this into our toxicology studies.

We don't do standalone local tolerability because really if you are doing a repeated dose study, there is not reason not to look at the injection sites from that study.

We routinely look at MF59 alone or the antigens plus MF59. A point, as I was reviewing the guidelines myself, that was pretty interesting -- the WHO guidance talks about sites inadvertently exposed, for example, eye exposure. And we never have done any kind of testing like this. And it's not something that really would have occurred to me.

But we did think about skin sensitization in a manufacturing setting. And so a dermal sensitization study was done in guinea pig, a classic Magnusson-Kligman Guinea Pig Test.



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I wanted to continue on talking about local tolerance. And this is in an experiment with New Zealand white rabbits using the clinical dose, the clinical volume, the clinical route of administration, which was intramuscular, the N+1 number of doses that were mentioned by Marion was done, dosing on Days 1, 15, and 29, so episodic but compressed dosing relative to routine clinical dosing.

Doses were alternated between hind limbs and necropsies were conducted two and 14 days following the last dose.

And it might be difficult for you to see this and I don't have any clever red boxes and only one pointer. So let me do -- I need new glasses. If you just focus on this column here, which is the MF59 alone, you can see that where there were animals with injection site findings, and this is histopathology, the findings were generally scored as minimal to mild, with an occasional

moderate.

This is the right-hand injection site. So this site had received two injections, one on Day 1 and one on Day 29. So we're looking at the injection site either two days post last dose or 14 days post last dose.

And if you just look down here, you can see that the number of observations are decreasing and the severities of the findings are approximately the same.

If we go to the left-hand injection site, which was only injected on Day 15, a single administration, you can see in the same column, which is the MF59 alone group, there are no observations left after 28 days, whereas two days post, there were the similar findings to the right injection site.

So basically minimal to mild types of effects in the muscle and reversible over time. And there's no association with granuloma formation with MF59.



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The induction of hypersensitivity and anaphylaxis, we did not conduct wither the PCA or the ASA test. I did mention that we looked at skin sensitization with the Magnusson-Kligman. And if we had seen a signal there, it's likely that we would have investigated further. But we didn't so we didn't.

There is the mention in the EMEA guideline that one may examine the adjuvant-induced increase of IgE against the antigen. We have not done this. So for the folks in the audience who are responsible for doing nonclinical programs, I would be interested to hear tomorrow who does, if any of us.

One thing I can say is that in repeat dose studies in various species, we've never had any signs or symptoms of hypersensitivity or anaphylaxis. And I think we will be able to say the same for people when you hear the clinical presentations tomorrow.



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Pyrogenicity was also mentioned as an endpoint that is important to assess. We test body temperatures in animals that are given the vaccine in our routine toxicology study.

And then as far as a release criterion, we don't test MF59 because it is not a product by itself. It is always formulated with an antigen so we do our pyrogenicity testing in the final vaccine product -- the pyrogenicity test that is the type for release of the vaccine.

For the reproductive and developmental toxicity, it is clear that by the time a vaccine is going to get to the market, if it is being used in women of childbearing potential, one would choose to investigate reproductive toxicology.

So MF59 alone we did pilot testing in rabbit through the C-section with 0.25X and half the clinical dose with dosing continuously on gestation Day 6 through 28.



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And then the definitive study was actually done with the MF59 plus antigen, the actual vaccine candidate versus a saline control.

We also did a full pre/postnatal development tox student in rodents, rats, with five or six injections that were administered over the time before, during, and up until the C-section and postnatal evaluations.

The individual MF59 adjuvanted vaccines are testing for repro tox if they are going to be used in women of childbearing potential, including adolescent populations. So if they are indicated for old people, like for Fluad, we didn't do it.

For genotoxicity, we did Ames testing and we did the Mice Micronucleus test in vivo and we followed the very standard paradigm that one would follow for a new chemical entity.

We went up to the 5,000 milligram per kilogram, which is the max dose specified, and in the Ames assay up to 5,000 micrograms

per plate. I guess you can assume that if I'm not saying that there was a positive result that it was negative.

Carcinogenicity, we have done no testing for the carcinogenicity of MF59 adjuvant or any of our preventive vaccines. We haven't done it and we don't plan to.

So I know that there is a comment in one of the guidelines that addresses carcinogenicity and that, perhaps, is something that we should discuss a little bit tomorrow.

And it may be more pertinent in the context of therapeutic vaccines if it is a vaccine that we would be giving for a long time -- and I know people have raised the issue where you get a flu vaccine every year. And so it is something that I think is a discussion point for tomorrow.

There is a lot of information in the guidelines on various aspects of the systemic toxicity assessments. And Sarah

actually did a very nice job covering a bunch of the points.

I wanted to focus in for the ending of my talk with looking at the multiples that we achieve in some of our programs. So this is just showing for MF59 alone this is really an NCE-like program.

We've got repeat dose toxicity, embryo/fetal development, AMES, Mouse, Micronuc, Magnusson-Kligman, and embryo/fetal development. But the place that it is different is in the repeat dose toxicity and in the embryo/fetal and developmental tox, we're only using low to X multiples of the clinical dose. So that is different.

And this is another slide just showing some more studies that have been done. This is the dog tolerability that I mentioned earlier. These are a bunch of different rabbit studies. There are repeat dose studies, single dose up to three to six administrations of MF59 adjuvanted antigens



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and MF59 alone. And, again, various multiple but tending to the low side.

Just overall, the toxicological findings with MF59 plus and minus adjuvant tend to be effects on white blood cells. In most studies, there are some elevations but sometimes you can see some decrease, depending on the exact time point when you take it if things have marginated.

But everything is reversible in a very short time frame. We do -- I think Marion had said in one of her -- or it is in the questions -- should we be measuring fibrinogen or C-reactive protein or things that might be associated with acute phase reactions?

We routinely measure fibrinogen because it actually is a nice little marker to show us that something is happening in rabbits. We tend to see up to a doubling up to two to three days post dose. And then it rapidly declines back to baseline.

Normally this is not associated with any sort of effect on prothrombin times. Very occasionally, we might see a slight shortening of PT.

Along with antigens certainly we see an elevation in globulins and changes into the Ag ratio because it is calculated. And I mentioned before and showed you the data on the injection site histopathology with MF59.

But we do evaluate a full panel of other things. And basically we have -- it is very well tolerated.

And one of the things that I'd like to point out that we have monitored over many years is we take a sample of bone, including the articular joint, to look at the cartilage and in case there is any sort of a signal for the adjuvant-induced arthritis. And we have not see a signal in any of our studies.

And we also have looked at uvea because historically uvea was an area where

some findings had been observed. Not with MF59, with other things.

So now I'm going to come to a comparison of exposures. And these are not your classic sort of pharmacokinetic exposures. That is why it is in quotes.

What we did was look at the smallest population that we think we would ever administer an adjuvant to. And so we based it on a six-month-old human infant. And we used the CDC growth charts.

The body weight, surface area -- sorry -- the body surface area calculation was done with a formula from John Current's paper. And then we used the animal BSAs form the Mike Derelenko's Toxicologist's Pocket Handbook.

So if my math is correct -- and you don't have to look at every number here -- looking across a panel of studies -- and this rat study is the embryo/fetal development. So this isn't a classic tox study. But I wanted to put the multiples in here just so we could



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see if you take a clinical dose, you put it into a rat, what does it look like.

So if you look across these three rabbit studies, you can see that based on body weight, it is true. You are basically having a XX to a XXXX multiple of your clinical dose that is being used in your toxicological study.

The rat, of course, it is smaller. And you end up with a higher multiple. But one of the things I thought about as I was thinking of what does the exposure really consist of is that in the tox study, you are really giving more doses, a shorter period of time in between doses. So I also looked at what the cumulative dosing would look like.

And so if we look at a study where we gave 14 daily doses, we end up with multiples that look a little bit more reasonable. They're not tremendously high. But, you've definitely got a multiple there based on cumulative dosing. And I think you

can see that there is a reasonable set of multiples across these studies.

Then looking at body surface area, I went through the same exercise. And you can see on a single dose basis it's not tremendously different because the infant is so small. So this is what you get if you are looking at animals, rats and rabbits, using a clinical dose or close to a clinical dose and comparing it to a human infant.

So now we come to the really hard stuff. The toxicity of components to classical target organs I think is fairly well understood from the standpoint of drug development. But when we come to trying to look at things like the autoimmunity discussions that we've had today, I think that this is an area that we are probably going to spend a lot of discussion tomorrow.

The first dose cytokine response, some of the cytokine storm, hypersensitivity, there are certain areas where we may be really

limited on what the animal models can tell us. And I think that this is a place that I would certainly like to hear people's ideas tomorrow.

One of the things that I was thinking about is what can we really expect from our animal studies and one of the questions from the last or the second to last session kind of touched on this point which is the diversity of people.

And this table just shows how many subjects you've got to study if you want to see a doubling of a rare event. So by the time you are looking for two out of a million instead of one out of a million, you have to be looking at 50,000 subjects.

So one of the things that I feel is pretty limiting is the ability for us to use a quantity of animals. It's not reasonable to think that we're going to be able to detect really, really rare events in animal models. So that's something that we

all struggle with.

And I think this is my second to last slide. I wanted to, again, provoke for tomorrow some discussion about are immunomodulators or things that are given separate from the vaccine really the same as an immunostimulant, an adjuvant, whatever you want to call it that you give at the same time.

I'd like to also acknowledge that the program of MF59 studies is a cast of hundreds. And if you include all of the human volunteers that have participated in our trials, thousands and thousands. So I just would like to thank all of those people and just acknowledge a great team that worked to bring this compound forward.

And thanks for the invitation and for listening.

(Applause.)

DR. GRUBER: Thank you, Dr. Novicki.



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Are there questions?

DR. PETROVSKY: Nick Petrovsky, Australia.

One of the issues of toxicity, I think, is the interaction between the administered compound and the genetics of the person receiving it. And obviously a lot of these preclinical studies are being done in inbred models where all the animals have the same genotype.

So obviously if the rare side effect is a relationship of a particular genotype with a compound, then you knock the chances of picking it up even if you study the million mice or rabbits of the same genotype is zero.

DR. NOVICKI: Toxicology studies generally are done with outbred strains or outbred animals. But they are not as outbred as we are.

DR. PETROVSKY: Yes.

DR. NOVICKI: All right?

DR. GRUBER: Okay. Thank you.

Okay, I think we're coming to the last two speakers of this afternoon's session.

As we mentioned this morning, in terms of thinking about improving and optimizing preclinical safety assessments of vaccines, what alternative methodologies can be used to supplement the currently ongoing nonclinical safety assessment programs?

And so it is a pleasure to announce today two speakers, the first of them Dr. Hana Golding from the Center for Biologics who is going to be talking about her research on the use of human cell lines for quantitative preclinical evaluation of vaccine adjuvant safety.

Thank you for being here, Hana.

DR. GOLDING: Thanks, Marion. And this is sort of a very special moment for me to be able to be a part of CBER and also to share with you some of our thoughts on how we move forward in this very important field of



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developing and testing new adjuvants.

And I think Dr. Fauci put it very well as well as Dr. Goodman. There's no question that there is a need for novel adjuvants to improve the immunogenicity of very challenging vaccines against emerging diseases and pathogens.

And clearly the development of adjuvants are an iterative process. And we heard a little bit from Derek O'Hagan about their screening program. There are other biotech companies that are involved in active screening of novel adjuvants.

My talk, and I think of the following talk, will kind of address the questions of what new tools we may need in the early screening, the early development of novel adjuvants that may give us a hint of what could be safety signals in vivo and will help us in the sort of screening and selection process.

So the rationale for the studies

that I am going to describe have actually been described before. We know that novel adjuvants may cause pyrogenicity and other short-term local or systemic toxicity. But also in the back of our mind is that immunomodulating activities or adjuvants may also promote unintended long-term consequences.

What we already heard today is the fact that adverse reactions observed during clinical trials of adjuvanted vaccines may not always be detected in preclinical studies in small animal models due to species' viability and pattern recognition receptors, including gene sequences as well as tissue distribution.

So we thought it would have been nice to concentrate on human-derived cells. And we have initiated a program for rapid evaluation of novel adjuvants and vaccine delivery systems based on what we would like to term human detector cell lines.

And I want to emphasize this is

very much a work in progress. And I'm going to share with you some of our plans and preliminary data.

So the couple of tests that I'm going to cover today is we're trying to develop an in vitro assay to measure proinflammatory cytokines such as IL6, IL1 beta, TNF alpha, and IL8 as a predictor of systemic toxicities in vivo.

We also would like to develop an assay to measure prostaglandin E2 because we know that this could be a very early mediator of temperature increase due to its ability to cross the blood brain barrier and work on the pre-optic interior hypothalamus and induce increasing temperature even in the absence of cytokines or before cytokines are induced.

Another sort of long-term goal is to develop an assay to measure elevation of intracellular calcium in astrocytes as a potential biomarker of indirect neurotoxic potential.



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And the fourth assay that I'm going to discuss with you is that assay to detect bacterial endotoxin in vaccine formulations containing novel delivery systems such as nanoparticles which are shown to interfere with the LAL test. And I'll give you a preliminary result on that as well.

So as far as the proinflammatory cytokines, of course one has to select the right type of cells and not one cell line is going to answer all the questions.

We started off with a cell line that had been described before, the MM6 ELISA, which is a promonocytic cell line with a known spectrum of TLRs. And we decided to try and use it to quantitate the levels of proinflammatory cytokines released in the presence of adjuvants.

Of course it is very important in order to quantitate it to have the right comparator. And we know from the literature that LPS in rabbits at a dose of .5 EU per ml

was defined as the pyrogenic threshold, namely this dose led to induction of increase in body temperature of more than .6 Celsius. Therefore, in all of our assays, as a positive control, we are using a USP reference endotoxin, the EC6 Lot G, and we run a complete dose response.

Now in order to sort of generate what I would call a proof of concept for the usage of this approach, it was important to start with several adjuvants that have been in the clinic, preferably in significant number of people, and had a known clinical safety profile.

And one can then ask well, how do these adjuvants behave in your own assays? Can you see a similar correlation in vitro to what was found in vivo?

So you heard a lot today about alum, which, you know, is licensed in both the U.S. and Europe, the mechanism of action in both antigen deposit and injection site

inflammasome induction, as you heard earlier in the talks, and it has a very excellent safety record to date.

You also heard quite a lot about MF59 water-in-oil emulsion that has been licensed in Europe, again, the mechanism of action has been unraveled. It includes APC maturation and antigen uptake. It has a good safety record as well.

The saponin QS21, which we also heard about earlier today, as you know it has been widely used in animal vaccines. It has been evaluated in several human trials as well but it has a mixed safety record including studies that have been interrupted due to adverse reaction.

So we thought this would be a nice sort of starting panel of adjuvants to test in the system. And as you can see -- and this is an example of how the system is done -- we are generating a dose response using different amounts of the endotoxin. And what is shown



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here in circles is the threshold of the .5 EU in blue circles and then in rectangular, this is the amount of cytokine proinflammatory that was produced.

So, for example, in this particular slide we are looking at IL6. And we are comparing different dose to different adjuvant. As you can see, at the various doses that we have used, the MF59 and the alum were basically inert.

And in with the QS21, we are starting to see an increase in IL6 production at the highest dose that was used in this experiment, which is the 20 microliter per ml.

However, I want to emphasize in both of these cases, even this IL6 production did not reach the level and the TNF alpha on the right did not reach the level that was seen with the LPS at this threshold. So we would not consider that an unsafe production of either TNF alpha or IL6.

On the other hand, when we looked

at other proinflammatory cytokines, especially IL1 beta and IL8, now if you compare the three adjuvants to the dose response with the LPS, you can see that definitely in the case of the IL8, the amount that is produced in response to QS21 exceed that that was found with the threshold dose of LPS. And also in the case of IL1 beta, it is very close to the threshold.

So just looking at those three adjuvants with clearly different clinical profiles, we found that one can see a differential proinflammatory cytokine produced whereby the QS21, which is the more reactogenic in the clinic did give elevated levels of at least two out of the four proinflammatory cytokines that are expected to be beyond the pyrogenic threshold of LPS.

With this kind of initial proof of concept, we are now starting to looking at different groups of novel adjuvants. First it was interesting to compare a mineral salt. In

addition to aluminum phosphate, calcium phosphate, you heard before, has been in the clinic before. And one would expect that it may have a similar mechanism to aluminum phosphate and the safety profile.

As you can see in this slide, on the other hand, the findings were quite different. So while alum is really inert, this is, again, the LPS dose response indicating the sort of threshold of the .5 EU response at either IL8 or IL1 beta.

And you can see that unlike aluminum phosphate, calcium phosphate actually generates much higher levels of both of these cytokines, suggesting, again, that this particular compound may be more reactogenic in vivo. But of course this needs to be corroborated.

We heard a lot about TLR agonists and, you know, a multiple of them. What we have started to do in this so far is to test several, the FS3, the TLR26 agonist, the

Pam3CS, which is the TLR12 agonist, Flagellin, which is a TLR5 agonist, and MPL. We've all heard a lot about about MPL today. The cell lines that we are currently using are not very appropriate for the TLR7,8,9 agonists.

So what did we find? Again, on the left, we are looking -- here we are looking at either IL6 or IL1 beta, the LPS dose response with the sort of threshold dose of LPS.

And as you can see, actually after different TLR agonists that we have tested so far, the PAM3, the Flagellin, and the FSC, all actually generated proinflammatory cytokines about the LPS threshold while MPL, which was the detoxified Lipid A, has been actually very inert in both of these assays, suggesting again that not all TLR agonists behave the same. And actually one would expect those three agonists, the TLR2 and the TLR5 to be much more reactogenic maybe than MPL.

So this is just sort of a general

summary of what I did show you and some data that I have not shown you. So I showed you the difference between alum and CAP in our system. The MF59 was very inert.

On the other hand, the QS21 definitely gave a signal that would be expected of more reactogenicity. Of the TLR adjuvants, those are the three that gave relatively strong signals.

We already tested a large number of delivery systems, including liposome, dendrimers, PLG, and colloidal gold, all of them were actually very inert in that particular cell line. And we started to test a few adenovirus vectors as well.

I also want to just share this. This is even more preliminary data in our attempts to quantitate PGE2 production. We are using a nice kit which involves FRET measurement whereby the donor is this molecule which is conjugated to anti-PGE2 at 620 nanomolar and the acceptor molecule, which is

linked to PGE2 at 665.

So you can have a very nice dose response comparing the emission at 665 over 620. And then you can now add a supernate from cells that had been activated with different adjuvants and determine whether they have any PGE2 in terms of inhibition of this particular dose response.

And I just want to show one preliminary data. The cell line that we have started using have been reported to be a good cell line for that purposes.

It is a U937 that had been activated with PMA, which then induced the differentiation into a macrophage adhering cell line. And if you now add LPS, it's a threshold .5 EU, you can now see that you can measure a significant amount of PGE2.

And we, of course, are going to extend the studies now to measure the complete dose response of LPS and other adjuvants and TLR agonists.

I just would like to then summarize this part of the talk. So what we have shown so far is that measurements of multiple proinflammatory cytokines released by human cell lines compared with LPS standard could be used as a first screen of novel adjuvants for predicting possible toxicity in vivo.

We would like to eventually develop some sort of alogrithm that will be called the safety score -- the number of proinflammatory cytokines the are induced and the levels.

We also are in the process of developing a test of PGE2 production in macrophage-like activated U937 cells which may provide an add-on information on potential cytokine-independent toxicity of adjuvants. And, of course, all of these studies need to be corroborated initially in rabbits, including measurement of circulating cytokines and PGE2 which are underway to provide



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correlation between the safety scores that we have generated in vitro and hopefully we will connect them to the in vivo system.

The last part of my talk will actually have to do with something that is Carl Alving already described and that's the potential problem with LAL testing of new adjuvants. And we know that the LAL test had been approved as a substitute for the rabbit pyrogenicity test in 1983.

However, since then several factors have been identified that could actually be interfering with the endotoxin measurement by LAL.

And those involve chemical inhibitors, physical inhibitors, and more recently it was found that nanoparticles, including liposomes, gold particles, and dendrimers may actually interfere with sensitivity of the LAL assay. Both enhancement and inhibition were recorded.

So when we became aware of that,

we thought it will be actually nice to see whether some of the cell-based assays that we have developed could be an additional approach.

This is just a very simple sort of depicting -- this slide depicts sort of the principle of the LPS detection in the Limulus amebocyte, lysate, and LSA whereby a proenzyme is converted to coagulate in the presence of the gram negative bacterial endotoxin. And this coagulate can then lead to the clotting -- to the self-association of coagulin.

So on the other hand, if one thinks of the way LPS is detected in mammalian cells, it is really binding to the TLR MDCD14 as was resolved recently by the crystal structure.

This is sort of a diagram of the LPS and most important of the lipid A, which is detected in the LAL assay. And the possibility that we wanted to test is that are the different parts of the lipid A, the core

versus the isolated lipid cytokines may be acting in the LAL versus the TLR MD2 system.

And in order to do that, we first wanted to make sure that our both MM6 ELISA and the LAL are very reproducible and you can see the cell assay that were conducted over one year and we got a very similar dose response. This is IL6. And also in the LAL we get a very good coefficient variation in most of the doses.

So now one can actually conduct what we call spiking experiment where different amount of LPS are added in either the LAL or the MM6 ELISA in the presence of different nanoparticles. And here we are looking at the colloidal gold at 59 nanomolar or DPPC liposomes, a very commonly used liposome in vaccine adjuvants.

And just to show you here, actually the colloidal gold can get an enhancement of the LAL assay, which will basically generate a false positive results.



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And in the case of the liposomes, many types of liposomes, especially catyonic liposomes but also neutral can actually cause interference in the LAL assay.

And that was, I think, discussed briefly recently whereby low levels of LPS may be missed in the presence of liposomes. As you can see here, using the MM6 ELISA was much less sensitive to interference by either DDPC lysosome or colloidal gold.

And at this point, this is a very early stage of this research. We just wanted to summarize by saying that the presence of nanoparticles in biological product can significantly the ability of endotoxin to activate the clotting enzyme cascade in the LAL assay.

A cell-based assay may then provide another approach. Prior to evaluation of products containing nanoparticles such as liposomes for endotoxin contamination, it may be important to test the ability of the

nanoparticles to interfere with LAL or with other biological assays by actually using spiking with LPS at multiple concentrations.

So this was kind of just a summary of our work in progress. And I really wanted to emphasize that all of the studies were supervised by Marina Zaitseva in our group. She is leading our adjuvant program.

Most of the work that you've seen was generated by Tatiana Romantseva and Oksana Blenova has recently joined the group and is working on the PGE2.

We are very thankful also to Marina at the NCL who works with us on the nanoparticles and Anu Puri and robert Blumenthal on liposomes.

We also were collaborated with the Canadian group on archaeosomes, which I haven't showed you. QS21 was provided by Antigenics. Novartis, especially Derek O'Hagan was very forthcoming in sharing with us several Novartis adjuvants, which I've



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showed you the results. And we have also established collaboration with the VRC and GenVec to look at different adenovirus vectors.

Thank you.

(Applause.)

DR. GRUBER: Well, thank you, Hana.

I think we have time for one or two questions. Yes, go ahead.

DR. SAHNER: Hello, again, David Sahner.

One comment and one question. The comment is obvious. Proinflammatory cytokines are a double-edged sword here obviously in the sense that they may correlate with toxicity but also may be integral to the mode of action of an adjuvant. So there is just that element of caution, obviously, and I'm sure you've thought about that quite intensely.

But secondly, given that, what is the -- can you comment on dose selection or



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concentration selection for your experiments because the concentrations locally within the interstitial fluid at the site of injection obviously are quite critical and may be very high and incite the sort of local proinflammatory response that one wants to see to enhance the endogenic response whereas the peripheral concentrations may be negligible. So there may be no meaningful impact on systemic levels of proinflammatory cytokines like TNF alpha and IL1 beta and so forth.

So can you comment on the dose selection and then perhaps on my comment as well.

DR. GOLDING: I think both your comment and your question are right on target. There's no question. And that is something that we are actually, you know, struggling with when we designed the experiment.

There is not question that there are many ways to evaluate in vitro the activity of adjuvants. And we specifically

actually shied away from using the traditional way of looking at dendritic cells that generate all the good cytokines, the IL12, the interferon alphas and so forth.

We tried to focus on cells that we think are involved in the more sort of proinflammatory that can lead to the reactogenicity of adjuvants. And of course we have to be very cognizant of the fact that some of the cytokines that are produced may be part of the normal immune response to these adjuvants.

And, therefore, we had a big effort of really including in our system an important comparator. Up until to now we worked mainly with LPS because at least for that particular TLR agonist, sort of the maximum tolerated dose is known.

And we are now in the process of establishing a similar type of maximum tolerated dose for other types of of TLR agonists.

Of course this is still, you know, work in progress. And computation will ultimately be very important. And we will have to then corroborate any finding that we find in vitro with some in vivo studies in animal models such as rabbit although we started out the project acknowledging that the animal models may be limiting.

As far as the doses that were used, this is an even more difficult problem. So first of all whenever we start with any new adjuvant, we are actually determining in our cell system what is the highest tolerated dose that does not effect the viability of the cells. And then we go down from there.

Now do we exactly -- in some cases in many of the adjuvants, we are actually able to work in a dose range that was not so different from what was used in the clinic, assuming that the local, the initial local concentration we tried to sort of mimic in vitro. But in other cases, of course, the

doses were not identical.

So this is not going to be something that will become part of any guidance documents anytime soon. Rather, we want to offer that as another way to start thinking of what we can use as a research tool and in the very early development, a screening of novel adjuvant.

And by making it at least standardized and quantitative with the appropriate type of internal control, we would hope that it will reach a stage that it will give us some predictive value for in vivo toxicities and, therefore, it can then be used if you really -- you know, as part of this iterative process of screening multiple adjuvants or even modification to adjuvants to screen out those that are the most reactogenic.

DR. GRUBER: Thanks, again, Hana.

At this point, I'd like to ask the last speaker of today or for today to come to

the podium. That is Dr. William Warren and he is from VaxDesign.

Yes, Dr. Warren, thank you very much for accepting our invitation.

And Dr. Warren's title of his presentation is VaxDesign's in vitro mimic of the immune system for evaluating adjuvants.

Thank you for being here.

DR. WARREN: Thank you very much.

For the last talk, Jay Slater gave me complete authorization to talk about anything that I wanted and to say whatever I wanted, right Jay?

Anyhow, thank you very much for the invitation to come.

I'd like to talk to you a little bit about some of the work that we have been doing on using our in vitro -- what we call a MIMIC system to look at various adjuvant studies.

The benefits to something like this are pretty clear compared to an animal



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model. Among other things is the fact that we can capture diversity in the population. We have a fully robotic platform to look at hundreds, if not thousands, of individuals to look at their various responses.

And some fo the benefits that we get are, of course, this is robotics so we can get a high throughput evaluation of what is occurring to look at multiple numbers of donors.

But also the thing that this system really affords is that one surrogate human really can contain all the controls whether it be no antigen, no adjuvant, vaccine, adjuvant alone, pathogen, et cetera, et cetera.

So one human or surrogate human can do this entire thing. And, of course, as we talked about, what we're hoping to get is faster cycle time in terms of understanding mechanistics and the mechanisms of action as well.

In terms of an overview of the MIMIC system, there are four main steps to this process. The first step is really blood collection from the donors that we get. We're located about -- a few minutes away from Florida's blood center, which is the fourth largest blood center in the U.S.

We've worked out all of the protocols for freezing and thawing the cells as well as purifying the PBMCs from the apheresis products that we have.

The next step is that we can take these PBMCs and then put them into the first module. So our system is very modular. And that we can dissect the mechanisms of immunity if we're looking at innate immunity, adaptive immunity, and then functional analysis as well.

So the first one is we talk about putting something into what we call the peripheral tissue module. This is really mimicking sort of skin or peripheral tissue.



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In this case we can look at innate immune responses such as reactogenicity as well as immunotoxicity.

This is where we pulse the antigen-presenting cells and we can take these and then put them into the second module, which we call the lymphoid tissue equivalent module, which is a culture of T cells, B cells, as well as follicular dendritic cells in which we can look at adaptive immune responses to look at antigen-specific T cells responses as well as B cell responses to either create CD4 help, CD8 cytotoxic T cells, or antibody responses.

We can then take these out of that module and put them into the third module, which is really a functional assay where we can look at hemoglutinin inhibition assays, microneutralization assays, or cytotoxic T cells assays.

So the MIMIC system compared to normal physiology is really shown in this

slide. It is fairly straightforward on why we've developed these various modules which can be put into a 96-well format. And all be robotically controlled.

And this way we can dissect, you know, the innate responses from the adaptive responses and also look at the entire system at the same time.

So in terms of the outline today, first I'll talk about the adjuvant studies that we've been able to look at in the PTE module and then we'll start to give an indication of how we would look at this in vitro system to dissect the mechanisms of action by looking at inflammation versus DC activation, looking at increased diapedesis that may occur across an endothelium, DC activation versus true antigen presentation in an antigen-specific way. And then even thinking about TH polarization as well.

We'll talk about how we can look at naked antigen versus adjuvanted and then

immunotoxicity of biologicals.

So in thinking about innate responses, the first one that we will talk about is the peripheral tissue equivalent module. In this case we can put PBMC -- what we have is we co-cast a collagen matrix inside of a 96-well format and grow a confluent and quiescent endothelium on top which sort of mimics blood vessels to first order.

We put PBMCs on top of the endothelium for about an hour and a half. And during that time monocytes primarily extravasate through the endothelium and then will spontaneously differentiate into antigen-presenting cells just like that occurs in vivo.

And what happens is these will differentiate into macrophages as well as dendritic cells. A large fraction of these dendritic cells will reverse transmigrate through the endothelium again, a process that mimics crossing the lymphatics on the way to

the lymph node. And this is typically where we will pulse these APCs with these dendritic cells with the adjuvant, the cosmetic formulation, the antigen, or the vaccine.

One of the things that we have been able to do is show that these cells are identical to dermal explants by doing extensive phenotypic analysis.

And by doing that, we found that there's primarily three types of antigen-presenting cells that come out of the peripheral tissue-equivalent module.

One is a subpopulation which is sort of immature CD14 positive dendritic cell precursors. The other one is an immature DC and the third type is a mature dendritic cell.

The ones that remain in the collagen matrix end up having more of a macrophage phenotype. And, again, we've done extensive phenotypic analysis and these are essentially identical to human dermal explants.

Now when we're looking at the effects of various adjuvants, what we wanted to do -- in this case we are looking at the effects of alum at various concentrations on the three types of dendritic cells, that reverse transmigrate out of the endothelium.

And you see that no matter -- when we have no treatment alum at various concentrations, the relative numbers remain about the same. But you see that we're not changing the APC phenotype by the addition of alum, which is consistent with what is found in the literature.

When we looked at alum -- and of course we can assess the supernatants to look at the generation of proinflammatory cytokines, and you can see that alum does indeed generate proinflammatory cytokines at lower concentrations.

As we increase the concentration significantly, we begin to see toxicity effects and the cells are beginning to die and

the cytokine production begins to diminish.

With alum, one of the things that came up is looking at the effects of alum versus alum with an antigen. And that's what this slide shows is when we have alum alone, we saw no change in the APC phenotype.

But when it is with an antigen, in this case it is with a plague vaccine where its fusion between the F1 and V antigens, you can clearly see by with different doses of the vaccine, we clearly see a change in the APC phenotype where it is changing to a more mature phenotype with the addition of alum with the antigen for this vaccine formulation.

So it brings up to what a lot of talks were about earlier today is looking at the adjuvant alone is important but just as important, looking at the adjuvant with the antigen is incredibly important as well because you get a different mechanism of action -- or you can.

With MF59, one of the things that

Hana Golding just showed in her talk and other talks by Dr. O'Hagan, mentioned the MF59 dose change. Indeed, the maturation state of the APCs, we see that in our in vitro MIMIC system as well.

Clearly you can see that by adding in MF59 at around 25 percent weight per weight is what is physiologically equivalent of what goes into a human, you see that we are getting more of a mature phenotype out of the dendritic cells.

And then with CpG, just looking at a total Aquaceptor 9 agonist, you can clearly see that we're getting a change in the APC phenotype as well. With the addition of CpG, we're seeing, again, a more mature phenotype at the expense of immature and DC precursors coming out of this peripheral tissue equivalent model, all consistent with what is in the literature.

And if we just look at relative comparisons between various types of cytokines

and chemokines that come out of -- that arise in the supernatant of the PTE module, you can clearly see that MF59 is relatively non -- does not secrete a lot of chemokines nor cytokines. Alum is somewhere in between. And CpG -- and this is on a log scale -- generates the largest concentration of proinflammatory cytokines and chemokines.

And when you put the whole story together, each of the mechanisms of action in and of themselves between MF59, alum, and this is aluminum hydroxide, I apologize, and CpG act quite a bit differently.

CpG, you get a large change in DC development as well as cytokine production. MF59, you are seeing large changes in DC development but very little change in cytokine generation. And alum, very little change in DC development but sort of middle-of-the-road -- sort of middlish cytokine generation.

So now just after giving some of these datasets with this in vitro system, I

wanted to start thinking about further dissecting the mechanisms of action of inflammation versus DC activation.

So this one, one of the things that we can do in the assay, and we just wanted to show, is that what we try to do is make this endothelium very quiescent to begin with.

And the way that we test this is by with neutrophil migration assays because typically you should only see about one percent of the neutrophils cross through the endothelium. And we can see that.

And we can also add in different types of proinflammatory cytokines on this to induce inflammation and induce neutrophil migration.

And that is really what this slide is showing is that we can have an inflammation model here as well where we can artificially make the peripheral tissue equivalent inflamed and, of course, we always want it quiescent



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when looking at adjuvants.

But when you are thinking about autoimmune diseases such as rheumatoid arthritis or other inflammatory diseases, this can be a useful inflammation model.

And one of the things that we typically do is well, for quality control, is that we can really show, in this case what we're looking at is the up-regulation of E-selectin by looking at the surface marker of CD62e, you can clearly see that when it is inflamed, we see an up-regulation of the surface marker on the endothelium.

And when at the control, you know, the types of error bars that we have, that we can get a very quiescent endothelium to begin. And, of course, the cytokines productions that come about when inflamed versus quiescent as well. And here we are artificially inflaming it, as I said, with a cytokine cocktail.

And one of the things we wanted to show is what MF59 is doing as just an example.

And what we are finding is that MF59, in and of itself, does not induce a lot of inflammation nor toxicity in this peripheral tissue equivalent module by looking at various different types of cytokines that are generated from the control versus MF59 at various concentrations from physiologically equivalent to below.

The next one is thinking about increased diapedesis because what you would anticipate is that if it is inflamed, you might open up the vasculature a little bit and you'll see increased diapedesis.

And so here is an example -- we're just giving a few examples here of MF59. And what we're showing here is when we have the no treatment.

So one of the things that we talked about is we put PBMCs on top of the endothelium. Mostly monocytes extravasate through but we're also getting some residual T and B cells as well as NK cells

transmigrating through the endothelium as well.

But what you can see is by -- with the addition of MF59, we're seeing a significant increase in diapedesis across the endothelium as well. So we can really start to dissect what may be occurring for a mechanism of action.

Last one is thinking about DC activation versus antigen presentation, and this one is just a small little cartoon that sort of shows that, you know, part of the fun of what we're doing is activating this DC and really getting it jazzed up and ready to go.

But really this DC means nothing if it can't find the right receptor match within the repertoire -- oh, it died. Oh, my gosh. I hope the computer didn't die. No, it didn't. Okay.

Sorry. This is really an R-rated movie. And given that we're near Washington, D.C., it is probably good that we stopped it

at G-rated.

(Laughter.)

DR. WARREN: Jay, I apologize for not showing the R-rated movie like you wanted, okay.

So anyhow, one of the things we wanted to do is show that we can pulse these antigen-presenting cells with the antigen and show specific T cell responses both to recall as well as primary T cells.

And here what we did is we looked at -- we're looking here at antigen-specific responses by looking at up-regulation of CD40 ligand as well interferon gamma through intracellular cytokine staining.

And we looked at standard PBMC assays. Standard PBMC assays were even added in DCs to sort of artificially get it going versus what we see when we put it in the MIMIC model.

And you can clearly see in the MIMIC model, whether we're looking at

secondary responses for influenza or tetanus toxoid, that the MIMIC model is giving significantly greater responses. And for primary responses, we looked at recombinant-protective antigen of Bacillus anthracis as well as MSP1 marozoite surface protein for malaria.

And clearly you can see that we are able to get antigen-specific T cell responses to both naive and recall responses.

Wanted to show that we can do it for multiple types of naive antigens, showing some of the hard core data. So those of you who don't believe pie charts can look at this.

And here we're looking at MSP1/AMA1 for malaria, KLH and GP120 from HIV. And we look at the culture and target conditions on top with no antigen, no antigen controls all the way to the far right where we have the specific antigen and the culture and the target as well.

You can clearly see we are above

the noise, any near noise floor, and looking at antigen-specific T cell responses in the MIMIC culture.

And we can also look at live attenuated vaccines. There was an excellent talk -- several talks today on yellow fever. And here just shows how we can look at live attenuated as well as inactivated vaccines as well.

And clearly we're seeing signals when looking at up-regulation of 154 and interferon gamma for antigen-specific T cell responses as well.

And just to give you an idea of the types of inter-donor variability that we see in the assay -- just wanted to show you for representative ten donors, the types of variabilities that we get within the assays.

Next we want to just talk about Th1/Th2 polarization because once this is in the lymphoid tissue equivalent, we can also assess the types of cytokines that are being

generated as well.

And here just wanted to show for yellow fever vaccine, well-known vaccine to generate a Th1 -- a balanced Th1/Th2 response, we are indeed showing this by the up-regulation of Th1 cytokines such as IL2 and interferon gamma as well as Th2-type cytokines such as interleukin 13 and IL5.

Next wanted to show differences between naked antigen versus adjuvanted antigen. In this case what we did is looked at RecombiVax, which is alum adjuvanted, versus the naked protein.

The thing to really note here -- and this is kind of a busy slide and I apologize -- but what we're looking at are the HPV -- or hepatitis B surface antigen-specific antibody responses, IgG via ELISPOT.

And we're testing it for RecombiVax. And note that antigen concentration is 50 nanograms per mil with the RecombiVax versus the naked antigen alone,



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which we had to go to five to ten micrograms, essentially a thousand times higher in concentration of the naked antigen, to get similar antibody responses, really showing that we can look at dose sparing issues as well as look at naked, you know, antigen and adjuvanted-antigen as well.

And last we'll talk about immunotoxicity of biologicals. We had two great presentations earlier in this session from Sanofi and Novartis talking about toxicity and how the -- and now we'll talk about how the MIMIC model might be used for this.

So what we wanted to do is look at in vitro reactogenicity and immunoregulatory or really immunomodulatory effects of various types of compounds. And we looked at various immunopotentiators such as Imiquimod. We've also looked at Gardiquimod, CpG, CpG control.

We've looked at different immunosuppressants such as cyclosporine

methotrexate as well as dexamethasone. And then we've also looked at monoclonal antibody therapeutics such as OKT3, which isn't really on the market any more, anti-CD154, CTLA-4, and anti-TNF-alpha.

And one of the things I just wanted to show really quickly the types of datasets that we can generate with the MIMIC system. Here we're looking at maturation of the dendritic cells in the model for the various different compounds that we looked at.

And you can see that the immunopotentiators generally led to up-regulation of maturation, which makes sense. That is what you would expect.

And CTLA-4 is really a human fusion protein that blocks the CD80/86 marker with respect to the interaction with the T cell. And you can clearly see that it is down-regulated which is, again, what you would anticipate to see.

We've looked at T cell



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proliferation as well as B cell proliferation. And, again, it comes as no surprises that, you know, things such as Imiquimod and CpG will induce B cell proliferation and immunosuppressants such as cyclosporine methotrexate would decrease it.

So we're seeing everything that is very consistent with the literature and expectations to sort of give proof of concept that the model is indeed behaving correctly. And, in fact, if we put the entire table together, and we have all the data to support this but just wanted to give it in a table form.

You can see for the -- like things such as CpG and Imiquimod, it's known to be more of an adjuvant, the MIMIC activity, it as showing as an immunopotentiator, cyclosporin methotrexate, immunosuppressants, and we're seeing immunosuppression of all the different types of aspects that we're seeing.

And for various of the monoclonal



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antibodies we are seeing things that are anticipated from the literature as well as from in vivo studies as well within this in vitro model.

And earlier today, Dr. O'Hagan from Novartis showed a slide and we talked with Jeff Ulmer, who also works at Novartis, and we asked him if we could modify the slide slightly to sort of fit our hypothesis or our thesis and where the MIMIC model would fit in to a lot of idea of adjuvant discovery when thinking about the various diverse libraries where we could do really rapid screens for immunogenicity.

And then find out if we have good hits, then follow on with immunotoxicity or toxicity in general. And then go on to understand structure/function relationships with this in vitro model.

And the nice thing is is that we can dissect things very easily. There was an earlier talk about the influence of Tregs.

We've looked at the influence of Tregs when we can pull them out of our assay or put them in to look at specific antigen-specific responses as well and we see marked changes going on there, too.

So in terms of conclusions, we understand that right now we believe that the MIMIC model does appear to replicate or at least be a biomimetic of human immunity. But we are also quite cognizant that validation is an ongoing process.

And each of our customers has a different way of validating the system. So it really is an ongoing process.

But the data does appear encouraging. Even though we've talked mostly about adjuvants today, we've done a lot of vaccine responses as well as immunotoxicity as well. And we see that some of the applications for adjuvants can be, with respect to optimizing formulation, thinking about QA/QC, dissecting the mechanisms of

action, as well as looking at immunotoxicity and immunoregulatory effects as well.

And with that, I'd like to thank the audience for staying for the very last talk as well as our funding agents and Amgen and Novartis for their respective works.

Thank you.

(Applause.)

PARTICIPANT: Yes, the T cell/B cell data looks quite good. I'm having a difficult time with the dendritic cell data with respect to the CpG effects because my understanding is in the human system, the TLR9 is really only expressed on human B cells and plasmacytoid DCs. And the type of DCs you are looking at are monocyte derived, from what I can understand in the system.

So I was just wondering have you been able to demonstrate TLR9 expression on those dendritic cells that are reverse transmigrating out of the endothelial layer because --



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DR. WARREN: Yes, that's a very good question. The thing that I forgot to mention is that in our model, we have indirect evidence that there are plasmacytoid DCs as well.

PARTICIPANT: Okay.

DR. WARREN: So it's not just monocytic-derived DCs. There are -- we don't have hardcore evidence because there are so few but we do have indirect evidence that plasmacytoid DCs are included in the assay as well.

PARTICIPANT: It's just that the facts, if you look at the conversion of those graphs, like 90 percent of the cells are up-regulating CD80. So it can't just be plasmacytoids that are doing it unless it is an indirect effect.

DR. WARREN: Yes. And as I mentioned -- so the other thing, which was not -- I didn't mention it as well as I could have is we do have a small number of residual B

cells that transmigrate into the endothelium or are stuck on the endothelium as well. So we do have the inclusion of B cells as well as plasmacytoid DCs in the model as well. And that can possibly explain some of the effects with CpG.

PARTICIPANT: Thank you.

DR. WARREN: Good observation. Thank you.

PARTICIPANT: Yes, I was quite excited to hear -- I'm right here.

DR. WARREN: Oh, okay. Sorry.

PARTICIPANT: I was quite excited to think of when you people came to visit us and also with the whole approach of VaxDesign to try to predict responses because we know how expensive it is to get in the clinic. And we'd like to be able to predict which thing we have that would work better for our particular antigens.

But I must say so far what you presented and what I've read, I'm not -- I



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don't know whether you have really convinced me anyway. Others may be quite convinced that your system will really predict for us what we need and whether you have done any critical studies that will give that answer.

I heard your talk. I heard all of your presentations. But I'm not, at this point, convinced.

DR. WARREN: Thank you for the comment even though I didn't want to hear that.

(Laughter.)

DR. WARREN: But I think that we always like the naysayers around because of the fact that part of our mission is to convert. But we can't show you all the data, unfortunately, because of the fact that the data that we can show is ones that we've conducted ourselves.

All of our data is owned by the customers. And they do not give us liberty many times to show the data.

But I think that if we are ever to unveil a lot of the datasets -- because each customer comes in -- because they all come in like you, which is very fair, is like hey, let me give this system a shot. I don't quite believe it. Let me test it.

And they come in with ten different compounds. And they want us to evaluate it, sometimes blinded, sometimes not. And then we have to sort of prove ourselves to each and every customer through a pilot program and work out way through that.

And, unfortunately, we're not allowed to publish a lot of those pilot projects because of the fact that sometimes they are formulations from the customers.

So it is a fair comment and fair observation. But, unfortunately, I think it is like most things. That you have to put your finger into the pudding and test it before you really can appreciate whether it is true or not. Or whether or not it tastes

good.

DR. GRUBER: Well, thank you very much. I found this presentation actually very stimulating.

I think there may be hope that we are on the way to perhaps define and develop alternative methods and methodologies to include in safety assessments of these vaccine products.

I wanted to ask Dr. Slater is there anything else? I wanted to give you the final word for this evening. He's coming up to the podium.

DR. SLATER: No. Just to wrap up more with housekeeping comments.

Please remember everybody, tomorrow morning we start at eight o'clock. If you are speaker at tomorrow's session, I would encourage you, if possible, to load your talks on to the computer either now or tomorrow morning a few minutes before eight o'clock.

Once we start, we will have a morning break and we will have a lunch break. But we will go directly from the first roundtable discussion into Session 4. So it would make things a little bit smoother if you get here a few minutes early and load your talks on.

Aside from that, it has been a long day. It's been an outstanding day. Thank you to all of the speakers today and thank you to all of the co-chairs.

Tomorrow promises to be just as long a day and probably more work because of the roundtable discussions which will involve a lot more give and take. So get a good rest tonight and we'll see you tomorrow morning.

(Applause.)

(Whereupon, the above-entitled workshop was concluded at 6:05 p.m.)

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