Research Activities

Project 1 - CD1d-binding NKT Stimulating Glycolipids as an Adjuvant for Malaria/HIV Vaccines

We aimed to identify a unique glycolipid which can exert potent stimulatory activity on invariant natural killer T (*i*NKT) cells and dendritic cells, while producing an adjuvant effect superior to that of α-GalCer. For this purpose, we performed step-wise screening assays on a focused library of approximately 100 α-GalCer analogs synthesized by our collaborator, Dr. Chi-Huey Wong's group at the Scripps Research Institute. Assays had included quantification of the magnitude of stimulatory activity on human *i*NKT cells *in vitro*, binding affinity to the invariant T cell receptor of human *i*NKT cells, and binding to human and murine CD1d molecules. Through this rigorous and iterative screening process, we have identified a clinical "lead" glycolipid, 7DW8-5, which exhibits a superior adjuvant effect as compared to α-GalCer on HIV and malaria vaccines in mice. This part of the project was summarized in our publication in *PNAS* in 2010 and reviewed in Clinical Immunology in 2011, and three US patents (7534434, 7923013, and 8163290B2) have been awarded based on this finding.

i) A glycolipid adjuvant against adenovirus-based human malaria vaccine

In collaboration with the U.S. Naval Medical Research Center in Maryland, we recently sought to determine whether 7DW8-5 would provide an adjuvant effect to a recombinant adenovirus (Ad) expressing a human malaria antigen in rhesus macaques. As a vaccine, we used the GMP grade of two recombinant Ad vectors, AdPfCS + AdPfAMA, one expressing the CS protein and another expressing an apical membrane antigen-1 (AMA-1) of *Plasmodium falciparum*, respectively. This combined vaccine has already been used in humans in a Phase I clinical trial with positive but modest results. Five groups of rhesus macaques received a single intramuscular (i.m.) immunization with 2 $x10^{10}$ virus particles of a 1:1 mixture of AdPfCS + AdPfAMA-1 with increasing doses of 7DW8-5 in each group: no adjuvant, 0.1μg, 1μg, 10μg, and 100μg. Macaques were then monitored for safety, innate immune responses and immunogenicity. We found that 7DW8-5 elicited dose-dependent local erythema at injection sites, but no evidence of systemic reactogenicity (fever, tachycardia, respiratory distress). As for the innate immune response, 7DW8-5 elicited activation of circulating monocytoid dendritic cells at 1 day post vaccination. Most significantly, 10 and 100 μg of 7DW8-5 appeared to optimally and consistently enhance malaria-specific CD8+ T cell responses by 10-fold, as shown in Fig. 1. The magnitude of response did not correlate with circulating NKT cell level, indicating that this approach may be useful across broad populations. Therefore, in conclusion, 7DW8-5 was found to provide a significant adjuvant effect on the cellular immunogenicity of an adenoviral vaccine in non-human primates. The manuscript was recently published in *PLoS ONE* in November, 2013. We are also preparing to conduct formal audited manufacturing, stability, and safety and toxicity studies of the adjuvant and antigen combination in anticipation of a Phase 1 clinical trial in humans.

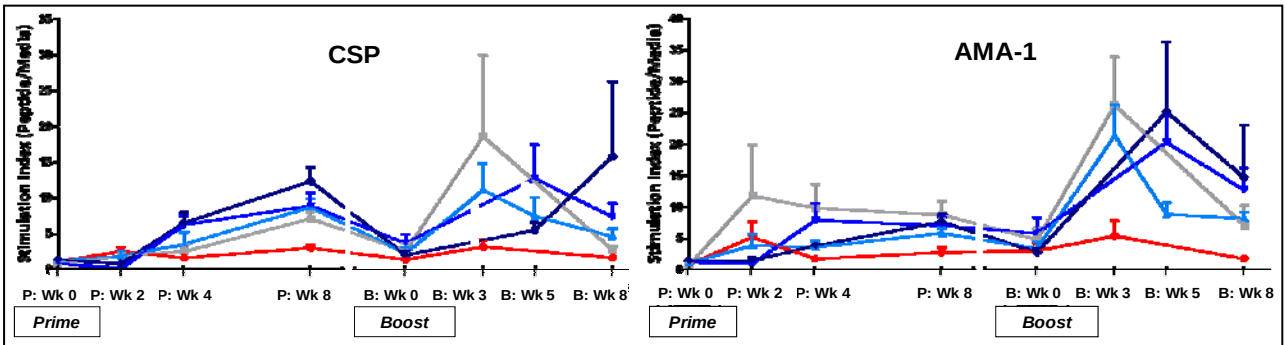

Fig 1. Adjuvant effect of 7DW8-5 on Ad-based malaria vaccine in monkeys. Rhesus macaques (5 per group) were immunized IM with 2x10^{10} v.p. of AdPfCS+AdPfAMA-1 with an ascending dose of 7DW8-5. Regimens: red lines (no adjuvant); gray lines (0.1μg adjuvant); light blue lines (1μg adjuvant); dark blue lines (10μg adjuvant); black lines (100μg adjuvant). The y-axis gives the stimulation index for the ELISpot assays equals (#spots with peptide/without peptide).

ii) Mechanisms underlying the adjuvant effect displayed by the glycolipids

In parallel, we investigated how 7DW8-5 can exert an adjuvant effect superior to α-GalCer. It is known that 7DW8-5 not only has a stronger stimulatory activity toward *i*NKT cells and DCs, but also has a higher binding affinity to CD1d molecules than α-GalCer. Much to our surprise, intramuscular (i.m.) administration of α-GalCer, but not 7DW8-5, induced a systemic production of cytokines including IFN-γ and IL-12, whereas both glycolipids induced a similar level of systemic cytokine response upon their intravenous (i.v.) administration (Fig. 2). To address the mechanisms underlying this disparate cytokine response upon different routes of administration, we set out to determine their biodistribution *in vivo*. For this purpose, we labeled each glycolipid with fluorophores without affecting their functions. We first introduced amino-linker to the 6'-OH position of its galactose head, which is known to be an unrelated site structurally for contacting the TCR of *i*NKT cells, and as expected, this did not affect the biological activity. We then labeled each modified glycolipid with AF680 or BODIPY-TR, through the amino-linker in collaboration with Dr. Akira Kawamura at Hunter college of CUNY. We found that the bioactivity of AF680-labeled, as well as BODIPY-labeled glycolipids against murine *i*NKT cells and human *i*NKT cells was almost identical to that of unlabeled glycolipids. Therefore, two different fluorophores, having a different excitation and emission wavelength, could be used for a whole body imaging by *in vivo* Imaging System (IVIS) and for a confocal microscopic analysis. In our IVIS studies, i.m. injection of AF680-labeled 7DW8-5 resulted in accumulating in the popliteal lymph nodes at 12 hr (Fig. 3, white arrows). However, AF680-7DW8-5 injected into CD1d/CD11c-deficient mice and AF680-α-GalCer injected into any mice failed to be localized in the draining lymph nodes upon i.m. injection. We then determined the bio-distribution of glycolipids at a cellular level using confocal laser scanning microscopic analyses. Having identified, by IVIS, the draining lymph as a target organ where the glycolipids get distributed, we dissected draining lymph nodes, at various time points, from 2 to 48 hours, after i.m. injection with BODIPY-TR labeled glycolipids and detected fluorophore-positive cells by a confocal laser scanning microscope. Lastly, we analyzed the percentage of CD11c+CD1d+DCs in popliteal lymph nodes upon i.m. (anterior tibialis) injection and found that 7DW8-5 injected by i.m. recruited DCs at the draining lymph nodes more rapidly than α-GalCer. We also found that 7DW8-5 administration resulted in increased expression level of CD1d by DCs than α-GalCer. These results have led to our hypothesis that the recruitment of DCs to the injection site and/or nearby draining lymphoid organs by 7DW8-5 contributes to a more localized cytokine response. One could conclude that 7DW8-5 is retained locally at the i.m. injection site and nearby draining lymph nodes due its higher binding affinity to CD1d molecules and/or the up-regulation of CD1d expression level on activated DCs by 7DW8-5. In the next step, we will investigate whether the local retention of 7DW8-5 or an increased activation of DCs induced by 7DW8-5 contribute to the superior adjuvant effect exerted by 7DW8-5, resulting in a stronger onset of the adaptive immune response elicited by vaccines.

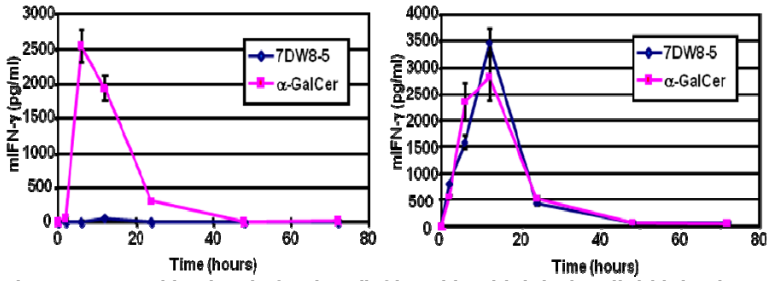

Fig 2. Sera cytokine level after i.m. (left) and i.v. (right) glycolipid injection. One μg of each glycolipid was administered by i.m. or i.v., and at indicated time, sera were collected and levels of IFN-γ determined by ELISA.

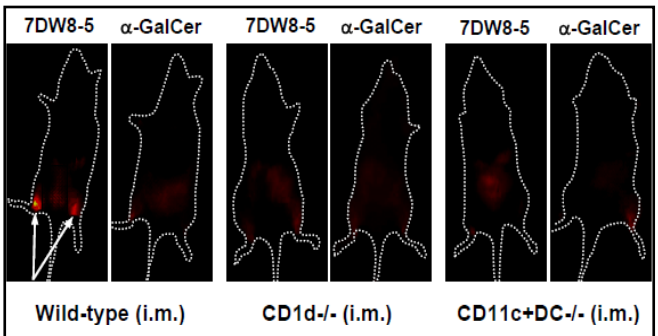

Fig 3. *In vivo* distribution of AF680-labeled glycolipids by IVIS. Five μg of AF680-labeled glycolipids were administered i.m. into wild-type, CD1d-/- or CD11c-/- BALB/c mice, and at 12 hr, the distribution of fluorophore was identified by IVIS.

Project 2 - Humanized Mice Model for Vaccine/Adjuvant Research

My laboratory has initiated the establishment of novel humanized mice that can mimic the human immune system (HIS), termed HIS mice. Basically, HIS mice have been made by infusing human hematopoietic stem cells (HSCs) to highly immuno-deficient NSG mice, which are made by backcrossing NOD/SCID mice to IL-2Rγ-deficient mice. These NSG mice lack almost in its entirety their mouse-derived immune system, including murine B cells, T cells and NK cells, and have impaired murine DCs and macrophages.

In order to develop a successful HIS mice model, we have utilized cutting-edge recombinant AAV-based gene transfer technologies. AAV is known to be one of the best vectors for delivering genes into mammals because of its many attractive features, including the characteristics of AAV being far less immunogenic and pathogenic than other viral vectors, while having the ability to infect both dividing and non-dividing cells. We have chosen AAV serotype 9 (AAV9) because, compared to AAV of other serotypes, AAV9 has additional advantages of not only transducing a high degree of transgenes into the host cells, but is also capable of infecting a wide range of different tissues in animals. We took two approaches to transferring human genes using AAV9 vectors. Firstly, we constructed AAV9 expressing various human cytokines that are known to facilitate the development and growth of human lymphocytes. After confirming the cytokine production by AAV9 vectors both *in vitro* and *in vivo*, we tested their effects on the development of human lymphocytes by administering them to NSG mice. As a source of HSCs, CD34+ cells were isolated from human fetal livers purchased from ABR Inc., and were infused i.v.to each of NSG mice that had received 150 rad of X-ray the day before. As shown in Fig. 4A, we identified three cytokines, i.e. human IL-3, IL-15 and GM-CSF, as the key cytokines that can facilitate the development of human lymphocytes expressing CD45 marker, a common leukocyte antigen, in NOG mice. We then determined that an administration of a cocktail of AAV9 expressing the 3 cytokines (AAV/hucytokines), further improved the reconstitution of human CD45+ cells in NSG mice (Fig. 4B). Secondly, we constructed AAV9 vector expressing HLA molecule. It has been shown that HLA-class I molecules can contribute to the development of human T cells from HSCs in HIS mice. Therefore, after having constructed AAV9 encoding HLA-A2 fused to human β2-microglobulin (AAV/A2), we administered it to NSG mice, followed by HSCs infusion. We found that the number of human CD45+ cells in NSG mice significantly increased upon intrathoracic (i.t.) and i.v. administration with AAV/A2 (Fig. 4C).

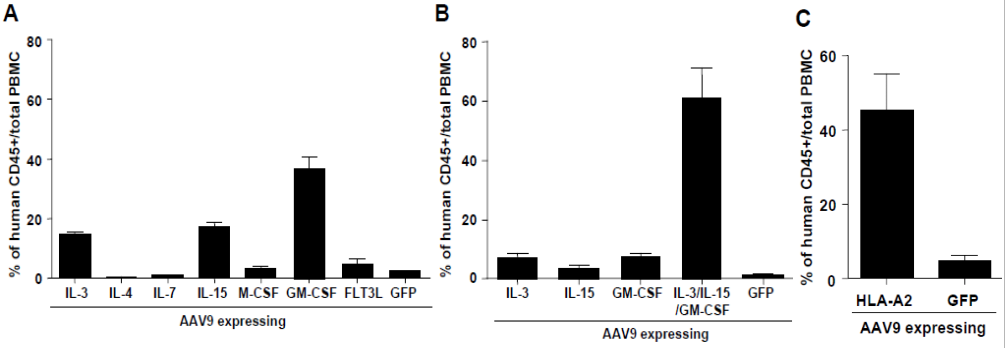

Fig 4. Reconstitution of HIS in NSG mice infected with AAV9 expressing selected human cytokines or AAV9 expressing HLA-A2. A group of NSG mice were infected i.v. with an optimal dose of each AAV9 expressing human cytokines (A) or in combination (B), or with AAV9 expressing HLA-A2 (C). Two-wk later, AAV9-infected NSG mice were sub-lethally irradiated and infused with human CD34+ HSCs. Fourteen-wk later, the percentage of human CD45+ cells engrafted among total PBMCs was determined by FACS.

Next, when we administered AAV/A2 in combination with AAV/hucytokines to NSG mice and compared to other HIS mice groups, as shown in Fig. 5A, we found that this combination was able to reconstitute almost 80% of human CD45+ cells, which was as good as that in A2-transgenic (A2-Tg) NSG mice, used as a positive control, at 14 weeks after infusion of HSC. When we determined the percentage of human T cells among human CD45+ population, the reconstitution level of human T cells in NSG mice receiving AAV/A2 and AAV/hucytokines (NSG-AAV/A2+hucytokines) was found to be even higher than that observed in A2-Tg NSG mice and close to that observed in human PBMCs (Fig. 5B). Therefore, we decided to vaccinate NSG-AAV/A2+hucytokines and other HIS mice groups with an adenovirus

expressing PfCS antigen, AdPfCS, and that expressing HIV-p24 antigen, Ad-p24, and measure the level of human CD8+ T cell response specific for PfCS antigen, and p24 antigen, respectively, by an ELISpot assay 2 weeks later. Briefly, we performed an ELISpot assay using peptides, corresponding to a CD8+ epitope of PfCS and p24 antigen. As shown in Fig. 5C, we found that more than 1,000 human T cells among a million splenocytes derived from NSG-AAV/A2+ hucytokines, were able to recognize the CD8+ peptide and secreted IFN-γ. This frequency was significantly higher than that induced in A2-transgenic NSG mice immunized with AdPfCS/Ad-p24. It is noteworthy that the level of human cytokines produced in HIS mice became undetectable at week 14 post AAV9 gene transfer, suggesting that there was no immune-modulating effect of human cytokines by the time when AdPfCS/Ad-p24 were vaccinated at 18 wk post AAV9 infection (at wk 16 post HSC infusion). The manuscript describing these results has just been published in *PLoS ONE* (February, 2014).

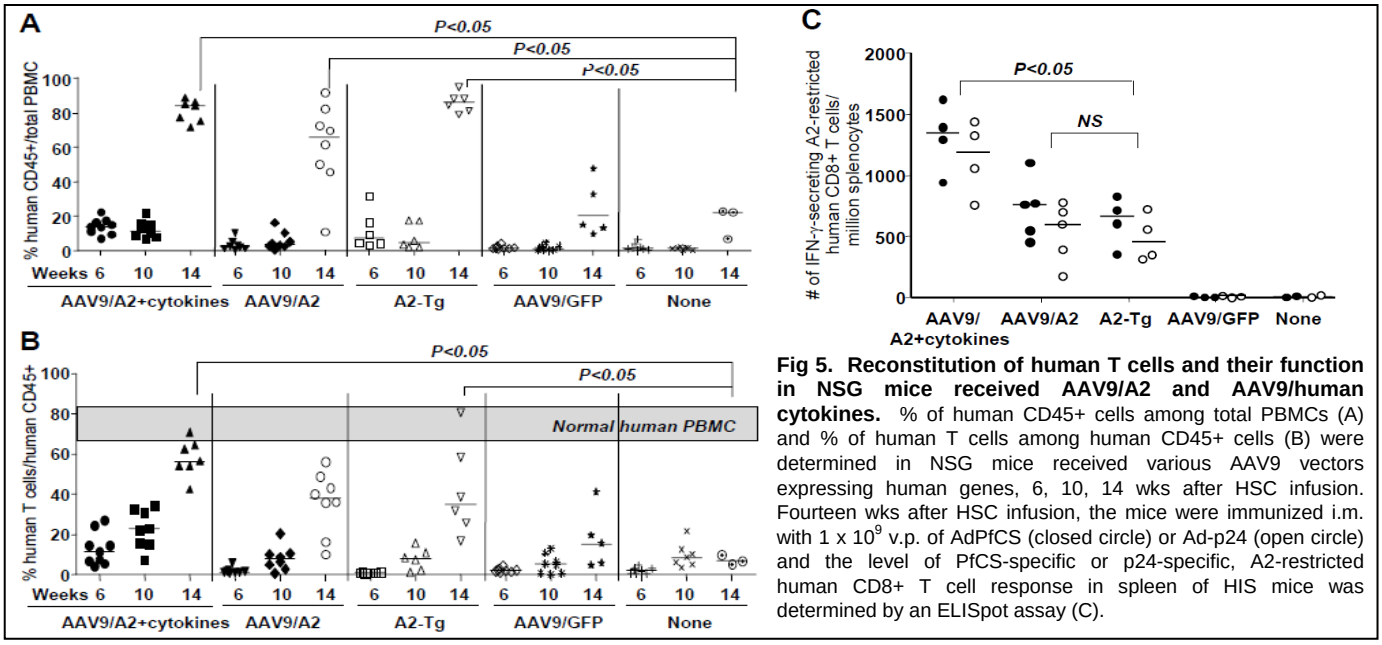

Fig 5. Reconstitution of human T cells and their function in NSG mice received AAV9/A2 and AAV9/human cytokines. % of human CD45+ cells among total PBMCs (A) and % of human T cells among human CD45+ cells (B) were determined in NSG mice received various AAV9 vectors expressing human genes, 6, 10, 14 wks after HSC infusion. Fourteen wks after HSC infusion, the mice were immunized i.m. with 1 x 10^9 v.p. of AdPfCS (closed circle) or Ad-p24 (open circle) and the level of PfCS-specific or p24-specific, A2-restricted human CD8+ T cell response in spleen of HIS mice was determined by an ELISpot assay (C).

Now that we have successfully developed HIS mice having functional CD8+ T cells, using funds from the Mark S. Bertuch AIDS Research Fund award and the Otsuka Pharmaceutical Co. Ltd., we have been seeking to establish HIS mice which possess human CD4+ T cells and B cells. For this purpose, we first constructed AAV9 encoding HLA class II molecules (AAV9/HLA-II) and tested whether the *in vivo* administration of AAV9/HLA-II could facilitate the engraftment and development of human CD4+ T cells in NSG mice upon HSCs infusion. Based on the anthropology data of HLA types among humans, we have identified HLA-DR1, DR4, DR7, DR11 and DR13 as class II ligands covering the make-up of nearly half of the world's population. As for human B cell engraftment and development, we plan to construct AAV9 vector expressing BAFF. BAFF is a B cell survival factor critical for B cell survival and differentiation. After confirming *in vivo* expression of various HLA-II and human BAFF molecules by the AAV9 vectors, we will infect NSG mice with them along with AAV9 vectors expressing various human cytokines listed above, and monitor not only the number of human CD4+ T and B cells upon HSCs infusion, but also the functionality of these cells by immunizing HIS mice with an antigen derived from human pathogen, followed by monitoring the induction of human IgG against the antigen. Ultimately, our HIS mice will be used to: 1) determine efficacy of candidate HIV/malaria vaccines with or without an adjuvant; and 2) study the mechanism of human protective immunity against HIV/malaria infection.

Project 3 – Discovery of Carbohydrate-Specific T Cell, "Tcarb"

Infection with Group B Streptococcus (GBS), also known as 'Streptococcus agalactiae', can cause serious illness and sometimes death, especially in newborn infants, the elderly, and patients with compromised immune systems. Approximately 20% of women are colonized with GBS in the vagina or rectum, and it can be harmful to both mother and the baby itself. Infection of GBS may result in neonatal death due to severe neonatal infection or occasionally in maternal death, by causing upper genital tract infection leading to septicemia. GBS is also a prominent veterinary pathogen, because it can cause bovine mastitis in dairy cows. Although the use of antibiotics has been a common practice to prevent the infection, GBS is increasingly becoming resistant to the antibiotics, thus efforts have been focused on the development of a vaccine against GBS. However, because of the unique nature of GBS, having capsular polysaccharide (PS) made up of the outermost layer of the bacterial surface, it is difficult to raise antibody against GBS. Therefore, glycoconjugate vaccines against GBS have been developed under the assumption that eliciting a potent humoral response to bacteria-derived PS requires coupling to a carrier protein that activates CD4+ T cells to help B cell producing the relevant antibodies (Fig. 6). In other words, it has long been thought that conventional CD4+ T cells with a variant T-cell receptor (TCR) could recognize peptides only in the context of major histocompatibility-class II molecules. Consequently, it has been normal practice to increase the immunogenicity of weak T-dependent antigens, such as glycans and haptens, by conjugating them to carrier proteins containing a number of dominant CD4+ T-cell epitopes. The traditional explanation for the mechanism of humoral response induction by glycoconjugate vaccines is that the carrier protein portion of the conjugated vaccine stimulates CD4+ T cells, which mediate B cell secretion of antibodies against glycans and/or haptens through a cognate interaction. However, in collaboration with Dr. Dennis Kasper's group at Harvard University, we

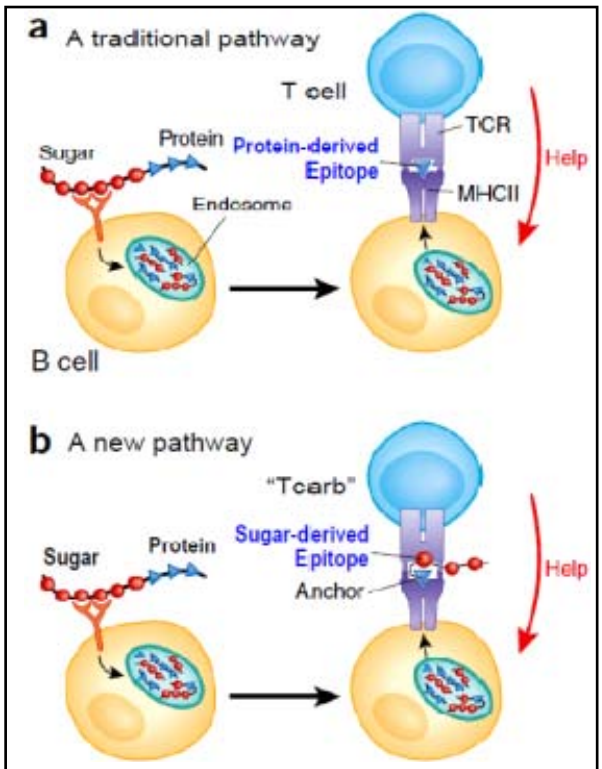

Fig 6. Two pathways of glycoconjugate-processing & presentation to T cells (Adapted from Rappuoli & De Gregolio, *Nat Med* 17; 1551-1552, 2011)

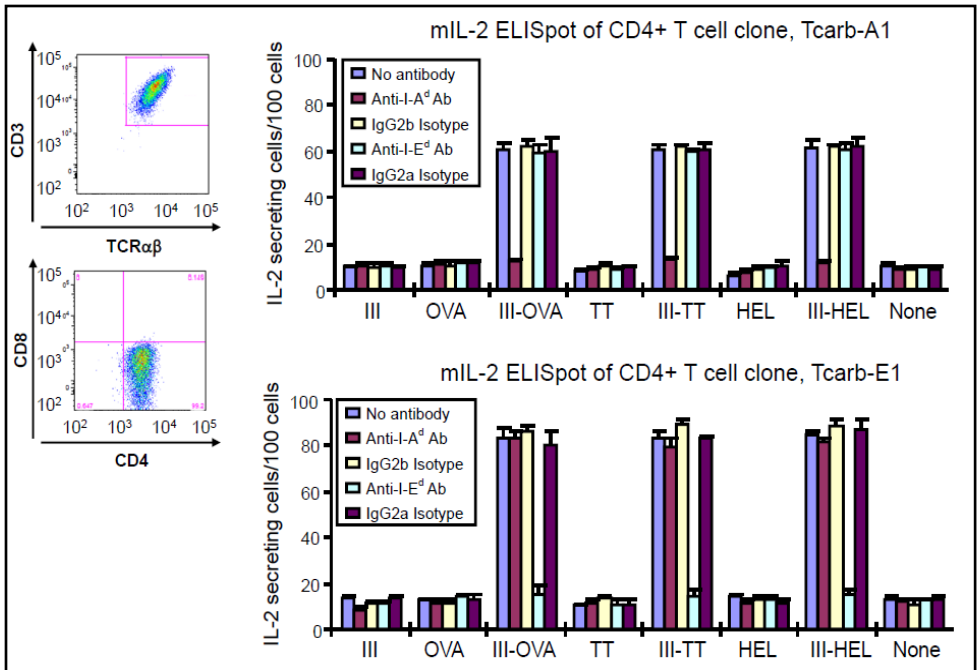

Fig 7. Antigen-specificity and MHC-restriction of two Tcarb clones. IL-2 and IL-4 ELISpot assays using two distinct CD4+ αβTcarb clones were conducted with various antigens, in the presence or absence of mAb to I-A^d or I-E^d or isotype controls. Irradiated naïve mouse splenocytes were used as APCs.

have recently published a study in *Nature Medicine* in December, 2011, showing that immunization of glycoconjugate vaccine can also induce CD4+ T cells that recognize the carbohydrate portion of the vaccine in addition to those recognizing the carrier protein (Fig. 7). In this study, my group was able to isolate two CD4+ T cell clones from lymphocytes of mice primed with a glycoconjugate, consisting of type III polysaccharide of group B *Streptococcus* (GBSIII) coupled to ovalbumin (OVA), followed by re-stimulation *in vitro* with GBSIII coupled to tetanus toxoid (TT). Our manuscript describing the detailed method of establishing Tcarb clones was most recently accepted for publication in *Nature Protocols* in November, 2012. These two CD4+ αβ T cell clones recognize the carbohydrate portion of the glycoconjugate vaccine, with one clone recognizing GBSIII in the context of I-A^d molecule, while the other, as GBSIII is presented by I-E^d molecule (Fig. 7). These Tcarb clones failed to recognize the linker region of the glycoconjugate vaccine (Fig. 8). Thus, my group has compellingly demonstrated for the first time the presence of carbohydrate-specific CD4+ T cells, which we named "Tcarb". Since then, we have been able to establish several more Tcarb clones that recognize carbohydrates. Interestingly, all Tcarb clones so far produce both IL-2 and IL-4 but not IFN-γ upon carbohydrate recognition (Table 1).

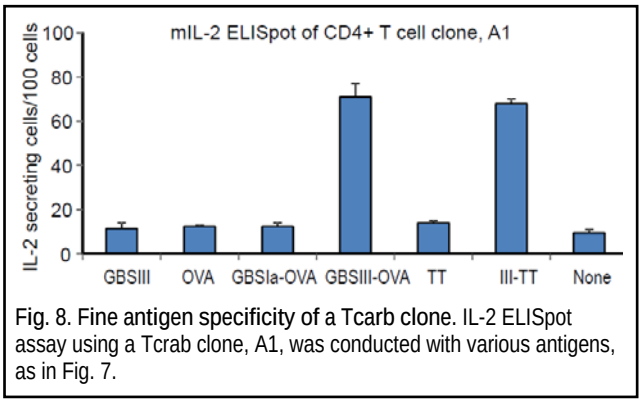

Fig. 8. Fine antigen specificity of a Tcarb clone. IL-2 ELISpot assay using a Tcrab clone, A1, was conducted with various antigens, as in Fig. 7.

The goal of this project is to determine the role of Tcarb cells in protection against streptococcal infection. Therefore, to accomplish this, we will first determine the physiological role of Tcarb, by investigating whether Tcarb can recognize and react with the GBS itself both *in vivo* and *in vitro*. We will then determine the role of Tcarb in promoting anti-glycan (GBSIII) antibodies *in vivo*, and whether Tcarb can mediate the protection against Streptococcal infection. We hope that our project will be able to change paradigm in T cell biology by shedding light on the important role played by Tcarb. Furthermore, in light of a recent finding by Dr. Kasper's group that an archetypical glycoconjugate vaccine, constructed to express high density carbohydrate epitopes, which are recognized by Tcarb, is 50-100 times more potent and significantly more protective than any currently used vaccine construct in an animal model of infection, we hope that our project can contribute to the future development of novel and more immunogenic carbohydrate-based vaccines against not only pathogens but also cancers.

Table 1. Properties of several Tcarb clones and OVA-specific CD4+ T cell clones.

Name	Ag specificity	MHC-restriction	CD4/CD8	Cytokines	Helper/ Protective Function
Tcarb-A1	GBSIII	A^d	CD4+	IL-2(+),IL-4(+), IFN-γ(-)	TBD
Tcarb-A2		A^d			
Tcarb-A3		A^d			
Tcarb-E1		E^d			
Tcarb-E2		E^d			
1-8	OVA	A^d		IFN-γ(+), IL-4(-)	
5.9.24					

Project 4 - Mechanisms of Induction of Protective Anti-Malarial CD8+ T Cells

The advancement in the development of malaria vaccines has been significant in the past several years. While a convincingly effective malaria vaccine has yet to be developed, the elucidation of the mechanisms of the protective anti-malaria immunity is imminent. The protective immunity against pre-erythrocytic stages of malaria, which include sporozoites and liver stages, has been known to be mediated in part by antibodies and in part by T cells, notably CD8+ T cells. The protective role of CD8+ T cells against the liver stages of malaria is now well established in a mouse model, in which rodent malaria parasites undergo a similar life cycle as that of human malaria parasites. Although the mechanisms by which the CD8+ T cells exert their anti-plasmodial activity have been extensively investigated and determined to some extent, it still remains largely unknown with regards to how or where malaria vaccines induce CD8+ T cells that can mediate protective anti-malaria immunity *in vivo*. In particular, in view of a recent study showing that vaccination of irradiated sporozoites (IrSp) by intravenous route, but not other routes, efficiently induced malaria-specific CD8+ T cell response in monkeys and mice and ultimately protective anti-malaria immunity in mice, it is imperative to address this key question in conjunction with different administration routes of selected T cell-based malaria vaccines (Fig. 9). In addition, another recent key study has shown that the administration of live *P. falciparum* sporozoites by the bites of mosquitoes, followed by chloroquine treatment induced significant malaria-specific pluripotent effector memory T cell responses and sterile immunity in all 10 vaccinated volunteers upon malaria challenge. This study indicates that sporozoites may need to be fully matured in hepatocytes in order to effectively induce protective CD8+ T cells (Fig. 9).

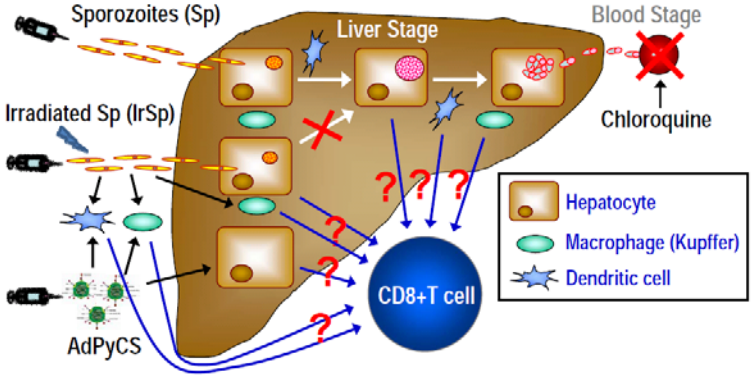

Fig. 9 Possible mode of induction of malaria-specific CD8+ T cells by pre-erythrocytic malaria vaccines. Live sporozoites injection followed by chloroquine administration allows parasites to develop through entire liver stages, whereas irradiated sporozoties (IrSp) vaccination allows parasites to infect hepatocytes but not replicate. Upon vaccination, AdPyCS infects various APCs that include DCs and macrophages, as well as hepatocytes, but its tropism depends on the vaccination routes.

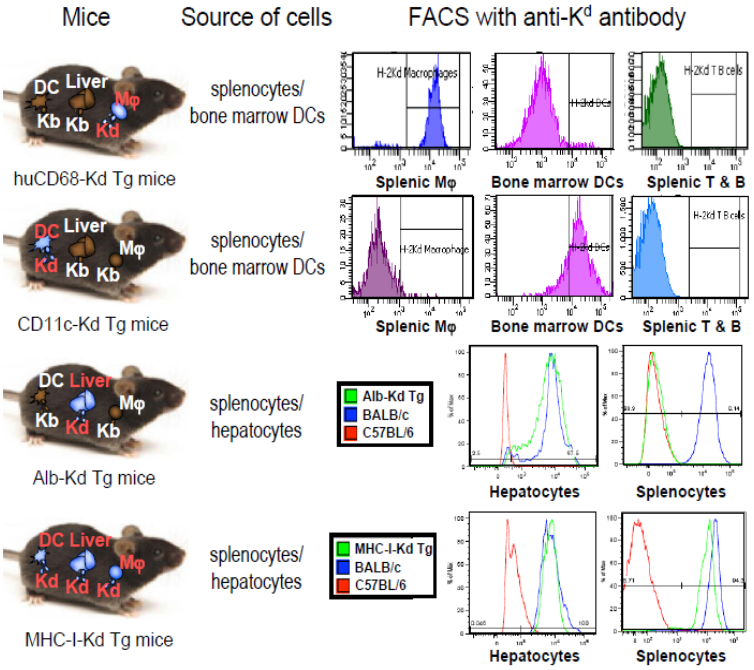

Fig. 10 Tissue-specific expression of H-2K^d molecules in various Tg mice. Splenocytes, hepatocytes and bone marrow DCs were isolated from each Tg mouse, and the level of K^d expression by specific cells was determined by gated on CD11c (DC), F4/80 (Mφ), CD3 (T), IgG (B) and albumin (hepatocyte).

Up to the present, there is only one known CD8+ epitope, SYVPSAEQI derived from the PyCS protein, which mediates the anti-malarial protection and is presented in the context of K^d molecule. Therefore, in order to provide answers to the mechanisms underlying the induction of protective anti-malarial CD8+ T cells, we have generated C57BL/6 transgenic (Tg) mice, in which K^d molecule is expressed only on hepatocyte (Alb-K^d), macrophage (huCD68-K^d) or dendritic cell (DC) (CD11c-K^d), by using albumin promoter, huCD68 promoter or CD11c promoter,

respectively (Fig. 10). We have also generated MHC-I-K^d Tg mice, which express K^d molecule under MHC-I promoter, as a positive control (Fig. 10). The description of the establishment of various K^d-Tg mice has been published in the *Journal of Immunological Methods* in January, 2013.

In order to determine that K^d molecule expressed by hepatocytes is sufficient for CD8+ T cells to exert their anti-plasmodial activity *in vivo*, *we* first immunized one MHC-I-K^d Tg mouse i.m. with AdPyCS and generated a PyCS-specific CD8+ T cell line, which were then adoptively transferred to MHC-I-K^d Tg mice (as a positive control), CD11c-K^d Tg mice, huCD68-K^d Tg mice and Alb-K^d Tg mice. All transferred mice were then challenged with live malaria parasites. Mice that did not receive the CD8+ T cell transfer were used as a negative control. Briefly, a PyCS-specific CD8+ T cell line was generated by FACS sorting with a K^d-SYVPSAQI tetramer complex (provided by the NIH tetramer core facility), from splenocytes of MHC-I-K^d Tg mouse immunized with AdPyCS. After expanding PyCS-specific CD8+ T cell population further and confirming the frequency of SYVPSAEQI-specific CD8+ T cells to be approximately 90% by the tetramer staining and ELISpot assay (Fig. 11A), we then adoptively transferred 5 x 10^6 cells of PyCS-specific CD8+ T cell line to each group of naïve K^d Tg mice, followed by a malaria challenge. The PyCS-specific CD8+ T cell line inhibited more than 90% of the parasite burden in the liver of Alb-K^d Tg, as well as MHC-I-K^d Tg mice, but only partially in CD11c-K^d Tg or huCD68-K^d Tg mice (Fig. 11B). These results indicate that K^d molecule expressed by hepatocytes, but not by DCs or macrophages, is sufficient in mediating the anti-parasitic effect of PyCS-specific CD8+ T cells *in vivo*.

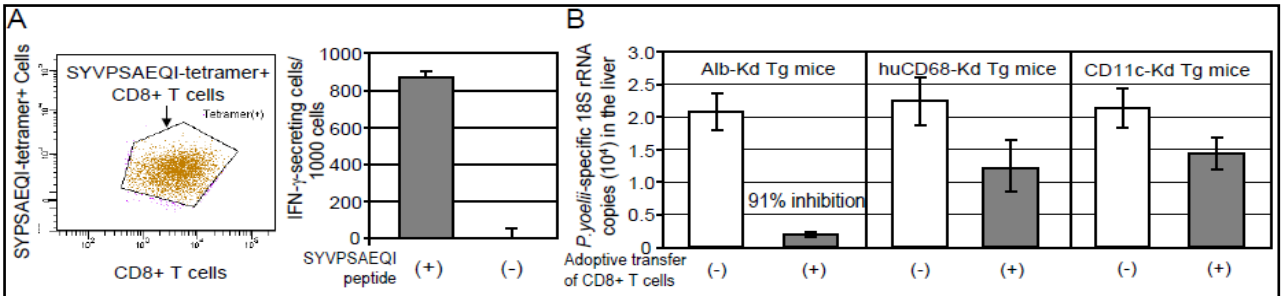

Fig. 11 Specificity and protective capacity of a PyCS-specific CD8+ T cell line. A PyCS-specific CD8+ T cell line was generated by FACS sorting with SYVPSAEQI-tetramer from spleens of 3 AdPyCS-immunized MHC-I-K^d Tg mice, followed by restimulation with SYVPSAEQI peptide 3 times with 10-day interval *in vitro*. In (A), 10 days after the last restimulation, the frequency of SYVPSAEQI-specific CD8+ T cells was determined by the tetramer staining and ELISpot assay. In (B), 5 million cells of the CD8+ T cell line were adoptively transferred to each naïve Alb-K^d Tg, huCD68-K^d Tg and CD11c-K^d Tg mouse. Mice received no cells were used as a negative control. One day after the adoptive transfer, all the mice were challenged with live malaria parasites and the inhibition of liver stage development was assessed by measuring the amount of parasites' rRNA in the liver by real-time RT-PCR.

In this project, we are currently immunizing these K^d Tg mice with various T cell-based malaria vaccines including IrSp, live sporozoites followed by treatment with chloroquine, or a recombinant Ad5 expressing the PyCS antigen (AdPyCS), by various routes. We will then determine the quantity, quality and durability of PyCS-specific CD8+ T cell response induced in each group of K^d Tg mice. We will also challenge these immunized K^d Tg mice with live malaria parasites at various time post vaccination to determine the level and persistence of protective immunity induced *in vivo*. In order to determine type(s) of APCs that can induce PyCS antigen-specific, protective CD8+ T cells in various K^d Tg mice that received malaria vaccines through different routes, we will isolate K^d-expressing (K^d+) cells from CD11c-K^d, huCD68-K^d, Alb-K^d Tg mice immunized with malaria vaccines through various different routes. We will then determine the ability of isolated K^d+ cells to activate and induce PyCS-specific CD8+ T cells *in vivo* and *in vitro*, and mount CD8+ T cell-mediated protective anti-malaria immunity *in vivo*. Lastly, we will isolate PyCS-specific CD8+ T cells from various organs of malaria-immunized, various K^d Tg mice and adoptively transfer them to naïve MHC-I-K^d Tg mice. Upon malaria challenge of the transferred mice, we will determine the protective capacity of the CD8+ T cells. Overall, we believe that the identification of the induction mechanisms of anti-malarial "protective" CD8+ T cells could ultimately lead to the vastly improved designs of potent T cell-based vaccines against human malaria.