A supramolecular chemistry approach for potentiating live attenuated whole-organism vaccines

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6

Summarizing Discussion

In this dissertation, the development of a strategy for chemically tuning SPZ immunogenicity has been presented. This strategy envisioned the use of supramolecularly complexed polymers to introduce adjuvants onto the SPZ cell surface. **Chapter 2** described adaptation of the strategy – previously used to functionalize eukaryotic cells (Rood et al., 2017) and microparticles (Spa et al., 2018) – to bacterial cells, and assessed its compatibility with immune functioning. In **Chapter 3**, the functionalization strategy's *in vivo* compatibility was gauged using a bacterial pre-targeting paradigm. **Chapter 4** added an adjuvant to the chemical system and investigated whether doing so could augment the immunogenicity of poorly immunogenic bacteria. **Chapter 5** outlined translation of this chemical augmentation strategy to malaria SPZ, and assessed their immunogenicity *in vitro* and *in vivo*. Altogether, the results demonstrate the conceptual feasibility of chemically tuning the immune response to SPZ, thus indicating a path forward to generating SPZ-based malaria vaccines of high efficacy for use in malaria-endemic areas.

A key step in developing a (supramolecular) strategy for chemically augmenting SPZ lay in adapting a recently developed supramolecular host-guest functionalization strategy. In this strategy, initial pre-functionalization of a cell surface with a supramolecular guest, adamantane (Ad), is followed by complexation of polymers bearing the cyclodextrin (CD) host – polymers that can theoretically serve as a vehicle for introducing other moieties of interest. Adaptation of the chemical strategy, to bacteria, was first described in **Chapter 2**. Here, pre-functionalization of bacterial cell membranes with the supramolecular guest Ad was achieved by conjugating it to a cationic antibacterial peptide (UBI_{29-41}) previously shown to have an affinity for bacterial cell surfaces (Welling et al., 2000). The UBI_{29-41}-Ad construct yielded excellent pre-functionalization of bacterial surfaces, with >98% of Gram-positive and -negative bacteria showing complexed polymer *in situ*. **Chapter 3** built on these findings by demonstrating the concept's compatibility *in vivo*: tissue-resident bacteria pre-functionalized with UBI_{29-41}-Ad led to a 16-fold higher accumulation of intravenously administered polymer in infected tissue. Ultimate adaptation of the chemical strategy to SPZ in **Chapter 5** involved another, different method of Ad pre-functionalization. Here, the target was the abundant circumsporozoite protein (CSP) sheathing malaria SPZ (Yoshida et al., 1980). Pre-functionalizing CSP with Ad was accomplished by chemically conjugating Ad to lysine residues of CSP, which yielded excellent functionalization of parasites *if* permanently immobilized by metabolic inactivation. In general, these findings suggest that supramolecular functionalization techniques can be flexibly adapted to different cell types given effective Ad pre-functionalization.

An important first test of the chemical strategy was its compatibility with (normal) immune functioning. To this end, **Chapter 2** investigated the response

of macrophages, immune cells important for initiating immune responses, to chemically functionalized bacteria. The classic response of macrophages to microbes is to engulf them. This process was unimpaired by functionalization: time-lapse confocal microscopy showed that macrophages effectively phagocytosed functionalized bacteria within 10 minutes of encounter. Furthermore, two key macrophage effector mechanisms – surface marker modulation and cytokine production – remained responsive to functionalized bacteria. Addition of adjuvant to the chemical system, for bacteria in **Chapter 4** and SPZ in **Chapter 5**, similarly yielded normal responses by macrophages. These findings validated supramolecularly complexed polymers as good vehicles for introducing immunomodulatory moieties onto microbial cell surfaces, at least in the context of attempting to modulate the immune responses of promiscuous players such as macrophages – further investigation in relation to more discerning immune cells, such as dendritic cells, is needed to fully assess the presented chemical system's general applicability.

Does introduction of immunomodulatory agents onto microbial cell surfaces in fact boost immunogenicity? This key question was the primary focus of **Chapter 4**, where the poorly immunogenic bacterium *Staphylococcus aureus* served as a model system. Here, addition of a TLR7 agonist-based adjuvant (CL307) to the chemical system introduced about 10^7 units of adjuvant per bacterium. These chemically augmented bacteria did indeed possess an improved immunogenicity, inducing four-fold increases in production of pro-inflammatory IL-6 by *in vitro* macrophages compared to wild-type bacteria. **Chapter 5** showed the same effect for SPZ, but to an even greater degree: SPZ chemically augmented with adjuvant induced large, 35-fold increases in IL-6 production by *in vitro* macrophages compared to control SPZ. These data confirmed the feasibility of boosting microbial immunogenicity by augmentation with immunomodulatory agents. Notably, for both bacteria and SPZ pro-inflammatory responses by macrophages were superior when adjuvant was complexed to the cell surface as compared to delivered in soluble form. This observation is in good keeping with previous adjuvanting studies for subunit and nanoparticle-based vaccines showing the benefit of physically co-localizing adjuvant (Cadoz, 1998; Francica et al., 2016; Lynn et al., 2020; Rutgers et al., 1988; Wilson et al., 2013), and is to our knowledge the first direct demonstration thereof for cells.

In vivo mouse models provided a more comprehensive assessment of chemically augmented SPZ's immunogenicity. As mentioned above, **Chapter 3** had shown the *in vivo* compatibility of the chemical system in a bacterial pre-targeting model, with good stability for up to 24 hours post administration. These findings supported the use of a well-established *in vivo* paradigm in **Chapter 5**, where a prime-boost immunization regimen with (chemically augmented) SPZ was followed by analysis of tissue-specific immune responses. This analysis, particularly

for the liver (where malaria infection begins), showed several indicators of an improved immune response. One was the reactivity of liver leukocytes towards a generic pro-inflammatory stimulus, with significantly increased production of several pro-inflammatory cytokines. More strikingly, SPZ re-stimulation of liver leukocytes from animals immunized with chemically augmented SPZ produced higher frequencies of IFN-γ^+ NK cells, $CD4^+$ T cells and $CD8^+$ T cells – cells shown to play an important role in malarial immunity (Ewer et al., 2013; Schofield et al., 1987; Wolf et al., 2017). These data indicated an improved liver immune response, especially towards SPZ, and confirmed the conceptual feasibility of chemically augmenting SPZ as a means to improving immunogenicity. Moreover, the specific increases in IFN-γ^+ cells induced by a chemically coupled TLR7 agonist, a response similar in nature to previous studies also using TLR7 agonists (Ahonen et al., 1999; Vascotto et al., 2019; Wille-Reece et al., 2005), suggest that chemical augmentation with specific adjuvants can boost specific immune responses. That being said, the functional benefit of these boosts – *i.e.*, improved protection from malaria challenge post immunization – remain to be elucidated in future studies of the concept, in order to more fully gauge the utility of chemical augmentation of SPZ-based malaria vaccines with adjuvants.

Future perspectives:

This dissertation has shown that chemically augmented SPZ engender improved immune responses. The next step in moving the concept forward will be assessing whether these improved immune responses translate into improved protection in an immunization-challenge model. Here, it will be particularly interesting to see how the variable of SPZ immobilization, which yielded robust chemical augmentation, factors into protection. Immobilized SPZ are recognized to be logistically preferable to motile parasites, given that no liquid nitrogen is required for storage and transport. However, their inability to initiate a liver infection appears to lower their protective efficacy (Doolan and Martinez-Alier, 2006). It will thus be interesting to see whether chemical augmentation could shore up this shortcoming, and actually exceed the efficacy of motile, unadulterated SPZ. Should this not be the case, a refinement of the chemical strategy here presented would involve adapting the method to motile, infectious SPZ.

To this end, heparin oligosaccharides appear potentially good candidate scaffolds for chemically augmenting motile SPZ. Mechanistic studies have demonstrated that SPZ's rapid invasion of the liver after entering the bloodstream is mediated by a strong affinity between the parasite's circumsporozoite protein (CSP) sheath and the highly sulfated glycans decorating hepatocytes (Cerami et al., 1992; Pancake et al., 1992). Logically, then, a highly sulfated glycan, such as heparin, should be able to bind SPZ – possibly, without adversely affecting

motility. Preliminary data supports this concept's feasibility. Heparins fluorescently labeled with Cy5 dyes (Figure 1a) appear to indeed have excellent affinity for SPZ, with confocal analysis showing strong Cy5 signals co-localized with SPZ (Figure 1b). Quantitation by flow cytometry similarly showed SPZ reliably bound with large quantities of heparin (Figure 1c). Crucially, SPZ with heparin bound showed unimpaired movement during time-lapse microscopy analysis (Figure 1d). These promising results indicate a path forward to chemically functionalizing SPZ whilst preserving motility, and offer another tool for continuing to optimize the immunogenic properties of SPZ in pursuit of more efficacious malaria vaccines for use in endemic areas.

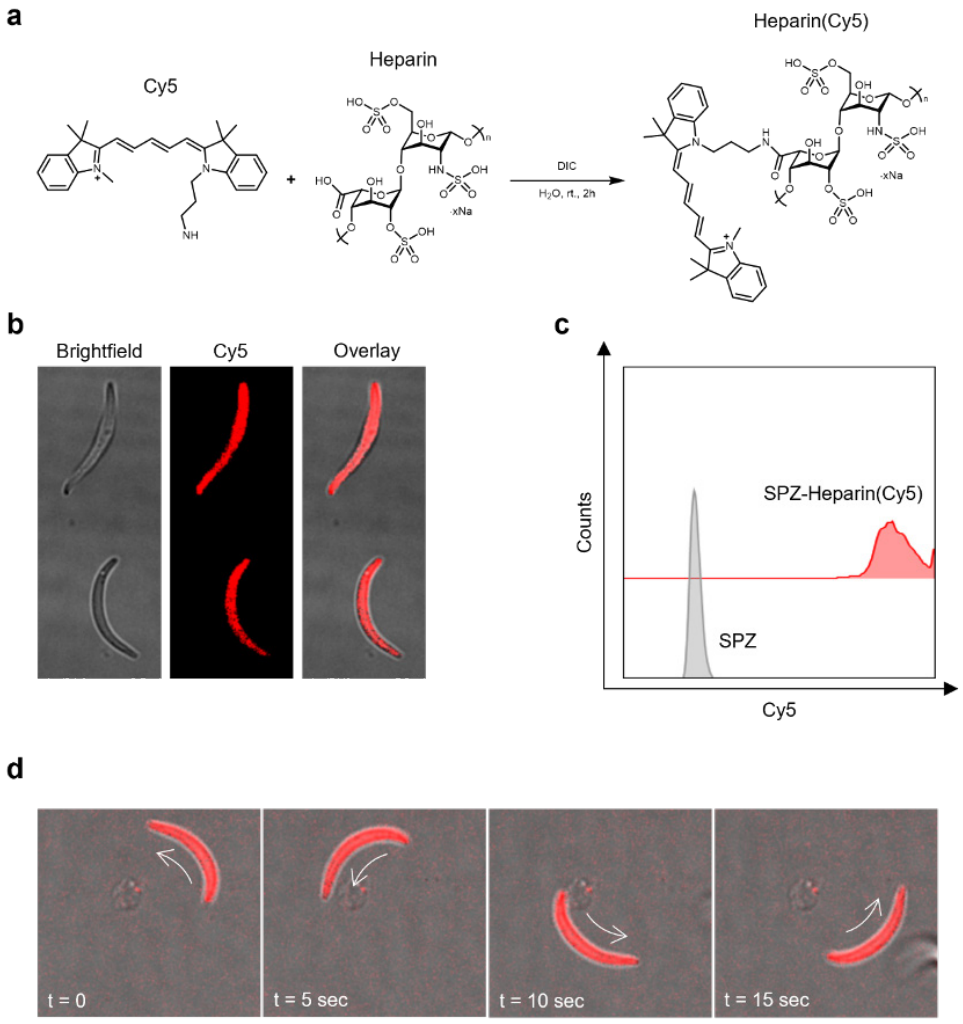

Figure 1: Heparin oligosaccharides can functionalize SPZ without perturbing motility. a) Reaction scheme showing synthetic route for generation of Cy5-labeled heparin construct. **b)** Confocal images showing co-localization of heparin(Cy5) construct with SPZ. **c)** Flow cytometry histogram showing Cy5 signal of heparin-bound SPZ (top: red) versus control SPZ (bottom: gray). **d)** Time-lapse confocal images showing a heparin-bound SPZ moving over the course of 15 seconds.

Beyond malaria, chemically augmenting cellular immunogenicity may also offer value for other (cell-based) vaccines lacking in efficacy. An obvious candidate here is the tuberculosis (TB) vaccine Bacillus Calmette-Guerin (BCG), derived

from an attenuated strain of bovine TB. BCG, the only licensed TB vaccine in the world, is reasonably effective in providing infants with protection, but its efficacy for adults is much lower (Mangtani et al., 2014). It stands to reason that (chemically) boosting BCG immunogenicity could offer a strategy for increasing that efficacy. On the oncological side, there is growing interest in developing vaccines that can trigger potent anti-tumor immune responses (Vermaelen, 2019). Many studies have examined the utility of antigen-based vaccines for overcoming this obstacle (Neek et al., 2019), and more recently cell-based vaccines have been explored also (Alson et al., 2020; Remic et al., 2022). For the latter, a chemical augmentation strategy like the one presented in this dissertation could provide a tool for increasing their immunogenicity, and with it anti-tumor efficacy.