Modulation of *B. pseudomallei* Immune Responses by Human-like T Cell Epitopes

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INTRODUCTION|

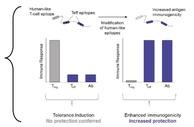
Burkholderia pseudomallei is a Gram negative, motile saprophyte endemic to tropical areas, with highest concentrations in Southeast Asia and northern Australia. Individuals exposed to *B. pseudomallei* suffer from melioidosis, a potentially fatal disease with a range of complications including widespread abscess formation, pneumonia, healing-resistant lesions of the skin and soft tissue, bacteremia and sepsis. Although average mortality rates have recently declined due to the advancements in diagnostics and antibiotic regiments, reoccurrence rates and resistance to treatment now appear to be on the rise. Given the environmental prevalence, ease of acquisition, and the lack of novel antibiotics on the horizon, the bacteria's potential as a biological threat is also abundantly clear. Despite the overwhelming need for prophylactic measures, we have yet to identify an efficacious and safe vaccine formulation for public use. Multi-agent vaccines against biological threats promise to efficiently induce protective immunity, reduce distribution and dispensing requirements and defend against an expanding bioweapon agent landscape to benefit the Warfighter and defense operations. Combination vaccines, however, run the risk that one vaccine component may interfere with the ability of another to stimulate immunity. Interference involves both vaccine extrinsic factors, such as dose and regimen, and intrinsic factors, including antigen composition. **Here, we consider effects of antigen-stimulated T cell cross-reactivity that works sometimes to the benefit and at other times to the detriment of vaccine protection. Induction of inhibitory responses can explain how an antigen may be fully or partially protective on its own but not protective when combined with another that contains regulatory T cell (T_{reg}) stimulating epitopes. This is a concern in biothreat vaccine development as intracellular bacterial agents suppress bacterial-specific effector T cells by stimulation of T_{regs}.**

OBJECTIVES|

The overarching goal of the study was to identify T_{reg} inducing epitopes within immunogenic *B. pseudomallei* proteins and to reduce the tolerogenic content as guided by immunoinformatic analysis, enhancing cellular and humoral immunity afforded by protein vaccines. Goals for individual legs of the study include:

- ✓ Confirm direct activation of T_{regs} in the course of *B. pseudomallei* infection
- ✓ Identify cross-conserved epitopes (human/*B. pseudomallei*) using the iVAX platform and select putative T_{reg} epitopes for *in vitro* testing
- ✓ Characterize suppressive capabilities of putative T_{reg} epitopes *in vitro* using PBMCs of melioidosis survivors

❑ Engineer confirmed tolerogenic motifs out of *B. pseudomallei* antigens and assess increased immunogenicity and immunity conferral *in vitro*



RESULTS|

Here **we identify unique expansion of the T_{reg} subsets in mice who exhibit uncontrolled *B. pseudomallei* infection**. Additionally we observe that increased T_{reg} activity, identified by IL-10 secretion, correlates with both the suppression of Th1 cytokine secretion and severe infections as indicated by lung bacterial burden. However, the balance of immunogenic and tolerogenic responses may be further compounded by increased PD-1 expression, commonly associated with exhaustion, particularly on T_{eff} cells. The likelihood of a direct T_{reg} induction by tolerogenic peptides is supported by several human-like epitopes identified in the *B. pseudomallei* proteome. **To assess regulatory responses in human donors**, we identified 90 *B. pseudomallei* antigens known to be seroreactive in humans and/or provide minimal protection through immunization despite demonstrating T cell immunogenicity in animal models. By refining our search to enrich for isolated human-like epitopes (TCR face) with increased likelihood of being tolerogenic while also being conserved in the related *B. mallei,* the cause of glanders and a tier-1 select agent, for use in a pan-species vaccine formulation, **we selected 10 peptides to test for recall responses in PBMCs** from *B. pseudomallei*-experienced donors. Through the use of our Tetanus Toxoid (TT) Bystander Suppression Assay, we identified that CD4+ T cells from melioidosis survivors (exhibiting no infection at time of sample collection) displayed dampened responses in both T_{eff} and T_{reg} compartments, most likely due to T cell exhaustion. Despite the limited range of response, both survivors and aged-matched control donors exhibited a significant suppression of TT – induced CD4+ proliferation when co-incubated with high doses of a control T_{reg}-activating peptide – thus this biological readout was further used to determine tolerogenic responses induced by our peptide set. Overall, 5 *B. pseudomallei* donors (1 additional donor's sample was not suitable for assessment and was excluded) and 6 age-matched control donors were assayed with each of the 10 selected peptides. As a whole, **melioidosis survivors exhibited stronger tolerogenic responses to the peptide set**, as 50% of peptide-donor pairs showed suppression of at least 20% of control (TT-only), with maximum suppression seen at 74%. This was in marked comparison to controls that primarily exhibited responses ranging from neutral to immunogenic. To highlight which peptides were the best candidates to move into detolerization studies, we characterized CD4+ T cell proliferation in those donors with suitable HLA-types. By scoring the overall degree of suppression, separation between *B. pseudomallei*-experienced and control donors (approaching significance in this small cohort), and direct correlation between predicted HLA-binding and CD4+ T cell suppression, **we confirmed half of our predicted peptide set was highly likely to induce tolerogenic recall responses and thus would be ideal targets for detolerization and subsequent immunization testing.**

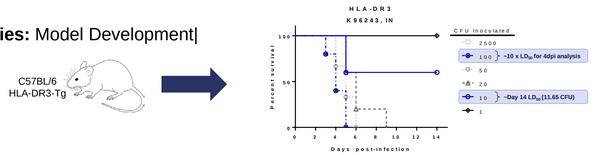
CONCLUSIONS|

For the first time, this study has confirmed the direct activation of T_{regs} following *B. pseudomallei* infection. Here we identify the rapid expansion and increased immunosuppressive function of FoxP3+ cells, in the absence of an exuberant pro-inflammatory response. As the indirect activation of T_{regs} in hyperinflammatory states also requires an extended timeline, we can conclude that the observed increase in tolerogenic responses stems from the direct ligation of regulatory TCRs via presentation of self-like peptides. Through immunoinformatic analysis, **we isolated 10 putative T_{reg} epitopes and confirmed that the majority of these peptides suppressed CD4+ T cell proliferation in select individuals according to their HLA-type, as predicted by our iVAX analysis platform.** However, clear immunosuppression in melioidosis survivors has reduced the power of our *in vitro* system. As we believe this immunosuppression to be linked to T cell exhaustion, as evidenced by PD-1 upregulation in our murine model, future directions plan to address reinvigorating these cells to more robustly identify regulatory recall responses to our predicted peptide set. With increased sensitivity, we will be able to confirm regulatory recall responses in melioidosis survivors and to more accurately characterize our detolerized epitopes. With the data presented here, [considering the degree of immunosuppression, the likelihood of a memory response (separation from control donors), and correlation to HLA restriction] we selected the 5 most responsive candidate peptides to **move forward into detolerization engineering and functional assessments with the ultimate goal being to formulate a protein vaccine with increased efficacy and optimal HLA coverage.**

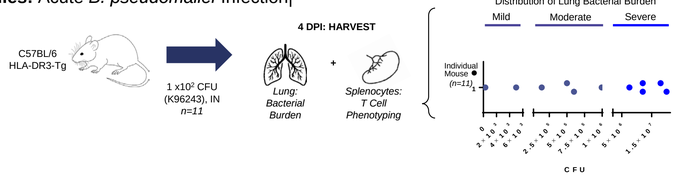
Human-like epitopes in *B. pseudomallei* antigens induce regulatory T cell-mediated tolerance & serve as novel targets for increasing vaccine efficacy in melioidosis prevention

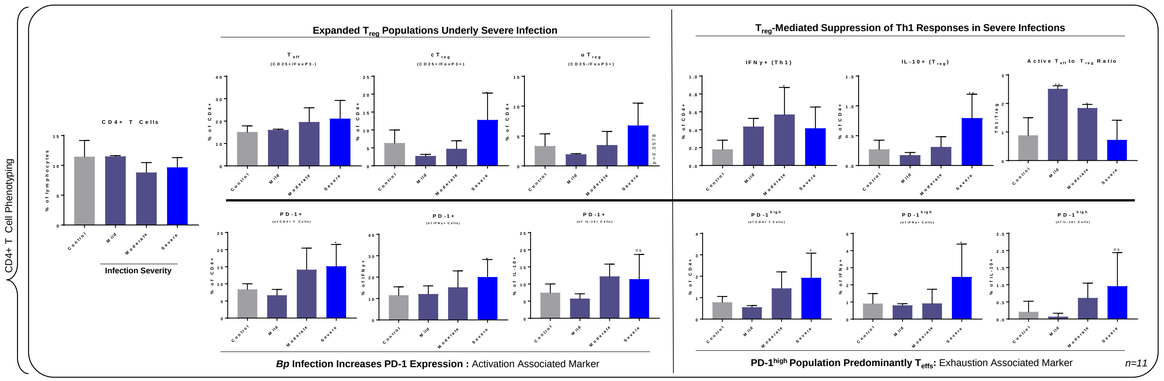
SUPPORTING DATA|

Mouse Studies: Model Development|

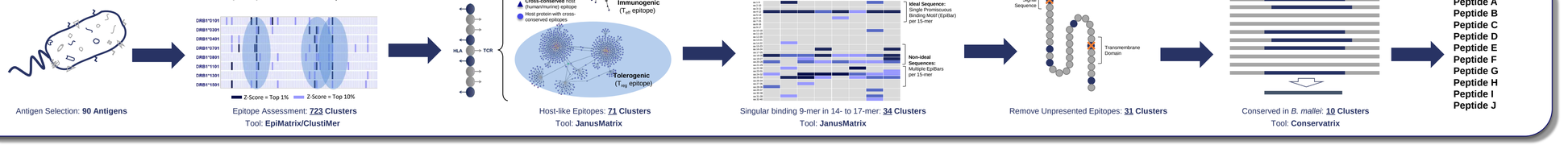


Mouse Studies: Acute *B. pseudomallei* Infection|





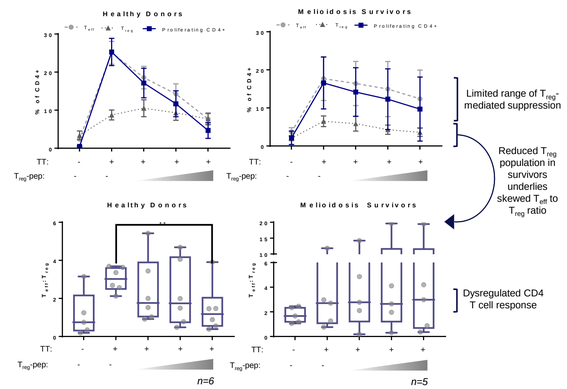
In-Silico Predictions: iVAX Guided Epitope Selection|



Human PBMCs: Memory Responses in Thai Melioidosis Survivors|

Characterizing Immunogenic and Tolerogenic Responses in Human Donors

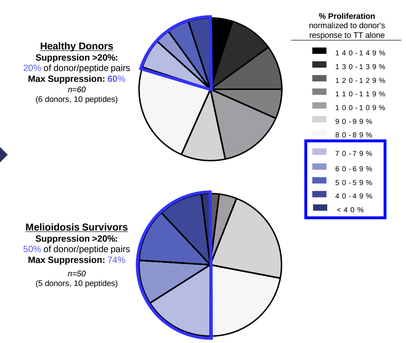
RATIONALE| The Tetanus Toxoid Bystander Suppression Assay (TT-BSA) tests potential T_{reg}-inducing epitopes by measuring suppression of TT-induced CD4+ T Cell Activation (Proliferation, Subset Frequency, T_{eff}-to-T_{reg} Inflammatory Index). Here we characterize the range of the immunosuppressive response with confirmed T_{reg} activating peptide (T_{reg}-pep) alongside TT exposure in melioidosis survivors and age-matched controls.



Test *Bp* Peptides

Immunosuppression Among ALL Peptide-Donor Pairs

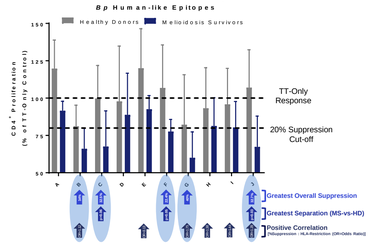
Suppression of TT-Induced CD4 T Cell Proliferation
(by individual peptide-donor pair)



Selected Peptides in HLA-RESTRICTED Donors

Predicted Sensitivity to *Bp* Human-like Epitope
(by HLA restriction)

Donor → / Peptide →	A	B	C	D	E	F	G	H	I	J
MS1	NO	YES	YES	YES	NO	YES	YES	YES	YES	YES
MS2	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES
MS3	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES
MS4	NO	YES	YES	NO	NO	YES	YES	NO	YES	YES
MS5	NO	YES	YES	YES	NO	YES	YES	YES	YES	NO
HD1	NO	YES	YES	YES	NO	YES	YES	YES	YES	YES
HD2	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES
HD3	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES
HD4	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES
HD5	YES	YES	YES	YES	NO	YES	YES	YES	YES	YES
HD6	NO	YES	YES	YES	NO	YES	YES	YES	YES	YES



SIGNIFICANT FINDINGS|

Mouse Studies:

- T_{reg} expansion & activity inhibit Th1 responses
- Early skewing of PD-1 expression (T_{eff}) may initially amplify the effect of T_{regs}

In Silico Predictions:

- The iVAX platform identified 155 putative *Bp* T_{reg} epitopes and guided selection of 10 candidates

Human PBMCs:

- PMBCs of melioidosis survivors display long-term immunosuppression
- Putative T_{reg} peptides were 2.5x as likely to elicit immunosuppressive responses from *Bp*-experienced PBMCs
- TT-BSA characterization & immunoinformatic analysis identified 5 peptides, likely inducing recall responses, to move into detolerization studies

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