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EDITORIAL

Brucella Lipopolysaccharide and pathogenicity: The core of the matter

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Brucellosis is one of the most prevalent zoonotic infections worldwide.[1] The half million reported new cases per year likely represents a gross underestimate related to challenges in diagnosis and incomplete reporting. In humans, brucellosis initially manifests as undulating fever accompanied by flu-like myalgias and arthralgias. However, chronic infection may lead to peripheral arthritis, sacroiliitis, orchitis, endocarditis and neurobrucellosis.[1,2] Treatment involves prolonged courses of multiple antibiotics and relapses occur in up to 10% of patients.[3] The causative agents, *Brucella* species, are Gram-negative facultative intracellular bacteria which infect herd animals such as goats, cattle and swine. In animals, *Brucella* species cause abortion, resulting in tremendous economic losses. Although animal vaccine strains such as Rev1, RB51 and S19 have been effective at controlling disease in the herds of nations with sufficient infrastructure and regulation, *Brucella* infections remain intractable in many parts of the world:[4] Brucellosis is endemic in Central and South America, Sub-Saharan Africa, the Middle East and Asia.[5] *Brucella* are primarily transmitted to human hosts by ingestion of contaminated unpasteurized dairy products, but *Brucella* also poses a risk to herders and abattoir workers. Because of the low number needed for infection (10-100 organisms), and ready aerosolization, *Brucella* is considered a bioterror threat.[6] Current vaccine strains cause disease in humans.[7–9] Thus at present, no safe and effective human vaccine exists.

With the goal of improving therapeutic and vaccine strategies, research has focused on understanding the pathogenic determinants that allow *Brucella* to establish successful chronic infections and evade immune eradication. Ultimately, immune control of *Brucella* involves the development of effective Th1 cellular immunity.[10,11] However, mobilization of *Brucella*-responsive T cells first requires recognition by "sentinel" dendritic cells of the innate immune system, and activation of these cells to become effective antigen presenting cells.[12] Innate immune cells detect pathogens via repeated cellular patterns (PAMPs) such as the Lipopolysaccharide (LPS) coat on Gram-negative bacteria. Intriguingly, one of the major *Brucella* virulence factors identified to date is its non-canonical LPS.[13]

LPS plays a vital role in the integrity of Gram-negative bacterial outer membranes. It comprises three regions: a Lipid A moiety composed of a disaccharide backbone linked to up to seven hydrophobic acyl chains that are embedded in the outer membrane, a species-conserved core polysaccharide linker, and the external most O-polysaccharide chain.[14] The inner core contains unusual sugars (e.g. 3-deoxy-D-manno-octulosonic acid (Kdo)), but the outer core has more common sugars such as hexoses and hexosamines. The O-polysaccharide, a long chain of repeating glycosyl subunits, exhibits the most variability, providing the means for strain differentiation in labs and antigenic stimulation of humoral immune responses. Innate immune cells recognize LPS via a heteromeric receptor composed of Toll-like receptor 4 (TLR4) and its binding partner myeloid differentiation-2 (MD2). MD2 contains a large hydrophobic pocket that accompanies 5 of the Lipid A acyl chains, whereas the LPS polysaccharide forms polar interactions with the rim of MD2 and TLR4.[15,16] Agonist activity (endotoxicity) is generally thought to reflect the acyl chain number, length, and chemical modifications.[14]

Brucella LPS counters innate immune defenses on multiple levels: the Lipid A moiety contains overly long C16-18 fatty acids (including up to C28), rather than the optimal 12–14 carbons, correlating with poor MD2

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binding and low endotoxicity.[13,17–19] The Lipid A component also kills neutrophils through an unclear mechanism.[20] The O-polysaccharide resists complement deposition and activation.[21] Inside cells, this outer polysaccharide is also important for evasion of lysosomal destruction. Indeed "rough" strains or mutants of *Brucella* lacking the external O-polysaccharide display significantly attenuated virulence.[22,23] More recently the core moiety of *Brucella* has come to the fore as a key modulator of virulence.[19] The *Brucella* core polysaccharide contains 2 Kdo sugars; one connects to the O-polysaccharide, and the other to an unusual branching side chain.[24,25] This side chain, with its positive charges, is thought to "shield" the more internal negative charges from effective interaction with the MD2/TLR4 receptor.[19,24] *Brucella* mutants deficient in the *wadC* mannosyltransferase enzyme required for this core saccharide branch display enhanced MD2 binding, and increased triggering of cytokine production. Interestingly, the *wadC* mutants are also more susceptible to complement and antibacterial cationic peptides – thus not all the serum resistance reflects O-polysaccharide or Lipid A composition.[21,26] Perhaps because of this increased susceptibility, plus the increased immune activation, the *wadC* mutant *Brucella* are attenuated *in vivo* in mice.[19] Conversely, the immune evasion properties of wild type *Brucella* LPS contribute to the prevailing conception of *Brucella* as a "stealthy pathogen".[27,28]

Nevertheless, there are some challenges to the notion that *Brucella* completely evades TLR4 detection. Although results are conflicting, some studies have documented a role for TLR4 in *Brucella* resistance.[29–31] Adjuvant properties of *Brucella* LPS have been reported.[32,33] Most of the studies documenting poor TLR4 stimulation of dendritic cells by *Brucella* LPS have used GM-CSF derived dendritic cells *in vitro*. However, dendritic cell heterogeneity has been increasingly recognized. Just within the spleen, subtypes include plasmacytoid dendritic cells, CD11b+ and CD8a+ conventional dendritic cells, and monocyte-derived dendritic cells.[34] These subtypes vary in TLR expression, capacity for viral-induced IFN responses, T-cell costimulation and cross-presentation of external antigen to CD8 T cells. The roles of these different subsets in response to *Brucella* infection have not been clearly defined. The study by Zhao et al. entitled "Immunomodulatory properties of *Brucella* melitensis lipopolysaccharide determinants in mouse dendritic cells in vitro and in vivo" supports the importance of evaluating different dendritic subsets when assessing LPS stimulatory capacity.[35]

In this study Zhao et al employed a "mix and match" approach, examining the effect of various purified LPS preparations sharing different features with *Brucella* LPS. For instance *Ochrobactrum anthropi 3331* LPS contains a Lipid A moiety similar to *Brucella*, whereas *Yersinia enterocolitica* O:9 shares a similar O-chain polysaccharide.[36,37] *E. coli* LPS served as the gold standard TLR4 agonist. The ability of these LPS preps to activate different types of dendritic cells, as assessed by cytokine production and cell surface markers (B7 molecules, CD40, MHC class II, PDL-1) were compared *in vitro* and *in vivo*. Several notable findings challenge current paradigms:

1) This study emphasized the importance of the *Brucella* core polysaccharide branch: surprisingly, *Brucella*-type lipid A and the O-polysaccharide were not in themselves problematic for TLR4 stimulation, as the *O. anthropi*, *Y enterocolitica* and the *wadC Brucella* mutant induced co-stimulator expression and cytokine production comparable to *E. coli* LPS in GM-CSF derived dendritic cells. Interestingly, only IL-10 production correlated with Lipid A structure. Thus a bulky Lipid A may be less of an issue for pro-inflammatory cytokine induction than previously thought. Indeed, despite the *Brucella*-type Lipid A, *O. anthropi 3331* LPS induced greater production of IL-12p70, TNF-α, IL-6 and IL-1β than equimolar *E. coli* LPS.
2) *Brucella* LPS is not completely inert: In Fms related tyrosine kinase 3 (Flt3) derived dendritic cells, *Brucella* LPS induces comparable TNF-α secretion and upregulation of MHC class II to other forms of LPS, and significant (though reduced) increases in co-stimulatory molecules. These findings challenge the notion that *Brucella* LPS is a universally poor TLR4 stimulus.

Their results also provide greater insight into how *Brucella* avoids potent activation of adaptive response: *In vitro*, *Brucella* LPS was deficient in stimulating Flt3 dendritic cell production of IL-12p70 and IFN-γ, 2 cytokines critical for the Th1 responses that control disease.[10,11] *Brucella* LPS also failed to activate Flt3 dendritic cells sufficiently to support CD4 or CD8 T cell proliferation *in vitro*. *In vivo*, unlike *E. coli* or the *wadC* mutant LPS, *Brucella* LPS induced very little splenic expansion of CD64+ DC-sign+ monocyte-derived dendritic cells. Interestingly, one of the few activation markers significantly up-regulated on CD11c^{hi} spleen cells was programmed death ligand (PDL-1), which would undermine adaptive responses by causing T cell exhaustion.[35] Consistent with this observation, CD8 T cell exhaustion has been reported in mouse models of brucellosis.[38,39]

Together, the results reported by Zhou et al. raise compelling questions regarding the nature of TLR4/MD2 recognition of LPS. It is not clear how TLR4/MD2

distinguishes between the individual types of LPS to induce a different cytokine profile. Are there subtle differences in oligomerization or structural conformation that result in altered recruitment of cytosolic adaptors and other signaling molecules? The exact mechanism by which the *Brucella* core polysaccharide branch "shields" recognition, as well as how these different bacterial forms of LPS bind the TLR4 receptor complex may require crystallographic definition.

One of the more intriguing issues raised by this study is the potential to use the *wadC Brucella* mutant as a vaccine. The *wadC* mutant LPS induces similar mobilization of CD11b BST-2+ monocyte derived dendritic cells as *E. coli* LPS and comparable induction of co-stimulatory marker expression, but displays attenuation *in vivo*. These authors have provided initial data that the *wadC* mutant *Brucella* may be at least as protective as the S19 vaccine strain.[40] However development of this mutant for vaccine purposes will require greater analysis of the effect of the whole bacteria *in vivo* (not just the LPS) and further exploration of the organism's protective capacity. Safety, particularly as it affects reproduction, will also need to be established.

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